**Prothymosin** **α activates type I collagen to develop a fibrotic placenta in gestational diabetes**

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**Clinical Perspectives**

(i) Why the study was undertaken

Although it is believed that placental dysfunction, including fibrosis, is related to gestational diabetes (GDM), factors that link these observations remain unknown. Prothymosin  (ProT) is expressed in the placenta and plays an important role in insulin resistance and fibrosis. However, the role of ProT in GDM is still unclear.

(ii) Summary of the results

Placental ProT expression and circulating ProT concentrations were significantly elevated in GDM patients. Hyperglycemia and AGEs might contribute to placental inflammation and the increased ProT expression. Increased ProT expression further induces Col-1 expression and contributes to placental fibrosis through an NF-κB-dependent pathway.

(iii) The potential significance of the results to human health and disease.

Although diagnosis of GDM is important in clinic, current diagnosis of GDM by oral glucose tolerance test is inconvenient. In the present study, our results revealed that ProT not only displayed a pathophysiological relevance to GDM, but also had a potential for the diagnosis of GDM.

**Abstract**

High-risk pregnancies, such as pregnancies with gestational diabetes (GDM), are becoming more common and as such, have become important public health issues worldwide. GDM increases the risks of macrosomia, premature infants, and preeclampsia. Although placental dysfunction, including fibrosis is associated with the development of GDM, factors that link these observations remain unknown. Prothymosin α (ProT) is expressed in the placenta and is involved in cell proliferation and immunomodulation. It also plays an important role in insulin resistance and fibrosis. However, the role of ProT in GDM is still unclear. In this study, we found that fibrosis-related protein expressions, such as type I collagen (Col-1) were significantly increased in the placentae of ProT transgenic mice. With elevated fibrosis-related protein expressions, placental weights significantly increased in GDM group. In addition, placental and circulating ProT levels were significantly higher in patients with GDM (*n*=39), compared with the healthy group (*n*=102), and were positively correlated with Col-1 expression. Mice with streptozotocin-induced GDM had increased ProT, fasting blood glucose, Col-1, and placental weight, whereas plasma insulin levels were decreased. ProT overexpression enhanced nuclear factor κB (NFκB) activation to increase fibrosis-related protein expressions in 3A-Sub-E trophoblasts, whilst treatment with an NF-κB inhibitor reversed the effect of ProT on fibrosis-related protein expressions. We further investigated whether ProT is regulated by hyperglycemia-induced reactive oxygen species. In conclusion, ProT increases the amount of placental connective tissue and thus contributes to the pathogenesis of placental fibrosis in GDM. Therefore, ProT may be a novel therapeutic target for GDM.

**Introduction**

Gestational diabetes mellitus (GDM) refers to the phenomenon of hyperglycemia first detected during pregnancy. GDM is becoming more common due to a greater number of women conceiving later in life and the obesity epidemic [1]. The prevalence of GDM varies among populations, ranging from 1.7% to 11.6% [2]. Numerous studies have established that GDM is associated with significantly higher risks of maternal and fetal complications, such as type 2 diabetes and cardiovascular disease [[3](#_ENREF_3),[4](#_ENREF_4)]. The current screening strategy for GDM of offering oral glucose tolerance test (OGTT) to high-risk patients is inadequate due to the inconvenience of OGTT. Therefore, biomarkers for predicting GDM are attractive approaches that require further investigation.

The pathogenic mechanisms of GDM remain elusive. The placenta acts as a natural selective barrier between the maternal and fetal circulatory systems. It is believed to be the main source of hormone and cell regulatory factors including placental growth factor, estrogen, glucocorticoids, and progesterone [5]. These hormones and regulatory factors antagonize the effects of insulin, leading to glucose metabolism dysfunction, low insulin sensitivity, and high blood glucose levels [6]. In addition, hyperglycemia creates hypoxia which causes placental damage and induces the production of proinflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) [7,8]. Moreover, histological changes, including marked hyperplasia of cytotrophoblasts, villous immaturity, villous fibrinoid necrosis, chorangiosis, and increased angiogenesis were observed in placentae from patients with poorly controlled GDM [9]. GDM placentae are enlarged, thick, and plethoric with an increased placental to fetal weight ratio [[10,11](#_ENREF_5)]. Although histological changes in GDM placenta are well documented and are related to the development of GDM, the underlying pathophysiology of GDM remains obscure [[9](#_ENREF_8)].

