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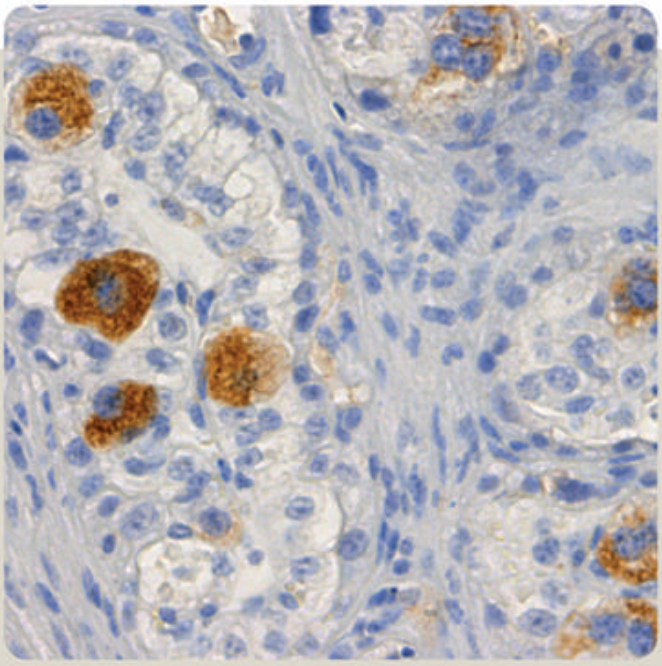
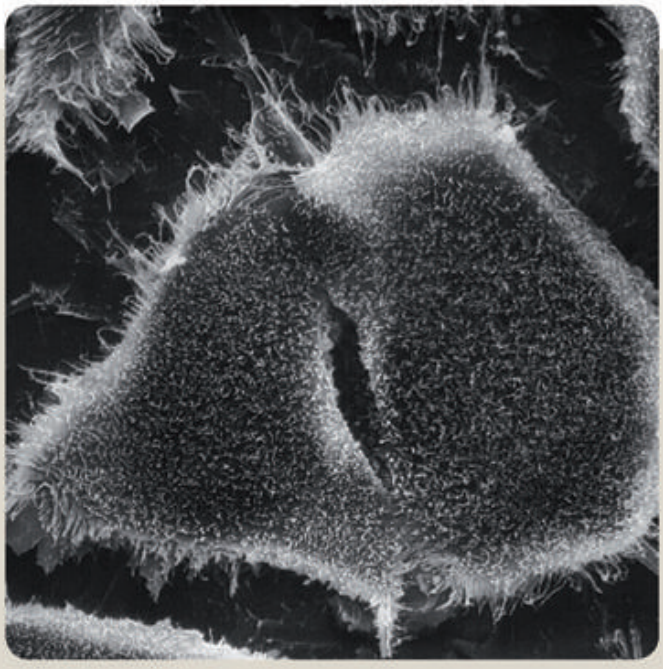
Marc Dilly

Expression of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in bovine placental cells *in vivo* and *in vitro*





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Department of Anatomy
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Expression of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in bovine placental cells *in vivo* and *in vitro*

THESIS

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Supervisor:	**Prof. Dr. Christiane Pfarrer**
Advisory committee:	**Prof. Dr. Christiane Pfarrer** **Prof. Dr. Gerhard Breves** **Prof. Dr. Dr. Stefan Arnhold**
1st Evaluation:	**Prof. Dr. Christiane Pfarrer** (Department of Anatomy, University of Veterinary Medicine Hannover, Foundation)
	Prof. Dr. Gerhard Breves (Department of Physiology, University of Veterinary Medicine Hannover, Foundation)
	Prof. Dr. Dr. Stefan Arnhold (Institute of Veterinary Anatomy, Histology and Embryology, University of Giessen)
2nd Evaluation:	**Prof. Dr. Vibeke Dantzer** (Section for Anatomy and Cell Biology, Dept of Basic Animal and Veterinary Sciences, University of Copenhagen, Denmark)
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Expression of matrix metalloproteinase (MMP)-2, MMP-14 and tissue inhibitor of matrix metalloproteinase (TIMP)-2 during bovine placentation and at term with or without placental retention
Theriogenology 2011 Jan, (Epub ahead of print)

Shenavai S, Hoffmann B, Dilly M, Pfarrer C, Ozalp GR Caliskan C, Seyrek-Intas K, Schuler G.
Use of the progesterone receptor antagonist aglepristone to characterize the role of progesterone withdrawal for parturition and placental release in cows
Reproduction 2010 Oct; 140(4):623-32

In vitro:

Dilly M, Hambruch N, Haeger JD, Pfarrer C.
Epidermal growth factor (EGF) induces motility and upregulates MMP-9 and TIMP-1 in bovine trophoblast cells
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Hambruch N, Haeger JD, Dilly M, Pfarrer C.
EGF stimulates proliferation in the bovine placental trophoblast cell line F3 via Ras and MAPK
Placenta 2010 Jan; 31(1):67-74

Haeger JD, Hambruch N, Dilly M, Froehlich R, Pfarrer C
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Oral presentations and abstracts at scientific meetings:

Dilly M, Hambruch N, Haeger JD, Pfarrer C.
Tight junctions and polarity in cultured bovine placental cells
Anatomia Histologia Embryologia 39 (4) Abstracts of the XXVIIIth congress of the European Association of Veterinary Anatomists (EAVA), Paris, France, 28th – 31st July 2010; Berlin: Blackwell, 2010 S. 282-283 (51)

Dilly M, Hambruch N, Haeger JD, Pfarrer C.
Epidermal growth factor (EGF) stimulates upregulation of MMP-9 and TIMP-1 in bovine placental cells via MAPK signalling pathway
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Poster presentations and abstracts at scientific meetings:

Dilly M, Shenavai S, Hambruch N, Schuler G, Özalp G, Seyrek-Intas K, Pfarrer C.
Matrix metalloproteinase 2 (MMP-2) may be activated by binding of tissue inhibitor of matrix metalloproteinase 2 (TIMP-2) to MMP-14 in bovine placentomes
Reproduction in Domestic Animals 44 (Suppl. 1) Abstracts for the 42nd Annual Conference of Physiology and Pathology of Reproduction and 34th Mutual Conference on Veterinary and Human Reproductive Medicine, Leipzig, Germany, 26th – 27th February 2009; Berlin: Blackwell S. 7-8 (16)

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Expression of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in bovine placentomes
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1 GENERAL REMARK

This thesis is submitted as a cumulative thesis with the main issue of elucidating potential functions and the regulation of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) in the bovine placenta. The thesis consists of two parts; each part being covered in one original paper published in peer reviewed journals. The first part analyses the involvement of MMPs/TIMPs in restricted trophoblast invasion/migration in respect to signal transduction, cell motility and proliferation in bovine trophoblast cells *in vitro*. The second part contains results of *in vivo* studies concerning the localization and expression of the MMP/TIMP system in different experimental groups and its possible involvement in the aetiology of retained fetal membranes (RFM).

2 GENERAL INTRODUCTION

2.1 THE BOVINE PLACENTA AND RETENTION OF FETAL MEMBRANES

The bovine placenta is classified according to its shape as cotyledonary type (Placenta cotyledonaria sive multiplex), where placentomes are formed of fetal cotyledons and maternal caruncles (Strahl 1906). The fetal and maternal tissue is in close contact to each other by interdigitation of fetal villi into maternal crypts (Mossmann 1987; Strahl 1906). Originally, the bovine placenta was classified as syndesmochorial by the number and form of layers between the fetal and maternal circulations (Grosser 1927). It was believed that the uterine epithelium disappeared and the trophoblast was apposed directly to the maternal connective tissue. Further studies demonstrated that the uterine epithelium persisted and therefore the bovine placenta was reclassified as an epitheliochorial placenta (Björkman 1954; Ludwig 1962; Steven 1975). The matter is further complicated by the fact that the chorionic epithelium consists of two populations of trophoblast cells, polarized uninucleated trophoblast cells and trophoblast giant cells (TGC). TGC are mostly binucleated, non-polarized and migrate through the chorionic epithelium to fuse with uterine epithelial cells (Wathes and Wooding 1980; Wimsatt 1951). As the resulting feto-maternal hybrid cells are indeed syncytia, it was recommended to classify the bovine placenta as synepitheliochorial (Wooding 1992).

The mostly binucleated TGC evolve from uninucleated trophoblast cells by acytokinetic mitosis and are able to migrate from the fetal into the maternal compartment (Klisch et al. 1999a; Wimsatt 1951). During this process TGC loose contact to the trophoblast, migrate through chorionic tight junctions and finally fuse with single maternal epithelial cells to form feto-maternal hybrid cells. Since the migration/invasion does not continue beyond the maternal basement membrane, this unique feature of the bovine placenta was termed “restricted trophoblast invasion/migration” (Pfarrer et al. 2003). The feto-maternal hybrid cells degenerate (Wimsatt 1951) and are phagocytized by uninucleated trophoblast cells (Klisch et al. 1999b). The main function of TGC is the production and delivery of proteins and steroid hormones into the maternal compartment (Wooding 1992). Thus,

placentomes are not only places of fetal-maternal exchange, but also of feto-maternal communication and signal transduction.

Supporting these functions TGC additionally contain a variety of signalling molecules, such as placental lactogen (Wooding and Beckers 1987) and pregnancy associated glycoproteins (Zoli et al. 1992). Furthermore, several growth factor systems as vascular endothelial growth factor, platelet-activating factor, fibroblast growth factor, and epidermal growth factor (EGF) are co-localized either in TGC or the uterine epithelium (Bucher et al. 2006; Pfarrer et al. 2006; Weise 2001), which implies autocrine and paracrine ways of action. In view of this synthetic capacity, TGC are prospective candidates for regulation of various biological effects such as migration, cell-adhesion, cell growth, differentiation and tissue remodelling.

For differentiation, migration and several other cell functions, the scaffolding extracellular matrix (ECM) plays a pivotal role beside its main function as tissue framework to give functional structure to organs (Ekblom and Timpl 1996; Humphries and Reynolds 2009; Stetler-Stevenson and Yu 2001; Werb 1997). Prior to cell migration, components of the ECM have to be degraded by proteases, such as matrix metalloproteinases (MMPs) (Brew and Nagase 2010; Itoh 2006; Seiki 2003; Stetler-Stevenson and Yu 2001). In the bovine placenta, the expression of the ECM proteins fibronectin, laminin, collagen types I, III, and IV, as well as MMPs and their tissue inhibitors of matrix metalloproteinase (TIMPs) has been demonstrated throughout pregnancy (Boos 2000; Pfarrer et al. 2003; Walter and Boos 2001). It has been suggested that the migration of TGC is accomplished by movement along laminin matrices (Pfarrer et al. 2003). Based on the observation that prior to parturition TGC cease to express the above mentioned growth factor systems, we hypothesize that TGC play an essential role in the release of fetal membranes by regulating the proteolytic activity of MMPs and the extracellular architecture at the end of gestation.

The tight connection between maternal crypts and fetal villi of each placentome which is essential during gestation must be terminated after expulsion of the fetus to ensure a healthy puerperium (Al-Sadi et al. 1994; Gross et al. 1986; Paisley et al. 1986). Loosing adherence at the feto-maternal interface is accompanied by a distinct ECM remodelling in late gestation. In parallel, a process termed placental maturation occurs, which includes reduction of the caruncular epithelium (Björkman 1954; Grunert 1985; Woicke et al. 1986) and decline in TGC numbers (Gross et al. 1991;

Shenavai et al. 2010; Williams et al. 1987). Therefore, the timely release of the fetal membranes after calving could depend on both placental maturation and the controlled reduction of feto-maternal adherence.

In cows the release of fetal membranes usually takes place less than 6 hours after expulsion of the fetus (Roberts 1986). Placental retention is most commonly defined as the condition in which the fetal membranes are not expelled from the uterus within 12-48 hours postpartum (Fourichon et al. 2000; Kelton et al. 1998). The retention of fetal membranes (RFM) is one of the major disorders in bovine reproduction. It affects the reproductive performance and leads to significant economic loss at the herd level (Joosten et al. 1988; Kossaibati and Esslemont 1997; Laven and Peters 1996; Peters and Laven 1996). A considerable number of factors have been implicated leading to RFM such as breed, dystocia, twin pregnancy, gestation length, season, herd management, environment, induction, nutrition and hormonal imbalances (Barnouin and Chassagne 1991; Bo et al. 1992; Claydon 1984; Dlamini et al. 1995; Garcia et al. 1992; Grunert et al. 1989; Kankofer et al. 2002; Takagi et al. 2002). However, despite an abundance of extensive studies, the regulatory mechanisms and pathogenesis of placenta retention are not completely understood. From a clinical point of view, a variety of methods have been used for the treatment of RFM (e.g. manual removal, ecbolic drugs). Whereas manual removal of the placenta remains a common practice, intrauterine antibiotic therapy in combination with manual removal is a more prospective treatment (Drillich et al. 2007; Drillich et al. 2003; Drillich et al. 2006; Peters and Laven 1996). First and foremost postpartum metritis is a frequent sequela of RFM, the use of antibiotics in cases of RFM is to prevent or treat metritis and subsequent negative effects on fertility (Paisley et al. 1986; Sheldon et al. 2009). To avoid side effects associated with manual removal, Eiler and Hopkins (1992) tested the effect of collagenase and/or hyaluroindase on sections of placentomes. They demonstrated that only collagenase had an effect on placental separation and injection of collagenase (into the umbilical vein) was effective in the treatment of RFM (Eiler and Hopkins 1992; Eiler and Hopkins 1993). Therefore it seems reasonable that proteolytic activity of degrading enzymes and the breakdown of ECM components could contribute the detachment of fetal membranes.

2.2 MATRIX METALLOPROTEINASES AND THEIR TISSUE INHIBITORS

The bovine placenta undergoes extensive growth and tissue remodelling from implantation and placentation until parturition. Likely candidates responsible for these dynamic changes in the extracellular architecture are matrix metalloproteinases (MMPs) and the tissue inhibitors of matrix metalloproteinases (TIMPs). The MMP/TIMP system acts to control the breakdown of ECM components and affects several reproductive processes, such as embryonic development, organ morphogenesis, cell growth, differentiation and migration (Curry and Osteen 2001)(Curry and Osteen 2003). These processes and MMP mediated structural changes can be influenced by various hormones, cytokines and growth factors (Brew and Nagase 2010; D'Alessio et al. 2008; Itoh 2006; Nagase et al. 2006; Woessner and Nagase 2000). Loss of control of the MMP/TIMP system can lead to a destructive degradation of the ECM as seen in cancer (Stetler-Stevenson and Yu 2001).

