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Original Article

mRNA expression profiling in cotyledons reveals significant up-regulation of the two bovine pregnancy-associated glycoprotein genes boPAG-8 and boPAG-11 in early gestation

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Abstract

The multigene family of pregnancy-associated glycoproteins (PAGs) belongs to a group of aspartic proteases that are exclusively expressed by trophoblast cells in the placenta of even-toed ungulates. In *Bovidae*, 22 different PAG genes (boPAGs) with a wide range of temporal and spatial expression- and glycosylation patterns have been reported to date. In this study we describe the mRNA expression patterns using real-time quantitative PCR (qPCR) for selected modern (boPAG-1, -9, -21) and ancient bovine PAGs (boPAG-2, -8, -10, -11, -12) in cotyledonary tissue. The highest mean expression was detected in boPAG-8 and lowest in boPAG-10 ($P < 0.05$). Furthermore, boPAG-8 and -11 were significantly greater expressed in early gestation compared with later pregnancy stages. The characterization of boPAG mRNA-expression levels gives important insights for further protein analyses which will be valuable information for the development of new pregnancy detection systems.

Keywords: bovine, pregnancy, pregnancy-associated glycoproteins, mRNA-expression, placenta.

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Introduction

Changes in gene expression are associated with the development of the blastocyst from the morula to the embryo. Some genes that are transcribed only in the trophectoderm are activated during pregnancy for the first time (Green *et al.* 2000).

Subsequent research in this field has revealed that multigene families are expressed in the reproductive tract of mammalian species during this period of life (Green 2004; Telugu *et al.* 2009). Pregnancy-associated glycoproteins (PAGs) are an example of this type of family. PAGs and PAG-like proteins can be found in numerous different species (e.g.de Sousa *et al.* 2006; Szafranska *et al.* 2006), but they are mainly expressed by the trophoblast cells of members of the *Cetartiodactyla* order (Wallace *et al.* 2015) where they represent one of the major secretory products (Szafranska *et al.* 1995; Xie *et al.* 1997a; Xie *et al.* 1997b; Garbayo *et al.* 1998; Green *et al.* 2000; Brandt *et al.* 2007).

PAGs belong to the vertebrate aspartic proteinase family. Therefore, they are directly related with a number of enzymes such as pepsin, chymosin, cathepsin D or renin (Hughes *et al.* 2003). Based on this relationship, a proteolytic activity in some PAGs has been discussed by different authors (Green *et al.* 1998; Telugu *et al.* 2009, 2010). They are suspected to play a role in the biochemical processing of latent growth factors at the placenta-uterine interface (Wooding *et al.* 2005). On the other hand, there are a lot of bovine PAGs (boPAGs) incapable of being enzymatically active due to amino acid substitutions around the catalytic site (Guruprasad *et al.* 1996; Xie

et al. 1997b; Wooding *et al.* 2005; Szafranska *et al.* 2006). These PAGs are hypothesized to bind other proteins or consist of an atypical binding site as described in several plasmepsins (Berry *et al.* 1999; Banerjee *et al.* 2002; Wooding *et al.* 2005). Despite the above-mentioned functions, PAGs have been proposed to have immunomodulatory and luteotrophic actions (Wallace *et al.* 2015).

In the *Bovidae* 22 different PAG genes with a wide range of temporal and spatial expression- and glycosylation patterns have been identified (Telugu *et al.* 2009; Wallace *et al.* 2015). Bovine PAG are the subject of intense research but the exact number and function of boPAG and their closely related variants remain unclear (Telugu *et al.* 2009). There are different scientific perspectives on grouping the 22 boPAGs. Phylogenetic analyses have indicated that PAGs can be clustered into at least two groups that were termed 'ancient' and 'modern' based on the time when each group arose (Hughes *et al.* 2000). The ancient group is thought to have arisen around 87 million years ago, whereas the modern one has arisen around 52 million years ago. Beside the difference in the date of origin both groups are transcribed in different cell types. The modern group (e.g. boPAG-1, -9, -21) is expressed only in a subset of trophoblast cells, the so-called binucleated cells (BNC). They are formed in the early stages of pregnancy and have been suspected to play an important role in implantation and placentation (Ishiwata *et al.* 2003; de Sousa *et al.* 2006). In addition, they are ideally positioned to adapt or regulate the maternal immune system to pregnancy (Wooding *et al.* 2005; Szafranska *et al.* 2006). Furthermore, boPAG-1 is the most commonly used PAG for pregnancy diagnosis in cattle (Wallace *et al.* 2015). The ancient group (e.g. boPAG-2, -8, -10, -11, -12) can be found in all trophoblast cells (Green *et al.* 2000; Hughes *et al.* 2000; Wooding *et al.* 2005; Telugu *et al.* 2009; Wallace *et al.* 2015). A group of PAG genes (e.g. boPAG-2) that are expressed mainly at the fetal–maternal interface may have an impact on the fetal-to-maternal surface attachment or the establishment of an immune barrier between both surfaces (Wallace *et al.* 2015).

