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# Effect of Ubiquitin-Proteasome Pathway on Mouse Blastocyst Implantation and Expression of Matrix Metalloproteinases-2 and -9[1]

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## ABSTRACT

**Previous studies have documented that ubiquitin-related proteins are present in human, baboon, rhesus monkey, cow, sheep, and mouse pregnant uteri, indicating that the ubiquitin-proteasome pathway (UPP) may be involved in the extensive uterine remodeling during mammalian early pregnancy, but there is still no direct evidence. A mouse intrauterine injection model was employed to study the direct effect of the UPP on mouse embryo implantation and its possible mechanisms. On Day 3 of pregnancy in each mouse, one of the uterine horns in each mouse was injected with different concentrations of lactacystin, a specific proteasome inhibitor, or anti-ubiquitin antibody, and the other side was used as a control. On Days 5, 6, and 7, the number of implanted embryos was counted and the expression and gelatinolytic activities of matrix metalloproteinase-2 (MMP-2) and MMP-9 were studied. Results presented here illustrate that injection of lactacystin and anti-ubiquitin antibody significantly inhibited mouse embryo implantation. Further investigations by reverse transcription-polymerase chain reaction and gelatin zymography showed that MMP-2 and MMP-9 mRNA expression, as well as the gelatinolytic activity of MMP-9 in the lactacystin-treated uterine horn, significantly decreased, whereas the activity of MMP-2 was not significantly affected. The results obtained from this study, together with previous reports, suggest that the UPP is involved in mouse embryo implantation, and UPP's effect on embryo implantation is achieved at least in part by regulating MMP-2 and MMP-9 mRNA expression and the gelatinolytic activity of MMP-9.**

*implantation, pregnancy, uterus*

## INTRODUCTION

Implantation of the embryo into the uterine endometrium is a highly regulated event critical for the establishment of pregnancy. Successful embryo implantation depends upon the synchronized development of both the invasiveness of the embryo and the receptivity of the endometrium [1]. This process is accompanied by extensive degradation and remodeling of the extracellular matrix (ECM). Numerous studies in mouse [2], primate [3], and human [4] have shown that matrix metalloproteinases (MMPs), which are responsible for the degradation of ECM, are key regulators of blastocyst implantation. Among these MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) have been extensively investigated. MMP-2 and MMP-9 are synthesized as latent proenzymes and must be activated in order to proteolytically degrade various components of ECM, including type IV, V, VII, and X collagens; fibronectin; and gelatin [5]. The activities of MMP-2 and MMP-9 are regulated by their tissue inhibitors (TIMPs), and/or other MMPs, such as MMP-14, the membrane type 1 MMP [6]. In some tissues, expression of MMP-2 and MMP-9 is also regulated by cytokines, growth factors, and proteolytic enzymes, including the ubiquitin-proteasome pathway [7–11].

The ubiquitin-proteasome pathway (UPP) is the cell's ATP-dependent, nonlysosomal proteolytic system, which can be divided into two consecutive processes: polyubiquitination of target proteins and degradation of ubiquitinated proteins by the 26S proteasome [12, 13]. The 26S proteasome [14, 15], a large protease complex of 1700 kDa, is composed of at least 30 kinds of proteins. It contains the 20S proteasome as a functional core and two 19S caps. The latter specifically recognize and bind with the polyubiquitin chains of the target protein conjugated with ubiquitin, modifying the configuration of the target proteins. With the help of ATP, the target proteins are then carried into the hollow cavity of the 20S proteolytic core, where they are degraded into short peptides or amino acids. By eliminating ubiquitin-targeted proteins, proteasomes play a number of biological roles including regulation of the cell cycle, presentation of the major histocompatibility complex (MHC)-I antigen, and degradation of some transcription factors, as well as the modification of some membrane proteins and the assembly of ribosomes. Proteasomes have also been shown to play a crucial role in tumorigenesis and metastasis [12].