Hyperglycemia induces mitochondria dysfunction and increases reactive oxygen species (ROS) production, and increased ROS level is associated with GDM [12,13]. Prior research has shown that placentae from women with GDM exhibit oxidative stress [14]. Furthermore, hyperglycemia-induced GDM placental changes, including increased MMP-9 expression, have been shown to be mediated by ROS [15]. Another study also demonstrated that placental MMP-9 activity is modulated by ROS which may result in the placental anomalies observed in diabetic pregnancies [16]. Although increased ROS level induces fibrosis, and fibrosis is observed in placentae from diabetic pregnancies [[1](#_ENREF_14)7], the factors that link these observations in GDM have yet to be elucidated.

Prothymosin α (ProT) is an acidic protein that plays roles in regulating cell fates, oxidative stress, and immunomodulation [[18,19](#_ENREF_15)]. ProT increases acetylation of histones and NFκB, and contributes to emphysema, a chronic disease characterized by inflammation and oxidative stress [20]. In addition, increased ROS might activate the apoptotic pathway, which further leads to the release of ProT into the circulation, and further induces insulin resistance through the toll-like receptor 4 (TLR4) pathway [21]. Although ProT expression can be detected in the placenta [22], the role of ProT in GDM is still unknown.

In this study, we compared the expression of ProT in both the placenta and blood in women with and without GDM. Furthermore, we investigated the role of ProT in the development of GDM.

**Material and Methods**

* 1. ***Human study***

This study was reviewed and approved by the Institutional Review Board of National Cheng Kung University Hospital (B-ER-106-416). The research has been carried out in accordance with the World Medical Association Declaration of Helsinki, and that all subjects provided written informed consent for their samples and data to be used. In total, 141 subjects (*n*=39 subjects with GDM and *n*=102 without GDM) were recruited for this study. All patients who attended the outpatient department of National Cheng Kung University Hospital for prenatal examination were screened. After an 8-h fast, study participants who were 24~28 weeks pregnant received OGTTs. Fasting, one hour-, and two hours- blood samples after loading of 75-g glucose were collected to measure blood glucose concentrations. GDM was diagnosed if one or more of the following criteria were met: fasting plasma glucose (FPG) of 5.1~6.9 mmol/l (92~125 mg/dl); one hour- plasma glucose of ≥10.0 mmol/l (180 mg/dl) following a 75-g oral glucose load; or two hour- plasma glucose of 8.5~11.0 mmol/l (153~199 mg/dl) following a 75-g oral glucose load. The body-mass index (BMI) (in kg/m2) was calculated as the weight (in kilograms) divided by the height (in meters) squared. For blood pressure measurements, subjects were asked to rest in a supine position in a quiet location, and measurements were obtained in a fasting state between 08:00 and 10:00 AM. Serum ProT concentrations were measured as previously described [[21](#_ENREF_18)]. We excluded women with preeclampsia, eclampsia, pregnancy-induced hypertension, preexisting hypertension or alcohol misuse prior to pregnancy. After delivery, the placentae were collected, weighed, and then fixed in 10% formalin overnight, dehydrated, and embedded in paraffin.

* 1. ***Animals***

All animal experiments were carried out at National Cheng Kung University in Taiwan, and approved by the Institutional Animal Care and Use Committee (IACUC no: 104101) of National Cheng Kung University. Ten to 12-week-old pregnant mice were purchased from the animal center of National Cheng Kung University. ProT transgenic mice with a friend virus B-type (FVB) background were backcrossed with mice with a C57BL/6J genetic background for ten generations [21]. After euthanasia by injection of pentobarbital (Sigma-Aldrich), the placental tissues of mice were collected and weighed on days 9, 11, 13, 15, 17, and 19 after the mice had become pregnant. Placental tissues were then fixed in 10% formalin overnight, dehydrated, and embedded in paraffin.