To date, there are only a few studies about detailed boPAG expression patterns throughout pregnancy. Comparison between these studies is difficult as the results are either inconsistent or show a low coverage of pregnancy days (e.g. only specific test days but no expression profiles). Although modern PAGs are well characterized in the maternal circulation (Sasser *et al.* 1986; Friedrich & Holtz 2010) detailed information about their mRNA expression up to day 60 *post conceptionem* (p.c.) are missing. The aim of this study was to illustrate detailed mRNA expression profiles for selected modern (boPAG-1, -9, -21) and ancient (boPAG-2, -8, -10, -11, -12) bovine PAGs with a high density of measurements, especially in early gestation and throughout pregnancy using real-time quantitative PCR (qPCR). The characterization of boPAG mRNA-expression levels provides important insights for further protein analyses which are valuable information for developing new pregnancy detection systems.

Materials and methods

Sample collection

Bovine cotyledonary tissue was collected at a local abattoir. Two samples from three different cotyledons per animal were taken as biological replicates. Cotyledons of similar size and location were collected by manual separation of placentomes within 30 min of slaughter. The pregnancy stage was estimated via fetal crown-rump-length (Rexroad *et al.* 1974) and divided into three groups: early pregnancy (day 20–90, $N = 12$), mid pregnancy (day 91–180, $N = 11$) and late pregnancy (day 181 - parturition, $N = 1$). Fifty mg of tissue per cotyledon were washed with 1xPBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L $Na_2HPO_4 \cdot 2H_2O$, 1.4 mmol/L KH_2PO_4) and transferred in 1.5 mL RNAlater (Sigma Aldrich, Germany), stored at 4°C overnight and at −20°C until RNA extraction.

RNA extraction

Total RNA was extracted using TriZol Reagent (Life Technologies, USA). The protocol was followed

according to the manufacturer's recommendation with minor modifications: Samples were removed from RNAlater and briefly washed with 1x PBS, made free from fetal and maternal membranes, dissected in Petri dishes and subsequently homogenized in 1 ml TriZol Reagent using a FastPrep® FP120 Cell Disrupter (Qbiogene, USA) two times for 15 sec at 4 m s^{-1}. RNA was precipitated in ice-cold isopropyl alcohol and then washed in 1 mL of 80% ethanol. RNA pellets were eluted in 50–150 μL RNase-free water (Ambion, USA) depending on the pellet size. The RNA quantity and quality was checked immediately after RNA extraction, samples were then reverse transcribed and stored at −80°C afterwards.

Assessment of RNA quantity and quality

The RNA concentration was measured at a wavelength of 260 nm, and the purity of RNA was assessed by the absorbance at 230 and 280 nm with a NanoPhotometer® P-360 spectrophotometer (Implen, Germany). The RNA quality number (RQN) was evaluated using a microchip electrophoresis on a Fragment Analyser (Advanced Analytical Technologies Inc., USA) in a core laboratory (Transcriptome and Genome Analysis Laboratory Goettingen, Germany).

