Mucin 15 is expressed in human placenta and suppresses invasion of trophoblast-like cells in vitro

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BACKGROUND: Trophoblast invasion is crucial for the development of normal placentas. Mucins are suggested to be involved in cancer invasion. However, the function of mucins in trophoblast invasion has never been reported. This study was to investigate the expression of mucin (MUC) 15 in human placenta and its role in trophoblast invasion.

METHODS: MUC15 mRNA in human tissues was analyzed by Northern blot. MUC15 mRNA and protein in human placenta were detected by real-time RT-PCR and Western blot, respectively. The distribution of MUC15 was revealed by immunohistochemistry. The effects of MUC15 on trophoblast invasion in vitro were analyzed by matrigel invasion assay in human choriocarcinoma JAR and JEG-3 cells. RESULTS: MUC15 was expressed most highly in human placenta. MUC15 mRNA and protein increased with gestational age (P < 0.05, first versus third trimester). Immunohistochemistry showed that MUC15 protein was expressed by both cytotrophoblasts and syncytiotrophoblasts, especially at the apical membrane of syncytiotrophoblasts. In addition, MUC15 was found to be present in the glandular epithelium of the decidua. Overexpression of MUC15 substantially decreased matrigel invasion of JAR and JEG-3 cells by 87.5 and 83.8%, respectively, versus control, which was closely associated with an increase in mRNA expression of tissue inhibitors of metalloproteinases (TIMP)-1 and TIMP-2. Knockdown of MUC15 with small interfering RNA significantly reversed these effects (P < 0.05). CONCLUSIONS: Differential expression of MUC15 in human placentas may play a critical role in the regulation of trophoblast invasion.

Keywords: mucin; trophoblast; invasion; tissue inhibitor of metalloproteinases; placenta

Introduction

During placental development, fetal cytotrophoblasts proliferate to form aggregates known as columns that anchor peripheral villi to the maternal decidua. From these columns, extravillous trophoblasts invade the uterine wall and remodel the maternal spiral arteries by displacing smooth muscle and endothelial cells (Pijnenborg et al., 1983). The stringent regulation of trophoblast invasion is required for appropriate blood vessel remodeling in the maternal–fetal interface and is essential for maintaining a normal pregnancy. Disturbed remodeling is associated with pre-eclampsia and intrauterine growth restriction (Gerretsen et al., 1981; Zhou et al., 1993). Trophoblast invasion shares common biochemical mechanisms with tumor invasion. In contrast to tumor invasion of a host tissue, trophoblast invasion during implantation is strictly spatiotemporally regulated. Identification of factors responsible for these regulatory processes will contribute to a better understanding of this unique developmental process.

Mucins (MUC) are heavily glycosylated proteins expressed by various epithelial cell types. According to the cellular localization, mucins are divided into two classes: membrane-bound mucins and secretory mucins (Singh and Hollingsworth, 2006). The secretory mucins, which lack a transmembrane domain and are secreted into extracellular spaces, include MUC2, MUC5AC, MUC5B, MUC6, MUC7, MUC8, and MUC19. The expression of secreted mucins is restricted to secretory organs and cell types. The membrane-bound mucins, which are type I membrane proteins, include MUC1, MUC3, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17, and MUC20 (Singh and Hollingsworth, 2006). Membrane-bound mucins are postulated to serve as sensors of the external environment. Signals from the extracellular matrix are transmitted to the interior of cells through the cytoplasmic tail of mucins. Alterations in membrane-bound mucin expression accompany the development of cancer and influence cellular growth, differentiation, transformation, adhesion, and invasion.
(Hollingsworth and Swanson, 2004). For example, MUC1 overexpression is associated with invasive and metastatic tumors of the colon, pancreas, gall bladder, and oral epithelium (Nakamori et al., 1994; Hiraga et al., 1998; Kashiwagi et al., 2000; Nitta et al., 2000; Lutteges et al., 2002). Furthermore, MUC1 can regulate the invasive and metastatic activities of tumor cells (Kohlgraf et al., 2003). In the female reproductive tract, MUC1 is expressed at the apical surface of the uterine epithelium under most conditions in many species (Carson et al., 1998) and is thought to regulate embryo attachment to the uterine epithelium via its anti-adhesive and/or adhesive activity (Carson et al., 1998; Carson et al., 2006; DeLoia et al., 1998). Of the known membrane-bound mucins, MUC3 has been reported to be expressed in trophoblasts of human placentas (Jeschke et al., 2002). MUC3 is most highly expressed in intestine and gallbladder (Audie et al., 1998). Of the known membrane-bound mucins, MUC3 can promote cell migration, inhibit apoptosis, and accelerate wound healing (Ho et al., 1998). MUC4 overexpression has been observed in many carcinomas of the breast, lung, pancreas, and colon (Moniaux et al., 2004; Nagy et al., 2005). Inhibition of MUC4 expression suppresses pancreatic tumor cell growth and metastasis (Singh et al., 2004). In addition, MUC4 was found to regulate cell behavior via modulating erythroid leukemia viral oncogene homolog 2 (ErbB2) and extracellular signal-regulated kinase (ERK) signaling (Carraway et al., 1999; Jeppson et al., 2002; Ramsauer et al., 2003, 2006; Pino et al., 2006). Furthermore, MUC20 mRNA was recently found to be highly expressed in kidneys and moderately expressed in human placentas by Northern blot (Higuchi et al., 2004a). Production of MUC20 reduces hepatocyte growth factor-induced matrix metalloproteinase (MMP) expression and proliferation of human embryonic kidney HEK293 cells (Higuchi et al., 2004b). Although mucins play important roles in many cellular properties, the roles of mucins in trophoblasts are still unknown.