* 1. ***GDM animal model***

The onset of pregnancy (gestational day 0) was defined by mating and detection of a copulation plug. Pregnant mice were then intraperitoneally injected with 80 mg/kg nicotinamide (Sigma-Aldrich, St. Louis, MO, USA), and 50 mg/kg streptozotocin (STZ; Sigma-Aldrich) for five consecutive days after 4-h starvation. On day 14, blood samples were collected, and glucose levels were determined using a commercial assay kit (Biosystems, Barcelona, Spain).

* 1. ***Cell culture***

The human 3A-Sub-E placental trophoblast cell line was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY, USA) containing heat-inactivated 10% fetal bovine serum (Gibco) at 5% CO2 and 37°C.

* 1. ***Reactive Oxygen Species (ROS) Detection***

Intracellular ROS were determined using 2'7'-dichlorofluorescin diacetate (DCFDA) (Thermo Fisher, Vantaa, Finland). 3A-sub-E (4×105/well) were grown in a 6-well plate and cultured at 37℃ and 5% CO2 until confluency. Cells were maintained in DMEM with high glucose (25 mM glucose) or low glucose (5.5 mM glucose). After 48 hours incubation, 25 M DCFDA were added into medium and incubated for 30 minutes. Cells were rinsed with serum free medium two times and analyzed using a fluorescence microscope (Olympus, Tokyo, Japan) with a 10X objective lens.

* 1. ***Ribonucleic acid (RNA) extraction***

The RNA of samples was extracted with 1.0 ml TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) at room temperature for 5 min until the cells had completely dissolved and then well mixed with 0.2 ml chloroform (Merck, Kenilworth, NJ, USA). After centrifugation (12,000 rpm for 10 min at 4 °C), the supernatant was removed, and then 0.2 ml 100% isopropanol (Merck) was added at room temperature for sedimentation of RNA. Absolute ethanol (Merck) was used to wash the RNA. The supernatant was removed, and RNA was air-dried and then reconstituted with DEPC-H2O.

* 1. ***Quantitative real-time polymerase chain reaction (qPCR)***

Five micrograms of complementary (c)DNA was added to 10 mM of a forward primer and 10 mM of a reverse primer, and then water was added to reach 12.5 µl. A quantitative (q)PCR was performed using a SYBR Green master mix (SYBR Green Premix Ex Taq, Takara, Japan). The reaction was performed for 25 cycles: initial denaturation at 95°C for 150 seconds, denaturation at 95°C for 15 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 7 min. Cepheid Smart Cycler vers. 2.0 software (Thermo Scientific, Rockford, IL, USA) was used to analyze the results.

* 1. ***Western blot analysis***

Proteins were extracted using a RIPA lysis buffer (VWR Chemicals, Sobn, OH, USA). After being centrifuged (12,000 rpm for 10 min at 4 °C), the supernatant was collected. The protein concentration was determined using a bicinchoninic acid protein assay kit (Thermo Scientific); protein lysates (30 µg) were separated using 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto nitrocellulose membranes. The membranes were blocked at room temperature for one hour in PBST (phosphate-buffered saline (PBS) + Tween 20) containing 10% skimmed milk, and probed with 1:1000 primary antibodies at 4°C overnight. Blots were rinsed with PBST and incubated with a 1:5000 dilution of secondary antibodies at room temperature for two hour, and then washed with PBST again. Protein bands were visualized using a chemiluminescence horseradish peroxidase substrate. The relative signal intensity was quantified using the BioSpectrum Imaging System (UVP, Upland, CA, USA).

