Effect of matrix metalloproteinase-26 (MMP-26) during embryo implantation in the mouse

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Abstract **Matrix metalloproteinase-26 (MMP-26, endometase and matrilysin-2), a novel member of the MMPs family, is detected not only in the placenta and uterus, but is widely expressed in malignant tumors from different sources as well as in diverse tumor cell lines. However, the function of MMP-26 in the reproductive system has never been reported. Expression of MMP-26 in mouse embryos and the function of the MMP-26 antibody during mouse embryo implantation was examined for the first time by injecting the uterine horn, immunohistochemistry, *in situ* hybridization, co-culture of mouse blastocysts and uterine monolayer epithelial cells, Western blot, RT-PCR, Northern blot and zymography. Our results show that there is strong expression of MMP-26 mRNA and protein in the mouse embryo. Furthermore, the MMP-26 antibody dramatically inhibited mouse embryo implantation and significantly inhibited adhesion and outgrowth of mouse blastocysts on *in vitro* uterine monolayer epithelial cells. At the same time, the MMP-26 antibody inhibited the expression of integrin αV mRNA and protein in a dose-dependent manner. These data suggest that MMP-26 may play a role in some of the tissue-remodeling events associated with the invasion of the endometrium by trophoblast cells and facilitate successfully embryo implantation.**

Keywords: matrix metalloproteinases (MMPs), MMP-26, embryo implantation, mouse.

Embryo implantation is the key event in a series of cell biology and molecular biology events during the process of mammalian reproduction. Matrix metalloproteinases (MMPs) play a pivotal role in cell invasion and metastasis by degrading all kinds of components of the extracellular matrix (ECM)[1]. To date, 23 different MMPs have already been found, of which MMP-26 (endometase and matrilysin-2) is a novel member of the MMPs family[2—9]. Previous results have shown that there was no MMP-26 mRNA expression in the human heart and kidney. But MMP-26 has been detected in the placenta, uterus and in malignant tumors from different sources as well as diverse tumor cell lines. The expression of MMP-26 mRNA in the human placenta and epithelial-origin tumor cells suggests that MMP-26 may be involved in some tissue-remodeling events. The MMP-26 gene is 930 bp encoding 260 amino acids and has many features of MMP-7. Although the gene and protein structures have been identified, its biological functions, particularly those involved in reproduction have never been reported. We studied the expression of MMP-26 in the mouse blastocyst during embryo implantation and intend to elucidate the effect of MMP-26 in this process.

1 Materials and methods

(ⅰ) Reagents. Rabbit anti-human MMP-26 polyclonal antibody and MMP-26 plasmid were generously provided by Prof. Qing-Xiang Amy Sang (Department of Chemistry and Biochemistry and Institute of Molecular Biophysics, Florida State University). Rabbit anti-mouse integrin αV polyclonal antibody was purchased from Santa Cruz Company (USA). Bovine serum albumin, paraformaldehyde and various kinds of restriction enzymes were purchased from Sigma Company (USA). F-12 medium, FBS and trypsin were from Gibco Company (USA).

(ⅱ) Animals. Adult (virginal; 22—25 g) mice of the outbred Kunming white strain were purchased from the Experimental Animal Center, Institute of Zoology, the Chinese Academy of Sciences, and raised at 25℃ in a constant photoperiod (light∶dark cycle, 12∶12 h). They were allowed free access to water and food.

(ⅲ) Intra-uterine horn injection. Each female mouse was caged together with one male to mate overnight as described above. Pregnant mice on day 4 were injected with 3 μg MMP-26 antibody in 3 μL TTBS into the lumen of the left uterine horn adjacent to the oviduct (experimental group), while that of the right horn was injected with 3 μg/3 μL rabbit IgG as control. On day 8, the treated animals were killed to count the numbers of embryos implanted.

