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Identification of a novel circular RNA specific to the bovine placenta from the pregnancy-associated glycoprotein gene

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Abstract

Circular RNAs (circRNAs) are covalently closed transcripts produced by back-splicing that regulate gene expression, including via microRNA (miRNA) sponge. However, trophoblast-specific circRNAs and their roles in the bovine placenta have not been well characterized. This study profiled circRNA expression in the bovine placenta during early gestation, validating abundant candidates and identifying circRNAs from trophoblast-specific genes. Sequencing of circRNAs from pooled placental tissues on day 65 of gestation revealed 13,958 circRNAs, 62% of which were derived from protein-coding regions, and were mostly 300–600 bases long. Highly expressed circRNAs were confirmed by divergent RT—PCR, Sanger sequencing of back-splice junctions, and RNase R assays, which revealed the enrichment of circRNAs. These circRNAs and their host mRNAs exhibited largely ubiquitous tissue expression, with circRNA/host ratios ranging from ~ 5–6% to > 20%. Among the circRNAs from trophoblast-specific genes, only bta_circ_2423 (from pregnancy-associated glycoprotein 4, PAG4) was confirmed to be circular and placenta-specific, showing a low circRNA/PAG4 mRNA ratio (0.85%) and peak abundance during mid-gestation. In silico analysis revealed that bta_circ_2423 binds to bta-miR-183, bta-miR-197, and bta-miR-3596, whose targets are enriched in membrane transport, metabolism, RNA regulation, and immune pathways. This study provides an early gestation bovine placental circRNA atlas and identifies a placenta-specific circRNA that potentially contributes to placental function via miRNA sponge activity.

Introduction

Circular RNA (circRNA) is a single-stranded RNA with a covalently closed circular structure formed by the joining of the 5' and 3' ends of one or more exons [1,2]. Unlike conventional splicing, which generates mRNA, circRNAs are produced through a unique mechanism called back splicing [1,2]. A loop junction connecting the 5' and 3' ends of the exons is formed, which is referred to as the back splicing junction (BSJ). Lacking terminal structures such as a 5' cap or 3' poly(A) tail, circRNAs are resistant to various ribonucleases and remain stable *in vivo* [1].

circRNAs regulate gene expression by sequestering microRNAs (miRNAs) [1]. Through this function, circRNAs are thought to influence various cellular behaviors and have been reported to be involved in the onset and progression of various diseases, as well as in physiological phenomena [3–5]. In human colorectal cancer, circSTK3, which is highly expressed, has been reported to promote tumor metastasis by inducing epithelial–mesenchymal transition and is suggested to function as a prognostic biomarker for colorectal cancer [6]. In addition, circ_0074673, expressed in human umbilical vein endothelial cells, promotes angiogenesis via the regulation of miR-1200, making it a promising candidate for elucidating the pathogenesis of gestational diabetes mellitus and as a therapeutic target [7]. In cattle, circADAMTS16 inhibits adipocyte differentiation in beef cattle [8], and circHIPK3 contributes to the proliferation of mammary epithelial cells in dairy cattle [9], have been reported. These circRNAs are expected to contribute to improvements in livestock productivity.

The placenta plays a crucial role in maintaining pregnancy and supporting fetal growth [10–12]. The trophoblast cells in the bovine placenta are responsible for the production and secretion of placenta-specific pregnancy-associated glycoproteins (PAGs) [11,13], placental lactogen [11,14], and prolactin-related proteins (PRP) [11,15,16]. These trophoblast cells are thought to play a role in the endocrine system during pregnancy through the secretion of placenta-specific proteins, although their physiological functions remain unclear. On the other hand, comprehensive analyses of circRNAs in the placenta have been reported in humans [17,18] and cattle [19]. In humans, circRNAs involved in trophoblast proliferation and invasion have been identified in various pathological conditions [20]. Su *et al.* (2019) conducted a comprehensive analysis of circRNA expression in normal and aberrant placentas generated by somatic cell nuclear transfer (SCNT) during late pregnancy in cattle, suggesting that aberrant circRNA expression may contribute to placental dysfunction in SCNT cattle. However, reports on circRNAs in the bovine placenta are limited to this study by Su et al. (2019), and trophoblast-specific circRNAs have not been identified in either humans or cattle.

