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Gene expression profiling of bovine peripartal placentomes: detection of molecular pathways potentially involved in the release of foetal membranes

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Abstract

The mechanisms underlying detachment of foetal membranes after birth in cows are still unclear. To address this problem in a systematic manner, we performed the first holistic transcriptome study of bovine placentomes antepartum (AP; $n=4$ cows) and intrapartum (IP; $n=4$ cows) using Affymetrix GeneChip Bovine Genome Arrays. Three placentomes were extracted from each cow, and tissue samples from the contact zones of the placentomes (foeto-maternal units) were recovered by systematic random sampling and processed for RNA extraction and for stereological quantification of cellular composition. Statistical analysis of microarray data (false discovery rate 1%) revealed 759 mRNAs with at least twofold higher levels in the samples of the AP group, whereas 514 mRNAs showed higher levels in the IP group. The differentially expressed genes were classified according to biological processes and molecular functions using the Functional Annotation Clustering tool of the DAVID Bioinformatics Resources. Genes with higher mRNA levels in the AP group were nearly completely related to mitotic cell cycle and tissue differentiation. During parturition, a complete shift occurred because the genes with higher mRNA levels in IP were nearly all related to three different physiological processes/complexes: i) apoptosis, ii) degradation of extra cellular matrix and iii) innate immune response, which play a fundamental role in placental detachment. These results are an excellent basis for future studies investigating the molecular basis of retained foetal membranes.

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Introduction

Anatomically, the bovine placenta is characterised as synepitheliochorial placenta (Wooding *et al*. 1997). It consists of 70–150 functional foeto-maternal units (Björkman & Sollen 1960), the placentomes. Each placentome is composed of a maternal caruncle and a foetal cotyledon. With ongoing pregnancy, the foetal villi invade intimately and extensively within the deeper branching caruncular crypts (reviewed in McNaughton & Murray (2009)).

Towards the end of pregnancy, placental maturation is a prerequisite for the subpartal detachment and release of foetal membranes. At the cellular level, the placental maturation is characterised by typical changes of the maternal crypt epithelium, which decreases in height and number of cells. Additionally, contacts with

foetal trophoblastic cells increase as well with the underlying maternal connective tissue (Boos *et al*. 2003). Areas denuded of epithelial cells also appear within the maternal epithelial lining of the crypts. Placental maturation results in a reduction of the distance between the foetal and maternal blood supplies to meet the growing nutritional demands of the foetus in the last months of pregnancy (Björkman 1954, Björkman & Sollen 1960, Woicke *et al*. 1986, Williams *et al*. 1987, Schoon 1989, Stallmach *et al*. 2001).

The initiation of parturition occurs as a result of activation of the foetal hypothalamus–pituitary–adrenal (HPA) axis. The reasons for its activation are still not elucidated, but different theories are discussed: maturation of the foetal hypothalamus; response of the foetal hypothalamus to placental hormones; and foetal

stressors due to hypoxia, hypocapnia, changes in blood pressure or blood glucose (reviewed in Wood (1999)). By activation of the HPA, the ACTH is released by the pituitary. In parallel, the binding capacity of the plasma for cortisol increases, which reduces the free cortisol and this reduces the negative feedback effect of ACTH to the pituitary. Additionally, the response of the foetal adrenal glands increases with foetal age (Glickman & Challis 1980). The increase in foetal cortisol concentration induces the endometrial prostaglandin synthesis by two different pathways (Whittle *et al.* 2000): oestrogen dependent as well as oestrogen independent. In the oestrogen-independent pathway, cortisol enhances the expression of prostaglandin synthase (PTGS2) within the foetal trophoblast cells of the placenta. Therefore, prostaglandin E_2 (PGE_2) is released in the placenta, which stimulates the conversion of pregnenolone to C19 steroids, which are aromatised by 17α-hydroxylase and aromatase to oestrogens. This increase in oestrogen levels leads to the activation of PTGS2 in the maternal endometrium, which increases the production and release of $PGF_{2\alpha}$. The effects of $PGF_{2\alpha}$ are different. In the myometrium, it lowers the threshold of the oxytocin receptor to respond to oxytocin and increases by this the myometrial contractions indirectly, but has also a direct effect on the myometrium (reviewed by Taverne & Noakes (2009)). Another effect of the increased $PGF_{2\alpha}$ production, which has been shown in cows and goats, is the induction of regression of the corpus luteum. Therefore, prostaglandins are main regulators of the initiation of parturition and are essential for initiating smooth muscle contractions.

Oestrogen has also a direct effect on the myometrium by increasing contraction-associated proteins (Olson 2003, Mendelson 2009), like connexins, which form gap junctions to coordinate myometrial contractions. Another essential effect of oestrogens is the softening of the birth canal, namely the cervix, the vagina and the associated tissues, by altering the structure of collagen fibres (reviewed by Taverne & Noakes (2009)).

The separation of the placental components involves a wide range of different processes. An increase in the proportion of collagen in the cotyledons throughout gestation in contrast to a decreased proportion in the maternal part was demonstrated (Sharpe *et al.* 1989). During labour, contractions of the myometrium result in pressure changes in the cotyledonary villi leading to alternating hyperaemic and ischaemic conditions and finally the physical separation of the foeto-maternal unit (reviewed in McNaughton & Murray (2009)). Furthermore, the cotyledonary villi collapse when the umbilical cord ruptures in parturition (Joosten & Hensen 1992), resulting in a reduced surface area. At the cellular level, this process is characterised by a number of morphological changes in the maternal crypt epithelium. Shortly before parturition, the number of epithelial cells increases in contrast to the number of giant trophoblast cells (GC)

that is reduced (Boos *et al.* 2003). It has also been described that the expression of major histocompatibility complex class II (MHC-II) in a subset of binucleate cells (BNC) in the near term placentomes is beneficial for the maturation process. These MHC-II antigens of the foetus are presented to the dam, which modulates the maternal immune system required for the placental separation (reviewed in McNaughton & Murray (2009)). The roles of single extracellular matrix-degrading enzymes, namely matrix metallopeptidases 2 (MMP2) and 9 (MMP9), as well as of the tissue inhibitor of metallopeptidase 2 (TIMP2) in placental maturation have been studied during parturition. It has been shown by immunohistological analysis as well as gene expression analysis that these genes are associated with the process of expulsion of the foetal membranes (Walter & Boos 2001, Boos *et al.* 2003, Takagi *et al.* 2007). Further, an increase in the number of apoptotic cells in maternal crypt epithelium, maternal stroma and foetal chorionic epithelium during pregnancy has been observed, suggesting an important role of regulated cell death in normal expulsion of the foetal membranes (Boos *et al.* 2003).

Retained foetal membranes (RFM) in cattle is defined as the failure to expel foetal membranes within a physiological time period of 8–12 h *post partum* (Sheldon 2004). RFM can lead to life threatening conditions in mammals including women. For dairy cows, there is an average incidence of 3–12% in industrialised countries (Esslemont & Kossaibati 1996, Sheldon 2004) also demonstrating the economic importance of this complication.

So far, many studies of single genes at the mRNA or protein level were performed that analysed only single aspects of this complex process. To obtain a more holistic view, a transcriptome analysis of well-defined tissue samples before and intraparturition was performed to identify changes in mRNA concentrations that indicate molecular pathways involved in the regulation of detachment of the foetal membranes. This improved understanding can help to facilitate the development of new strategies in the prevention and therapy of RFM.

Results

Morphometrical analysis

To ensure the comparability of the tissue samples with regard to cellular composition, the volume fraction of the epithelial cells of each sample was determined by morphometrical analysis. Whereas the variation in the volume fraction of epithelial cells between individual tissue samples was relatively high (Vv 0.02–0.47 epithelial cells), the variation between animals (mean volume fraction of epithelial cells of all nine tissue samples per animal Vv 0.15–0.35) was clearly reduced due to the pooling of samples per animal. There was no significant difference between the experimental groups ($P > 0.5$; Fig. 1).
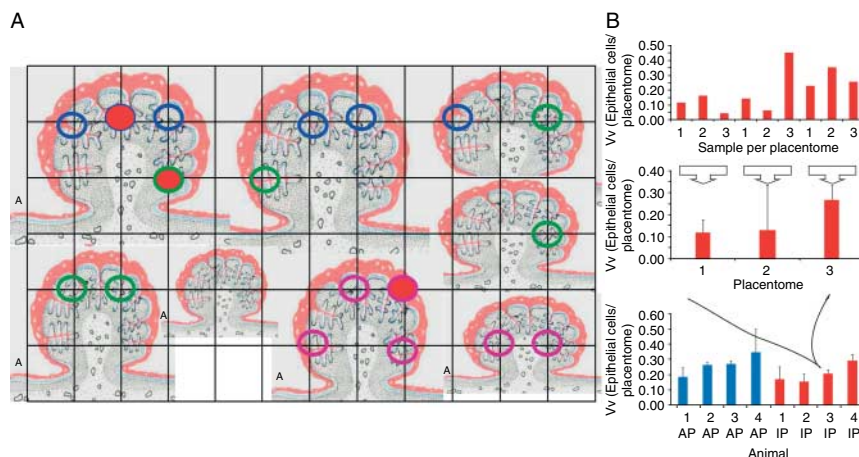
Figure 1 (A) Scheme of systematic random sampling. Cross sections of a placentome covered by a grid (1×1 cm) are shown; grey: epithelial tissue of the caruncle; light grey: caruncle stroma; red: epithelial tissue of the cotyledon. Ovals indicate the crosses of the grid lying on the zone of foeto-maternal contact. Filled ovals indicate the locations of tissue sampling (adapted, with permission, from Fig. 8.9 in **Rüsse I & Sinowatz F** 1991 *Lehrbuch der Embryologie der Haustiere*. Berlin: Paul Parey Verlag). (B) Mean volume density (Vv$_{\text{(Epithelial cells/Placentome)}}$) of the epithelial cells. The s.d. is marked by a bar. In the upper diagram, three placentomes of one animal (three IP) were shown. Three placentomes were sampled per animal. In the middle diagram, the mean volume density of each placentome per animal was shown. In the lowest diagram, the mean volume density of epithelial cells is shown for all the eight animals.

Microarray analysis

To identify peripartal transcriptome changes in the foeto-maternal unit, samples recovered 12 days before the estimated day of calving (antepartum (AP), $n=4$) and samples taken immediately after delivery of the calf (intrapartum (IP), $n=4$) were analysed using Affymetrix GenChip Bovine Genome Arrays. In total, eight Affymetrix microarrays were hybridised and evaluated. A heatmap of the pair-wise distances of the single data sets showed high correlation between the animals of the individual experimental groups (data not shown). Statistical analysis (SAM, two-class unpaired (Tusher *et al.* 2001)) revealed 1650 probe sets with significant differences (false discovery rate 1%, ≥twofold) in their hybridisation signals between the placentomes of the AP group vs the IP group. These probe sets corresponded

to 1226 different genes, 743 with higher transcript levels AP and 483 with higher levels IP (Supplementary Table 2, see section on supplementary data given at the end of this article). Twelve selected genes were verified by quantitative real-time RT-PCR (Table 1). The corresponding primer sets are shown in Supplementary Table 1, see section on supplementary data given at the end of this article.