Genomic DNA contamination was tested by endpoint PCR using intron spanning primers specific to bovine *CDH1* and PAG-genes (Table 1).

cDNA synthesis

Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). A fixed amount of 500 ng total RNA was reverse transcribed at 37°C in a final volume of 20 μL containing 2x RT-Buffer, 2x RT Random Primers, 50 U MultiScribe™ Reverse Transcriptase, 4 mmol/L dNTPs and 20 U RNase Inhibitor. CDNA samples were stored at −20°C.

qPCR analysis

Relative expression levels of selected PAG-genes in cotyledons were determined using real-time quantitative PCR (qPCR) with EvaGreen detection. Cadherin-1 (*CDH1*) and Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Gamma (*YWHAG*) were selected as reference genes for normalization. Primer pairs for CDH1, boPAG-1, -2, -8, -9, -11, -12 and -21 were designed using primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) and their specificity checked with NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers for *YWHAG* and boPAG-10 were obtained from Telugu *et al.* (2009). Each PCR-fragment was sequenced and aligned to the corresponding reference sequence. The specificity was evaluated by Nucleotide-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) prior to qPCR-analyses to assess the amplification of the correct gene. All primers used in this study including their applications are given in Table 1.

PCR-products were amplified using 2 μL of cDNA, 5 mmol/L dNTPs (Roche, Switzerland), 10 μmol/L of each primer (Sigma Aldrich, Germany), 0.5 μL EvaGreen (JenaBioscience, Germany) and 1.5 U FastStart Taq-Polymerase in 1x PCR buffer containing $MgCl_2$ and 1x GC-Solution (Roche, Switzerland) in a final volume of 25 μL. For amplification of boPAG-11 1x Sulfolane (Sigma Aldrich, Germany) was added to the reaction mix.

PCRs were performed in a Stratagene Mx3005P real-time cycler (Agilent, USA) with the following thermal profile: 38 cycles of 30 s at 95°C, 30 s at 56°C and 30 s at 72°C with an additional 10-min denaturation in the first cycle. The amplification was followed by a melting curve measurement with 1 min at 95°C, 30 s at 70°C then heating the samples to 95°C, where the change in fluorescence was measured at each 0.5°C rise.

Amplification efficiencies were calculated using standard curves generated by serial dilutions of known concentrations of the target amplicons with six orders of magnitude. Samples were quantified by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001) or Pfaffl method (Pfaffl 2001), respectively. For each transcript, PCR was performed in triplicate as technical replicates.

Table 1. Oligonucleotides used for qPCR to measure relative expression levels of bovine PAG-genes during pregnancy

Gene	Accession Number	Primer	Sequence (5′-3′)	Product size (bp)	
				cDNA	gDNA
boPAG-1	NM_174411.2	for	TAGGCTCATCGGTGCCATAC	116	203
		rev	GACCTGGCACTGGGTAGTTG		
boPAG-2	NM_176614.1	for	TCCTGGAGGAACAAGCTTACA	149	1111
		rev	TGAGCCTGTGTCAAAGACGA		
boPAG-8	NM_176619.3	for	GACACCGGCTCATCTGACTT	139	229
		rev	CAGAGCCGTAGACGAGTTCA		
boPAG-9	NM_176620.2	for	TGAAGTGGATTGTGCTCCTC	149	1228
		rev	CTGGGACAGTCTGTAAGGATGC		
boPAG-10	NM_176621.3	for	TTGAGCAGTCAGAAAGAGAACG	137	-
		rev	TTCATGGAGATGCTGTCTATGTTT		
boPAG-11	NM_176623.2	for	GAAGATGACAGGAGGCAGGATAC	138	221
		rev	GTGGATACCGGGACATCACT		
boPAG-12	NM_176622.1	for	TCCTGGAAGAACGAGCTTACA	149	1113
		rev	TGAGCCTGTGTCAAAGACGA		
boPAG-21	NM_176630.2	for	TACAGGCTCATCTGACTTTTGG	141	240
		rev	CTCCCAGATCCATAGGTGATGC		
CDH1	NM_001002763.1	for	TGCCCAGAAAATGAGAAAGG	99	225
		rev	TTGGCCAGTGATGCTGTAGA		
YHWAG	NM_174793.2	for	AGCACATGCAGCCCACTC	121	-
		rev	TCGTCGAAGGCGGTCTTG		

Statistical analysis

The experimental results were analysed with R 3.2.2 (R Development Core Team, Austria). Expression levels were compared within different PAG-genes (boPAG-1, boPAG-2, boPAG-8, boPAG-10, boPAG-11, boPAG-12, boPAG-21) and different pregnancy stages (early, middle and late) with PAG-gene-pregnancy stage interactions using a two-way ANOVA. Post hoc evaluation was performed with Tukey's Honest Significant Difference (HSD) Test.