MUC15 is a membrane-bound mucin which was originally isolated from bovine milk fat globule membranes (Pallesen et al., 2002). The mRNA of MUC15 has been detected by RT–PCR in various human tissues (Pallesen et al., 2002; Russo et al., 2006). The predicted protein product of human MUC15 (334 amino acids) contains an extracellular domain, a small transmembrane domain, and a highly conserved cytoplasmic tail. In addition, a splice variant of MUC15 encoding a secreted mucin has been detected (Pallesen et al., 2002). But, there is no documentation of the existence of the splice variant at the protein level. So far, physiological functions of MUC15 have never been investigated.

Although membrane-bound mucins have been studied in various cancers (Hollingsworth and Swanson, 2004), little is known about their expression and function in placenta, where their cell invasion and signaling properties could be important in early embryonic development. Here, we analyzed mRNA expression of membrane-bound mucins in human placenta and found that MUC1, MUC3, MUC15, and MUC20 were highly expressed. The tissue distributions and biological functions of MUC15 were poorly understood. We found that, among the tissues tested, MUC15 was expressed at the highest level in human placenta. We therefore investigated the expression pattern of MUC15 in human placenta and its role in trophoblast invasion.

Materials and Methods

Northern blot

Multiple Tissue Northern (MTN™) Blot was purchased from Clontech (BD Biosciences, CA, USA). The blot was probed with 32P-labeled random-primed full-length MUC15 complementary DNA (cDNA). Hybridization was performed at 65°C overnight. The blot was washed at 55°C for 1 h in a buffer containing 2× standard saline citrate and 0.5% sodium dodecyl sulphate (SDS), and signals were visualized by autoradiography using Kodak BioMax film.

Clinical tissue collection

The first trimester (n = 7, 8–13 weeks gestation), second trimester (n = 7, 17–25 weeks gestation), and third trimester (n = 13, 33–40 weeks gestation) placentas were obtained from the Department of Obstetrics and Gynecology, National Taiwan University Hospital. The use of human placentas for this study was approved by the local hospital ethic committees and written consent was obtained from patients before the collection of samples. The placental tissue sections were obtained by dissecting a 1.5-cm square segment, 0.5-cm thick, from the maternal part of placentas. For immunohistochemistry, specimens were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) at −20°C and for use in the RT–PCR and Western blot analysis, the samples were stored in RNA later (Qiagen, CA, USA) and at −80°C, respectively, until use.

Cell lines and cell culture

Human choriocarcinoma cell lines JEG-3 and JAR were purchased from BCR (Hsinchu, Taiwan). Cells were maintained with Dulbecco’s modified Eagle’s medium (DMEM) (Biowest, Florida, USA) containing 10% fetal bovine serum (FBS) (PAA Laboratories, Linz, Austria), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Biowest) in a humidified tissue culture incubator at 37°C, in 5% CO2 atmosphere.

RNA extraction and RT–PCR

Total RNA was extracted with Trizol reagent (Invitrogen, California, USA) according to the manufacturer’s protocol. Two micrograms of total RNA were reverse-transcribed into cDNA in a total volume of 25 µl using StrataScript reverse transcriptase (Stratagene, CA, USA). In both conventional and quantitative PCR, we used a 2-µl aliquot of the cDNA and the following primer sets. PCR primers are indicated in Table 1 (Bernacki et al., 1999; Williams et al., 1999; Argueso et al., 2002, 2003; Gipson et al., 2003; Higuchi et al., 2004a; Huang et al., 2006). PCR products were run on a 2% agarose gel and visualized with ethidium bromide.

