Regulation of membrane-type matrix metalloproteinase 1 (MT1-MMP) in human placenta

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Carmen Kar Sum Tam-Amersdorfer

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Ich erkläre ehrenwörtlich, dass ich die vorliegende veröffentlichten wissenschaftlichen Publikationen entsprechend der Richtlinie der Herausgeber erfüllt habe.

Graz, am 7. November 2014

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Name: Carmen Tam-Amersdorfer

Institute: Institute of Pathophysiology & Immunology

Commit myself to adhering to the Standards of Good Scientific Practice valid at the time of my research related activities at the University.

Graz, 7th of November 2014
Membrane-Type Matrix Metalloproteinase 1 Regulates Trophoblast Functions and Is Reduced in Fetal Growth Restriction

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Fetal growth restriction (FGR) results from placental insufficiency to adequately supply the fetus. This insufficiency involves impaired cytotrophoblast functions, including reduced migration and invasion, proliferation, and syncytium formation. Membrane-type matrix metalloproteinase 1 (MT1-MMP) is a key enzyme in these cellular processes. MT1-MMP exists in various forms: a 63-kDa proenzyme is synthesized as primary translation product, which is cleaved into a 57-kDa membrane-anchored active form. We hypothesized that reduced placental MT1-MMP in FGR impairs trophoblast functions. MT1-MMP mRNA and active enzyme was quantified in placentas from FGR and age-matched control pregnancies. MT1-MMP protein was localized in first-trimester and term placentas. Putative MT1-MMP functions in trophoblasts were determined using two blocking antibodies for measuring migration and proliferation, as well as fusion of primary trophoblasts and trophoblast-derived cells. MT1-MMP was expressed predominantly in the syncytiotrophoblast and the villous and extravillous cytotrophoblasts. In FGR placentas, levels of MT1-MMP mRNA and of active MT1-MMP protein were reduced (−34.2%, P < 0.05, and −21.5%, P < 0.01, respectively), compared with age-matched controls. MT1-MMP blocking antibodies diminished migration, proliferation, and trophoblast fusion. We conclude that reduced placental MT1-MMP in FGR may contribute to the impaired trophoblast functions associated with this pathology. (Am J Pathol 2013, 182: 1563–1571; http://dx.doi.org/10.1016/j.ajpath.2013.01.011)

Fetal growth restriction (FGR) is a failure to achieve the genetically determined growth potential of the fetus. The pathogenesis of FGR is still not fully understood, but placental insufficiency is a well-established contributor. It results in inadequate fetal supply and therefore also inadequate fetal growth.1,2 Proper placental function depends on several key processes executed by cytotrophoblast cells. In early pregnancy, placental cytotrophoblasts invade the maternal uterus and acquire a distinct phenotype.3 These extravillous, invasive cytotrophoblasts remodel maternal uterine spiral arteries into dilated, low-resistance vessels. Thus, under normal conditions, uteroplacental blood flow is established, allowing adequate fetal nutrient and oxygen supply.3,4 Throughout pregnancy, proliferation of villous cytotrophoblasts largely determines placental growth. Villous cytotrophoblasts also fuse to form the multinucleated syncytiotrophoblast, which represents the classical fetomaternal barrier, through which nutrients and oxygen have to be transported to the fetal circulation. Steady proliferation and fusion of underlying cytotrophoblasts maintains and enlarges the syncytiotrophoblast throughout pregnancy, because the syncytiotrophoblast is mitogenically inactive.3,4

In a pregnancy complicated by FGR, these key functions of trophoblast cells are impaired, and transcription factors

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regulating differentiation processes seem to be involved.\textsuperscript{3,6} Trophoblast invasion and remodeling of maternal uterine arteries are diminished (reviewed by Kaufmann et al\textsuperscript{7}).

Several studies also report reduced cytotrophoblast proliferation\textsuperscript{2,5,8} and increased apoptosis,\textsuperscript{9} and recent data indicate disturbed trophoblast fusion associated with FGR in mice\textsuperscript{10} and in humans.\textsuperscript{11,12} The human studies demonstrate perturbations in the ratio of cytotrophoblast to syncytiotrophoblast nuclei, as well as a reduced ability of isolated cytotrophoblasts to form syncytiotrophoblast nuclei, as well as a reduced ability of isolated cytotrophoblasts to form syncytiotrophoblast nuclei. Both human and mouse studies suggest that reduced expression of genes involved in syncytium formation contributes to placental insufficiency and consequently, to FGR; however, the mechanisms underlying impaired trophoblast functions are yet to be identified.

All these trophoblast functions depend on proteolysis and extracellular matrix degradation. Invasion requires tissue degradation in front of the moving cells.\textsuperscript{13,14,15} Also migration (ie, the movement of cells along the invasion path),\textsuperscript{16} proliferation,\textsuperscript{15} and fusion\textsuperscript{17} crucially depend on adequate turnover of structural proteins inside and outside the cell and therefore also on the presence of proteases.

A key enzyme for invasion, migration, proliferation, and fusion is membrane-type matrix metalloproteinase 1 (MT1-MMP). It belongs to a small family of membrane-anchored matrix metalloproteinases (MMPs) with a particular wide range of substrates. MT1-MMP degrades extracellular matrix components such as collagens, fibronectin, laminin, and vitronectin.\textsuperscript{18} It also activates other MMPs (eg, MMP-1, MMP-2, and MMP-13) and is able to activate or inactivate various cytokines and chemokines by cleaving their pro-forms, such as protransforming growth factor-β (pro-TGF-β), protumor necrosis factor-α (pro-TNF-α), or active forms, such as IL-8 and TNF-α.\textsuperscript{19,20} MT1-MMP exists in various protein forms; it is synthesized as an inactive, 63-kDa pro-form, which is transported to the cell membrane and cleaved at a furin recognition motif into the active 57-kDa enzyme.

The role of MT1-MMP in migration,\textsuperscript{21} invasion,\textsuperscript{22,23} proliferation,\textsuperscript{24} and fusion\textsuperscript{17} has been demonstrated in various cell types. However, the contribution of MT1-MMP to these processes in trophoblasts has remained unknown, although the expression of MT1-MMP in invading first-trimester trophoblasts indeed suggests that MT1-MMP is involved in trophoblast invasion.\textsuperscript{25,26}

In the present study, we tested the hypothesis that FGR, a pathology primarily associated with placental insufficiency, is associated with reduced placental MT1-MMP levels in the third trimester. We localized MT1-MMP expression in placental cells and analyzed active MT1-MMP protein during normal pregnancy and FGR. Because the trophoblast and its subpopulations are the main expression sites of MT1-MMP in the first- and third-trimester placenta, we aimed at identifying potential consequences of reduced MT1-MMP activity in FGR placenta. Key processes of trophoblast function were chosen as endpoints: migration, proliferation, and syncytium formation.

### Materials and Methods

#### Placenta Samples

Placentas were collected after caesarean section and were immediately put on ice. Two tissue samples were taken from each placenta, both within 3 cm of the cord insertion site (to avoid position effects) and were snap-frozen in liquid nitrogen. Informed consent of the patients was obtained. The study was approved by the institutional review board and ethical committee of the Medical University of Graz and of the Medical Faculty, University of Milan.