(ⅳ) Co-culture of mouse blastocysts and uterine monolayer epithelial cells. On day 4 of pregnancy, collection of mouse blastocysts and preparation of a monolayer of uterine epithelial cells were carried out using the method reported by Zeng and Cao[10], the protocol in brief was as follows:

7.5 IU PMSG and 5 IU hCG were injected routinely into the adult female mice to induce superovulation. The injected female mice were caged with the same strain male overnight. On the following morning, those with vaginal plugs were designated as being in the first day of pregnancy. Uteri from day 4 pregnant mice were split to expose the epithelial cell surface, and digested with trypsin solution. Then the epithelial cell suspension was adjusted to 1×10^6 cells/mL, put on 24-well plates, and incu-

bated at 37℃, 5% atmospheric CO_2 in a humidified chamber.

Preimplantation blastocysts were flushed from the uterus of day 4 pregnant mice with Hank's solution and incubated at 37℃, 5% atmospheric CO_2 in a humidified chamber.

The next morning, a monolayer of uterine epithelial cells had formed. Blastocysts that developed well were selected and transferred into wells. Then, the co-culture medium was added, which contained F-12 medium supplemented with a specific concentration of MMP-26 antibody. The MMP-26 antibody experiment was composed of 4 groups: 0.00 μg/mL (T1, as control), 0.01 μg/mL (T2), 0.1 μg/mL (T3) and 1 μg/mL (T4).

The attachment or outgrowth was observed at 24 or 48 h after culture using phase-contrast microscopy, if the blastocyst was found to stay in the same place, this blastocyst was designated "attached"; if not, it was designated "unattached". After attachment, blastocysts began to grow outwards. When primary giant trophoblast cells were visible around the attachment site of the attached blastocysts, we designated the blastocyst as an "outgrowth".

(v) Indirect immunocytochemistry. Polyclonal specific antibodies against MMP-26 were produced and characterized as in our previous reports. Embryos were cultured in chamber slides. After 48 h in culture, embryos were fixed for 30 min in freshly prepared 4% paraformaldehyde (PFA, Sigma, Deisenhofen, Germany) containing 0.2% Triton X-100. After rinsing several times in 0.01 mol/L PBS (pH 7.4), embryos were incubated in 5% BSA to block non-specific binding of the antibodies for 45 min at room temperature. Then the BSA solution was aspirated with filter paper, and embryos were incubated with the primary antibody against MMP-26 diluted 1:100 in PBS at 4℃ overnight. After rinsing in PBS, embryos were incubated for 60 min in FITC-conjugated secondary antibody (Boehringer, Mannheim, Germany) at 37℃, then incubated in PI for 10 min. Finally, embryos were viewed under a fluorescent microscope (Leica, Heidelberg, Germany). Parallel experiments were performed with embryos using rabbit IgG from the same rabbit as a negative control.

(vi) *In situ* hybridization. The MMP-26 plasmid was kindly provided by Prof. Qing-Xiang Amy Sang (Department of Chemistry and Biochemistry and Institute of Molecular Biophysics, Florida State University). *In situ* hybridization was performed as previously described[11]. Breifly, embryos were treated with proteinase K prehybridized, and hybridized overnight with digoxigenin labeled antisense transcripts from a MMP-26 cDNA. After hybridization, RNase treatment and 3 stringent washes were performed. Sections were incubated with mouse antidigoxigenin antibodies (Boehringer) followed by incubation with biotin-conjugated secondary mouse anti-rabbit antibodies (Dako, Hamburg, Germany). The colorimetric detection was performed by a standard, indirect streptavidin-biotin immunoreaction method. Parallel experiments were performed using a sense probe as a negative control.

(vii) Western blot. Firstly, protein obtained from mice uteri was subjected to a Western blot test to detect the MMP-26 antigen. Secondly, protein isolated from embryos cultured in different media was also subjected to a Western blot test to detect the αV integrin protein expression using the α_v integrin polyclonal specific antibody (Gibco). Briefly, proteins obtained from cell lysates were boiled in an SDS/β-mercaptoethanol sample buffer and about 10 μg proteins were loaded onto each lane of the 12% acrylamide-PAGE gels. The proteins were separated by electrophoresis and the proteins in the gels were blotted onto nylon membranes by electrophoretic transfer in 25 mmol/L Tris, 192 mmol/L glycine buffer, pH 8.3. Blots were blocked in 10% non-fat milk for 1 h. Primary antibodies were diluted 1∶100 in TTBS (30 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.1% Tween20). After overnight incubation with the primary antibodies at 4℃, the blots were washed 4×15 min in TTBS, then, incubated for 1 h in goat anti-rabbit IgG (Promega) diluted 1:500 in TTBS. The blots were then washed 4×15 min in TTBS and 2×15 min in TBS, then the bands were visualized by NBT-BCIP.

