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Deficiency of the Oxidative Stress-Responsive Kinase p70S6K1 Restores Autophagy and Ameliorates Neural Tube Defects in Diabetic Embryopathy

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Abstract

BACKGROUND—Autophagy is highly active in neuroepithelial cells of the developing neuroepithelium, and impaired autophagy leads to neural tube defects (NTDs). We have demonstrated that maternal diabetes induces NTDs, and that impaired autophagy and consequent cellular imbalance, including the endoplasmic reticulum (ER), where critical events occur leading to the induction of diabetic embryopathy. Because the mammalian target of rapamycin (mTOR) pathway suppresses autophagy, we hypothesize that p70S6K1 (70 kDa ribosomal protein S6 kinase 1), a major downstream effector of mTOR, mediates the inhibitory effect of maternal diabetes on autophagy in the developing neuroepithelium.

OBJECTIVE—We investigated whether p70S6K1 mediates the inhibitory effect of maternal diabetes on autophagy during neurulation. We also examined if p70S6K1 deficiency restores autophagy and thus relieves ER stress and inhibits maternal diabetes-induced apoptosis, which leads to reduction in NTD incidence in diabetic embryopathy.

STUDY DESIGN—Female p70S6K1 heterogeneous knockout (p70S6K1^{+/-}) mice were bred with male p70S6K1 heterogeneous knockout (p70S6K1^{+/-}) mice to generate wild type (WT), p70S6K1^{+/-} and p70S6K1 knockout (p70S6K1^{-/-}) embryos. Embryos at embryonic day 8.5 (E8.5) were harvested for the assessment of indices of autophagy, ER stress and apoptosis. NTDs incidence was determined in E10.5 embryos. For in vitro studies, siRNA knockdown of p70S6K1 in C17.2 mouse neural stem cells were used to determine the effect of p70S6K1 deficiency on autophagy impairment and ER stress under high glucose conditions.

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RESULTS—Knockout of the *Rps6kb1* gene, which encodes for p70S6K1, ameliorated maternal diabetes-induced NTDs and restored autophagosome formation in neuroepithelial cells suppressed by maternal diabetes. Maternal diabetes-suppressed conversion of LC3-I (Microtubule-associated protein 1A/1B-light chain 3) to LC3-II, an index of autophagic activity, in neurulation stage embryos was abrogated in the absence of p70S6K1. p70S6K1 knockdown in neural stem cells also restored autophagosome formation and the conversion of LC3-I to LC3-II. The activation of the major unfolded protein response (UPR), indicated by phosphorylation of IRE1 α , PERK and eIF2 α , and the increase of the endoplasmic reticulum (ER) stress marker, CHOP, were induced by maternal diabetes in vivo and high glucose in vitro. UPR and ER stress induced by maternal diabetes or high glucose were diminished by *Rps6kb1* deletion or p70S6K1 knockdown, respectively. *Rps6kb1* knockout blocked maternal diabetes-induced caspase cleavage and neuroepithelial cell apoptosis. The SOD mimetic Tempol abolished high glucose-induced p70S6K1 activation.

CONCLUSION—We revealed the critical involvement of p70S6K1 in the pathogenesis of diabetic embryopathy.

Keywords

maternal diabetes; neural tube defects; p70S6K1; autophagy; ER stress; apoptosis; diabetic embryopathy

Introduction

Autophagy (here referred to macroautophagy) is a cellular process by which eukaryotic cells degrade damaged proteins and dysfunctional organelles, and sustain bio-energetic demands under stress conditions¹. Autophagy is initiated with the formation of specialized double-membrane autophagosomes, which engulf protein aggregates or dysfunctional organelles and subsequently fuse with lysosomes to enable degradation^{1–3}. Autophagy is important for cellular balance between anabolic and catabolic processes in sustaining cell viability^{4–7}, and thus, autophagy is crucial for cells to adapt to stressors, such as oxidative stress, parasite invasion or chemotherapeutic exposure^{1, 7–18}. Thus, mild oxidative stress induces adaptive responses in stimulating autophagy which can remove oxidative stress-damaged cellular components. However, severe oxidative stress, such as maternal diabetes-induced oxidative stress, suppresses autophagy leading to intracellular imbalance and cell apoptosis^{19, 20}. Autophagy is an effective and highly regulated process in maintaining cellular homeostasis through the removal of unnecessary or defective components^{8, 21, 22}. It is essential for preimplantation development²³. Our previous studies have shown that restoring autophagy inhibits maternal diabetes-induced neural tube defects^{19, 20}. Others have shown that autophagy deficiency due to a gene deletion result in neural tube defects (NTDs)²⁴.

Mammalian target of rapamycin (mTOR), a serine/threonine protein kinase, is a negative autophagy regulator²⁵. Inhibiting mTOR activity is a crucial step for autophagy induction under nutrient starvation²⁵. p70S6K1 is a downstream kinase of the mTOR signaling pathway, indicating that p70S6K1 may mediate the inhibitory effect of mTOR on autophagy. However, mTOR suppresses autophagy by directly phosphorylating (or inactivating) the protein of autophagy related gene 1 (ATG1)²⁵. We propose a new function of p70S6K1 in

diabetic embryopathy: activation of p70S6K1 suppresses autophagy in neuroepithelial cells leading to cellular organelle stress.

Our previous studies have demonstrated that autophagy in the developing neuroepithelium is suppressed by hyperglycemia of diabetes during pregnancy^{19, 20, 26, 27}. Maternal diabetes disrupts neurulation through oxidative stress leading to neural tube defects (NTDs)^{28–39}, while restoring autophagy ameliorates maternal diabetes-induced NTDs^{19, 20}. However, it is still unclear how maternal diabetes represses autophagy. Maternal diabetes predominantly induces exencephaly, triggers neuroepithelial cell apoptosis and suppresses autophagy in the region between the forebrain and midbrain of the developing embryo^{31, 40–51}. We hypothesize that knockout of the *Rps6kb1* gene, encoding for p70S6K1, reverses maternal diabetes-induced autophagy impairment, cellular organelle stress and apoptosis, leading to a reduction of NTD formation.

Cellular organelle stress, including ER stress and mitochondrial dysfunction is manifested in diabetic embryopathy^{19, 29, 30, 44, 45, 49, 50, 52–55}. The ER is morphologically and functionally perturbed in cells of developing organs that are susceptible to maternal diabetes^{19, 48, 56, 57}. Maternal diabetes activates the major unfolded protein response (UPR) sensors, Inositol requiring enzyme 1 alpha (IRE1 α) and protein kinase R-like endoplasmic reticulum kinase (PERK), which induce the pro-apoptotic effects of ER stress leading to NTDs⁴⁸. Thus, activation of the UPR and ER stress mediates the teratogenicity of maternal diabetes⁴⁸. Autophagy restores intracellular balance by relieving ER stress. Therefore, if p70S6K1 is critically involved in maternal diabetes-induced autophagy impairment, it will impact maternal diabetes-induced UPR and ER stress in the developing embryo.

Our previous studies found maternal diabetes-induced neuroepithelial cell apoptosis^{41, 50}. p70S6K1 deficiency blocks hepatocyte undergoing apoptosis⁵⁹, suggesting that p70S6K1 may induce apoptosis. Apoptosis and autophagy are two inter-related cellular processes that regulate cell fate. Some studies show that their regulation is intimately connected and the same regulators can sometimes control both autophagy and apoptosis^{60, 61}. p70S6K1 may be one of these regulators to impact both maternal diabetes-induced autophagy impairment and apoptosis in the developing embryo.