In this study, we elucidated the expression profiles of circRNAs in the bovine placenta during early gestation and examined their roles in placental function. Furthermore, we attempted to identify circRNAs derived from bovine trophoblast-specific genes.

Results

CircRNA-seq analysis and characterization of highly expressed circRNAs in bovine placenta

From the circRNA-seq results, 31,079,039 raw reads and 30,612,824 clean reads were obtained, of which approximately 93% were mapped to the reference genome, resulting in the detection of 13,958 circRNAs. More than half of the detected circRNAs originated from protein-coding regions (Fig. 1a). Most sequences measured fewer than 2,000 bases in length, with sequences ranging from 300 to 600 bases comprising more than half of the total number of sequences (Fig. 1b). The most highly expressed circRNA was bta_circ_9746 (Table 1). bta_circ_9746 is hosted by the ARL13B gene, and exons 2 and 3 of ARL13B form BSJ.

Table 1
Top 10 highly expressed circRNAs in bovine placenta

circRNA ID	Host gene		Exon Count	Index of exon	Exon sizes (bases)
	Transcript ID	Symbol			
bta_circ_9746	ENSBTAT00000006796	*ARL13B*	2	2, 3	71, 250
bta_circ_8048	ENSBTAT00000071398	*STK3*	2	5, 6	165, 168
bta_circ_1913	ENSBTAT00000075879	*ILRUN*	2	3, 2	198, 155
bta_circ_6638	ENSBTAT00000001668	*HNRNPC*	3	3, 4, 5	278, 76, 48
bta_circ_6403	ENSBTAT00000007892	*CAMSAP1*	2	3, 2	162, 263
bta_circ_543	ENSBTAT00000014872	*IPMK*	3	2, 3, 4	86, 97, 173
bta_circ_1512	ENSBTAT00000063586	*BTBD7*	2	3, 2	1,080, 188
bta_circ_568	ENSBTAT00000085809	*TNKS2*	3	7, 8, 9	67, 187, 122
bta_circ_2675	ENSBTAT00000033783	*SLC26A4*	3	3, 4, 5	111, 185, 165
bta_circ_11134	ENSBTAT00000064161	*ZNF292*	3	4, 3, 2	136, 79, 155

The order is in descending order of the expression level. The exon count indicates the number of circRNA-involved exons. The index of exon indicates the index of each exon in the transcript, and Arabic numerals represent the location of exons in the transcript (2 indicates that it is the second exon in this transcript). The reverse order entries in the Index of exon column indicate those arising from antisense DNA strands in the genome. Exon sizes indicate exon length.

To detect bta_circ_9746 via RT—PCR, cDNA was synthesized from bovine placental RNA in the presence or absence of SuperScript IV reverse transcriptase (RT). PCR yielded amplicons with the anticipated molecular weights of approximately 200 bp for circ_9746 S and a larger product of approximately 500 bp for circ_9746 L, as depicted in Fig. 2a. No amplification products were detected in samples without RT. The host gene of bta_circ_9746, ARL13B, was also detected by RT—PCR (Fig. 2a). After subcloning circ_9746 S and circ_9746 L, which were detected by RT-PCR, their nucleotide sequences were analyzed using Sanger sequencing (Fig. 2b). In circ_9746 L, a longer isoform than circ_9746 S, two BSJs were found, where the 5 ‘and 3' terminals of exons 2 and 3 of ARL13B were joined. A single BSJ sequence was identified in circ_9746 S.