Generation of MUC15 recombinant proteins in Escherichia coli

RT–PCR was performed for cloning the partial human MUC15 (GenBank Accession No. BC020912) from normal human colon total RNA. The sense primer was 5’-GGg gta ccG ACA tAA ACA CAA CAC AG-3’, and the anti-sense primer was 5’-CGG tgc gAG GGG ATC TGA CGT ATT TGG-3’. The PCR products were cloned into pET30a(+) (Novagen, Madison, WI, USA) to generate the MUC15-His fusion gene. The insert was confirmed to be correct by DNA sequencing. To express MUC15 recombinant proteins, pET30a/MUC15 plasmids were transformed into E. coli BL21 and MUC15-His expression was induced by 1 mM
The anti-sense primer was 5'-GAA GTA CG-3'.

RT–PCR was performed for cloning the full-length human MUC15 (St. Louis, MO, USA).

Cell invasion assays were analyzed in a BD BioCoat Invasion Chamber (BD Biosciences, Massachusetts, USA) according to the manufacturer’s protocol and as described previously (Huang et al., 2007). Briefly, JAR (5 × 10^5) or JEG-3 (2 × 10^5) cells were transiently transfected with 5 μg of pcDNA3.1/myc-His control plasmids (Mock) or MUC15/pcDNA3.1/myc-His (MUC15) with Lipofectamine 2000 (InVitrogen). After 48 h of transfection, cells were harvested for experiments.

**Construction of plasmids**

RT–PCR was performed for cloning the full-length human MUC15 (BC020912) from normal human colon total RNA. The sense primer was 5'-CGG GAT CCC GAC AAT GTT GGC CTT AGC C-3', and the anti-sense primer was 5'-GCT CTA GAG CCG TTC CAT ACA-3'.

**Matrigel invasion assays**

Cell invasion assays were analyzed in a BD BioCoat Matrigel Invasion Chamber (BD Biosciences, Massachusetts, USA) according to manufacturer’s protocol and as described previously (Huang et al., 2007). Briefly, JAR (5 × 10^5) or JEG-3 (2 × 10^5) cells were transiently transfected with 4 μg of pcDNA3.1/myc-His control plasmids (Mock) or MUC15/pcDNA3.1/myc-His (MUC15) with Lipofectamine 2000 (InVitrogen). After 48 h of transfection, cells were harvested for experiments.

**Table 1: Primers used for PCR analysis**

<table>
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<tr>
<th>Gene</th>
<th>Primer sets</th>
<th>Product size, bp</th>
<th>Reference</th>
<th>GenBank accession number</th>
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<td>MUC1</td>
<td>F: 5'-CGA TCG TAG CCC CTA TGA GA-3'</td>
<td>220</td>
<td></td>
<td>BC12094</td>
</tr>
<tr>
<td></td>
<td>R: 5'-TGA GCA GCC CAC CTG AAC TC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUC3</td>
<td>F: 5'-CCT CAT TGC AAA CTT CAC TC-3'</td>
<td>234</td>
<td>Bernacki et al. (1999)</td>
<td>AF857194</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AGC CCA CAT TTT CTG TAC TG-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUC4</td>
<td>F: 5'-GCC CAA GCT ACA GTG TGA ACT CA-3'</td>
<td>102</td>
<td>Argueso et al. (2002)</td>
<td>AF588803</td>
</tr>
<tr>
<td></td>
<td>R: 5'-ATG GTG CCG TTT TAA TTT GTT GT-3'</td>
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<tr>
<td>MUC12</td>
<td>F: 5'-TGA AGG GCC ACA ACG CTC CTC-3'</td>
<td>511</td>
<td>Williams et al. (1999)</td>
<td>XM_499351</td>
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<td></td>
<td>R: 5'-TAC AGG AGG CTC TTG GCC ATG TTG-3'</td>
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<tr>
<td>MUC13</td>
<td>F: 5'-TGC TTC TAT CCC CAC GAA AAT GGA-3'</td>
<td>73</td>
<td>Gipson et al. (2003)</td>
<td>AF286113</td>
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<td>MUC15</td>
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<td>347</td>
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<td>BC020912</td>
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<tr>
<td></td>
<td>R: 5'-GCA ATG AGA AGA CCA GAA TA-3'</td>
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<td>MUC15-Ex4</td>
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<td>Membrane-bound: 255</td>
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<td>NM_145650</td>
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<tr>
<td></td>
<td>R: 5'-CAT AAG GGT CCG GTG CAT TG-3'</td>
<td>secreted: 102</td>
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<td>MUC16</td>
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<td>114</td>
<td>Argueso et al. (2003)</td>
<td>AF361486</td>
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<td>R: 5'-GTT GTT ACC CCA TGG CTT TTG-3'</td>
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<td>MUC17</td>
<td>F: 5'-GGG CCA GGA TAG CTA CGA-3'</td>
<td>91</td>
<td>Gipson et al. (2003)</td>
<td>AF430017</td>
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<td>MUC20</td>
<td>F: 5'-AAC TCC ACC CCC ACG CCG CT-3'</td>
<td>360</td>
<td>Higuchi et al. (2004a)</td>
<td>AB098731</td>
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<td></td>
<td>R: 5'-GGA AGC ACA CAG ATG GGT G-3'</td>
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<td>MMP-2</td>
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<td>R: 5'-TAC TCT ACA CGG ACC ACT TG-3'</td>
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<tr>
<td>MMP-9</td>
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<td>242</td>
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<td>XM_00113262</td>
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<tr>
<td>β-actin</td>
<td>F: 5'-GCT CGT GTG CGA CAA CGG CT-3'</td>
<td>326</td>
<td>Huang et al. (2006)</td>
<td>NM_001101</td>
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</table>

isopropyl-beta-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, MO, USA).