(viii) Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA isolated from embryos cultured in F-12 media was subjected to a reverse transcription-polymerase chain reaction (RT-PCR) using mouse MMP-26 specific primer: (5′-primer: TTC TTG CCC TGG TGT TTC GC-3′ (20 bp); 3′-primer: 5′-GCC CAA AGA ATG CCC AAT CT-3′ (20 bp)) to detect MMP-26 mRNA expression. Secondly, RNA isolated from embryos cultured in different media was subjected to semi-quantitative RT-PCR to detect αV integrin mRNA expression. αV integrin primers: upper: 5′-CCG CCG GTG CCA GCC CAT TGA G-3′; lower: 5′-GCT ACC AGG ACC ACC GAG AAG T-3′, the estimated fragment is 337 bp[12]. β-actin sense primer: 5′-GTG GGG CGC CCC AGG CAC CA-3′, β-actin antisense primer: 5′-CTT CCT TAT TGT CAC GCA CGA TTT C-3′, the estimated fragment is 540 bp. RNA was reverse transcribed (RT) by oligo $(dT)_{15}$ priming and AMV reverse transcriptase (Promega Corporation, Madison, WI). PCR amplification was carried out on 1/10 of the cDNA product. PCR cycles were as follows: 95℃×5′ followed by 35 cycles for cell samples of 95℃×45 s, 54℃×45 s, 72℃×1 min. Ten microliters of the PCR products were visualized under ultraviolet light on 1.0% agarose gels containing 1 μg/mL ethidium bromide.

(ix) RNA blot. Isolated RNA was subjected to a

Northern blot test to detect MMP-26 mRNA expression.

The MMP-26 probe was a PCR fragment of pCR3.1MMP-26 plasmid. The MMP-26 specific primer was: upper: 5′-acctacttgacatgcagatgc-3′ (21 bp); lower: 5′-gaggtcctaaaggtcttaaacc-3′ (22 bp), PCR fragment is 351 bp, 94℃ for 1 min, 60℃ for 1 min, 72℃ for 1 min, 35 cycles, then 72℃ for 5 min. The MMP-26 probes were labeled using a Random Primer DNA labeling System (Gibco) in the presence of ^{32}P-dCTP and purified using Nick Columns according to the manufacturers instructions. Membranes were incubated in pre-hybridization buffer (1 mol/L NaCl, 50 nmol/L Tris, 2.2 nmol/L sodium pyrophosphate, 10 g/L SDS, 1×Denhardt's reagent, 10 mg/L denatured salmon sperm DNA, pH 7.5) for 3 h at 65℃. Labeled probe (25 ng, specific activity approximately 1 Ci/mg), denatured by being placed in boiling water for 5 min and snap-cooled on ice, was added and hybridization was carried out overnight at 65℃. After hybridization, the membranes were washed twice for 15 min in wash buffer 1 (1 g/L SDS, 2×SSC), then twice in wash buffer 2 (1 g/L SDS, 0.2×SSC), at 65℃. After autoradiography at –70℃ with X-ray film, probes were removed from the membranes.

(ⅹ) Statistics. Results were presented as the average ±SE of at least three separate experiments. Statistical differences were evaluated with Student's *t*-test. Values of $P<0.05$ were accepted as significant.