The differentially expressed genes were analysed with the Functional Annotation Clustering tool of the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources. The result of this integrated analysis of different functional databases (e.g. GO, KEGG pathways, SP-PIR keywords, etc.) are clusters of quantitatively enriched functional terms with overlapping sets of genes with a significant cluster enrichment score (geometric mean in log scale

Table 1 Results of quantitative real-time RT-PCR.

	Mean C_T		s.d.		qPCR		Array	
Gene	IP	AP	IP	AP	FC IP/AP	*P* value	FC IP/AP	*P* value
B2M	19.1	19.9	0.4	0.7	1.9	0.037	1.3	0.004
CD14 [a]	28.5	30.7	0.8	1.6	5.8	0.014	2.8	<0.001
CXCL2	25.5	27.8	0.5	0.7	4.4	0.001	4.5	<0.001
FGF2	31.6	33.6	1.0	0.8	3.3	0.021	2.3	<0.001
GPX3	22.1	23.3	0.5	0.7	2.6	0.002	2.4	<0.001
HPGD [a]	26.3	27.3	1.2	0.5	2.4	0.051	2.4	0.005
MT2A	23.9	25.7	1.0	0.6	3.8	0.005	2.4	<0.001
PTX3	31.9	34.0	0.6	1.2	3.4	0.021	2.7	<0.001
SDS [a]	27.4	28.9	0.9	0.5	3.5	0.010	3.1	<0.001
KLF5	26.3	25.0	0.6	0.8	−2.9	0.014	−2.1	<0.001
LBP	34.6	33.0	1.1	0.9	−3.7	0.036	−2.1	<0.001
TRIB3	24.3	21.4	0.8	0.4	−8.6	0.001	−5.1	<0.001

FC, fold change.
[a]Roche master mix, all others with ABI master mix.

Table 2 Results of functional annotation clustering of genes up-regulated 12 days antepartum.

Functional annotation cluster description[a]	Enrichm. score[b]	Genes[c]
Cell cycle (63, 4.2), mitotic cell cycle (54, 4.6), cell division (45, 6.2), M phase of mitotic cell cycle (45, 5.3), mitosis (38, 8.2)	19.15	91
Cell cycle (63, 4.2), regulation of cell cycle (41, 2.1)	12.72	90
Chromosome (35, 2.4), chromosome pericentric region (19, 6.9), centromere (14, 11.4)	6.65	35
Chromosome organisation and biogenesis (24, 1.7), chromosome segregation (14, 6.0), mitotic sister chromatic segregation (10, 8.8)	4.98	25
Microtubule cytoskeleton (37, 2.4), cytoskeleton (71, 1.6), intracellular non-membrane-bound organelle (107, 1.4)	4.48	107
Cytoskeleton organisation and biogenesis (39, 2.0), microtubule cytoskeleton organisation and biogenesis (12, 3.9), spindle organisation and biogenesis (7, 9.5)	3.75	37
Substrate-specific transmembrane transporter (43, 1.3), symporter activity (19, 3.3), solute: sodium symporter activity (11, 6.0), carboxylic acid transmembrane activity (9, 3.4), organic acid: sodium symporter activity (7, 7.9)	2.88	46
Enzyme regulator activity (48, 1.6), regulation of signal transduction (38, 1.8), GTPase regulator activity (29, 2.0), small GTPase regulator activity (22, 2.4), Ras protein signal transduction (20, 2.1), Rho protein signal transduction (12, 2.8), Ras guanyl nucleotide exchange factor activity (11, 3.4), Rho guanyl-nucleotide exchange factor activity (10, 3.6)	2.40	82
Cytoskeletal part (49, 1.8), microtubule (20, 2.3), microtubule-based process (19, 2.3), cytoskeleton-dependent intracellular transport (10, 2.1)	2.01	54
Purine nucleotide binding (89, 1.3), nucleotide binding (75, 1.4), ATP-binding (63, 1.5), nucleotide phosphatase-binding region: ATP (53, 1.8), kinase activity (53, 1.5), protein amino acid phosphorylation (36, 1.4)	1.97	190
Cellular lipid metabolic process (33, 1.4), steroid metabolic process (15, 2.2), lipid synthesis (9, 2.9), steroid biosynthesis (7, 5.4)	1.87	39
Development process (152, 1.3), cell differentiation (87, 1.3), cell development (65, 1.4), apoptosis (43, 1.5), programmed cell death (43, 1.5), regulation of apoptosis (27, 1.4), negative regulation of apoptosis (17, 2.0)	1.83	152
Endosome (18, 2.4); endosome membrane (8, 2.4)	1.81	18
Oxidoreductase (33, 1.6), iron ion binding (18, 1.6)	1.58	45
Calmodulin binding (10, 2.1), IQ calmodulin-binding region (8, 2.7)	1.39	11
Cytoskeleton protein binding (26, 1.7), actin cytoskeleton (18, 1.8), actin binding (16, 1.5)	1.35	33

[a]Based on the most meaningful terms. [b]Geometric mean (in $-\log_{10}$ scale) of member's P values of the corresponding annotation cluster. [c]Total number of different genes in a functional annotation cluster; in brackets: number of genes and fold enrichment of the functional term.

of member's Fisher Exact P values in a corresponding annotation cluster). Before parturition, most significant clusters were related to cell cycle and cell division, transmembrane transporters and regulation of signal transduction (Table 1). In parturition, the highest enrichment scores were obtained for clusters related to immune response, extracellular proteins, regulation of apoptosis and different categories involved in tissue remodelling (Table 2). The results of the text mining of Medline abstracts (CoPub analysis) overall confirmed the results of the DAVID analysis. For the genes with higher mRNA levels in the AP placentome samples, almost all overrepresented keywords were related to cell cycle (46 genes) and the different phases of mitosis, including DNA replication (24 genes) and its regulation (six genes; Supplementary Table 3, see section on supplementary data given at the end of this article). The analysis of the IP up-regulated genes showed overrepresented keywords related to innate immune response (20 genes), membrane degradation (11 genes), inhibition of matrix metallopeptidases (MMPs; five genes) and angiogenesis (13 genes; Supplementary Table 4, see section on supplementary data given at the end of this article).

Using Pathway Architect software, interaction networks were built for genes up-regulated during parturition. DAVID clusters with similar functional terms were further integrated resulting in three large functional groups: immune response (clusters 1, 2, 5, 10,

11, 14, 22, 25), combination of different clusters related to tissue remodelling (clusters 4, 6, 8, 9, 13, 17, 27) and apoptosis (cluster 7). Each of these functional groups was analysed by CoPub and the overrepresented genes were used in the interaction networks. Networks were drawn based on different types of interactions like binding, regulation, expression, transport, assignment to a protein family or a biological process. Proteins were assigned to their primary cellular localisation. Figure 2 shows the interaction network for the genes involved in immune response (31 genes assigned). The network of genes involved in apoptosis (15 genes assigned) is shown in Fig. 3. Figure 4 shows the network of genes related to tissue remodelling (37 genes assigned; Tables 3–6).

Localisation of selected proteins

To examine the expression pattern and the localisation of PTGS2, MMP14, TIMP1 nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA), BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3) and X-linked inhibitor of apoptosis (XIAP), we have carried out immunohistochemical investigations using paraffin sections from formalin-fixed bovine placentomes, which were taken AP or during birth (IP; Fig. 5 and Table 7).

Staining intensity for the antibody against PTGS2 (alias COX2) was moderate to distinct positive in the
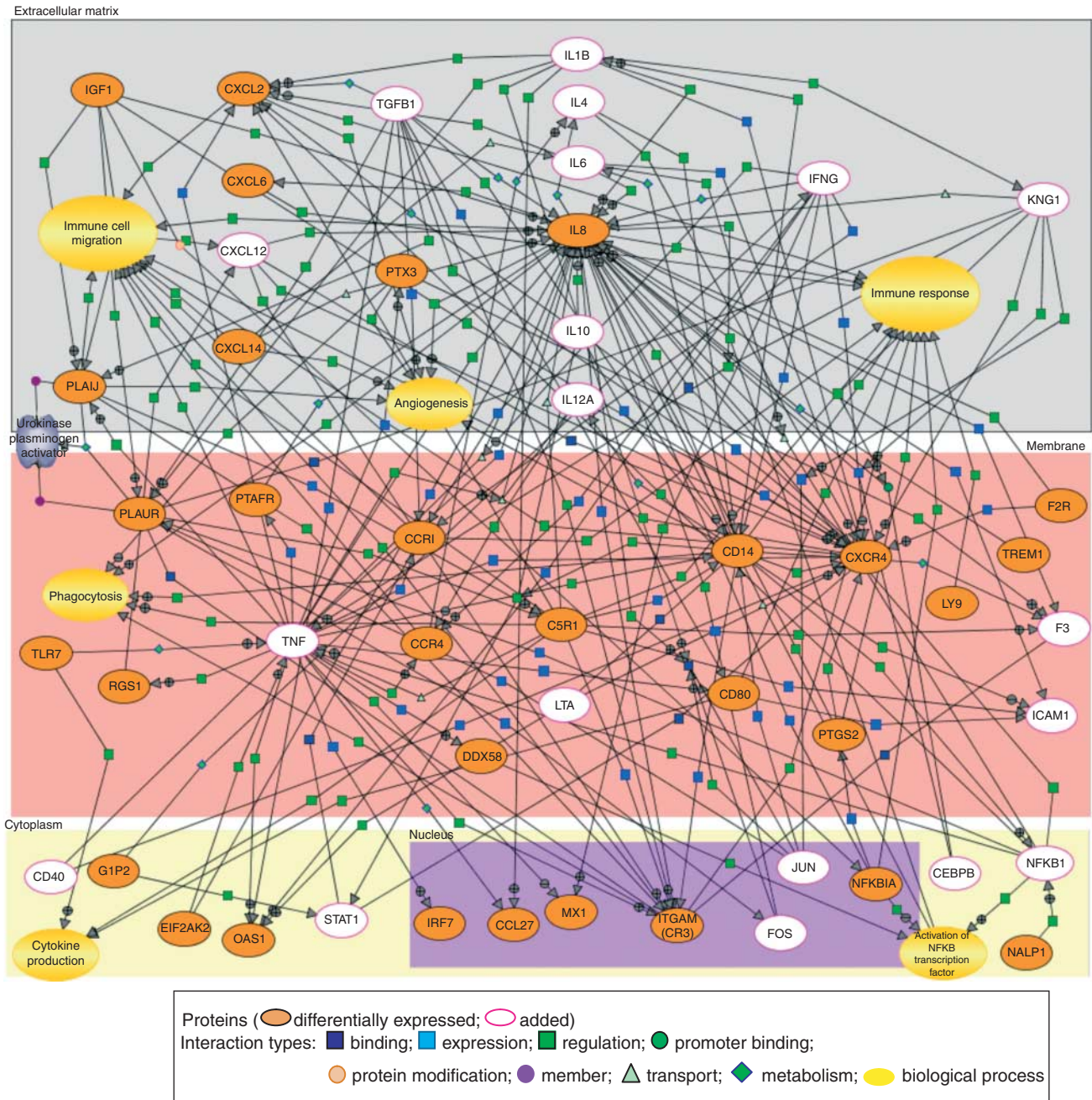
Figure 2 Interaction network of genes with higher mRNA levels in intrapartum related to immune response processes. Proteins (orange if differentially expressed and white with a pink outline if added) are assigned to their primary cellular localisations. Interaction types: dark blue squares: binding; light blue squares: expression; green squares: regulation; green circles: promoter binding; orange circles: protein modification; purple circles: member; green triangle: transport; cyan diamond: metabolism; yellow ovals: biological process.

uninucleated trophoblast cells (UNC) during the AP period, whereas in samples from IP placentomes the cytoplasmic expression of PTGS2 was increased and could be estimated as strong. No distinct immunostaining for PTGS2 could be observed in GC at both time points. The caruncular epithelial cells covering the basal plate and the septa displayed a weak to moderate cytoplasmic expression for PTGS2. No PTGS2 staining was found in stromal cells of the uterus AP and IP. IP,

a few PTGS2-positive mesenchymal cells, could be detected in the foetal stroma.