* 1. ***Immunohistochemistry***

At the end of the experiments, each group of the mice was euthanized, and the placentae were removed. Tissue samples were fixed in 10% formaldehyde at 4°C, and fixed specimens were then dehydrated and embedded in paraffin. Specimens were cut into 5 µm-thick sections at 50 μm intervals and stained with hematoxylin and eosin (Muto Pure Chemicals, Tokyo, Japan) or picro-Sirius red stain (Abcam, Cambridge, UK), and sections were observed under a microscope (100×). In addition, the target proteins in the sections were quantified using TissueFAXS Plus (TissueGnostics, Vienna, Austria), and analyzed by HistoQuest (TissueGnostics).

***1.10. Statistical analysis***

Data were analyzed with two-way repeat-measures analysis of variance (ANOVA) and unpaired Student’s *t*-test. All results are presented as the meanstandard error of the mean (SEM), and *p*<0.05 was considered as significant.

**Results**

***Expressions of fibrosis-related genes and proteins are increased in the placentae of GDM patients***

As shown in Table 1, age- and BMI-matched subjects with (*n*=39) or without (*n*=102) GDM were enrolled in this study. Blood glucose levels, including FPG, and plasma glucose levels at one or two-hour post-loading with oral glucose showed significant differences between GDM and Normal groups, while there were no differences in their blood pressure. Consistent with previous studies and observations, we found that the weight of the placenta after delivery was significantly heavier in GDM patients (*n*=39) compared to normal pregnant women (*n*=102) (Figure 1A). To identify the possible sources that contributed to the increased placental weight, we tested the placental expression of Col-1. Figure 1B shows that the increased protein expression of Col-1 in the placenta of GDM patients by immunohistochemical staining (Figure 1B). In addition, the gene expressions of Col-1 (Figure 1C), transforming growth factor  (TGFβ) (Figure 1D), and  smooth muscle actin (SMA) (Figure 1E) expressions were significantly increased in GDM patients.

***Levels of ProT are increased in GDM patients and positively correlated with Col-1 expression in the placenta***

Because ProT plays an important role in fibrogenesis, we investigated the relationship between ProT and placental fibrosis. As shown in Figure 2A, Col-1 expression was significantly increased in the placentae of ProT transgenic mice compared to wild-type mice. From another perspective, both the protein (Figure 2B) and RNA (Figure 2C) expression levels of ProT, as well as the ProT concentration in the plasma (Figure 2D), were also significantly elevated in GDM patients. We further investigated the relationship between placental fibrosis and ProT expression. Figure 2E shows that ProT expression in the placenta was positively correlated with Col-1 levels implying that ProT may play a role in enriching the placental extracellular matrix.

***Expression of ProT is increased in the placenta of GDM mice***

To elucidate the role of ProT in the increased GDM placenta weight, we first investigated changes in the expression of placental ProT during pregnancy in mice. As shown in Figure 3A and 3B, ProT expression was gradually increased during pregnancy, and reached a peak on day 15 of pregnancy, but then dramatically decreased before delivery. In view of the increment in ProT expression during pregnancy and the fact that ProT plays an important role in insulin resistance, we speculated that the increased placental ProT expression might be related to the development of GDM. We established a GDM mouse model to test this hypothesis further. After the injection of STZ into pregnant mice, decreased plasma insulin levels (Figure 3C) led to hyperglycemia (Figure 3D). In addition, the weight of the placenta significantly increased in pregnant mice with GDM (Figure 3E). Moreover, placental ProT expression was significantly higher compared to the control group at the same stage during pregnancy, indicating that the increased ProT expression may play a crucial role in the enlargement of the placenta in GDM and the development of GDM (Figure 3F). In addition, the expressions of fibrosis-related proteins, such as Col-1, TGFβ andSMA, were significantly increased in placentae at gestational day 19 of pregnant mice (Figure 3G).

