

Altered Matrix Metalloproteinases Expression in Placenta from Patients with Gestational Diabetes Mellitus

Rong Ding¹, Xi-Mei Liu¹, Yu-Qian Xiang^{1,2}, Yong Zhang^{1,2}, Jun-Yu Zhang^{1,2}, Fei Guo¹, Zhi-Wei Liu¹, Jian-Xia Fan¹

¹Department of Obstetrics and Gynecology, International Peace Maternity and Child Health Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200030, China

²Institute of Embryo-Fetal Original Adult Disease, Shanghai Jiao Tong University School of Medicine, Shanghai 200030, China

To the Editor: Gestational diabetes mellitus (GDM) is defined as glucose intolerance with the onset of pregnancy and is related to increased maternal and fetal risks. Incidence of GDM varies in different countries and the prevalence ranges between 4% and 17%.^[1] GDM is one of the most common medical complications of pregnancy, negatively affecting fetal development and metabolic balance. If untreated, GDM can cause fetal malformation, macrosomia, fetal growth retardation, and even intrauterine fetal death. Recent studies have shown correlations between GDM and postpartum insulin resistance, pregnancy-induced hypertension, and dyslipidemia. GDM has also been shown to be associated with placental abnormalities including altered placental function, oxidative stress, and vascular dysfunction.

The placenta undergoes functional adaptations when exposed to high glucose levels. For example, it can lead to functional and structural abnormalities including increasing vascularization and immature villous. These changes cause a thickening of the basement membrane due to impaired gaseous or nutrition exchange, eventually affecting the health of the fetus as well as the mother. The underlying mechanisms involved in placental pathology remain unclear. A number of genetic and signaling pathways are involved in different developmental stages of the placenta during GDM. We explore the role of matrix metalloproteinases (MMPs) in the deregulation in GDM.

During placental development, angiogenesis and trophoblast invasion into maternal decidua necessitate remodeling and breakdown of extracellular proteolytic enzymes. MMPs are the key molecules responsible for the degradation of extracellular matrix components and participate in the formation of the placenta. During pregnancy, dysregulation of MMP expression is involved in aberrant remodeling of the uteroplacental artery as well as trophoblast. Deregulated MMP2 expression, together with inflammation and oxidative factors, brings about the dysfunction of placental trophoblastic cells.^[2] Although studies have demonstrated altered MMPs expression in embryos from diabetic rats, MMPs in placenta from GDM have not been extensively investigated. In the present study, we investigated the changes in the expression of MMPs in placenta from patients with GDM and examined the relationship between the altered expression of MMPs and the levels

of glucose in GDM. Our studies will be helpful in ascertaining the role of MMPs in the complications of GDM.

Pregnant women at 24–28 weeks of pregnancy were screened for GDM using oral glucose tolerance test (OGTT). GDM was diagnosed using the IADPSGs criteria: (1) fasting blood glucose level >92 mg/dl (5.1 mmol/L), 1-h glucose level >180 mg/dl (10.0 mmol/L), and 2-h glucose level >153 mg/dl (8.5 mmol/L).

Placentas were collected from October 2015 to December 2016 in the International Peace Maternity and Child Health Hospital under a protocol granted by the Ethical Committee. Placental villous tissues were collected at term after C-section (no labor) from women with GDM (defined as GDM group). Women with *in vitro* fertilization, maternal diabetes history, twins (multiple) pregnancy, preeclampsia, or other pregnancy complications were excluded from the study. Placental tissues were preserved in RNA-later solution (Life Technologies, Grand Island, NY, USA) and stored at –80°C.

Total RNA was collected from GDM placental specimens with TRIzol (Life Technologies, Grand Island, NY, USA) reagent. cDNA was synthesized using a PrimeScript™ First-Strand cDNA Synthesis Kit (Takara, Dalian, China) with random or oligo-dT primers. Relative expression of cDNA was quantified by a real-time polymerase chain reaction (PCR) using QuantiNova SYBR Green PCR kit (Qiagen, German). For clinical data, the 2-ΔCT method normalized against glyceraldehyde-3-phosphate dehydrogenase was used to calculate relative cDNA expression level. The primers are shown in Supplemental Table 1.