In the present study, we investigated the effect of p70S6K1 deficiency on maternal diabetes-induced autophagy impairment, ER stress and apoptosis in diabetic embryopathy. We found that deleting the *Rps6kb1* gene abrogated maternal diabetes-induced autophagy impairment, resolved cellular homeostatic imbalance by preventing UPR activation and ER stress, inhibited maternal diabetes-induced apoptosis, and ultimately reduced NTD formation in the embryos of diabetic pregnant mice.

Materials and Methods

Study design

To investigate whether the oxidative stress-responsive kinase p70S6K1 mediates the inhibitory effect of maternal diabetes on autophagy during neurulation, we used the streptozotocin (STZ)-induced type 1 diabetes mouse model. Diabetes was induced in Wild-

type (WT) C57BL/6J mice and heterozygous p70S6K1 knockout (KO) mice in the same background. Indices of autophagy, cellular stress and apoptosis in the developing neuroepithelium were analyzed in WT and KO embryos from nondiabetic and diabetic dams at embryonic day 8.5 (E8.5), a critical time point for mouse neurulation. NTD incidence was morphologically examined at E10.5. For *in vitro* studies in the C17.2 mouse neural stem cell line, p70S6K1 knockdown by transfection of siRNAs was achieved for the evaluation of autophagy and cellular stress. Cells were cultured under normal (5 mM glucose) and high glucose (25 mM glucose) conditions and transfection of the scramble siRNA (the untargeting siRNA) served as a control group. For a separate *in vitro* experiment, cells were treated with the antioxidant Tempol to determine whether oxidative stress is responsible for high glucose-induced p70S6K1 activation (phosphorylation).

Animals and Reagents

WT C57BL/6J mice (median body weight 22 g) were purchased from The Jackson Laboratory (Bar Harbor, ME). p70S6K1 KO mice on C57BL/6J background was previously described⁶². STZ (Sigma Chemical Company, St. Louis, MO) was dissolved in sterile 0.1mol/L 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

Our mouse model of diabetic embryopathy has been described previously^{20, 41, 47}. In the diabetic group, female mice were intravenously injected daily with 75 mg/kg STZ over 2 days to induce diabetes before pregnancy was established. Diabetes was defined as a 12-h fasting blood glucose level of greater than or equal to 13.9 mM. Then, these female diabetic mice were mated with nondiabetic male mice. In the nondiabetic group, female mice were treated with vehicle injection. On E8.5, mice were euthanized and conceptuses were dissected out of the uteri for analyses.

Cell Culture and Transfection

C17.2 mouse neural stem cells, originally obtained from the European Collection of Cell Culture (Salisbury, UK), were maintained (more than 3 passages) in DMEM (5 mM glucose) supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO₂. p70S6K1 siRNA and control siRNA were obtained from Invitrogen. Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used according to the manufacturer's protocol for transfection of siRNA into the cells. After seeding for 12h, the cells were transfected with siRNA and cultured in 1% FBS + DMEM for 8h. Then cells were cultured in 10% FBS + DMEM and cells were harvested after 48h for analyses.

Immunofluorescent staining

Cells were maintained under standard tissue culture conditions at 37 °C, 5% CO₂ in a humidified incubator. After 48h in different treatment conditions, cells were washed with DPBS. 1ul of Cyto-ID Green Detection Reagent was added to 1 ml cell culture medium. After Cyto-ID staining, cells were incubated with DAPI (4',6-diamidino-2-phenylindole) for 10 min for cell nuclei staining. Fluorescence images were taken on a Nikon H600L

microscope with the IP Lab imaging system (Scientific Instrument Company, Campbell, CA).

Whole embryos were fixed in 4% paraformaldehyde (PFA) (pH7.4) for 30 min at room temperature and fixed embryos were embedded in optimum cutting temperature medium (OCT) compound (Sakura Finetek, Torrance, CA). Then, 10- μ m embryonic frozen sections were obtained and permeabilized with 0.25% Triton-X100 (Sigma, St. Louis, MO) for 10 min. Samples were blocked for 30 min in 10% donkey serum in PBS and incubated with the LC3 antibody (1:200, Cell Signaling Technology, Danvers, MA) overnight at 4°C. After they were washed with PBS, samples were incubated with Donkey anti-Rabbit Secondary Antibody, Alexa Fluor 488 (1:1000, Invitrogen) for 2h, followed by DAPI cell nuclear counterstaining for 10 min and coverslip mounting with aqueous mounting medium. For confocal fluorescent imaging, images were captured by a laser scanning microscope (LSM 510 META, ZEISS, Germany).

Immunoblotting

Immunoblotting was performed as previously described⁵⁰. To extract protein, samples (embryos or C17.2 cells) were sonicated in lysis buffer (Cell Signaling Technology) containing a protease inhibitor cocktail (Sigma). Equal amounts of protein and the Precision Plus Protein standards (Bio-Rad, Portland, ME) were resolved by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Billerica, MA). Membranes were incubated in 5% nonfat milk for 1h and then incubated overnight at 4°C with primary antibodies at their respective dilutions. p-p70S6K1(1:1000, Cell Signaling Technology), p70S6K1(1:1000, Cell Signaling Technology), LC3(1:1000, Cell Signaling Technology), β -actin(1:10,000, Abcam, Cambridge, MA), caspase-3(1:500, Millipore), caspase-8(1:1000, Enzo Life Sciences, Farmingdale, NY), p-S6(1:1000, Cell Signaling Technology), S6(1:1000, Cell Signaling Technology). Membranes were exposed to HRP-conjugated goat anti-rabbit or goat anti-mouse (Jackson Immuno Research Laboratories, West Grove, PA) secondary antibodies. Signals were detected using the Super Signal West Femto Maximum Sensitivity Substrate kit (Thermo Fisher Scientific, Halethorpe, MD). Chemiluminescence emitted from the bands was directly captured using a UVP Bioimage EC3 system.

TUNEL assay

The TUNEL assay was performed using the ApopTag Fluorescein in Situ Apoptosis Detection kit (Chemicon) as previously described^{48, 63}. Briefly, 10- μ m frozen embryonic sections were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and incubated with TUNEL reaction agents. Three embryos from three different dams ($n = 3$) each group were used, and two sections per embryo were examined. TUNEL-positive cells in an area (about 200 cells) of the neuroepithelia were counted. The percentage of TUNEL-positive cells was calculated as a fraction of the total cell number, multiplied by 100 and averaged within the sections of one embryo.

Statistical analysis

Data are presented as means \pm SEM (standard error). Embryonic samples from each replicate were from a different dam. For overall statistical significance, one-way ANOVA

was performed using the Sigma Stat 3.5 software (Systat Software Inc., San Jose, CA). In multiple comparisons, the *Tukey-test* was used to estimate the significance of the difference between groups. Significant difference between groups in NTDs incidences was analyzed by the Fisher Exact Tests. Statistical significance was accepted at $P < 0.05$.

Results

p70S6K1 knockdown restores autophagy suppressed by high glucose

Our previous study demonstrated that high glucose represses autophagy²⁰. To investigate whether removing p70S6K1 restores normal autophagy, even under high glucose conditions, we utilized a specific siRNA to knockdown p70S6K1 expression in C17.2 neural stem cells. Immunoblotting showed that p70S6K1 siRNA reduced p70S6K1 protein levels (Fig. 1A), indicating the effectiveness of the siRNA-mediated p70S6K1 mRNA silencing. The amount of LC3-II, the lipidated form of LC3-I as an autophagic activity index, was not affected by p70S6K1 siRNA knockdown under normal glucose conditions, but was significantly reduced by high glucose (Fig. 1A). High glucose-reduced LC3-II abundance was abrogated by p70S6K1 siRNA knockdown (Fig. 1A). Using the Cyto-ID staining method to detect autophagosomes, we observed inhibition of autophagy by high glucose (Fig. 1B). Under normal glucose conditions, the number of Cyto-ID staining puncta did not differ between the control group and the p70S6K1 siRNA transfected group (Fig. 1B). However, in the high glucose conditions, the number of Cyto-ID staining puncta was significantly reduced, and p70S6K1 knockdown reversed this puncta reduction (Fig. 1B).