***ProT induces fibrosis through an NFκB-dependent pathway***

Following the investigation of the role of ProT in the development of GDM, we then investigated the mechanism of ProT-induced Col-1 expression in trophoblasts. Overexpression of ProT using lentivirus-mediated gene delivery in 3A-sub-E trophoblasts significantly increased the expressions of fibrosis-related proteins, such as Col-1, TGFβ andSMA (Figure 4A), whereas knockdown of ProT expression by different lentiviral vectors containing short hairpin (sh)RNA targeted to ProT significantly decreased the expressions of fibrosis-related proteins (Figure 4B). We further investigated the mechanism of increased ProT expression in GDM in detail. As shown in Figure 5A, we found increased macrophage infiltration in placentae from GDM patients, as determined by CD68 staining. Furthermore, expressions of proinflammatory cytokines in the placenta, such as IL-1β, IL-6, TNFα, and monocyte chemoattractant protein-1 (MCP-1) were significantly higher in GDM patients compared to the normal group (Figure 5B). Given the observed inflammation in the placentae of GDM patients and the fact that ProT activates NFκB to induce insulin resistance, we evaluated the role of NFκB in ProT-induced fibrosis-related protein expressions. Overexpression of ProT in 3A-sub-E trophoblasts increased the activity of NFκB p65 (Figure 5C), and inhibition of NFκB activity by the NFκB inhibitor Bay117082 significantly reversed the effect on fibrosis-related protein expressions by ProT, indicating that ProT induces fibrosis through an NFκB pathway (Figure 5D).

***Hyperglycemia-induced ROS regulates ProT expression***

In this study, we found that high glucose at a concentration of 25 mM significantly induced ROS production in trophoblasts (Figure 6A). We then investigated the effects of hyperglycemia on ProT expression. As shown in Figure 6B, high glucose significantly increased the expression of both ProT and Col-1 in 3A-sub-E trophoblasts. On the other hand, pretreatment with N-acetyl-L-cysteine (NAC) to inhibit ROS reversed methylglyoxal, an advanced glycation end-product (AGE) that induces ProT expression, indicating that ROS plays roles in regulating ProT expression in GDM (Figure 6C).

**Discussion**

To the best of our knowledge, this is the first report to investigate the physiological effects of ProT on the placenta and the pathological role of ProT in the development of GDM. In this study, we found that ProT expression in the placenta and circulating ProT concentrations were both significantly elevated in GDM patients. Furthermore, hyperglycemia and AGEs might contribute to placental inflammation and the increased expression of ProT. Increased ProT expression further induces Col-1 expression and contributes to placental fibrosis through an NFκB-dependent pathway. This effect may be associated with the increased placental mass observed in GDM.

An abnormal secretion of placental hormones may be attributed, in part, to the pathogenesis of gestational trophoblastic diseases, gestational diabetes, and preterm delivery. However, few biochemical and biophysical markers from the placenta have sufficient sensitivity as valid screening tools for clinical application [[23](#_ENREF_20)]. Although we provide a novel target for the screening of GDM in the present study, the use of plasma ProT examinations to improve the early diagnosis of gestational diseases still needs further longitudinal studies to evaluate changes in ProT expression from the first trimester to delivery.

Consistent with a previous study on GDM rats, fetal weights were significantly lower and placental weights higher along with increased placental fibrosis and ischemia [2[4](#_ENREF_21)]. Moreover, both GDM and preexisting diabetes in pregnancy change the structure of the placenta, including increased calcium and fibrin deposits, and is associated with higher incidences of placental infarction, hematoma, and fibrosis [[17](#_ENREF_22)]. Although certain factors, such as the cystic fibrosis transmembrane regulator, regulate the functions of trophoblasts, the crosstalk between placenta fibrosis and GDM is unknown. In the present study, we provide mechanistic evidence for the role of ProT in the link between placenta fibrosis and GDM. ProT is ubiquitously expressed in various cell types and tissues [[2](#_ENREF_23)5]. In the lung, ProT contributes to the pathogenesis of emphysema. ProT inhibits the association of histone deacetylases with histones and NFκB, and ProT overexpression increases the expression of NFκB-dependent matrix metalloproteinase-2 (MMP2) and MMP9, which are found in the lungs of patients with the chronic obstructive pulmonary disease [[20](#_ENREF_17)]. In the liver, the silencing of hepatic ProT expression ameliorates high-fat diet-induced insulin resistance in C57BL/6 mice through a TLR4/NFκB-dependent pathway. Serum ProT levels of patients with type 2 diabetes are significantly higher than those of normal individuals [[21](#_ENREF_18)]. In the present study, we investigated a novel role of ProT in the placenta that contributes to fibrosis and its link with GDM.