Total protein was extracted from human placental tissues using RIPA Lysis and Extraction buffer (Thermo Fisher Scientific, Waltham,

Address for correspondence: Dr. Jian-Xia Fan, Department of Obstetrics and Gynecology, International Peace Maternity and Child Health Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200030, China
E-Mail: fanjianxia122@126.com

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Received: 17-01-2018 **Edited by:** Qiang Shi

How to cite this article: Ding R, Liu XM, Xiang YQ, Zhang Y, Zhang JY, Guo F, Liu ZW, Fan JX. Altered Matrix Metalloproteinases Expression in Placenta from Patients with Gestational Diabetes Mellitus. Chin Med J 2018;131:1255-8.

Access this article online

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DOI:
10.4103/0366-6999.231530

Massachusetts, USA) that containing phenylmethanesulfonyl fluoride (1:100) and protease inhibitors (Sigma-Aldrich, USA). Antibodies recognizing MMP2 (1:1000, 4022s, CST, Boston, USA) were used to determine protein levels using Western blot. Anti- β -actin antibody (1:1000, Yisheng, China) was detected as a loading control.

Immunohistochemical staining was performed as previously described. Human placenta tissues were labeled with rabbit anti-MMP2 (ab37150; Abcam, USA) antibodies.

HTR-8/SVneo cells, which was derived from human invasive extravillous trophoblast cells. The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 (Gibco, California, USA), containing 10% fetal bovine serum (FBS, Gibco); primary trophoblasts were isolated by trypsin-DNase I digestion. The cells were cultured in DMEM/F12 with 10% FBS. Under conditions mimicking hyperglycemia, the cells were cultured in the medium with RPMI 1640, 25 mmol/L D-glucose, or 50 mmol/L D-glucose for 24 h, 48 h, and 72 h, respectively. For the time course study, proteins were separately collected from cells cultured in 50 mmol/L D-glucose at 4, 8, 12, 16, 20, and 24 h, respectively.

The methylation levels of selected site-specific CpG sites from MMP2 were determined by bisulfite sequencing. Approximately 1 μ g of DNA extracted with All-Prep Kit (Qiagen) was used for bisulfite treatment with the Epitect reagent (Qiagen) according to manufacturer's specifications. The CpG island was amplified using the primers designed by PyroMark Assay Design 2.0. DNA methylation was detected by Pyrosequencing detector (PyroMark Q96 ID, Qiagen) to analyze the methylation status of each site.

All statistical analyses were performed with SPSS 17 (SPSS Inc., Chicago, Illinois, USA). Data are reported as a mean \pm standard error or mean \pm standard deviation. A two-tailed Student's *t*-test and analysis of variance followed by least significant difference or Dunnett's test were performed. The correlation between different variables was analyzed with Pearson's correlation coefficient. Differences were considered to be statistically significant at $P < 0.05$.

Clinical characteristics of the patients involved in the investigation are shown in Supplemental Table 2. There were no significant differences in maternal age, gestational age at delivery, gestational body mass index, hemoglobin A1c, placental weight (PW) or birth weight (BW) between control and GDM groups. The fasting, 0-h, and 2-h plasma glucose levels at OGTT were significantly higher in the GDM group compared with those in control group.

Changes in MMP2 levels have been reported to be altered in the placental tissue of gestational hypertension. In the present study, the expression of MMPs was detected by real-time PCR; we show that the mRNA levels of MMP2 and MMP14 were upregulated in GDM group ($n = 18$) compared with those in normal pregnant women (CON) group ($n = 16$) [Figure 1a]. On the other hand, we found a significant decrease in MMP12 in the placental tissue of GDM group [Figure 1a]. Other MMP genes investigated showed no significant difference between the CON and GDM group [Supplemental Figure 1a]. Subsequently, we assessed the protein levels of MMP2, MMP12, and MMP14 by Western blotting in GDM group ($n = 6$) and CON group ($n = 6$). Of note, MMP2 gave two immunostained bands as previously reported in other studies. We found that an increased MMP2 protein expression in the placenta of GDM group compared with that in CON group [Figure 1a], consistent with the mRNA expression levels. However, there were no significant differences in the

protein levels of MMP12 and MMP14 between CON and GDM group [Figure 1a].

