Bovine endometrial metallopeptidases MMP14 and MMP2 and the metallopeptidase inhibitor TIMP2 participate in maternal preparation of pregnancy

Susanne E. Ulbrich, Swanhild U. Meyer, Karina Zitta, Stefan Hiendleder, Fred Sinowatz, Stefan Bauersachs, Mathias Büttner, Thomas Fröhlich, Georg Arnold, Horst-Dieter Reichenbach, et al.

Title: Bovine endometrial metallopeptidases MMP14 and MMP2 and the metallopeptidase inhibitor TIMP2 participate in maternal preparation of pregnancy

Authors: Susanne E. Ulbrich, Swanhild U. Meyer, Karina Zitta, Stefan Hiendleder, Fred Sinowatz, Stefan Bauersachs, Mathias Büttner, Thomas Fröhlich, Georg Arnold, Horst-Dieter Reichenbach, Eckhard Wolf, Heinrich H.D. Meyer

PII: S0303-7207(10)00470-3
DOI: doi:10.1016/j.mce.2010.09.009
Reference: MCE 7640

To appear in: *Molecular and Cellular Endocrinology*

Received date: 27-10-2009
Revised date: 18-9-2010
Accepted date: 21-9-2010

Please cite this article as: Ulbrich, S.E., Meyer, S.U., Zitta, K., Hiendleder, S., Sinowatz, F., Bauersachs, S., Büttner, M., Fröhlich, T., Arnold, G., Reichenbach, H.-D., Wolf, E., Meyer, H.H.D., Bovine endometrial metallopeptidases MMP14 and MMP2 and the metallopeptidase inhibitor TIMP2 participate in maternal preparation of pregnancy, *Molecular and Cellular Endocrinology* (2010), doi:10.1016/j.mce.2010.09.009

Bovine endometrial metallopeptidases MMP14 and MMP2 and the metallopeptidase inhibitor TIMP2 participate in maternal preparation of pregnancy

Susanne E. Ulbrich[1*)], Swanhild U. Meyer[1)], Karina Zitta[2,7)], Stefan Hiendleder[2,8)], Fred Sinowatz[4)], Stefan Bauersachs[2,3)], Mathias Büttner[5)], Thomas Fröhlich[3)], Georg Arnold[3)], Horst-Dieter Reichenbach[6)], Eckhard Wolf[2,3)] and Heinrich H.D. Meyer[1)]

[1]Physiology Weihenstephan, Technical University Munich, Freising, Germany
[2]Chair for Molecular Animal Breeding and Biotechnology, and [3]Laboratory for Functional Genome Analysis (*LAFUGA*), Gene Center, Ludwig-Maximilians-Universität München, Munich, Germany
[4]Institute of Veterinary Anatomy, Histology and Embryology, Ludwig-Maximilians-University Munich, Munich, Germany
[5]Bavarian Health and Food Safety Authority, Oberschleißheim, Germany
[6]Bavarian State Research Center for Agriculture, Institute of Animal Breeding, Grub, Germany
[7]*present address*: Department of Anaesthesiology and Intensive Care Medicine, University Hospital Schleswig-Holstein, Campus Kiel, Germany
[8]*present address*: JS Davies Epigenetics and Genetics Group, School of Agriculture, Food & Wine and Research Centre for Reproductive Health, The University of Adelaide, Roseworthy Campus, SA 5371, Australia

Grant support: This study was supported by the German Research Foundation (UL 350/1-2, FOR 478).

[*]Correspondence and reprint requests: Susanne E. Ulbrich, Physiology Weihenstephan, Technical University of Munich, Weihenstephaner Berg 3, 85354 Freising, Germany; Telephone: +49 8161 71 4429; FAX: +49 8161 71 4204; E-mail: ulbrich@wzw.tum.de

Keywords: matrix metallopeptidases, metallopeptidase inhibitor, preimplantation, pregnancy, (Bos taurus), (endometrium)

ABSTRACT

Early embryonic development is critically dependent on both maternal preparation and embryonic signalling of pregnancy. Matrix metallopeptidases (MMP) contribute to spatial and temporal matrix remodeling in the bovine endometrium. In this study we observed distinct changes of MMP2, MMP14 and the metallopeptidase inhibitor TIMP2 between different phases of the estrous cycle indicating an endocrine regulation. A distinct increase of TIMP2 protein abundance was ascertained in the uterine lumen during the time of embryo elongation. The expression pattern and cellular localization correlate well with the assumed effects of MMPs on release and activation of cytokines and growth factors directing cell migration, differentiation, and vascularization during this pivotal period of development. Specifically, active MMP2 in the endometrium may determine the allocation of growth factors supporting conceptus development. Due to the presence of a day 18 conceptus *in vivo* and day 8 blastocysts *in vitro* endometrial *TIMP2* mRNA expression was induced. The results imply that *TIMP2* is involved in very early local maternal recognition of pregnancy. Matrix metallopeptidases are likely to participate in remodeling processes preparing a receptive endometrium for a timely precise regulation of embryo development.

Figure 1:

Transcript abundance of matrix metallopeptidases and their inhibitor in bovine endometrium.

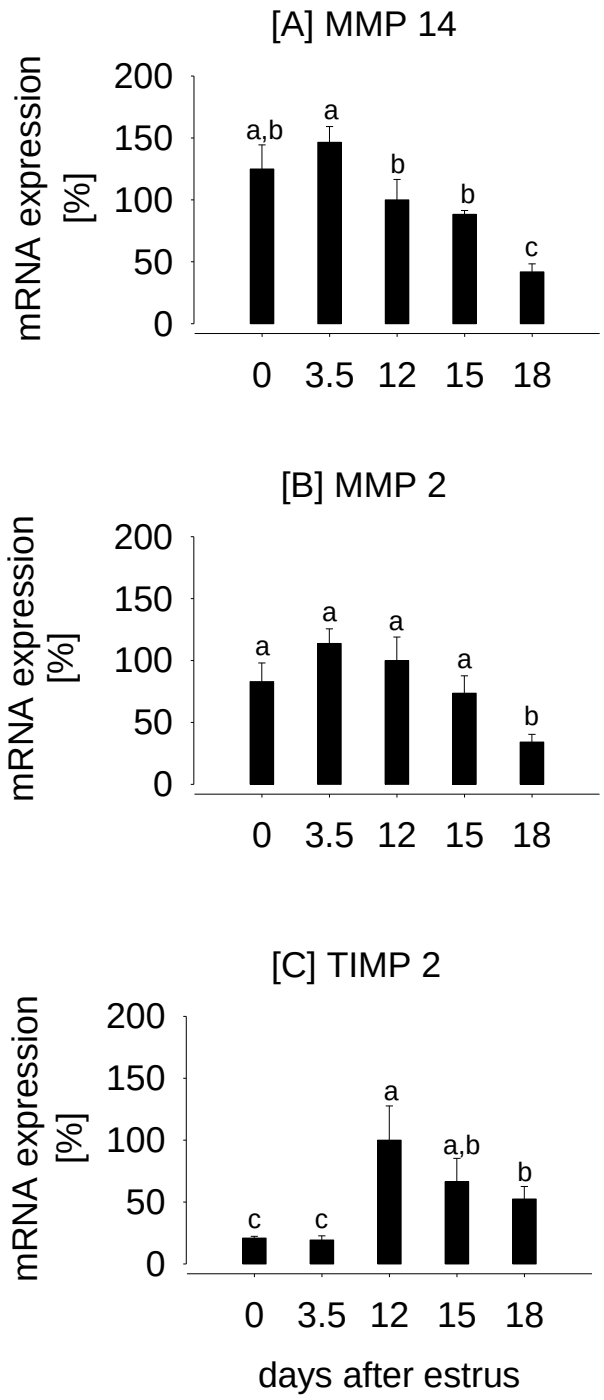


Figure 2:

Immunohistochemical localization of MMP14, MMP2 and TIMP2 in bovine endometrium.