In IP samples, MMP14 was mostly expressed in the UNC and GC. The immunostaining varied from distinct to strongly positive. In the epithelium and the stroma of the caruncular epithelium, a weak to moderate immunostaining for MMP14 could be detected. The mesenchymal cells of the chorionic plate and the primary villi showed a weak positive reaction with the antibody
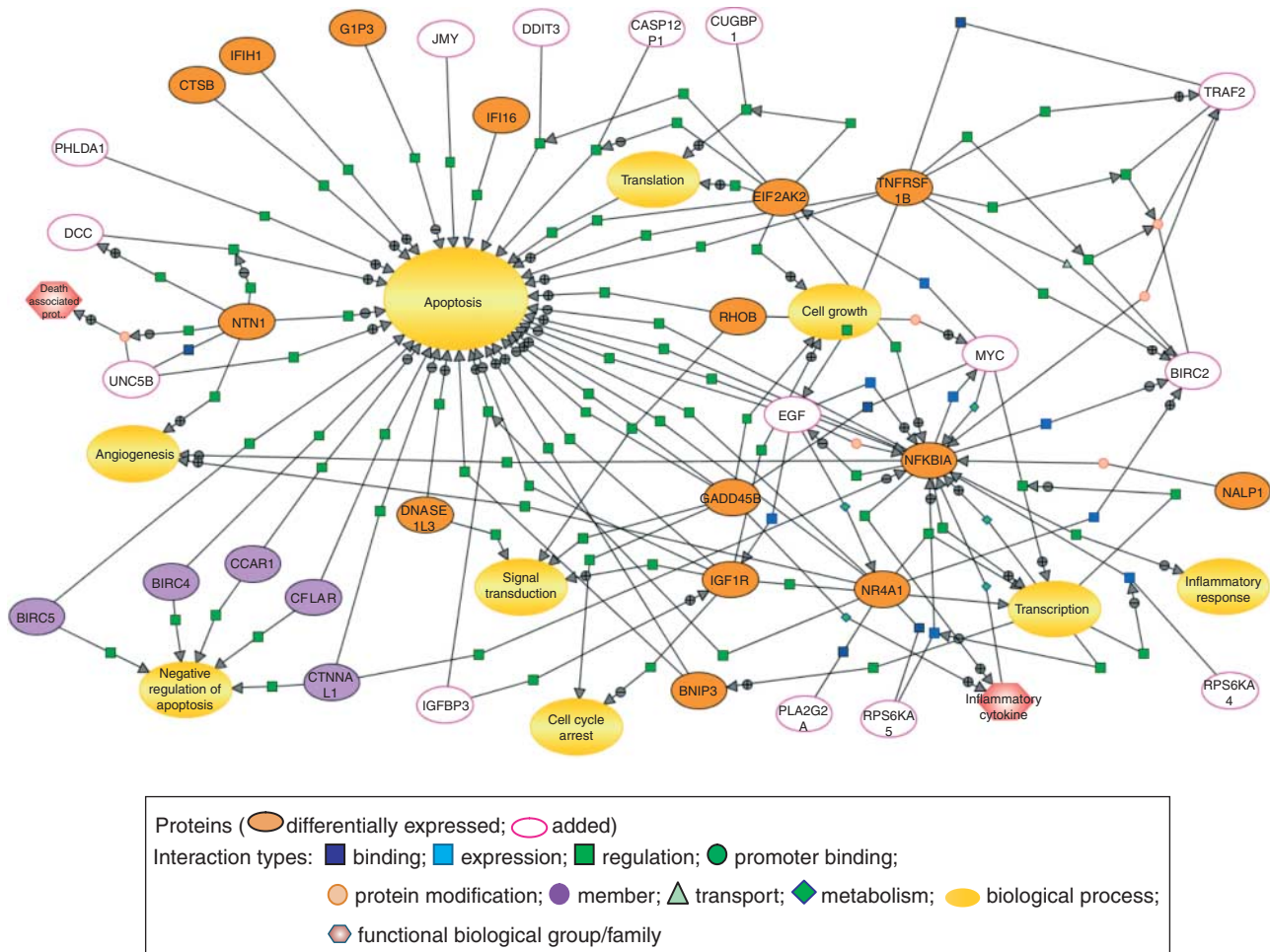
Figure 3 Interaction network of genes with highest mRNA levels intrapartum according to DAVID clusters associated with cell death. Proteins (orange if differentially expressed and white with a pink line, if added) are assigned to their primary cellular localisations. Interaction types: dark blue squares: binding; light blue squares: expression; green squares: regulation; green circles: promoter binding; orange circles: protein modification; purple circles: member; green triangle: transport; cyan diamond: metabolism; yellow ovals: biological process; red hexagons: functional biological group/family.

against MMP14. In AP samples, no difference for the staining intensity for MMP14 in the caruncular epithelium was observed. UNC expressed MMP14 moderately, whereas the GC generally showed no or only weak expression of MMP14. The endotheliums of the foetal or maternal blood vessels were usually distinct positive for MMP14. The foetal stroma cells of all villi displayed a weak positive reaction for MMP14. In contrast, the maternal stromal cells were negative.

TIMP1 immunostaining in the UNC appeared weak to moderate positive in AP samples, whereas the GC expressed TIMP1 with a moderate to distinct intensity. In IP, the staining intensity increased and could be considered as distinct to strong positive in both cell types. In the foetal stroma, the staining intensity was stronger in AP. The caruncular epithelial cells showed a moderate staining in IP, but somewhat attenuated immunostaining for TIMP1 was seen in the specimen obtained from AP. The maternal stromal cells showed

a negative to weak expression before birth, which further increased to moderate expression in the IP samples.

NFKBIA was localised in the UNC and GC in both AP and IP bovine placentomes, but the staining intensity of the UNC appeared more distinct in IP samples. The maternal and foetal stroma showed a weak or moderate staining at both time points. The expression in the caruncular epithelium was weak to distinct positive and no differences between the AP and IP samples could be detected. Interestingly, during the antepartal phase, the endothelium of the blood vessels was immuno-positive for NFKBIA, but appeared negative during IP.

BNIP3 was expressed only weakly in some scattered UNC and GC in IP samples. No immunostaining was observed in UNC and GC in AP samples. Caruncular epithelium and foetal stroma showed no BNIP3 immunostaining at both stages, whereas the staining intensity appeared as increased in uterine stromal cells of samples taken during birth.
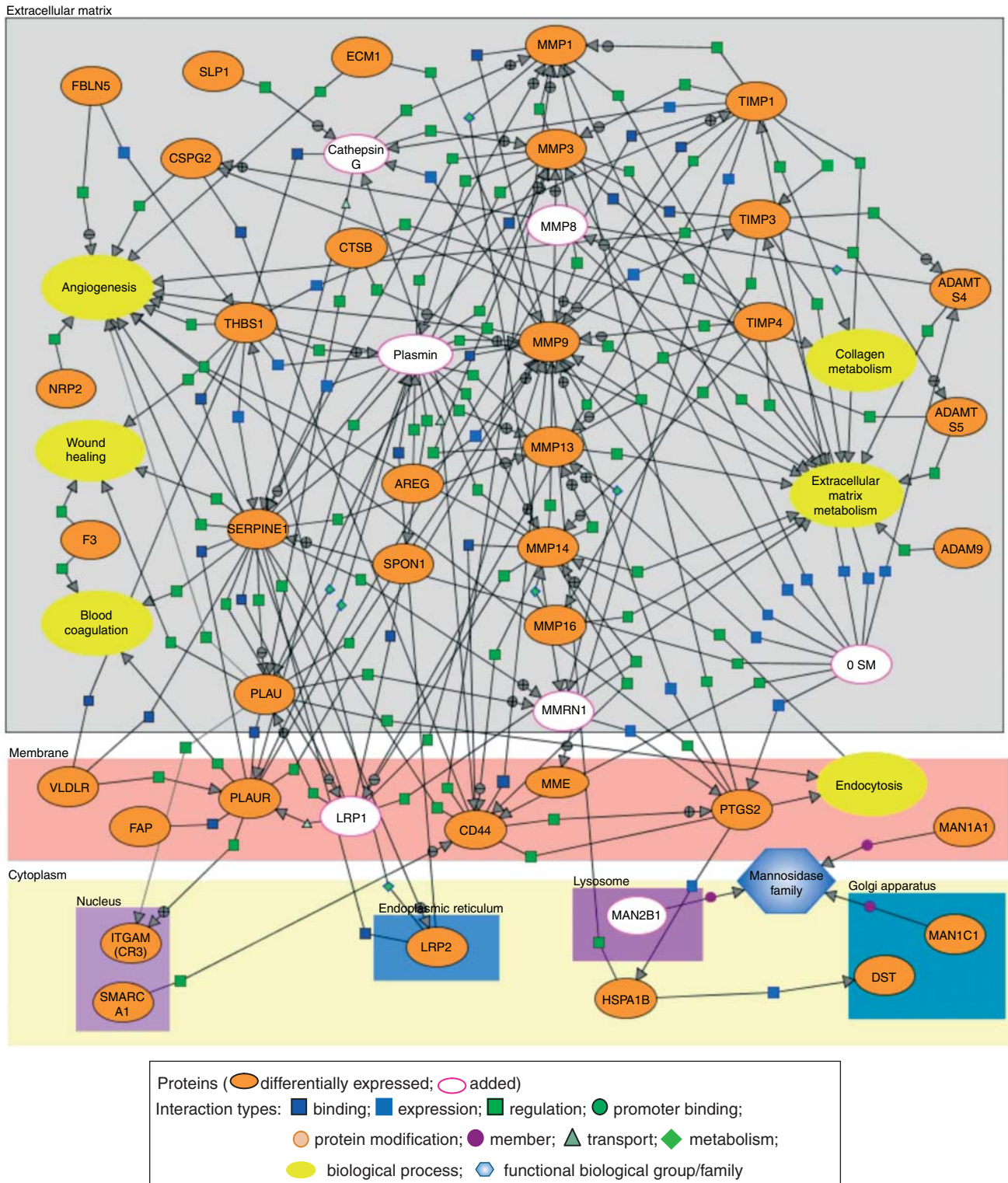
Figure 4 Interaction network of genes with higher mRNA levels in intrapartum related to extracellular matrix remodelling. Proteins (orange if differentially expressed and white with a pink outline if added) are assigned to their primary cellular localisations. Interaction types: dark blue squares: binding; light blue squares: expression; light green squares: regulation; green circles: promoter binding; orange circles: protein modification; purple circles: member; green triangle: transport; cyan diamond: metabolism; yellow ovals: biological process; blue hexagons: functional biological group.

