Comparative Medicine
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Vol 62, No 3
June 2012
Pages 179–184

Original Research

Expression of Maspin in the Early Pregnant Mouse Endometrium and Its Role during Embryonic Implantation

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Maspin (serpin B5), a tumor-suppressing member of the serine protease inhibitor family, participates in cell migration, adhesion, invasion, and apoptosis. These processes are also critical for embryo implantation, but the role of maspin in embryo implantation remains poorly understood. The aim of the present study was to investigate the spatiotemporal expression of maspin in early pregnant mouse endometrium and its role in embryo implantation. Real-time quantitative PCR analysis, immunohistochemistry, and Western blotting were used to detect mRNA and protein expression of maspin in the endometria of nonpregnant and early pregnant (days 0 to 7) mice. On day 3 of pregnancy, mice in the treated group (n = 20) were injected in the left uterine horn with antimaspin polyclonal antibody and in the right horn with purified rabbit IgG; control mice (n = 20) were injected only with purified rabbit IgG in the right uterine horn. Implanted embryos were counted on pregnant day 8. The mRNA and protein expressions of maspin were higher in the endometria of pregnant mice than nonpregnant mice; these levels gradually increased from day 1 of pregnancy, peaked on day 5, and then decreased on days 6 and 7. The mice treated with antimaspin polyclonal antibody group had far fewer implanted embryos than did the control group. Taken together, these results suggest that maspin, a tumor suppressor, may play an important role in embryo implantation.

Maspin, a tumor suppressor gene that was identified in 1994, is a member of the serpin (serine protease inhibitors) family.[38] Maspin is located on human chromosome 18q21.3 and mouse chromosome 1 and encodes a 375-amino-acid protein. Maspin is expressed in many tissues including prostate, mammary gland, skin, stomach, and uterus. The protein has distinct biologic functions, including inhibition of tumor cell migration and invasion through cell surfaces and inhibition of tumor angiogenesis and metastasis.[1,4,20,30,36] These functions depend in part on the fact that maspin induces the adhesion of cells to extracellular matrix.[1,30,31] Although the exact role of maspin in human gestation is unclear, in vitro data suggest that the protein regulates trophoblastic invasion.[9,10] Indeed, knockout mouse studies have shown that maspin plays an essential role in early embryonic development.[15] Together, these early studies have highlighted the roles of maspin in early embryonic development, trophoblastic invasion, and tumor development.[9,10,15,38]

Embryo implantation, the first step in a successful pregnancy, is a crucial process in mammal reproduction.[8,11] This complex physiologic process is regulated by numerous genes and proteins[2,7] and relies on diverse precisely coordinated interactions between embryos and the maternal uterine milieu.[6,22] All of these interactions include apposition of the blastocyst, adhesion, and invasion of trophoblasts.[6,33] Synchronization between blastocyst development and modification of the maternal endometrium is crucial for successful embryonic implantation.[2,8,11,19] The uterine epithelium around the embryo undergoes apoptosis in response to the presence of the blastocyst.[21,27,33] The adhesive and invasive abilities of trophoblastic cells are regulated by extracellular matrix, matrix metalloproteinases, cell adhesion molecules, and various growth factors.[2,6,7] This process of embryonic implantation is similar to that of tumor cell metastasis.[5,14]

As a tumor suppressor, the role of maspin in tumor progression and invasion has been proposed and studied widely,[1,4,10,20,30,31] but its effect on blastocyst implantation is still unknown. The present study investigated the expression of maspin in endometrium and its contribution to early pregnancy of mice by using real-time fluorescent quantitative PCR analysis, immunohistochemistry, Western blotting, and in vivo functional experiments.