#### Subject Characteristics

Gestational age was calculated from the last menstrual period and was confirmed by an ultrasonographic examination performed before week 20 of gestation. Infants with morphological malformations at birth and/or chromosomal abnormalities were excluded from the study. Mothers were excluded if they had obstetrical complications (hypertension, diabetes mellitus, or gestational diabetes) or factors predisposing to FGR (eg, autoimmune and endocrine disease, chronic hypertension, or pregnancy-induced hypertension). Maternal alcohol or drug consumption was also an exclusion criterion. Control pregnancies were those of women who were delivered by elective caesarean section because of placenta praevia or after spontaneous amniorrhexis and who gave birth to healthy term neonates with a birth weight between the 10th and 90th percentile according to Italian standards for birth weight and gestational age.\textsuperscript{27} Each FGR placenta was compared with one or two control placentas of the same gestational age and was matched for mode of delivery. FGR was defined as deviation from growth trajectory in two consecutive ultrasound examinations (separated by a minimum of 2 weeks). FGR pregnancies showed no fetal hemodynamic changes. Characteristics of the subjects are listed in Table 1.

#### qPCR

One microgram of total RNA isolated with TRI reagent (MRC, Cincinnati, OH) was reversely transcribed to cDNA using

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clinical Characteristics of Study Subjects</th>
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<tr>
<td>Characteristics</td>
<td>Control</td>
</tr>
<tr>
<td>Sample size</td>
<td>( n = 11 )</td>
</tr>
<tr>
<td><strong>Mother</strong></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>29.2 ± 7.2</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>37.8 ± 2.5</td>
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<tr>
<td>Prepregnancy BMI</td>
<td>22.3 ± 3.3</td>
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<tr>
<td>Postgravid BMI</td>
<td>29.6 ± 5.0</td>
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<tr>
<td><strong>Fetus</strong></td>
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<tr>
<td>Fetal weight (g)</td>
<td>3065 ± 659</td>
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<tr>
<td>Placental weight (g)</td>
<td>591 ± 115</td>
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Data are expressed as means ± SD.
*\( P < 0.01 \) versus control group.
BMI, body mass index.
SuperScript II Reverse Transcriptase (Life Technologies—Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. cDNA (100 ng) was subjected to real-time quantitative PCR (qPCR) using FAM-labeled TaqMan gene expression assays (MT1-MMP: Hs00237119_m1; HPRT1: Hs02806955_m1) and TaqMan universal PCR master mix (Life Technologies—Applied Biosystems). Components were mixed according to the manufacturer’s instructions and amplified in 20 μL total volume per well (96-well plates; Life Technologies—Applied Biosystems) using an ABI 7900 real-time cycler (Life Technologies—Applied Biosystems, Foster City, CA). C_T values were automatically generated by the associated software (SDS version 2.2; Life Technologies—Applied Biosystems), and relative gene expression was calculated by the standard 2^-ΔΔC_T method, using gene expression of hypoxanthine phosphoribosyltransferase 1 (HPRT1) as the reference. Statistical analyses used the ΔC_T values.

Immunolocalization

Studies were conducted on two different samples of 4% paraformaldehyde-fixed, paraffin-embedded first-trimester (week 7) and term human placenta villous tissue, sectioned at 4 μm. Slides were deparaffinized in xylene and were rehydrated with decreasing concentrations of ethanol according to standard methods. Antigen retrieval was used for tissue sections submerged in 10 mmol/L sodium citrate buffer, 0.05% Tween 20 (pH 6.0) for 10 minutes in a microwave oven at 700 W to 95°C. Slides were allowed to cool for 45 minutes at room temperature before being rinsed in wash buffer [Tris-buffered saline, Tween-20 (TBST), pH 7.4]. Sections were blocked with UV ultra block (Lab Vision; Thermo Fisher Scientific, Kalamazoo, MI) for 7 minutes before primary antibody incubation at 4°C overnight (10 μg/mL; rabbit anti-human MT1-MMP (Abcam, Cambridge, MA); 0.5 μg/mL mouse anti—HLA-G (MEM-G/1; BioVendor, Candler, NC); and 0.1 mg/mL mouse anti-CD34, 0.21 mg/mL mouse anti-CK7, and 0.58 μg/mL mouse anti-human vWF (DakoCytomation, Carpinteria, CA)). Negative controls were incubated with non-specific IgG1 (Vector Laboratories, Burlingame, CA) or with non-specific polyclonal rabbit antibodies (DakoCytomation) as isotope controls. All incubation steps were performed in a dark, humidified chamber at room temperature. After 5 minutes of TBST wash, secondary antibodies (goat anti-rabbit DyLight 488 1.88 μg/mL and goat anti-mouse Cy-3 1.75 μg/mL) were applied for 30 minutes. After another TBST wash, 5 mg/mL DAPI was added to the slides for 20 minutes as a nuclear counterstain. Sections were rinsed again with TBST before mounting with Vectashield mounting medium (Vector Laboratories).

To acquire and analyze computerized images of sections and cells, a Leica DM4000 B microscope equipped with a Leica DFC 320 video camera (Leica Microsystems Cambridge, Cambridge, UK) was used.

Cell Culture

To investigate the role of MT1-MMP in proliferation and invasion, we used ACH-3P cells, a hybrid cell line of choriocarcinoma cells and primary trophoblasts, which are a model for invading and proliferating trophoblasts.ACH-3P cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. The role of MT1-MMP in trophoblast syncytium formation was determined by measuring fusion of BeWo cells (a widely used model for trophoblast fusion), which were purchased from the European Collection of Cell Cultures (ECACC, London, UK) and cultured as described previously.32 The role of MT1-MMP was further determined in primary trophoblasts isolated from term placentas as described previously.33

Because chemical MMP inhibitors often interact with other proteases and are not absolutely specific, we used blocking antibodies for the functional assays. To increase credibility of the results, two different clones of blocking antibodies were used: LEM-2/15.8 and LEM-2/63.1 (Milipore, Billerica, MA).34 Nonspecific mouse IgG1 served as an isotype control. All antibodies were used at a concentration of 15 μg/mL.

To observe fusion of BeWo cells and trophoblasts, BeWo cells (100,000/well) and freshly isolated trophoblasts (200,000/well) were seeded in a 24-well plate. Fresh medium with blocking antibodies or control IgG1 was applied 1 day after seeding. Cells and supernatant were used for analysis after 48 hours. For migration and proliferation assays, 20,000 ACH-3P cells treated with Accutase (Innovative Cell Technologies, San Diego, CA) were incubated with blocking and control antibodies for 30 minutes and then were seeded into the assay wells.

To test antibodies for cytotoxicity, cells were treated similarly as for the experiments, culture supernatants were collected, and the release of lactate dehydrogenase (LDH) was determined. Supernatants of ACH-3P cells were analyzed after 12 hours; supernatants of BeWo cells and primary trophoblasts were analyzed after 48 hours. The concentration of LDH released into the supernatant was measured with a commercial LDH cytotoxicity detection kit (TaKaRa Bio, Otsu, Japan) according to the manufacturer’s instructions. There were no differences in released LDH between cells incubated with the blocking antibodies and the nonspecific IgG1 control (data not shown).