Malignant tumors initiate invasion by breaking down the basement membrane. MMP-2 and MMP-9, as the proteases that hydrolyze type IV collagen (the main components of the basement membrane), play essential roles in this process [16]. Increased expression of MMP-2 or MMP-9 has been demonstrated in malignant tumor tissues [17], and the ubiquitin-proteasome pathway has been documented to have some regulatory effect on the expression of MMPs and their tissue inhibitors in some malignant tissues [9]. In

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cultured squamous carcinoma cells, ALLN, an inhibitor of proteasomes, strongly down-regulates MMP-9 activity and the expression of NF-κB, a transcription factor regulating MMP-9 gene expression [9]. The p53 gene is a tumor suppressor that regulates the expression of genes required for cell cycle arrest or apoptosis. Degradation of p53 is closely related to the UPP. It has been reported that both the wild-type p53 and p53 mutant could inhibit expression of TIMP-3 mRNA [18]. Blastocyst invasion shares many invasive characteristics with tumors, and MMPs-TIMPs are extensively involved in uterine tissue remodeling during blastocyst implantation [19, 20]. Thus, investigations of the UPP in tumor invasion will provide insights into the effect of the UPP on blastocyst implantation.

Ubiquitin-related proteins have been reported to be present in the human, baboon, rhesus monkey, cow, sheep, and mouse pregnant uteri [21–25], and may be essential for endometrial modification and placental development during early pregnancy. However, there is still no direct evidence to show whether the UPP is involved in embryo implantation and has a regulatory effect on mRNA expression and the activities of MMP-2 and MMP-9.

In this study, a mouse intrauterine injection model was employed to study the direct effect of ubiquitin and proteasomes on mouse embryo implantation and the expression of MMP-2 and MMP-9 in order to explore the possible relationship between MMPs and the UPP during embryo implantation.

## MATERIALS AND METHODS

### *Animals and Tissue Collection*

Investigations were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals for the Study of Reproduction filed by the State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences.

Adult mice of the Kunming white strain (12 wk old, weighing 25–30 g) were provided by the Experimental Animal Center of Institute of Genetics, Chinese Academy of Sciences. Animals were bred at room temperature (about 25°C) with controlled light cycles (12L:12D) and allowed free access to food and water.

The method of mouse intrauterine injection was detailed previously by Wu and Gu [26]. In brief, female mice were allowed to mate with male mice of the same strain (two females for one male). The morning when the vaginal sperm plug was observed was designated as Day 1 of pregnancy. Pregnant mice were randomly divided into several groups. On Day 3 of pregnancy, animals were anesthetized and one uterine horn in each received one of the following treatments: 1. 5 μl of 0.05 ng/μl (n = 17) or 0.1 ng/μl (n = 9) lactacystin (Sigma Chemical Co., St. Louis, MO), a proteasome inhibitor, in distilled water; 2. 1 μg (n = 10) or 2 μg (n = 10) polyclonal rabbit anti-ubiquitin antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA); or 3. 2 μg (n = 8) or 3 μg (n = 8) monoclonal mouse anti-ubiquitin antibody (Santa Cruz Biotechnology Inc.). Uterine horns serving as controls received equal volumes of distilled water, rabbit preimmune serum (Zhong Shan Corp., Beijing, China), or normal mouse IgG (Santa Cruz Biotechnology Inc.), respectively. On the morning of Day 5, animals were anesthetized, and the implantation sites were defined by tail intravenous injection of 0.5 ml 0.1% trypan blue 5 min before dissection of the reproductive tract. On Days 6 and 7, the implantation sites were visually distinct. Animals were killed on the morning of Days 5, 6, and 7 of pregnancy, and the uteri were excised and trimmed, and appropriate implantation sites were separated. The uteri were snap-frozen in liquid nitrogen and stored at −80°C until use.