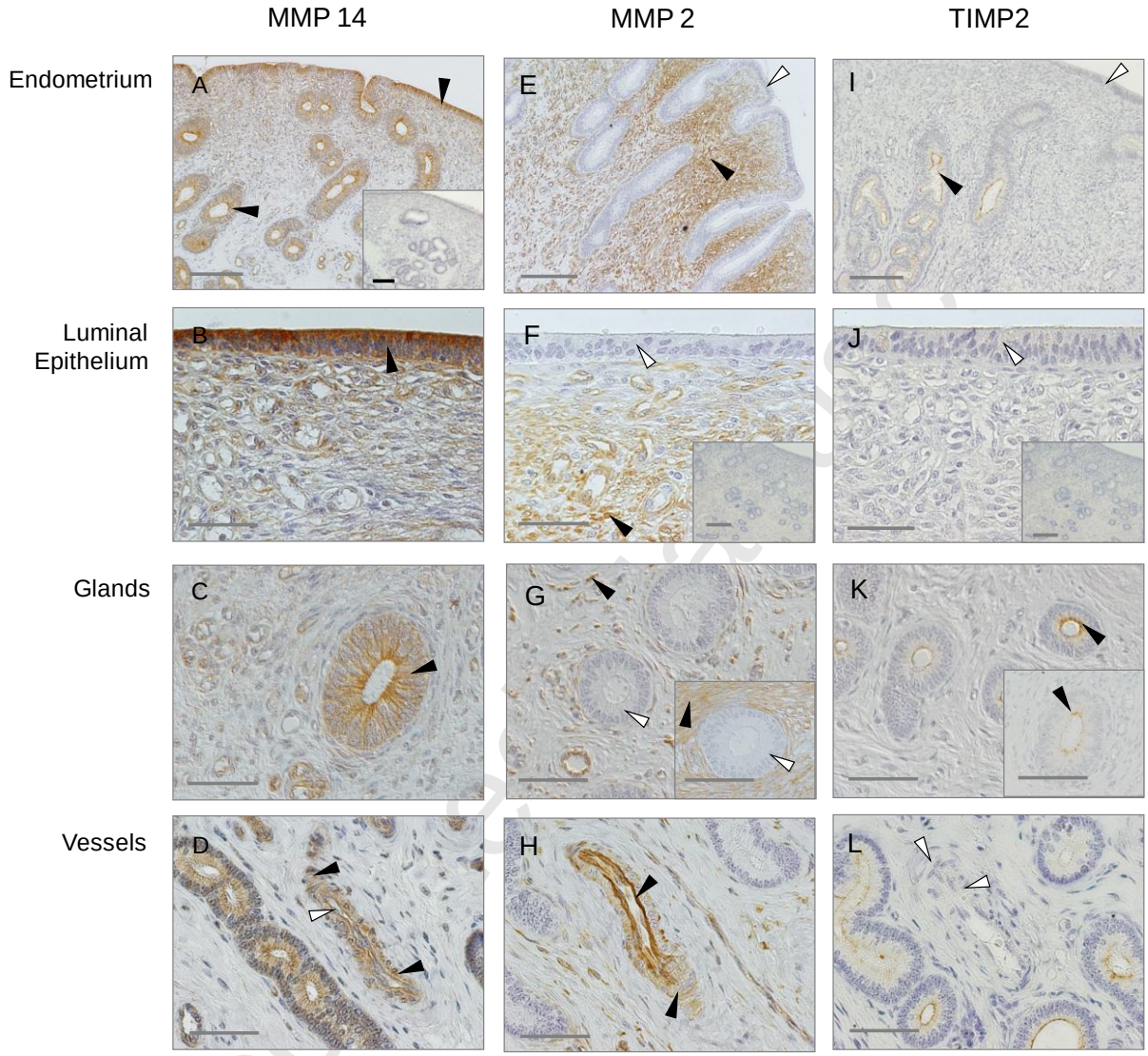
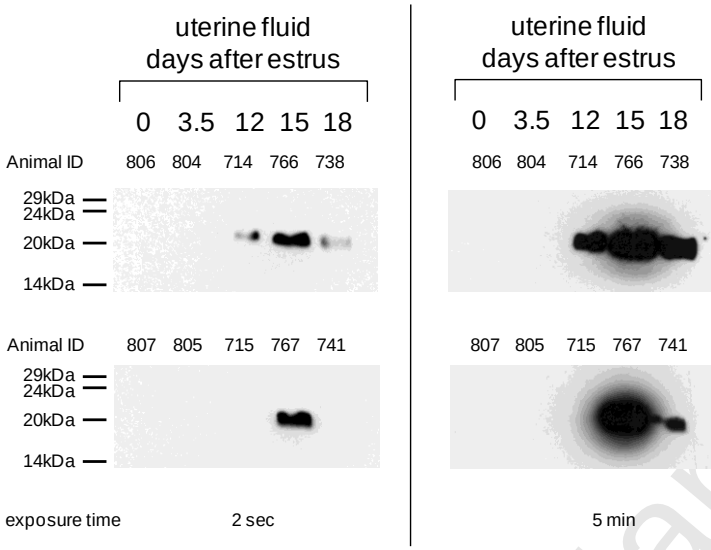


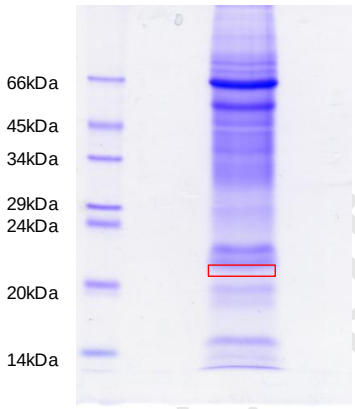
Figure 3:

Protein abundanceof TIMP2 in selected uterine fluid samples during the estrous cycle and LC-MS identification of TIMP2 protein.

[A]



[B]



Acc. No. AAI02711
Bos taurus TIMP metallopeptidase inhibitor 2 [TIMP2].

MGAAARSLPL AFCLLLLGTL LPRADACSCS PVHPQQAFCN
ADIVIRAKAV NKK**EVDSGND IYGNPIKRIQ YEIKQIK**MFK
GPDQDIEFIY TAPAAAVCGV SLDIGGKK**EY LIAGK**AEGNG
NMHITLCDFI VPWDTLSATQ KKSLNHRYQM GCECKITRCP
MIPCYISSPD ECLWMDWVTE KNINGHQAK**F FACIKRSDGS**
CAWYRGAAPP KQEFLDIEDP

[C]

EVDSGNDIYGNPIK

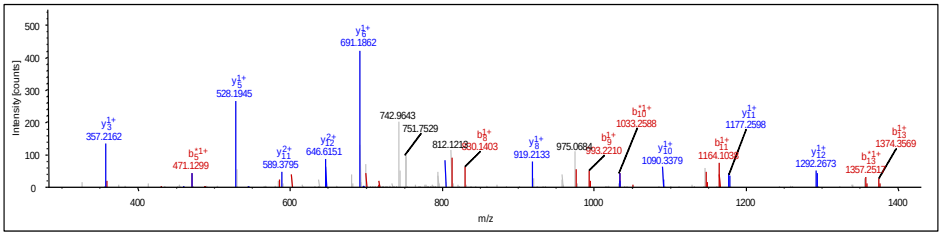


Figure 4:

Enzyme activity of MMP2 in bovine endometrium.

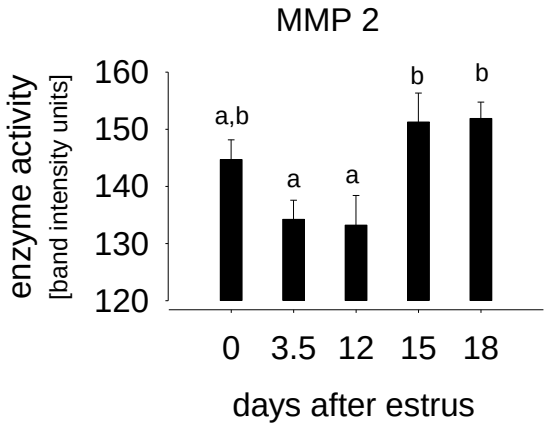


Figure 5:

Messenger RNA expression of *MMP14 [A]*, *MMP2 [B]* and *TIMP2 [C]* in endometrial tissue during the estrous cycle (black bars) and early pregnancy (grey bars).

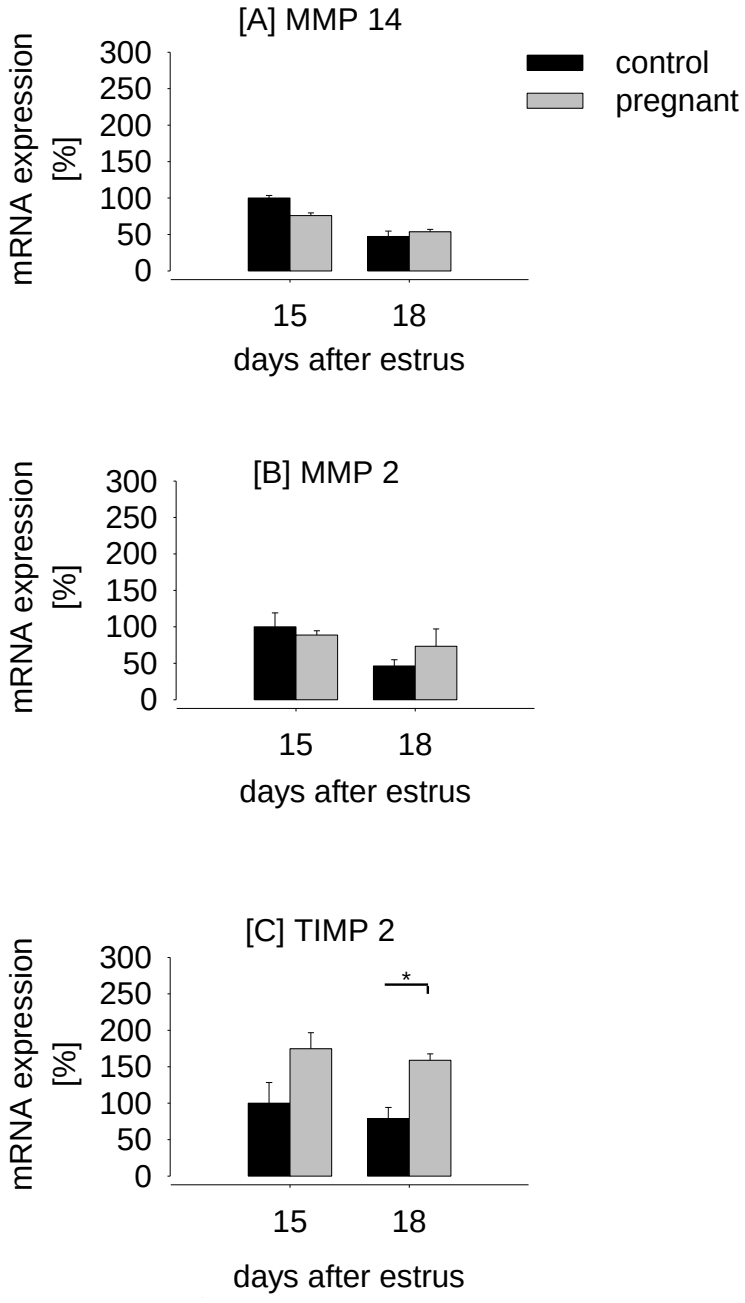
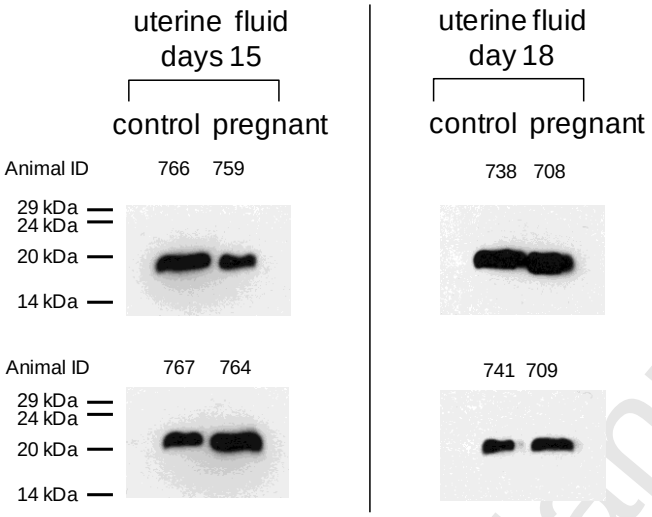


Figure 6:

TIMP2 western blot of selected uterine fluid samples [B] and gelatine zymography showing active MMP2 at 62 kDa [B] of selected endometrium samples during early pregnancy compared to non-pregnant control animals. No differences appeared.