2 Results

(ⅰ) Expression of MMP-26 protein. The expression of MMP-26 protein in the mouse blastocyst was detected by immunocytochemistry and Western blot. As shown in Plate Ⅰ-1 the cytoplasm of trophoblast cells, not the inner mass of mouse blastocysts, was able to produce MMP-26 protein. Furthermore, Western blot revealed 29 kD (pro-MMP-26) and 25 kD (activated MMP-26) bands in the embryo lysates (fig. 1). Hence we conclude that MMP-26 protein was present in the mouse blastocysts.

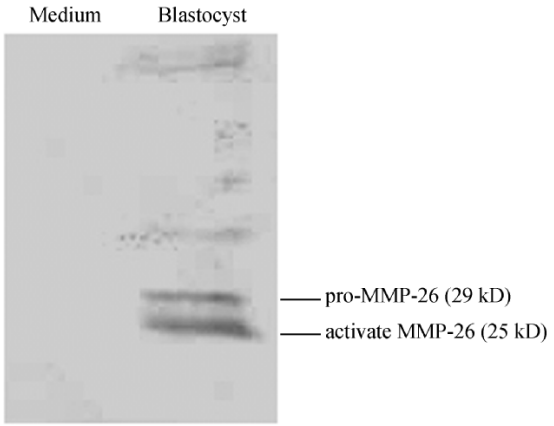

Fig. 1. MMP-26 protein was detected by Western blot in mouse blastocysts.

(ⅱ) Gene expression of MMP-26. The mRNA expression of MMP-26 in the mouse blastocyst was detected by *in situ* hybridization and RT-PCR. As shown in fig. 2, MMP-26 mRNA was expressed in the trophoblast cells of mouse embryos by *in situ* hybridization. Furthermore, a 600 bp band was detected in the mouse blastocyst by RT-PCR (Plate Ⅰ-2) and a 930 bp band by RNA blots (fig. 3). The PCR result was compatible with the expected size of the DNA fragment and indicated the precision and repeatability of this study. The PCR fragment was sequenced to confirm that it was the MMP-26 gene. Hence, from the results of fig. 2 and Plate Ⅰ-2 we conclude that MMP-26 mRNA was expressed in the mouse blastocyst.

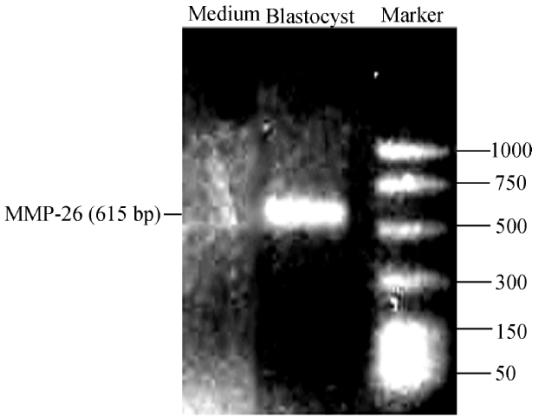

Fig. 2. MMP-26 mRNA was detected by RT-PCR in mouse blastocysts.

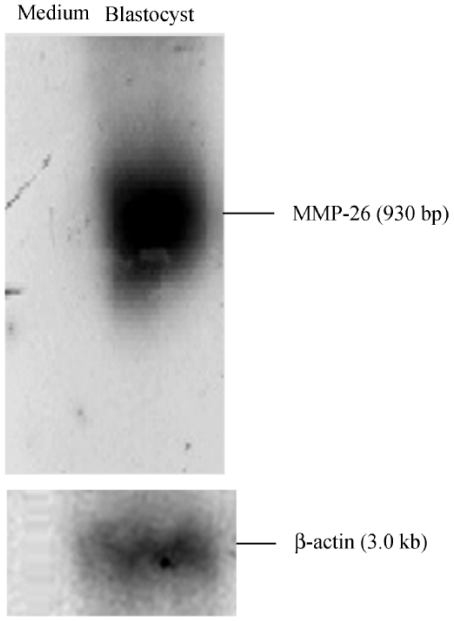

Fig. 3. Expression of MMP-26 mRNA detected by Northern blot in mouse blastocyst.