MMPs are zinc-dependent endopeptidases capable of degrading essential components of the ECM. To date the MMP family (matrixin subfamily of zinc metalloprotease family M10) encompasses at least 25 related proteolytic enzymes that include four classes (Nagase et al. 2006; Nagase and Woessner 1999; Visse and Nagase 2003; Woessner and Nagase 2000): collagenases, gelatinases, stromelysins, and membrane type enzymes (MT-MMPs). MMPs show several common features, for instance the presence of zinc in the active site of the catalytic domain. Furthermore, MMPs are synthesized and secreted as proenzymes, which have to be activated for the cleavage of ECM components. The enzyme activity of MMPs is specifically inhibited by TIMPs in the extracellular environment.

The TIMP family consists of four members TIMP-1, -2, -3 and -4, which can bind MMPs in a 1:1 stoichiometry (Bode et al. 1999; Brew and Nagase 2010; Nagase et al. 2006; Visse and Nagase 2003; Woessner and Nagase 2000). Despite the fact that all members of the TIMP family are able to inhibit MMP activity, selective inhibition and functional diversity have been observed (Brew and Nagase 2010; Stetler-Stevenson 2008; Stetler-Stevenson and Seo 2005). For example, although TIMP-1 is a prototypic inhibitor for the gelatinases (MMP-2 and MMP-9), it is a poor inhibitor of the MT-MMPs (Baker et al. 2002). Furthermore, TIMP-2 functions to both inhibit MMP activity and promote activation of pro-MMP-2 by MT1-MMP (Wang et al. 2000; Zucker et al. 1998). MT1-MMP, also termed MMP-14, has been described as

possible “master switch” that can control ECM remodelling in several organs and species (Bai et al. 2005a; Bai et al. 2005b; Bakke et al. 2002; Rabot et al. 2007; Uekita et al. 2004; Wang et al. 2001). The key to many tissue remodelling processes is a delicate balance of MMPs and counteracting TIMPs controlling formation and dissolution of extracellular matrix (ECM) and thus the composition of the ECM. In the bovine placenta the distribution and activity of MMP-2, MMP-9 and TIMP-2 was demonstrated (Maj and Kankofer 1997; Walter and Boos 2001), but functional evidence that MMP-14 is the decisive molecule whether an activation or inactivation takes place has not been presented yet. Previous studies in the goat demonstrated the expression of MMP-14, MMP-2 and TIMP-2 during pregnancy and hypothesized a regulated ECM breakdown (Uekita et al. 2004). In addition, the MMP gene expression is transcriptionally regulated by different extracellular stimuli (Westermarck and Kahari 1999) including growth factors (Tian et al. 2007), which are also expressed in the bovine placenta (Pfarrer et al. 2006; Weise 2001). Beside other growth factors, EGF is a well described candidate for remodelling of extracellular matrix, invasion and migration by activating key signalling molecules like the mitogen-activated protein kinases (MAPKs) (Oda et al. 2005), Akt and phosphatidylinositol 3-kinase (PI3K) (LaMarca et al. 2008; Qiu et al. 2004a; Qiu et al. 2004b) In addition, migration and invasion are active processes in which proteases and degradation of extracellular matrix (ECM) play a pivotal role (Pilcher et al. 1997; Stetler-Stevenson and Yu 2001). In human trophoblast cells EGF activates the degradation of ECM by the stimulation and secretion of MMP-9 (Anteby et al. 2004) and also promotes cell motility (Qiu et al. 2004a). *In vivo* studies have shown that the MMP-9 protein is expressed in trophoblast cells of the synepitheliochorial sheep placentae throughout the last third of gestation and during the whole gestational period in the cow (Vagnoni et al. 1998; Walter and Boos 2001). In the human placenta, a strong enzymatic activity for MMP-9 and MMP-2 was detected at various regions of the feto-maternal interface, suggesting a pivotal role of MMPs in the separation of the placenta from the uterine wall after birth (Demir-Weusten et al. 2007). Altogether, these findings support our idea, that MMPs can be involved in placental tissue remodelling and the release of bovine fetal membranes.

Fact is that placental remodelling has to occur when fetal membranes disengage from the maternal surface and ECM proteins are due to degrade. The capacity of MMPs to degrade components of the ECM could be a precondition for tissue

remodelling and migration of TGC throughout gestation as well as the release of fetal membranes after birth.

General purpose of this thesis was to gain more information on the regulation and aetiology of placental retention. To achieve our aims, we used two approaches 1) to prove the hypothesis *in vitro* that growth factors, such as EGF, are involved in the regulation of the MMP/TIMP balance and could influence placental functions in several ways including TGC migration and tissue remodelling, and 2) to test the hypothesis that the expression of MMP-14, MMP-2 and TIMP-2 is involved in the ECM turnover during pregnancy and in the regulatory mechanisms leading to the release of fetal membranes in the bovine placenta *in vivo*.

3 PAPER I

Epidermal growth factor (EGF) induces motility and upregulates MMP-9 and TIMP-1 in bovine trophoblast cells

Abstract

Differentiation and restricted invasion/migration of trophoblast cells are crucial for feto-maternal communication in the synepitheliochorial placenta of cattle. EGF is expressed in the bovine placenta and likely regulates these cell properties. As cell migration and motility rely on the degradation of extracellular matrix we hypothesize that EGF is involved in the regulation of the MMP-9/TIMP-1 balance and thus could influence trophoblast migration, tissue remodeling, and the release of the fetal membranes after parturition. The aim of this in vitro study was to examine EGF-mediated effects on cell motility, proliferation, and MMP-9 and TIMP-1 expression in cultured bovine trophoblast cells. We used a trophoblast cell line (F3) derived from bovine placentomes to examine the influence of EGF on MMP-9 and TIMP-1 expression by semiquantitative RT-PCR and MMP activity by zymography. Migration assays were performed using a Boyden chamber and cell motility was measured by time-lapse analyses. To identify the involved signaling cascades, phosphorylation of mitogen-activated protein kinase (MAPK) 42/44 and Akt was detected by Western blot. EGF treatment increased both the abundance of MMP-9 and TIMP-1 mRNAs and the proteolytic activity of MMP-9. Furthermore, EGF stimulated proliferation and migration of F3 cells. Addition of specific inhibitors of MAPK (PD98059) and/or PI3K (LY294002) activation abolished or reduced EGF-induced effects in all experiments. In conclusion, EGF-mediated effects stimulate migration and proliferation of bovine trophoblast cells and may be involved in bovine placental tissue remodeling and postpartum release of fetal membranes.

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RESEARCH ARTICLE

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Epidermal Growth Factor (EGF) Induces Motility and Upregulates MMP-9 and TIMP-1 in Bovine Trophoblast Cells

M. DILLY,† N. HAMBRUCH,† J.-D. HAEGER, AND C. PFARRER*

Department of Anatomy, University of Veterinary Medicine Hannover, Hannover, Germany

SUMMARY

Differentiation and restricted invasion/migration of trophoblast cells are crucial for feto-maternal communication in the synepitheliochorial placenta of cattle. EGF is expressed in the bovine placenta and likely regulates these cell properties. As cell migration and motility rely on the degradation of extracellular matrix we hypothesize that EGF is involved in the regulation of the MMP-9/TIMP-1 balance and thus could influence trophoblast migration, tissue remodeling, and the release of the fetal membranes after parturition. The aim of this in vitro study was to examine EGF-mediated effects on cell motility, proliferation, and MMP-9 and TIMP-1 expression in cultured bovine trophoblast cells. We used a trophoblast cell line (F3) derived from bovine placentomes to examine the influence of EGF on MMP-9 and TIMP-1 expression by semiquantitative RT-PCR and MMP activity by zymography. Migration assays were performed using a Boyden chamber and cell motility was measured by time-lapse analyses. To identify the involved signaling cascades, phosphorylation of mitogen-activated protein kinase (MAPK) 42/44 and Akt was detected by Western blot. EGF treatment increased both the abundance of MMP-9 and TIMP-1 mRNAs and the proteolytic activity of MMP-9. Furthermore, EGF stimulated proliferation and migration of F3 cells. Addition of specific inhibitors of MAPK (PD98059) and/or PI3K (LY294002) activation abolished or reduced EGF-induced effects in all experiments. In conclusion, EGF-mediated effects stimulate migration and proliferation of bovine trophoblast cells and may be involved in bovine placental tissue remodeling and postpartum release of fetal membranes.

* Corresponding author:
Department of Anatomy
University of Veterinary Medicine Hannover
Bischofsholer, Damm 15, 30173
Hannover, Germany.
E-mail: christiane.pfarrer@tiho-hannover.de

† M. Dilly and N. Hambruch contributed equally to this work.

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INTRODUCTION

The trophoblast of the bovine synepitheliochorial placenta is composed of two cell populations, polarized uninucleated trophoblast cells and nonpolarized trophoblast giant cells (TGC). Throughout gestation TGC evolve from uninucleated trophoblast cells by acytokinetic mitosis, invade the maternal epithelium, and fuse with single cells to form feto-maternal hybrid cells. The ability to undergo this differentiation, genome duplication, migration, and fusion is shared by all uninucleated trophoblast cells (Wooding and Wathes, 1980; Klisch et al., 1999). The processes of restricted migration and fusion result in the delivery of substances to the maternal compartment and are of major importance for feto-maternal communication and maintenance of pregnancy in the bovine placenta (Klisch et al., 2006; Hashizume et al., 2007). Since the underlying mechanisms regulating these processes are barely understood, we recently established a trophoblast cell line (F3) from bovine placentomes. This tool allows the evaluation of possible differences and regulatory mechanisms in both compartments in vitro (Bridger et al., 2007; Hambruch et al., 2010).

Migration and invasion are active processes in which proteases and degradation of extracellular matrix (ECM) play a pivotal role (Pilcher et al., 1997; Stetler-Stevenson and Yu, 2001). Matrix metalloproteinases (MMPs) are

zinc-dependent endopeptidases capable of degrading essential components of the ECM. Most of the MMPs are secreted as inactive pro-enzymes, which become activated upon cleavage of an N-terminal propeptide. Among the MMPs identified, MMP-9 is well described in a wide array of physiological and pathophysiological events, including cellular migration, inflammation, metastasis/invasion, and tissue remodeling (Lemaitre and D'Armiento, 2006). In the bovine placenta, MMP-9 has been localized in the trophoblast throughout gestation (Walter and Boos, 2001). Its capacity to degrade components of the ECM could be a precondition for tissue remodeling and migration of TGC throughout gestation as well as the release of fetal membranes after birth. Therefore, it is important to investigate the regulatory mechanisms and involved pathways for a better understanding of bovine reproductive disorders such as retained placenta, which is a common disease resulting in considerable economic loss (Laven and Peters, 1996).

The activity of MMPs is strictly regulated by counteracting tissue inhibitors of MMPs (TIMPs) (Nagase et al., 2006). Several members of the TIMP family have been identified for maintaining the delicate balance of active and inactive MMPs. Among them TIMP-1 is the preferential inhibitor of MMP-9 (Curry and Osteen, 2003). In addition to the regulation of MMP activity by TIMPs, the expression of MMP genes is transcriptionally regulated by different extracellular stimuli (Westermarck and Kahari, 1999) including epidermal growth factor (EGF) (Tian et al., 2007), which is also expressed in the bovine placenta (Weise, 2001). EGF can activate key signaling molecules like the mitogen-activated protein kinases (MAPKs) (Oda et al., 2005), Akt and phosphatidylinositol 3-kinase (PI3K) (Qiu et al., 2004b; LaMarca et al., 2008) and is therefore a likely regulatory candidate for a variety of cell properties such as growth, differentiation, remodeling of ECM, invasion, and migration. In human trophoblast cells, EGF activates the degradation of ECM by the stimulation and secretion of MMP-9 (Anteby et al., 2004) and also promotes cell motility (Qiu et al., 2004b). Based on the findings from these in vitro studies and the fact that MMP-9 is expressed in bovine trophoblast throughout gestation (Walter and Boos, 2001), we hypothesized that EGF is involved in the regulation of the MMP-9/TIMP-1 balance and thus could influence bovine placental function in several ways. This includes specifically TGC migration, tissue remodeling, and the release of the fetal membranes after parturition.

Therefore, the aim of this in vitro study was to examine EGF-mediated effects on cell motility, proliferation, and MMP-9 and TIMP-1 expression in cultured bovine placental cells. The identification of the underlying mechanisms is particularly interesting because key pathways of EGF signaling are functional in cultured bovine trophoblast cells (Hambruch et al., 2010).

RESULTS

EGF Increases mRNA Levels of MMP-9 and TIMP-1

Stimulation with EGF significantly increased the abundance of MMP-9 (378 bp) and TIMP-1 (231 bp) mRNAs (Fig. 1). To examine whether MAPK and/or PI3K signaling pathways are involved in the upregulation of MMP-9 and TIMP-1 mRNAs, cells were treated with EGF (50 ng/ml) for 24 hr with or without pretreatment (for 45 min) with inhibitors PD98059 (PD, 50 μM) and LY294002 (LY, 10 μM). The EGF -induced increase in mRNA abundance of MMP-9 and TIMP -1 was reduced by the presence of specific inhibitors of MAPK (PD98059) or PI3K (LY294002).

EGF Induces MMP-9 Secretion and Activity

Zymographic analysis of F3 cell supernatant after EGF treatment revealed an increase in MMP-9 activity (Fig. 2). After treatment with EGF (50 ng/ml) the predominant form of MMP-9 was the active form (82 kDa) compared to pro-form of MMP-9 (92 kDa). In order to examine whether or not the regulation of MMP-9 activity involves MAPK or PI3K, cells were treated with two specific inhibitors (PD98059 and LY294002), as described above. The presence of either the inhibitor of MAPK (PD980053, 50 μM) or PI3K (LY294002, 10 μM) abolished the effect of EGF on MMP-9 activation.