RNase R, which does not act on circular RNA and degrades only linear RNA, was applied to placental RNA, and cDNA was synthesized. The levels of bta_circ_9746 and ARL13B in cDNA derived from both RNase R-treated and untreated RNA were quantified using RT—qPCR (Fig. 2c). There was no difference in

the amount of bta_circ_9746 between RNase R-treated and untreated samples, whereas ARL13B decreased upon RNase R treatment compared with that in the untreated group. Examination of bta_circ_9746 and ARL13B expression in various bovine tissues revealed no distinct tissue distribution (Fig. 2d). Although the expression levels of bta_circ_9746 varied among tissues, they were approximately 20–50% of those of the host gene, ARL13B.

Similarly, for bta_circ_8048, bta_circ_1913, and bta_circ_6638, which also presented high expression levels, each circRNA and host gene mRNA were detected via divergent primers, BSJ detection by sequence analysis, and quantification of circRNA and host gene mRNA with or without RNase R treatment were performed. The results were almost identical to those of bta_circ_9746 (Supplementary Figures S1, S2, and S3). For bta_circ_8048, bta_circ_1913, and bta_circ_6638, expression was not particularly distinctive in the placenta, and the same trend was observed for host gene mRNA (Supplementary Figure S4). The ratio of circRNA to host gene expression was approximately 6% for bta_circ_8048, 5% for bta_circ_1913, and 24% for bta_circ_6638 in this study.

The miRNA-binding sites within the nucleotide sequence of bta_circ_9746 were analyzed using two data search algorithms, miRWalk, and miRanda. Each algorithm detected 12 types of miRNA-binding sites, with bta-miR-1306 being common to both (Supplementary Figure S5a and Supplementary Table S4). A search for target genes of bta-miR-1306 using miRWalk identified 1,590 genes (Supplementary Table S5). GO analysis of these genes using the PANTHER program revealed the GO terms shown in Supplementary Figure S5b.

Similarly, when searching for bta_circ_8048, bta_circ_1913, and bta_circ_6638, the miRNAs identified by both algorithms were bta-miR-2303, bta-miR-331-3p, and bta-miR-2416, bta-miR-2882, respectively (Supplementary Figures S6a, S7a, and S8a, and Supplementary Table S4). The number of target genes detected was 110 for bta-miR-2303, 1,795 for bta-miR-331-3p, 1,470 for bta-miR-2416, and 1,297 for bta-miR-2882 (Supplementary Tables S6, S7, S8 and S9). GO analysis of these genes using PANTHER revealed the GO terms shown in Supplementary Figures S6b, S7b, S7c and S8b.

Identification of bovine trophoblast-specific circRNAs

Upon close examination of the circRNA-seq results, three types, bta_circ_2015, bta_circ_2421, and bta_circ_2423, were identified, each using a bovine trophoblast-specific gene as its host (Table 2). bta_circ_2015 is a circRNA derived from prolactin-related protein 6 (PRP6), bta_circ_2421 from pregnancy-associated protein (PAG) 2, and bta_circ_2423 from PAG4 gene as the host gene mRNA.

Table 2
List of circRNAs derived from bovine trophoblast-specific genes

circRNA ID	FPM	Host gene		Exon Count	Indexof exon	Exon sizes (bases)
		Transcript ID	Symbol			
bta_circ_2015	0.035	ENSBTAG00000025302	*PRP6*	3	4, 3, 2	180, 108, 182
bta_circ_2421	0.018	ENSBTAG00000033096	*PAG2*	4	8, 7, 6, 5	99, 142, 117, 194
bta_circ_2423	0.035	ENSBTAG00000053183	*PAG4*	4	7, 6, 5, 4	99, 142, 117, 194

Fragments mapped to back-spliced junctions per million mapped fragments (FPMs) indicate the normalized circRNA expression levels. The exon count indicates the number of circRNA-involved exons. The index of exon indicates the index of each exon in the transcript, and Arabic numerals represent the location of exons in the transcript (2 indicates that it is the second exon in this transcript). The reverse order entries in the Index of exon column indicate those arising from antisense DNA strands in the genome. Exon sizes indicate exon length.