***Rps6kb1* gene knockout ameliorates maternal diabetes-induced NTDs**

To determine whether p70S6K1 plays a role in the induction of NTDs in diabetic pregnancy, we used p70S6K1 knockout mice and examined NTD formation. As shown in Table 1. Under diabetic conditions, 10 out of 31 embryos (32.3%) from Wild-Type (WT) diabetic dams had NTDs. Only 2 of 29 *Rps6kb1* gene-deleted embryos (6.9%) exhibited NTDs (Table 1) and this NTD rate was significantly lower than that of WT embryos from diabetic dams (Table 1). *Rps6kb1* gene deletion knockout significantly reduced NTD formation in diabetic pregnancy. Under nondiabetic condition, none of *Rps6kb1* gene-deleted embryos exhibited NTDs (Table 1), indicating that *Rps6kb1* gene deletion knockout did not affect embryonic development. These findings support our hypothesis that p70S6K1 deficiency ameliorates maternal diabetes-induced NTDs.

***Rps6kb1* gene knockout rescues autophagy suppressed by maternal diabetes**

Because impaired autophagy is a critical mechanism by which NTDs occur in offspring of diabetic pregnant dams, we investigated the impact of *Rps6kb1* gene knockout on autophagy activity. *Rps6kb1* gene knockout prevented maternal diabetes-induced reduction in autophagosome numbers in neuroepithelial cells (Fig. 2A). Immunofluorescent staining showed that the number of autophagic puncta in neuroepithelial cells was not significantly different in WT embryos compared with *Rps6kb1* gene deleted embryos under nondiabetic conditions (Fig. 2A). In WT embryos of diabetic dams, the number of autophagic puncta was significantly reduced (Fig. 2A). This reduction of autophagic puncta by diabetes was reversed by *Rps6kb1* gene knockout (Fig. 2A). The lipidation of LC3-I into LC3-II in

neurulation stage embryos was significantly reduced by maternal diabetes (Fig. 2B), and *Rps6kb1* gene knockout abrogated the suppression of LC3-II expression by maternal diabetes (Fig. 2B).

p70S6K1 deficiency inhibits UPR activation and ER stress

Autophagy can effectively resolve ER stress¹. Because *Rps6kb1* gene knockout re-activated autophagy in the developing neuroepithelium, we sought to determine whether deleting the *Rps6kb1* gene blocks diabetes-induced UPR activation and ER stress. Maternal diabetes triggered the phosphorylation of two major UPR sensors, IRE1 α and PERK, and their downstream effectors, CHOP and eIF2 α , respectively, in neurulation stage embryos (Fig. 3). Deleting *theRps6kb1* gene abolished maternal diabetes-induced phosphorylation of IRE1 α , PERK and eIF2 α (Fig. 3), and blocked the increase of CHOP expression (Fig. 3).

To further determine whether p70S6K1 accounts for UPR and ER stress, we tested the effect of reducing p70S6K1 expression *in vitro* under high glucose conditions. High glucose triggered the phosphorylation of IRE1 α , PERK and their downstream effectors, CHOP and eIF2 α , while p70S6K1 siRNA knockdown abolished high glucose-induced IRE1 α , PERK and eIF2 α phosphorylation (Fig. 4). Additionally, p70S6K1 siRNA knockdown prevented the increase of CHOP expression (Fig. 4).

***Rps6kb1* gene knockout blocks maternal diabetes-induced apoptosis**

Maternal diabetes induces the neuroepithelium apoptosis through caspase-dependent signaling pathway^{29, 64}. We used the TUNEL assay to detect apoptotic cells in the developing neuroepithelium. We found the apoptotic cell number in the neuroepithelia of WT embryos from diabetic dams was significantly higher than that in WT embryos from nondiabetic dams (Fig. 5A). Under diabetic conditions, the apoptotic cell numbers in neuroepithelia of *Rps6kb1* gene knockout embryos were significantly lower compared to those in the neuroepithelia of the WT embryos (Fig. 5A). Thus, *Rps6kb1* gene knockout prevented maternal diabetes induced-excessive cell apoptosis. To determine whether maternal diabetes induces caspase activation in the neuroepithelium, cleaved caspase 3 and caspase 8 were assessed. Maternal diabetes increased the abundance of both cleaved caspase 3 and caspase 8 in WT embryos, whereas *Rps6kb1* gene knockout blocked maternal diabetes-induced caspase cleavage (Fig.5B). These results suggest that *Rps6kb1* gene knockout inhibits maternal diabetes-induced apoptosis in the developing neuroepithelium.

The superoxide dismutase (SOD) memetic Tempol inhibits high glucose-induced p70S6K1 activation

We sought to determine whether mitigating high glucose-induced oxidative stress using the antioxidant enzyme SOD memetic Tempol would block the activation (phosphorylation) of p70S6K1 and its downstream kinase S6. Indeed, we found that levels of phosphorylated-(p-) p70S6K1 and S6 were increased by high glucose, whereas total protein expression of p70S6K1 and S6 was not affected by high glucose (Fig. 6). The ratios of p-p70S6K1/p70S6K1 and p-S6/S6 were significantly increased by high glucose (Fig. 6). Temple treatment blocked high glucose-induced phosphorylation of p70S6K1 and S6 (Fig. 6). These findings demonstrate that oxidative stress is responsible for the activation of p70S6K1 and

its major downstream effector S6 induced by high glucose, and further contribute to the oxidative stress hypothesis in the induction of diabetic embryopathy.

Comment

Autophagy maintains cellular homeostasis and promotes cell survival during embryonic development²³. During mouse development, two peaks of massive autophagy occur at the time of fertilization and in the early neonatal period²³. At the tissue level, autophagy participates in cell differentiation and tissue patterning. Autophagy in neuroepithelial cells is suppressed by maternal diabetes in pregnancy and may be a primary cause for diabetes-induced NTDs^{19, 20}.

In the present study, we elucidated a mechanism by which maternal diabetes suppresses autophagy in neuroepithelial cells leading to NTD formation. Our evidence *in vivo* and *in vitro* supports the hypothesis that p70S6K1 is a negative regulator of autophagy and mediates the inhibitory effect of maternal diabetes on autophagy.

p70S6K1 regulates important biological processes, including protein synthesis, maintenance of cellular energy states, cell growth, proliferation and survival⁶⁵. It has been demonstrated that p70S6K1 activates anabolic pathways and inhibits catabolic processes⁶⁵. The role of p70S6K1 in autophagy regulation has been controversial. p70S6K1 negatively regulates autophagy in rat hepatocytes⁶⁶. Other data have indicated that p70S6K1 is necessary for autophagy in the fat body of *Drosophila melanogaster*^{67,68}. p70S6K1 is a downstream effector of mTOR signaling, which inhibits autophagy. There is no report in pre-gestational diabetes-induced p70S6K1 activation. However, higher basal phosphorylation of p70S6K1 was observed in gestational diabetes versus control subjects, which is associated with impaired insulin receptor signaling⁶⁹. In the current study, we observed that p70S6K1 knockdown by specific siRNA increased the abundance of LC3-II and restored autophagosome formation suppressed by high glucose. Results obtained from our *in vivo* studies showed that removing p70S6K1 restores LC3-II expression and autophagosome formation. Furthermore, deleting the *Rps6kb1* gene restored autophagy in the developing neuroepithelium and blocked cellular stress. Not surprisingly, removing p70S6K1 ultimately abrogated maternal diabetes-induced neuroepithelial cell apoptosis and consequent NTD formation.