As shown in Figure 1b, MMP2 protein was identified to be immunolocalized in syncytiotrophoblast and extravillous trophoblast cells. MMP2 appeared as brown or buffy particles by immunohistochemical staining. MMP2 staining was significantly enhanced in GDM placenta ($n = 6$) compared with that in normal placenta ($n = 6$), either in the extravillous trophoblast or syncytiotrophoblast. Compared with control group, enhanced MMP2 staining was shown in extravillous trophoblast cells (GDM: 0.32 ± 0.01 vs. control: 0.24 ± 0.02 , $P < 0.01$) and syncytiotrophoblast cells (GDM: 0.36 ± 0.01 vs. control: 0.29 ± 0.01 , $P < 0.01$) in GDM group.

The correlation between placental MMP2 expression and maternal glucose concentration was further evaluated. Among the 34 cases of placental tissue, the results showed that the mRNA expression of MMP2 and the levels of glucose in OGTT, PW, and PW/BW ratio were positively correlated, using Pearson's correlation analysis. As shown in Figure 1c, increased MMP2 correlated with higher fasting glucose ($r = 0.4300$, $P < 0.05$), higher 1-h ($r = 0.5273$, $P < 0.01$), 2-h ($r = 0.5209$, $P < 0.01$) blood glucose concentration at OGTT, PW ($r = 0.3855$, $P < 0.05$), and PW/BW ratio ($r = 0.4370$, $P < 0.01$), using Pearson's correlation analysis.

We further examined the association of glucose levels and MMP2 expression by carrying out an *in vitro* experiment. The HTR8-Svneo cell lines and isolated primary trophoblast were treated with high glucose concentration (25 mmol/L and 50 mmol/L), and then western blotting was used to determine the MMP2 protein level. As shown in Figure 1d and 1e, the MMP2 expression was significantly increased by high glucose treatment, in a dose-dependent manner. Besides, the expression of MMP2 increased in a time-dependent manner [Supplemental Figure 1b]. These *in vitro* results were in accordance with the clinical consequences.

To assess whether changes of the methylation levels of MMP2 contributed to the increased expression of MMP2 in GDM placenta, we amplified, purified, and sequenced the promoter region of MMP2 [Supplemental Figure 1c] and evaluate the methylation levels of the site-specific selected CpG islands in MMP2. However, there were no significant differences in site-specific selected CpG sites in MMP2s promoter between GDM placenta ($n = 3$) and control placenta ($n = 3$).

MMPs especially MMP2 expression are present in most vascularized tissues, including the placenta, and are involved in diabetes-associated complications. In the present study, we investigated the expression of MMPs in maternal placenta from GDM patients and normal pregnant women. We also demonstrated that the expression of placental MMP2 increased significantly with the increase in the glucose levels. This finding was further verified in HTR8-Svneo cell cultures subjected to different glucose concentrations and time periods. In addition, we found that the MMP2 expression level was positively correlated with PW and PW/BW ratio. However, with the increased gene expression of MMP2 in the placenta of GDM patients, no correlation was observed with the methylation status of the MMP2 promoter region. The key finding is the upregulation of MMP2 expression and is associated with increased glucose levels and hyperglycemia can cause an upregulation of MMP2 expression in the placenta of women with GDM.

GDM is a pregnancy-associated complication without a clear cause and mechanisms involved. In previous investigations,