### *Reverse Transcription-Polymerase Chain Reaction (RT-PCR)*

The presence of mRNAs for MMP-2 and MMP-9 in mouse uteri injected with lactacystin or the control solution was examined by RT-PCR. Total RNA of approximately 100 mg of mouse uterus was isolated using Trizol reagent (Gibco BRL, Grand Island, NY). The integrity of the purified RNA was confirmed by visualization of the 28S and 18S rRNA bands after the electrophoresis of RNA through a 1% agarose-formaldehyde gel. The quantity of total RNA was determined by spectrophotometric measurement at 260 nm. Complementary DNA was obtained from 2 μg RNA using superscript II reverse transcriptase (Gibco BRL). The resulting cDNA samples were amplified by PCR. Specific primer pairs used in this study are as follows: MMP-2, upstream 5′-GCT GAT ACT GAC ACT GGT ACT-3′, downstream 5′-CAA TCT TTT CTG GGA GCT C-3′, giving a PCR product of 216 bp; MMP-9, upstream 5′-TGA GTC CGG CAG ACA ATC C-3′, downstream 5′-CCT TAT CCA CGC GAA TGA CG-3′, to amplify a PCR product of 432 bp. Amplification of the human β-actin gene transcripts was used to control the efficiency of RT-PCR among the samples. Sequences of antisense and sense primers for β-actin were 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′ and 5′-GTG GGG CGC CCC AGG CAC CA-3′, respectively, and the expected size of the PCR product was 548 bp. PCR was conducted in the exponential range of amplification for each set of primers. The ranges in which the exponential amplification was observed were 26 cycles for MMP-2 and 29 cycles for MMP-9. Therefore, PCR was performed by 26 cycles for MMP-2, and 29 cycles for MMP-9. The parameters used for PCR amplification were as follows: MMP-2, denaturation, 2 min at 94°C; annealing, 45 sec at 56°C; extension, 2 min at 72°C. For MMP-9, denaturation, 45 sec at 94°C; annealing, 45 sec at 56°C; extension, 45 sec at 72°C. The products of the amplification were separated on 1% agarose containing ethidium bromide and photographed under a UV transilluminator. As internal controls for the RT, samples without RNA or without reverse transcriptase were prepared in parallel, and these yielded no amplification products. As negative controls for the PCR, samples without reverse-transcribed cDNA or without Taq enzymes were used. As positive controls, samples containing MMP-2 or MMP-9 cDNA were performed (data not shown).

### *Gelatin Zymography*

Protein extraction was performed according to the method provided by Trizol reagent (Gibco-BRL Life Technologies Inc., New York). Protein extract (50 μg) was mixed with 4-strength sample buffer (8% SDS [w:v], 0.04% bromophenol blue [w:v], 40% glycerol [v:v], 0.25 M Tris) and incubated at 37°C for 30 min, then subjected to electrophoresis in 10% polyacrylamide gel containing 0.5 mg/ml gelatin (Difco Laboratories, Detroit, MI). The gel was washed in 2.5% Triton X-100, 50 mM Tris-HCl, at pH 7.5 for 1 h to remove SDS, and incubated for 18 h in calcium assay buffer (50 mM Tris, 200 mM NaCl, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, 1% Triton X-100, pH 7.5) at 37°C. MMP specificities were determined by adding activator (0.5 mM APMA) or inhibitor (7.2 mg/ml EDTA) to MMPs. The former thickened the bands, whereas the latter made the bands disappear (data not shown). Staining was done with 0.2% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid, and the gel was destained with 10% acetic acid. Estimation of molecular weight was possible owing to the concurrent electrophoresis of molecular weight markers.

### *Statistics*

The quantity of the PCR products was determined by densitometric analysis of the intensities of the bands (MetaView Image Analyzing System, version 4.50, Universal Imaging Corp., Downingtown, PA). The relative levels of MMP-2 and MMP-9 mRNAs normalized to β-actin mRNA were calculated. Activities of MMP-2 and MMP-9 detected by gelatin zymography were quantified by densitometric analysis of the intensities of the bands.

Statistical analysis was performed using the Statistical Package for Social Science (SPSS for Windows package release 10.0; SPSS Inc., Chicago, IL). The Student *t*-test was used to analyze the statistical differences between the treated horns and their corresponding controls. $P < 0.05$ was considered to be statistically significant.

## RESULTS

### *Effect of Intrauterine Injection of Lactacystin on Mouse Embryo Implantation*

Our study was aimed at investigating whether proteasomes are involved in mouse embryo implantation. On Day 3 of pregnancy, 5 μl of 0.05 ng/μl (n = 17) or 0.1 ng/μl (n = 9) lactacystin was injected into one of the mouse uterine horns, and the contralateral horn was injected with

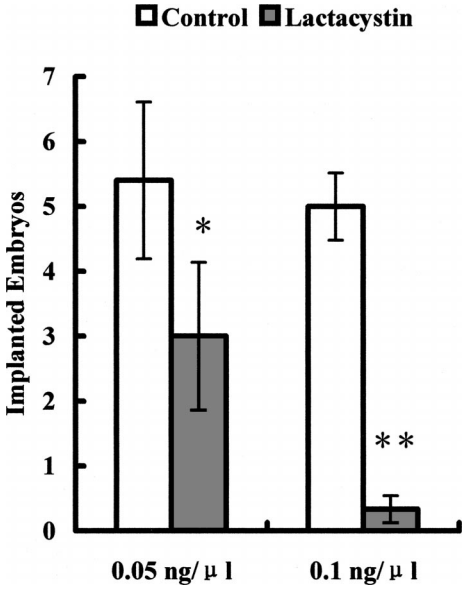


FIG. 1. The effect of intrauterine lactacystin injection (0.05 ng/μl, n = 17; 0.1 ng/μl, n = 9) on the mouse embryo implantation compared with its control, which was injected with 5 μl of distilled water: $*P < 0.05$; $**P < 0.01$.