Materials and Methods

Animal preparation. Adult ICR mice (25 to 30 g; age, 6 to 8 wk) were obtained from the Laboratory Animal Center of Chongqing Medical University in China (certificate SCXK[YU] 2005002). All of the mice were kept under SPF conditions and provided a complete diet. Excluded agents included: *Pasteurella pneumotropica*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, β-hemolytic *Streptococcus*

Received: 19 Jul 2011. Revision requested: 26 Aug 2011. Accepted: 17 Dec 2011.
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spp., pneumonia virus of mice, reovirus type 3, minute parvovirus of mice, mouse encephalomyelitis virus, mouse adenovirus, polyomavirus, flagellates, and infusoria. Female mice were evaluated twice daily for 1 wk to determine their estrous cycle phase (proestrus, estrus, metestrus, or diestrus). Vaginal secretions were collected by swabbing or aspiration, smeared on a slide, and stained with Harris hematoxylin. Identification of the estrous phase depended on the cell types present in the samples.[32] Pregnancy (day 1) was confirmed by vaginal smears or the presence of a vaginal plug. Subsets of mice were euthanized on pregnancy days 1 through 7 by cervical dislocation at 0800 to 0900. Endometrial tissues were collected, rapidly frozen, and stored at −80 °C for fluorescent qualitative PCR and Western blotting. The uteri of 20 mice in each group were fixed in 4% paraformaldehyde for immunohistochemistry. In addition, on the morning of day 4, 40 pregnant mice were randomly divided into 2 groups (n = 20 per group) to investigate the function of maspin in embryo implantation. All animal procedures were approved by the IACUC of Chongqing Medical University and in accordance with the policy of the Ethical Committee, State Key Laboratory of Reproductive Biology (Institute of Zoology, Chinese Academy of Sciences).

PCR analysis. Total RNA was extracted from frozen endometrial tissues by using Trizol reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. Quantification and assessment of purity were performed by using a UV spectrophotometer; acceptable samples had OD_{260}/OD_{280} values of 1.8 or greater. The extracted RNA was reverse-transcribed into cDNA in a total volume of 20 µL. Master Mix reagents (Toyobo, Osaka, Japan) were used, as follows: 12 µL RNA (1 mg/mL), 4 µL 5× RT buffer, 2 µL dNTP mixture (10 mM), 1 µL RNAase inhibitor (10 U/µL), and 1 µL ReverTra Ace. The RT reaction was carried out as follows: 30 °C for 10 min, 42 °C for 30 min, 95 °C for 10 min, and 4 °C for 5 min.

Each 50-µL PCR reaction (Toyobo) comprised 10 µL cDNA, 5 µL 10× PCR buffer, 0.5 µL *Taq* DNA polymerase (2.5 U/µL), 1 µL maspin or GAPDH primers (10 pmol/µL), and diethylpyrocarbonate-treated water as needed. Primers for maspin were 5′ GCC CTG AGA CTG GCA AAT TCA 3′ (forward) and 5′ GCG CAA GGG ACA GAG AAG TA 3′ (reverse; amplicon size, 121 bp); those for GAPDH were 5′ AGG TCG GTG TGA ACG GAT TTG 3′ (forward) and 5′ TGT AGA CCA TGT AGT TGA GGT CA 3′ (reverse; amplicon size, 113 bp; Invitrogen, Shanghai, China). A BLAST search was performed to verify the specificity of these primers' sequences. The PCR reaction conditions were 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 20 s; with a final extension at 72 °C for 10 min.

Quantification of the transcripts was carried out in the Rotorgene 3000 Real-Time PCR System (Corbett, Victoria, Australia). The reactions were prepared in 96-well MicroAmpoptical plates by the addition of 20 µL PCR master mixture consisting of 1 µL primers (10 mmol/L), 10 µL SYBR Green PCR Master Mix (TaKaRa) and 8 µl ddH2O and 1 µL cDNA. The PCR conditions were: initial denaturation at 95 °C for 1 min; 40 cycles of 95 °C for 30 s, 58 °C for 20 s, and at 72 °C for 30 s; with a final extension at 72 °C for 3 min. Gene copy numbers for maspin and GAPDH were derived from a standard curve generated by plotting the cycle threshold values for 2-fold serial dilutions of 10^0 to 10^2 copies of a standard. The gene expression rate was obtained by normalizing the amount of maspin mRNA with that of GAPDH.