(iii) Effect of the MMP-26 antibody on mouse embryo implantation. Fig. 4 summarizes the results of the injection of the MMP-26 antibody into a uterine horn of pregnant mice on day 4. The number of embryos implanted in treated uterine horns decreased significantly compared with that in the control horns ($P < 0.01$). This suggests that the MMP-26 antibody inhibits mouse embryo implantation.

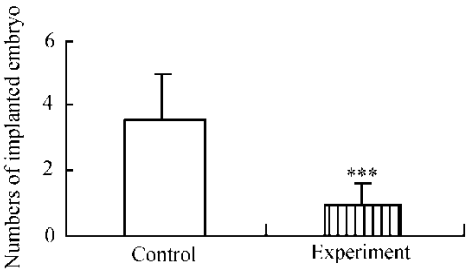

Fig. 4. Effect of MMP-26 antibody on the numbers of implanted embryos. Experimental uterine horn was treated with 3 μg/3 μL anti-MMP-26 IgG. Control horn was treated with 3 μg/3 μL normal rabbit IgG (n=25). *** $P < 0.001$.

(iv) Anti-MMP-26 IgG inhibits adhesion and outgrowth of mouse blastocysts on uterine monolayer epithelial cells. As shown in fig. 5, anti-MMP-26 IgG significantly inhibited the adhesion and outgrowth of mouse blastocysts on uterine monolayer epithelial cells. There was a negative correlation between the percentage of blastocysts with outgrowths and the dosage/concentration of the MMP-26 antibody at co-culture times of 24 and 48 h.

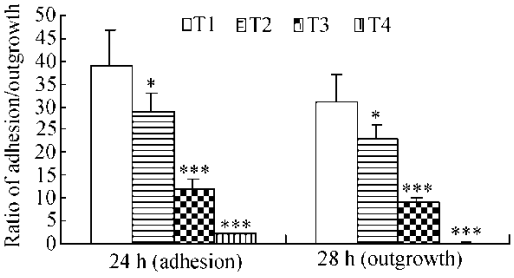

Fig. 5. Effect of anti-MMP-26 IgG on adhesion and outgrowth of mouse blastocysts on uterine monolayer epithelial cells. The concentrations of anti-MMP-26 IgG in T1, T2, T3 and T4 are 0.00 (control), 0.01, 0.1 and 1 μg/mL (n=50). * $P < 0.05$; *** $P < 0.001$.

(v) Effect of anti-MMP-26 IgG on mRNA and the protein expression of integrin αV. As shown in fig. 6, different concentrations of anti-MMP-26 IgG all influenced the expression of αV integrin mRNA and protein. Furthermore, there was a negative correlation between the percentage of blastocysts with outgrowths and the dosage/concentration of MMP-26 antibody.