MAPK 42/44 and PI3K Signaling Pathways Are Involved in EGF-Mediated Proliferation, Migration, and Motility of the Bovine Trophoblast Cell Line F3

We have previously shown that EGF-induced F3 proliferation can be abolished by the MAPK inhibitor PD98059 (Hambruch et al., 2010). Additionally, we report here that the EGF-dependent increase in cell growth (67% compared to serum-free medium) could not only be blocked in full by PD98059 but also by LY294002, a PI3K inhibitor (Fig. 3A). Since EGF is also known to be a potent migratory factor, the regulatory mechanisms involved in EGF-mediated cell motility were investigated by time-lapse analyses. EGF was consistently able to enhance F3 cell motility by 66% whereas 10% FCS had no significant effect. EGF-stimulated motility was completely inhibited by pretreatment with specific inhibitors to MAPK (PD98059) or PI3K (LY294002) (Fig. 3B). In the Boyden Chamber assay, addition of EGF (50 ng/ml) or 10% FCS significantly increased migration of F3 cells by 68% (EGF) or 28% (10% FCS) compared to serum-free conditions (Fig. 3C).

Detection of EGF-R and Effect of EGF Stimulation on Signaling Molecule Activation

The presence of EGF-R protein in F3 cells was confirmed by Western blot. Homogenate from bovine placentome served as positive control (Fig. 4A). The signaling involved in the regulation of F3 cell migration and proliferation was analyzed using pharmacological inhibitors. For this purpose, Western blot analysis was performed for phospho-p42/44 MAPK and phospho-Akt (Ser^{473}). While addition of PD98059 completely suppressed the EGF-induced MAPK activation, no effect on the phospho-Akt content was observed. Treatment with LY294002 consistently reduced only the phosphorylation of Akt after growth factor stimulation and did not affect the phospho-p42/44 MAPK level (Fig. 4B).

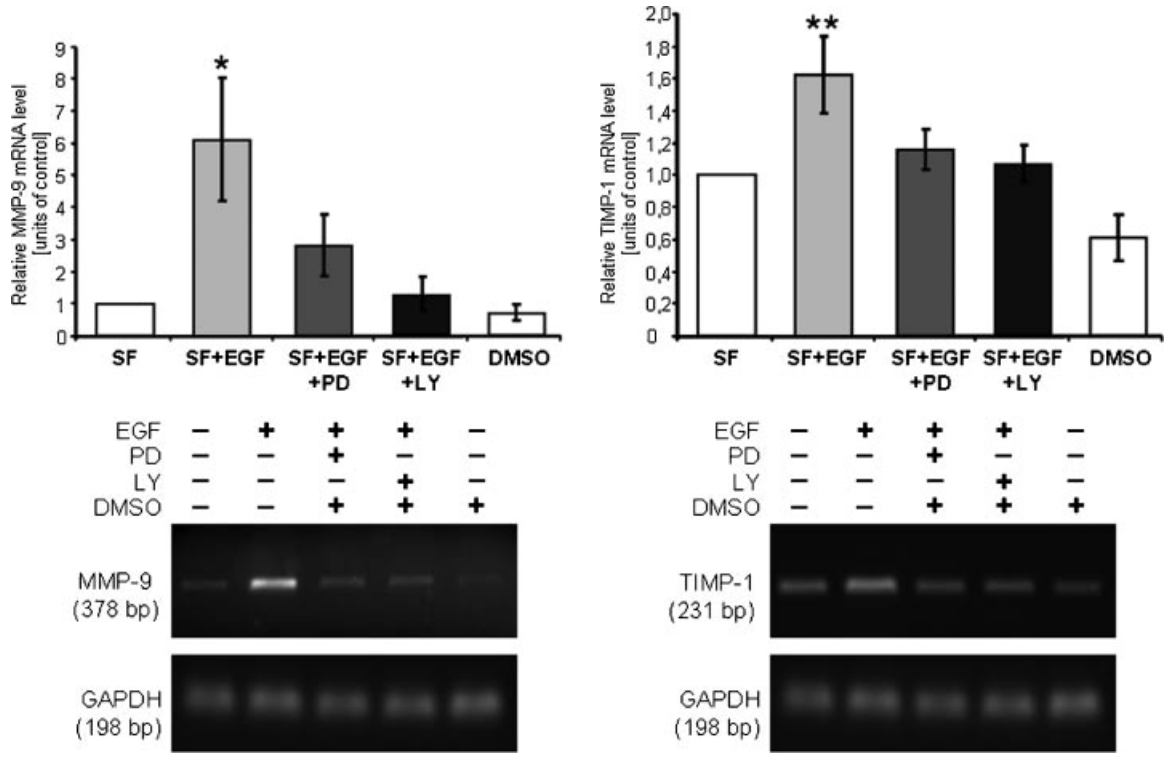


Figure 1. Effect of EGF and inhibitors of MAPK and PI3K on the abundance of MMP-9 and TIMP-1 mRNA. Agarose gel electrophoresis demonstrates the presence of RT-PCR products of MMP-9 (378 bp), TIMP-1 (231 bp), and GAPDH (198 bp) in bottom panels. Top panels show the influence of specific inhibitors of MAPK (PD980053, PD, 50 μM) and PI3K (LY294002, LY, 10 μM) on EGF-induced (50 ng/ml) mRNA levels of MMP-9 and TIMP-1. EGF significantly increases mRNA levels of MMP-9 and TIMP-1 in F3 cells compared to control (SF, serum-free medium). Values represent means ± SEM of three independent experiments, one asterisk represents $P < 0.05$, two asterisks indicate $P < 0.005$ compared to control (SF), dimethyl sulfoxide (DMSO) served as vehicle control.

DISCUSSION

Several signaling pathways have been investigated in connection with cell migration and differentiation, yet the mechanisms regulating motility of bovine trophoblast are still unknown. Utilizing the well-characterized trophoblast cell line F3 derived from a bovine placentome, we demonstrated for the first time that EGF is involved in the upregulation of MMP-9 and TIMP-1 mRNAs in bovine trophoblast cells and enhances MMP-9 activity. Furthermore, we have shown that MAPK 42/44 and Akt activation are required for proliferation, migration, and motility in F3 cells in response to EGF, suggesting that EGF plays a pivotal role in the differentiation and migratory activity of bovine trophoblast cells.

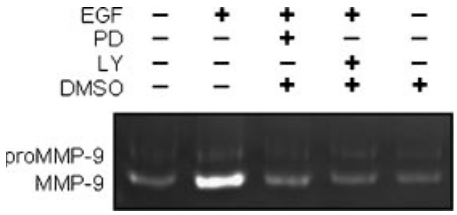
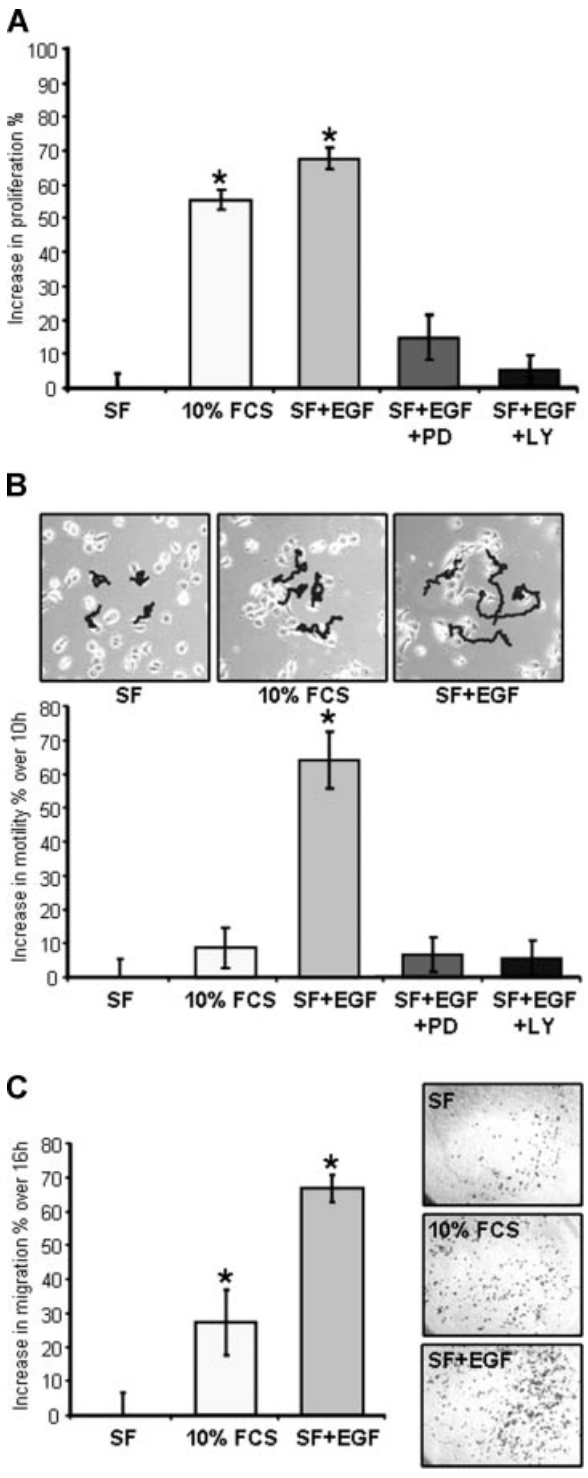


Figure 2. Effect of EGF and inhibitors of PI3K and MAPK on MMP activity. Zymographic analysis of MMP-9 activity in conditioned media (after stimulation for 24 hr). EGF increases the expression of active MMP-9 in fetal cells (F3). This effect could be abolished in the presence of inhibitors PD98059 (PD, 50 μM) and LY294002 (LY, 10 μM), respectively.

Influence of EGF on MMP Expression

As EGF is a potent inductor of MMP-9 expression and activation, it can upregulate invasion and motility in different cell types. For instance, EGF is involved in the upregulation of MMP-9 expression in human breast cancer cells (Kondapaka et al., 1997). A recent study of Rothhut et al. (2007) demonstrated that EGF increases MMP-9 gene expression and enhances motility in human follicular thyroid carcinoma cells by distinct signaling pathways. In the present study, we have shown that a significant upregulation in the abundance of MMP-9 mRNA in response to EGF correlates with an increased motility and proliferation of F3 cells. Furthermore, both EGF-mediated migration and MMP-9 activity require the MAPK and PI3K pathway. Hence, the upregulation of MMP-9 expression and activity could be involved in the process of migration in bovine trophoblast cells. These findings are in accordance with previous studies where EGF induced the secretion of MMP-9 and motility in human trophoblast cells (Qiu et al., 2004b; LaMarca et al., 2008).

While the upregulation of MMPs by single cytokines such as tumor necrosis factor (TNF) alpha in bovine luteal cells, as well as transforming growth factor (TGF) alpha and TGF beta in human and bovine endometrial cells, was reported by several authors (Hashizume et al., 2003; Zhang et al., 2005; Braundmeier et al., 2006), others have demonstrated that a synergistic effect of growth factors is needed to increase MMP gene expression (Tian et al., 2007). We have shown



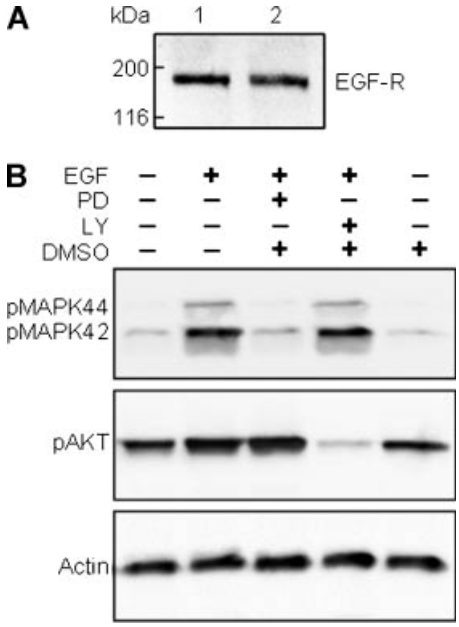


Figure 4. Detection of EGF-R and effect of EGF on MAPK42/44 and Akt phosphorylation in the absence or presence of PD98059 or LY294002. **A**: Western blot of protein samples derived from F3 cells (1) and bovine placentome (2) detecting EGF-R (170 kDa). **B**: F3 cells were serum-starved for 4 hr and treated with EGF (50 ng/ml) for 24 hr. PD98059 (50 μM) or LY294002 (10 μM) was added 45 min before EGF treatment as indicated in the figure. Whole cell lysates were used for immunoblotting to detect pMAPK42/44 and pAkt. Actin (42 kDa) was used as loading control. PD specifically abolishes EGF-dependent activation of MAPK, while LY exerts the same effect on Akt phosphorylation. The presented experiments were repeated three times.

Figure 3. Involvement of mitogen-activated protein kinases (MAPK) and phosphatidylinositol 3 kinase (PI3K) signaling in F3 proliferation, migration, and motility. **A**: The growth response of F3 cells was determined by the MTT assay. Serum-starved F3 cells were pretreated with 50 μM PD98059 (specific inhibitor to MAPK) or 10 μM LY294002 (specific inhibitor to PI3K) for 45 min and afterwards incubated 10% FCS or 50 ng/ml EGF for 24 hr. Treatment with either LY294002 or PD98059 significantly inhibited EGF-induced proliferation. **B**: For motility measurement, time-lapse digital microscopy was performed. Serum-starved F3 cells were pretreated with 50 μM PD98059 (specific inhibitor to MAPK) or 10 μM LY294002 (specific nhibitor to PI3K) for 45 min. After stimulation with 10% FCS or 50 ng/ml EGF, motility was monitored over 10 hr. Tracks (black lines) of individual motile F3 cells (60 cells per experiment) were recorded and the accumulated distance was analyzed with the help of Image J software. Treatment with either LY294002 or PD98059 significantly inhibited EGF-induced motility. **C**: For analysis of migration, a Boyden chamber was used. Serum-starved F3 cells were exposed to 10% FCS or 50 ng/ml EGF in the Boyden chamber. The cells that migrated through the porous membrane were stained, and the average number of migrating cells was determined. Representative images (10×) of the stained membranes of cells exposed to serum-free medium (SF), 10% FCS in SF or 50 ng/ml EGF are depicted on the right. Both 10% FCS and EGF significantly stimulated F3 migration. The data presented in each panel were normalized relative to the control (SF, serum-free medium) and then averaged (mean ± SEM). Asterisks represent $P < 0.001$ compared to control. Each experiment was repeated three times.

that EGF alone significantly upregulates mRNA levels of MMP-9 and TIMP-1 in bovine trophoblast cells. The simultaneous upregulation of MMP-9 and TIMP-1 by EGF may appear contradictory. However, upregulation of TIMP1 by EGF is very reasonable because TIMP1 promotes cell growth independently of its inhibitory capacity (Bertaux et al., 1991; Hayakawa et al., 1992; Stetler-Stevenson, 2008). The fact that TIMPs are multifunctional proteins suggests that other TIMPs potentially also have the ability to inactivate MMP-9 (Brew and Nagase, 2010, for review). For instance TIMP-2, which is expressed in the bovine placenta throughout gestation (Walter and Boos, 2001), is able to block growth factor induced migration and proliferation (Murphy et al., 1993; Oh et al., 2004) and thus could be involved in the regulation of restricted trophoblast invasion and differentiation in the bovine placenta. Nevertheless, it has to be considered that, besides EGF, other growth factors could participate in the control of bovine trophoblast migration and differentiation. Such an involvement has been shown for TNF-α, vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF), which, in addition to the expression and secretion of active MMP-9, can activate MMP-9 protease activity in human trophoblast cells (Anteby et al., 2004; Cohen et al., 2006). As members of the FGF and VEGF systems are localized in the bovine trophoblast, these factors are also likely to play a role in trophoblast differentiation and migration (Pfarrer et al., 2006).