Immunohistochemistry. Uterine sections (5 µm) were cut, deparaffinized, rehydrated, and treated with 3% H_2O_2 in distilled water for 10 min and followed by a rinse in distilled water. For antigen retrieval, sections was placed in 10 mmol/L citrate buffer (10 mM citric acid, 0.05% Tween 20; pH 6.0), heated in a microwave oven for 20 min, and cooled at room temperature. Nonspecific binding was blocked by incubating in 10% normal goat serum at 37 °C for 30 min. Primary antibody diluted in blocking serum (50 µL antimaspin, 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) then was added to the slides and incubated overnight at 4 °C in a humidified chamber. For some sections, antimaspin antibody was replaced with purified nonimmune rabbit IgG at the same final concentration as a negative control. After 3 washes in PBS for 3 min each, tissue sections were incubated for 30 min with biotinylated antirabbit antibody (3 µg/mL; Boster, Wuhan, China). Subsequently, slides were washed with PBS and incubated for 30 min with avidin–biotin complex reagent (Boster) containing horseradish peroxidase. Slides were washed with PBS 3 times for 3 min each, and color development was achieved by using DAB substrate (Beijing Zhongshan Golden Bridge, Beijing, China). The tissue sections were counterstained with hematoxylin.

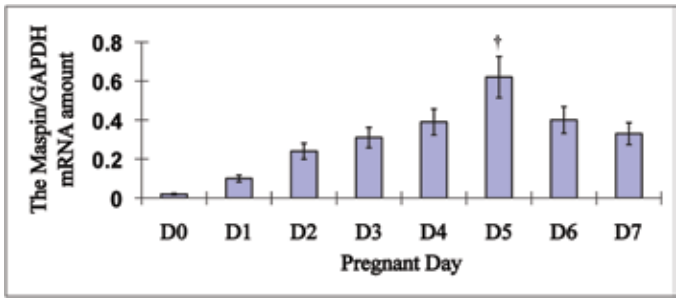

Figure 1. Fluorescent qualitative PCR analysis of maspin mRNA expression in mice endometria. D0, estrus; D1–D7, days 1 through 7 of pregnancy. The expression of maspin mRNA was gradually increased from day 0, peaked on day 5, and then decreased on days 6 and 7. Expression of maspin mRNA did not differ between days 3 and 7 or days 4 and 6, but the differences among other groups were significant ($P < 0.05$). †, $P < 0.01$ (n = 20) compared with values on days 0, 1, 2, 3, 4, 6, and 7.

Western blot analysis. Mouse endometria was lysed in 300 µL RIPA buffer (Pierce, Rockford, IL) containing 2 mM phenylmethylsulphonyl fluoride, 10 µg/mL aprotinin, 10 µg/mL leupeptin, and 2 mM sodium vanadate. After sonication, the lysates were centrifuged at 13,000 × g for 30 min at 4 °C. Protein concentrations were determined by using the bicinchoninic acid assay kit (Pierce, Rockford, IL), with albumin as a standard. Laemmli sample buffer then was added to 40 µg protein and the sample heated in a boiling water bath for 5 min. Equal amounts of protein from each sample were fractionated by SDS–10% polyacrylamide gel electrophoresis. The fractionated protein samples were transblotted onto a nitrocellulose membrane (Pierce) by using a Semi-Dry Transfer Cell (Bio-Rad, Mississauga, ON), and nonspecific binding was blocked by incubating the blot in 5% nonfat dry milk for 1 h. After extensive washing with Tris-buffered saline, blots were probed with antimaspin polyclonal antibody (1:300 dilution; Santa Cruz Biotechnology) in Tris-buffered saline containing 1% bovine serum albumin and 0.1% Tween 20 for 24 h at room temperature with gentle shaking. The blots then were probed with a 1:10,000 dilution of goat antirabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology) for 30 min at room temperature with gentle shaking. Subsequently, the blots were further washed and signals detected by using enhanced chemilu-