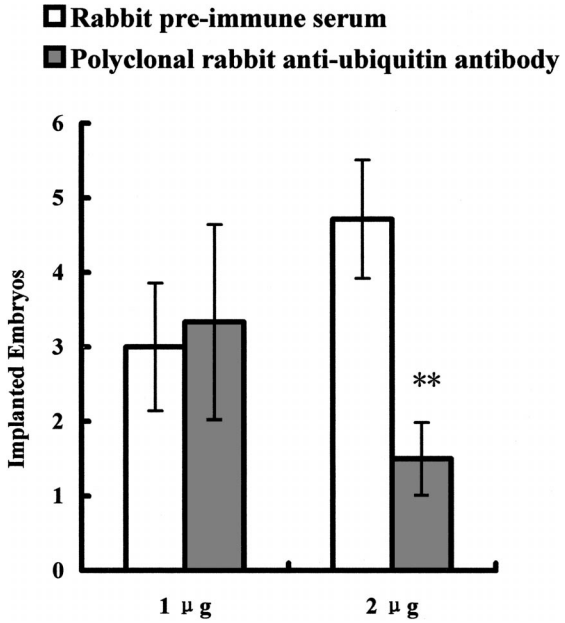


FIG. 2. The effect of intrauterine injection of polyclonal anti-ubiquitin antibody (1 μg, n = 10; 2 μg, n = 10) on the mouse embryo implantation compared with its corresponding control, which was injected with 5 μl and 10 μl of rabbit pre-immune serum, respectively. $**P < 0.01$.

an equal volume of distilled water, the control solution. On Day 7 of pregnancy, mice were killed and the number of implanted embryos in each uterine horn was counted. As shown in Figure 1, intrauterine injection of 5 μl of 0.05 ng/μl or 0.1 ng/μl lactacystin decreased ($P < 0.05$, $P < 0.01$, respectively) the number of implanted embryos. In subsequent studies on the modulatory effect of lactacystin on the expression of MMP-2 and MMP-9, 0.1 ng/μl of lactacystin was used.

### *Effect of Intrauterine Injection of Polyclonal Rabbit Anti-Ubiquitin Antibody on Mouse Embryo Implantation*

Compared with the control (intrauterine injection with rabbit preimmune serum), intrauterine injection of 2 μg polyclonal rabbit anti-ubiquitin antibody decreased the number of implanted embryos significantly ($P < 0.01$; Fig. 2), whereas 1 μg of this antibody had no significant ($P > 0.05$) effect on the number of implanted embryos (Fig. 2).

### *Effect of Intrauterine Injection of Monoclonal Mouse Anti-Ubiquitin Antibody on Mouse Embryo Implantation*

Results in Figure 3 show that compared with the control horn, injected with normal mouse IgG, intrauterine injection of 2 μg monoclonal mouse anti-ubiquitin antibody did not significantly ($P > 0.05$) decrease the number of implanted embryos, but 3 μg of this antibody decreased ($P < 0.05$) the number of implanted embryos.

### *Effect of Lactacystin on the Expression of MMP-2 and MMP-9 mRNAs and the Activities of MMP-2 and MMP-9 During Mouse Embryo Implantation*

*Effect of lactacystin on the expression of MMP-2 and MMP-9 mRNAs during mouse embryo implantation.* Mouse uterine horns were collected on Days 5, 6, and 7 of pregnancy after the intrauterine injection of 5 μl of 0.1 ng/μl lactacystin on Day 3 of pregnancy. Using RT-PCR, mRNA for MMP-2 was detected in the total RNA samples from the mouse uteri treated with lactacystin or the control solution on Days 5, 6, and 7 of pregnancy. Representative pictures of PCR-amplified products are shown in Figure 4A. MMP-2 mRNA was significantly ($P < 0.01$) decreased in the lactacystin-treated uterine horn compared with that in the control horn on Days 5, 6, and 7 of pregnancy (Fig. 4B).