Table 3 Results of functional annotation clustering of genes up-regulated intrapartum.

Functional annotation cluster description[a]	Enrichm. score[b]	Genes[c]
Response to stress (64, 2.2), response to external stimulus (54, 3.1), defence response (43, 2.7), response to wounding (40, 3.4), response inflammatory (28, 3.4)	9.15	86
Response to stimulus (135, 1.6), immune system process (75, 2.3), immune response (60, 2.3), defence response (43, 2.7)	9.13	144
Membrane (172, 1.3), glycoprotein (170, 1.7), signal (157, 2.1), transmembrane (144, 1.3), signal peptide (138, 1.7), secreted (91, 2.5), extracellular region (79, 2.2)	8.35	267
Extracellular region (79, 2.2), extracellular matrix (28, 3.0), proteinaceous extracellular matrix (27, 2.9)	7.68	80
Multi-organism process (28, 3.2), response to biotic stimulus (25, 2.9), response to virus (15, 5.5)	5.34	35
Development process (134, 1.5), cell differentiation (85, 1.7), cellular development process (85, 1.7), cell death (47, 2.1), apoptosis (44, 2.0), regulation of programmed cell death (31, 2.1), positive regulation of apoptosis (19, 2.7)	4.09	146
Organ morphogenesis (25, 2.2), vascular development (18, 3.4), anatomical structure formation (17, 3.3), angiogenesis (16, 4.1)	4.00	34
Hydrolase activity (88, 1.4), protease (29, 2.4), zymogene (18, 3.6), metalloendopeptidase activity (14, 4.6)	3.96	95
Response to chemical stimulus (46, 2.8), behaviour (25, 2.8), cytokine–cytokine receptor interaction (19, 1.7), chemotaxis (17, 4.2), G-protein-coupled receptor binding (10, 4.9), chemokine activity (7, 5.3)	3.66	63
Carbohydrate binding (22, 2.6), pattern binding (13, 4.2), polysaccharide binding (12, 4.2), glycosaminoglycan binding (11, 4.0)	3.31	23
Carboxylic acid metabolic process (35, 2.2), organic acid metabolic process (35, 2.2), cellular lipid metabolic process (35, 2.0), monocarboxylic acid metabolic process (22, 3.3), amine metabolic process (21, 1.7), fatty acid metabolic process (17, 3.5)	3.26	63
Enzyme inhibitor activity (17, 2.3), endopeptidase inhibitor activity (14, 3.2), protease inhibitor activity (14, 3.2), serine-type endopeptidase inhibitor activity (9, 3.1)	2.63	34
Regulation of development process (16, 2.2), hemopoietic or lymphoid development (15, 2.7), immune system development (15, 2.5), regulation of cell differentiation (13, 3.0), myeloid cell differentiation (11, 4.4)	2.62	23
EGF-like region (22, 2.7), EGF-like, type 3 (16, 2.9), EGF-like calcium binding (9, 3.3), aspartic acid and asparagines hydroxylation site (9, 3.2)	2.44	42
Regulation of body fluid levels (12, 3.6), wound healing (10, 2.8), blood coagulation (9, 3.3), haemostasis (9, 3.1)	2.36	13
Membrane organisation and biogenesis (18, 2.2), membrane invagination (15, 2.8), endocytosis (15, 2.8)	2.30	25
Cell communication (144, 1.3), signal transduction (131, 1.3), intracellular signalling cascade (59, 1.4), cell surface receptor-linked signal transduction (59, 1.1)	2.30	144
Membrane fraction (37, 1.9), endoplasmic reticulum (20, 1.4), vesicular fraction (13, 2.7), microsome (12, 2.6)	2.21	46
Immune effector process (11, 3.5), adaptive immune response (8, 3.6), lymphocyte-mediated immunity (7, 3.4)	2.16	12
Reproduction (21, 1.4), reproductive process (18, 2.1), reproductive process in a multicellular organism (8, 4.0)	2.05	21
Signal transducer activity (87, 1.3), receptor activity (64, 1.2), transmembrane protein (39, 2.4)	2.05	134
Leukocyte activation (13, 2.3), lymphocyte activation (12, 2.4), T-cell activation (8, 2.5)	1.74	14
Chemical homeostasis (17, 2.3), cellular ion homeostasis (13, 2.2), metal ion homeostasis (9, 2.5), cytosolic Ca ion homeostasis (7, 3.7)	1.66	21
Cellular structure morphogenesis (23, 1.7), cell morphogenesis (23, 1.7), cell growth (11, 2.1), regulation of cell growth (9, 2.1)	1.46	26
Regulation of molecular function (25, 1.6), regulation of catalytic activity (23, 1.7), regulation of protein kinase activity (13, 2.1), positive regulation of protein catalytic activity (13, 2.0)	1.39	33

[a]Based on the most meaningful terms. [b]Geometric mean (in $-\log_{10}$ scale) of member's *P* values of the corresponding annotation cluster. [c]Total number of different genes in a functional annotation cluster; in brackets: number of genes and fold enrichment of the functional term.

UNC and GC displayed a distinct to strong immunostaining for XIAP in IP samples, which appeared weaker in AP samples. At both time points, the expression for XIAP was weak in the caruncular epithelium and moderate positive in foetal stromal cells. Maternal stromal cells were always negative for XIAP.

There were no staining reactions in all used negative controls (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Discussion

The detailed molecular correlates of the process of release of foetal membranes and the early steps of uterine involution are unknown. Therefore, we performed the first holistic transcriptome analysis of foeto-maternal

units recovered from a defined animal model AP and IP. This study facilitated a global detection of differential gene expression and the identification of potentially involved molecular pathways.

Animal model and tissue sampling

In this study, bovine placentome samples collected before the expected date of delivery (group AP) and samples taken from IP-extracted placentomes (group IP) were compared. Samples of group AP were collected 12–15 days before estimated term after undisturbed gestation with an active Cl ($P_4 > 2$ ng/ml) because a decrease in P_4 induces a significant shift in steroid secretion (Takagi *et al.* 2002) and decreases epithelial proliferation significantly. The AP samples were

Table 4 Genes of the interaction network 'immune response' (Fig. 2) that were differentially expressed.

Entrez gene ID	Human gene symbol	Human Entrez gene description	Gene ID	Entrez gene (bovine)	Entrez gene description	FC (IP/AP)	P value (%)	Score (d)
10850	CCL27	Chemokine (C-C motif) ligand 27	508933	CCL27	Chemokine (C-C motif) ligand 27	4.31	0.42	1.96
1230	CCR1	Chemokine (C-C motif) receptor 1	407771	CCR1	Chemokine (C-C motif) receptor 1	2.23	0.00	4.91
1233	CCR4	Chemokine (C-C motif) receptor 4	408019	CCR4	Chemokine (C-C motif) receptor 4	2.07	0.10	2.91
929	CD14	CD14 molecule	281048	CD14	CD14 molecule	2.85	0.00	5.04
941	CD80	CD80 molecule	407131	CD80	CD80 molecule	2.25	0.00	8.66
9547	CXCL14	Chemokine (C-X-C motif) ligand 14	511771	CXCL14	Chemokine (C-X-C motif) ligand 14	2.12	0.22	2.48
2920	CXCL2	Chemokine (C-X-C motif) ligand 2	613667	CXCL2	Chemokine (C-X-C motif) ligand 2	4.55	0.00	5.20
6372	CXCL6	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	281735	CXCL5	Chemokine (C-X-C motif) ligand 5	5.60	0.00	4.74
7852	CXCR4	Chemokine (C-X-C motif) receptor 4	281736	CXCR4	Chemokine (C-X-C motif) receptor 4	2.53	0.12	2.82
23586	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	504760	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	2.85	0.03	3.63
5610	EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2	347700	EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2	2.04	0.12	2.71
2149	F2R	Coagulation factor II (thrombin) receptor	526585	F2R	Coagulation factor II (thrombin) receptor	2.23	0.12	2.83
3479	IGF1	Insulin-like growth factor 1 (somatomedin C)	281239	IGF1	Insulin-like growth factor 1 (somatomedin C)	2.95	0.03	3.91
3576	IL8	Interleukin 8	280828	IL8	Interleukin 8	5.92	0.00	8.23
3665	IRF7	Interferon regulatory factor 7	100125591	IRF7	Interferon regulatory factor 7	2.08	0.00	4.34
3684	ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)	407124	ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)	2.15	0.12	2.83
4063	LY9	Lymphocyte antigen 9	528926	LY9	Lymphocyte antigen 9	2.15	0.05	3.25
4599	MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	280872	MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	4.62	0.00	6.01
4792	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	282291	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, alpha	2.45	0.00	7.10
22861	NLRP1 (NALP)	NLR family, pyrin domain containing 1	788205	LOC788205	Hypothetical protein LOC788205	2.06	0.03	3.58
4938	OAS1	2',5'-Oligoadenylate synthetase 1, 40/46 kDa	654488	OAS1	2',5'-Oligoadenylate synthetase 1, 40/46 kDa	3.81	0.76	2.94
5328	PLAU	Plasminogen activator, urokinase	281408	PLAU	Plasminogen activator, urokinase	2.49	0.05	3.52
5329	PLAUR	Plasminogen activator, urokinase receptor	281983	PLAUR	Plasminogen activator, urokinase receptor	2.36	0.00	5.71
5724	PTAFR	Platelet-activating factor receptor	518283	PTAFR	Platelet-activating factor receptor	3.15	0.00	6.92
5743	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	282023	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	2.41	0.05	4.26
5806	PTX3	Pentraxin 3, long	541148	PTX3	Pentraxin 3, long	2.69	0.00	4.64
5996	RGS1	Regulator of G-protein signalling 1	540836	RGS1	Regulator of G-protein signalling 1	4.44	0.00	4.38
51284	TLR7	Toll-like receptor 7	493686	TLR7	Toll-like receptor 7	2.02	0.03	3.69
54210	TREM1	Triggering receptor expressed on myeloid cells 1	404547	TREM1	Triggering receptor expressed on myeloid cells 1	2.58	0.00	4.24

Table 5 Genes of the interaction network 'ECM remodelling' (Fig. 4) that were differentially expressed.