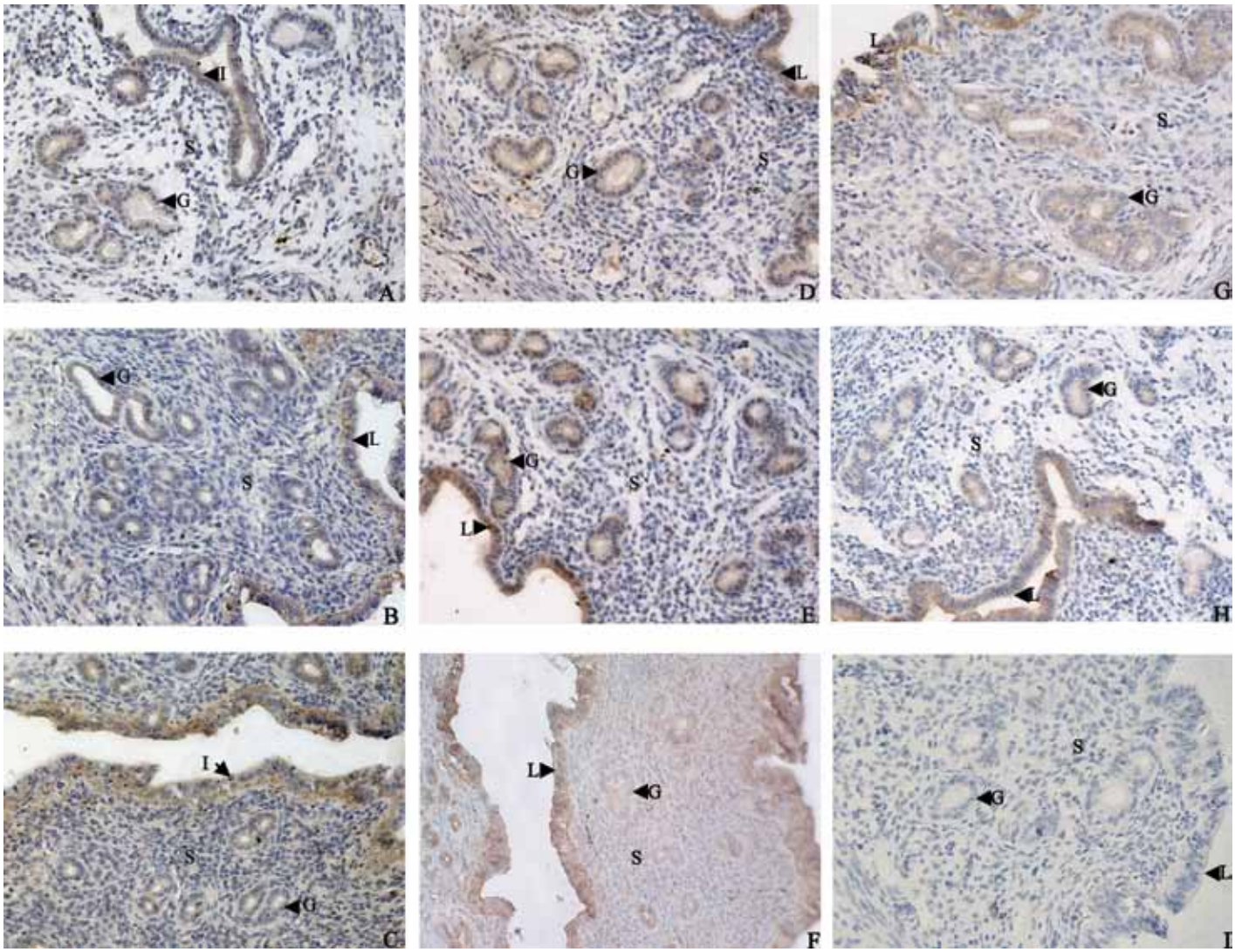

Figure 2. Expression of Maspin detected by immunohistochemistry of mouse endometrium during early pregnancy. The strongest signals for maspin were found in the luminal epithelium (L), glandular epithelium (G), and stromal cells (S) on pregnant day 5. D, Day of pregnancy. (A) D0. (B) D1. (C) D2. (D) D3. (E) D4. (F) D5. (G) D6. (H) D7. (I) Negative control (no primary antibody was absent). Magnification, 20×.

minescence (ECL System, Amersham, Arlington Heights, IL). The expression of maspin was quantified by using Scion Image Alpha (version 4.0.3.2, Scion, Frederick, MD).

Role of maspin in embryo implantation. To determine whether maspin plays a role in embryonic implantation, 40 pregnant mice were divided randomly into 2 groups (n = 20 per group). On pregnancy day 3 at 0800 to 0900, 20 mice (treatment group) were anesthetized with pentobarbital sodium (50 mg/kg), anti-maspin polyclonal antibody (5 µL, 5 µg/mL) was injected into the left horn of uterus, and purified rabbit IgG (5 µL, 5 µg/mL) into the right horn of uterus. In addition, 20 mice (control group) were anesthetized and injected with purified rabbit IgG (5 µL, 5 µg/mL) into the right uterine horn, with no treatment of the left horn. Beginning immediately after the operation, buprenorphine (0.5 mg/kg) was administered subcutaneously every 12 h for 72 h for analgesia. All mice were euthanized at 0800 to 0900 on day 8 of pregnancy, and then the number of implantation sites was counted.[13]

Statistical analysis. All data are presented as mean ± 1 SD and were analyzed by using the SAS 9.1 statistical software package (SAS, Cary, NC). One-way ANOVA and the SNK-q test were used to evaluate the PCR and Western blotting data. Data from the functional study were analyzed by Student t test; P values less than 0.05 were considered significant.

Results

Endometrial expression of maspin mRNA. The ratio of maspin mRNA to GAPDH mRNA in the endometrium was higher ($P < 0.05$) in pregnant mice than nonpregnant mice. Maspin mRNA expression in the endometria of pregnant mice gradually increased from pregnancy days 0 to 5, peaked on day 5, and decreased on days 6 and 7 (Figure 1). The expression of maspin mRNA did not differ between days 3 and 7 or between days 4 and day 6, but all other differences between groups were significant ($P < 0.05$; Figure 1).