ER stress and mitochondrial dysfunction are causally involved in diabetic teratogenesis^{20, 57}. Embryos exposed to diabetes exhibit impaired autophagy^{19, 20}. It is well established that ER stress stimulates autophagy⁷⁰. However, in diabetic embryopathy, ER stress does not enhance autophagy; in fact, autophagy is diminished in neuroepithelia exposed to maternal diabetes^{20, 26, 27}. The signal transduction mechanism underlying enhanced ER stress and diminished autophagy in diabetic embryopathy is distinct to other systems. p70S6K1 activation-induced protein overproduction may override the ER capacity of posttranslational processing resulting in ER stress. ER stress negatively affects mitochondrial function⁵⁰. Suggesting that p70S6K1 may play a role in the induction of mitochondrial dysfunction. The present study demonstrated that p70S6K1 was required for autophagy impairment in diabetic embryopathy and removing p70S6K1 restored autophagy leading to suppression of

cellular stress induced by maternal diabetes. Thus, p70S6K1 is a key autophagy regulator and cellular stress enhancer in the pathogenesis of diabetic embryopathy.

Apoptosis is directly involved in the induction of diabetic embryopathy^{41, 71}. We have consistently shown that maternal diabetes-induced neuroepithelial cell apoptosis is caspase 8-dependent^{20, 41}. It has been reported that ER stress induces caspase 8 activation in other systems⁷². The *Rps6kb1* gene knockout relieves ER stress and reduces maternal diabetes-induced apoptosis.

The C17.2 cell line may not truly reflect the cell biology of the embryonic neuroepithelium. This cell line was derived from neonatal cerebellum, which is developmentally more differentiated than neuroepithelium of the neurulation-stage embryo⁷³. Because using primary neuroepithelial cells is impossible due to the very small size of the neuroepithelium, cell lines are the choices for some experiments that are essentially being done *in vitro*. Our previous studies have shown that high glucose mimics the *in vivo* condition of maternal diabetes to trigger cellular stress, suppress autophagy and induce apoptosis in C17.2 cells^{20,27, 57}. Therefore, the C17.2 cell line is suitable for the current study.

In summary, our study reveals a mechanism underlying maternal diabetes-suppressed autophagy in the neuroepithelium leading to NTD formation. We demonstrated that p70S6K1 negatively regulates autophagy. Future studies may aim to reveal the importance of autophagy in other morphogenetic processes that are affected by maternal diabetes.

Clinical Significance

Our studies show that impaired autophagy is critically involved in the pathogenesis of maternal diabetes-induced NTDs. Clinical research has demonstrated that defective autophagy is involved in the pathogenesis of various pregnancy-related diseases. For example, altered autophagy is associated with preterm delivery, intrauterine growth restriction, stillbirth, low birthweight, aberrant placental metabolism and syncytial knot formation^{74–83}. Intrauterine inflammation suppresses autophagy in the fetal brain⁷⁴. Autophagy is an important contributor to the pathogenesis of preterm birth because altered autophagy is observed in preterm birth caused by fetoscopic laser surgery in twin-twin transfusion syndrome⁸⁴. Autophagy is inhibited in vaginal epithelial cells infected by Group B streptococcus⁷⁶. It has been demonstrated that stillbirth and late-term placentas contain larger and arrested autophagosomes than those in 37 to 39-week placentas, indicating inhibition of the autophagic process in these placentas⁸¹. Oxidative stress^{85–88} and pro-apoptotic kinases that negatively regulate autophagy in diabetic pregnancy are critical etiological factors in the pathogenesis of many maternal-fetal complications^{20, 41, 47, 48}. It is still unclear whether autophagy impairment is a significant contributor or just a secondary effect associated with adverse pregnancy outcomes in various maternal conditions. Therefore, further work may determine the potential involvement of autophagy impairment in the induction of specific pregnancy complications.

Research Implications

Autophagy sustains cellular homeostasis and is essential for cell survival during embryonic development²³. Thus, determining the cause of autophagy dysregulation is an important area

in maternal-fetal complications. Our study sets an example in this research area by demonstrating that autophagy in neuroepithelial cells of the developing embryo is suppressed by maternal diabetes leading to diabetes-induced NTDs^{19, 20}.

p70S6K1 regulates many important biological processes, including protein synthesis, maintenance of cellular energy states, cell growth, proliferation and survival⁶⁵. There is no report in pre-gestational diabetes-induced p70S6K1 activation. Thus, the present study provides the novel role of p70S6K1 in the etiology of diabetic embryopathy through repressing autophagy.

ER stress and mitochondrial dysfunction are causally involved in diabetic teratogenesis^{20, 57}. Embryos exposed to diabetes exhibit impaired autophagy, ER stress and mitochondrial dysfunction^{19, 20}. The relationship among these three cellular processes in diabetic embryopathy is unclear. It is well established in other system that ER stress stimulates autophagy. However, autophagy is diminished in neuroepithelia exposed to maternal diabetes^{20, 26, 27}. The signal transduction mechanism underlying diabetes-enhanced ER stress and diminished autophagy in diabetic embryopathy is distinct to other system. p70S6K1 activation-induced protein overproduction may override the ER capacity of posttranslational processing leading to ER stress. ER stress negatively affects mitochondrial function⁵⁰, suggesting that p70S6K1 may play a role in the induction of mitochondrial dysfunction. The present study demonstrated that p70S6K1 was required for autophagy impairment in diabetic embryopathy and removing p70S6K1 restored autophagy leading to suppression of ER stress and restoration of mitochondrial function. Thus, p70S6K1 is a key autophagy regulator and cellular stress enhancer in the pathogenesis of diabetic embryopathy and warrants further study.

Apoptosis is directly involved in the induction of diabetic embryopathy^{41, 71}. Apoptosis and autophagy are two inter-related cellular processes that regulate cell fate. Studies have suggested that their regulation is intimately connected and the same regulators can sometimes control both autophagy and apoptosis^{60, 61}.

We elucidated a mechanism by which deleting the gene encoding p70S6K1 alleviates diabetic embryopathy by resorting autophagy and inhibiting apoptosis, suggesting that autophagy is essential for neuroepithelial cell viability. Future studies may aim to reveal the importance of autophagy in cell survival processes that are affected by maternal diabetes.

Strengths and Weaknesses

Strengths of our study are 1) the use of the *Rps6kb1* gene deletion mouse model in determining the role of p70S6K1 in diabetic embryopathy; 2) the delicate design of the animal studies in revealing the cause of diabetic embryopathy, and 3) the detailed analyses of cellular stress processes including impaired autophagy, ER stress and mitochondrial dysfunction. The findings in these well-designed molecular studies provide the mechanistic basis for autophagy dysregulation-associated pathogenesis.

One of the weaknesses of our study is that there is no immediate clinical impact. Our study is not a human study and animal experiments in the present study may not faithfully reflect

the complex human conditions. However, the underlying etiology of maternal diabetes-induced neural tube defects in the fetus is still elusive. Additionally, diabetic embryopathy is a significant health problem affecting both the mother and her newborn. The current study has potential translational value and provides a mechanistic basis for future clinical research.

Conclusions

Our findings show that p70S6k1 mediates the inhibitory effect of maternal diabetes on autophagy in the developing neuroepithelium. p70S6K1 decreases autophagy and induces NTD formation in diabetic embryopathy. Furthermore, p70S6K1 deficiency relieves ER stress and inhibits maternal diabetes-induced apoptosis. Our study reveals a mechanism underlying maternal diabetes-suppressed autophagy in the neuroepithelium leading to NTD formation.