Representative pictures of PCR-amplified products of MMP-9 mRNA in mouse uteri treated with lactacystin or the control solution for 2, 3, and 4 days are shown in Figure 5A. On Day 5 of pregnancy, expression of MMP-9 mRNA in the lactacystin-treated uterine horn showed no significant

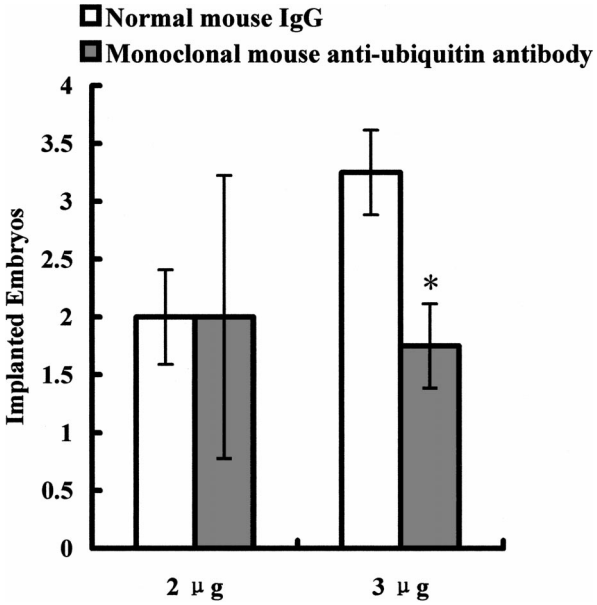


FIG. 3. The effect of intrauterine injection of monoclonal anti-ubiquitin antibody (2 μg, n = 8; 3 μg, n = 8) on the mouse embryo implantation compared with its corresponding control, which was injected with 10 and 15 μl of normal mouse IgG, respectively. $*P < 0.05$.

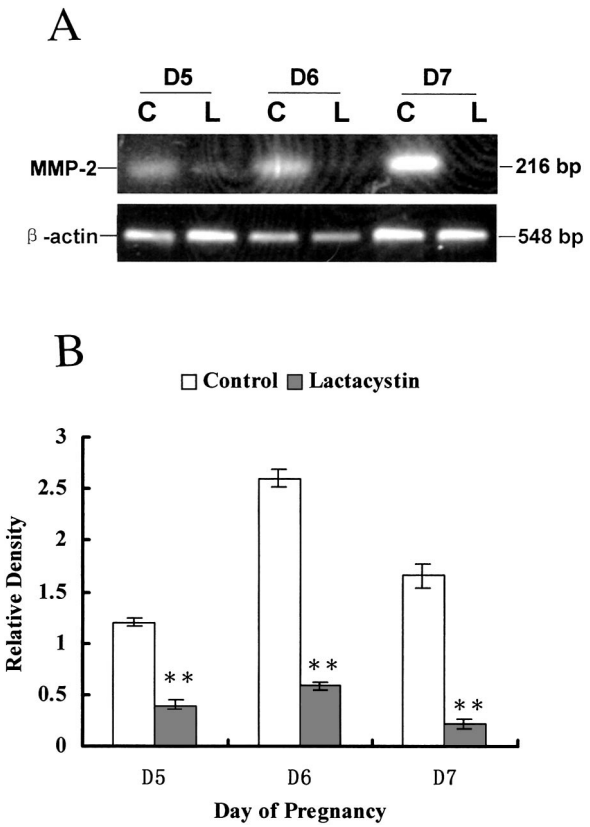


FIG. 4. RT-PCR analysis of MMP-2 transcripts in the mouse uteri on Days 5, 6, and 7 of pregnancy after treatment with lactacystin (L) or the control solution (C) on Day 3 of pregnancy. **A**) Agarose-gel electrophoresis of MMP-2 and β-actin (used as an internal control) on Days 5 (D5), 6 (D6), and 7 (D7) of pregnancy (indicated above the gel). The PCR product sizes in base pair (bp) are shown on the right. **B**) MMP-2 mRNA relative levels in the mouse uteri on Days 5, 6, and 7 of pregnancy treated with lactacystin or the control solution. The relative level for MMP-2 in each tissue was determined as the ratio of MMP-2 mRNA:β-actin mRNA measured by densitometry. Compared with its corresponding control, $^{**}P < 0.01$.