Hyperglycemia is a key symptom of GDM, while high glucose level is related to inflammation and increased ROS. However, the role of ProT in ROS production remains unclear. ProT regulates the expression of oxidative stress-protective genes [[2](#_ENREF_24)6] by increasing endothelial nitric oxide (NO) synthase expression and NO release, as well as reducing ROS production by endothelial cells [[2](#_ENREF_25)6]. Moreover, ProT enhances the antioxidant defense system of cells via its interaction with the Keap1 protein [27]. On the other hand, ProT exhibits immunomodulatory activity in neutrophils to increase phagocytosis and ROS production [[2](#_ENREF_27)8]. In the present study, we found that both hyperglycemia and AGEs induced ROS production and enhanced ProT expression in trophoblasts. Increased ProT in hyperglycemia might be a compensatory effect against glucotoxicity in GDM. In order to protect against ROS-induced cell death, ProT expression is increased in trophoblasts, which may further increase the expression of antioxidant proteins. In addition, ProT can increase proinflammatory cytokine expression to activate inflammatory responses, such as macrophage infiltration to remove debris from glucotoxicity-induced cell death in the placenta. However, the increased ProTlevels induced placental fibrosis which impaired placental functions, and might further contribute to the development of GDM.

Taken together, we have identified a novel pathophysiological mechanism of ProT in the development of GDM. Hyperglycemia induces the expression of ProT, and increases ProTinduced fibrosis in placenta. ProTmight increase the size of placenta and impair placental function, which further contribute to GDM. Furthermore, plasma ProT levels may be a useful biomarker for the diagnosis of GDM and a candidate target for the treatment of GDM.

**Authors’ contributions**

Hung-Tsung Wu, Lin Kang, Yu-Chu Su, Fu-Yu Chan, and Yi-Shiang Wang designed and performed the experiments, analyzed the data, and wrote the manuscript. Horng-Yih Ou, Yi-Cheng Chen, and Bing-Hua Su wrote the manuscript and edited the text. Chao-Liang Wu provided technical assistance. Ai-Li Shiau and Pensée Wu designed and supervised the study and wrote the manuscript. All authors read and approved the final manuscript.

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Table 1. Clinical characteristics of study subjects with and those without gestational diabetes mellitus (GDM)

|  |  |  |
| --- | --- | --- |
|  | Normal | GDM |
| *N* | 102 | 39 |
| Age (years) | 30.34±4.52 | 31.96±6.84 |
| Body mass index (kg/m2) | 26.78±2.55 | 27.89±5.43 |
| SBP (mmHg) | 120.47±12.98 | 124.50±17.01 |
| DBP (mmHg) | 73.94±12.55 | 75.77±9.43 |
| FPG (mg/dL) | 80.13±7.51 | 95.15±12.53\*\*\* |
| OGTT 1hPG (mg/dL) | 130.47±8.09 | 180.48±20.33\*\*\* |
| OGTT 2hPG (mg/dL) | 123.55±19.96 | 169.41±32.12\*\*\* |

Data are presented as the mean±standard deviation. \*\*\* *p*<0.001 compared to the Normal group. SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; OGTT-1h-PG, plasma glucose at one-hour during an oral glucose tolerance test; OGTT-2h-PG, plasma glucose at two-hour during an oral glucose tolerance test.

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**Figure Legends**

**Figure 1. Placental weight and fibrosis-related gene expressions are increased in patients with gestational diabetes mellitus (GDM).** After delivery, placentae were collected from study participants with (*n*=39) and those without (*n*=102) GDM. The placentae were weighed (A), and stained type 1 collagen (Col-1) protein by immunohistochemistry (B). The mRNA was extracted to detect Col-1 (C), Transforming growth factor  (TGFβ) (D), and  smooth muscle actin (SMA) (E) expressions by qPCR. \*\*\* *p*<0.001.