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Glossary of Terms

Diabetic embryopathy

Pregestational maternal diabetes-induced birth defects are collectively termed as diabetic embryopathy

Neural tube defects (NTDs)

A severe structural birth defects with an open neural tube resulting from the failure of neurulation, a process involving the formation of the primitive brain and spinal cord during embryonic development

Autophagy

Autophagy (or macroautophagy) is the natural, regulated, and destructive mechanism of the cell that disassembles unnecessary or dysfunctional components. Autophagy allows the orderly degradation and recycling of cellular material. In macroautophagy, targeted cytoplasmic constituents are isolated from the rest of the cell within a double-membrane vesicle known as an autophagosome. The autophagosome eventually fuses with lysosomes, resulting in the degradation and recycling of its contents. Three forms of autophagy are commonly described: macroautophagy, microautophagy, and chaperone-mediated autophagy. In disease, autophagy has been viewed as an adaptive response to stress to promote 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 energy levels

p70S6K1

(Ribosomal protein S6 kinase beta-1) 70 kDa ribosomal protein S6 kinase 1 is encoded by the *Rps6kb1* gene in humans. It is a serine/threonine kinase that acts downstream of mTOR, phosphatidylinositol (3,4,5)-trisphosphate and phosphoinositide-dependent kinase-1 in the phosphatidylinositol 3-kinase pathway. p70S6K1 phosphorylates the S6 ribosomal protein, which induces protein synthesis

LC3

LC3 (Microtubule-associated protein 1 light chain 3) is also called Atg8, which is a homologue of Apg8p in yeast. The conversion of cytosolic LC3-I (18 kDa) to autophagosome membrane-bound LC3-II (16 kDa) is a critical event in autophagy

C17.2

C17.2 is a transformed cell line of NSCs (neural stem cells) isolated from the external germinal layer of neonatal mouse cerebellum. It has potential to differentiate into a variety of cell types, such as neurons, oligodendrocytes, and astrocytes, when transplanted into the appropriate part of the central nervous system *in vivo* or induced by neural factors *in vitro*. Therefore, it is an ideal cell model for assessing the *in vitro* effect of high glucose

Endoplasmic reticulum (ER) stress

The endoplasmic reticulum (ER) is the cellular organelle that is critical for protein folding and secretion, calcium homeostasis, and lipid biosynthesis. Under various conditions, called ER stress, protein folding in the ER is impaired leading to the accumulation of misfolded proteins. Multiple cellular disturbances can cause ER stress including disturbances in redox regulation, calcium regulation, glucose deprivation, and viral infection. The ER stress response is initiated when the capacity of ER-resident chaperone proteins is exceeded by the load of misfolded proteins. Prolonged ER stress typically results in cell death by apoptosis

mTOR

The mammalian target of rapamycin, is a kinase that is encoded by the *Mtor* gene. mTOR belongs to the phosphatidylinositol 3-kinase-related kinase family of protein kinases. mTOR regulates an array of cellular function including cell growth, cell proliferation, cell motility, cell survival, protein synthesis, autophagy, and transcription. mTOR also promotes the activation of insulin receptors and insulin-like growth factor 1 receptors. The mTOR pathway, a central pathway that regulates metabolism and physiology, play important roles in the function of tissues including liver, muscle, white and brown adipose tissue, and the brain, and its dysregulation is manifested in human diseases, such as diabetes, obesity, depression, and certain cancers. p70S6K1 is a downstream effector of mTOR because mTOR activation leads to p70S6K1 activation (phosphorylation)

Autophagosome

is a spherical structure with double layer membranes. It is the key structure in macroautophagy, the intracellular degradation system for cytoplasmic contents (e.g., abnormal intracellular proteins, excess or damaged organelles, invading microorganisms). After formation, autophagosomes deliver cytoplasmic components to the lysosomes. The outer membrane of an autophagosome fuses with a lysosome to form an autolysosome. The

lysosome's hydrolases degrade the autophagosome-delivered contents and its inner membrane

siRNA

Small interfering RNA, sometimes known as short interfering RNA or silencing RNA, is a class of double-stranded RNA non-coding RNA molecules, 20–25 base pairs in length, similar to miRNA, and operating within the RNA interference (RNAi) pathway. It interferes with the expression of specific genes with complementary nucleotide sequences by degrading mRNA after transcription, preventing translation

UPR

The unfolded protein response is a cellular stress response related to the endoplasmic reticulum (ER) stress. The UPR is activated in response to an accumulation of unfolded or misfolded proteins in the lumen of the endoplasmic reticulum. In this scenario, the UPR has three aims: initially to restore normal function of the cell by halting protein translation, degrading misfolded proteins, and activating the signaling pathways that lead to increasing the production of molecular chaperones involved in protein folding. If these objectives are not achieved within a certain time span or the disruption is prolonged, the UPR aims towards apoptosis

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Condensation

Deficiency of the oxidative stress-responsive kinase p70S6K1 ameliorates diabetic embryopathy via restoring autophagy.

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AJOG at a Glance

Why was this study conducted?

- Our previous studies have demonstrated that autophagy in the developing neuroepithelium is suppressed in diabetic embryopathy. Maternal diabetes disrupts neurulation leading to neural tube defects (NTDs). However, it is unclear how maternal diabetes inhibits autophagy leading to NTD formation. Because oxidative stress is critically involved in the induction of diabetic embryopathy, we aim to determine the role of an oxidative stress-responsive kinase, p70S6K1 (70 kDa ribosomal protein S6 kinase 1), in maternal diabetes-suppressed autophagy.

Key Findings

- Deleting the *Rps6kb1* gene encoding for the oxidative stress-responsive kinase p70S6K1 (70 kDa ribosomal protein S6 kinase 1) reverses maternal diabetes-inhibited autophagy.
- Autophagy rescued by the deletion of the *Rps6kb1* gene blocks endoplasmic reticulum stress and neuroepithelial cell apoptosis leading to amelioration of diabetic embryopathy.
- An antioxidant blocks high glucose-induced p70S6K1 activation.

What does this add to what is known?

- The findings reveal the critical involvement of p70S6K1 in the pathogenesis of maternal diabetes-induced NTDs and demonstrate that p70S6K1 is responsible for maternal diabetes-repressed autophagy in neuroepithelial cells of the developing embryo.