Results

Sample collection and RNA-extraction

Cotyledonary tissue from 24 animals was collected and used for RNA extraction.

RNA-samples were observed to have mean spectrometry values of 1.99 ± 0.09 for absorbance ratios A260/A280, on the other hand mean OD A260/A230 ratios were 1.4 ± 0.4. RQN values derived from a Fragment Analyser (Advanced Analytical Technologies Inc., USA) were between 5.8 and 9.9. After quality assessment a total of 21 samples with two or three biological replicates remained in the study (early pregnancy: $N = 11$; mid pregnancy: $N = 9$; late pregnancy: $N = 1$).

qPCR

Standard curves of all genes had efficiencies in the range of 95.1% to 103% and slopes in the range of −3.24 to −3.45. Gene expression ratios for boPAG-8, -9, -11, -12 and -21 were determined by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001) and according to Pfaffl (2001) for boPAG-1, -2 and -10 due to higher differences in primer efficiencies between reference genes and boPAG-genes of interest (Fig. 1).

All boPAG-genes of interest were detectable and could be quantified throughout the available pregnancy stages. A 2-way ANOVA indicated significant differences in the expression levels between PAGs ($P < 0.001$) and an interaction between boPAG-gene and pregnancy stage ($P < 0.001$) on the relative expression level. A significant overall pregnancy stage effect was not detected. In the next step a