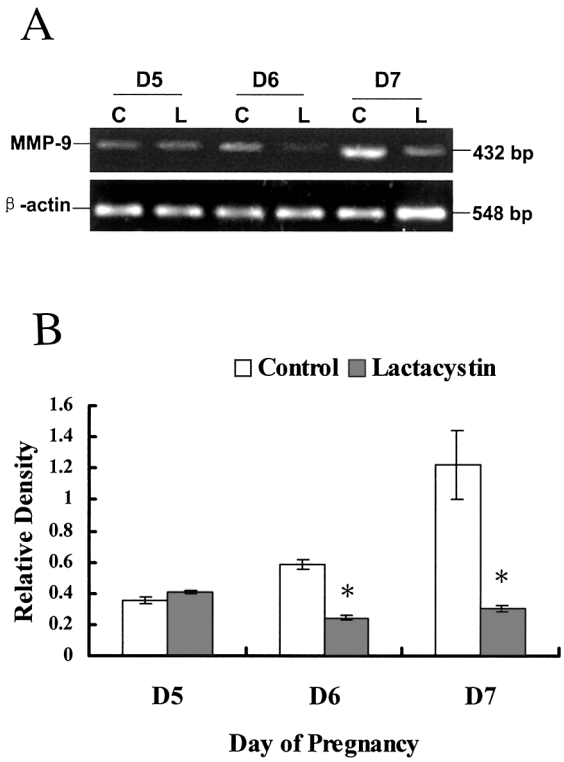


FIG. 5. RT-PCR analysis of MMP-9 transcripts in the mouse uteri on Days 5, 6, and 7 of pregnancy after treatment with lactacystin (L) or the control solution (C) on Day 3 of pregnancy. **A**) Agarose-gel electrophoresis of MMP-9 and β-actin (used as an internal control) on Days 5 (D5), 6 (D6), and 7 (D7) of pregnancy (indicated above the gel). The PCR product sizes in base pair (bp) are shown on the right. **B**) MMP-9 mRNA relative levels in the mouse uteri on Days 5, 6, and 7 of pregnancy treated with lactacystin or the control solution. The relative level for MMP-9 in each tissue was determined as the ratio of MMP-9 mRNA:β-actin mRNA measured by densitometry. Compared with its corresponding control, $^{*}P < 0.05$.

($P > 0.05$) difference from the control side (Fig. 5B). However, on Days 6 and 7 of pregnancy, expression of MMP-9 mRNA in the lactacystin-treated uterine horn was significantly ($P < 0.05$) decreased compared with that in the control side (Fig. 5B).

*Effect of proteasomes on the gelatinolytic activities of MMP-2 and MMP-9 during mouse embryo implantation.* As shown in Figure 6, proteins from the control side uterine horn contained both gelatinases, MMP-2, and MMP-9, as detected by lytic zones in the zymogen gels. The lower migrating bands at 72 and 64 kDa are representative of latent (pro) MMP-2 and active MMP-2, respectively. The 88 and 92 kDa bands represent the active MMP-9 and pro-MMP-9, respectively (Fig. 6A). In the uterine horn injected with lactacystin, three bands of gelatin activity at 92, 72, and 64 kDa were detected, and these are consistent with MMP-9 and the latent and active forms of MMP-2 (Fig. 6A). Furthermore, the band at 92 kDa in the lactacystin-treated uterine horn was less evident ($P < 0.01$) than that in the control side, and the gelatinolytic activity at 88 kDa was hardly detectable in the uterine horn injected with lactacystin (Fig. 6B; $P < 0.01$). On the contrary, the gelatinolytic activity of MMP-2 did not show a significant difference ($P > 0.05$) between the lactacystin-treated set and the control set (Fig. 6B).

## DISCUSSION

Remodeling of extracellular matrix is one aspect of embryo implantation. Cell proliferation and differentiation is another indispensable event for successful implantation. In recent years, proteolytic pathways related to cell proliferation and differentiation, such as the UPP, have been a focus of studies to define the complicated regulatory network of implantation [21].

It has been reported that human cultured term decidual cells secret ubiquitin [27]. Ubiquitin is also found in the bovine uterine flushings of early pregnancy [28]. Furthermore, anti-ubiquitin immunoreactivity was up-regulated in human and baboon endometrial stromal cells as they decidualized at the beginning of pregnancy [21]. All previous results suggest that the UPP is involved in the extensive tissue remodeling occurring during early pregnancy [29], but there is no direct evidence thus far for the exact mechanism of the UPP's regulatory effect on embryo implantation in early pregnancy. In this study, a mouse intrauterine injection model was used to study the effect of the UPP on embryo implantation and the expression of MMP-2 and MMP-9 mRNAs and proteins.