For these three circRNAs, detection was attempted using divergent primers specific to each; however, no amplification products of the expected strand length were observed for bta_circ_2015 (Supplementary Figure S9). For bta_circ_2421, an expected circRNA product of approximately 200 bp was detected (Supplementary Figure S10a). Sequence analysis of the amplified circ_2421 S revealed a single BSJ (Supplementary Figure S10b). However, no BSJ was detected in the sequences of circ_2421 L1 and L2, which were longer than those of circ_2421 S.

We conducted a similar validation for bta_circ_2423. RT—PCR targeting the sequence including the BSJ revealed the expected amplification product of approximately 200 bp (circ_2423 S) and an approximately 700 bp product (circ_2423 L) (Fig. 3a). The host gene mRNA of bta_circ_2423, PAG4 mRNA, was also detected in the placental cDNA. Sequence analysis of circ_2423 S and circ_2423 L revealed one BSJ site in circ_2423-S and two BSJ sites in circ_2423 L (Fig. 3b). The expression levels of bta_circ_2423 and PAG4 in cDNA derived from placental RNA with and without RNase R treatment were quantified using RT—qPCR (Fig. 3c). The amount of bta_circ_2423 tended to decrease following RNase R treatment compared with that in untreated samples, whereas linear PAG4 mRNA was markedly reduced by RNase R treatment.

Examination of bta_circ_2423 and PAG4 expression in various bovine tissues revealed that both were exclusively expressed in placental tissue (Fig. 3d). The bta_circ_2423/PAG4 mRNA ratio in the placenta was 0.85%. RPL27 mRNA, a housekeeping gene, was ubiquitously expressed in all examined tissues. The quantification of bta_circ_2423 and PAG4 mRNA was performed using samples of placentomal cotyledonary villi from Japanese Black cattle in early (54–65 days), middle (120–156 days), and late (224–250 days) gestation. bta_circ_2423 had the highest expression at mid-gestation, whereas PAG4

mRNA expression peaked at late gestation; both genes had the lowest expression during early pregnancy (Fig. 3e).

A search using miRWalk for miRNAs that can bind to the nucleotide sequence of bta_circ_2423 identified 104 miRNAs, each with one or more binding sites (Fig. 4a and Supplementary Table S10). In addition, a search using miRanda detected 24 miRNAs. miR-183, miR-197, and miR-3596 were commonly identified by both algorithms as miRNAs with a single binding site for bta_circ_2423 (Fig. 4b). A search using miRWalk for the target genes of bta-miR-183, bta-miR-197, and bta-miR-3596 identified 886, 1,556, and 511 target genes, respectively (Supplementary Tables S11, S12, and S13). GO analysis of the target genes was conducted using the PANTHER database. Among the target genes of bta-miR-183, in the Biological Process, numerous GO terms related to protein endosomal localization, protein polymerization, and ion membrane transport were detected (Fig. 4c). In the Molecular Function category, several GO terms associated with ion membrane transporter activity, ion binding, protein binding, and small molecule binding were identified. In the Cellular Component, GO terms related to the endosomal inner membrane, neurons, and sarcoplasmic reticulum were observed. In the target genes of bta-miR-197, GO terms such as response to triacyl lipopeptide, metabolic pathways of acetylcholine and related compounds, and vitamin D receptor signaling pathway were detected in the Biological Process (Fig. 4d). In terms of Molecular Function, O-acetyl-ADP-ribose deacetylase activity, acetylcholinesterase activity, and various phosphatase activities were observed. In addition, GABA transporter activity was also detected. In the Cellular Component, the piccolo histone acetyltransferase complex, interchromatin granule, and eIF4F complex were characteristically observed. For the target genes of bta-miR-3596, GO terms related to interleukin-22 were prominently observed in the Biological Process and Molecular Function categories, with no other notable features detected (Fig. 4e).