In vivo studies have shown that the MMP-9 protein is expressed in trophoblast cells of the synepitheliochorial sheep placentae throughout the last trimester of gestation and throughout the whole gestational period in the cow (Vagnoni et al., 1998; Walter and Boos, 2001). However, the localization of MMPs alone is not sufficient as it does not give any information on MMP activity. In our study, we demonstrated for the first time that EGF induces the activation of MMP-9 protease in bovine placental cells. This suggests that such activation may also take place in vivo. So far the active and latent forms of MMP-9 have only been distinguished by zymography in the sheep placenta (Vagnoni et al., 1998). These authors found MMP-9 activity in conditioned media of all compartments of the ovine placenta and concluded that MMP-9 and TIMP-1 are involved in angiogenesis and tissue remodeling during gestation. In the human placenta, a strong enzymatic activity for MMP-9 was detected at various regions of the feto-maternal interface, including anchoring villi, basal plate, and decidua, suggesting a pivotal role of MMP-9 in the separation of the placenta from the uterine wall after birth (Demir-Weusten et al., 2007). These findings support our idea that MMP-9 plays a role in placental tissue remodeling and the release of bovine fetal membranes besides its presumed functions for trophoblast migration and differentiation.

Implications of EGF Utilized Pathways

Treatment with EGF in our study consistently led to an increase in the active form of MMP-9 in F3 cells while the inhibition of MAPK or Akt activation blocked this effect. These results confirm that the MAPK and the PI3K/Akt signaling pathways are involved in the secretion and activation of MMP-9 in bovine trophoblast cells. Utilization of the same signaling cascades has been reported for human trophoblast cells, which require the activation of both MAPK and PI3K pathways for the regulation of migration and secretion of MMP-9 and TIMP-1 (Qiu et al., 2004a,b). Furthermore, we demonstrated that MAPK 42/44 and Akt were phosphorylated in response to EGF in F3 cells. Additionally, both MAPK and PI3K inhibitors abolished the EGF-induced activation of these signaling pathways as well as the induction of motility and proliferation. This is in agreement with other studies, which describe EGF-stimulated migration and invasion through these pathways using human trophoblast cell lines (Qiu et al., 2004a; LaMarca et al., 2008). In a previous study, we demonstrated the activation of the small GTPase Ras upon stimulation with EGF (Hambruch et al., 2010). The involvement of this classical mitogenic Raf/MEK/ERK cascade in the regulation of MMP-9 expression is well documented (Rothhut et al., 2007; Tian et al., 2007). Moreover, EGF has been shown to have a proliferative effect on cultured human trophoblast cells (Iguchi et al., 1993; Li and Zhuang, 1997) and can inhibit apoptosis and mediate differentiation in human cytotrophoblasts (Morrish et al., 1997; Smith et al., 2002). Our experiments examining the growth response of F3 cells to EGF indicate that the activation of both MAPK and PI3K/Akt pathways is essential for trophoblast proliferation and motility. It remains to be determined whether NFkB and AP-1 are part of the downstream signaling cascade (Bancroft et al., 2002) and if activation of these transcription factors is crucial for promoting proliferation and/or MMP-9 expression.

In addition to the various biological effects of the EGF system, we were also able to confirm the presence of EGF-R in F3 cells. These findings are in accordance with in vivo studies where the EGF-R was localized in the trophoblast as well in the maternal epithelium of the bovine placenta throughout gestation (Weise, 2001). Therefore, we speculate that the effect of EGF on the activity of MMP-9 in trophoblast cells and the subsequent degradation of ECM might be involved in the process of tissue remodeling throughout the whole gestational period as well as the release of fetal membranes. This latter point is of particular interest due to the fact that the underlying mechanisms of placenta release might be far too complicated to be clarified by in vivo studies. Further studies will be necessary to gain more insight into the regulatory events in the process of loosening adherence between the fetal and maternal compartment of the bovine synepitheliochorial placenta.

Based on the in vitro results presented and above-mentioned published data, we developed our current working concept that in vivo EGF produced by TGC and/or uninucleated trophoblast cells stimulates MMP-9 and TIMP-1 secretion and activation (via MAPK and PI3K/Akt) in an auto- or paracrine fashion. Protease activity is necessary for the detachment of cells from the basal membrane and surrounding cells prior to migration, while the balance of protease activity and inhibition influences the number of migrating cells and thus participates in the control of restricted trophoblast migration/invasion in the bovine placenta.

MATERIALS AND METHODS

Cell Culture and Stimulation Experiments

The bovine trophoblast cell line F3 was derived from placentomes and has been well characterized (Hambruch et al., 2010). Cells were cultured in full supplemented medium (FSM: Dulbecco's modified Eagle medium (DMEM)/Ham's F12 containing 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine (PAA, Coelbe, Germany) at 37°C in 5% CO_2. For stimulations, F3 cells were seeded in 3.5 cm tissue culture dishes (TPP, Trasadingen, Switzerland; 1×10^5 cells) and cultivated for 48 hr in FSM. Afterwards cells were serum-starved for 4 hr (SF medium: DMEM/Ham's F12 containing 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine) before being treated with inhibitors of MAPK activation (PD98059, 50 μM in DMSO) or inhibitor of PI3K activation (LY294002, 50 μM in DMSO). After 45 min EGF 50 ng/ml, human recombinant EGF (Biomol, Hamburg, Germany) was added and the cells were incubated for 24 hr. Incubations with SF and SF with DMSO (vehicle control for inhibitors) served as controls. All experiments were designed in double and repeated three times. From each experiment, cells were lysed for analysis via Western blot as well as for isolation of total RNA. Furthermore, the medium was collected prior to lyses to determine MMP-9 activity.

RNA Isolation and Semiquantitative RT-PCR Analyses

Total RNA from stimulated cells was isolated with SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer's instructions. Reverse transcription was performed using 0.5 μg of total RNA in a 20 μl reaction volume. Briefly, RNA and random hexamers were mixed and incubated for 5 min at 70°C, then chilled on ice for 3 min. The reaction mix (1× ImProm-II reaction buffer, 3 mM $MgCl_2$, 0.5 mM each dNTP, 20 U RNasin ribonuclease inhibitor, 1 μl ImProm-II reverse transcriptase) was added to the mixture and incubated according to the manufacturer's instructions. Primer pairs (purchased from MWG Biotech, Ebersberg, Germany) for cDNA amplification (in the 5′-3′direction) were as follows: AGATTCCAGACCTTTGAGGGCGAA (forward) and TTGCCCAGAGACCACAACTCTTCA (reverse) for bovine MMP-9; CATCTACACCCCTGCCATG (forward) and CAGGGGATGGATGAGCAG (reverse) for bovine TIMP-1; GTCTTCACTACCATGGAGAAGG (forward) and TCATGGATGACCTTGGCCAG (reverse) for bovine GAPDH. The expected fragment length of MMP-9, TIMP-1, and GAPDH was 326, 231, and 198 bp, respectively. PCR amplifications were performed on a PeqStar Cycler (PeqLab, Erlangen, Germany) using GoTaq Hot Start Polymerase (Promega) for 28 cycles for TIMP-1 and GAPDH and 35 cycles for MMP-9. GoTaq Hot Start Polymerase was activated at 95°C for 2 min before the beginning of the cycle (94°C for 30 sec for denaturing, 58°C for annealing, 72°C for extension). PCR products were visualized under UV transillumination on 1.5% agarose gel containing ethidium bromide. The ratios of MMP-9 and TIMP-1 to GAPDH were analyzed by densitometric measurement and quantification using BIO-1D software (Vilber Lourmat, Eberhardzell, Germany).

Gelatin Zymography

After stimulation, enzymatic activity of MMP-9 in the supernatant was determined by zymography. Equal volumes of culture supernatants were mixed with 4× sodium dodecyl sulfate (SDS)-loading buffer (RotiLoad®, Roth, Karlsruhe, Germany) and incubated for 10 min at 37°C and resolved on 10% SDS–polyacrylamide gels containing 0.1% gelatin (Sigma, Steinheim, Germany) as substrate. After electrophoresis, the gels were washed in renaturing buffer (Invitrogen, Darmstadt, Germany) for 30 min at room temperature (RT), followed by incubation in developing buffer (Invitrogen) for 30 min at RT. Thereafter, developing buffer was changed and gels were incubated over night at 37°C. Gels were stained with 0.5% Coomassie blue R-250 in 10% acetic acid/50% methanol, and destained with 10% acetic acid/40% methanol to reveal discrete areas of gelatine degradation. The proteolytic activity appeared as clear bands on a blue background. The images were scanned by using Vision Capt software (Vilber Lourmat).

Western Blot Analyses

After stimulation cells were placed on ice, washed once with cold PBS, and were lysed with a buffer containing 50 mM Tris–HCl at pH 7.4, 150 mM NaCl, 40 mM NaF, 5 mM EDTA, 1% (v/v) Nonidet P40, 0.1% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS supplemented with protease and phosphatase inhibitor cocktail (75 μl per 35 mm; 300 μl per 60 mm dish). Lysates were centrifuged for 5 min at 13,000*g* (4°C), and the protein concentration of the supernatant was determined. For stimulation experiments, equal amounts of protein (15 μg/slot) were denaturated in Laemmli sample buffer (5 min at 95°C) and analyzed with 12% SDS–polyacrylamide gels. Protein derived form unstimulated F3 cells and from bovine placentome homogenate were used for the detection of EGF-R and analyzed with a 10% SDS–polyacrylamide gel. Afterwards Western blots were performed as suggested by the suppliers of the antibodies anti-phosphoMAPK (M8159, 1:12,500, Sigma), anti-phosphoAkt (4060, 1:4,000, Cell Signaling, Frankfurt, Germany), anti-EGF-R (1234-1. Epitomics, Burlingame, CA, USA), and anti-beta-actin (sc-47778, 1:5,000, Santa Cruz, Santa Cruz, CA, USA) and detected with chemiluminescence (SuperSignal West Pico, Pierce, Rockford, IL, USA).

Migration Assay

For analysis of cell migration, fibronectin-coated 8 μm polycarbonate filters in a Boyden chamber (Neuro Probe, Gaithersburg, MD, USA) were used according to the manufacturer's information. Briefly, cells were starved for 4 hr and a total number of 4,000 cells in SF medium were added to the upper compartment. Fifty nanograms EGF or 10% FCS as a positive control were added to the lower chamber, and the system was incubated at 37°C for 16 hr in 5% CO_2. After incubation and fixation, the nonmigrating cells were removed with a cotton swab and the remaining cells were stained with Crystal Violet (0.2% in 2% ethanol) for 15 min. The number of cells in four microscopic fields was counted and the median of six wells was determined. The experiment was repeated three times.

Cell Motility Assay

F3 cells (2.5×10^5 cells per well) were allowed to adhere in 12-well tissue culture test plates (TPP) for 4 hr in culture medium. Prior to stimulation cells were serum starved for 2 hr and pretreated for 45 min with 50 μM PD98059 or 10 μM LY294002. Afterwards, the cells were stimulated with 10% FCS or 50 ng/ml EGF. The plates were placed in an environmental chamber (Cell Observer System, Zeiss MicroImaging, Jena, Germany) and pictures were taken every 25 min for 10 hr. Sixty cells were chosen at random per treatment and the accumulated distance cells moved was quantified using ImageJ software over the entire time period (Abramoff et al., 2004). The experiment was repeated three times.

Statistical Analyses

Data are presented as the mean ± SEM. Data from treated groups were compared with control groups and significant differences were determined by one-way analysis of variance followed by Tukey's honestly significant difference (HSD) test using SPSS software. A *P*-value of <0.05 was defined as significant.

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MRD

4 PAPER II

Expression of matrix metalloproteinase (MMP)-2, MMP-14 and tissue inhibitor of matrix metalloproteinase (TIMP)-2 during bovine placentation and at term with or without placental retention

Abstract

Matrix metalloproteinases (MMPs) and counteracting tissue inhibitors of metalloproteinases (TIMPs) are balancing extracellular matrix (ECM) formation and degradation. The latter is believed to be an important aspect for the detachment of fetal membranes postpartum when loosening the feto-maternal connection which is a prerequisite to avoid placental retention a common disease in cows leading to considerable economic loss. Membrane-type (MT) MMPs have been suggested as potential activators controlling ECM remodelling. In particular, MT1-MMP (MMP-14) is able to degrade ECM substrates and activate MMP-2 through binding TIMP-2 at the cell surface. Since the connection between the trophoblast and the maternal caruncular epithelium is supported by integrin receptors bound to ECM, we hypothesize that impaired modulation of the ECM by TIMPs/MMPs participates in the aetiology of bovine retained fetal membranes. To analyse this involvement, placentomes were collected from cows after term parturition and timely release of fetal membranes (n = 4) and cows with retained fetal membranes after various treatments for the induction of parturition using progesterone antagonist (aglepristone), PGF(2α) analogue, glucocorticoid, and after elective caesarean sections (each group n = 3). The expression of MMP-14, MMP-2 and of TIMP-2 was examined by real-time-PCR, immunohistochemistry, Western blot and zymography. The relative mRNA expression levels of MMP-14 remained unchanged, while the expression levels of TIMP-2 and MMP-2 partly increased in animals with induced parturition and retention of fetal membranes compared to animals without placental retention. MMP-14 protein was expressed in cells of the uninucleated trophoblast, the fetal mesenchyme and maternal stroma. TIMP-2 was present exclusively in trophoblast giant cells, while MMP-2 could be detected in uninucleated trophoblast cells and the fetal mesenchyme. The presence of the activated enzyme was confirmed by zymography. In conclusion, MMP-14, MMP-2 and TIMP-2 are co-

localized in the fetal compartment and therefore could influence the timely release of fetal membranes in cattle.