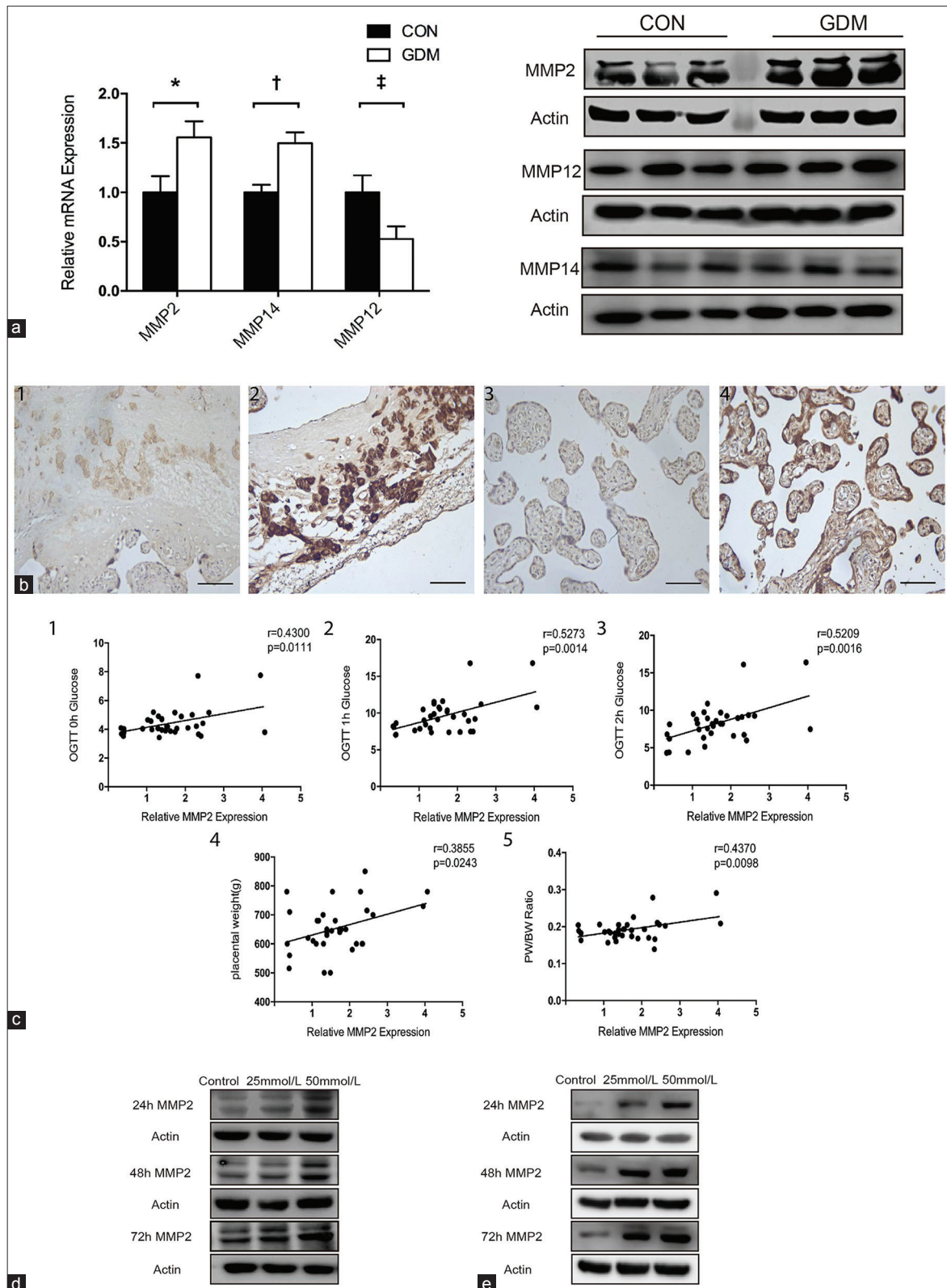


Figure 1: The significance of aberrant expression of MMP2 in GDM. (a) Comparison of MMPs mRNA and protein levels in placenta between normal pregnant women (CON) and GDM patients. *Represents that the difference is statistically significant in MMP2 ($P < 0.05$, CON vs. GDM); †represents that the difference is statistically significant in MMP14 ($P < 0.01$, CON vs. GDM); ‡represents that the difference is statistically significant in MMP12 ($P < 0.05$, CON vs. GDM). (b) A more distinct staining was observed in the extravillous trophoblasts and syncytiotrophoblast from the normal placenta (1 and 3) and the GDM placenta (2 and 4). (standard streptavidin-peroxidase method, scale bar = 100 μ m). (c) The expression of MMP2 mRNA is positively correlated with the maternal blood glucose concentration, PW, and PW/BW ratio ($n = 34$). (d and e) Expression of MMP2 in HTR8-Svneo cell and isolated primary trophoblast upon treatment with high glucose at different time intervals. MMP: Matrix metalloproteinase; GDM: Gestational diabetes mellitus; PW/BW: Placental weight/birth weight.