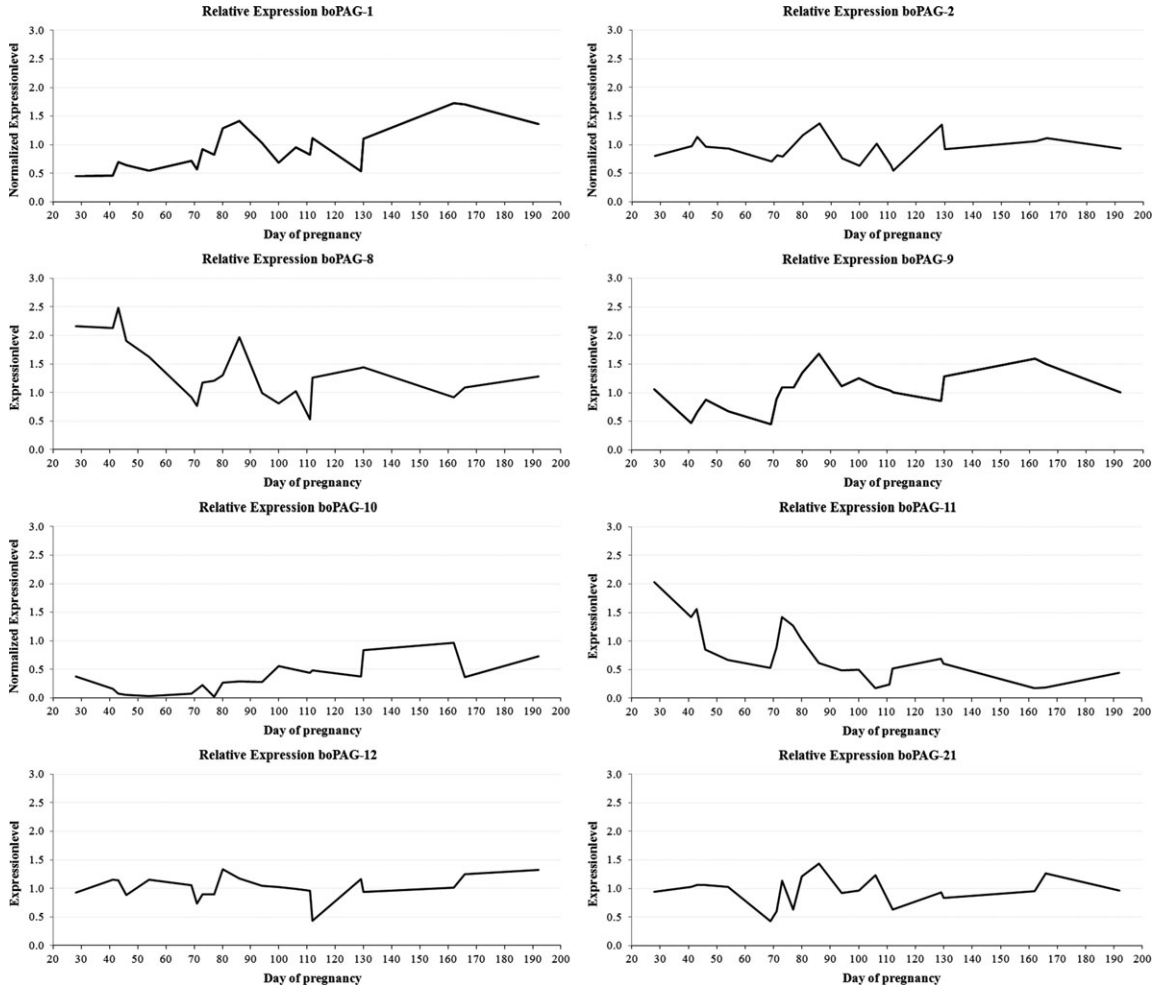

Fig. 1. Relative expression patterns of boPAG-1, -2, -8, -9, -10, -11, -12, -21. Expression levels for boPAG-1, -2 and -10 derived from efficiency normalization by Pfaffl method. Expression levels for boPAG-8, -9, -11, -12 and -21 were determined by $2^{-\Delta\Delta CT}$ method.

pairwise comparison using Tukey's HSD test was performed.

BoPAG-1 shows highest expression levels around the end of the first and second trimester. A similar pattern is given in boPAG-9 and, for the early pregnancy, in boPAG-21, which are members of the modern boPAG-group.

BoPAG-2 and -12 are the most closely related PAG-members in this investigation. Nevertheless, the expression patterns are only slightly similar with an increase towards the end of the early pregnancy and a decline between day 110 and 120.

BoPAG-8 has the highest mean expression level and boPAG-10 the lowest mean expression level, a significant difference in the mean expression level was observed in comparison with the other investigated PAGs ($P < 0.05$).

Besides that, boPAG-8, -10 and -11 transcripts show a mirror-inverted expression in cotyledonary tissue where boPAG-8 and -11 expressions are higher in the early gestation while boPAG-10 expression increases throughout pregnancy. BoPAG-1, -2, -9, -11, -12 and -21 are expressed on similar level throughout the different pregnancy stages (Fig. 2).

Pairwise comparison revealed that boPAG-8, which had the highest mean expression level in early pregnancy stage, decreases significantly from early pregnancy to mid pregnancy ($P < 0.05$) and

remained at steady-state levels between mid pregnancy and late pregnancy. A similar effect to boPAG-8 was observed in boPAG-11 with a significant decrease in mean expression between early pregnancy and mid pregnancy ($P < 0.001$) (Fig. 3). In all other examined boPAGs no significant differences between the relative expression levels within the pregnancy stages were observed.

Discussion

To our knowledge, this is the first study describing mRNA expression profiles of both ancient and modern boPAG genes in cotyledons before day 60 p.c.. In contrast to other studies that examined expression levels from different boPAGs, we did not examine specific pregnancy days but collected samples continuously. Therefore we have been able to illustrate detailed patterns of boPAG expression, especially in early gestation from day 28 p.c. onwards.

Some of the first studies on the relative quantification of bovine PAG transcripts in placental tissue were reported by Patel *et al.* (2004) and Telugu *et al.* (2009). Patel *et al.* (2004) observed a significantly higher expression of boPAG-9 on day 30 of gestation compared to boPAG-1. Our results correspond to these findings, regarding a generally higher relative expression of boPAG-9 in cotyledonary tissue although we could not determine any significant differences. Furthermore, our results do not indicate a continuous increase of boPAG-9 expression from day 30 to day 60 as reported by Patel *et al.* (2004). Instead there is a decrease in-between.