Lactacystin, a microbial metabolite, has been reported to be a specific proteasome inhibitor [30, 31]. In this study, lactacystin was injected into the mouse uterus, and its effect on mouse embryo implantation was examined. The results of statistical analysis indicated that implanted embryos in the lactacystin-treated horn were fewer than in the control

side, suggesting that the inhibitor of proteasomes has an inhibitory effect on mouse embryo implantation. These results provide strong evidence that proteasomes may be involved in mouse embryo implantation, and that active proteasomes are a prerequisite for successful embryo implantation.

To further investigate UPP's modulatory effect on mouse embryo implantation, antibodies against ubiquitin were injected into uterine horns. Antibody against a specific factor of a proteolytic pathway is a useful reagent to study the involvement of this pathway in certain cellular functions. The secretory characteristic of ubiquitin [27] makes it feasible to study the effect of ubiquitin on embryo implantation by the method of intrauterine injection of antibodies. In this study, it was found that both monoclonal and polyclonal anti-ubiquitin antibodies decreased the number of implanted embryos. Antibodies to ubiquitin injected into the lumen of the uterus will interact with ubiquitinated proteins in the extracellular matrix or on the cell membrane, thus preventing them from being recognized and degraded by the UPP. The inhibitory effect of anti-ubiquitin antibodies on embryo implantation strongly suggests that the degradation of some ubiquitinated proteins in the ECM of the mouse uterus or some signaling pathway initiated from the recognition between the antigen and the antibody to ubiquitin occurring on the cell membrane is necessary for successful implantation, and the UPP is involved in mouse embryo implantation.

MMP-2 and MMP-9 play an important role during mammalian embryo implantation. In nonreproductive tissues, UPP has been reported to have a regulatory effect on the expression of MMPs [9]. Therefore, it is possible that this pathway may also function in regulating MMP expression during embryo implantation. In this study, the expression of MMP-2 and MMP-9 mRNAs and the gelatinolytic activities of MMP-2 and MMP-9 in the pregnant mouse uteri injected with lactacystin were examined.

The results indicate that when the activity of proteasomes was inhibited during embryo implantation, the expression of MMP-2 and MMP-9 was decreased at the transcriptional level, suggesting that proteasomes are involved in the regulation of the gene expression of MMP-2 and MMP-9 during embryo implantation. It is possible that the UPP has a regulatory effect on the gene expression of MMP-2 and MMP-9, since the UPP is mainly a cellular proteolytic pathway. It has been reported that there are binding sites for NF-κB and AP-1 in the promoter region of the MMP-9 gene [32–34]. NF-κB [35] is an important transcription factor. Under basal nonstimulated conditions, the association between the inhibitors of nuclear factor κB (I-κB) and NF-κB molecules masks the nuclear localization site on the NF-κB molecules, thus preventing movement to the nucleus. Conversely, under stimulation by cytokines, degradation of I-κB by the UPP leads to the nuclear translocation of NF-κB to stimulate expression of its target genes, including MMP-9 and MMP-14. Activation of NF-κB is closely related to the UPP [36, 37], and NF-κB binding to the promoter of MMP-9 is an important mechanism in regulating MMP-9 expression [38]. Thus the fact that injection of lactacystin decreased MMP-9 gene expression during embryo implantation suggests that the proteasome's regulatory effect on MMP-9 gene expression may be through NF-κB. Similar results were reported in the cultured human squamous carcinoma cells [9]. There is no evidence that the UPP regulates the gene expression of MMP-2 thus far, but in this study we found that the proteasome inhibitor decreased the expression of MMP-2. No NF-κB binding sites have been reported on the promoter region of the MMP-2 gene. Therefore, UPP's regulatory effect on the expression of MMP-2 mRNA may be achieved via some other transcription factors. Further investigations on possible transcription factors of MMP-2 and their relationship with the UPP will provide clues on how lactacystin inhibits the expression of MMP-2 mRNA.