Pustovrh *et al.*^[3] have found that MMP2 and MMP9 were upregulated in placenta of diabetic rats and were strongly expressed in both labyrinth and junctional zones. Interestingly, in our study, we found significant alterations in MMP2 expression in placenta tissue from GDM; we also showed that MMP2 was abundantly present in the syncytiotrophoblast and extravillous trophoblast. In the course of placental development, MMPs are secreted as an inactive form, which degrade the extracellular matrix and are involved in pathologic tissue remodeling processes. Studies performed in mouse carotid occlusion model suggest that the knockout of MMP2 and MMP9 is associated with tissue remodeling by decreasing vascular smooth muscle cell migration and neointima formation. Altered expression of MMPs has been demonstrated to contribute to various kinds of changes during placental development. For example, decreased MMPs were correlated with poor trophoblast invasiveness and placental ischemia in preeclampsia; conversely, increased levels of MMPs are related with abnormal invasion and proliferation of the trophoblast such as in gestational trophoblastic diseases. Upregulated membrane type (MT) 1-MMP may be responsible for GDM-associated changes in the vascularization, angiogenesis, and arterial enlargement, which can alter placental blood flow and transfer efficiency.^[4] Therefore, the increased MMP2 production and the localization of MMP2 in the GDM placentas may result in an increased proliferative and remodeling activity, giving rise to an increased vascularization and an increase in the amount of immature villi in the placenta.

The placenta is a significant organ from fetus and mother, and its development and function are pivotal for the maternal and fetal health. Changes to the intrauterine metabolic environment induced by GDM have been recently reported to result in the morphology changes in the placenta, resulting in increased PW. Our data show that increased MMP2 expression is positively correlated with PW and PW/BW ratio. Previous studies have showed that increased PW and PW/BW ratio were related to adverse pregnancy outcomes as well as conditions such as preeclampsia, heavier BW, and impaired Apgar score.^[5] Therefore, MMP2 might be implicated in increased risk for mother and fetus in GDM by having a direct influence on the PW and PW/BW ratio.

Recent studies have also established that cultured trophoblast cell lines exposed to hyperglycemic conditions increased the expressions of perlecan, cytokine, the angiogenesis, and apoptosis-associated molecules. In the present study, we show that the hyperglycemic conditions induce the expression of MMP2 in trophoblast cells, indicating that MMP2 may play an important role in trophoblastic cells under hyperglycemic conditions. During invasive events, MMPs are secreted by the decidual cells and by invasive trophoblasts, particularly during implantation. Besides, MMPs especially MMP2 have been reported to be involved in the pathophysiology of pregnancy-associated complications such as spontaneous early pregnancy failure, premature rupture of membranes, and pre-eclampsia. One of the studies has confirmed that MT-MMPs are critical in the late embryonic development and postnatal life and catalyze the formation of syncytiotrophoblasts in early placental development. Our present study showed that the cell-associated MMP2 expression increased gradually upon treatment with high glucose in a dose-dependent and time-dependent manners. Furthermore, we demonstrated that the expression of MMP2 in GDM placenta was positively correlated with maternal glucose levels in OGTT, PW, and PW/BW ratio,

indicating that MMP2 might be associated with maternal and fetal complications by altering placental function during GDM.

In the present work, we show that maternal placental tissues from GDM patients and trophoblasts, when exposed to high glucose concentrations, had an upregulation of MMP2 expression. Given the positive correlation of MMP2 and hyperglycemia, MMP2 may be used as a clinical indicator to detect the severity of diabetes. Furthermore, it is of vital importance to regulate blood glucose to normal levels because the level of MMP2 increases with increasing blood glucose concentrations, which may cause placental structure malformation such as heavier placenta and higher PW/BW ratio. However, the methylation status of MMP2 did not cause any significant changes in the MMP2 gene expression. Therefore, further research is necessary to fully understand the relevant pathways involved in the alterations of MMP2 expression and its consequences on the placental function under conditions of maternal hyperglycemia.