[A] TIMP2



[B] MMP2

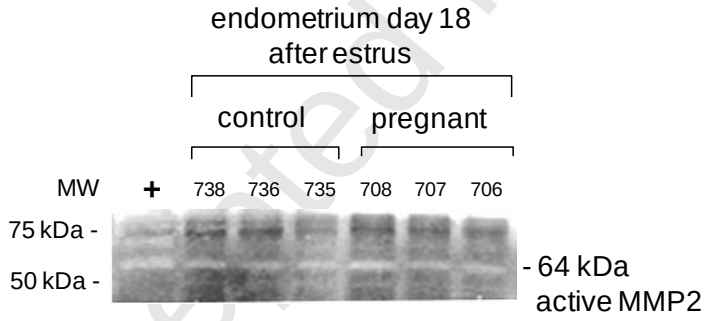
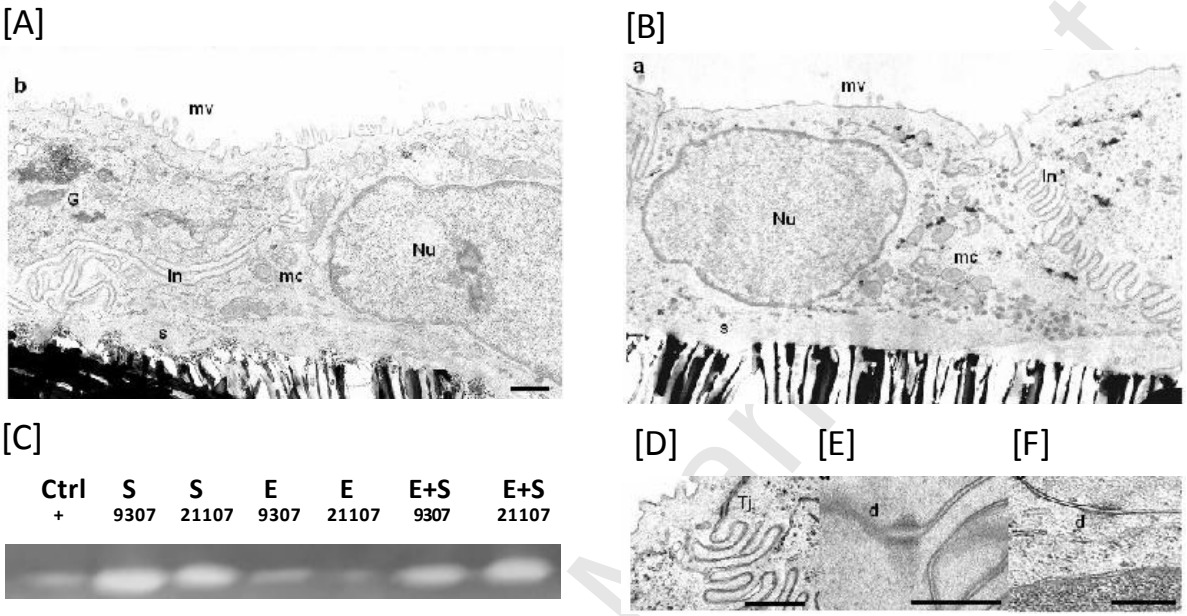


Figure 7:

Representative transmission electron microscopy of endometrial epithelial cells in culture [A-B, D-F]. Gelatine zymography of MMP2 in cell culture supernatants [C].



INTRODUCTION

Functional changes occurring during the bovine estrous cycle are of specific importance for the preparation of the reproductive tract hosting final gamete maturation, fertilization, and embryonic development. Embryonic losses occur most prevalently during the preimplantation period (Thatcher et al., 2006). A well-synchronized maternal environment must therefore be present to allow normal development.

Matrix metallopeptidases (MMPs) and the tissue metallopeptidase inhibitors (TIMPs) are most important mediators in the process of remodeling extracellular matrices (ECM) (Visse and Nagase 2003). Built by a complex network of fibrous matrix proteins, the ECM not only drives form and polarity of cellular arrangement, but also impairs growth and development as well as blood vessel formation and stabilization. It forms a dynamic intercellular net, predominated by collagen and proteoglycans, through which ions, nutrients, metabolites and peptides like growth factors may diffuse. As MMPs degrade ECM components and cell surface molecules, they are involved in the release and activation of cytokines and growth factors which direct cell migration, differentiation, and vascularization (Nagase et al., 2006).

MMPs and TIMPs are involved in the regulation of key steps in reproduction, such as ovulation and luteolysis, endometrium function, growth and development of fetal membranes, cervix dilation during parturition and postpartum regression of the uterus. Uterine receptivity requires timely and spatially controlled changes of the ECM in which MMPs are involved. The transcription of MMPs may be stimulated through cytokines, growth factors, hormones, and cell-to-cell or cell-matrix interactions (Nagase et al., 2006; Nagase and Woessner, Jr. 1999). The activity of the MMPs is low in tissues which are in a dynamic equilibrium.

MMPs consist of highly conserved functional domains which direct substrate specificity and comprise binding sites for MMP-specific inhibition by TIMPs. MMPs are synthesized as pre-proenzymes and processed to proenzymes which are eventually secreted into the extracellular space. The activation of the proenzyme in the ECM is of specific regulatory importance, as this is a prerequisite for exhibiting proteolytic activity (Nagase et al., 2006). Due to the presence of specific inhibitors, proteolytic degradation, binding to a specific substrate or to a specific site of action and endocytosis (Remacle et al., 2003) are control mechanisms directing MMP activity. The natural inhibitors of MMPs, TIMPs 1-4, are involved in the maintenance of balanced ECM remodeling. They bind with different affinities to the catalytically active domain and thereby exert their specific proteinase inhibiting activity. TIMP2 additionally participates in activating pro-MMP2 by binding pro-MMP2 to form a

trimolecular complex with membrane bound MMP14. An adjacent MMP14 molecule is then able to cleave the TIMP2 bound pro-MMP2 (Itoh and Seiki 2006). The concentration of all three molecules is important as pro-MMP2 may only be cleaved in the presence of a neighboring non-TIMP2 bound MMP14 molecule and free available TIMP2. In case TIMP2 is present in excess or absent, pro-MMP2 is not cleaved (Curry, Jr. and Osteen 2003).

Recently we have applied a holistic transcriptomic approach to define changes in endometrial gene expression during the bovine estrous cycle (Mitko et al., 2008) and to compare pregnant and non-pregnant endometrium prior to implantation (Bauersachs et al., 2006; Klein et al., 2006). Herein, a high number of genes related to extracellular matrix remodeling were found to be differentially expressed. In the present study, we characterized the expression of a prominent subset of candidate genes thereof in detail including their cellular distribution and protein activity. Additionally, *in vitro* co-culture of endometrial cells with embryos was performed to address the question whether endometrial matrix metallopeptidases and the metallopeptidase inhibitor TIMP2 contribute to maternal preparation and endometrial recognition of pregnancy.

MATERIALS AND METHODS

Pretreatment of Animals and Collection of Endometrial Tissue Samples

All experiments were performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as promulgated by the Society for the Study of Reproduction and with the European Convention on Animal Experimentation.

Study A

Cyclic heifers (Deutsches Fleckvieh, Simmental) were cycle synchronized at diestrus as described previously (Ulbrich et al., 2009). In brief, animals were observed for sexual behavior to determine standing heat. Blood samples were taken at day 0 of the estrous cycle and just before slaughter to determine serum progesterone (P_4) levels (Prakash et al., 1987). Animals of the pregnant groups (n=5 per group) were inseminated with cryo-preserved sperm (ejaculate + diluter 1:10) at estrus and slaughtered either at day 15 or at day 18 after insemination. Animals only entered the study if an intact conceptus was visually detected in the uterine lumen. Control animals (n=5 per group) were inseminated with the supernatant of centrifuged sperm and slaughtered at estrus or 3.5, 12, 15, or 18 days after insemination. No concepti were present in control uteri. The obtained uteri were flushed with 100 mL PBS (~21 °C). Flushing fluid was centrifuged at 800 g for 10 min at 5 °C and stored at -20 °C until further investigation. The uterine horns were opened longitudinally as described previously (Bauersachs et al., 2005). Samples were carefully taken from the intercaruncular endometrium of the middle part of the ipsilateral uterine horn with a scalpel and immediately transferred into liquid nitrogen (zymography) or vials containing 4 ml RNAlater (Ambion, Cambridgeshire, UK) and incubated at 4°C overnight (quantitative PCR). Samples were then stored at -20°C until further processing. For immunohistochemistry, uterine tissue samples were transferred to Bouin's fixation solution prior to embedding in paraffin. Tissue samples for qPCR, immunohistochemistry and zymography were collected from the same animals.