Entrez gene ID	Human gene symbol	Human Entrez gene description	Gene ID	Entrez gene (bovine)	Entrez gene description	FC (IP/AP)	Pvalue (%)	Score (d)
374	AREG	Amphiregulin	538751	AREGB	Amphiregulin B	5.47	0.29	2.33
5054	SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	281375	SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	4.17	0.00	6.40
9507	ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4	286806	ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4	4.00	0.00	5.41
10516	FBLN5	Fibulin 5	535185	FBLN5	Fibulin 5	3.70	0.00	5.12
4036	LRP2	Low-density lipoprotein receptor-related protein 2	533181	LRP2	Low-density lipoprotein receptor-related protein 2-like	2.84	0.05	3.54
57134	MAN1C1	Mannosidase, alpha, class 1C, member 1	615012	MAN1C1	Mannosidase, alpha, class 1C, member 1	2.83	0.00	4.91
4312	MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	281308	MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	2.79	2.75	1.05
4314	MMP3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	281309	MMP3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	2.68	1.71	1.23
4318	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	282871	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	2.66	0.12	2.72
7057	THBS1	Thrombospondin 1	281530	THBS1	Thrombospondin 1	2.64	0.03	3.80
4311	MME	Membrane metalloendopeptidase	536741	MME	Membrane metalloendopeptidase	2.53	0.00	4.25
5328	PLAU	Plasminogen activator, urokinase	281408	PLAU	Plasminogen activator, urokinase	2.49	0.05	3.52
960	CD44	CD44 molecule (Indian blood group)	281057	CD44	CD44 molecule (Indian blood group)	2.46	0.00	4.23
6590	SLPI	Secretory leukocyte peptidase inhibitor	512548	SLPI	Secretory leukocyte peptidase inhibitor	2.45	0.07	3.12
5743	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	282023	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	2.41	0.05	4.26
5329	PLAUR	Plasminogen activator, urokinase receptor	281983	PLAUR	Plasminogen activator, urokinase receptor	2.36	0.00	5.71
2191	FAP	Fibroblast activation protein, alpha	508882	FAP	Fibroblast activation protein, alpha	2.34	0.70	1.74
4322	MMP13	Matrix metallopeptidase 13 (collagenase 3)	281914	MMP13	Matrix metallopeptidase 13 (collagenase 3)	2.30	1.53	1.35
1462	VCAN (CSPG2)	Versican	282662	VCAN	Versican	2.29	0.07	3.12
1893	ECM1	Extracellular matrix protein 1	524222	ECM1	Extracellular matrix protein 1	2.27	0.07	3.04
8754	ADAM9	ADAM metallopeptidase domain 9	281600	ADAM9	ADAM metallopeptidase domain 9	2.26	0.01	3.92
11096	ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5	286805	ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5	2.22	0.21	2.51
4323	MMP14	Matrix metallopeptidase 14 (membrane inserted)	281915	MMP14	Matrix metallopeptidase 14 (membrane inserted)	2.21	0.08	3.06
7078	TIMP3	TIMP metallopeptidase inhibitor 3	282094	TIMP3	TIMP metallopeptidase inhibitor 3	2.19	0.00	4.94
7436	VLDLR	Very low-density lipoprotein receptor	282123	VLDLR	Very low-density lipoprotein receptor	2.18	3.84	0.85
4121	MAN1A1	Mannosidase, alpha, class 1A, member 1	530027	MAN1A1	Mannosidase, alpha, class 1A, member 1	2.18	0.07	3.19
7076	TIMP1	TIMP metallopeptidase inhibitor 1	282092	TIMP1	TIMP metallopeptidase inhibitor 1	2.17	0.00	5.15
4325	MMP16	Matrix metallopeptidase 16 (membrane-inserted)	282886	MMP16	Matrix metallopeptidase 16 (membrane-inserted)	2.17	1.72	1.39
1508	CTSB	Cathepsin B	281105	CTSB	Cathepsin B	2.17	0.22	2.47
3684	ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)	407124	ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)	2.15	0.12	2.83
8828	NRP2	Neuropilin 2	541004	NRP2	Neuropilin 2	2.13	0.03	4.00
7079	TIMP4	TIMP metallopeptidase inhibitor 4	317694	TIMP4	TIMP metallopeptidase inhibitor 4	2.06	0.05	3.41
6594	SMARCA1	SWI/SNF-related, matrix associated, actin-dependent regulator of chromatin, subfamily a, member 1	535439	SMARCA1	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 1	2.06	0.00	6.43
667	DST	Dystonin	535297	DST	Dystonin	2.04	0.05	3.53
10418	SPON1	Spondin 1, extracellular matrix protein	282866	SPON1	Spondin 1, extracellular matrix protein	2.03	0.07	3.15

Table 6 Genes of the interaction network 'apoptosis' (Fig. 3) that were differentially expressed.

| Entrez gene ID	Human gene symbol	Human Entrez gene description	Gene ID	Entrez gene (bovine)	Entrez gene description	FC (IP/AP)	P value (%)	Score (d)
1776	DNASE1L3	Deoxyribonuclease I-like 3	512512	DNASE1L3	Deoxyribonuclease I-like 3	33.12	0.00	7.41
2537	IFI6 (G1P3)	Interferon, alpha-inducible protein 6	512913	IFI6	Interferon, alpha-inducible protein 6	4.50	0.00	4.51
3428	IFI16	Interferon, gamma-inducible protein 16	506759	IFI16	Interferon, gamma-inducible protein 16	3.30	0.00	4.29
9423	NTN1	Netrin 1	522767	NTN1	Netrin 1	3.12	0.22	2.42
64135	IFIH1	Interferon induced with helicase C domain 1	535490	IFIH1	Interferon induced with helicase C domain 1	2.65	0.11	2.89
4616	GADD45B	Growth arrest and DNA damage inducible, beta	618405	GADD45B	Growth arrest and DNA damage inducible, beta	2.59	0.00	5.94
4792	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	282291	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	2.45	0.00	7.10
3164	NR4A1	Nuclear receptor subfamily 4, group A, member 1	528390	NR4A1	Nuclear receptor subfamily 4, group A, member 1	2.23	0.05	3.44
1508	CTSB	Cathepsin B	281105	CTSB	Cathepsin B	2.17	0.22	2.47
388	RHOB	Ras homologue gene family, member B	515118	RHOB	Ras homologue gene family, member B	2.14	0.00	4.49
3480	IGF1R	Insulin-like growth factor 1 receptor	281848	IGF1R	Insulin-like growth factor 1 receptor	2.10	0.00	4.88
22861	NLRP1 (NALP1)	NLR family, pyrin domain containing 1	788205	LOC788205	Hypothetical protein LOC788205	2.06	0.03	3.58
5610	EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2	347700	EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2	2.04	0.12	2.71
7133	TNFRSF1B	Tumour necrosis factor receptor superfamily, member 1B	338033	TNFRSF1B	Tumour necrosis factor receptor superfamily, member 1B	2.02	0.03	3.76
55749	CCAR1	Cell division cycle and apoptosis regulator 1	767587	CCAR1	Cell division cycle and apoptosis regulator 1	−2.05	0.03	−3.69
332	BIRC5	Baculoviral IAP repeat containing 5	414925	BIRC5	Baculoviral IAP repeat containing 5	−2.37	0.00	−5.75
331	XIAP (BIRC4)	X-linked inhibitor of apoptosis	281647	XIAP	X-linked inhibitor of apoptosis	−3.03	0.00	−5.14
8837	CFLAR	CASP8 and FADD-like apoptosis regulator	497199	CFLAR	CASP8 and FADD-like apoptosis regulator	−3.80	0.00	−9.86
8727	CTNNAL1	Catenin (cadherin-associated protein), alpha-like 1	515749	CTNNAL1	Catenin (cadherin-associated protein), alpha-like 1	−5.48	0.00	−7.80
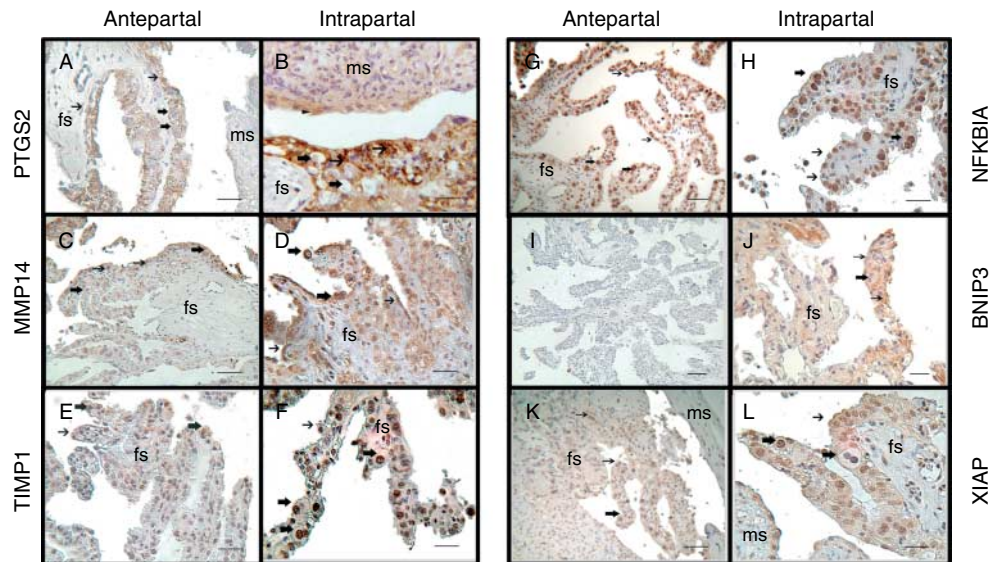
Figure 5 Localisation of expression of selected proteins in antepartum (AP) and intrapartum (IP) placentomes: (A) immunostaining for PTGS2 in AP placentomes: the staining intensity for PTGS2 of the uninucleated trophoblast cells (UNC) was moderate to distinct positive (thin arrows). In contrast, all giant trophoblast cells (GC) were negative (bold arrows). fs, foetal stroma; ms, maternal stroma; sb=50 µm; (B) immunostaining for PTGS2 in IP placentomes: strong staining for the antibody against PTGS2 is restricted to the UNC (thin arrows), whereas the TGC are negative (bold arrows). The cytoplasm of caruncular epithelium cells showed a weak to moderate expression for PTGS2 (arrowhead); sb=50 µm; (C) immunostaining for MMP14 in AP placentomes: TGC were only weak positive stained or negative (bold arrows), whereas the UNC (thin arrows) expressed MMP14 moderately; sb=100 µm; (D) immunostaining for MMP14 in IP placentomes: strong immunostaining signals were detected in the UNC (thin arrows), whereas the TGC exhibit a distinct staining intensity (bold arrows). A distinct to strong expression for MMP14 was localised in the fs cells. sb=50 µm; (E) immunostaining for TIMP1 in AP placentomes: TIMP1 was detected in the UNC (thin arrow) and the TGC (bold arrows) with a weak to distinct staining pattern; sb=50 µm; (F) immunostaining for TIMP1 in IP placentomes: the immunostaining for TIMP1 increased in the UNC (thin arrows) and the TGC (bold arrows) to strong positive; sb=50 µm; (G) immunostaining for NFKBIA in AP placentomes: UNC demonstrated a distinct expression for NFKBIA (thin arrows). In contrast, TGC expressed NFKBIA strong (bold arrows); sb=100 µm; (H) immunostaining for NFKBIA in IP placentomes: the immunostaining appeared strong in the UNC (thin arrows) and moderate to distinct in the TGC (bold arrows); sb=50 µm; (I) immunostaining for BNIP3 in AP placentomes: no positive signals for BNIP3 were observed in the UNC and TGC; sb=150 µm; (J) immunostaining for BNIP3 in IP placentomes: some UNC (thin arrows) and TGC (bold arrow) demonstrated a weak expression for BNIP3, whereas the staining intensity increased in the uterine stromal cells during birth; sb=50 µm; (K) immunostaining for XIAP in AP placentomes: the UNC (thin arrows) and TGC (bold arrow) showed weak to moderate XIAP expression; sb=100 µm; (L) immunostaining for XIAP in placentomes of intrapartal cattle: the staining intensity for XIAP was distinct to strong in the UNC (thin arrow) and the TGC (bold arrows). The fs were stained moderate to distinct positive. sb=50 µm.

compared to placentome samples extracted in labour immediately after the end of the expulsion phase of parturition (maximum of 15 min). The sampling was done after expulsion of the calf to avoid interference with the expulsion process and induction of RFM. We strictly defined the experimental model to minimise influences that alter physiological parturition including the stage of placental expulsion, like prolonged parturition, hypocalcaemia or low oestrogen levels (Schuler *et al*. 2002, McNaughton & Murray 2009). Therefore, gestations as well as the parturition were controlled over the whole period.