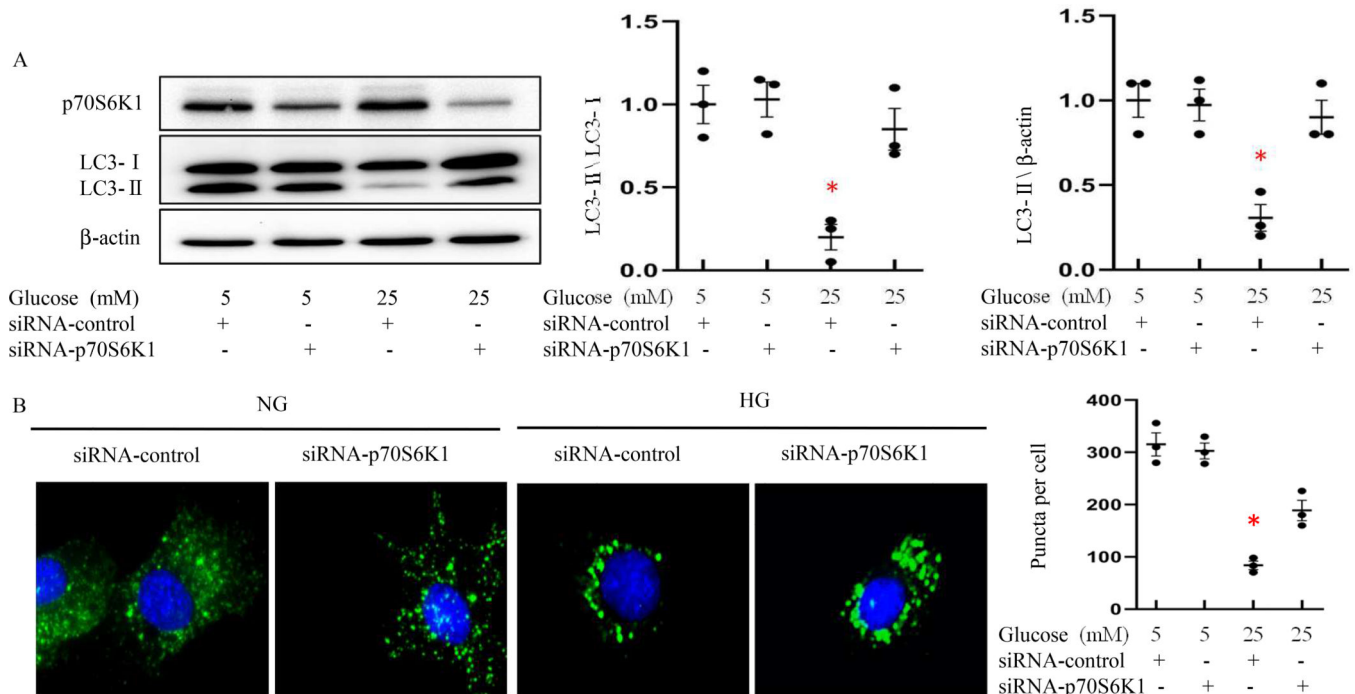
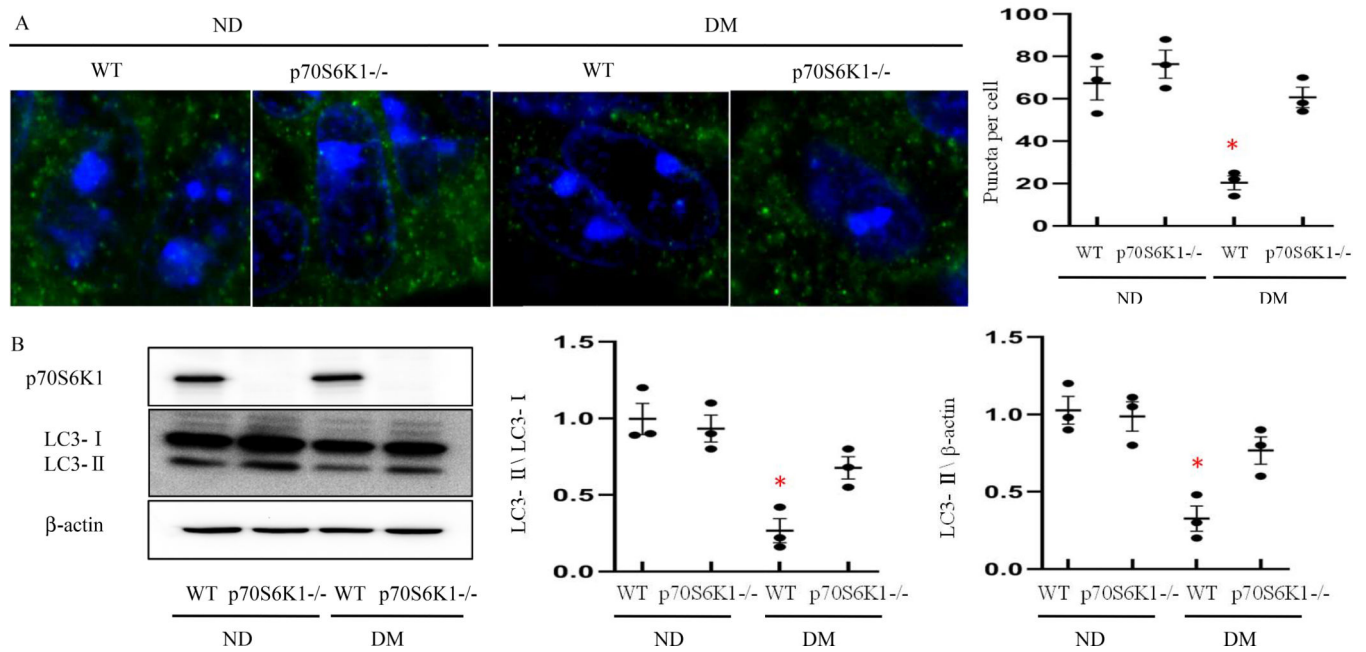


Figure 1. p70S6K1 knockdown reverts high glucose-suppressed autophagy.

A: Quantification of relative protein levels of p70S6K1 and LC3-II (Microtubule-associated protein 1 light chain 3 II, the lipidation form of LC3-I, is an index of autophagy activation) versus LC3-I in normal glucose (NG; 5 mM glucose) and high glucose (HG; 25 mM glucose) culture conditions. **B:** Representative Cyto-ID staining (green) images and quantification of autophagosomes (green puncta) in C17.2 neural stem cells in normal glucose and high glucose culture conditions. C17.2 cells were transfected with p70S6K1 siRNA. Scramble siRNA (the un-targeting siRNA) was used as a control. In B, cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI), bars = 15 μ m.

Experiments were repeated three times ($n=3$); * indicates significant difference compared to the other groups ($P < 0.05$).