**Antibody generation**

The anti-human MUC15 polyclonal antibody was generated by immunizing rabbits with keyhole limpet hemocyanin (Pierce, Illinois, USA) conjugated with a synthetic N-terminal peptide of MUC15: hSPLNLPNSSHGITDIFS (MDBio Inc., Taipei, Taiwan). Briefly, 0.5 mg of conjugated peptide was used for each immunization, and nine boosts were performed to enhance titer. Anti-serum was affinity purified with CNBr-sepharose beads conjugated with the MUC15 peptide.

**Immunohistochemistry**

Human placental sections were de-paraffinized in xylene and re-hydrated in a series of graded alcohols. After rehydrating the sections with distilled water, the sections were rinsed three times with PBS and then incubated with 5% non-fat milk/PBS for 30 min to reduce non-specific bindings. Sections were incubated with primary antibodies, an anti-MUC15 polyclonal antibody (1:400), anti-cytokeratin (CK) 7 monoclonal antibody (1:100; Santa Cruz Biotechnology, CA, USA), or anti-Ki67 monoclonal antibody (1:100; Santa Cruz Biotechnology), diluted with 5% non-fat milk/PBS for 16 h at 4°C. After rinsing twice with PBS, Super Sensitive link-Label IHC detection System (BioGenex, California, USA) was used and the specific immuno-staining was visualized with 3,3-diaminobenzidine liquid substrate system (Sigma). All sections were counterstained with hematoxylin for 1 min and mounted with UltraKitt (J.T. Baker, Deventer, Holland). Negative controls were performed by replacing primary antibodies with an isotype matched control IgG at the same concentration.
**Quantitative real-time PCR**

Quantitative PCR System Mx3000P (Stratagene) was used to analyze MUC15 expression in human placentas according to manufacturer’s protocol. Briefly, reactions were performed in a 25-μl volume with 2 μl cDNA, 400 nM each of sense and anti-sense primers, and 12.5 μl Brilliant®SYBR®Green QPCR Master Mix (Stratagene). PCRs were incubated for 15 min at 95°C followed by 40 amplification cycles with 30-s denaturation at 95°C, 50-s annealing at 54°C, 30-s extension at 72°C. Samples were analyzed in triplicate, and product purity was checked through dissociation curves at the end of real-time PCR cycles. Relative quantity of MUC15 expression normalized to β-actin was analyzed with MxPro Software (Stratagene).

**Western blot**

Human placental tissues were homogenized in lysis buffer containing 1% Triton X-100, 20 mM Tris–HCl (pH 8.0), 160 mM NaCl, 1 mM CaCl₂, 1 mM phenylmethylsulfonyl fluoride (Huang et al., 2006). Insoluble material was removed by centrifugation at 10 000 g for 15 min. Fourty micrograms of proteins were separated by electrophoresis on a 6% SDS-polyacrylamide gel and transferred to a Hybond enhanced chemiluminescence nitrocellulose membrane (GE Healthcare, UK). MUC15 proteins were detected with rabbit anti-MUC15 polyclonal antibodies. Actin was used as an internal control and detected with anti-actin monoclonal antibodies (BD Pharmigen, California, USA). Bands were visualized by incubation with horse-radish peroxidase conjugated secondary antibodies (Vector Laboratories, California, USA) and chemiluminescence reagents (GE Healthcare). The MUC15 signals were quantified and normalized to actin signals by ImageQuant 5.1 software (Molecular Dynamics, CA, USA).

**SiRNA knockdown of MUC15 expression**

Small interfering RNA (siRNA) oligonucleotides against MUC15 and control siRNA were synthesized by Dharmacon Research, Inc. (Illinois, USA) using their custom SMARTpool and non-targeting siRNA pool, respectively. For knockdown of MUC15, JAR and JEG-3 cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen) with a final concentration of 100 nmol siRNA. The cells were incubated for 4 h and then serum-free DMEM medium was replaced with complete DMEM medium (10% FBS). After 48 h incubation, cells were harvested for analysis. The knockdown of MUC15 expression was confirmed by real-time RT–PCR and western blot.