During sampling, the foetal part of the placenta was not separated from the maternal part to facilitate detection of transcriptome changes of the whole placental unit. Additionally, contaminations from the counterpart cannot be excluded when placentomes are manually separated. To ensure a similar tissue composition in all samples, systematic random sampling was

performed and nine samples from three placentomes were pooled. Estimation of the volume fraction of epithelial cells revealed large variations between individual placentome samples that would lead to much higher variation between animals due to tissue sampling if only a single sample per animal was analysed. As described previously (Mayhew 2008), the sampling method is essential to avoid any bias in generating gene expression data because differences of the tissue composition or localisation of the tissue sampling directly influence the results of gene expression analyses (Wyatt *et al*. 2005).

Overrepresented functional terms for the genes with higher mRNA levels in AP placentome samples

Analysis of the mRNAs with higher levels in placentome samples of the AP group revealed strongest overrepresentation of functional categories related to cell

Table 7 Results of immunohistochemistry analyses (Fig. 5).

Antibody	UNC	GC	FS	CE	MS	BV
PTGS2						
Antepartal	+/++	−	−	−+/+	−	+
Intrapartal	++/+++	−	−/−+	−+/+	−	+
MMP14						
Antepartal	+/++	−/−+	−+	+	−	++
Intrapartal	++/+++	++	++/+++	−+/+	−+/+	++
TIMP1						
Antepartal	−+/+	+/++	+/++	−+/+	−/+	+
Intrapartal	++	++/+++	−+/+	+/++	−+/+	+/++
NFKBIA						
Antepartal	++	++/+++	−+/+	−+/++	−+	++
Intrapartal	+++	+/++	−+/+	−+/++	−+	−
BNIP3						
Antepartal	−	−	−	−	+	−
Intrapartal	−+	−+	−	−	++/+++	−
XIAP						
Antepartal	−+/+	−+/+	+	−+	−	+/++
Intrapartal	++/+++	++/+++	+/++	−+	−	+/++

UNC, uninucleated trophoblast cells; GC, giant trophoblast cells; CE, caruncular epithelium; FS, foetal stroma; MS, maternal stroma; BV, blood vessels (− negative, −+ weak, + moderate, ++ distinct, +++ strong).

cycle and cell division (e.g. cell cycle, cell division, mitosis, microtubule cytoskeleton, chromosome, etc.). This observation is in line with histochemical studies showing a decrease in (maternal) epithelial proliferation near parturition (Boos *et al.* 2003). Cellular proliferation and cell death are inversely proportional during gestation and near parturition, where apoptosis increases and proliferation declines (Boos *et al.* 2003). The functional term 'regulation of apoptosis' was also found in the DAVID analysis, whereas genes with higher mRNA levels in AP were predominantly related to negative regulation of cell death and those with higher levels in IP with positive regulation of cell death. Genes involved in the regulation of apoptosis are discussed in detail. In addition, a large number of genes coding for different transmembrane transporters (e.g. for glucose, amino acids, fatty acids, sodium iodide, potassium/ chloride, zinc, vitamins, neurotransmitters, etc.) were identified. Also a number of genes typically associated with the establishment and maintenance of pregnancy showed higher mRNA levels in the AP samples, e.g. members of the pregnancy-associated glycoprotein family (*Pag3*, *PAG5*, *PAG6*, *PAG7*, *LOC100336184*, *PAG9*, *PAG15*, *PAG17*, *PAG18* and *PAG21*) and members of the family of prolactin-related proteins (*PRL*, *PRP-VII*, *PRP2* and *PRP3*). Expression of PAGs and PRPs has been described in BNC and a role in placenta formation and differentiation has been postulated (Gootwine 2004, Hashizume 2007, Haig 2008). Down-regulation of these genes might be a result of decreasing number of BNC. The decline in number of BNC shortly before parturition is typical in cases of physiological release of the foetal membranes (Gross *et al.* 2001).

Overrepresented functional terms for the genes with higher mRNA levels in IP placentome samples

Genes related to immune response

Regulation of different pro-inflammatory cytokines is essential for uterus activation and normal parturition (Christiaens *et al.* 2008). Especially, in the last stage of parturition, pro-inflammatory reactions are essential for proper release of the foetal membranes and for a healthy puerperal stage (Kelly 1996). At the level of mRNA, we found elevated concentrations in the IP placentome samples for mRNAs coding for CD14 molecule (2.8-fold higher), CD36 molecule (thrombospondin receptor; threefold higher), chemokine (C-X-C motif) ligand 2 (CXCL2; 4.9-fold higher), CXCL14 (2.1-fold higher), chemokine (C-C motif) receptor 4 (CCR4; 2.1-fold), macrophage expressed 1 (*MPEG1*; 2.5-fold) and macrophage scavenger receptor 1 (*MSR1*; 2.5-fold).

Up-regulation of these genes indicates increased numbers of monocytes and/or macrophages (MPEG1 and MSR1) in the IP samples compared with AP placentomes since specific expression in monocytes/ macrophages (e.g. CD14) or in macrophages (CXCL14) has been shown (Oliveira & Hansen 2009). Also for *MPEG1* and *MSR1*, macrophage-specific expression has been shown (Spilsbury *et al.* 1995 PMID: 7888681, Freeman 1990 PMID: 1978939). A functional role of bovine placental macrophages during parturition for the release of foetal membranes has been suggested by Miyoshi *et al.* (2002). Oliveira & Hansen (2009) demonstrated that CD14(+) and CD68(+) macrophages are located in the maternal crypts but not in the foetal part of the placentome. CD14 is an important binding protein for lipopolysaccharides of *Escherichia coli*, the typical pathogen in the *post partum* uterus

(Sheldon *et al.* 2008). In addition to its role in innate immune responses to bacterial and other microbial structures, CD14 also functions as a receptor involved in the recognition and phagocytosis of cells undergoing apoptosis (Gregory 2000). Thus, the role of endometrial macrophages could be on one side clearing of apoptotic cells and pathogen defence after parturition.

Nuclear factor kappa B (NFKB) is a key transcription factor that regulates transcription of, e.g. different cytokine genes involved in inflammatory reactions, such as *TNFA*, *IL1* and also expression of *PTGS2* (Baeuerle & Baichwal 1997). The activity of NFKB is regulated by several specific inhibitors such as NFKB inhibitor alpha (NFKBIA), which prevents translocation of NFKB to the nucleus (Baeuerle 1998). In the presented study, a 2.4-fold higher expression of *NFKBIA* was observed in IP placentomes. On protein level, staining intensity in UNC was higher in IP samples. Interestingly, distinct staining was found in or around caruncular blood vessels of AP samples but not in IP placentomes. This result could indicate a differential regulation of leukocyte infiltration of the placentomes, via NFKB regulation.

Macrophages have been shown to be the major producers of pro-inflammatory cytokines like interleukin 1 beta (IL1B) and tumour necrosis factor alpha (TNFA) as well as the chemokine IL8, which were described as essential regulatory mediators during labour (Elliott *et al.* 1998, Goldenberg *et al.* 2000). For *IL8*, higher mRNA levels (5.9-fold higher) were found in IP placentomes. IL8 is one of the major mediators of the inflammatory response and has been shown both to attract and to activate PMNs (Colditz *et al.* 1989), the most prominent postpartal uterine immune cells. It has been demonstrated that IL8 can be produced by many cell types, e.g. macrophages, endothelial cells and even phagocytising PMNs (Bazzoni *et al.* 1991). IL8 expression is down-regulated during gestation by high progesterone (P_4) concentrations (Barclay *et al.* 1993, Kelly *et al.* 1994). In human placenta, IL8 production is increased after spontaneous labour and was localised in the perivascular area of foetal vessels in third-trimester placenta (Elliott *et al.* 1998). The increase in *IL8* mRNA levels corresponds to the influx of PMNs and other immune cells like macrophages that has been found immediately before parturition (Yellon *et al.* 2003) and its known synergism with the effects of PGE_2 in attracting PMNs to skin sites (Foster *et al.* 1989, Colditz 1990) suggests a possible role for IL8 in initiation and progression of labour through activation of PMNs, which release proteolytic enzymes, such as collagenases and elastases that degrade ECM contributing together with apoptosis to foetal membrane rupture (Osmers *et al.* 1992, Winkler & Rath 1996, Moore *et al.* 2006).

In the context of prostaglandin metabolism, two mRNAs coding for prostaglandin synthases, namely hydroxyprostaglandin dehydrogenase 15-(NAD; HPGD) and PTGS2 (alias COX2), were identified as up-regulated in the IP placentome samples (HPGD: 2.4-fold, PTGS2: 2.4-fold). PTGS2 protein was mainly detectable in the uninuclear trophoblast cells but also to a lesser extent in the caruncular epithelium and blood vessels. This is in line with investigations of the PTGS2 expression around parturition (Whittle *et al.* 2000) and its essential role in the production of prostaglandins $PGF_{2\alpha}$ and PGE_2, which are involved in the regulation of every step of parturition: membrane rupture, cervical dilatation, myometrial contractility, placental separation and uterine involution (Olson *et al.* 1995). Myometrial contractions at parturition are a product of uterine activation and increased levels of genes involved in this process, including PTGS2 and HPGD (Olson 2003, Mendelson 2009), also described as uterine activation proteins (UAPs). An additional indication for the activation of the innate immune system was found by up-regulation of many typical interferon-induced genes (e.g. interferon-induced protein 44 like (*IFI44*; 4.8-fold higher) and interferon induced with helicase C domain 1 (*IFIH1*; 2.7-fold higher)) and of members of the complement system (e.g. complement component 1, r subcomponent (*C1R*; 2.2-fold higher) and complement component 7 (*C7*; 3.6-fold higher).