Study B

In a confirming survey, five monozygotic twin pairs generated by embryo splitting were sampled as described previously (Klein et al., 2006). Briefly, seven days after standing heat, two in vitro-produced bovine blastocysts (Day 7 after in vitro fertilization) in 200 µL culture medium were transferred into the ipsilateral uterine horn of one twin of each pair. In vitro production of embryos was performed as described previously (Stojkovic et al., 1999b), with minor modifications (Klein et al., 2006). The corresponding twin served as a control and received a sham transfer of the same amount of transfer medium without embryos. The

animals were monitored for serum progesterone as described above, and the two groups showed no differences (mean 3.7 ng/ml). At day 18 after estrus the animals were slaughtered. Samples from the ipsilateral intercaruncular endometrium were recovered and processed for mRNA analyses as described above.

RNA extraction and reverse transcription

Total RNA from endometrial tissue samples was isolated using Trizol™ reagent (Invitrogen, Karlsruhe, Germany) as described previously (Bauersachs et al., 2006). Quantitative real-time PCR (qPCR) was performed to evaluate the influence of the different extraction methods on the mRNA yield according to the MIQE guidelines (Bustin et al., 2009). The integrity of total RNA was determined by the Agilent 2100 Bioanalyzer (RNA 6000 Nano Assay Kit). RNA integrity numbers (RIN) ranged between 7 and 10. Quantity was spectrophotometrically determined at 260 nm by the Nanodrop 1000. A two-step qPCR was undertaken as described recently (Bauersachs et al., 2006). Quantitative real-time PCR reactions were performed using the LightCycler® DNA Master SYBR Green I protocol (Roche). Specific primers were used to amplify fragments referring to the genes of interest as depicted in Table 1. All amplified PCR fragments were commercially sequenced to verify the resulting PCR products (MWG-Biotech, Ebersberg, Germany). Thereafter the specific melting point of the amplified product determined within the LightCycler® standard qPCR protocol served as verification of the product identity. The cycle number required to achieve a definite SYBR Green fluorescence signal was calculated by the second derivative maximum method (LightCycler software version 3.5.28).

Data Analysis of qPCR

Results are presented as means ± SEM (n=4) of relative mRNA expression levels. The crossing point (CP) determined for the target genes were normalized against the housekeeping gene *Histone H3* (∆CP) (Livak and Schmittgen 2001). Results are presented as % expression compared to day 12 (Fig. 1) and to day 15 controls (Fig. 4). For statistical analysis the SAS program package release 9.1.3 (2002; SAS Institute, Inc., Cary, NC, USA) was used. The normal distribution was tested by the Kolmogorow-Smirnov method. The effect of the cycle day on mRNA expression was analyzed using one-way ANOVA and localized using the Least Significant Difference test (LSD). Student´s *t*-test was employed to compare the transcript abundance in endometrium samples of pregnant versus control group at day 15 and day 18, respectively. Differences were considered significant at $P<0.05$. Graphs were plotted with SigmaPlot 8.0 (SPSS Software GmbH, Munich, Germany).

Immunohistochemistry

Serial sections (4 µm) were cut on a Leitz microtome. To expose antigenic sites dewaxed sections were heated four times to 95°C in a 600-W microwave oven in 10 mM sodium citrate buffer (pH 6.0) for 5 min. Endogenous peroxidase activity was then eliminated by incubation in 0.5 % (v/v) H_2O_2 solution in absolute methanol for 15 min at 20°C. Non-specific protein binding was eliminated by incubation in 10 % normal goat serum in phosphate buffered saline (PBS) for 1 h at 20°C. Sections were then incubated with, a polyclonal rabbit antibody against MMP14 (RB-1544), a monoclonal mouse antibody against MMP2 (MS-806, Ab-4) (all purchased from Lab Vision, Newmarket, Suffolk, UK) (Kliem et al., 2007) as well as a monoclonal mouse antibody against TIMP2 (clone 3A4, antibodies-online GmbH, Aachen, Germany). Each antibody was used at a dilution of 4.0 µg/mL. Incubation was performed for 18 h at 4°C in a humidified chamber. This was followed by incubating the sections with HRP-labeled anti-rabbit or anti-mouse IgG at a concentration of 2.5 µg/mL (Sigma-Aldrich) for 1 hr. The secondary antibody was made visible by reaction with 0.05 % 3,3-DAB and 0.01 % H_2O_2 in PBS for 15 min. Sections were counterstained in Mayer's hematoxylin, dehydrated, cleared, and mounted. Controls were performed by either replacing primary antibody with buffer or non-immune serum, or incubating with DAB reagent alone to exclude the possibility of non-suppressed endogenous peroxidase activity. Lack of detectable staining in the controls demonstrated the specificity of the reactions. Images were captured on an inverse microscope (LEICA DM IRB, Wetzlar, Germany) equipped with an Olympus DP-72 camera (Hamburg, Germany) using differential interference contrasting technique.

Western blotting

Prior to Western blot analysis, uterine flushings were concentrated using Amicon Ultra-4 (Millipore, Bedford, MA) ultrafiltration devices. The devices were prepared according to the manufacturers instructions, pre-rinsed with 4000 µl water and centrifuged for 10 min at 4000 g using a Beckman GS-6KR (Beckman Coulter, Brea, CA, USA) centrifuge. 2000 µl of uterine flushing was applied to the prepared centrifugal filters and centrifuged for 15 min at 4000 g to obtain approximately 150 µl of concentrate. Protein concentration within the concentrate was determined using the Bradford (Bradford 1976) assay. Aliquots of the samples were subjected to gel electrophoresis and Western blotting as described in (Ulbrich et al., 2009). As a control, the transferred proteins were visualized by Ponceau S staining (0.2 % w/v Ponceau S, 3 % trichloroacetic acid, 3 % sulfosalicylic acid in water). The nitrocellulose membrane was blocked overnight in 5 % BSA PBS-T (80 mM di-sodium hydrogen phosphate dehydrate, 20 mM sodium dihydrogen phosphate monohydrate, 100 mM sodium chloride and 0.1 % Tween 20). For immunodetection, a 1:500 dilution of TIMP2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA. U.S.A.) in PBS-T was used.

Membranes were incubated for 1 hour at room temperature, washed once for 10 min and twice for 15 min in PBS-T, and incubated for 1 hour in a 1:2000 dilution of horse raddish peroxidase conjugated anti-mouse secondary antibody (polyclonal goat anti-mouse immunoglobulins, Dako, Hamburg, Germany). The substrate (SuperSignal West Pico Chemiluminescent, Thermo Fisher Scientific , Rockford, USA) was used as recommended by the manufacturer. The membrane was exposed to X-ray film (BioMax XAR, Kodak, Rochester, USA).

Mass spectrometric identification of TIMP2

The SDS gel for TIMP2 identification was prepared using exactly the same conditions as for the Western blot detection of the protein. Instead of a semidry transfer the gel was Coomassie stained overnight (50% v/v methanol, 0.05% w/v Coomassie brilliant blue R-250, 10% v/v acetic acid). After destaining (5% (v/v) Methanol with 7% (v/v) acetic acid) the relevant gel band was excised using a scalpel. Tryptic in gel digestions and extraction of the peptides was performed as described in (Ulbrich et al., 2009). Chromatographic separation of peptides was performed on a nano-HPLC system (Ettan MDLC, GE Healthcare). Peptides were loaded on a reversed phase trap column with a flow-rate of 10µl per min (Loading buffer: 0.1 % formic acid; Trap column: C18 PepMap 100, 5µm bead size, 300 µm i.d., 5mm length, LC Packings) and subsequently separated with an analytical column (Dr. Maisch, Reprosil-Pur C18 AQ, 3 µm; 150 mm x 75 µm, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) with a 120-min gradient (A: 0.1% formic acid, B: 84% acetonitrile and 0.1% formic acid) at a flow rate of 280 nl/min. The gradient was 0-30% B in 80 minutes, 30-60% B in 30 minutes, 100% B for 10 minutes. Mass spectrometry was performed on a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) online coupled to the nano-HPLC system. For electrospray ionization a distal coated SilicaTip (FS-360-50-15-D-20) and a needle voltage of 1.4 kV was used. The MS method consisted of a cycle combining one full MS Orbitrap scan (Resolution: 60,000; Mass range: 300-2000 m/z) with five data dependant LTQ MS/MS scans (acquisition parallel to Orbitrap scans, 35% collision energy). The dynamic exclusion was set to 30 s. Finally the MS/MS data were searched with Mascot Version: 2.1.03 (Matrix Science, Boston, USA) using IPI-bovine database (Release 3.52) and the following parameters: i) Enzyme: Trypsin, ii) Fixed Modification: Carbamidomethyl (C), iii) Variable modifications: Oxidation (M), Methyl (K), N-acetyl (protein); iv) Peptide tol.: 10 ppm, v) MS/MS tol.: 0.8 Da, vi) Peptide charge 1+, 2+ and 3+, vii) Instrument: ESI-TRAP and viii) Allow up to 1 missed cleavages.

Gelatine zymography

Zymography was performed as described previously (Kleiner and Stetler-Stevenson 1994). Briefly, tissue homogenates from 100 mg endometrial samples or cell epithelial cell supernatants were homogenized in PBS using a MagnaLyzer (Roche, Penzberg, Germany). Following a 1 min centrifugation step at 1000 g, tissue supernatants were concentrated 5-fold using 10 kDa Microcon centrifugal filter devices YM-10 (Millipore, Billerica, USA). 30 µg total protein (evaluated by BioRad protein DC assay) was separated on 7 % SDS – page gels (containing 1 mg/ml gelatine) under non-reducing conditions. After electrophoresis, the gels were soaked in 2.5 % Triton X-100 for 30 min to remove SDS and incubated in Tris-HCl (50 mmol/l, pH 7.5), containing $CaCl_2$ (5 mmol/l), and $ZnCl_2$ (1 mmol/l) overnight at 37°C. Finally, gels were stained with Coomassie blue. After Coomassie blue staining white bands of lysis indicate gelatin digestion by MMPs. Densitometric analysis was performed using the ImageJ 1.41o software (ImageJ, NIH, USA).