Touzard *et al.* (2013) described lowest boPAG-1 levels at day 60 of gestation and a significant increase until day 80. This expression pattern is in line with the present data, but boPAG-1 abundance increases from the beginning of gestation until day 60.

In former studies performed by Telugu *et al.* (2009) boPAG-2 was found as the most abundant transcript. Although this cannot be confirmed by our results regarding relative expression levels, the absolute expression of boPAG-2 is higher than of other transcripts. Its most closely related transcript - boPAG-12 - did not show significant differences in relative expression patterns but it's absolute abundance is lower (results not shown). So far, boPAG-21 expression was analysed in only one study with alternating up- and down-regulation (Touzard *et al.* 2013). Our results verify previous findings and additionally show that boPAG-21 is more highly expressed in the very early pregnancy and down-regulated until day 60 p.c.

The regulation of PAG transcription has not yet been elucidated (Wallace *et al.* 2015). The pre-transcriptional regulatory sequences are highly conserved within the gene family and include potential binding sites for transcription factors that might be involved in transcriptional regulation (Telugu *et al.* 2009).

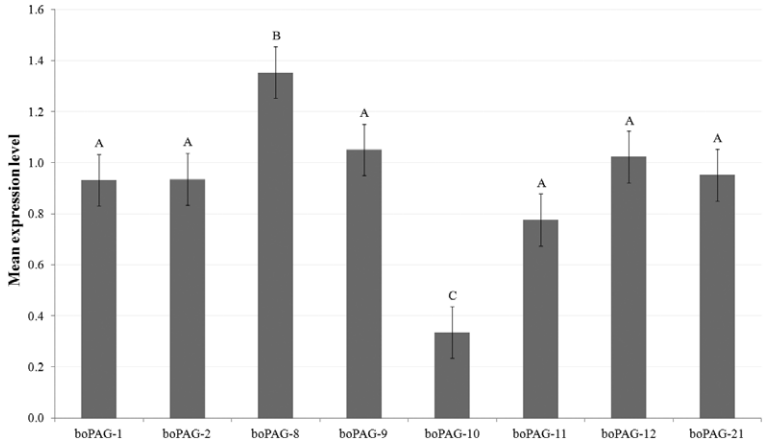

Fig. 2 Differences in gene expression levels between observed boPAG-genes. Letters indicate significant differences ($P < 0.05$).

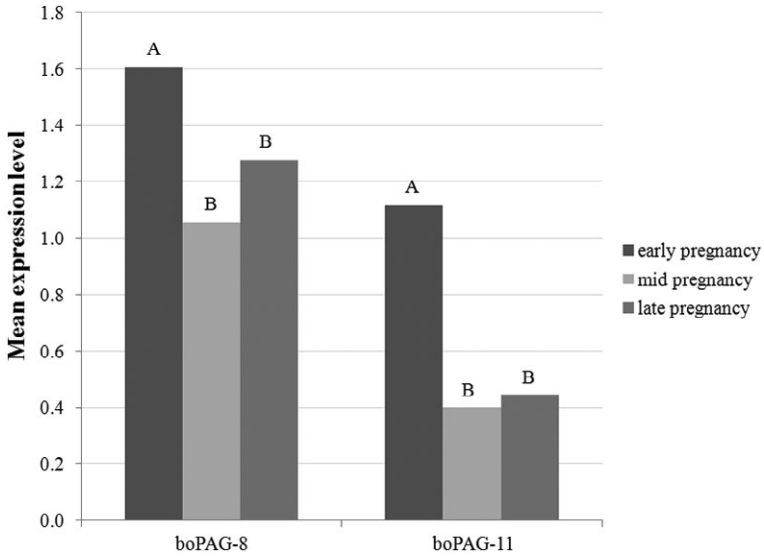

Fig. 3 Differences in gene expression levels between different pregnancy stages. Letters indicate significant differences between pregnancy stages within boPAG-8 ($P < 0.05$) and boPAG-11 ($P < 0.001$).

Our results show that the relative expression of boPAG-8 is significantly higher throughout the whole pregnancy compared to the other PAG-genes. This confirms the study of Touzard *et al.* (2013) who also described the highest expression in boPAG-8.