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Expression of matrix metalloproteinase (MMP)-2, MMP-14 and tissue inhibitor of matrix metalloproteinase (TIMP)-2 during bovine placentation and at term with or without placental retention

M. Dilly[a], N. Hambruch[a], S. Shenavai[b], G. Schuler[b], R. Froehlich[a], J.-D. Haeger[a], G.R. Ozalp[c], C. Pfarrer[a,*]

[a] *Department of Anatomy, University of Veterinary Medicine Hannover, Hannover, Germany*
[b] *Clinic for Obstetrics, Gynecology and Andrology of Large and Small Animals, Faculty of Veterinary Medicine, Justus-Liebig-University, Giessen, Germany*
[c] *Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Uludag University, Bursa, Turkey*

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Abstract

Matrix metalloproteinases (MMPs) and counteracting tissue inhibitors of metalloproteinases (TIMPs) are balancing extracellular matrix (ECM) formation and degradation. The latter is believed to be an important aspect for the detachment of fetal membranes postpartum when loosening the feto-maternal connection which is a prerequisite to avoid placental retention a common disease in cows leading to considerable economic loss. Membrane-type (MT) MMPs have been suggested as potential activators controlling ECM remodelling. In particular, MT1-MMP (MMP-14) is able to degrade ECM substrates and activate MMP-2 through binding TIMP-2 at the cell surface. Since the connection between the trophoblast and the maternal caruncular epithelium is supported by integrin receptors bound to ECM, we hypothesize that impaired modulation of the ECM by TIMPs/MMPs participates in the aetiology of bovine retained fetal membranes. To analyse this involvement, placentomes were collected from cows after term parturition and timely release of fetal membranes (n = 4) and cows with retained fetal membranes after various treatments for the induction of parturition using progesterone antagonist (aglepristone), $PGF_{2\alpha}$ analogue, glucocorticoid, and after elective caesarean sections (each group n = 3). The expression of MMP-14, MMP-2 and of TIMP-2 was examined by real-time-PCR, immunohistochemistry, Western blot and zymography. The relative mRNA expression levels of MMP-14 remained unchanged, while the expression levels of TIMP-2 and MMP-2 partly increased in animals with induced parturition and retention of fetal membranes compared to animals without placental retention. MMP-14 protein was expressed in cells of the uninucleated trophoblast, the fetal mesenchyme and maternal stroma. TIMP-2 was present exclusively in trophoblast giant cells, while MMP-2 could be detected in uninucleated trophoblast cells and the fetal mesenchyme. The presence of the activated enzyme was confirmed by zymography. In conclusion, MMP-14, MMP-2 and TIMP-2 are co-localized in the fetal compartment and therefore could influence the timely release of fetal membranes in cattle.

Keywords: Bovine placenta; Matrix metalloproteinases

1. Introduction

In the synepitheliochorial bovine placenta a tight feto-maternal connection is established in so-called placentomes where fetal cotyledons interdigitate with maternal caruncles [1]. While the uterine or caruncular epithelium (CE) is a homogeneous cell population, the bovine trophoblast characteristically consists of two populations of trophoblast cells, uninucleated trophoblast cells (UTC) and mostly binucleated trophoblast giant cells (TGC). TGC migrate and fuse with singular uterine epithelial cells to form feto-maternal hybrid cells throughout gestation [2,3]. A firm anchorage is guaranteed firstly by the complementary interdigitation

* Corresponding author. Tel.: +49-511-856-7211; fax: +49-511-856-7683.
E-mail address: Christiane.Pfarrer@tiho-hannover.de (C. Pfarrer).

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of fetal villous trees with maternal crypts and secondly by the interdigitation of the apical microvilli from UTC and CE directly by cell-cell or indirectly by cell-matrix contacts [4]. This tight connection is essential during gestation, but must be terminated after expulsion of the fetus to ensure a healthy puerperium. Therefore distinct remodelling and loosing adherence at the feto-maternal interface takes place in late gestation. A process, termed placental maturation, includes a reduction of the CE and a decline in TGC numbers, and is required for the release of the bovine fetal membranes [5–9].

Impaired regulation of the process may lead to one of the major reproductive disorders in cattle, the retention of fetal membranes (RFM). This disturbance is defined as the condition in which the fetal membranes are not expelled from the uterus within 12–48 h postpartum [10,11]. RFM can affect the reproductive performance and lead to considerable economic loss at the herd level [12–14]. Several risk factors are associated with RFM, as are shortened gestation, caesarean section and induced parturition [15–17]. With regard to the latter factor it is reported that hormonal changes and prostaglandins play an important role in the regulation of placental separation and maturation [18–24]. Therefore, an impairment of placental separation likely occurs after induction of parturition.

Furthermore, local factors might be involved in loosening the adherence of the fetal membranes from the maternal compartment. Both, the maternal and fetal compartment are subject to rapid growth, angiogenesis and tissue remodelling during gestation. These processes, as well as the proper release of fetal membranes require proteolytic enzymes and subsequent degradation of extracellular matrix (ECM) components [25,26]. Matrix metalloproteinases (MMPs) are considered to play a pivotal role in the processes of tissue remodelling and breakdown of the ECM during placentation and implantation in several species [27–29]. MMPs are zinc-dependent endopeptidases capable of degrading essential components of the ECM. Most MMPs are secreted as inactive pro-enzymes, which become activated upon cleavage of an N-terminal propeptide. Their activity is strictly regulated by counteracting tissue inhibitors of MMPs (TIMPs). Among the MMP family, two members (gelatinases MMP-2 and MMP-9) are well characterized in ruminants. However, the enzymatic activity of MMP-2 seems to play a more important role in the synepitheliochorial placenta of ruminants, than the activity of MMP-9 [30–35]. Thus, the regulation and activation of MMP-2 is of particular interest for our study. It has been reported that the latent form of MMP-2 (proMMP-2) is mainly activated through formation of a trimolecular complex between MMP-14, TIMP-2, and proMMP-2 [36]. The membrane bound MMP-14, also called membrane type-1 MMP (MT1-MMP), interacts with proMMP-2/TIMP-2 to form a ternary complex. The proteolytical activation of this complex is triggered by cleavage of the propeptide of MMP-2 by a second MMP-14 molecule. Thus, TIMP-2 can serve as an inhibitor and activator of MMPs depending on the quantity of surrounding components of the MMP/TIMP system [37]. So far, information about enzyme activity and potential activation of MMP-2 via MMP-14 and TIMP-2 is lacking for the bovine placenta. The spatial regulation of ECM degradation and subsequent release of fetal membranes could be mediated by the fetal compartment itself and/or by the maternal compartment. Furthermore, the underlying mechanisms involved in the loosing of adherence of fetal membranes postpartum are barely understood in the bovine placenta. We hypothesize that the process of loosing adherence between the maternal and fetal compartment is modulated by TIMPs/MMPs.

Therefore, the aim of our study was to test this hypothesis and to gain more information about the regulatory mechanisms leading to the release of fetal membranes. As induced parturition leads to RFM in most cases, the expression of MMP-14, MMP-2 and TIMP-2 was compared in placentomes from animals with induced parturition (by progesterone receptor antagonist aglepristone, $PGF_{2\alpha}$ analogue, glucocorticoid) and placentomes collected during preterm caesarean sections as well as after spontaneous parturition.

2. Materials and methods

2.1. Sample collection and fixation

All animal experiments were approved by the committee on the use of animals for research purposes at the regional council (Regierungspraesidium Giessen, no.V54-19c-20-15(I) Gi 18/14-Nr.41/2007; LAVES, 33.9-42502-04-09/1634) according to the German animal protection law. Placental tissues were collected from Holstein cows (n = 16). The cows were divided into five different groups as follows (I) preterm elective caesarean section at day 272 of gestation (n = 3), (II) induced parturition by injection of dinoprostum, $PGF_{2\alpha}$ analogue [Dinolytic/Dinoprost® 25 mg i.m.] at day 272 of gestation (n = 3), (III) induction of parturition by treatment with dexamethasone, glucocorticoid [Dexafort® 0.06 mg/kg i.m.] at day 272 of gestation (n = 3), (IV)

Table 1

Gene	Sequence of forward and reverse primer (5′→ 3′)	Fragment size (bp)	Accession number or Reference
MMP-2	CCCAGACAGTGGATGATGC (for) TTGTCCTTCTCCCAGGGTC (rev)	248	NM_174745 [41]
MMP-14	ACTTGGAAGGGGGACACC (for) AGGGGGCATCTTAGTGGG (rev)	235	AF144758 [41]
RPS-9	AAACGTGAGGTCTGGAGGGTCAAA (for) GCAACAGGGCATTACCTTCGAACA (rev)	117	NM_001101152 [42]
TIMP-2	GGGTCTCGCTGGACATTG (for) TTGATGTTCTTCTCCGTGACC (rev)	255	NM_174472 [41]

induced parturition by injection of progesterone receptor blocker [aglepristone 5 mg/kg s.c.] at day 270 and day 271 of gestation (n = 3) and (V) term parturition (spontaneous parturition) and release of fetal membranes within a period of 12 h after calving (n = 4). In groups II and III caesarean sections were performed within 24-36 h after injections, while parturition occurred within 24 h after second treatment in group IV. All animals from groups I–IV retained the fetal membranes for more than 24 h.

Immediately after expulsion of vital calves three placentomes were collected randomly from each animal per vagina or caesarean section, respectively. For immunohistochemistry placentomes were cut into slices of 0.5 cm thickness and fixed in 4% (v/v) neutral buffered formaldehyde solution according to Lillie for 24 h. Tissue samples were subsequently dehydrated in a graded ethanol series and finally embedded in paraffin. Every tissue block includes the total height of the placentome from allantochorion to caruncular stalk. For extraction of mRNA, gelatinase assay (zymography) and western blot analysis, respectively, further tissue samples from the central part of the placentomes were cut into smaller pieces (approximately 0.3 cm edge length) and shock frozen immediately in liquid N_2 [38].

Additionally, placentomes from 18 generally healthy, pregnant cows were collected at a slaughterhouse, perfusion-fixed with Bouins solution and paraffin embedded for immunohistochemistry. The animals were assigned to the following groups: early gestation (≤3 mo, n = 6, 4^{th} mo, n = 3 and 5^{th} mo, n = 2) and late gestation (6th mo, n = 2; 7^{th}–8^{th} mo, n = 2, and >8 mo, n = 3) [39]. The gestational age was assessed according to fetal crown-rump length [40].

2.2. Total RNA extraction and reverse transcription

Total RNA was extracted from placentome tissue using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions. To quantify the amount of total RNA extracted, the optical density (260 nm) was determined with a spectrophotometer (SmartSpec, Bio-Rad, CA, USA) for two different dilutions of the final RNA preparations. Reverse transcription was performed using 1 μg of total RNA in a 20 μl reaction volume. Briefly, RNA (or nuclease free water for negative control) and random hexamer primers were mixed and incubated for 5 min at 70 °C then chilled on ice for 3 min. The reaction mix (1x ImProm-II reaction buffer, 3 mM $MgCl_2$, dNTP (0.5 mM each), 20 U RNasin ribunuclease inhibitor and 1 μl ImProm-II reverse transcriptase) was added to the mixture and incubated according to the manufacturer's instructions. All components were purchased from Promega.

2.3. Quantitative real-time PCR

Specific primer sequences and size of resulting fragments for reference and target genes are shown (Table 1). Each polymerase chain reaction was carried out with 200 ng cDNA sample and 5 nM primer in a final reaction volume of 25 μl using SYBR Green PCR Master Mix (Applied Biosystems, Forster City, CA, USA). A negative control containing the reaction mix and no cDNA was included in each assay. Thermocycling was performed in a StepOnePlus Real Time PCR system (Applied Biosystems) using the following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60°C for 60 s with fluorescence detection during the annealing/extension step. The efficiency of primers (90.1–93.5%) were analysed and quality of amplification was verified by subsequent melt curve analysis. The amplicons were checked by gel electrophoresis and commercial sequencing. The values were normalized to reference gene ribosomal protein S9 (RPS-9) using ΔΔCT threshold method.

2.4. Immunohistochemistry

Proteins were localized in paraffin embedded placenta tissue sections (4–5 μm) using EnVision+™ anti-rabbit or anti-mouse immunoglobulin conjugated to peroxidase labelled dextran polymer (DAKO, Glostrup, Denmark). Sections were deparaffinised and hydrated in graded ethanol. Detection of immunoreactive protein was performed according to the manufacture's instructions. Antibodies used for immunohistochemistry included rabbit anti-MMP-14 (1:150, Ab-2) and rabbit anti-MMP-2 (1:75, Ab-7) from Thermo Fisher Scientific (Fremont, CA, USA) and mouse anti-TIMP-2 (1:2500, MAB3310) from Chemicon (Billerica, MA, USA). Primary antibodies were incubated in a humidified chamber over night at 4 °C. Finally, sections were washed with PBS and peroxidase activity was detected with DAB (Sigma, Steinheim Germany) as substrate for 5 min at room temperature. Sections were counterstained with hemalum, dehydrated and mounted with DPX (Fluka, Buchs, Switzerland). To analyse unspecific binding, primary antibodies were replaced by rabbit IgG and mouse IgG (Sigma) respectively, at the same concentration of the primary antibody.

2.5. Western blot analyses

Immunoblotting was performed to verify specificity of the used antibodies with bovine placental tissue. Tissue samples were cut into small pieces and manually homogenized in ice-cold protein extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 40 mM NaF, 5 mM EDTA, 5 mM EGTA, 1 mM Na_3VO_4, 1% (v/v) nonidet, 0.1% (w/v) natriumdesoxycholat and 1 mM PMSF). Protein content was determined using DC Protein Assay Kit™ (BioRad, Hercules, CA, USA). Equal amounts of samples, normalized to 40 μg total protein concentration, were mixed with loading buffer under reducing conditions and subjected to 8% SDS-polyacrylamid gels. SDS-PAGE was performed according to manufacture's instructions using a BioRad electrophoresis unit. Broad range molecular weight marker (Sigma) was used to determine molecular weights. After electrophoresis, separated proteins were transferred onto a nitrocellulose membrane (Roth, Karlsruhe, Germany) in a blotting device (BioRad). After blocking non-specific binding sites with BSA (3% in TBS/Tween for 45 min at room temperature) the membranes were incubated with primary antibodies over night at 4 °C (anti-MMP-14 1:500, anti-MMP-2 1:500 and anti-TIMP-2 1:2000). After washing the secondary antibodies were incubated for 45 min at room temperature. The bands were visualized using chemiluminescence (SuperSignal West Pico, Pierce, Rockford, IL, USA).