Endometrial cell culture

Bovine uteri, approximately 8 days after estrus, were collected at a slaughterhouse and kept on ice until further processing in the laboratory (n=5). The physiological status of the uteri was estimated by examination of ovarian morphology (Ireland et al., 1980). The uterine horn ipsilateral to the functional corpus luteum was removed from the uterus, was washed two times with PBS without Ca^{2+}/Mg^{2+} (PBSA), ligated at the horn tip, and filled with 0.76 % EDTA solution. It was then placed in PBSA and incubated for 60 min at 37°C. The epithelial cells were scrapped directly from the surface and placed in 5 ml Dulbecco modified Eagle medium (DMEM) / F12 with 10 % fetal bovine serum supplemented with 10,000 U/ml penicillin and 10 mg/ml streptomycin. The samples were incubated for three hours in 5 % CO_2 in air to allow the attachment of contaminating stromal cells and to improve the purity of the epithelial cell fraction. The unattached epithelial cells were collected and seeded on 24-well culture plates. The endometrial tissue without epithelial cells was further dissected and digested with 0.05 % collagenase (digestion activity 454 U/mg) in DMEM/F12. After 3 h of incubation the suspension was filtered to separate the cells from tissue debris using re-usable syringe filter holders (Sartorius, Göttingen, Germany) equipped with 250 µm filters (Bückmann, Mönchengladbach, Germany). The filtrate was loaded on a second filter (33 µm) to separate the stromal cells from the uterine glands. Isolated stromal and epithelial cells were >95 % viable as shown by trypan blue exclusion. Immunocytochemistry with specific antibodies against cytokeratin (for epithelial cells), and vimentin (for the stromal cells), in combination with propidium iodide staining for nuclei, showed that the purity of each cell population was greater than 90 % (data not shown). Approximately 10^6 viable cells were seeded per well. Stromal cells were placed directly on the plastic surface of a 24-well culture plate (Nunc, Roskilde, Denmark) and epithelial cells were seeded on inserts (Nunc) coated with matrigel

(BD Biosciences) according to the manufacturer's specifications. The inserts were filled with 400 µL medium (DMEM/F12, 10 % FBS) and then placed into the wells containing the seeded stromal cells in 400 µL of the same medium. To improve purity of the stromal cell preparation, the medium was changed 18 h after plating, at which time selective attachment of stromal cells had occurred. The medium was changed every other day until confluence was reached; generally after 8 days in culture at 37°C in a humidified atmosphere of 5 % CO_2 in air.

Transmission electron microscopy

Endometrial epithelial cells were cultured for transmission electron microscopy in inserts with or without stromal cells and were subsequently fixed in 2.5 % glutaraldehyde and 2 % formaldehyde in 0.1 M Na-cacodylate buffer over night at 4°C. After washing the samples four times in 0.1 M Na-cacodylate buffer the samples were contrasted with 2 % osmium tetroxide and 1.5 % potassium ferrocyanide. The samples were then washed three times in 0.1 M Na-cacodylate-buffer, and gradually infiltrated with ethanol (50 % to 100 %). Following two changes of propylene oxide the samples were embedded in Epon for 24 h at 60°C. Semithin and ultrathin sections were sliced on a Reichert Ultracut E microtome. The ultrathin sections were stained with uranyl acetate and lead-citrate (REYNOLDS 1963). All samples were analyzed with a Zeiss 902 electron microscope.

In vitro production of bovine embryos for cell co-culture

Embryos were produced based on procedures described previously (Stojkovic et al., 1999a). Briefly, ovaries were obtained from slaughtered cows. Cumulus-oocyte complexes (COCs) were obtained by aspiration, washed in oocyte maturation medium (TCM-199) with Earle salts containing 22 mg/ml FSH and 8 mg/ml LH and supplemented with 5 % (v/v) estrous cow serum (ECS). Oocytes were matured for 20–22 h at 39°C. After maturation, COCs were washed once in IVF-TALP (modified Tyrode stock solution supplemented with 6 mg/ml essentially fatty-acid-free BSA, 0.022 mg/ml pyruvic acid, and 0.01 mg/ml heparin) and transferred to four-well plates containing 400 µL IVF-TALP per well. Spermatozoa were purified by swim-up procedure in Sperm-TALP (modified Tyrode stock solution supplemented with 6 mg/ml BSA and 0.11 mg/ml pyruvic acid) at 39 °C in 5 % (v/v) CO_2 in humidified air for 1 h. Oocytes were fertilized with 25 µl spermatozoa suspension (~1 x 10^6 spermatozoa/ml). Approximately 18 h after fertilization, presumptive zygotes were denuded of cumulus cells by vortexing in 1 ml synthetic oviduct fluid (SOF) (SOF stock was modified on the day of use by adding 5 % (v/v) ECS, 4 % (v/v) essential amino acids and 1 % (v/v) nonessential amino acids) for 3 min. Groups of 30-35 presumptive zygotes were placed in 400 µL SOF overlaid

with mineral oil and cultured at 39°C in a humidified atmosphere of 5 % (v/v) CO_2, 5 % (v/v) O_2, and 90 % (v/v) N_2 for seven days.

Day 8 hatched blastocysts and zonae pellucidae (day 8, n=5 per well) were added to the cultures which had been in medium without serum for 24 h. Empty zonae pellucidae (day 8, n=5 per well) were added to wells that served as controls under the same conditions. After a coculture of 24 h both blastocysts and empty zonae pellucidae were taken out of the wells. In additional co-culture wells, recombinant bovine interferon-τ instead of embryos was applied for 4 hours (antiviral activity 4.8 x 10^3 U/mL medium, PBL Biomedical Laboratories, Piscataway, NJ, USA) with medium containing the respective diluent serving as respective control. For RNA isolation, epithelial cells were washed with PBS before 500 µL TriZol was added to the inserts or wells that contained the cells. The solution was pipetted up und down vigorously, transferred to a 1.5 ml tube and stored at -20°C until RNA isolation.

Biological assay for Interferon-τ bioactivity

Interferon production was quantified by means of a highly sensitive bioassay based on the inhibition of the cytopathic effect of vesicular stomatitis virus (Indiana strain) on Madin–Darby bovine kidney (MDBK) cells (Rubinstein et al., 1981). The NIH recombinant human interferon-α_2 reference preparation (No. Gxa01-901-535, NIH-Research Reference Reagent Note No. 31, 1984) was included in each assay. The antiviral activity was shown to be mediated by Interferon-τ, as the effects of supernatant and an appropriate control Interferon-τ preparation were blocked by specific anti-interferon sera (kindly provided by Dr. R.M. Roberts, University of Missouri, Columbia, MO) (Stojkovic et al., 1999a). Culture medium that did not contain blastocysts showed no antiviral activity.

RESULTS

Endometrial mRNA expression of *MMP14, MMP2* and *TIMP2* during the estrous cycle

The day of the cycle had a significant effect on endometrial transcript abundance of *MMP14 (p=0.0002), MMP2 (p=0.007)* and *TIMP2 (p<0.0001)* (Fig. 1).

The mRNA expression of *MMP14* was high at estrus and day 3.5 and subsequently declined. At day 18 the transcript abundance was 2.5-fold (*p<0.01*) lower compared to day 12 (Fig. 1B).

A similar mRNA expression pattern as seen for *MMP14* was measured for *MMP2* (Fig. 1C) showing highest expression following estrus. At day 18 the level of expression was 3.3-fold *(p<0.001)* lower as compared to day 3.5.

In contrast, *TIMP2* mRNA expression was low at estrus and day 3.5 (Fig 1D), increased 5-fold towards day 12, and then decreased towards day 18 (Fig. 1D).

Endometrial MMP14, MMP2 and TIMP2 protein localization

The cytoplasm of luminal epithelial cells stained positive for MMP14 protein (Fig. 2A-D), and there was an observable change of intensity during the cycle with predominant expression during the luteal phase (Fig. 2B) and a rather faint epithelial staining at estrus exemplified in Fig. 2B (insert). Glandular epithelial cells were positive for MMP14 (Fig. 2C). In both, the sub-epithelial stroma near the endometrial lumen and the zona basalis encompassing the glands, the tunica media of blood vessels (Fig. 2D) displayed MMP14 protein, whereas there was no staining in endothelial cells. There was abundant MMP2 in the endometrium (Fig. 2E-H), especially in the sub-epithelial stroma of the zona functionalis (Fig. 2E, 2G insert). Both luminal (Fig. 2F) and glandular epithelium (Fig. 2G, 2G insert) were devoid of MMP2 protein. Endothelial cells as well as the tunica media of vessels in the zona basalis (Fig. 2H) displayed prominent staining.

TIMP2 protein was localized to the apical part of the glandular epithelial cells, and only faintly visible on the luminal surface of the epithelial cells (Fig. 2I-L). Stromal cells and vessels were devoid of TIMP2 protein (Fig. 2K,L).

TIMP2 protein in the luminal fluid during the estrous cycle

TIMP2 protein consistently appeared distinctly at day 15 in uterine flushing fluid (Fig. 3A) while it was less prominently present at days 12 and 18. A LC-MS-identification of the corresponding protein band verified the specificity of the antibody (Fig. 3B,C).

Endometrial MMP2 enzyme activity during the estrous cycle

The enzyme activity of active MMP2 in endometrial tissue homogenates was significantly lower at days 3.5 and 12 than at days 15 and 18 ($p<0.05$) (Fig. 4). Day 0 displayed an intermediate enzyme activity.