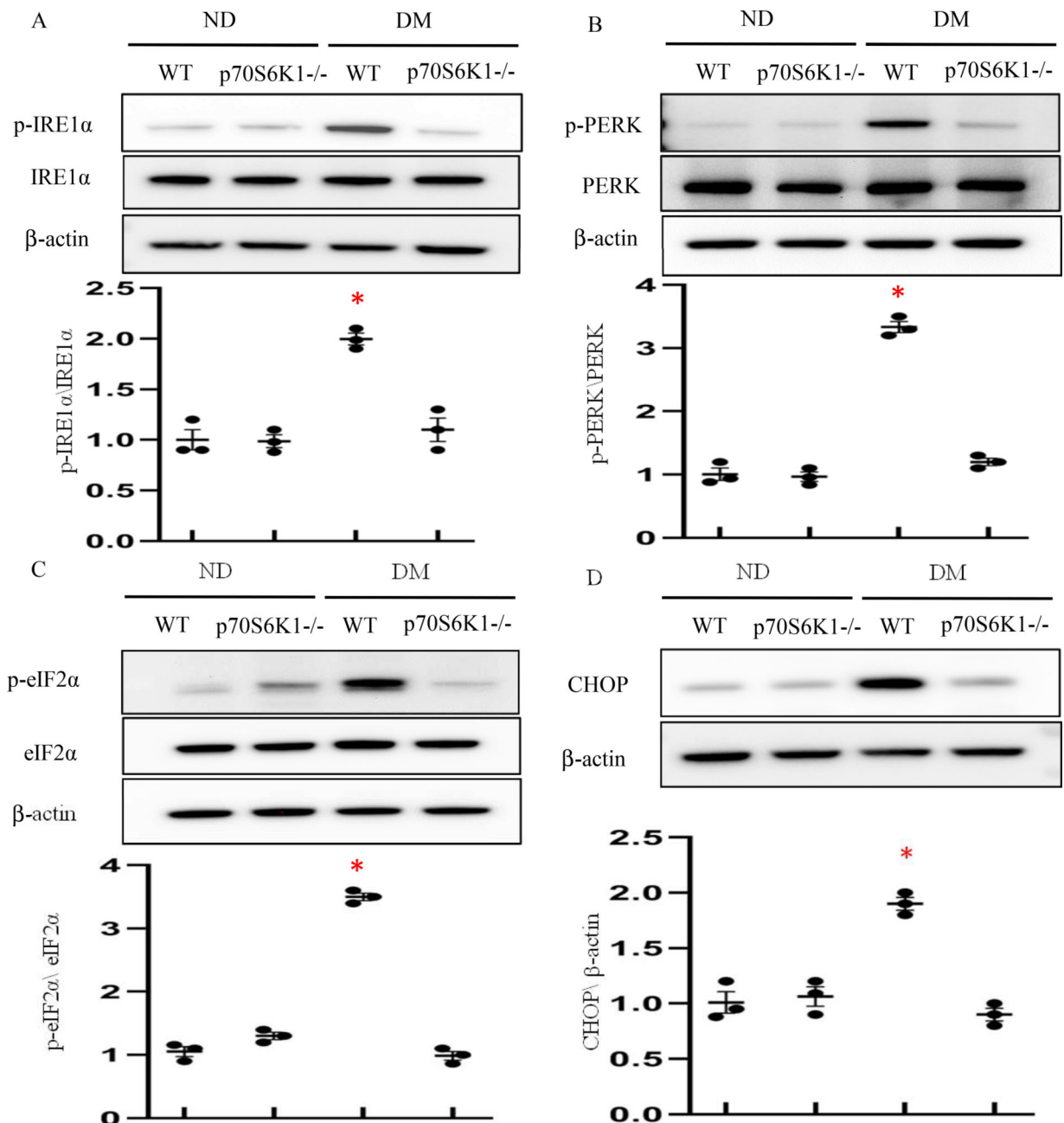


Figure 3. *Rps6kb1* gene knockout blocks the unfolded protein response (UPR) activation and ER stress.

(A-D) Protein levels of ER stress in E8.5 Wild-Type (WT) and *Rps6kb1* gene deleted embryos from nondiabetic or diabetic dams. Quantification of relative expression levels of phosphorylation of IRE1α, PERK and eIF2α versus total IRE1α, PERK and eIF2α and CHOP were shown in the graphs. Experiments were performed using three embryos from three different dams per group (n=3); * indicates significant difference compared to the other groups ($P < 0.05$). ND: Nondiabetic; DM: Diabetes Mellitus.

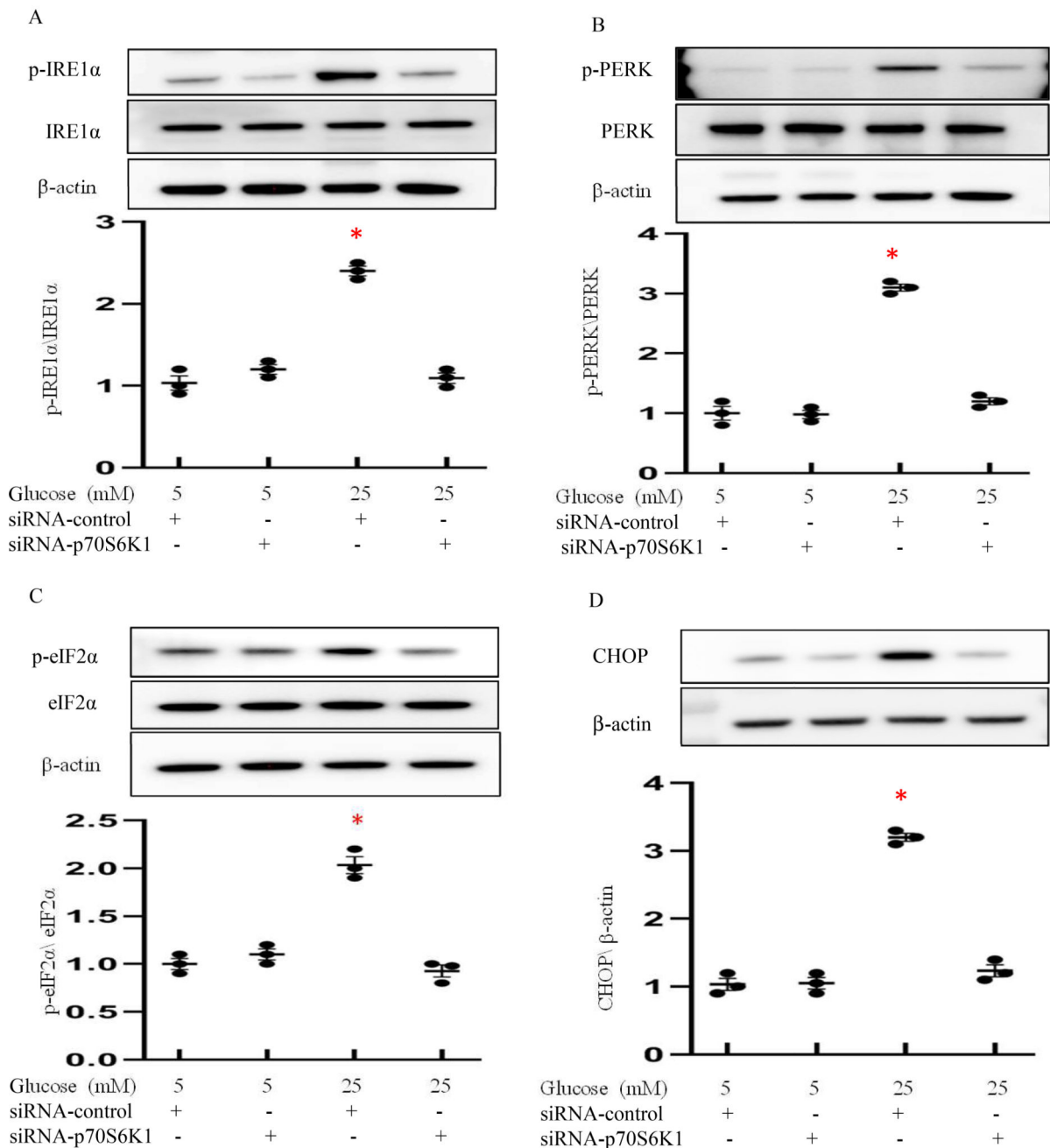


Figure 4. p70S6K1 Knockdown inhibits UPR activation and ER stress.

(A-D) Protein levels of ER stress in normal glucose (NG; 5mM glucose) and high glucose (HG; 25mM glucose) culture conditions. Quantification of relative expression levels of phosphorylation of IRE1 α , PERK and eIF2 α versus total IRE1 α , PERK and eIF2 α , and CHOP were shown in the graphs. Experiments were repeated three times (n=3); * indicates significant difference compared to the other groups ($P < 0.05$).