Migration Assay

Migration of ACH3P cells was observed in real time using a real-time cell analyzer single-plate (RTCA SP) instrument, an xCelligence system (Roche Applied Science), and CIMPlate 16 permeable support arrays. The upper chambers of these assay wells are sealed at the bottom with a microporous polyethylene terephthalate membrane with a mean pore size of 8 μm. The bottom side of the membrane is covered.
by gold electrodes. The electrical impedance is monitored in real time by measuring electrical impedance across the microelectrodes. This impedance measurement provides quantitative information about the cells that have migrated through the membrane and reached the gold electrodes at the bottom side of the membrane. The cell index, a dimensionless parameter derived as a relative change in measured electrical impedance to reflect the migration of the cells, is proportional to the number of cells that migrated through the pores.

ACH-3P cells were pretreated with blocking and control antibodies and were seeded into the upper chamber in medium without fetal calf serum. The lower chambers contained medium with 10% fetal calf serum as a chemo-attractant for the cells. After approximately 5 hours, all cells were attached. Thereafter, the migration was continuously monitored for 24 hours at 37°C.

**Impedance-Based Proliferation Assay**

ACH-3P cells were used for impedance-based xCelligence proliferation assay using an RTCA SP instrument and 16-well E-plate arrays (Roche Applied Science). The assay plates have gold electrodes at the bottom of the wells, and impedance across these microelectrodes is measured. Cell number correlates with impedance; the assay thus provides quantitative information. ACH-3P cells were pretreated with the blocking and control antibodies and were seeded into each well of the 16-well E-plate arrays. After approximately 5 hours, all cells were attached onto the bottom. Proliferation is reflected by an increase of the integrated cell index values (ie, a relative change in measured electrical impedance\(^{35}\)), which is proportional to the number of cells. Proliferation was continuously monitored for 24 hours at 37°C.

**WST-1 Cell Proliferation Assay**

The WST-1 cell proliferation assay is based on enzymatic cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases, which are active only in viable cells. ACH-3P cells were incubated with blocking or control antibodies and seeded in each well of a 96-well plate. After 24 hours, 10 μL of WST-1 reagent (Roche Applied Science) was added and the cells were incubated for another 2 hours at 37°C. Absorbance at 450 nm was then measured spectrophotometrically using an enzyme-linked immunosorbent assay (ELISA) plate reader (ThermoMax ELISA plate reader; Molecular Devices, Sunnyvale, CA).

**Fusion of BeWo Cells and Primary Trophoblasts**

Intercellular fusion of BeWo cells was induced with forskolin (Sigma-Aldrich, St. Louis, MO), which was supplemented to the cell culture medium at a final concentration of 20 μmol/L (10 mmol/L stock in dimethyl sulfoxide). The same volume of dimethyl sulfoxide (0.2%) was added to the control cells. The MT1-MMP-blocking antibodies or nonspecific IgG1 were added to the culture medium. After 48 hours of incubation, cells were lysed for immunoblot analysis and E-cadherin detection. To exclude the detection of different E-cadherin levels as a result of E-cadherin shedding from the cell surface, we used an antibody directed against the cytoplasmatic domain of the protein. The cell culture supernatant was used to determine the concentration of human β-chorionic gonadotropin (β-hCG; Dade Behring, Deerfield, IL) after 48 hours. Each experiment was run in triplicate and independently repeated four times.

**Immunoblot Analysis**

Tissue was homogenized and cells were washed and lysed in buffer containing 10 mmol/L Tris pH 7.4, 1% SDS, 1 mmol/L Na-orthovanadate, and Complete protease inhibitor (Roche Applied Science) mixed with an equal volume of Laemmli sample buffer (Sigma-Aldrich). Before electrophoresis, samples were centrifuged and boiled for 5 minutes at 99°C. Equal amounts of protein were determined using the Lowry assay with SDS–PAGE on a 10% gel (Pierce; Thermo Fisher Scientific, Rockford, IL). After electrophoretic transfer according to the manufacturer’s instructions, membranes were stained with Ponceau S to check for blotting efficiency and then were photographed. Next, the membranes were blocked for 1 hour with 5% (w/v) nonfat dry milk (Bio-Rad Laboratories, Hercules, CA) and 0.1% (v/v) Tween-20 (Sigma-Aldrich) in 0.14 mol/L Tris-buffered saline pH 7.3 at room temperature; this solution was also used for subsequent washings and as an antibody diluent. The membranes were incubated overnight at 4°C with antibodies against the intracellular region of E-cadherin (1:1000; no. 4065; Cell Signaling Technology, Danvers, MA), against β-actin (1:10,000; Amersham; GE Healthcare, Little Chalfont, UK), or against active MT1-MMP (0.5 μg/mL; LEM-2/15.8; Millipore, Billerica, MA). This MT1-MMP antibody recognizes the active center of MT1-MMP. After a wash, membranes were incubated with the secondary antibody (1:1000; Bio-Rad Laboratories) for 1 hour at room temperature. Immunolabeling was visualized using a Pierce SuperSignal CL-HP substrate system (Thermo Fisher Scientific). Membranes were exposed to Amersham Hyperfilm (GE Healthcare) and were densitometrically scanned using a digital camera and AlphaDigiDoc 1000 software (Alpha Innotech Corp., San Leandro, CA) within the linear range of film and camera. MT1-MMP and E-cadherin signals were normalized either to the overall signal of the whole lane on the Ponceau S–stained membranes or to β-actin.

**Statistical Analysis**

SigmaStat software version 3.1 (Jandel Scientific, San Rafael, CA) was used for statistical analyses. Student’s t-test or two-way analysis of variance with Holm–Šidák or Duncan’s method as post hoc test was used to test for
differences as appropriate. Significance was accepted at a level of $P < 0.05$.

**Results**

Placental MT1-MMP in Normal Pregnancy and in FGR

Comparison of mRNA expression between normal and FGR placentas by qPCR revealed 34% lower levels of MT1-MMP in FGR (Figure 1A). To determine whether this difference was present also for the active MT1-MMP enzyme, an antibody directed against the active catalytic domain of MT1-MMP was used for immunoblot analysis. Indeed, active MT1-MMP levels were lower also in FGR placentas, compared with controls of similar gestational age (Figure 1, B and C).

Localization of MT1-MMP in the Placenta throughout Gestation

The placenta is a multicellular tissue. We therefore next localized the main placental expression sites of MT1-MMP activity at different stages of gestation by immunofluorescence and colocalized MT1-MMP with markers for specific placental cells or subpopulations of cells (Figure 2). Cytokeratin 7 (CK7) is a general marker for trophoblast cells and stains all trophoblast subpopulations, including proliferating villous cytotrophoblasts and syncytiotrophoblasts, which result from fusion of villous cytotrophoblasts and the invasive extravillous cytotrophoblasts that invade the maternal uterus particularly in the first trimester of pregnancy. HLA-G is a marker for the majority of extravillous cytotrophoblasts. Endothelial cells were stained for CD34 and von Willebrand factor (vWF). The trophoblast compartment showed intensive MT1-MMP staining throughout pregnancy. In first-trimester placenta, the invasive HLA-G—positive extravillous cytotrophoblasts, the syncytiotrophoblasts, and the villous cytotrophoblasts were stained, predominantly at the plasma

![Figure 1](image1.png)

**Figure 1** Expression of MT1-MMP mRNA and enzyme in FGR placentas and age-matched controls. A: qPCR of MT1-MMP expression. B: Representative immunoblot (one of five) for active MT1-MMP and the corresponding β-actin staining of the membrane. C: Quantification of active MT1-MMP in control ($n = 11$) and FGR ($n = 8$) placentas over time. ANOVA, analysis of variance. Data in A are expressed as means ± SEM.