2.6. Zymographic analysis

Enzymatic activity of MMP-2 was determined by gelatine zymography. Proteins were extracted in ice-cold sample buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$, 1% (v/v) nonidet) and protein concentration was determined. Samples of different groups (1–5) were normalized to 80 μg total protein concentration per sample. Samples were mixed with loading buffer containing 2% SDS and resolved on an 8% SDS-polyacrylamide gel containing 0.1% gelatine (Sigma, Germany) as substrate. After electrophoresis, the gels were washed in renaturing buffer (Invitrogen, Darmstadt, Germany) for 30 min, followed by immersion in developing buffer (Invitrogen) for 30 min. Developing buffer was changed and gels were incubated over night at 37 °C. Gels were stained with 0.5% Coomassie blue R-250 (10% acetic acid/50% methanol), staining was removed with 10% acetic acid/40% methanol to reveal discrete areas of gelatin degradation. The proteolytic activity was indicated by clear bands on a blue background.

2.7. Data analyses

All data are expressed as the mean ± standard error (SEM). After testing for normality and equal variance, the relative mRNA expression of each gene from every experimental group (three animals per group, three placentomes per animal) was separately analyzed by a one-way ANOVA followed by multiple pair-wise comparisons using the Tukey's HSD (Honestly Significant Difference) test. All experiments were repeated three times and data were analyzed with SPSS software (Chicago, IL, USA) and a P value of <0.05 was defined as significant.

3. Results

3.1. mRNA expression profiles obtained with quantitative real time PCR

Quantitative real time PCR of placentome homogenates from animals with induced parturitions and term parturition was performed to evaluate the relative mRNA expression of MMP-14, TIMP-2 and MMP-2. The expression levels of MMP-14 were not significantly different between the analysed groups, while the expression levels of TIMP-2 and MMP-2 were increased in animals with induced parturition and elected

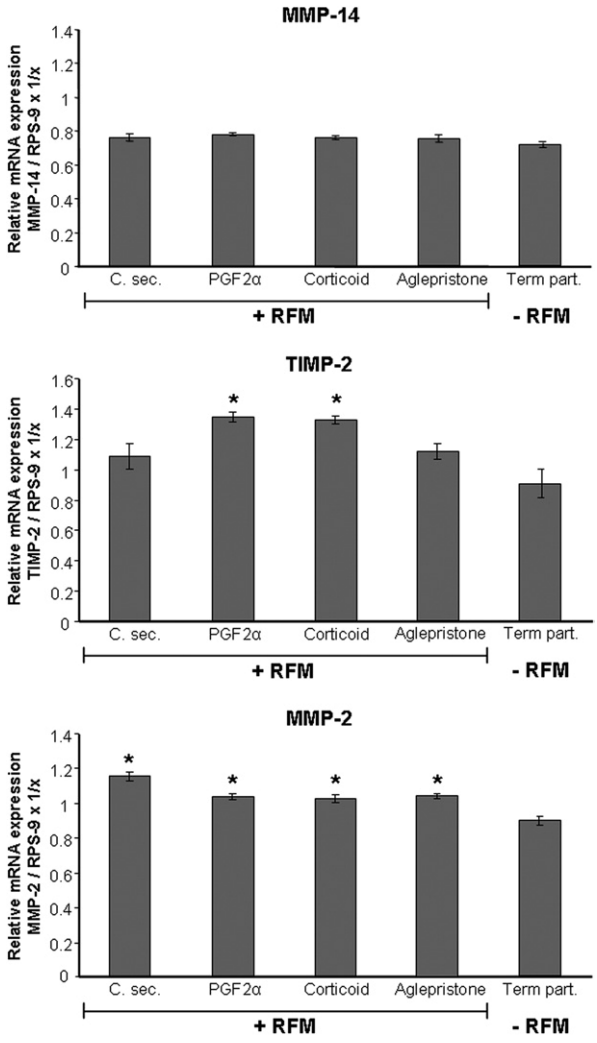

Fig. 1. Relative gene expression of MMP-14, TIMP-2 and MMP-2 from placentomal homogenates were determined to mRNA expression of ribosomal protein S9 (RPS-9) as reference gene. Data are expressed as means ± SEM; asterisks represent $P < 0.05$ by Tukey's HSD test.

C. sec., premature caesarean section (n = 3); $PGF_{2\alpha}$ (n = 3); Corticoid, glucocorticoid (n = 3); Aglepristone (n = 3); Term part., term parturition (n = 4); RFM, retained fetal membranes.

caesarean section with RFM (+RFM) compared to animals at term parturition without placental retention (-RFM, Fig. 1). For the TIMP-2 expression a significant higher value ($P < 0.05$) could be demonstrated at term parturition in comparison to $PGF_{2\alpha}$ induced parturition as well as induction of parturition by glucocorticoid treatment. The expression of MMP-2 was significantly higher in all induced parturitions and elected caesarean section including RFM (+RFM), than in animals at term without RFM (-RFM).

3.2. Localization of MMP-14, TIMP-2 and MMP-2 in bovine placentomes during gestation

To gain insight in the localization and expression patterns of MMP-14, TIMP-2 and MMP-2 in the course of gestation, we performed immunohistochemistry of sections from bovine placentomes (Fig. 2).

In early gestation (days 60–150), MMP-14 was primarily expressed in fetal mesenchyme, with highest intensities in the chorionic plate and stem villi (Fig. 2A). Both uninucleated trophoblast cells and TGC were devoid of any MMP-14 immunoreactivity. In the caruncular epithelium and stroma of maternal crypts only weak staining occurred. Staining for MMP-14 in the caruncular epithelium decreased and vanished with ongoing pregnancy. In late gestation (days 180–278), immunoreactivity for MMP-14 increased and was detected in the fetal mesenchyme of secondary and tertiary villi (Fig. 2B). Uninucleated trophoblast cells moderately expressed MMP-14 near term while TGC demonstrated no staining. Fetal and maternal blood vessels showed a weak staining or no specific staining for MMP-14 throughout gestation. TIMP-2 is exclusively expressed in TGC during the whole gestational period (Fig. 2C, D). In early pregnancy, MMP-2 is restricted to the mesenchyme of the chorionic plate and primary and secondary villi (Fig. 2E). The caruncular epithelium is negative for MMP-2, while the maternal stroma showed a weak staining. At the beginning of gestation the immunoreactivity for MMP-2 is weakly positive in the uninucleated trophoblast, but became strongly positive from the 4th until the 9th month of gestation (Fig. 2F). TGC showed no positive staining for MMP-2 throughout pregnancy. Several endothelia of blood vessels are positive for MMP-2 in the fetal and maternal stroma, smooth muscle cells of blood vessel walls showed a weak or no staining (data not shown).

3.3. Localization of MMP-14, TIMP-2 and MMP-2 in bovine placentomes in induced parturition and term parturition

All cows with induced parturition by injection of aglepristone, $PGF_{2\alpha}$ analoque or glucocorticoid and premature caesarean section (n = 12) retained the fetal membranes (+RFM) for more than 24 h. Immunolocalization of the analysed proteins in these groups with induced parturition showed consistent patterns among all animals. The results were compared to the expression patterns of cows with spontaneous birth of a vital calf and following release of fetal membranes (-RFM) within 12 h (n = 4).

MMP-14 protein was localized in the fetal mesenchyme, while the maternal stroma showed a moderate

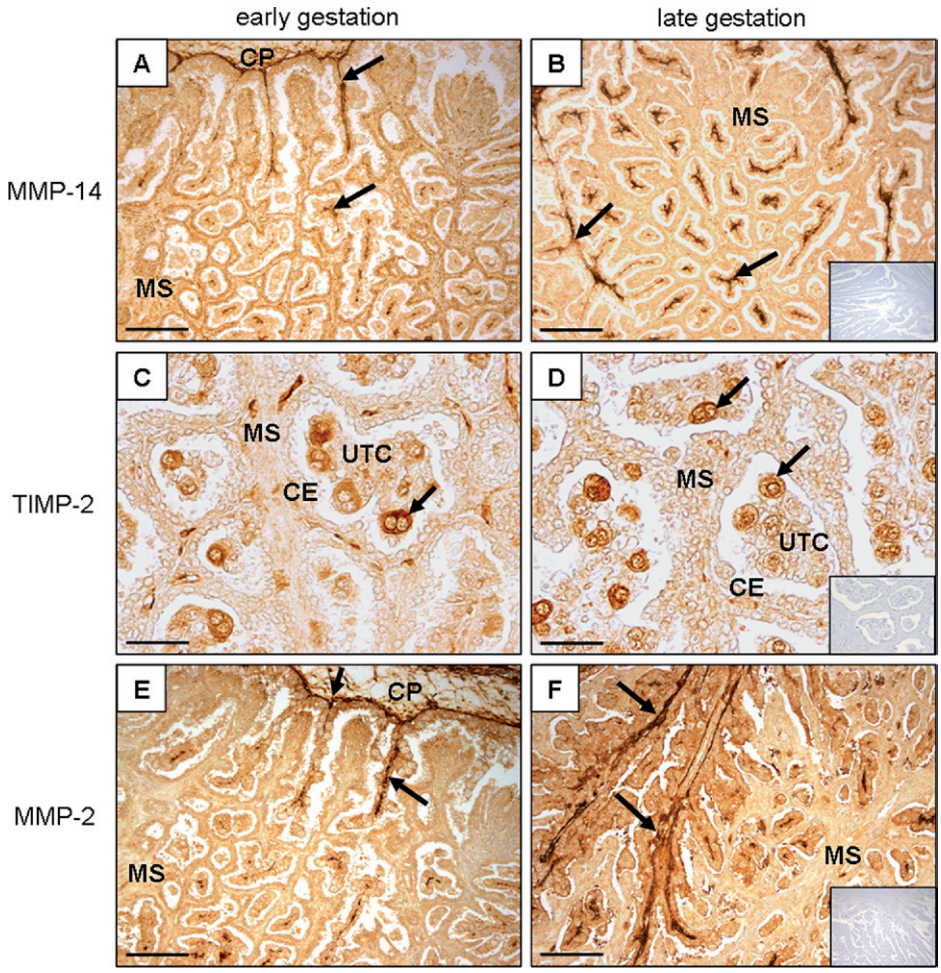

Fig. 2. Immunostaining for MMP-14, TIMP-2 and MMP-2 in bovine placentomes throughout pregnancy (A–F). Staining for MMP-14 is localized in the chorionic plate (CP) and the fetal mesenchyme of basal and apical primary villi (arrows) in early gestation (day 120, A). With ongoing pregnancy (day 270, B), immunoreactivity increased in the fetal villi (arrows) and uninucleated trophoblast cells moderately expressed MMP-14. Maternal stroma (MS) showed a weak staining throughout pregnancy. TIMP-2 is exclusively expressed by TGC (arrows) in early gestation (day 110, C) and late gestation (day 270, D). MMP-2 is restricted to the chorionic plate (CP) and the fetal villi (arrows) in early gestation (day 100, E) while in late gestation (day 275, F) uninucleated trophoblast moderately and the maternal stroma (MS) weakly expressed MMP-2.

Inserts show negative controls with rabbit and mouse IgG, respectively. Chorionic plate (CP), maternal stroma (MS), uninucleated trophoblast cells (UTC), caruncular epithelium (CE). Scale bars = 200 μm (A, B, E, F); scale bars = 50 μm (C, D).

staining in animals with RFM (Fig. 3A). In cows with release of fetal membranes, MMP-14 was expressed in cells of the uninucleated trophoblast, the fetal mesenchyme and maternal stroma (Fig. 3B). Fetal and maternal blood vessels showed a weak staining or no specific staining for MMP-14. Only trophoblast giant cells demonstrated a positive staining for TIMP-2 which was not affected by RFM (Fig. 3C, D). The fetal mesenchyme and the uninucleated trophoblast expressed MMP-2 in animals with induced parturition (Fig. 3E). In contrast, in cows with placental release, the staining intensities for MMP-2 were higher in the uninucleated trophoblast, but absent in the fetal mesenchyme (Fig. 3F). Maternal stroma cells showed a consistent weak staining in animals of all groups. Furthermore, trophoblast giant cells were negative for MMP-14 and MMP-2 immunoreactivities.

3.4. Western blot and gelatin zymography

Western blot analyses verified the specificity of the antibodies against MMP-14 (latent proform 64 kDa/active form 54 kDa), MMP-2 (latent proform 72 kDa/active form 62 kDa), and TIMP-2 (24 kDa) for bovine placental tissue (Fig. 4A). The abundance of the pre-

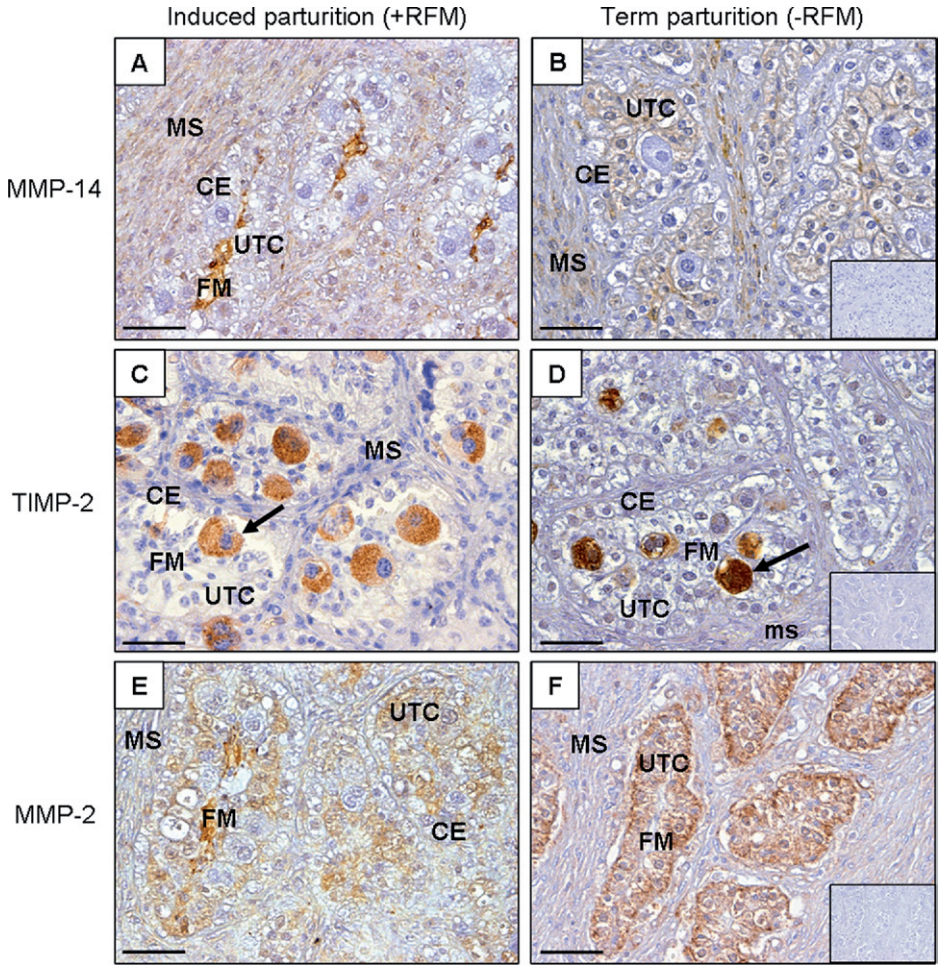

Fig. 3. Immunostaining for MMP-14, TIMP-2 and MMP-2 in bovine placentomes at induced and term parturition (A–F). Representative sections for induced parturition are shown for the aglepristone group, as all induced parturitions retained the fetal membranes (+RFM) and showed comparable results. MMP-14 was detected in the fetal mesenchyme (FM) of induced parturition (A) and in the uninucleated trophoblast (UTC) of term parturition (B). Maternal stroma (MS) reacts positive in both. TIMP-2 is restricted to TGC (arrows, C, D). MMP-2 is localized in the fetal mesenchyme (FM) of induced parturition and the uninucleated trophoblast (UTC) of both induced and term parturition (E, F).