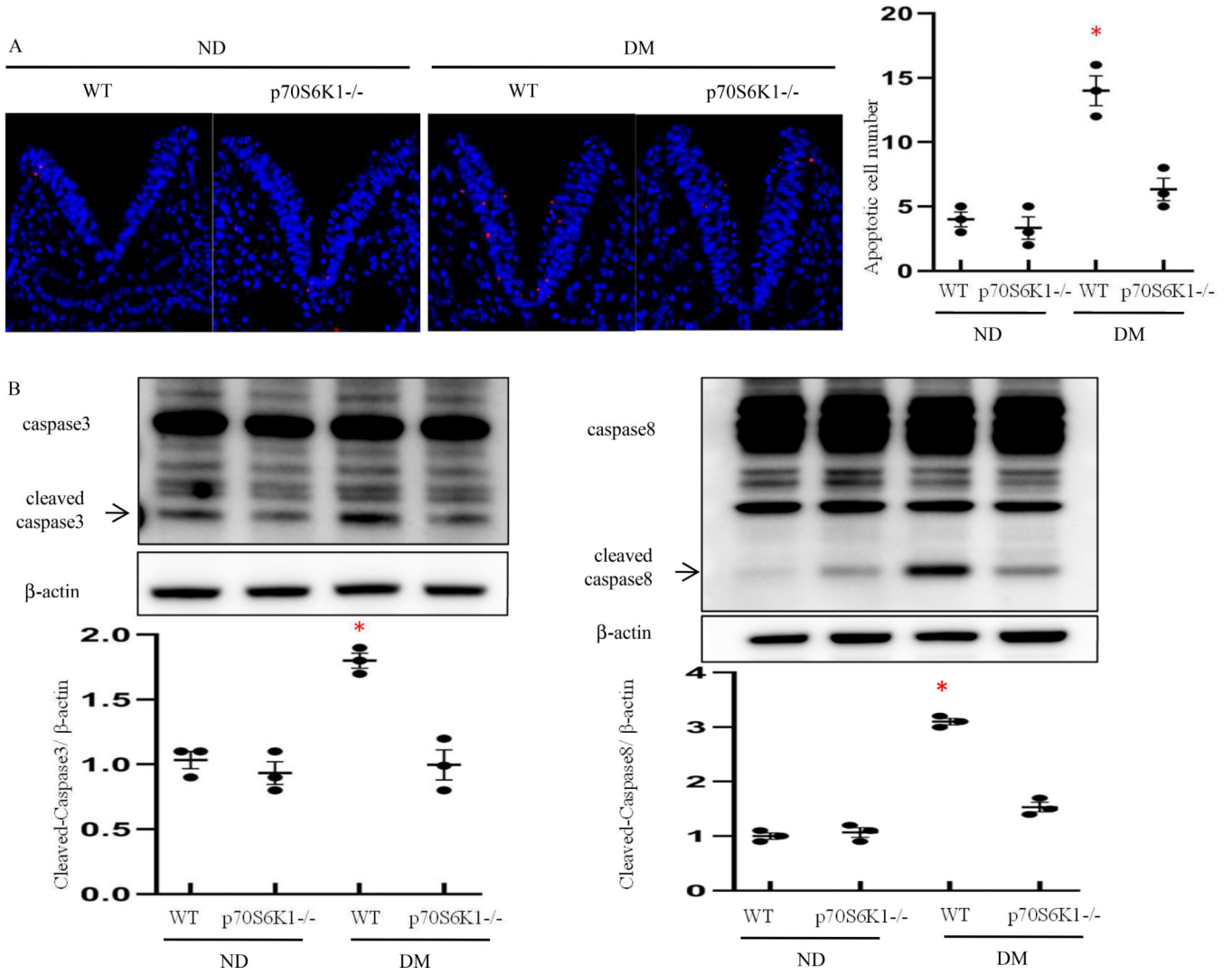


Figure 5. *Rps6kb1* gene knockout reduces maternal diabetes-induced apoptosis in the developing neuroepithelium.

(A) Representative TUNEL assay images showing apoptotic cells (red dots) in the E8.5 neuroepithelium. Cell nuclei were stained with DAPI (blue), bars = 30 μ m. Quantification of TUNEL positive cells per section. Experiments were performed using three embryos from three different dams per group (n=3). (B) Protein levels of cleaved caspase8 and caspase3 in E8.5 embryos. Experiments were repeated three times (n=3); * indicates significant difference compared to the other groups ($P < 0.05$). ND: Nondiabetic; DM: Diabetes Mellitus; WT: Wild-Type.

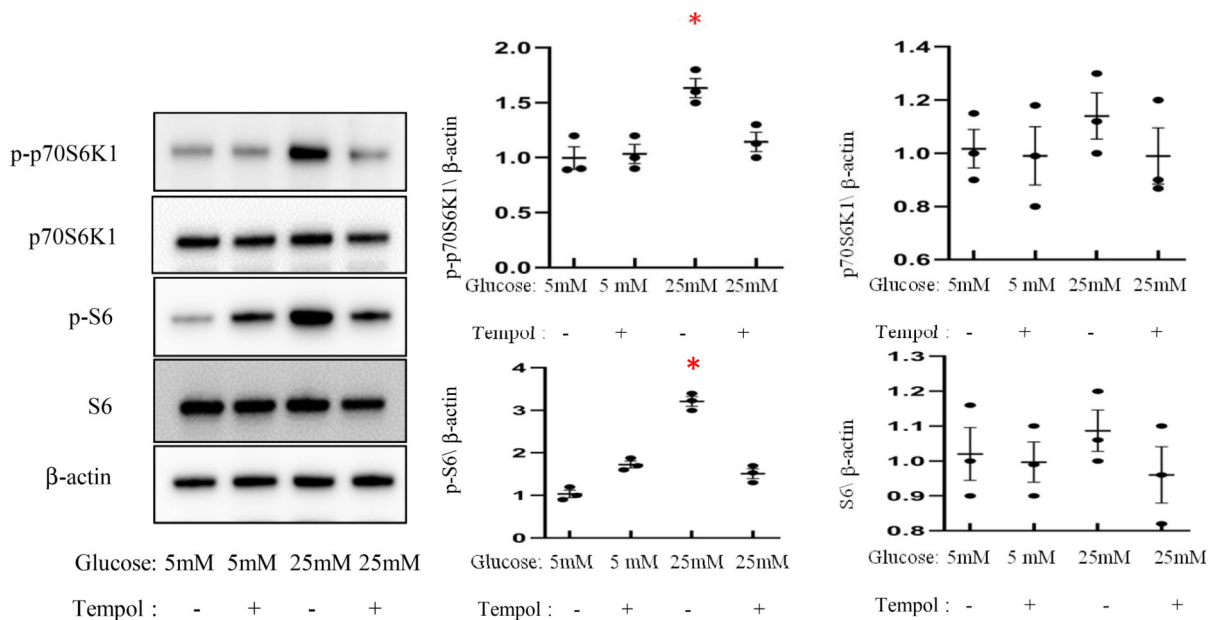


Figure 6. Tempol inhibits high glucose-induced p70S6K1 activation.

Protein levels and quantification data of phosphorylation of p70S6K1, S6 and total p70S6K1 and S6 in normal glucose (NG; 5mM glucose) with Tempol and high glucose (HG; 25mM glucose) with Tempol culture conditions. Experiments were repeated three times (n=3); * indicates significant difference compared to the other groups ($P < 0.05$).

Table1.

p70S6K1 deficiency ameliorates maternal diabetes-induced neural tube defects.

Experimental group	Glucose level (mg/dl)	Genotype	embryos	NTD Embryos (NTDs rate %)
ND p70S6K1 ^{+/-} male × p70S6K1 ^{+/-} female (11 litters)	144.8±12.6	WT	20	0(0.0%)
		p70S6K1 ^{+/-}	39	0(0.0%)
		p70S6K1 ^{-/-}	15	0(0.0%)
DM p70S6K1 ^{+/-} male × p70S6K1 ^{+/-} female (15 litters)	381.8±12.5	WT	31	10(32.3%)
		p70S6K1 ^{+/-}	53	10(18.9%)
		p70S6K1 ^{-/-}	29	2(6.9%) *

* indicates significant difference compared with the other groups in Fisher Exact Tests ($P < 0.05$)

NTDs: neural tube defects; ND: Non- diabetes Mellitus; DM: Diabetes Mellitus; WT: Wild-Type. 11 litters from ND mice and 15 litters from DM mice were used for evaluation of each genotype.

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