Supplementary information is linked to the online version of the paper on the Chinese Medical Journal website.

Declaration of patient consent

We certify that we have obtained all appropriate patient consent forms. In the form, the patients have given their consent for their clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity.

Financial support and sponsorship

This work was supported by grants from the National Natural Science Foundation of China (No. 81471516, No. 81501274, and No. 81501276), Shanghai Pujiang Program (No. 15PJ1408900), the Shanghai Municipal Commission of Science and Technology Program (No. 15411966700), the Shanghai Municipal Commission of Health and Family Planning (No. 15GWZK0701 and No. 20154Y0039).

Conflicts of interest

There are no conflicts of interest.

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Supplemental Table 1: Sequences of quantitative polymerase chain reaction primers used in this study

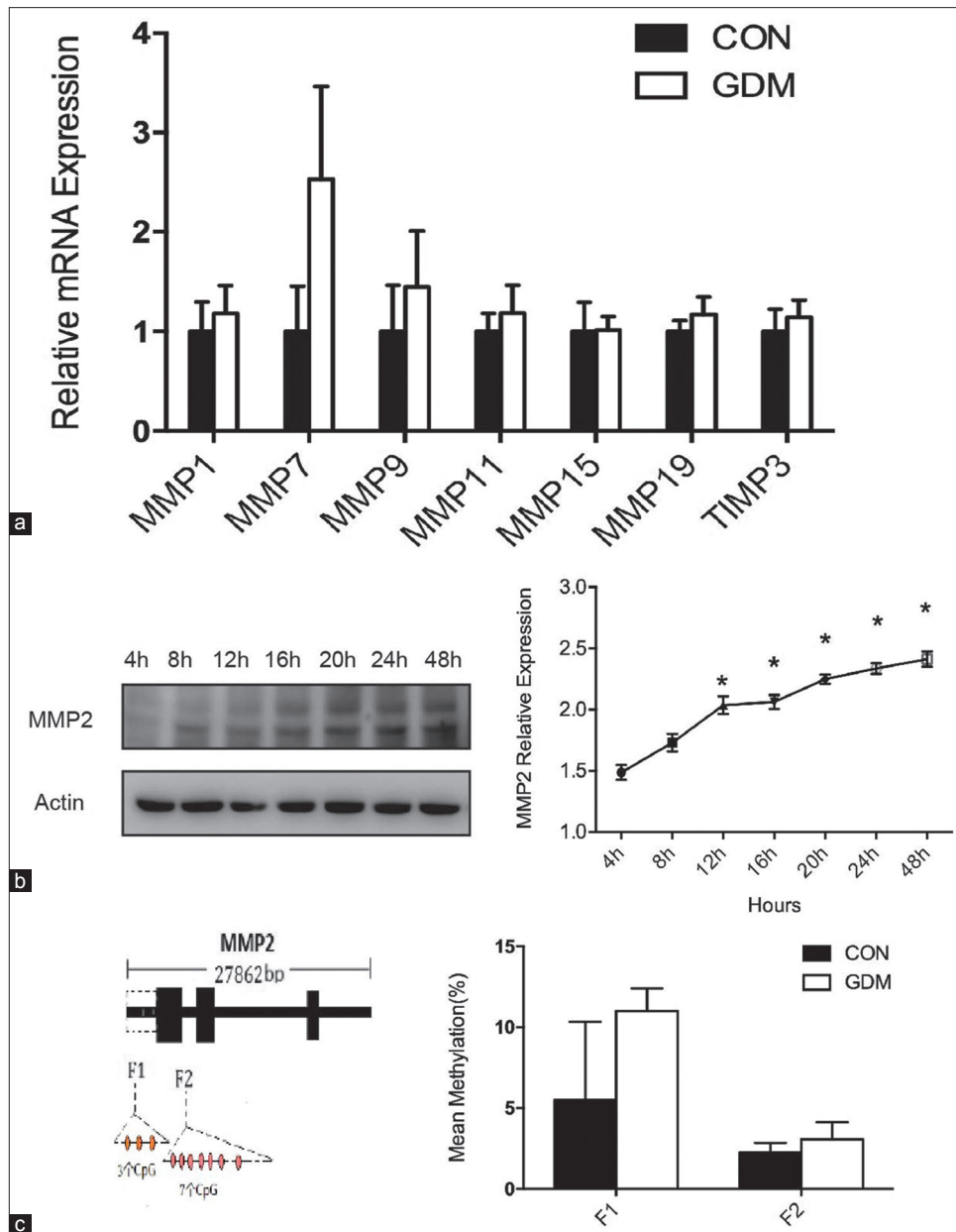
Gene	Forward primer	Reverse primer
<i>MMP1</i>	5'-GCGCACAAATCCCTTCTACC-3'	5'-ACCGGACTTCATCTCTGTTCG-3'
<i>MMP2</i>	5'-GCTCAGATCCGTGGTGAGAT-3'	5'-ACACAGCCTTCTCCTCCTGT-3'
<i>MMP7</i>	5'-CATGATTGGCTTTGCGCGAG-3'	5'-ATCTCCTCCGAGACCTGTCC-3'
<i>MMP9</i>	5'-TCCAGTACCGAGAGAAAAGCC-3'	5'-CATAGGTCACGTAGCCCACT-3'
<i>MMP11</i>	5'-CCGCAACCGACAGAAGAGG-3'	5'-ATCGCTCCATACCTTTAGGGC-3'
<i>MMP14</i>	5'-GGTGGTCTCGGACCATGTCT-3'	5'-GCTGTGTGTGGGTACGTAGG-3'
<i>MMP15</i>	5'-GAACTGGCTGCGGCTTTATG-3'	5'-CCTTGGTCTCTTCGTCGAGC-3'
<i>MMP19</i>	5'-CCAGAAGATATCACCGAGGCT-3'	5'-GGATCCTTAGGCCACAACG-3'
<i>TIMP3</i>	5'-TGAAGATGTACCGAGGCTTCAC-3'	5'-GGTACTTGTGACCTCCAGCTT-3'
<i>GAPDH</i>	5'-TGGAGTCCACTGGCGTCTTC-3'	5'-TGCTGATGATCTTGAGGCTGTTG-3'

MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Supplemental Table 2: Clinical characteristics of subjects with normal pregnant women (*n* = 16) and GDM patients (*n* = 18)

Characteristics	Control (<i>n</i> = 16)	GDM (<i>n</i> = 18)	<i>P</i>
Age (years)	31.19 ± 0.67	32.17 ± 0.20	0.329
Gestational age (days)	272.31 ± 4.89	272.83 ± 4.70	0.773
BMI (kg/m ²)	20.73 ± 0.24	20.84 ± 0.37	0.793
OGTT 0 h (mmol/L)	3.93 ± 0.40	4.86 ± 1.12	0.004
OGTT 1 h (mmol/L)	8.00 ± 0.65	10.94 ± 2.47	<0.001
OGTT 2 h (mmol/L)	6.60 ± 1.46	10.15 ± 2.41	<0.001
HbA1c (%)	5.02 ± 0.21	5.21 ± 0.61	0.253
BW (g)	3385.88 ± 107.40	3575.88 ± 72.02	0.145
PW (g)	631.3 ± 24.7	675.6 ± 14.1	0.119
PW/BW ratio	0.187 ± 0.004	0.200 ± 0.008	0.159

Data are the mean ± SD. GDM: Gestational diabetes mellitus; BMI: Body mass index; OGTT: Oral glucose tolerance test; HbA1c: Hemoglobin A1c; PW/BW: Placental weight/birth weight; SD: Standard deviation.



Supplemental Figure 1: (a) MMPs (MMP1, 7, 9, 11, 15, and 19) and TIMP3 showed no significant difference between normal pregnant women (CON) and GDM groups. (b) HTR8-Svneo cells were cultured in 50 mmol/L D-glucose media for different time intervals (4, 8, 12, 16, 20, 24, and 48 h). *Represents that the difference is statistically significant in MMP2 ($P < 0.05$; 8, 12, 16, 20, 24, and 48 h vs. 4 h). Representative Western blot bands are indicated. (c) Methylation levels of the two site-specific selected CpG sites from MMP2. MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinase; GDM: Gestational diabetes mellitus.