**Gelatin zymography**

Conditioned media of transfected cells cultured in serum-free DMEM for 48 h were electrophoresed on an 8% SDS-polyacylamide gel co-polymerized with 1% gelatin. After electrophoresis, the gels were washed twice for 30 min in 2.5% Triton X-100 and incubated for 16 h in developing buffer (50 mM Tris, 200 mM NaCl, 10 mM CaCl₂, and 0.05% Brij35, pH 7.5). After incubation, gels were stained with Coomassie Brilliant Blue R-250 (0.5% in 50% methanol, 10% glacial acetic acid) for 2 h and destained in 10% glacial acetic acid and 20% methanol.

**Statistical analysis**

For statistical analyses, Mann–Whitney U-test or student’s t-test was used. Data are presented as mean ± SD. P < 0.05 was considered statistically significant.

**Results**

**Expression patterns of MUC15 mRNA in human tissues**

To determine the membrane-bound mucin profile of human placentas, gene transcripts for MUC1, 3, 4, 12, 13, 15, 16, 17, and 20 were analyzed by RT–PCR (Table 1, Fig. 1A). We found that MUC1, 3, 15, and 20 were highly expressed in term placentas. MUC13 and 17 were expressed at moderate levels. In contrast, MUC4, 12, and 16 were not detected.

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**Figure 1:** Expression of MUC15 in human tissues

(A) Expression of membrane-bound mucins in the human placenta at term. RT–PCR was used to detect membrane-bound mucins MUC1, 3, 4, 12, 13, 15, 16, 17, and 20, as indicated. (B) Northern blot of MUC15 in human tissues. Multiple human tissue Northern blot (MTN I) was probed with a 32P-labeled probe corresponding to full-length MUC15 complementary DNA. Note that MUC15 mRNA was expressed most abundantly in the placenta. (C) Expression of membrane-bound and secreted forms of MUC15 mRNA in human term placentas. Membrane-bound (m) and secreted (s) forms of MUC15 transcripts were detected by RT–PCR, as indicated by arrows (255 bp and 102 bp). Markers are shown at the left
forms of primers which can amplify membrane-bound and secreted human placenta. Secreted forms of MUC15 this was significantly higher (expression reached its peak at the third trimester and MUC15 formed. The results revealed that mRNA for both membrane-bound and secreted forms of MUC15 during placental development. Indication that MUC15 mRNA is highly expressed in the human placenta.

Next, we analyzed whether human placentas expressed secreted forms of MUC15 transcripts. We designed specific primers which can amplify membrane-bound and secreted forms of MUC15 with different sizes by RT–PCR (Table 1, Fig. 1C). Our results suggest that human placentas express mRNA for both membrane-bound and secreted forms of MUC15.

Expression of MUC15 mRNA in human placentas: real-time RT–CR analysis
To investigate expression levels of MUC15 in the development of human placentas, quantitative real-time RT–PCR was performed. The results revealed that MUC15 mRNA gradually increased with gestational week (Fig. 2A). The expression of MUC15 mRNA at the second trimester was significantly higher \( (P < 0.05) \) than that of the first trimester. And, the MUC15 expression reached its peak at the third trimester and this was significantly higher \( (P < 0.05) \) than that of the first and second trimester. The representative amplification plots of real-time RT–PCR were shown in Fig. 2B. These results indicate that MUC15 expression is significantly increased during placental development.

Differential expression of MUC15 protein in human placentas
Next, we analyzed expression of MUC15 protein in placentas in early pregnancy and at term by western blot. First, we tested the specificity of anti-MUC15 polyclonal antibodies by using E. coli lysates with or without induction of recombinant MUC15 proteins \( (~37 \text{kDa}) \). Our results showed that anti-MUC15 polyclonal antibodies only recognized E. coli lysates with the induction of MUC15 protein expression \( (+ \text{ IPTG}, \text{Fig. 3A}) \), indicating the high specificity of the anti-MUC15 polyclonal antibodies. The results of western blot showed that placentas at term expressed much higher levels of MUC15 than those in early pregnancy (Fig. 3B and C). In agreement with the results from real-time RT–PCR, placental tissues in early pregnancy expressed relatively low levels of MUC15. In addition, the protein band of MUC15 in placentas was smear and located at \( ~120 \text{kDa} \), which is consistent with previous findings. These results further demonstrate that MUC15 is differentially expressed in human placentas at different gestational ages.