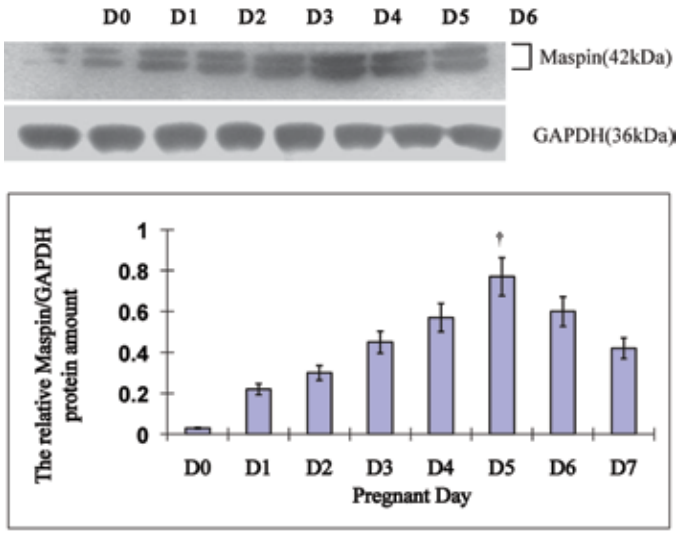

Figure 3. Protein expression of maspin detected by Western blotting of mouse endometrium during early pregnancy. GAPDH blots were used as controls. Endometrial tissues were divided into 8 groups (D0–D7); each group was evaluated 20 times by using 20 different samples from 20 pregnant and nonpregnant mice per day. Maspin expression was significantly ($P < 0.05$) on day 5 of pregnancy. Expression levels did not differ between days 3 and 7 or days 4 and 6, but the differences among other groups were significant ($P < 0.05$). The data in the graph are those from densitometrical analysis of the Western blots and are the means ± SEM of 8 independent experiments. †, $P < 0.01$ compared with values from other groups.

Expression and location of maspin protein. Immunohistochemistry showed that maspin protein was present in the luminal and glandular epithelia of mice during pregnancy days 1 through 7 and was predominant in stromal cells on pregnancy days 4 through 6. The strongest immunoreactivity occurred on pregnancy day 5. Only faint signals were detected in the luminal and glandular epithelia of nonpregnant mice; the negative control did not show any positive immunostaining (Figure 2). Western blotting demonstrated maspin protein expression in all mice in the present study. Changes in the expression of maspin protein paralleled those in maspin mRNA expression (Figure 3). Maspin protein levels were higher ($P < 0.05$) in the endometria of pregnant than nonpregnant mice, reaching a peak value on pregnancy day 5 ($P < 0.01$) and then declining on days 6 and 7. Amounts of maspin protein did not differ between days 3 and 7 or between days 4 and 6, but all other differences among groups were significant ($P < 0.05$; Figure 3).

Role of maspin in embryo implantation. Among mice in the treatment group, the number of implanted blastocysts in the horn treated with maspin antibody (left; 2.42 ± 1.15) was lower ($P < 0.01$) than that in the horn treated with rabbit IgG (right; 8.43 ± 1.31). In the control mice, the numbers of implanted blastocysts did not differ between the untreated horn (left; 8.91 ± 1.28) and that treated with rabbit IgG (right; 8.23 ± 1.20; Figure 4; Table 1).

Discussion

In the present study, we demonstrated for the first time that maspin is expressed in the endometria of early-pregnant mice. Real-time fluorescent quantitative PCR, immunohistochemistry, and Western blotting showed that mRNA and protein expression of maspin in the endometria were higher in early-pregnant mice than in nonpregnant mice. Both mRNA and protein expression of maspin in pregnant mice peaked on pregnancy day 5. An in vivo functional experiment indicated that injection of antimaspin polyclonal antibody into the uterine horn could significantly inhibit embryonic implantation, with significantly lower numbers of implanted embryos in mice that received antimaspin antibody. These results suggest that maspin plays an important role in the regulation of early embryonic implantation.

Maspin (serpin B5), a tumor suppressor, has an important role in the regulation of cell motility, adhesion, invasion, apoptosis, and angiogenesis.[1,4,10,20,30,31,38] Recent studies have indicated that maspin is expressed in breast cancer, endometrial cancer, trophoderm cell tumors, cervical carcinoma, liver cancer, lung cancer, esophageal cancer, and other malignant tumors.[4,26,35,38] Maspin may inhibit tumor cell motility by mediating the signal pathway of the small G proteins Rac1 and Pakl (a p21-activating enzyme).[26,34] In addition, maspin participates in the regulation of tumor cell migration, adhesion, apoptosis, and proliferation through PI3K and ERK pathways or the Bax–Bcl2 signal pathway.[26,37] Maspin promotes the expression of Bax but markedly inhibits that of Bcl2,[12,24] to selectively control Bax–Bcl2 protein stability, thereby mediating cell apoptosis and inhibiting tumor growth.[12,18,23,38]