Inserts show negative controls with rabbit and mouse IgG, respectively. Fetal mesenchyme (FM), maternal stroma (MS), uninucleated trophoblast cells (UTC), caruncular epithelium (CE), retained fetal membranes (RFM). Scale bars = 50 μm.

dominant latent proform of MMP-2 at 72 kDa correlates with enzyme activity observed by zymography. Zymograms revealed only small amounts of activated enzymes and predominantly the latent form of MMP-2 in supernatants of homogenates from bovine placentomes from animals after induced and spontaneous parturitions (Fig. 4B). No differences were observed within and between the analysed groups.

4. Discussion

In this study, the expression of MMP-14, TIMP-2 and MMP-2 as well as the protein activity of MMP-2 was analysed on mRNA and protein level for the first time during gestation and in conjunction with placental retention after induction of birth or premature section in cattle. We were able to demonstrate that the gene expression of TIMP-2 and MMP-2 was significantly higher in animals with RFM after induction of birth or premature section in comparison to animals releasing the fetal membranes. MMP-14, TIMP-2 and MMP-2 proteins were located in neighbouring cell populations of the fetal compartment showing spatiotemporal alterations in the course of pregnancy. Additionally, localization of MMP-14 and MMP-2 differed between animals with/without retained fetal membranes. The

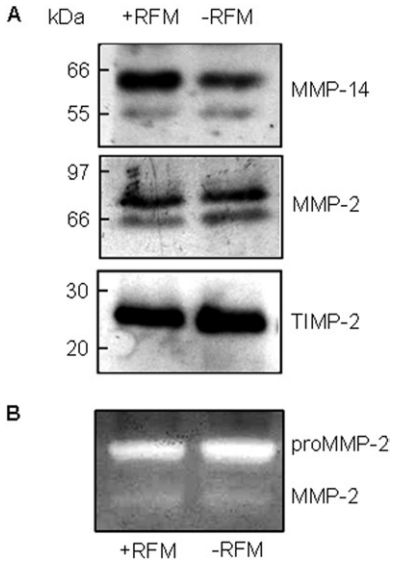

Fig. 4. Western blot and Zymography. (A) Western blot analyses of bovine placental homogenates detecting MMP-14 (64/54 kDa), MMP-2 (72/62 kDa) and TIMP-2 (23 kDa). (B) Gelatin zymography was performed from supernatants of bovine placental homogenates. In all groups the proform of MMP-2 was the predominant form. For representative illustration, samples derived aglepristone group for induced parturition with RFM (+RFM) and from animals after term parturition and release of fetal membranes (-RFM).

apparent alterations we have shown at the transcriptional level together with a specific localization of MMP-14, TIMP-2 and MMP-2 imply that the function of the MMP/TIMP system is altered in RFM following induction of birth or premature caesarean section and thus may be involved in the process of placental retention.

In the present study, all cows with induced parturition or elected caesarean section retained their fetal membranes for more than 24 h. Even though, it is know that treatment with glucocorticoids or $PGF_{2\alpha}$ analogue is associated with a high incidence of RFM [43–45] it is commonly applied in veterinary practice to induce parturition in cattle. One reason for placental retention could be an insufficient placental maturation, which is a prerequisite for a timely release of fetal membranes and can be influenced by several substances [9,20,46]. The induction of parturition using $PGF_{2\alpha}$, glucocorticoids or preterm caesarean section and the known complications concerning the physiological release of fetal membranes [16,47], provides a useful model to investigate the underlying mechanisms of placental retention.

Physiologically, the mRNA expression levels of MMP-2 and TIMP-2 decrease at the end of gestation in the placenta of the closely related goat [32]. For bovine placentomes it has been suggested that the reduction of MMP-2 and TIMP-2 mRNA expression in conjunction with an impaired placental steroid synthesis might be related to RFM [48]. In our study, the mRNA expression of TIMP-2 and MMP-2 was significantly higher in bovine placentomes after certain regimes of induced parturition (+RFM) in comparison to spontaneous parturition (Fig. 1). This implies that the degree of maturity possibly correlates with hormonal changes and MMP/TIMP gene expression in bovine placentomes.

In our study, the mRNA expression profiles of MMP-2 are not directly correlated with the respective enzyme activity. The inactive proform of MMP-2 was predominant in all groups comparing cows with retention of fetal membranes and cows with proper release of fetal membranes. However, the active form of MMP-2 could play a role in the tissue remodelling of bovine placentomes. Similar to our findings, the majority of MMP-2 protein in ovine placental homogenates corresponded to the latent proform at term [31]. In contrast, zymographic analysis of the goat placenta demonstrated that MMP-2 is predominantly present in the active form at the end of gestation [32]. It has also been reported that the latent proform of MMP-2 is predominantly expressed in the maternal part while the active form of MMP-2 is found in the fetal part of the bovine placenta [30]. The discrepancy in the detected amounts of inactive or active MMP-2 is probably due to different species and/or homogenate preparations used in the studies. The tissue preparation itself could cause the release of both forms of MMP-2 as well as their specific inhibitors. In connection with the apparent discrepancy in the activity between the latent proform and the active form of MMP-2, it has been suggested that proMMP-2 is secreted and probably accumulated in the ECM [32,49].

Several components of the ECM are expressed in the bovine placenta throughout gestation [50,51]. Beside fibronectin, collagen type III is the predominant extracellular matrix protein in the fetal compartment of bovine placentomes, whereas collagen types I and IV are expressed in the maternal as well as in the fetal compartment [50,52]. It is reported that MMP-2 is able to degrade collagen types IV, V, VII, X, gelatine, elastin and fibronectin. MMP-14, besides cleaving its substrate proMMP-2, is capable of digesting collagen types I, II, III, IV, laminin and fibronectin (for review see [53,54]). In view of its distribution in the fetal stroma, the uninucleated trophoblast and maternal stroma, the MMP-14 protein may function as an activator for

proMMP-2 as well as a proteolytic active enzyme itself. With ongoing pregnancy the immunoreactivity of MMP-14 and MMP-2 increased in the mesenchyme of the chorionic plate and fetal villi suggesting a function during fetal expansion, growth and tissue remodelling. The fact that uninucleated trophoblast cells (UTC) moderately expressed MMP-14 and MMP-2 at the end of gestation suggests that the fetal compartment functions as the regulatory side for the separation of fetal membranes. In addition, we demonstrated the localization of MMP-14 and MMP-2 in the uninucleated trophoblast of placentomes, which expel the fetal membranes. On the contrary, uninucleated trophoblast of placentomes retaining the fetal membranes showed no or only weak staining for MMP-14 and MMP-2 indicating a minor degree of maturation. It is believed that the co-localization of MMP-14, TIMP-2 and MMP-2 leads to the activation of MMP-2 in the placenta of several species [32,52,55–57]. Our finding that bovine TGC constantly expressed TIMP-2 protein is in accordance with previous studies [35]. Hence, TIMP-2 is a prospective candidate for the regulation of MMP-2 activity in the fetal compartment. For example previous studies demonstrated MMP-2 activity only in the fetal part of bovine placentomes [30]. On the contrary, our results revealed the latent form of MMP-2 is predominant in placentomal homogenates. Similar to these findings other studies have demonstrated that the latent form of MMP-2 is the prevailing form in the maternal and fetal part of the bovine placenta from day 150 to day 250 [34]. However, the balance between inhibitors and MMPs is dynamic and the ECM degrading capacity of cells have been shown to be dependent on the ratio of TIMP to MMP [58]. Furthermore, TIMP-2 can exert inhibitory and activating effects which are regulated in a dose dependent manner [59]. It has also been hypothesized that loss of TGC, which expressed TIMP-2 throughout gestation, is necessary for a regular release of fetal membranes [7,35]. This hypothesis is sustained by the finding that TGC possess glucocorticoid receptors as well as steroidogenic enzymes [60,61]. Furthermore, the maternal compartment should not be underestimated, since almost all cell types express a variety of hormone receptors and could therefore react on preterm hormonal and cellular changes [46,60,62–64].

It can be assumed that both, the activities of proteolytic enzymes and changes at the intercellular interface play a role in the complete release of fetal membranes in cattle. During mouse placentation and human parturition a change in the integrin expression is observed, which influences differentiation, invasion and interaction of trophoblast cells at the cell-ECM level [65,66]. Additionally, it is reported that integrin subunits are involved in the migration of TGC and the anchoring of epithelial cells to the basement membrane by integrins $\alpha_2\beta_1$ (collagen) and $\alpha_6\beta_1$ (laminin) in bovine placentomes [50]. Thus, possible alterations in the integrin expression at the feto-maternal interface can influence the detachment/separation of fetal membranes in the cow.

5. Conclusions

In view of the expression patterns of MMP-14, TIMP-2 and MMP-2 throughout gestation, we can conclude that the fetal compartment can act as the regulatory/effective side for a timely release of fetal membranes. Moreover, the co-localization of MMP-14, TIMP-2 and MMP-2 proteins in the fetal compartment, the fetal mesenchyme and the trophoblast in relation to correlating degrees of maturation probably reflects the functional involvement of these factors during the release/retention of fetal membranes. Further studies have to clarify the role of other MMP related molecules, stimulators, precursors, and inhibitors.

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Disclosure statement

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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5 GENERAL DISCUSSION AND CONCLUSIONS

Based on the results presented and published data we developed our current working concept that growth factors and MMPs produced by TGC and/or uninucleated trophoblast cells participate in the control of restricted trophoblast migration/invasion and the release of fetal membranes in the bovine placenta. A delicate balance of protease activity and inhibition is necessary for the detachment of cells from the basal membrane and surrounding cells prior to migration/invasion, as well as the detachment of the fetal cotyledon from the maternal caruncle to ensure a healthy puerperium.

Several growth factors and MMPs have been studied in connection with cell migration and differentiation *in vitro*. We could demonstrate that EGF is involved in the upregulation of the MMP/TIMP system in bovine trophoblast cells and enhances MMP activity. Furthermore, we have shown that MAPK 42/44 and Akt activation are required for proliferation, migration and motility in cultured bovine trophoblast cells (F3 cells) in response to EGF, suggesting that EGF plays a pivotal role in the differentiation and migration of F3 cells.

EGF is a potent inductor of MMP expression and activation, it upregulates invasion and motility in different cell types by distinct signalling pathways (Kondapaka et al. 1997; Rothhut et al. 2007). We have shown that a significant increase in the abundance of MMP-9 mRNA in response to EGF correlates with an enhanced motility and proliferation of F3 cells. Furthermore, both EGF-mediated migration and MMP-9 activity require the MAPK and PI3K pathway. Hence, the upregulation in MMP-9 expression and activity could be involved in the process of migration in bovine trophoblast cells. While the activation of MMPs by single cytokines as tumor necrosis factor (TNF) alpha in bovine luteal cells, as well as transforming growth factor (TGF) alpha and TGF beta in human and bovine endometrial cells, was reported by several authors (Braundmeier et al. 2006; Hashizume et al. 2003; Zhang et al. 2005), others have demonstrated that a synergistic effect of growth factors is needed to increase MMP gene expression (Tian et al. 2007). We have shown that EGF alone significantly upregulates mRNA levels of MMP-9 and TIMP-1 in bovine trophoblast cells. Nevertheless, it has to be considered that, besides EGF, other growth factors could participate in the control of bovine trophoblast migration and differentiation.

Such an involvement has been shown for TNF-α, vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF), which can activate MMP-9 in human trophoblast cells (Anteby et al. 2004; Cohen et al. 2006). As members of the FGF and VEGF systems are localized in the bovine trophoblast, these factors are also likely to play a role in trophoblast differentiation and migration (Pfarrer 2006). Treatment with EGF consistently led to an increase of the active form of MMP-9 in F3 cells while the inhibition of MAPK or Akt activation blocked this effect. These results confirm that the MAPK and the PI3K/Akt signalling pathways are involved in the secretion and activation of MMP-9 in bovine trophoblast cells. Furthermore, MAPK and PI3K inhibitors abolished the EGF-induced activation of these signalling pathways as well as the induction of motility and proliferation. In a previous study it has been demonstrated that stimulation with EGF leads to the activation of the small GTPase Ras (Hambruch et al. 2010). The involvement of this classical mitogenic Raf/MEK/ERK cascade in the regulation of MMP-9 expression is well documented (Rothhut et al. 2007; Tian et al. 2007). Moreover, EGF has been shown to have a proliferative effect on cultured mouse and human trophoblast cells (Iguchi et al. 1993; Li and Zhuang 1997) and can inhibit apoptosis and mediate differentiation in human cytotrophoblasts (Morrish et al. 1997; Smith et al. 2002). Our experiments examining the growth response of F3 cells to EGF indicate that the activation of both, MAPK and PI3K/Akt pathways is essential for trophoblast proliferation and motility.
In addition to the various biological effects of the EGF system, we confirmed the presence of EGF-R in F3 cells. Therefore, we suggest that EGF produced by TGC and/or uninucleated trophoblast cells stimulates MMP-9 and TIMP-1 secretion and activation (via MAPK and PI3K/Akt) in auto- or paracrine fashion. Thus, proteolytic activity and degradation of ECM might be involved in the control of restricted trophoblast migration/invasion in the bovine placenta and the process of tissue remodelling throughout pregnancy as well in the release of fetal membranes.