![Figure 2](image2.png)

**Figure 2** Immunofluorescent staining of MT1-MMP and markers for all trophoblast populations (CK7), extravillous cytotrophoblasts (HLA-G), and endothelial cells (CD34 and vWF). A, D, G, J, and M: Green staining of MT1-MMP in first-trimester (A, D, G, and J) and third-trimester (3 and M) placental villi. B, E, H, K, and N: To allow identification of cells showing MT1-MMP expression, the sections were costained for the cell markers CK7 (B), HLA-G (E), and CD34 (H) in first-trimester placenta and for CK7 (K) and vWF (N) in third-trimester placenta, C, F, I, L, and O: Overlay imaging shows a merge of the two stainings. Nuclei were counterstained with DAPI (blue). P and Q: Negative controls for first-trimester (P) and third-trimester (Q) placental tissue were treated with nonspecific mouse IgG1 fractions and nonspecific polyclonal rabbit IgG. Original magnification, ×400. EC, endothelial cell; evCT, extravillous cytotrophoblast; ST, syncytiotrophoblast; T, trophoblasts; vCT, villous cytotrophoblast.
membrane (Figure 2, A–F). Also, developing fetoplacental vessels were immunolabeled for MT1-MMP (Figure 2, G–I). At term of gestation, trophoblast invasion was diminished. The syncytiotrophoblasts and the subjacent villous cytotrophoblasts were immunolabeled for MT1-MMP (Figure 2, J–L), similar to the first trimester. Furthermore, the endothelial cells of the fetal vessels were stained in the term placenta (Figure 2, M–O).

MT1-MMP Function in Trophoblasts

The Effect of MT1-MMP Blocking Antibodies on Migration of Trophoblast Cells

The migration assay demonstrated reduced migration of the first-trimester trophoblast model ACH-3P cells in the presence of the blocking antibodies (Figure 3A). The difference between the control (nonspecific IgG1) and the blocking antibodies appeared immediately after cell attachment, and became statistically significant after 8 hours. Both blocking antibodies reduced migration to approximately 50% at 24 hours.

The Effect of MT1-MMP Blocking Antibodies on Proliferation of Trophoblast Cells

To determine the role of MT1-MMP in trophoblast proliferation, two different experimental approaches were used. First, an impedance-based array (xCelligence system) was used to monitor the first-trimester trophoblast model ACH-3P cells from 0 to 24 hours after attachment (Figure 3B). In a second approach, the WST-1 proliferation assay was used to measure mitochondrial activity at 24 hours after seeding (Figure 3C). Assays were performed in the presence of MT1-MMP–blocking antibodies or nonspecific IgG1. Both approaches demonstrated a proliferation decrease, by 46% with the WST-1 assay and by 12% with the impedance-based array (Figure 3, B and C). In contrast to the rapid effect of the MT1-MMP–blocking antibodies on migration, proliferation was affected only 12 hours after attachment of the cells in the impedance-based system.

The Effect of MT1-MMP Blocking Antibodies on Fusion of BeWo Cells and Primary Trophoblasts

BeWo cells were stimulated to fuse with forskolin in the presence of MT1-MMP–blocking antibodies or nonspecific IgG1. Loss of the cell adhesion molecule E-cadherin is a well-documented marker and proxy measure for the process of trophoblast fusion. Moreover, secreted β-hCG as a marker for trophoblast differentiation was measured in the supernatant. Immunoblot analysis demonstrated an approximately 30% decrease (P = 0.001) of cellular E-cadherin after forskolin-induced fusion of BeWo cells (Figure 4, A and B). The decrease was abolished in the presence of either MT1-MMP–blocking antibody or nonspecific IgG1. Because of their intrinsic ability to form syncytia, primary trophoblasts did not require forskolin treatment to stimulate fusion.

Similar to BeWo cells, primary trophoblasts exhibited a reduced amount of both E-cadherin and secreted β-hCG in the presence of MT1-MMP–blocking antibodies (Figure 4, C and D).

Figure 3 Migration and proliferation of trophoblast model cells (ACH-3P) in the presence of MT1-MMP–blocking antibodies. A: Migration was analyzed by measuring the electrical impedance of the cells that had migrated through the pores of the permeable support membranes after pretreatment with MT1-MMP–blocking antibodies (LEM-2/15.8 and LEM-2/63.1) and nonspecific IgG1 in the same concentration. B and C: Proliferation was analyzed by measuring the electrical impedance of the cells at the bottom of the assay wells (B) and by WST-1 assay (C). Reduced proliferation was observed after pretreatment with MT1-MMP–blocking antibodies (LEM-2/15.8 and LEM-2/63.1) in the impedance-based (cell index—based) assay after 12 hours and in the WST-1 assay at 24 hours. Data are expressed as means ± SD. n = 6. *P = 0.05, **P = 0.01, and ***P = 0.001 versus control. A450, absorbance at 450 nm.
Discussion

In the present study, FGR was associated with reduced levels of MT1-MMP enzyme in the placenta. MT1-MMP was present in the trophoblast compartment throughout pregnancy and participated in migration, proliferation, and fusion of cytotrophoblasts.

Both MT1-MMP mRNA expression and active MT1-MMP enzyme were reduced in FGR placentas. To identify physiological consequences of the decreased MT1-MMP activity in the placenta in FGR, we determined potential functions of MT1-MMP in trophoblast cells. Migration, proliferation, and fusion were chosen as established key processes of trophoblasts.

Trophoblast invasion in the first trimester of pregnancy crucially determines pregnancy outcome and success, because shallow invasion is associated with early pregnancy loss, subsequent FGR, and pre-eclampsia. The migration of cells along the invasive path is a central step within the invasion process and was therefore used as a functional endpoint. Blocking MT1-MMP immediately reduced trophoblast migration in the impedance-based migration assay; however, the impedance-based proliferation assay revealed a reduction of proliferation only 8 hours after attachment. The results of the migration assay were therefore not a result of altered cell proliferation in the presence of blocking antibodies.

In contrast to the first trimester, the invasive potential of trophoblasts is not a predominant feature in the third trimester. Rather, the placenta has to grow and enlarge its surfaces. We therefore hypothesized a potential third-trimester role for MT1-MMP in cytotrophoblast proliferation and fusion. Both are required to enlarge and maintain the syncytiotrophoblast throughout pregnancy. To establish a role in proliferation, we used two methods, based on different biological properties, namely, the electrical impedance of the cell monolayer and the mitochondrial activity. Both approaches demonstrated reduced proliferation when MT1-MMP activity was blocked. The exact mechanism by which MT1-MMP promotes proliferation in trophoblasts is not clear, but it may be that cleavage products of MT1-MMP substrates in the extracellular space induce or augment proliferation, similar to cancer cells.