Endometrial mRNA expression of *MMP14, MMP2* and *TIMP2* during early pregnancy

At day 15, pregnant animals in study A already showed a 1.7-fold increase of endometrial *TIMP2* mRNA compared to the non-pregnant group. At day 18 the difference in endometrial *TIMP2* mRNA abundance between pregnant and non-pregnant animals was 2.0-fold ($p=0.03$) (Fig. 5). Neither *MMP14* nor *MMP2* were differentially expressed in day 15 or day 18 pregnant vs. non-pregnant endometrium in study A. In study B, the mRNA expression of *TIMP2* was 2.8-fold higher ($p=0.01$) in day 18 pregnant animals compared to their non-pregnant sisters whereas no significant differences in mRNA abundance were detected for *MMP2* or *MMP14*.

TIMP2 protein in the luminal fluid and endometrial MMP2 enzyme activity during early pregnancy

TIMP2 protein was distinctly present in the luminal fluid of pregnant and non-pregnant day 15 and day 18 animals (Fig. 6A) with no differences between groups. Gelatinase zymography revealed active MMP2 protein in day 18 pregnant as well as control endometrial homogenates (Fig. 6B) with no differences in protein activity between the two groups (controls 169.9±2.8 units vs. pregnant 161.8±5.7 units, $p=0.4$).

Endometrial cell culture

The morphology of epithelial cells in monolayers (Fig. 7A) and co-cultured with stromal cells (Fig. 7B) was evaluated after 8 days of culture. Transmission electron microscopy showed the presence of epithelial cell characteristics, including microvilli on the upper cell surface and interdigitations in the lateral part between cells (Fig. 7A,B). This confirmed the polarity of epithelial cells in both culture systems. However, desmosomes and tight junctions were only present in co-cultured epithelial cells and not in epithelial cell mono-cultures (Fig. 7D-F). Moreover, the microvilli in pure epithelial cells were smaller and fewer in comparison with co-cultured cells. Importantly, the morphology of co-cultured epithelial cells was more similar to epithelial cells obtained *ex vivo* (data not shown): mitochondria showed large numbers of cristae and other subcellular structures like the Golgi apparatus and endoplasmic reticulum were also more frequent in co-cultured epithelial cells than in epithelial cell mono-cultures. Overall these results indicate a positive effect of co-culture with stromal cells on morphology and activity of epithelial cells.

MMP2 activity in uterine epithelial cell co-culture

Active MMP2 protein was present in epithelial cell supernatants co-cultured with stromal cells (Fig. 7C), most probably due to the contribution of stromal cells in co-culture, as stromal cells showed a distinctly higher MMP2 activity than epithelial cells if cultured alone (Fig. 7C), The intensity of enzymatic digestion was not different due to the presence of day 8 hatched blastocysts (controls 54.7±5.8 units vs. blasocyst co-culture 45.4±5.8 units, *p=0.3*).

***TIMP2* mRNA expression in uterine epithelial cell co-culture**

In endometrial epithelial cells co-cultured with stromal cells derived from uteri of five different animals the mRNA expression of *TIMP2* was 2.6-fold (*p<0.01*) increased in the presence of day 8 hatched blastocysts. As positive control for the stimulatory effect of the embryos in our culture system, interferon-stimulated gene 15 mRNA levels were determined (Klein et al., 2006) and found 13.0-fold (*p<0.0001*) increased, demonstrating the ability of cultured epithelial cells to respond physiologically to the interferon-τ released by the hatched blastocysts. The epithelial cells did not reveal a change in *TIMP2* gene expression if stimulated with recombinant interferon-τ (1.1-fold, *p=0.9*), although interferon-stimulated gene 15 mRNA was up-regulated 57.7-fold (*p<0.0001*).

Antiviral activity in uterine epithelial cell co-culture supernatant

The concentration of interferon-τ was below the detection limit of the bioassay in control cultures of endometrial epithelial cells co-cultured with stromal cells (<0.4±0.0 U/mL), whereas significantly higher amounts were assessed in the presence of day 8 blastocysts (6.4±1.8 U/mL) (*p<0.0001*).

DISCUSSION

Matrix metallopeptidases seem likely to participate in the preparation of a receptive endometrium in cattle, as both mRNA and protein abundances varied significantly during the estrous cycle.

MMP14 and *MMP2* showed similar expression profiles during the estrous cycle, with high transcript abundance around estrus followed by a gradual decline during the luteal phase. Supporting the present findings, progesterone has shown an inhibitory influence on *MMP14* mRNA and protein expression in human endometrium (Plaisier et al., 2006; Zhang et al., 2000). Both MMP14 and MMP2 are involved in endometrial shedding during human menstruation (Goffin et al., 2003) after the luteal phase. As the bovine endometrium does not undergo endometrial shedding, the function of MMPs may be linked to obtaining a balance between proliferation and differentiation around estrus (Nagase et al., 2006). As membrane-type MMP, MMP14 is specifically involved in the proteolytic activity affecting the close surrounding in a paracrine manner in processes of cell migration and invasion (Itoh and Seiki 2006; Schenk et al., 2003). The participation of MMP14 in promoting angiogenesis in the bovine endometrium is discussed controversially, as MMP14 does not appear in endothelial cells (Plaisier et al., 2006; Zhang et al., 2000). The remainder MMP14 localization in glands and perivascular muscle cells however is in line with findings in humans (Plaisier et al., 2006).

Most prominently present in stromal cells, blood vessels and the basal lamina, MMP2 is likely to be involved in vascular remodeling processes as shown in human tissue (Zhang et al., 2000). Knockout mice have demonstrated that MMP2 is essential for angiogenesis in tumors (McCawley and Matrisian 2000), and angiogenic stimuli like vascular endothelial growth factor VEGF and FGF increased MMP2 production from endothelial cells (Lamoreaux et al., 1998; Zucker et al., 1998). The angiogenic action of FGF in turn can be affected by MMP2 since MMP2 is able to release the ectodomain of FGF receptor which is a prerequisite for activation (McCawley and Matrisian 2000; Moses 1997). In accordance to our findings in bovine, pro-MMP2 has been detected in stromal cells of the human endometrium and MMP2 was not present in luminal and glandular epithelium (Goffin et al., 2002). Although in human endometrial stromal *MMP2* transcripts appear to be constantly expressed during all phases of the menstrual cycle, a specific function has been ascribed facilitating menstrual proteolyis (Goffin et al., 2003; Zhang et al., 2000). The highest MMP2 enzyme activity coincides with the time of conceptus elongation. Thus, MMP2 in bovine endometrium might facilitate and regulate the release of growth factors likely of importance during the window of implantation.

The temporal expression pattern of the natural MMP inhibitor *TIMP2* appears inversely compared to the expression of *MMP2* and *MMP14*. The highest mRNA levels were detected at day 12, probably due to a stimulatory effect of progesterone. The results are in line with previous findings in cattle (Bauersachs et al., 2005; Ledgard et al., 2009; Mitko et al., 2008), sheep (Hampton et al., 1995) and human (Goffin et al., 2003) suggesting that ovarian hormones may control *TIMP2* expression. Nuclear receptors and their ligands including retinoids and glucocorticoids may participate in the regulation of *TIMP2* as well (Schroen and Brinckerhoff 1996). The demonstrated protein abundance is in line with *in vivo* studies that found co-expression of mRNA and protein (Freitas et al., 1999). Interestingly, TIMP2 was only marginally detectable in endometrial homogenates (data not shown), but clearly in the uterine fluid. Confirmatively, TIMP2 protein has been found abundantly in the non-pregnant and pregnant uterine luminal fluid (Ledgard et al., 2009), suggesting that TIMP2 localized on the apical edge of the glandular epithelium is directly secreted. Others (Ledgard et al., 2009) have also detected luminal epithelial and sub-epithelial stromal as well as blood vessel staining in bovine as shown for human endometrium displaying TIMP2 mostly in arterioles and fibroblasts (Freitas et al., 1999; Stetler-Stevenson and Seo 2005). This disparity may be due to the antibody used.

TIMP2 binds pro-MMP2 with high affinity, so in case of low TIMP2 concentrations at the beginning of the luteal phase only limited amounts of unbound TIMP2 might be available. This could stabilize the endometrium during the secretory phase in a definite homeostasis possibly of relevance for the early formation of the uterine fluid. Around the mid-luteal phase TIMP2 increases extensively. If TIMP2 was present at the interface of epithelial and stromal cell, this increase could allow activation of MMP2 by the TIMP2/MMP14/pro-MMP2 complex at the cell surface into the extracellular space (Curry, Jr. and Osteen 2003). Thus TIMP2 could stimulate the MMP2-mediated release of growth factors furthering maternal receptivity when embryo elongation commences. Towards estrus, this induction may cease as all three molecules decrease after prolonged P4 exposure at the end of the luteal phase (Curry, Jr. and Osteen 2003).

Interferon-τ, produced in large amounts by the elongating embryo, has been shown to inhibit the expression of pro-MMP1 and pro-MMP2 (Salamonsen et al., 1995) as well as the activity of MMP2 (Hashizume et al., 2003), but in our study the expression of neither *MMP14* nor *MMP2* mRNA was reduced due to pregnancy. In sheep, interferon-τ has been shown to trigger the increase of *TIMP2* (Chen et al., 2007; Gray et al., 2006), and *TIMP2* mRNA expression has been shown to remain elevated in ovine and bovine early pregnancy (during

implantation until day 31) (Ledgard et al., 2009). Because the increase in *TIMP2* mRNA at day 18 was moderate in our study A, we confirmed the data using a second experimental set-up with identical twins from which one had received an embryo and the other a seminal plasma control insemination (Klein et al., 2006). Both studies thus clearly confirm an embryo induced up-regulation of *TIMP2*. Direct physiological consequences of elevated endometrial *TIMP2* could include an effective block of proliferation, migration and invasion of endothelial cells and inhibition of endothelial growth factor stimuli (Lambert et al., 2004; Murphy et al., 1993) thus limiting epithelio-chorial placentation. However, the endometrial *TIMP2* mRNA increase was not accompanied by a distinct increase of TIMP2 protein in the luminal fluid. Even if a minor quantitative increase of endometrial TIMP2 protein did occur, TIMP2 did not further the cleavage of pro-MMP2 as gelatinase zymography revealed that active endometrial MMP2 remained unchanged at day 18 of pregnancy. Thus a physiological conclusion of the up-regulated endometrial *TIMP2* mRNA during early pregnancy remains to be shown. However, the presence of TIMP2 in the pregnant luminal fluid was clearly demonstrated using a specific antibody as well as a mass spectrometry based approach. As mass spectrometry signals for TIMP2 peptides were intense, significant amounts of TIMP2 were present in the uterine lumen during early pregnancy, most probably in close contact with the developing embryo. As part of the uterine milk, TIMP2 may contribute to providing a shielded environment from ECM degrading enzymes allowing the rapid elongating of the trophoblast.