Remodelling of the extracellular matrix and regulation of angiogenesis

DAVID analysis also revealed enrichment of genes coding for proteins involved in ECM remodelling for the mRNAs with higher levels in IP placentome samples. The main groups of these genes were i) matrix metallopeptidases: *MMP1*, *MMP3*, *MMP9*, *MMP13*, *MMP14* and *MMP16*; ii) TIMP metallopeptidase inhibitors: *TIMP1*, *TIMP3* and *TIMP4* and iii) ADAM metallopeptidases: *ADAM9*, *ADAM19*, *ADAMDEC1*, *ADAMTS4*, *ADAMTS5* and *ADAMTSL4*. The up-regulation of MMP2 and MMP9 protein in relation to onset of labour has been described in various species (Ulug *et al.* 2001, Walter & Boos 2001, Goldman & Shalev 2003, Takagi *et al.* 2007). Especially, the up-regulation of MMP9 protein, partially released by PMNs in the foetal membranes (Osmers *et al.* 1995) with beginning contractions (Tsatas *et al.* 1999, Goldman & Shalev 2003), is linked to the rupture of the foetal membranes implicating an important role in placental separation. MMP9 degrades type IV and V collagens and has been shown to be present in tertiary granules of neutrophils and MMP9 release can be induced by IL8 via activation of chemokine (C-X-C motif) receptor 2 (CXCR2; Chakrabarti & Patel 2005). Additionally, the expression of MMP9 is regulated by a PTGS2–PGE_2–EP4 receptor axis in macrophages. Macrophages are able to secrete active MMP9 after exposure to MMP1 or MMP3; both of them are up-regulated in the IP group (2.8- and 2.7-fold respectively). These exposures also triggered

a quick release of TNFA, which could be blocked by inhibitors of MMP1 and MMP3 (Steenport *et al.* 2009). Pro-MMP9 is also activated by MMP3 and indirectly by MMP14 (on protein levels also detected, see Fig. 5 and Table 7), which activates MMP13 that also cleaves pro-MMP9 (Pap *et al.* 2000, Elnemr *et al.* 2003, Dreier *et al.* 2004). The localisation and the increase in MMP14 protein in IP samples (foetal stroma, UNC) is in line with recent findings and gives additional hints about the importance of MMP14 in the process of foetal membrane release (Dilly *et al.* 2011).

Furthermore, plasminogen activator, urokinase (PLAU), whose mRNA is also up-regulated (2.5-fold) in IP samples, is known to play a major role in degradation of ECM (Tsatas *et al.* 1999). The function of PLAU is to convert plasminogen to plasmin, which subsequently activates MMPs, such as MMP3 and MMP9. PLAU is also able to directly activate MMP9 (Zhao *et al.* 2008). The simultaneous up-regulation of PLAU receptor (*PLAUR*, 2.4-fold) mRNA and serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (*SERPINE1*, 4.2-fold) mRNA indicates tight regulation of ECM degradation in the placentomes immediately after expulsion of the calf. Interestingly, prostaglandins PGE$_2$ and PGF$_{2\alpha}$ are also able to increase the expression of MMPs, especially MMP2 and MMP9, and therefore the production of prostaglandins by PTGS2 (also up-regulated in IP samples) could play a role in the separation and release of the placenta (Weinreb *et al.* 1997, Yoshida *et al.* 2002). MMP16 (2.2-fold up-regulated) is an activator of pro-MMP2 and it could be demonstrated that the activation of pro-MMP2 is enhanced by TIMP3 (2.2-fold) and by TIMP2 (Zhao *et al.* 2004). A disintegrin and metallopeptidases (ADAMs) and ADAMs with thrombospondin motifs (ADAMTS) are membrane-bound peptidases closely related to MMPs. These multifaceted molecules bear metallopeptidase and disintegrin domains endowing them with features of both peptidases and adhesion molecules. Peptidases of the ADAM family are associated with various physiological and pathological processes like trophoblast invasion and matrix degradation during pregnancy, angiogenesis and inflammation, neurodegeneration and fibrosis, through shedding of the apoptosis-inducing FAS ligand (van Goor *et al.* 2009). It was shown that catalytically active ADAM9 (2.3-fold), together with ADAM15 and ADAM17, is associated with CD68+ monocytes derived cells in inflammatory tissues (Oksala *et al.* 2009). Since MMPs and ADAMs are very potent collagenases, they have to be tightly regulated to avoid uncontrolled tissue damage. Three mRNAs for inhibitors of different MMPs, *TIMP1*, *TIMP3* (both 2.2-fold) and *TIMP4* (2.1-fold) were also identified as up-regulated in the IP placentome samples. TIMP1 is the direct inhibitor of MMP1 (Vallon *et al.* 1997). Strongest expression of TIMP1 protein was detected in GC (TGC) but also in all other areas of the

placentomes. Except for foetal stroma, staining intensity was always higher in IP samples, which correlates with up-regulation of *TIMP1* mRNA in IP samples. The broad expression could indicate a regulatory function in fine-tuning the activity of MMPs such as MMP1, MMP2 and MMP3. It has been shown for TIMP3 that expression is strictly balanced with expression of MMP9 during early gestation in the mouse (Teesalu *et al.* 1999). The process of parturition is also associated with the regulation of vascular remodelling-related genes like *SERPINE1*, *FBLN5*, thrombospondin 1 (*THBS1*; 2.6-fold; Haddad *et al.* 2008), angiopoietin 2 (*ANGPT2*; 4.4-fold) and angiopoietin-like 4 (*ANGPTL4*; 2.7-fold). This process of remodelling is also represented by the differential regulation of different genes all described to be involved in the process of angiogenesis and regulation of angiogenesis (e.g. fibroblast growth factor 2 (basic; *FGF2*; 2.3-fold), platelet/endothelial cell adhesion molecule (*PECAM1*; 2.3-fold), vascular endothelial growth factor A (*VEGFA*; −2.7-fold) and vasohibin 2 (*VASH2*; −2.4-fold)). Interestingly, *VEGFA* mRNA has lower levels in IP samples, which could be a mechanism to reduce vascular permeability (Zachary 1998, Farmer *et al.* 2002). Since many genes involved in the biological processes of ECM remodelling were also described in the context of angiogenesis, the regulation of vascular remodelling could also play a role in the release of foetal membranes.

Apoptosis-related genes

DAVID analysis showed enrichment for apoptosis-related genes for both the IP up-regulated and the IP down-regulated genes. The majority of the genes with lower levels in the IP placentome samples are known or assumed to have negative effects on apoptosis whereas the genes with increased mRNA levels are predominantly positive regulators of programmed cell death. Apoptosis has been shown to be an essential process during the release of the foetal membranes and maturation of the placenta before parturition (Boos *et al.* 2003, Keelan *et al.* 2003). The observed up-regulation of typical monocyte/macrophage markers in IP placentomes could also be associated with increased apoptosis in the placentomes, since macrophages play an important role in clearance of apoptotic cells (Erwig & Henson 2007). In addition to pro-apoptotic signals, the reduction of the blood supply to the placenta after umbilical cord rupture could be a possible reason for induction of apoptosis (Runic *et al.* 1998, McLaren *et al.* 1999). One important gene family involved in the regulation of apoptosis is the TNF receptor family (Baker & Reddy 1998). For two members of this gene family, namely TNF receptor superfamily, member 1B (*TNFRSF1B*; twofold) and TNF receptor superfamily, member 6b, decoy (*TNFRSF6B*; 4.2-fold) higher mRNA levels were found in IP placentomes. Furthermore, a number of

genes associated with the TNF signalling pathway were down-regulated in IP placentome samples, such as TNF receptor-associated factors 3 and 6 (*TRAF3*, *TRAF6*; −3.3-fold, −2.1-fold), Fas (TNFRSF6)-associated via death domain (*FADD*; −2.6-fold) and Fas-associated factor family member 2 (*FAF2*; −3.3-fold). Some of the mRNAs with higher levels in the IP group compared with the AP group are growth arrest and DNA damage-inducible, beta (*GADD45B*), *BNIP3*; DnaJ (Hsp40) homologue, subfamily A, member 3 (*DNAJA3*), eukary-otic translation initiation factor 2-alpha kinase 2 (*EIF2AK2*; Table 7 and Supplementary Figure 1, see section on supplementary data given at the end of this article for the corresponding protein detections). Increased expression of BNIP3 protein in UNC, GC and uterine stromal cells during birth could indicate increased apoptosis since BNIP3 a pro-apoptotic factor of the Bcl-2 family was localised in IP maternal stroma. The presence of this protein was also shown in human placenta (Stepan *et al.* 2004). The expression of it is associated with hypoxic situation, e.g. in necrotic regions of malignant tumours (Sowter *et al.* 2001).

Genes with lower levels in IP samples that are associated with cell survival and anti-apoptotic functions are, e.g. CASP8 and FADD-like apoptosis regulator (*CFLAR*; −3.8-fold), cell death-inducing DFFA-like effector A (*CIDEA*; −4.8-fold) and *XIAP* (−3-fold; Table 7 and Supplementary Figure 1, see section on supplementary data given at the end of this article for the corresponding protein detections). In contrast to the findings of gene expression, expression of XIAP protein, a known inhibitor of apoptosis (Straszewski-Chavez *et al.* 2004), was higher in IP samples. However, mRNA expression was only analysed in two time points and the

expression profile between these time points is not known, and the half-life of mRNA and protein is different. Interestingly, XIAP was found in all different parts of the placentome (UNC, GC, CE and BV) except for the maternal stroma, where BNIP3 expression was found. This indicates complex spatial regulation of apoptosis in the placentome during birth.

This study is the first systematic analysis of differential gene expression of bovine placentome samples collected from contact zones before parturition compared with the time immediately after expulsion of the calf. Figure 6 summarises the findings of this study to illustrate the complex processes in the placentome during birth. The identified transcriptome changes and protein localisations revealed a complex regulation and interaction of immune system-related processes, vascular, tissue and ECM remodelling, and apoptosis. Particularly, the identified immune-related genes suggest an important role of inflammatory cells, such as macrophages and neutrophils, in the process of release of foetal membranes. Furthermore, the results of this study provide a basis for investigations of the pathophysiology of disturbed release of foetal membranes.