The trophoblast cell line BeWo is a model for study of trophoblast fusion. However, because the role of MT1-MMP in trophoblast fusion had not been described in the literature, we also verified this effect in primary trophoblasts. In the present study, we demonstrated that both forskolin-induced

Figure 4 Fusion of trophoblast model cells (BeWo) and isolated primary trophoblasts in the presence of MT1-MMP-blocking antibodies. Fusion was stimulated with forskolin in BeWo cells; primary trophoblasts isolated from term placentas spontaneously fuse without stimulation. A–D: Reduction of E-cadherin protein expression as indicator for cell fusion was determined by immunoblot analysis 48 hours after addition of 15 μg/mL blocking antibodies (LEM-2/15.8 and LEM-2/63.1) in BeWo cells and trophoblasts. Nonspecific IgG1 was added to the controls in the same concentration (15 μg/mL) as the blocking antibodies. A and C: Representative blots. B and D: Quantification of the immunoblots. E and F: The secretion of β-hCG into the supernatant by BeWo cells (E) and primary trophoblasts (F) was measured as a second parameter reflecting fusion. Data are expressed as means ± SEM of three independent experiments each performed in triplicate (BeWo cells) or duplicate (trophoblasts). *P < 0.01 versus dimethyl sulfoxide–treated IgG1 control; †P < 0.01 versus forskolin-treated IgG1 control. DMSO, dimethyl sulfoxide; mlU, milli-international units.
fusion of BeWo cells and intrinsic fusion of primary trophoblasts is reduced by MT1-MMP—blocking antibodies, indicating a contribution of MT1-MMP to this process. MT1-MMP promotes macrophage fusion through signaling events, independent of its proteolytic activity.37 The syncytium formation and syncytial differentiation—related decrease in E-cadherin and β-hCG secretion, however, was blocked by neutralizing antibodies and therefore required MT1-MMP activity.

It was surprising for us that MT1-MMP seems involved in all three key processes of trophoblast function: migration, proliferation, and fusion. These processes are tightly associated with actin reorganization and site-directed degradation.39–41 MT1-MMP is anchored in the plasma membrane, making it ideally suited for interaction with the cytoskeleton. Thus, MT1-MMP can be specifically transported and located at sites of proteolytic events.42,43 This particular feature of MT1-MMP may predestine it to participate in distinct key processes of trophoblast differentiation and function.

A limitation of the present study is the fact that only placentas at the end of FGR pregnancies were analyzed. These placentas reflect only the outcome of disturbed or inadequate placentation that had occurred earlier in pregnancy. FGR is clinically diagnosed in the second half of gestation, and potential causes for invasion defects that may have happened in early pregnancy are therefore difficult to observe. With analysis at the endpoint of pregnancy, any extrapolations to earlier stages have to be made with caution. For example, we do not know whether MT1-MMP levels are already reduced in first-trimester placentas of pregnancies that will become complicated by FGR later in pregnancy.

In the present study, we have demonstrated that key processes of trophoblast function (ie, migration, proliferation, and fusion) depend on MT1-MMP activity. However, which of these processes is indeed impaired in cytrophoblasts of FGR placentas as a result of reduced levels of MT1-MMP enzyme will have to be the focus of further studies.

References

Fetal Insulin and IGF-II Contribute to Gestational Diabetes Mellitus (GDM)-Associated Up-Regulation of Membrane-Type Matrix Metalloproteinase 1 (MT1-MMP) in the Human Feto-Placental Endothelium


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Context: Gestational diabetes mellitus (GDM)-associated hormonal and metabolic derangements in mother and fetus affect placental development and function. Indeed, in GDM, placentas are characterized by hypervascularization and vascular dysfunction. The membrane-type matrix metalloproteinase 1 (MT1-MMP) is a key player in angiogenesis and vascular expansion.

Objective: Here, we hypothesized elevated placental MT1-MMP levels in GDM induced by components of the diabetic environment. Therefore, we measured placental MT1-MMP in normal vs. GDM pregnancies, identified potential functional consequences, and investigated the contribution of hyperglycemia and the insulin/IGF axis.

Design: Immunohistochemistry identified placental cell types expressing MT1-MMP. MT1-MMP was compared between normal and GDM placentas by immunoblotting. Quantitative PCR of MT1-MMP in primary feto-placental endothelial cells (fpEC) and trophoblasts isolated from both normal and GDM placentas identified the cells contributing to the GDM-associated changes. A putative MT1-MMP role in angiogenesis was determined using blocking antibodies for in vitro angiogenesis assays. Potential GDM-associated factors and signaling pathways inducing MT1-MMP up-regulation in fpEC were identified using kinase inhibitors.

Results: Total and active MT1-MMP was increased in GDM placentas (41% and 54%, respectively, \( P < 0.05 \)) as a result of up-regulated expression in fpEC (2.1-fold, \( P = 0.02 \)). MT1-MMP blocking antibodies reduced in vitro angiogenesis up to 25% (\( P = 0.03 \)). Pathophysiological levels of insulin and IGF-II, but not IGF-I and glucose, stimulated MT1-MMP expression in fpEC by phosphatidylinositol 3-kinase signals relayed through the insulin, but not IGF-I, receptor.

Conclusions: GDM up-regulates MT1-MMP in the feto-placental endothelium, and insulin and IGF-II contribute. This may account for GDM-associated changes in the feto-placental vasculature. (J Clin Endocrinol Metab 97: 3613–3621, 2012)
and function. Despite the profound increase of GDM and the long-term consequences on the fetus, only little is known about how GDM may affect placental structure.

Maternal hyperglycemia is the hallmark of GDM and results also in fetal hyperglycemia, fetal hyperinsulinemia, and other metabolic and hormonal changes in the fetal circulation, such as elevated levels of IGF (3, 4). The maternal and the fetal circulations are in contact with two different placental surfaces. The syncytiotrophoblast is formed by fusion of cytotrophoblast cells, covers the placental villous structure, and is in contact with the maternal blood. The feto-placental vasculature within the placental villi is continuous with the fetal vasculature, lined by endothelial cells, transports the fetal blood and hence is exposed to endocrine and metabolic influences similar to the vasculature of the fetus proper.

Therefore, diabetic derangements will primarily affect these placental surfaces. Indeed, both surfaces are enlarged. The syncytiotrophoblast surface is increased and the villi are hypervascularized resulting in a larger feto-placental endothelial surface. The feto-placental vasculature is characterized by an increased diameter and hypervascularization (5, 6), and signs of endothelial and vascular dysfunction have been described (7).

The morphological and functional changes of the placenta may contribute to the altered fetal development in GDM. Short-term consequences of GDM for the fetus may include excessive fat accretion (8). Recent data show that the diabetic intrauterine environment in GDM can program the fetus and determine the development of the offspring into adulthood. Long-term consequences of GDM include a higher risk for developing metabolic disorders and vascular dysfunction (2). Genetic inheritance of risk factors as well as fetal programming in utero contribute to the increased risk (9).