**Figure 2. Prothymosin** **α (ProT) expression is positively correlated with the expression of type 1 collagen (Col-1) in the placenta.** Col-1 expression was determined in ProT transgenic mice (ProTTg) compared to wild-type (WT) mice (*n*=8 for each group of mice) (A). ProT protein expression in the placenta from study participants with (*n*=8) and without (*n*=15) gestational diabetes mellitus (GDM) after delivery were also determined by immunohistochemistry (B). ProT mRNA was detected by qPCR in participants with (*n*=8) and without (*n*=15) GDM (C). Serum ProT concentrations in subjects with (*n*=39) and without (*n*=102) GDM were determined by ELISA (D). The relationship between ProT and Col-1 was analyzed using the linear regression analysis (E). \*\*\* *p*<0.001.

**Figure 3. Prothymosin α (ProTα) levels in the placenta of mice with gestational diabetes mellitus (GDM).** Pregnant mice were sacrificed to collect placentae at the indicated time points to determine ProT expressions by immunohistochemistry, and quantified by TissueFAXS (*n*=8~10 for each pregnancy time point of mice) (A). The placentae collected at the indicated time points were also used for the determination of ProT expressions by Western blots (B). GDM was induced in pregnant mice by an intraperitoneal injection of 80 mg/kg nicotinamide and 50 mg/kg streptozotocin for 5 consecutive days after 4 h of starvation. Plasma insulin (C) and glucose (D) levels and placental weights (E) were measured to confirm GDM in mice. Expressions of ProT at different pregnancy time points of wild-type (WT) and GDM mice was determined by western blot analysis (*n*=13 for each group of mice) (F).Placentae at gestational day 19 of pregnant mice were collected to detect type 1 collagen (Col-1), Transforming growth factor  (TGFβ) and  smooth muscle actin (SMA) expressions by Western blots (G).\* *p*<0.05, \*\* *p*<0.01, \*\*\* *p*<0.001, compared to the indicated group.

**Figure 4. Prothymosin α (ProTα) enhances the expressions of fibrosis-related proteins in trophoblasts.** 3A-sub-E trophoblasts were transfected with lentiviral vectors containing the ProT gene (A) or different short hairpin (sh)RNAs targeted to ProT (B). The cell lysates were collected to determine type 1 collagen (Col-1), Transforming growth factor  (TGFβ), and  smooth muscle actin (SMA) expressions by Western blots (n=6 for each group). \*\*\* *p*<0.001.

**Figure 5. Prothymosin α (ProTα) causes inflammation in the gestational diabetes mellitus (GDM) placenta and upregulates fibrosis-related protein expressions through a nuclear factor (NF)-κB pathway.** Placenta samples were collected from healthy and GDM pregnant women for immunohistological staining of CD68 (A), and expression of proinflammatory cytokine was quantified by real-time PCR (n=20 for each group) (B). 3A-sub-E trophoblasts were transfected with lentiviral vectors containing the ProT gene to determine phosphorylated P65 (pP65) expression (n=4 for each group) (C). Trophoblasts were treated with 1 µM Bay117082 (Bay) in ProT-overexpressing trophoblasts for 24 h and then harvested for the detection of type 1 collagen (Col-1), Transforming growth factor  (TGFβ), and  smooth muscle actin (SMA) expressions by Western blots (n=5 for each group) (D). \*\* *p*<0.01, \*\*\* *p*<0.001

**Figure 6. Hyperglycemia-induced reactive oxygen species (ROS) regulates prothymosin α (ProTα) expression.** 3A-sub-E trophoblasts were maintained in the indicated glucose concentrations for 48 h and then stained with dichlorofluorescein diacetate for the detection of reactive oxygen species (A). The cells were then harvested to determine the expression of type 1 collagen (Col-1) and ProT (B). Trophoblasts were pretreated with N-acetyl-L-cysteine (NAC) for 30 min and then treated with methylglyoxal (MG) for an additional 24 h. Cell lysates were collected to detect ProT expression (C). Results were obtained from at least four individual experiments. \* *p*<0.05, \*\*\* *p*<0.001.