Immunohistochemistry of MUC15 in the human placenta and decidua
To investigate MUC15 protein expression in placental development, human placental tissues at different gestational ages were analyzed by immunohistochemistry. In first trimester, we found that syncytiotrophoblasts and cytotrophoblasts showed positive staining of MUC15 protein (Fig. 4A–C). CK7 and Ki67 were used as a cytotrophoblast marker (Fig. 4B) and proliferation marker (Fig. 4C), respectively. In second trimester placentas, MUC15 was expressed in syncytiotrophoblasts, but not extravillous trophoblasts (Fig. 4D–E). The negative control did not show any staining (Fig. 4F). In third trimester placentas, MUC15 protein was intensely stained on the apical surface of syncytiotrophoblasts (Fig. 4G). No staining was observed in stromal cells of the placental villi. In the decidua, MUC15 was not detected in the

Figure 2: Quantitative real-time RT–PCR analysis of MUC15 expression in human placentas at different gestational ages
(A) Real-time RT–PCR revealed that MUC15 mRNA expression in placentas was increased from the first (first Tri) to the third trimester (third Tri). The real-time RT–PCR signals of MUC15 were normalized to β-actin and analyzed with MxPro Software. \( n \) indicates the placenta number. Mann–Whitney U-test was used for statistical analyses of relative MUC15 mRNA expression. Results are presented as mean \( \pm \) SD. \* \( P < 0.05 \); \** \( P < 0.01 \). (B) Representative amplification plots are shown. The arrows indicate the amplification plots of MUC15 for the first (first Tri), second (second Tri), and third trimester (third Tri). β-actin signals were used as internal controls.
MUC15 overexpression inhibits invasion of trophoblast-like JAR and JEG-3 cells

To investigate the effects of MUC15 expression on trophoblast invasion, matrigel invasion assays were carried out in choriocarcinoma JAR and JEG-3 cells, which express very low levels of MUC15 and are widely used cell lines for studying trophoblast invasion. JAR and JEG-3 cells were transiently transfected with pcDNA3.1 (Mock), MUC15/pcDNA3.1 (MUC15), MUC15/pcDNA3.1 + control siRNA (MUC15 + control siRNA), or MUC15/pcDNA3.1 + MUC15 siRNA (MUC15 + MUC15 siRNA) for 48 h and seeded into invasion chambers for another 48 h. The overexpression and knockdown of MUC15 mRNA and protein were confirmed by real-time RT–PCR and western blot (data not shown). We found that overexpression of MUC15 substantially decreased invasion of JAR and JEG-3 cells by 87.5 ± 1.1 and 83.8 ± 5.7%, respectively (Fig. 5), and this decrease was significantly (P < 0.05) blocked by MUC15 siRNA, but not control siRNA. These results suggest that MUC15 overexpression can suppress trophoblast-like cell invasion.

MUC15 overexpression increases TIMP-1 and TIMP-2 expression, but does not affect MMP-2 and MMP-9 activity

To investigate the effect of MUC15 on MMP activity in JAR and JEG-3 cells, gelatin zymography was performed to detect the gelatinase MMP-2 and MMP-9 activity. We found that MUC15 overexpression did not significantly affect MMP-2 and MMP-9 activity in both JAR and JEG-3 cells, as determined by gelatin zymography (Fig. 6A and B). In addition, MMP-2 and MMP-9 mRNA were not significantly changed as revealed by real-time RT–PCR (data not shown).

Our data showed that MUC15 overexpression suppressed cell invasion. However, we could not detect significant changes of MMP-2 and MMP-9 activity. Since TIMP-1 and TIMP-2 are the major natural inhibitors of MMP-9 and MMP-2, respectively, we therefore examined whether MUC15 expression can affect TIMP-1 and TIMP-2 expression. Interestingly, results of real-time RT–PCR showed that MUC15 overexpression increased TIMP-1 and TIMP-2 mRNA expression in JAR cells by an average of 4.1- and 3.1-fold, respectively (Fig. 6C). In addition, TIMP-1 and TIMP-2 mRNA expression in JEG cells were increased by an average of 4.7- and 3.7-fold, respectively (Fig. 6D). To confirm the effect of MUC15 overexpression on the induction of TIMP-1 and TIMP-2 mRNA expression, MUC15 siRNA smart pool was used to knockdown MUC15 overexpression in JAR and JEG-3 cells. We found that the induction by MUC15 of TIMP-1 and TIMP-2 expression in both JAR and JEG-3 cells was significantly blocked by the knockdown of MUC15 expression (Fig. 6C and D). These results suggest that MUC15 overexpression can induce TIMP-1 and TIMP-2 mRNA expression in both JAR and JEG-3 cells.

Discussion

In the present study, MUC15 is most abundantly expressed in the placenta, moderately in the kidney and lung, whereas little or no expression is found in the other tested tissues. This implies that MUC15 plays a crucial role in placentas. This study demonstrates that MUC15 is differentially expressed in human placentas throughout gestation. Both MUC15 mRNA and protein are maximally expressed in term placentas. Results of immunohistochemistry further reveal that trophoblasts of placentas in early pregnancy express lower levels of MUC15 than those at term. It has been demonstrated that trophoblasts isolated from placentas in early pregnancy have higher invasive ability than those from term placentas (Dokras et al., 2002). These results indicate that expression levels of MUC15 are inversely correlated with luminal epithelium, blood vessels, and decidual cells (Fig. 4H). In contrast, MUC15 was present in the glandular epithelium (Fig. 4I). Similar results were also found in placental tissues stained with another two polyclonal antibodies, which were generated by immunizing rabbits with C-terminal peptide and recombinant protein of MUC15 (data not shown). These results indicate that the expression of MUC15 protein in syncytiotrophoblasts is increased during placentation development.