We furthermore performed *in vitro* experiments to examine whether the presence of early embryos at the blastocyst stage would locally induce *TIMP2* as well and effect MMP2 enzyme activity, thus evidencing their participation in very early embryo-maternal mechanisms of communication. The rationale of developing an *in vitro* model was because due to its small size prior to elongation, *in vivo* studies are difficult to perform. Especially finding the site where the embryo possibly exerts important local paracrine interactions with the maternal epithelium is impossible. A co-culture system of primary epithelial and stromal endometrial cells was used clearly mimicking the *in vivo* situation as cells maintained ultra-structural characteristics and morphological hallmarks such as numerous microvilli, tight junctions and desmosomes among the cells, a monolayer with clear polarity, and well shaped organelles such as Golgi apparatus, endoplasmic reticulum and mitochondria. Moreover, the co-culture showed higher basal active MMP2 in supernatants than epithelial cells cultured alone, thus demonstrating that the stromal cells in culture resembled those *in vivo* by producing the majority of MMP2. Only in such a co-culture setup the effects of an early embryo may be reasonably analyzed (Ulbrich et al., 2010). After co-culture of hatched day 8 blastocysts for 24 h, cell culture supernatants contained significant amounts of

interferon-τ (Stojkovic et al., 1999a) inducing ISG15 ubiquitin-like modifier as internal control gene. The epithelial cells also responded to the stimulus of the co-cultured blastocysts by significantly increasing *TIMP2*. Because the transcriptional response did not lead to enhanced pro-MMP2 activation in the culture supernatants, *TIMP2* in this context might resemble an interferon-dependent gene which does not induce further downstream effects. But interestingly and contrary to findings in sheep endometrium (Chen et al., 2007), a direct stimulation of recombinant interferon-τ did not affect *TIMP2* gene expression in our bovine cell culture, indicating species specific peculiarities (Spencer et al., 2008). Thus, both the causing variable, most probably a factor secreted by the early embryo, and the purpose of the enhancement remain to be shown. By cautiously extrapolating *in vitro* findings, these results point towards an earlier maternal recognition of pregnancy appearing locally than previously described (Evans et al., 2008). Further studies are indicated.

The regulation of MMPs and TIMPs is a complex interplay of many participating factors. Although the impact of pregnancy is much less pronounced in ruminants than in primates due to the non-invasive placentation, the principal importance of matrix metallopeptidases during the preimplantation period is established in cattle as well (Salamonsen 1999). In this study, we have characterized a subset of matrix metallopeptidases involved in cleavage of different substrates and participating in various regulatory mechanisms. Taken together we find pronounced variations in their abundance during the cycle. Interestingly, a high activity of endometrial MMP2 during the elongation phase of the trophoblast may determine the allocation of growth factors supporting conceptus development. Moreover, abundant TIMP2 in the uterine fluid during trophoblast elongation may participate in regulating a well-balanced ECM integrity, thus indicating that MMPs may actively take part in maternal preparation of a receptive endometrium.

ACKNOWLEDGMENTS

We thank Dr. Katrin Mitko for help with tissue sampling, Angela Sachsenhauser and Miwako Kösters for technical assistance and Nadine Waldschmitt for excellent performance of immunohistochemistry.

REFERENCES

Bauersachs, S., Ulbrich, S. E., Gross, K., Schmidt, S. E., Meyer, H. H., Einspanier, R., Wenigerkind, H., Vermehren, M., Blum, H., Sinowatz, F., and Wolf, E. 2005. Gene expression profiling of bovine endometrium during the oestrous cycle: detection of molecular pathways involved in functional changes. J. Mol. Endocrinol. **34**:889-908.

Bauersachs, S., Ulbrich, S. E., Gross, K., Schmidt, S. E., Meyer, H. H., Wenigerkind, H., Vermehren, M., Sinowatz, F., Blum, H., and Wolf, E. 2006. Embryo-induced transcriptome changes in bovine endometrium reveal species-specific and common molecular markers of uterine receptivity. Reproduction **132**:319-331.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248-254.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., and Wittwer, C. T. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. **55**:611-622.

Chen, Y., Antoniou, E., Liu, Z., Hearne, L. B., and Roberts, R. M. 2007. A microarray analysis for genes regulated by interferon-tau in ovine luminal epithelial cells. Reproduction **134**:123-135.

Curry, T. E., Jr. and Osteen, K. G. 2003. The matrix metalloproteinase system: changes, regulation, and impact throughout the ovarian and uterine reproductive cycle. Endocr. Rev. **24**:428-465.

Evans, A. C., Forde, N., O'Gorman, G. M., Zielak, A. E., Lonergan, P., and Fair, T. 2008. Use of microarray technology to profile gene expression patterns important for reproduction in cattle. Reprod. Domest. Anim **43 Suppl 2**:359-367.

Freitas, S., Meduri, G., Le Nestour, E., Bausero, P., and Perrot-Applanat, M. 1999. Expression of metalloproteinases and their inhibitors in blood vessels in human endometrium. Biol. Reprod. **61**:1070-1082.

Goffin, F., Frankenne, F., Beliard, A., Perrier, D. S., Pignon, M. R., Geenen, V., and Foidart, J. M. 2002. Human endometrial epithelial cells modulate the activation of gelatinase a by stromal cells. Gynecol. Obstet. Invest **53**:105-111.

Goffin, F., Munaut, C., Frankenne, F., Perrier, D. S., Beliard, A., Fridman, V., Nervo, P., Colige, A., and Foidart, J. M. 2003. Expression pattern of metalloproteinases and tissue inhibitors of matrix-metalloproteinases in cycling human endometrium. Biol. Reprod. **69**:976-984.

Gray, C. A., Abbey, C. A., Beremand, P. D., Choi, Y., Farmer, J. L., Adelson, D. L., Thomas, T. L., Bazer, F. W., and Spencer, T. E. 2006. Identification of endometrial genes regulated by early pregnancy, progesterone, and interferon tau in the ovine uterus. Biol. Reprod. **74**:383-394.

Hampton, A. L., Butt, A. R., Riley, S. C., and Salamonsen, L. A. 1995. Tissue inhibitors of metalloproteinases in endometrium of ovariectomized steroid-treated ewes and during the estrous cycle and early pregnancy. Biol. Reprod. **53**:302-311.

Hashizume, K., Takahashi, T., Shimizu, M., Todoroki, J., Shimada, A., Hirata, M., Sato, T., and Ito, A. 2003. Matrix-metalloproteinases-2 and -9 production in bovine endometrial cell culture. J. Reprod. Dev. **49**:45-53.

Ireland, J. J., Murphee, R. L., and Coulson, P. B. 1980. Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. J. Dairy Sci. **63**:155-160.

Itoh, Y. and Seiki, M. 2006. MT1-MMP: a potent modifier of pericellular microenvironment. J. Cell Physiol **206**:1-8.

Klein, C., Bauersachs, S., Ulbrich, S. E., Einspanier, R., Meyer, H. H., Schmidt, S. E., Reichenbach, H. D., Vermehren, M., Sinowatz, F., Blum, H., and Wolf, E. 2006. Monozygotic twin model reveals novel embryo-induced transcriptome changes of bovine endometrium in the preattachment period. Biol. Reprod. **74**:253-264.

Kleiner, D. E. and Stetler-Stevenson, W. G. 1994. Quantitative zymography: detection of picogram quantities of gelatinases. Anal. Biochem. **218**:325-329.

Kliem, H., Welter, H., Kraetzl, W. D., Steffl, M., Meyer, H. H., Schams, D., and Berisha, B. 2007. Expression and localisation of extracellular matrix degrading proteases and their inhibitors during the oestrous cycle and after induced luteolysis in the bovine corpus luteum. Reproduction **134**:535-547.

Lambert, E., Dasse, E., Haye, B., and Petitfrere, E. 2004. TIMPs as multifacial proteins. Crit Rev. Oncol. Hematol. **49**:187-198.

Lamoreaux, W. J., Fitzgerald, M. E., Reiner, A., Hasty, K. A., and Charles, S. T. 1998. Vascular endothelial growth factor increases release of gelatinase A and decreases release of tissue inhibitor of metalloproteinases by microvascular endothelial cells in vitro. Microvasc. Res. **55**:29-42.

Ledgard, A. M., Lee, R. S., and Peterson, A. J. 2009. Bovine endometrial legumain and TIMP-2 regulation in response to presence of a conceptus. Mol. Reprod. Dev. **76**:65-74.

Livak, K. J. and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25**:402-408.

McCawley, L. J. and Matrisian, L. M. 2000. Matrix metalloproteinases: multifunctional contributors to tumor progression. Mol. Med. Today **6**:149-156.

Mitko, K., Ulbrich, S. E., Wenigerkind, H., Sinowatz, F., Blum, H., Wolf, E., and Bauersachs, S. 2008. Dynamic changes in messenger RNA profiles of bovine endometrium during the oestrous cycle. Reproduction. **135**:225-240.