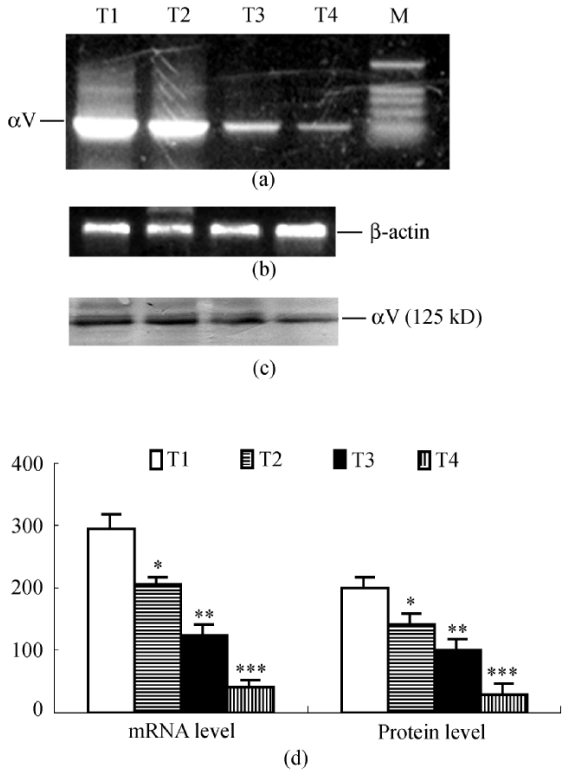

Fig. 6. Anti-MMP-26 IgG downregulated the mRNA and protein levels of integrin αV. (a) RT-PCR product of integin αV (337 bp); (b) RT-PCR product of β-actin (540 bp). M, PCR marker: 2000, 1000, 750, 500, 300 bp; T1, control; T2, T3 and T4, contain 0.01, 0.1 and 1 μg/mL anti-MMP-26 IgG; (c) the result of integrin αV immunoblot. (d) Mean± SEM (n=3—4) folds of increase in the levels of mRNA and protein expression of integrin αV. * $P < 0.05$, ** $P < 0.01$. *** $P < 0.001$.

3 Discussion

Embryo implantation, including its associated series of cell ular and molecular events, is a key process in mammalian reproduction[17—19]. Trophectoderm differentiates into primary trophoblast giant cells that invade the decidual matrix after blastocysts have adhered to the uterine epithelial cells. This occurred at day 4.5 in pregnant mice. At day 7.5, polarized trophectoderm proliferates into ectoplacental cones and differentiates into secondary trophoblast giant cells invading the uterine endomerium together with primary trophoblast giant cells after 3 d. The process of invasion and the establishment of the placenta is complete by day 11.5 and day 12, respectively.

The complex process by which trophoblast cells invade the endometrium involves numerous regulators and has been divided into 3 steps: adhesion, degradation and migration[13—15]. This process includes 3 important events: angiogenesis, proteinase degradation and programmed cell death (PCD). During the process of trophoblast cell invasion of the uterus endometrium, trophoblast cells adhere to the extracellular matrix (ECM) component and

then invade and degrade ECM. Matrix metalloproteinase (MMPs) are a family of genes that may play a role in ECM degradation and tissue remodelling during placentation and also in tumor metastasis[16]. It has been reported that MMPs take part in the process of angiogenesis directly or indirectly. MMPs play a pivotal role in cell invasion and metastasis as MMPs can degrade all kinds of ECM components. To date, 23 different MMPs have been identified since Gross and Lapiere found the first MMPs in 1962. MMPs have been divided into 5 groups: collagenase, gelatinase, stromalysin, membrane type MMPs and others. MMP-26 is the newest member of the MMPs family. The structure of the MMP-26 gene and protein have been identified, however, its biological functions, particularly its role in the reproductive system have never been reported. This study is the first to detect the expression of MMP-26 during mouse embryo implantation using intra-uterine injection, immunohistochemistry, Western blot, RT-PCR, *in situ* hybridization and RNA blots. Our results showed that there is MMP-26 mRNA and protein expression in the mouse blastocyst. *In vivo* study showed that intra-uterine injection of anti-MMP-26 IgG significantly inhibited embryo implantation, suggesting that MMP-26 has an effect on mouse embryo implantation. Anti-MMP-26 IgG inhibited the outgrowth of blastocysts on mouse uterine monolayer epithelial cell by neutralizing the effect of MMP-26. Interestingly, anti-MMP-26 IgG inhibits the adhesion of mouse embryos on uterine monolayer epithelial cells. So we detected the influence of anti-MMP-26 IgG on the expression of integrin αV mRNA and protein. Our results indicate that the MMP-26 antibody inhibits the mRNA and protein level of integrin αV in a dose-dependent manner. It is not difficult to explain why MMP-26 antibody inhibits mouse embryo implantation. At the same time, we detected the activity of MMP-26 by gelatin zymography. The band produced by MMP-26 is weaker than that produced by MMP-2 and MMP-9 because the ability of MMP-26 to degrade gelatin is very weak (data not shown). This suggests that MMP-26 degrades ECM by activating other MMPs directly or indirectly. The hypothesis that MMP-26 activates other MMPs and the regulation mechanism of MMP-26 during embryo implantation needs further investigation.

In summary, both the MMP-26 gene and the protein expressed in the mouse blastocyst indicate that MMP-26 may be involved in the process of mouse embryo implantation by degrading ECM or other MMPs.

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