The developmental processes of the placenta, i.e. angiogenesis and growth of the feto-placental vasculature as well as invasion and fusion of the cytotrophoblast require proper remodeling and breakdown of extracellular matrix. Key molecules herein are matrix metalloproteinases (MMP) (10). Dysregulation of some members of this protein family has been implicated in various pathologies as MMP1 (13, 14).

Feto-placental angiogenesis and vascular enlargement as well as trophoblast fusion occur throughout pregnancy until term (15). Therefore, these processes may be affected by the diabetic environment in GDM, which clinically manifests as glucose intolerance in the second half of gestation. However, studies demonstrate hormonal derangements both in mother and fetus already in wk 11–15. These changes include altered levels of insulin, IGF-I, and leptin (16–18), indicating that placental development may already be affected by these hormonal changes before the onset of GDM.

Membrane-type MMP1 (MT1-MMP) is a membrane-anchored MMP and plays an outstanding role in structural remodeling. Established examples are angiogenesis (19), tube formation (20), and arterial enlargement (21), all of which are key features of endothelial cells. MT1-MMP is synthesized as an inactive, 63-kDa pro-form, transported to the cell membrane, and cleaved at a furin recognition motif into the active 57-kDa enzyme. Apart from the degradation of extracellular matrix components such as collagens, fibronectin, laminin, and vitronectin (22), MT1-MMP activates other MMP, i.e. MMP1, MMP2, and MMP13. Moreover, it activates or inactivates various cytokines and chemokines by cleaving their pro-forms, e.g. pro-TGF-β and pro-TNF-α, or active forms such as IL-8 and TNF-α (23).

In this study, we tested the hypothesis that GDM is associated with elevated placental MT1-MMP levels in the third trimester. We localized MT1-MMP-expressing cells in the third-trimester placenta, identified the cells accounting for MT1-MMP expression changes, and characterized consequences of the stable alterations identified in GDM. Moreover, we discovered components of the diabetic environment and signaling pathways by which they contribute to the MT1-MMP changes.

### Subjects and Methods

**Placenta samples**

The study was approved by the institutional review board and ethical committee of the Medical University of Graz and of the Medical Faculty, University of Milan. Informed consent of the patients was obtained. After cesarean section, placentas were collected after normal and GDM pregnancies and immediately put on ice. A tissue sample of each placenta was taken within 3 cm of the cord insertion site of each placenta and snap frozen in liquid nitrogen.

**Subject characteristics**

Control pregnancies (n = 14) were nonsmoking women, with a negative 100-mg oral glucose tolerance test, free of medical or obstetrical disorders. All neonates were delivered by elective cesarean section and had a birth weight between the 10th and 90th percentile.

GDM was diagnosed at 28–32 wk of gestation with a 100-g oral glucose tolerance test with two or more values of the plasma glucose exceeding the fasting value of 5.2 mmol/L, the 1-h value of 10 mmol/L, the 2-h value of 8.6 mmol/L, or the 3-h value of 7.7 mmol/L. After diagnosis, all patients were followed according to a clinical protocol (24). The GDM women (n = 9) monitored their capillary glucose levels (memory reflectance meters, Accu-Chek; Roche Diagnostics, Mannheim, Germany) at least four times a day.
They began appropriate diet providing 25–40 kcal/day-kg according to prepregnancy body mass index. Compliance was checked by measuring maternal glycemia and fetal growth every 2 wk. If maternal blood glucose values exceeded mean fasting and postprandial values of 5 mmol/L and postprandial values of 6 mmol/L, insulin therapy was instituted (n = 2). All GDM patients were in good metabolic control with glycosylated hemoglobin below 5% and achieved target preprandial below 5 mmol/L and postprandial below 6.6 mmol/L glucose values until the last glucose control at the day before delivery. None of the women showed signs of hypertension or any other disease. The subject characteristics are shown in Table 1.

### Immunohistochemistry

Immunohistochemistry used the Ultra Vision LP detection system (Thermo Scientific, Fremont, CA) with the horseradish peroxidase polymer and the 3-amino-9-ethylcarbazole substrate following manufacturer’s instructions. Tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5 μm) were cleared with xylene, and rehydration was followed by antigen retrieval with citrate buffer (pH 6.0). Sections were incubated with monoclonal mouse anti-MT1-MMP (LEM-2/15.8 and LEM-2/63.1; Millipore). Then 8000 cells per well were added onto polymerized Matrigel in 96-well plates and incubated for 12 h at 37°C to allow for capillary-like network formation. Mitomycin C (5 μg/ml) were cleared with xylene, and rehydration was followed by antigen retrieval with citrate buffer (pH 6.0). Sections were incubated with monoclonal mouse anti-MT1-MMP (LEM-2/15.8 and LEM-2/63.1; Millipore). The fpEC were cultured and used for experiments up to passage 5.

### In vitro angiogenesis

In vitro network formation was induced using growth factor-reduced Matrigel basement membrane matrix (BD Biosciences, Bedford, MA) as recommended by the manufacturer. Primary fpEC were preincubated (30 min at 37°C) in EBM (2% FCS) containing 15 μg/ml nonspecific mouse IgG1 (BD PharMingen, Bedford, MA) or 15 μg/ml MT1-MMP blocking antibodies (Pierce, Rockford, IL). After overnight incubation, samples were centrifuged and boiled for 5 min at 99°C. The tissue was homogenized and cells were washed and lysed in buffer containing 10 mM Tris (pH 7.4), 1% sodium dodecyl sulfate, 1 mM Na-orthovanadate, and Complete protease inhibitor (Roche) mixed with an equal volume of Laemmli sample buffer (Sigma Chemical Co., St. Louis, MO). Before electrophoresis, samples were centrifuged and boiled for 5 min at 99°C. Equal amounts of protein were used for SDS-PAGE on a 10% gel (Pierce, Rockford, IL). After electrophoresis according to the manufacturer’s instructions, membranes were stained with Ponceau S to check for blotting efficiency and densitometrically scanned. Then they were blocked for 1 h with 5% (wt/vol) nonfat

### Isolation of trophoblasts and feto-placental endothelial cells (fpEC)

Primary trophoblasts and fpEC from third-trimester placentas were isolated after uncomplicated vaginal delivery from normal and GDM pregnancies as described previously (25, 26).

Trophoblasts were cultured in DMEM (5.5 mM d-glucose; Life Technologies, Inc., Paisley, UK) supplemented with 10% fetal calf serum (FCS) and tested for viability by measuring β-human chorionic gonadotropin levels secreted into the medium (Dade Behring, Deerfield, IL). Purity was determined by immunocytochemical staining for the trophoblast marker cytokeratin 7 (25). Only preparations with a purity of at least 99% and the characteristic kinetics of human chorionic gonadotropin secretion (27) were used. The fpEC were cultured on 1% (vol/vol) gelatin-coated plates using endothelial basal medium (EBM, Cambrex; Clonetics, Walkersville, MD) supplemented with EGM-MV BulletKit (Clonetics). They were characterized by internalization of acetylated low-density lipoprotein and immunohistochemical staining for the endothelial cell marker von Willebrand factor and absent staining for fibroblast-specific antigen and smooth muscle actin (26). The fpEC were cultured and used for experiments up to passage 5.