Figure 3: Expression of MUC15 protein in human placenta
(A) Specificity of anti-human MUC15 polyclonal antibodies. Lysates from E. coli (BL21) with (+) or without (−) IPTG induction of MUC15 recombinant protein expression (37 kDa) were western blotted with anti-MUC15 polyclonal antibodies. Only E. coli lysates with MUC15 recombinant protein expression showed positive signals. The arrow indicates MUC15 recombinant proteins. The arrow head indicates the aggregated MUC15. Marker scales (M) are shown at the left. (B) Western blot of MUC15 in human placenta. Protein extracts of human placental tissues from the first trimester (gestational age: 8, 9, 10, and 13 weeks) and third trimester (gestational age: 38, 39, 39, and 38 weeks) were subjected to SDS-PAGE analysis under reducing conditions and western blotted with anti-human MUC15 polyclonal and anti-actin monoclonal antibody. A representative immunoblot is shown. Molecular weight markers are shown at the right. Actin was used as an internal control (lower panel). (C) The MUC15 signals were quantified and normalized to actin signals by ImageQuant 5.1 software. *P < 0.05, compared with the signal of first trimester (first Tri) placenta
trophoblast invasive ability. Furthermore, MUC15 overexpression significantly suppresses invasion of trophoblast-like JAR and JEG-3 cells, suggesting that MUC15 could act as a negative regulator of trophoblast invasion in vivo. Given that, these results suggest that low levels of MUC15 expression may be critical for trophoblast invasion in early placental development, whereas high levels of MUC15 expression may provide a suppressive signal for trophoblast invasion at the end of gestation. However, it has to be noticed that although MUC15 overexpression in vitro decreases trophoblast invasion, the invasive extravillous trophoblast in vivo does not express MUC15. Therefore, the physiological significance of MUC15 for the extravillous trophoblast invasion in vivo remains to be further investigated.

To date, mucins have been reported to participate in various physiological functions, such as immune reactions, and cell adhesion, migration, and invasion (Hollingsworth and Swanson, 2004). Here, we demonstrate that overexpression of MUC15 significantly suppresses invasion of both trophoblast-like JAR and JEG-3 cells. In addition to MUC15, membrane-bound mucin MUC1 has been suggested to serve as a molecular sensor on the cell surface and regulate the invasive and metastatic activities (Kohlgraf et al., 2003). The present study, for the first time, demonstrates that mucins could play a critical role in regulating trophoblast cell invasion.

Among members of MMP family, MMP-2 and MMP-9 were suggested to regulate trophoblast invasion (Staun-Ram et al., 2004). We found that MUC15 overexpression can suppress invasion of both JAR and JEG-3 cells. However, MMP-2 and

**Figure 4:** Immunohistochemistry of MUC15 in the human placenta

Human placenta from the first (A–C), second (D–F), and third trimester (G–I) were stained with anti-MUC15 polyclonal antibodies (A, D, G, H, and I), anti-CK7 (B and E), anti-Ki67 (C) monoclonal antibodies, or without primary antibodies (F), as indicated. Scale bars = 50 μm. Original magnification: × 200. (A) In the representative first trimester placenta (9 weeks gestation), MUC15 was weakly stained in cytotrophoblasts (CT) and syncytiotrophoblasts (ST). (B) CT were positively stained for CK7. (C) CT were stained for Ki67, which is a proliferation marker in the nucleus. (D) MUC15 was present in ST, but not extravillous trophoblasts (ET), in the second trimester placenta (17 weeks gestation). (E) Extravillous trophoblasts (ET) in the decidua were positive for CK7 staining. (F) Negative (-) control did not show any signals. (G) In the representative term placenta (38 weeks gestation), MUC15 was highly expressed on the apical membrane of ST. (H) In the decidua, MUC15 was not detected in the luminal epithelium (LE), blood vessels (V), and decidual cells (DC). (I) MUC15 was expressed in the glandular epithelium (GE)
MMP-9 activity were not significantly affected by MUC15 overexpression. Two distinct MMP inhibitors, TIMP-1 and TIMP-2, have been demonstrated to be expressed in human trophoblasts (Huppertz et al., 1998). Since MMPs and TIMPs in SDS-PAGE are separated, inhibition of MMP activity by TIMPs could not exactly be revealed by gelatin zymography. We therefore performed real-time RT–PCR and found that MUC15 overexpression significantly increased TIMP-1 and TIMP-2 expression in both JAR and JEG-3 cells. There is evidence to support that both TIMP-1 and TIMP-2 can decrease cytotrophoblast invasion in vitro (Librach et al., 1991). Induction of TIMPs by transforming growth factor (TGF)β1 and TGFβ2 has also been demonstrated to decrease invasion of first trimester cytotrophoblasts (Graham and Lala, 1991). These results suggest that the inhibition of trophoblast invasion by MUC15 could partly result from the induction of TIMP-1 and TIMP-2 expression.