Embryonic implantation is an important physiologic process in mammalian reproduction.[2,8] The invasion of trophoblastic cells into the endometrium and formation of the placenta are the key steps in embryonic implantation.[5,14,17] The 'dialogue' among trophoblasts, endometrial epithelial cells, and stromal cells leads to precise regulation of the differentiation and proliferation of trophoblast and endometrial decidualization.[6,28] In addition, the invasion process involves the interaction and regulation of adhesion molecules, proteolytic enzymes, growth factors, cytokines, and vasoactive factors.[2,6-8,22] Comparative research has revealed striking similarities in the behavioral patterns of invasive trophoblastic cells and cancer cells.[11,14] Homozygous deletion of the maspin gene can result embryonic death shortly after implantation.[15] Although the exact role of maspin in human gestation is still unclear, in vitro data suggested that maspin regulates trophoblastic invasion [9,10] and plays an essential role in early embryonic development.[15] Furthermore, apoptosis is known to be induced during embryo implantation and decidualization in rodents.[3,21,27] Early pregnancy in rodents is characterized by progressive interaction between the embryo and the maternal compartment. Various studies have shown that the PI3K–Akt and ERK1–ERK2 pathways also play an important role in the control of endometrial proliferation and differentiation during embryo implantation.[16,29,25] These pathways—in which maspin is involved—play key roles in tumor cell development, which shows striking similarities to aspects of embryonic implantation.

The expression of maspin in our current study gradually increased during the early days of pregnancy in mice and reached the maximum at the time of implantation. On pregnancy days 3 and 4, maspin expression increased in the luminal epithelium compared with stromal cells. On pregnancy day 5, maspin was expressed strongly both in luminal epithelium, glandular epithelium, and stromal cells. All of these findings imply that maspin is involved in trophoblastic invasion and degradation of the extracellular matrix during the invasive stage of implantation and induces epithelial cell apoptosis and stromal cell decidualization through the Bax–Bcl2, PI3K–Akt, and ERK1–ERK2 pathways. We