### Values are presented as mean ± SD. BMI, Body mass index.
FIG. 1. Identification of placental cells expressing MT1-MMP in the normal third-trimester placenta. Immunohistochemical staining of active MT1-MMP demonstrated high expression in the syncytiotrophoblast and cytotrophoblasts and in the endothelium of the feto-placental vessels (IV). A, overview of MT1-MMP staining in a placental villous cross-section with staining in the syncytiotrophoblast (ST), the cytotrophoblast (CT), and the feto-placental endothelium (E). Magnification, ×200. The inset (a) shows the negative control using unspecific mouse IgG. B, higher magnification more clearly shows the staining of endothelial cells (×400) marked with an arrowhead. C, Quantification (immunoblotting) of MT1-MMP protein in isolated trophoblasts (T) vs. isolated fpEC revealed expression in both cell types. D, One representative immunoblot of five. Data are presented as mean ± SEM.

Dry milk (Bio-Rad, Hercules, CA) and 0.1% (vol/vol) Tween 20 (Sigma) in 0.14 mol/liter Tris-buffered saline (pH 7.3) at room temperature, which was also used for subsequent washings and as an antibody diluent. The membranes were incubated overnight at 4 C with polyclonal antibodies against MT1-MMP (hinge region; Chemicon, Bedford, MA; 0.5 μg/ml), β-actin (Amersham, Buckinghamshire, UK; 150 ng/ml), phospho-ERK1/2 Tyr202/204 (no. 9101; Cell Signaling, Danvers, MA; 1:1000), or phospho-protein kinase B (PKB)Ser473 (no. 4050; Cell Signaling; 1:1000). After washing, membranes were incubated with the secondary antibody (Bio-Rad; 1:1000) for 1 h at room temperature. Immunolabeling was visualized using the SuperSignal chemiluminescent substrate for horseradish peroxidase substrate system (Pierce). Membranes were exposed to Hyperfilm (Amersham) and densitometrically scanned using a digital camera and the AlphaDigiDoc 1000 software within the linear range of film and camera. Signals were normalized to β-actin or to the total protein amount in the respective molecular weight range of MT1-MMP.

Real-time PCR
One microgram of total RNA isolated with TriReagent (MRC, Cincinnati, OH) was reverse transcribed to cDNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. cDNA (100 ng) was subjected to real-time PCR using FAM-labeled TaqMan gene expression assays (MMP14, Hs00237110_m1; RPL30, Hs00263497_m1) and TaqMan Universal PCR Mastermix (Applied Biosystems, Branchburg, NJ). Components were mixed according to the manufacturer’s instructions and amplified in 20 μl total volume/well (96-well plates; Roche) using an ABI7900 (Applied Biosystems) real-time cycler. Cycle threshold (Ct) values were automatically generated by the software (SDS version 2.2), and relative gene expression was calculated by the standard 2−ΔΔCt method (29) using gene expression of ribosomal protein L30 (RPL30) as reference. Statistical analyses used the ΔCt values.

Insulin/IGF and glucose treatment of fpEC
For the different treatments 40,000 cells per well were seeded in gelatin-coated 24-well plates and cultured in EBM supplemented only with 2% (vol/vol) FCS. After 24 h, medium was replaced by the same medium supplemented with insulin (0.1 and 1 nM), IGF-I (6.7 and 13 nM), IGF-II (22 and 44 nM), or d-glucose (17 and 25 mM). The basal glucose concentration of EBM was 5.5 mM, and the insulin concentration in EBM supplemented with 2% FCS was 0.002 nM. To identify receptors and pathways involved, inhibitors for the IGF-I receptor (IGF-IR) [picrotoxinin (PPP), 100 nM], phosphatidylinositol 3-kinase (PI3K) (wortmannin; 100 nM), or the ERK1/2 pathway (U0126; 10 μM) were added 1 h before treatment. IGF-I and IGF-II were purchased from R&D Systems (Minneapolis, MN) and insulin and the inhibitors from Calbiochem (Darmstadt, Germany).

To confirm that the concentrations of wortmannin and U0126 indeed blocked insulin and IGF-II-induced phosphorylation of PKb and ERK1/2, respectively, signaling experiments using antibodies against phospho-ERK1/2 Tyr202/204 and phospho-PKB Ser473 were performed. The fpEC were seeded as described above, serum starved overnight, pretreated with wortmannin (100 nM), U0126 (10 μM), or dimethylsulfoxide (DMSO) alone for 1 h, and stimulated with insulin or IGF-II (both 10 nM). After 15 min, cells were harvested and used for immunoblotting.

Data and statistical analysis
For data analysis, the immunoblots obtained from the insulin/IGF and glucose treatment of fpEC were band intensity harmonized for inter-blot variation. Each immunoblot replicate contained the same sequence of untreated (control) and treated (insulin, IGF-I, IGF-II, glucose, and inhibitor) samples, but from different cell isolations. The average signal of each blot was adjusted to 100. This allowed calculation of mean ± SD for controls and treatments as well as statistical analysis.

Statistical analysis used Sigma Stat version 3.1 (Jandel Scientific, San Rafael, CA) software. After testing for normal distribution (Kolmogorov-Smirnov), Student’s t test was used. For testing the effect of different insulin, IGF-I, and IGF-II concentrations, one-way ANOVA and Dunn’s method as post hoc test was performed. For testing the effect of the inhibitors, two-way ANOVA and Holm-Sidak post hoc test was used. Significances were accepted at a level of P < 0.05.

Results
Localization of MT1-MMP in the placenta in the third trimester of gestation
Immunohistochemistry was used to identify the cell types expressing MT1-MMP in the normal third-trimester placenta. The syncytiotrophoblast, subjacent cytotrophoblasts, and the feto-placental endothelium showed intensive and specific MT1-MMP staining (Fig. 1, A and B). Immunoblotting revealed that MT1-MMP protein was also expressed in primary trophoblasts and fpEC isolated from third trimester placentas with 90% higher levels (P < 0.001) in fpEC. (110 ±
18 arbitrary units) compared with trophoblasts (58 ± 28 arbitrary units) (Fig. 1, C and D).

**MT1-MMP expression in normal and GDM placentas**

MT1-MMP protein is expressed in the third-trimester placenta. Comparison of MT1-MMP in placentas from GDM vs. normal pregnancies by immunoblotting revealed about 50% higher levels ($P < 0.05$) in total MT1-MMP in GDM. When pro- (63 kDa) and active (57 kDa) MT1-MMP were analyzed separately, only the approximately 55% increase in the active form was significant ($P = 0.03$) (Fig. 2, A and B).

**MT1-MMP levels in fpEC and trophoblasts in normal vs. GDM pregnancies**

Furthermore, we aimed to identify whether trophoblasts, fpEC, or both account for the increased MT1-MMP levels in GDM. Therefore, primary fpEC and trophoblasts were isolated from normal and GDM pregnancies, and their MT1-MMP expression was compared using quantitative RT-PCR (Fig. 2C). The only significant change was up-regulation of MT1-MMP in fpEC in association with GDM (fold change 2.1; $P = 0.02$).