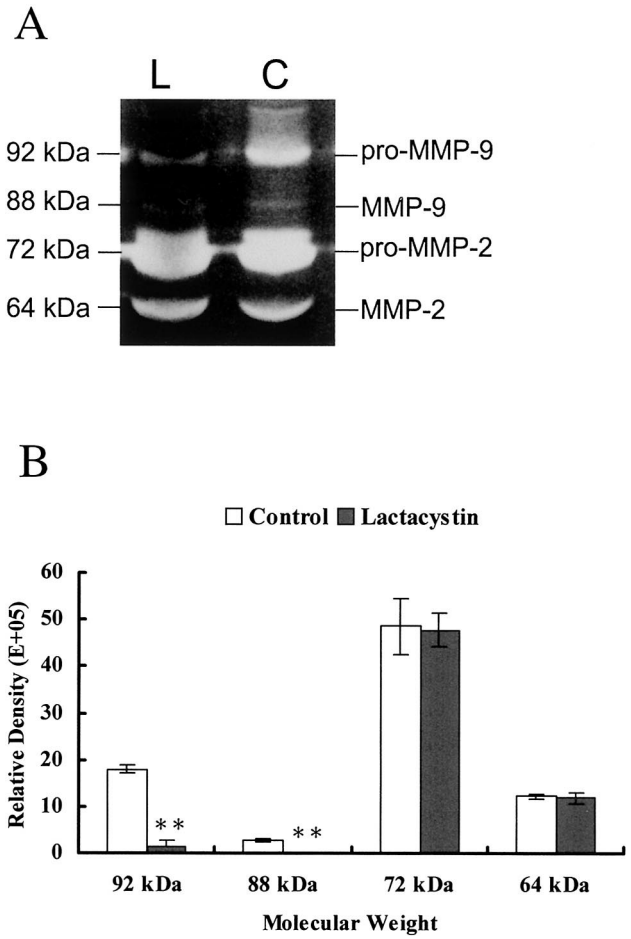


FIG. 6. Zymographic analysis of the gelatinolytic activities of MMP-2 and MMP-9 in the mouse uteri on Day 7 of pregnancy after treatment with lactacystin (L) or the control solution (C) on Day 3 of pregnancy. **A**) Representative gelatin zymogram of mouse uterine extracts on Day 7 of pregnancy treated with L or C. The bands are bright against a dark background, and the molecular size of each band in kDa is demonstrated on the left. C, on Day 7 of pregnancy, the uteri injected with distilled water showed gelatinolytic activities at four bands at 64, 72, 88, and 92 kDa, which represent MMP-2, pro-MMP-2, MMP-9, and pro-MMP-9, respectively. L, in the uterine horn injected with L, three bands of gelatin activity at 92, 72, and 64 kDa were detected. Furthermore, the band of 92 kDa in the L-treated uterine horn was less evident than that in the control side, and the gelatinolytic activity at 88 kDa was hardly detectable in the uterine horn injected with L. **B**) Activities of MMP-2 and MMP-9 detected by gelatin zymography were quantified by computer-aided densitometry. Values are means ± SEM of three experiments. Compared with its corresponding control, $^{**}P < 0.01$.

The activities of MMP-2 and MMP-9 are regulated at several levels including gene expression, stability of mRNA, and activation of zymogen [33]. Regulation of MMP-2 and MMP-9 at the zymographic level was also examined in this study. The activity of MMP-9 was significantly decreased in the uterine horn treated with lactacystin, but the activity of MMP-2 was not significantly affected, suggesting that proteasomes are involved in regulating the

gelatinolytic activity of MMP-9, but not MMP-2, during mouse embryo implantation.

MMP-2 and MMP-9 zymogens are activated by numerous factors including non-MMPs, other MMPs, or TIMPs [39]. TIMP-1 and TIMP-2 are tissue inhibitors of MMPs, but they also function in the activation of MMP-2 and MMP-9, with TIMP-2 preferentially binding with pro-MMP-2, and TIMP-1 preferentially binding with pro-MMP-9 [40]. Binding of TIMP-2 and TIMP-1 with pro-MMP-2 and pro-MMP-9 prevents their degradation. It has been reported that activation of TIMP-1 is related to the UPP [41], hinting that UPP's effect on the gelatinolytic activity of MMP-9 was fulfilled through their inhibition of TIMP-1 during embryo implantation. Activation of MMP-2 zymogen was under the control of MMP-14 and TIMP-2. Expression of MMP-14 was mediated by NF-κB [42]. Philip et al. [43] found that osteopontin could stimulate mouse melanoma cell growth and pro-MMP-2 activation by regulating the expression of MMP-14 activated by NF-κB. However, our study did not show that the proteasome regulated the gelatinolytic activity of MMP-2. The possible mechanism in this process remains to be further investigated.

In summary, UPP is involved in mouse embryo implantation, and this pathway's effect on embryo implantation is mediated in part by regulating MMP-2 and MMP-9 mRNA expression and the gelatinolytic activity of MMP-9.

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