MUC15 protein is highly conserved in its cytoplasmic domain (74 amino acids), which shares 81.1% identity and 94.6% similarity between human and mouse amino acid sequences. The cytoplasmic tail contains four conserved tyrosine and seven serine/threonine phosphorylation sites. Recently, it was found that COS-7 cells infected with MUC1 lacking all tyrosines in the cytoplasmic tail display increased invasion, which is associated with changes in the ERK 1/2 and nuclear factor-κB signaling pathways (Thompson et al., 2006). In addition, the MUC1 cytoplasmic tail can interact with β-catenin and promotes invasiveness of breast cancer cells (Schroeder et al., 2003). We therefore speculate that the potential phosphorylation sites in MUC15 could regulate the interactions of its cytoplasmic tail with proteins involved in signal transduction and thereby modulate trophoblast invasion.

In addition to signaling, mucins have numerous functions in the glycocalyx. Their high degree of glycosylation provides lubrication, prevents dehydration, and offers protection from proteolysis and microbial infection (Brayman et al., 2004). For example, MUC1 is suggested to play a critical barrier role in microbial infection in the female reproductive tract (DeSouza et al., 1999). We found that MUC15 is predominantly expressed on the apical membrane of syncytiotrophoblasts. Therefore, it is reasonable to speculate that MUC15 in syncytiotrophoblasts could form a protective barrier on the surface, which is similar to the protective function of other mucin molecules in the gastrointestinal, respiratory, or genitourinary tract. In addition, MUC15 has been found to be expressed on the surface of endometrium and suggested to regulate embryo attachment (Carson et al., 1998, 2006). However, we did not observe MUC15 expression on the

**Figure 5:** MUC15 overexpression inhibits invasion of trophoblast-like JAR and JEG-3 cells
For matrigel invasion assays, JAR and JEG-3 cells were transiently transfected with pcDNA3.1 (Mock), MUC15 pcDNA3.1 (MUC15), MUC15/pcDNA3.1 + control siRNA (MUC15 + control siRNA) or MUC15/pcDNA3.1 + MUC15 siRNA (MUC15 + MUC15 siRNA) for 48 h. JAR (5 × 10⁴) or JEG-3 (2 × 10⁵) cells were seeded in each chamber and incubated for another 48 h. Invaded cells were fixed with 100% methanol and stained with 0.5% crystal violet. The invaded cells from six fields were counted under a phase-contrast microscope. The representative images of invaded JAR (A) and JEG-3 (B) are shown, magnification ×200. At least four independent experiments were performed. The numbers of invaded JAR (C) and JEG-3 cells (D) are shown as mean ± SD (lower panel). **P < 0.01

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endometrial surface. Instead, MUC15 is present in the glandular epithelium of endometrium. Therefore, our data suggest that MUC15 may not play a significant role in embryo attachment to the endometrium. The physiologic role of MUC15 in the endometrium remains to be further elucidated.

In conclusion, our results demonstrate that MUC15 expression in the human placental villi is increased during the progression of pregnancy. MUC15 overexpression suppresses trophoblast-like cell invasion, which is associated with increased TIMP-1 and TIMP-2 expression. These findings open new insights into the understanding of trophoblast invasion during placental development. For further investigation, it will be of great interest to study the roles of MUC15 in pregnancy disorders, such as pre-eclampsia and intrauterine growth restriction, which are related to dysregulation of trophoblast invasion.

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References

Figure 6: Effects of MUC15 overexpression on MMP activity and TIMPs expression
JAR and JEG-3 cells were transiently transfected with Mock, MUC15, MUC15 + control siRNA or MUC15 + MUC15 siRNA. Gelatin zymography showed that MUC15 overexpression did not significantly affect MMP-2 and MMP-9 activity in the conditioned media of JAR (A) and JEG-3 (B) cells. Real-time RT–PCR showed that MUC15 overexpression increased TIMP-1 and TIMP-2 mRNA expression in both JAR (C) and JEG-3 (D) cells. The induction of TIMP-1 and TIMP-2 was significantly blocked by MUC15 siRNA, but not control siRNA. Data are presented as mean ± SD. from four independent experiments. *P < 0.05