Moses, M. A. 1997. The regulation of neovascularization of matrix metalloproteinases and their inhibitors. Stem Cells **15**:180-189.

Murphy, A. N., Unsworth, E. J., and Stetler-Stevenson, W. G. 1993. Tissue inhibitor of metalloproteinases-2 inhibits bFGF-induced human microvascular endothelial cell proliferation. J. Cell Physiol **157**:351-358.

Nagase, H., Visse, R., and Murphy, G. 2006. Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc. Res. **69**:562-573.

Nagase, H. and Woessner, J. F., Jr. 1999. Matrix metalloproteinases. J. Biol. Chem. **274**:21491-21494.

Plaisier, M., Koolwijk, P., Hanemaaijer, R., Verwey, R. A., van der Weiden, R. M., Risse, E. K., Jungerius, C., Helmerhorst, F. M., and van Hinsbergh, V. W. 2006. Membrane-type matrix metalloproteinases and vascularization in human endometrium during the menstrual cycle. Mol. Hum. Reprod. **12**:11-18.

Prakash, B. S., Meyer, H. H., Schallenberger, E., and van de Wiel, D. F. 1987. Development of a sensitive enzymeimmunoassay (EIA) for progesterone determination in unextracted bovine plasma using the second antibody technique. J. Steroid Biochem. **28**:623-627.

Remacle, A., Murphy, G., and Roghi, C. 2003. Membrane type I-matrix metalloproteinase (MT1-MMP) is internalised by two different pathways and is recycled to the cell surface. J. Cell Sci. **116**:3905-3916.

REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17**:208-212.

Rubinstein, S., Familletti, P. C., and Pestka, S. 1981. Convenient assay for interferons. J. Virol. **37**:755-758.

Salamonsen, L. A. 1999. Role of proteases in implantation. Rev. Reprod. **4**:11-22.

Salamonsen, L. A., Nagase, H., and Woolley, D. E. 1995. Matrix metalloproteinases and their tissue inhibitors at the ovine trophoblast-uterine interface. J. Reprod. Fertil. Suppl **49**:29-37.

Schenk, S., Hintermann, E., Bilban, M., Koshikawa, N., Hojilla, C., Khokha, R., and Quaranta, V. 2003. Binding to EGF receptor of a laminin-5 EGF-like fragment liberated during MMP-dependent mammary gland involution. J. Cell Biol. **161**:197-209.

Schroen, D. J. and Brinckerhoff, C. E. 1996. Nuclear hormone receptors inhibit matrix metalloproteinase (MMP) gene expression through diverse mechanisms. Gene Expr. **6**:197-207.

Spencer, T. E., Sandra, O., and Wolf, E. 2008. Genes involved in conceptus-endometrial interactions in ruminants: insights from reductionism and thoughts on holistic approaches. Reproduction **135**:165-179.

Stetler-Stevenson, W. G. and Seo, D. W. 2005. TIMP-2: an endogenous inhibitor of angiogenesis. Trends Mol. Med. **11**:97-103.

Stojkovic, M., Buttner, M., Zakhartchenko, V., Riedl, J., Reichenbach, H. D., Wenigerkind, H., Brem, G., and Wolf, E. 1999a. Secretion of interferon-tau by bovine embryos in long-term culture: comparison of in vivo derived, in vitro produced, nuclear transfer and demi-embryos. Anim Reprod. Sci. **55**:151-162.

Stojkovic, M., Westesen, K., Zakhartchenko, V., Stojkovic, P., Boxhammer, K., and Wolf, E. 1999b. Coenzyme Q(10) in submicron-sized dispersion improves development, hatching, cell proliferation, and adenosine triphosphate content of in vitro-produced bovine embryos. Biol. Reprod. **61**:541-547.

Thatcher, W. W., Staples, C. R., Danet-Desnoyers, G., Oldick, B., and Schmitt, E.-P. 2006. Embryo health and mortality in sheep and cattle. Journal of animal science **72**:16-30.

Ulbrich, S. E., Schulke, K., Groebner, A. E., Reichenbach, H. D., Angioni, C., Geisslinger, G., and Meyer, H. H. 2009. Quantitative characterization of prostaglandins in the uterus of early pregnant cattle. Reproduction **138**:371-382.

Ulbrich, S. E., Zitta, K., Hiendleder, S., and Wolf, E. 2010. In vitro systems for intercepting early embryo-maternal cross-talk in the bovine oviduct. Theriogenology **73**:802-816.

Visse, R. and Nagase, H. 2003. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ. Res. **92**:827-839.

Zhang, J., Hampton, A. L., Nie, G., and Salamonsen, L. A. 2000. Progesterone inhibits activation of latent matrix metalloproteinase (MMP)-2 by membrane-type 1 MMP: enzymes coordinately expressed in human endometrium. Biol. Reprod. **62**:85-94.

Zucker, S., Mirza, H., Conner, C. E., Lorenz, A. F., Drews, M. H., Bahou, W. F., and Jesty, J. 1998. Vascular endothelial growth factor induces tissue factor and matrix metalloproteinase production in endothelial cells: conversion of prothrombin to thrombin results in progelatinase A activation and cell proliferation. Int. J. Cancer **75**:780-786.

Table 1:

Sequences of primer pairs used, corresponding references as well as annealing temperatures (AT), fluorescence acquisition (FA), melting points (MP) and the resulting fragment length.

Gene	reference [acc. no.]	forward primer [5´-... -3´]	reverse primer [5´-... -3´]	PCR-product [bp]	AT [°C]	FA [°C]	MP [°C]	PCR Efficiency
Histon H3	AF469469	ACTGCTACAAAAGCCGCTC	ACTTGCCTCCTGCAAAGCAC	233	60	82	86	1.9
MMP14	AF144758	ACTTGGAAGGGGGACACC	AGGGGGCATCTTAGTGGG	236	60	84	88	1.9
MMP2	NM_174745	CCCAGACAGTGGATGATGC	TTGTCCTTCTCCCAGGGTC	248	60	84	88	1.7
TIMP2	AF144764	GGGTCTCGCTGGACATTG	TTGATGTTCTTCTCCGTGACC	256	60	84	88	1.6

FIGURE LEGENDS

Figure 1: Transcript abundance of matrix metallopeptidases and their inhibitor. Messenger RNA expression of *MMP14* (A), *MMP2* (B) and *TIMP2* (C) in endometrial tissue (n=5-7 animals per group) during the estrous cycle is shown as means % ± SEM relative to day 12 (100 %). Different superscript letters indicate significant differences ($p<0.05$) among day 0, 3.5, 12, 15 and 18 following estrus, thus same superscript letters indicate that there is no significant difference.

Figure 2: Immunohistochemical localization of MMP14 (A-D), MMP2 (E-H) and TIMP2 (I-L) in bovine endometrium. The luminal epithelium (B,F,J), glands (C,G,K) and vessels (D,H,L) are specifically depicted. Panel G depicts MMP2 localization in a section of the zona basalis of the endometrium, whereby the insert shows the clear absence of MMP2 in glandular epithelial cells of the sub-epithelial stroma, which are distinctly MMP2-positive. Panel K exemplifies TIMP2 localized to the apical edge of glandular epithelial cells in both zona basalis (K) and zona functionalis of the sub-epithelial stroma (K insert). The inserts in (A, F, J) represent negative controls. Black arrowheads point at immunopositive cells, white arrowheads indicates lack of staining. Tissue sections were counterstained with Mayer's haemalaun. Bar in (A), (E) and (I) = 300 µm, and 50 µm in all other figures.

Figure 3: Representative Western blot of TIMP2 protein (two samples per cycle stage) of uterine fluid during the estrous cycle [A] with short (left) and longer (right) exposure time. A corresponding protein gel stained with Ponceau S is shown in [B] indicating a band segment which was cut out for the LC-MS-identification of TIMP2. Peptides highlighted in red were identified. A representative MS/MS spectrum of peptide EVDSGNDIYGNPIK (corresponding to aa 54 to 67 of bovine TIMP2 NCBI accession AAI02711) is shown in [C].

Figure 4: Gelatine zymography of MMP2 enzyme activity in bovine endometrium during the estrous cycle. Band intensity units of active MMP2 are shown as means ± SEM with different superscript letters indicating significant differences ($p<0.05$) among day 0, 3.5, 12, 15 and 18 following estrus. Same superscript letters indicate that there is no significant difference.

Figure 5: Messenger RNA expression of *MMP14* (A), *MMP2* (B) and *TIMP2* (C) in endometrial tissue (n=5-7 per group) during the estrous cycle (black bars) and early pregnancy (grey bars). Data are shown as means % ± SEM relative to day 15 controls (100 %). * indicates a significant difference ($p<0.05$).

Figure 6: Gelatine zymography showing active MMP2 at 62 kDa of selected endometrium samples [A] and TIMP2 western blot of selected uterine fluid samples [B] during early pregnancy compared to non-pregnant control animals. There were no differences between the pregnant and control group.

Figure 7: Representative transmission electron microscopy of endometrial epithelial cells in mono- [A] or co-culture with stromal cells [B, D-F]. Epithelial cells after 8 days in mono- [A] and co-cultures with stromal cells [B] grown on a membrane support (s). The following structures are visible throughout the culture period: microvilli (mv), mitochondria (mc), Golgi apparatus (G), nucleus (Nu) and interdigitation between cells (In). Only epithelial cells co-cultured with stromal cells [B,D,E,F] displayed tight junctions (Tj) and desmosomes (d). These structures were not present in epithelial cells in mono-cultures [A]. Bar = 2 µm. Gelatine zymography showing active MMP2 [C] in cell culture supernatants of stromal (S), epithelial cells (E) and epithelial cells co-cultured with stromal cells (E+S).