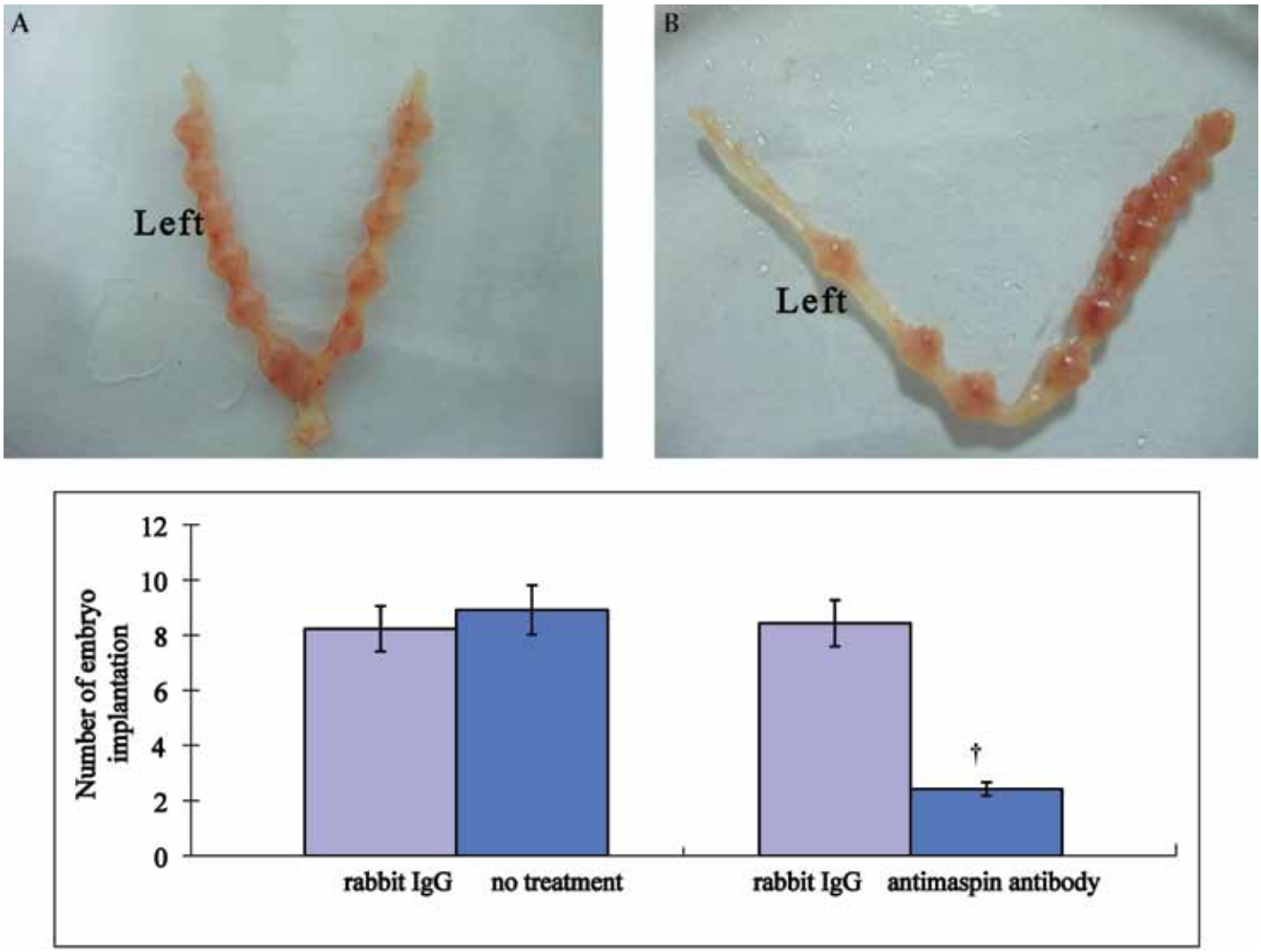

Figure 4. Functional analysis of maspin. (A) Control group. (B) Treatment group. The number of embryos that implanted in the right (rabbit IgG) compared with left (no treatment) horn did not differ in the control group but differed significantly ($P < 0.05$; rabbit IgG versus antimaspin antibody) in the treatment group. †, $P < 0.01$ ($n = 20$) compared with IgG value.

Table 1. Functional analysis of maspin during embryo implantation

		No. of embryos implanted		
Group	Uterine horn	Total	Mean ± SEM	*P*
Control ($n = 20$)	Left (untreated)	180	8.91 ± 1.28	0.2598
	Right (rabbit IgG)	163	8.23 ± 1.20	
Treated ($n = 20$)	Left (antimaspin antibody)	52	2.42 ± 1.15	<0.0001
	Right (rabbit IgG)	167	8.43 ± 1.31	

therefore speculated that maspin plays an essential role in cell apoptosis, cell adhesion, and degradation of the extracellular matrix during early pregnancy, especially in the process of embryo implantation.

In conclusion, the expression of maspin followed a spatiotemporal pattern in the mouse endometrium during early pregnancy. The high levels of maspin in the mouse endometrium suggest that maspin may be a novel molecule involved in the early processes of pregnancy, especially in embryonic implantation. However, further studies are needed to understand the precise mechanism underlying the role of maspin in embryonic implantation.

Acknowledgments

We thank Professors Liang-Ming Liu (Third Military Medical University), Ying-Xiong Wang, Mao-Sheng Yang, and Yi Tan (Chongqing Medical University) for their constructive comments on the manuscript and Dr Huan Yang (Medicine Laboratory Center, Shanghai Fudan University) for her technical assistance.

This work was supported by Science and Technology Research Project of Chongqing Education Committee, China (KJ110307).

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