Materials and Methods

Animal model

Each experimental group (AP and IP) consisted of four cows (*Bos taurus*; Simmental breed), with two primiparous and two polyparous in the second or third pregnancy. None of the polyparous animals suffered from RFM in earlier calvings. All pregnancies were monitored by regular clinical examinations, and blood samples were taken and investigated to
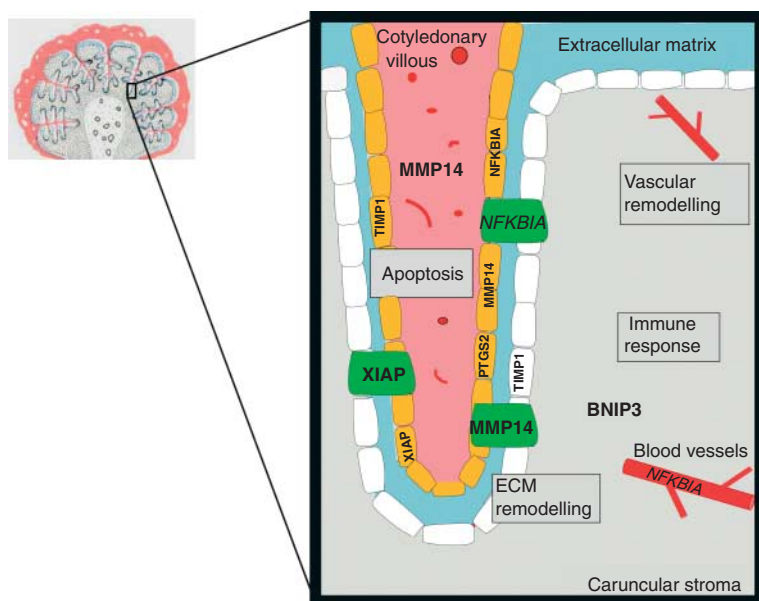

Figure 6 Scheme demonstrating the findings of this microarray study. The foetal maternal contact zone is shown exemplarily. In the boxes (light grey) biological processes, to which genes in the IP group are associated with: apoptosis, ECM remodelling, vascular remodelling and immune response. The locations of the proteins according to the results of immunohistochemistry (Table 7). Proteins with stronger appearance IP (in bold) and AP (in italic). Light blue: extracellular matrix; orange: uninucleated trophoblast cells; rose: cotyledonary villous, including foetal stroma; red: blood vessels; green: giant trophoblast cells; white: caruncular epithelium; light grey: caruncular stroma.

exclude predisposing factors of dystocia or RFM, such as subclinical hypocalcaemia or ketosis. Plasma P_4 levels were determined (Prakash *et al.* 1987), which were stable throughout the last 3 weeks of gestation (>2 ng/ml). Animals of the AP group underwent caesarean section 272 ± 3 days after artificial insemination (AI). The animals of the IP group calved 288 ± 7 days after AI. In all IP animals, parturition occurred physiologically without any problems and the foetal membranes were delivered in <6 h after calving.

All experiments with animals were conducted with permission from the local veterinary authorities and in accordance with the accepted standards of humane animal care.

Tissue sampling

After rupture of the umbilical cord, three placentomes per animal were collected. In the AP group, the placentomes were collected under visual control. In the IP group, the placentomes were recovered transvaginally by using an effeminator by Reisinger (Heuwieser *et al.* 1985) within 15 min after rupture of the umbilical cord. The tissue sampling was done systematically (Herbach *et al.* 2005; Fig. 1) as follows: the placentomes were analysed as a foeto-maternal unit, therefore maternal and foetal parts were not separated. Every placentome was cut perpendicular to the luminal surface in 0.5 cm-thick slices, which were placed with the right cut surface facing downwards on a sterile RNase-free surface. The slices were covered with a grid (1×1 cm) and every point hitting the visual foeto-maternal connection was counted. These connecting points were divided by three resulting in $n=x$, and a random number between 1 and x was determined, where tissue samples were collected. Tissue cuboids ($\sim0.6\times0.6\times1.0$ cm) were cut out and divided into three parts of $\sim0.2\times0.2\times0.3$ cm. The middle part was transferred into RNAlater (Ambion, Huntingdon, Cambridgeshire, UK), incubated overnight at 4 °C and then stored at -20 °C until further processing. The other samples were fixed in formalin (3.7%) and paraformaldehyde (4.2%), respectively, and routinely processed and embedded in paraffin.

Morphometric analysis

Sections were stained with haematoxylin and eosin (H&E) and evaluated using unbiased model-independent stereological methods as described (Herbach *et al.* 2005). Morphometric evaluation was carried out on a Videoplan image analysis system (Zeiss-Kontron, Eching, Germany) attached to a microscope by a colour video camera. Images were displayed on a colour monitor at a $850\times$ final magnification, and the profiles of placentomes, epithelial cells and non-epithelial cells were measured planimetrically by circling their contours with a cursor on the digitising tablet of the image analysis system. The volume density of epithelial cells in the placentomes ($Vv_{(Epithelial\ cells/Placentome)}$) was calculated by dividing the sum of cross-sectional areas of epithelial cells by the sum of cross-sectional areas of the placentome, the volume density of other cells ($Vv_{(Non-epithelial\ cells/Placentome)}$) was calculated analogue to the $Vv_{(Epithelial\ cells/Placentome)}$. The morphometric data were

analysed by a Kruskal–Wallis test (SPSS 15.0; IBM Corporation, Armonk, NY, USA).

Microarray hybridisation and data analysis

Total RNA was isolated from the tissue samples of the placentomes using Trizol Reagent (Invitrogen GmbH) according to the manufacturer's instructions. Tissue samples ($n=3$) of individual placentomes ($n=3$) were pooled before isolation of the RNA. Quantity and quality of total RNA were determined by spectrometry and agarose gel electrophoresis. For every animal, equal amounts of total RNA of the single placentomes ($n=3$) were pooled for microarray analysis. Preparation of hybridisation probes for Affymetrix GeneChip Bovine Genome Arrays (Affymetrix, Santa Clara, CA, USA) started from 10 μg total RNA and was done using the One-Cycle Target Labelling and Control Reagent package (Affymetrix). For the hybridisation, wash and staining processes, the GeneChip Hybridization, Wash and Stain Kit (Affymetrix) was used. All steps were done according to the manufacturer's protocol. The processed arrays were scanned with a GeneChip Scanner 3000 7G with AutoLoader (Affymetrix).

Affymetrix CEL files were processed using log scale robust multi-array analysis (RMA; Irizarry *et al.* 2003). Quality control was done using different BioConductor tools, such as pseudo images (BioC package AffyPLM), RNA degradation plots (BioC package Affy), box plots, distance matrices and heatmaps. After RMA processing probe sets were filtered based on expression calls. Only such probe sets were used that had present calls in at least three of four samples in at least one of the experimental groups. Significance analysis was performed using the 'significance analysis of microarrays method' (SAM, two-class unpaired; Tusher *et al.* 2001). If a transcript was represented on the array by more than one probe set, the mean signal ratio was calculated.

Identification of quantitatively enriched functional categories was done using the 'Functional annotation clustering' tool of the DAVID (Dennis *et al.* 2003). The text-mining tool CoPub (Frijters *et al.* 2008) was used to identify biological keywords associated with the differentially expressed genes. These analyses were all based on the Entrez Gene ID of the putative human orthologous genes. Interaction networks were built for genes of selected functional groups using Pathway Architect software (version 3.0.1, Stratagene, Heidelberg, Germany). Most of the interactions and cellular localisations provided by the Pathway Architect databases were checked. Additional interactions found in the literature were assigned to the network.

Quantitative real-time RT-PCR

The same RNA samples as for microarray analysis were used for quantitative real-time RT-PCR (qPCR). First-strand cDNA was synthesised starting from 1 μg total RNA with the Sprint RT Complete-Double PrePrimed (6×8 well) kit (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France). qPCR was performed on a StepOne Real-Time PCR System, 48-well (Applied Biosystems, Langen, Germany) by the use of the Power SYBR Green PCR Master Mix (Applied Biosystems) or the FastStart Universal SYBR Green Master (Rox; Roche) in

Table 8 Antibodies for immunohistochemistry.

Primary antibody dilution, incubation time, incubation temperature	Clonality	Secondary antibody dilution, incubation time, incubation temperature	Company primary antibody	Company secondary antibody
PTGS2 1:400, 20 h, 6 °C	Polyclonal	Anti-goat IgG rabbit 1:400, 30 min, rt	Abcam plc	Dako Deutschland GmbH
MMP14 1:50, 20 h, 6 °C	Monoclonal	Anti-mouse IgG rabbit 1:300, 30 min, rt		
TIMP1 1:100, 20 h, 5 °C	Polyclonal	Anti-rabbit IgG swine, 1:300, 30 min, rt		
NFKBIA 1:100, 20 h, 5 °C	Polyclonal	Anti-rabbit IgG swine, 1:300, 30 min, rt		
BNIP3 1:400, 20 h, 6 °C	Monoclonal	Anti-mouse IgG rabbit 1:300, 30 min, rt		
XIAP 1:100, 20 h, 5 °C	Polyclonal	Anti-rabbit IgG swine, 1:300, 30 min, rt	Abnova GmbH c/o EMBLEM	

rt, room temperature.

a final volume of 20 µl. Primer sequences are shown in Supplementary Table 1, see section on supplementary data given at the end of this article. Annealing temperature was 60 °C for all PCRs. As control for DNA contamination, RNA without RT was used as PCR template ($-$RT control). The cycle number (C_T) required to achieve a definite SYBR Green fluorescence signal (C_T threshold) was calculated (StepOne Software v2.1, Applied Biosystems). The C_T is correlated inversely with the logarithm of the initial template concentration. The C_T determined for the target genes was normalised against the housekeeping genes *RPS23* and *PPP2R5B* (ΔC_T; Livak & Schmittgen 2001). Relative expression difference between the IP and the AP samples was calculated from the difference of the mean C_T values ($\Delta\Delta C_T$). All PCR fragments were analysed on a DNA1000 Labchip (Agilent Technologies, Waldbronn, Germany) to check amplification of a single product and sequenced to verify the obtained PCR product.

Immunohistochemistry

For immunohistochemical studies, 5 µm serial sections of each placentome sample were collected on slides (SupraFrost Ultra Plus, Menzel-Gläser; Gerhard Menzel Glasbearbeitungswerk GmbH & Co KG, Braunschweig, Germany), which were coated with aminopropyltriethoxysilane. Sections were pre-incubated with proteinase XXIV 0.1% (Sigma, P8038) for 15 min and washed with PBS buffer pH 7.4. Endogenous peroxidase activity was blocked with 0.1% H_2O_2 at room temperature for 10 min.

Antigen localisation was achieved using the avidin–biotin complex technique (ABC-technique; Hsu *et al*. 1981) according to the following protocol. Paraffin sections (5 µm) were dewaxed and subjected to the following immunohisto-chemical staining schedule: i) elimination of endogenous peroxidase activity with 0.5% H_2O_2 in PBS for 15 min at 20 °C; ii) elimination of non-specific protein binding by incubation at 20 °C with 10% normal goat serum for 1 h; iii) incubation overnight at 4 °C with one of the following

Table 9 Non-specific antibodies for isotype controls for immuno-histochemistry.

Goat IgG	Purified (UNLB)	BiOZOL Diagnostica
Mouse IgG$_{2b}$		
Mouse IgG$_3$		
Rabbit IgG		

primary antibodies (Table 7) against PTGS2, MMP14, TIMP 1, NFKBIA, BNIP3 and XIAP; iv) incubation with a corresponding secondary biotin-conjugated antibody (Table 8); v) incubation with HRP-conjugated ABC for 1 h at 20 °C and vi) treatment with 0.05 mg/ml diaminobenzidine in PBS. Sections were left unstained or counterstained in Mayer's haematoxylin, dehydrated and mounted with DePeX Eukitt (Riedel de Haen, Seelze, Germany).

Protein Block Serum-Free solution (Dako, X0909, Hamburg, Germany) was used to inhibit non-specific staining of primary and secondary antibodies during the immunohistochemical detection of antigens. As negative control, buffer (Dako) was used instead of the specific primary antibody. As positive controls, tissues were used, which is known to show specific immunostaining with the antibodies used in this study. For further negative control experiments, non-specific antibodies (Table 9) diluted to the same final protein concentration like the used specific antibodies were substituted for the primary antibodies.

The used non-specific antibodies were matched to the host species and isotype of the specific antibodies.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-11-0204.

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