**MT1-MMP function in in vitro angiogenesis of fpEC**

To identify potential consequences of the up-regulated MT1-MMP in the feto-placental endothelium, we determined the role of MT1-MMP in fpEC. Angiogenesis as a key process of endothelial cells was chosen as an endpoint, and in vitro angiogenesis assays were performed with and without inhibition of MT1-MMP action. Because chemical MMP inhibitors often interact also with other proteases and are thus not absolutely specific, we used blocking antibodies. To increase credibility of the results, two different clones of blocking antibodies were used. Unspecific mouse IgG of the same isotype was added to the controls. Addition of both MT1-MMP blocking antibodies significantly reduced the total tube length by 20.0 and 22.5% (Fig. 3A) as well as the number of branching points by 21.0 and 24.8%, respectively (Fig. 3B).

**Regulation of MT1-MMP expression in fpEC by insulin, IGF-I, IGF-II, and glucose**

We further aimed to identify potential factors of the diabetic environment to account for MT1-MMP up-regulation in the feto-placental endothelium in the third trimester. The concentrations of several metabolites are altered in the fetal compartment resulting from maternal GDM, e.g. glucose, amino acids, and nonesterified fatty acids (24, 30, 31). In GDM, the fetus may be exposed to hyperglycemia, which may result in a deregulation of the insulin/IGF axis. High glucose as well as insulin/IGF are known to alter, directly or indirectly, expression of some MMP in different cell types (13, 14). Therefore, fpEC were cultured in presence of insulin, IGF-I, IGF-II, or glucose. Only the 57-kDa band of the active MT1-MMP was detected when isolated cells were analyzed by immunoblotting. Insulin and IGF-II increased MT1-MMP expression in fpEC by 22 ± 18 and 157 ± 26%, whereas IGF-I and glucose had no effect (Fig. 4, A and B). This insulin and IGF-II effect was blocked by pretreatment with the PI3K inhibitor wortmannin but not by the ERK1/2 pathway inhibitor (U0126). Wortmannin indeed blocked insulin- and IGF-II-stimulated phosphorylation of PkB at...
Ser473, whereas U0126 fully abolished any ERK1/2 phosphorylation at Tyr202/204 (Fig. 4C). To identify which receptor signals the IGF-II effect, the IGF-IR inhibitor PPP was added. Currently, no inhibitor for the insulin receptor is available. The blocking effect of PPP on IGF-IR signaling was demonstrated by the inhibition of IGF-stimulated in vitro angiogenesis of fpEC in presence of PPP (data not shown). PPP did not abolish the IGF-II-induced MT1-MMP up-regulation; thus, the insulin receptor, but not the IGF-IR, accounted for the IGF-II effect (Fig. 4, D and E), which, like the insulin effect itself, involves pathways downstream of PI3K.

**Discussion**

The present study reveals two major findings. 1) GDM affects the fetal environment in such a way that MT1-MMP, a key player in vascularization, angiogenesis, and arterial enlargement, is up-regulated. 2) MT1-MMP is elevated only in the feto-placental endothelium, suggesting the diabetic environment in the fetal rather than maternal circulation induces its up-regulation.

One of the potential functions of MT1-MMP in endothelial cells is its contribution to angiogenesis, which was shown by MT1-MMP overexpression in bovine aortic en-
endothelial cells (32). Angiogenesis is a key function of fpEC in the placenta throughout pregnancy (15) and might involve MT1-MMP. This hypothesized role of MT1-MMP in fpEC was indeed verified by reduced two-dimensional network formation using MT1-MMP blocking antibodies. In vitro angiogenesis is a useful tool to determine and compare the effect of treatments on individual endothelial cell isolations, in which the untreated cells serve as internal control. However, basal in vitro angiogenesis varies profoundly between different cell isolations, thus precluding its comparison between fpEC from normal vs. GDM pregnancies.

The observed changes represent long-term effects of maternal GDM on the feto-placental endothelial cells because changes were still present after five passages in culture. It was already shown that endothelial cells isolated from diabetic donors remember their endothelial dysfunction in culture (33). GDM can induce epigenetic changes in various fetal organs (9), including the placenta (34). However, whether this is the underlying cause for the stable up-regulation of MT1-MMP in fpEC isolated from GDM pregnancies will be the focus of additional studies. Because hyperglycemia and insulin/IGF regulate some MMP (13, 14), the effect of hyperglycemia and the insulin/IGF axis on MT1-MMP expression of fpEC in vitro was determined. The insulin, IGF-I, and IGF-II concentrations chosen were to mimic the physiological and pathological concentrations of the growth factors in cord plasma of normal and GDM pregnancies (3, 4). The glucose levels chosen are commonly used for experiments mimicking hyperglycemia (35). Both insulin and IGF-II stimulated MT1-MMP production in endothelial cells. This effect was dependent on the PI3K/PkB pathway. The IGF-IR inhibitor PPP did not abolish the IGF-II effect. This strongly suggests that the IGF-II signal is predominantly relayed by the insulin receptor of which particularly the isoform A (IR-A) is a signaling receptor for IGF-II (36). The contribution of the IGF-IR, if any, will be rather minor, which is supported by an absent MT1-MMP increase by IGF-I treatment, a growth factor that exclusively binds to the insulin receptor isoform A (IR-A) is a signaling receptor for IGF-II (36). The role of PI3K/PkB signaling in the regulation of MT1-MMP expression is in line with data from tumor cells, in which mammalian target of rapamycin was identified as a downstream link between PI3K and MT1-MMP regulation (37).

The study is limited by the fact that tissues and cells were obtained at the end of pregnancy, and any extrapolations to earlier stages have to be made with caution. It may well be that placental development is altered already before GDM clinically manifests in the second half of gestation because some metabolic alterations occur already in earlier phases of gestation as indicated by higher levels of maternal glucose, insulin, and growth factors already in the first trimester (17, 18). Furthermore, we previously demonstrated that placental MT1-MMP is sensitive toward the alterations of insulin and IGF-II levels in T1D already during the first trimester (13). Because placental vascularization is most pronounced in the third trimester (15), it is likely to be sensitive to changes in the in utero environment, which will manifest as hypervascularization found in all studies at the end of diabetic pregnancies. Placental vascularization may thus be affected by the diabetic environment in the first and third trimester.

The study clearly shows that not only the GDM-associated changes in the maternal environment but also the resulting derangements in the fetal circulation can induce modifications in the placenta. Besides insulin and IGF-II as identified here, other alterations including fetal hypoxia and hyperleptinemia as well as elevated fetal fibroblast growth factor 2 levels may contribute (38). On the basis of the present data, it is tempting to speculate that the MT1-MMP elevation in placental endothelial cells of GDM pregnancies may lead to morphological and functional changes also in the fetus vasculature. Hence, this study fuels into the growing body of evidence that maternal diabetes contributes to fetal endothelial and vascular dysfunction. This notion is supported by studies using animal models as well as human feto-placental and umbilical endothelial cells and vessels (39, 40). Investigating these endothelial and vascular changes may help in understanding the impact of diabetes on endothelial cell function.
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