In several species it is believed that co-localized MMP-14, TIMP-2 and MMP-2 lead to the activation of MMP-2 during placentation (Bai et al. 2005a; Bai et al. 2005b; Bjorn et al. 1997; Uekita et al. 2004; Wang et al. 2001). We demonstrated that cows with the release of fetal membranes differed in the MMP-14 and MMP-2 expression compared to cows with induced parturition or elected caesarean section, which all subsequently retained their fetal membranes for more than 24 hours. Even though, it

is know that treatment with glucocorticoids or $PGF_{2\alpha}$ analogue is associated with a high incidence of RFM (Claydon 1984; Johnson and Jackson 1982; Rasmussen et al. 1996), this treatment is commonly applied in veterinary practice to induce parturition in cattle. In our *in vivo* study, we assumed that different degrees of placental maturation were represented by different experimental groups and treatments, respectively. Therefore, animals with caesarean section represented the premature placenta, whereas animals with spontaneous parturition and release of fetal membranes were considered to have mature placentae. The placentae derived from induced parturition with glucocorticoids, $PGF_{2\alpha}$, and aglepristone represented an incomplete placental maturation.

The mRNA expression and protein localization of MMP-14, TIMP-2 and MMP-2 as well as the enzymatic activity of MMP-2 was analysed during gestation and in conjunction with placental retention in cattle. We were able to demonstrate that the gene expression of TIMP-2 and MMP-2 was significantly increased in animals with RFM in comparison to animals releasing the fetal membranes. This implies that the degree of maturity correlates with MMP/TIMP gene expression in bovine placentomes. Additionally, MMP-14, TIMP-2 and MMP-2 proteins were located in neighbouring cell populations of the fetal compartment showing spatiotemporal alterations in the course of pregnancy. Furthermore, MMP-14 and MMP-2 protein expression differed between animals with/without retained fetal membranes, while TIMP-2 consistently stained TGC. In animals with RFM, MMP-14 was localized exclusively in the fetal mesenchyme, whereas MMP-2 was expressed in the fetal mesenchyme and uninucleated trophoblast cells. However, in cows with release of fetal membranes, MMP-14 and MMP-2 were co-localized in uninucleated trophoblast cells. The apparent alterations we have shown at the transcriptional level together with a specific localization of MMP-14, TIMP-2 and MMP-2 imply that the function of the MMP/TIMP system is altered in RFM and thus may be involved in the process of placental retention. Moreover, the process of placental maturation could be insufficient in animals with induced parturition (Boos et al. 2003; Grunert et al. 1989; Shenavai et al. 2010). In addition, it has also been hypothesized that the loss of TGC, which expressed TIMP-2 throughout gestation, is necessary for a regular release of fetal membranes (Walter and Boos 2001; Williams et al. 1987).

Thus, we conclude that both, the activities of proteolytic enzymes and changes at the intercellular interface play a role in the complete release of fetal membranes in cattle.

In view of the expression patterns of MMP-14, TIMP-2 and MMP-2 throughout gestation, we can conclude that the fetal compartment can act as the regulatory/effective side for a timely release of fetal membranes. Moreover, the co-localization of MMP-14, TIMP-2 and MMP-2 proteins in the fetal compartment, the fetal mesenchyme and the trophoblast in relation to correlating degrees of maturation reflects the functional involvement of these factors during the release/retention of fetal membranes.

6 SUMMARY

Marc Dilly

Expression of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in bovine placental cells *in vivo* and *in vitro*

The cumulative thesis presented characterizes the expression and functional significance of matrix metalloproteinases (MMPs) and their tissue inhibitors of metalloproteinases (TIMPs) in bovine placental cells *in vitro* and *in vivo* with special reference to trophoblast giant cell (TGC) invasion/migration and placental retention.

The bovine synepitheliochorial placenta is characterized by restricted trophoblast invasion/migration, a unique feature of which the regulatory mechanisms are not completely understood. The activity of MMPs in the extracellular space is specifically inhibited by counteracting TIMPs to serve and control cell migration and tissue remodelling. MMP-9 is present in the bovine placenta throughout gestation; its proteolysis is believed to be predominantly regulated by the action of endogenous TIMP-1. Epidermal growth factor (EGF), as regulator of fundamental cell properties, is expressed in the bovine placenta and capable to up-regulate MMP-9 activity in a variety of cells types. Aim of this *in vitro* study was therefore to examine the influence of EGF on cell motility, proliferation, as well as MMP-9 and TIMP-1 expression in cultured bovine trophoblast cells.

The effect of EGF on MMP-9 and TIMP-1 expression was examined in a trophoblast cell line (F3) by semiquantitative RT-PCR. The proteolytic activity of MMP-9 was determined by zymography. Migration assays were performed using a Boyden chamber and cell motility was measured by time-lapse analyses. To identify the involved signalling cascades, phosphorylation of mitogen-activated protein kinase (MAPK) 42/44 and Akt was detected by Western blot. EGF treatment increased both the abundance of MMP-9 and TIMP-1 mRNAs and the proteolytic activity of MMP-9. Furthermore, EGF stimulated proliferation and migration of F3 cells. Addition of specific inhibitors of MAPK (PD98059) and/or phosphatidylinositol 3-kinases (LY294002) activation abolished or reduced EGF-induced effects in all experiments.

The results of the *in vitro* study suggest that EGF could also be responsible for stimulating migration and proliferation of bovine trophoblast cells *in vivo*, and thus may be involved in bovine placental tissue remodelling and postpartum release of fetal membranes by the upregulation MMP-9 and TIMP-1.

The retention of fetal membranes is one of the most common reproductive diseases in cattle causing considerable economic loss (e.g. reduced milk yield, poorer fertility). To allow a physiological release of fetal membranes and avoid placental retention, the tight feto-maternal connection established by fetal cotyledonary villi interdigitating with maternal caruncles must be separated.

Membrane-type MMPs have been suggested as potential activators controlling extracellular matrix (ECM) degradation and remodelling. In particular, MMP-14 is able to degrade ECM substrates and activate MMP-2 through binding TIMP-2 at the cell surface. We hypothesize that impaired modulation of the ECM by MMPs/TIMPs participates in the aetiology of bovine retained fetal membranes.

This involvement was analysed *in vivo* comparing placentomes from cows at term parturition and timely release of fetal membranes and cows with retained fetal membranes after various treatments for the induction of parturition, and after elective caesarean sections. The expression of MMP-14, MMP-2 and TIMP-2 was examined by real-time-PCR, immunohistochemistry, Western blot and zymography.

The relative mRNA expression levels of MMP-14 was similar in all groups, while the expression levels of MMP-2 and TIMP-2 were higher in most animals with induced parturition and retention of fetal membranes compared to animals without placental retention. In cows with placental retention, MMP-14 protein was expressed in cells of the fetal mesenchyme and maternal stroma, whereas in cows with release of fetal membranes MMP-14 was localized in uninucleated trophoblast cells. MMP-2 could be detected in uninucleated trophoblast cells and the fetal mesenchyme, while TIMP-2 was present exclusively in trophoblast giant cells. The enzyme activity was confirmed by zymography.

The co-localization of MMP-14, MMP-2 and TIMP-2 in the fetal compartment, especially in trophoblast cells at term, allows the modulation of ECM composition at the feto-maternal interface and therefore could influence the timely release of fetal membranes in cattle.

In conclusion, the specific expression of MMPs and TIMPs in bovine trophoblast cells *in vitro* and *in vivo* suggests an involvement of the MMP/TIMP system in TGC migration/invasion and in the aetiology of placental retention. The capability of growth factors, in particular EGF, to induce proteolysis by MMPs and changes in the MMP/TIMP system itself can lead to alterations of the ECM composition and therefore support the release of fetal membranes.

7 ZUSAMMENFASSUNG (GERMAN)

Marc Dilly

Expression von Matrix-Metalloproteinasen und ihren Inhibitoren in bovinen plazentaren Zellen *in vivo* und *in vitro*

Die vorgelegte kumulative Arbeit charakterisiert die Expression und funktionelle Bedeutung von Matrix-Metalloproteinasen (MMPs) und ihren Inhibitoren (TIMPs) in bovinen Plazentazellen unter Berücksichtigung der Invasion/Migration von Trophoblastriesenzellen (TGC) und der Nachgeburtsverhaltung des Rindes.

Die bovine synepitheliochoriale Plazenta ist durch eine eingeschränkte Trophoblasteninvasion/-migration gekennzeichnet, eine Besonderheit deren regulative Mechanismen nicht vollständig geklärt sind. Die Aktivität von MMPs im extrazellulären Raum wird durch entgegenwirkende TIMPs spezifisch inhibiert, um Zellmigration und Gewebeumbau zu unterstützen und kontrollieren. MMP-9 ist während der gesamten Trächtigkeit in der bovinen Plazenta vorhanden; seine Proteolyse wird vorwiegend durch die Aktivität von endogenem TIMP-1 reguliert. Der epidermale Wachstumsfaktor (EGF), als Regulator grundlegender Zelleigenschaften, wird in der bovinen Plazenta exprimiert und kann die Aktivität von MMP-9 in einer Vielzahl von Zellarten hoch regulieren. Ziel dieser *in vitro* Studie war es daher, den Einfluss von EGF auf die Zellmotilität, Zellproliferation sowie die Expression von MMP-9 und TIMP-1 in kultivierten bovinen Trophoblastzellen zu untersuchen.
Der Effekt von EGF auf die Expression von MMP-9 und TIMP-1 wurde mittels semiquantitativer RT-PCR in einer Trophoblastzelllinie (F3) untersucht. Die proteolytische Aktivität von MMP-9 wurde mittels Zymographie bestimmt. Migrationsuntersuchungen wurden in der Boyden Chamber durchgeführt und die Zellmotilität wurde mit Hilfe von „time-lapse“ Messungen bestimmt. Zur Identifizierung der beteiligten Signalkaskaden, wurde die Phosphorylierung der mitogen-activated protein kinase (MAPK) 42/44 und Akt mittels Western Blot detektiert. EGF führte zu einem Anstieg der mRNA Expression von MMP-9 und TIMP-1 sowie zum Anstieg der proteolytischen Aktivität von MMP-9. Weiterhin stimulierte EGF die Proliferation und Migration von F3 Zellen. Die Zugabe von spezifischen Inhibitoren für die Signalwege

MAPK (PD98059) und/oder Phosphoinositid-3-Kinase (LY294002) führte zu einer Reduzierung oder Aufhebung aller durch EGF induzierter Effekte und Aktivierungen in allen Experimenten.
Die Ergebnisse der *in vitro* Studie lassen vermuten, dass EGF auch für die Stimulation der Migration und Proliferation von bovinen Trophoblastenzellen *in vivo* verantwortlich ist, und somit über die Hochregulation von MMP-9 und TIMP-1 am plazentaren Gewebeumbau und dem Ablösen der Nachgeburt postpartum beteiligt sein könnte.

Die Nachgeburtsverhaltung (Retentio secundinarum) ist eine der häufigsten Reproduktionskrankheiten des Rindes, welche bedeutende ökonomische Verluste verursacht (z.B. reduzierte Milchleistung, geringere Fertilität). Um eine physiologische Ablösung der Nachgeburt zu ermöglichen und eine Nachgeburtsverhaltung zu verhindern, muss die enge feto-maternale Verbindung, welche durch die Interdigitation von fetalen kotyledonären Zotten mit maternalen Karunkeln gebildet wird, von einander getrennt werden. Membrane-type MMPs wurden als potentielle Aktivatoren für den Abbau und Umbau der extrazelluläre Matrix (ECM) vorgeschlagen. Insbesondere MMP-14 ist fähig ECM-Substrate abzubauen und MMP-2 mittels Bindung von TIMP-2 an der Zelloberfläche zu aktivieren. Wir nehmen an, dass eine gestörte Modulation der ECM durch MMPs/TIMPs an der Ätiologie der Nachgeburtsverhaltung beim Rind beteiligt ist.
Diese Beteiligung wurde *in vivo* an Plazentomen von Rindern nach fristgerechter Geburt und zeitgerechten Abgang der Nachgeburt, sowie Plazentomen von Rindern nach verschiedenen Geburtseinleitungen und nach Kaiserschnitt mit Nachgeburtsverhaltung analysiert. Die Expression von MMP-14, MMP-2 und TIMP-2 wurde mittels quantitativer real-time PCR, Immunhistochemie, Western Blot und Zymographie untersucht.
Die relative MMP-14 mRNA Expression war in allen Gruppen ähnlich, während die Expression von MMP-2 und TIMP-2 in den meisten Tieren mit Geburtseinleitung und Nachgeburtsverhaltung erhöht waren. Bei Rindern mit Nachgeburtsverhaltung wurde MMP-14 Protein in Zellen des fetalen Mesenchyms und maternalen Stromas exprimiert, wohingegen MMP-14 in Rindern bei denen die Nachgeburt fristgerecht abgegangen war, im uninukleären Trophoblasten detektiert wurde. MMP-2 konnte in uninukleären Trophoblastzellen und im fetalen Mesenchym nachgewiesen werden,

während TIMP-2 ausschließlich in Trophoblastriesenzellen lokalisiert war. Die Enzymaktivität wurde mittels Zymographie bestätigt.
Die Kolokalisation von MMP-14, MMP-2 und TIMP-2 im fetalen Kompartiment, insbesondere den Trophoblastzellen zum Zeitpunkt der Geburt, erlaubt eine Modulierung der ECM Komposition an der feto-maternalen Kontaktfläche und könnte so den fristgerechten Abgang der Nachgeburt beim Rind beeinflussen.

Schlussfolgerung: Die spezifische Expression von MMPs und TIMPs in bovinen Trophoblastzellen *in vitro* und *in vivo* weist auf eine Beteiligung des MMP/TIMP Systems bei der TGC Migration/-Invasion sowie der Ätiologie der Nachgeburtsverhaltung des Rindes hin. Die Fähigkeit von Wachstumsfaktoren, insbesondere EGF, Proteolysen durch MMPs und Veränderungen im MMP/TIMP System selbst zu induzieren, kann zu Veränderungen der ECM Komposition führen und somit eine Ablösung der Nachgeburt unterstützen.

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