Furthermore, we confirm earlier studies on expression patterns of ancient boPAG-genes (Telugu *et al.* 2009): the expression of boPAG-8 and boPAG-11 is significantly higher in the early gestation and declines with progression of pregnancy with a short increase around day 80. In contrast to that boPAG-10 expression peaks in the end of second trimester, beginning of the last trimester, respectively. Nevertheless, it is the least expressed transcript in our study. In general, the present study confirms further findings, e.g. by Patel *et al.* (2004), Telugu *et al.* (2009) and Touzard *et al.* (2013) and expands the knowledge by more detailed information about boPAG-expression in early gestation. We collected samples from an abattoir and did not slaughter animals at fixed timepoints but determined the age of the fetuses by measuring the crown-rump-length which is an approximate standard for the normality of Holstein–sFriesian fetal growth (Rexroad *et al.* 1974). Nevertheless, we obtained similar expression patterns as previously described. This shows that the method by Rexroad *et al.* (1974) is still a reliable tool for estimating the age of bovine fetuses, although breeding progress took place in the Holstein-Friesian cattle bread, e.g. increased body size (Schönmuth & Löber 2006).

The low amount of samples for late pregnancies in this study is due to several reasons: on the one hand we generally observed better RNA-quality in samples from early gravidities compared to samples from mid or late pregnancies. This might be a result of higher time exposure during sample collection as the conjunction of placentomes is stronger and the detachment of cotyledonary tissue is more complicated. Previous studies already described that there is an effect of time exposure during tissue handling and RNA-integrity (Copois *et al.* 2007; Fajardy *et al.* 2009). The variability of gene expression profiles is dependent on the homogeneity of the starting material. The placenta consists of a large pattern of different cell types, fetal and maternal areas including blood of both individuals (Mondon *et al.* 2005; Fajardy *et al.* 2009). This leads to different expression patterns even in samples from one organism according to the (cell) composition of each sample. As a consequence samples or biological replicates, especially from pregnancies > 100 days, were excluded from the analyses to ensure reliable results. Nevertheless, a

minimum of two biological replicates per sample remained in the study. Another reason is a law that prohibits slaughtering healthy mammals (except goats and sheep) in the last third of gestation for commercial reasons, which came into force in September 2017 (TierErzHaVerbG, §4). On the other hand we had a high coverage of measurement points especially for <100 days of pregnancy.

According to the different expression patterns of the analysed boPAG genes, we suppose that boPAGs with a higher expression in early pregnancy (boPAG-8 and boPAG-11) seem to be important factors during placentation. Touzard *et al.* (2013) pointed out the restriction of boPAG-11 to binucleate cells located in the chorionic plate of the cotyledon. Therefore, they are ideally situated to accumulate at the placenta-uterine interface by acting as bridging molecules during cell–cell adhesion (Wallace *et al.* 2015).

The expression levels of boPAG-1, boPAG-9 and boPAG-10 are higher in the mid/late gravidity, which leads us to the suggestion that these PAGs might play a crucial role in maintenance of pregnancy. For boPAG-1, luteotrophic capabilities have already been described (Weems *et al.* 1998). Similar functions can be assumed for boPAG-2, boPAG-12 and boPAG-21.

Conclusion

In summary, it was possible to derive distinct longitudinal mRNA expression patterns and varying overall expression levels of eight different bovine PAGs in cotyledonary tissue across subsequent pregnancy stages. Although late pregnancy stages were underrepresented in our data, clear differences between early (pregnancy days 20–90) and later stages were found, especially for boPAG-8 and -11 indicating a possible role of these PAGs in placentation and the maintenance of early pregnancy.

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Conflict of interest

The authors declare that they do not have any financial and personal relationships with other people or organizations that could inappropriately bias or influence their work.

Ethical statement

The study is in accordance with the German legal and ethical requirements of appropriate animal procedures. Animals were not purposely euthanized for this study. Samples were taken during the conventional slaughter process.

Contributions

CK, IW and JT designed the study, TK collected samples, IW an NM performed molecular genetic analyses, TK analyzed the data, IW, TK and JT drafted the paper.

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