Discussion

This study aimed to elucidate the expression profile of circRNAs in the bovine placenta during early pregnancy and examine their roles in placental development and function. The main findings of this study are as follows:1) The circRNA expression profile in the placenta during early pregnancy was elucidated using next-generation sequencing, and for highly expressed circRNAs, their circular structure was verified via RT—PCR validation, sequence confirmation, and RNase R assays. 2) A circRNA derived from the bovine trophoblast-specific gene PAG4 was identified, and its placenta-specific expression pattern was demonstrated.

In this study, 13,958 types of circRNAs were detected in placental chorionic villi in early pregnancy, of which 62% had sequences corresponding to the protein-coding regions. More than half of these had nucleotide lengths ranging from 300 to 600 bases. In experiments in which late-gestation placentas from bovine somatic cell nuclear transfer fetuses were used, approximately 10,000 types of circRNAs were identified [19]. RNA-seq analysis of circRNA in bovine testes revealed 12,048 circRNAs in neonatal testes and 18,340 in adult testes, with approximately 75% of circRNAs being less than 1,000 bases in length [21]. In circRNA analysis using RNA-seq in bovine mammary glands, 4,000–5,000 circRNAs were detected in

lactation samples [22]. These reports support the results of the circRNA-seq analysis using early pregnancy placental samples in the present study.

To validate the results obtained from circRNA-seq, RT—PCR was performed using placental cDNA as a template and a divergent primer designed to amplify the region surrounding the BSJ of bta_circ_9746. Although the bands appeared as a smear, two major bands were detected. Sequence analysis of these PCR products revealed that the lower-molecular-weight circ_9746 S had one BSJ site, whereas the higher-molecular-weight circ_9746 L had two BSJ sites. According to the design of the divergent primers, the expected amplified product was the lower-molecular-weight circ_9746 S. In this experiment, because a reverse transcriptase lacking RNase H activity (SuperScript IV) was used, the higher molecular weight circ_9746 L was considered to be based on reverse transcription-rolling circle amplification [23]. That is, reverse transcription of circRNA by an RNase H-deficient reverse transcriptase is suggested to generate multimeric cDNA through a rolling-circle mechanism, resulting in the detection of amplified products containing multiple BSJs [24]. For bta_circ_8048, 1913, and 6638, the results were almost identical to those of bta_circ_9746. Based on the above results, bta_circ_9746, 8048, 1913, and 6638 possessed BSJs specific to each circRNA, allowing partial validation of the findings obtained by circRNA-seq. In subsequent RT—qPCR, only monomeric cDNA was amplified via reverse transcriptase with RNase H activity, which was used for quantification and other analyses, only monomeric cDNA was amplified.

Next, to verify the circular structure of circRNAs, an RNase R treatment experiment was conducted. RNase R is an exoribonuclease derived from *Escherichia coli* that degrades linear RNA from the 3’ end to the 5’ end; thus, linear mRNA is degraded by RNase R [25]. However, because circRNA possesses a circular structure with no ends, it is resistant to RNase R. When placental RNA, either treated or untreated with RNase R, was used as a template, the amounts of each circRNA and host gene mRNA were quantified using RT—qPCR. Compared with those of the untreated group, the levels of bta_circ_9746, bta_circ8048, bta_circ1913, and bta_circ_6638 were nearly unchanged following RNase R treatment, whereas the amount of each host gene mRNA was significantly reduced. These results following RNase R treatment were consistent with the results obtained for circRNAs and the host gene mRNAs expressed in the bovine mammary gland [22]. These findings indicate that bta_circ9746, bta_circ8048, bta_circ1913, and bta_circ_6638 are RNAs that maintain a circular structure.

To clarify the tissue distribution of these circRNAs expressed in the placenta, we examined their expression in various bovine tissues, along with that of their host gene mRNAs. The expression levels of bta_circ9746, bta_circ8048, bta_circ1913, and bta_circ_6638 were ubiquitous across all tissues. Furthermore, the expression patterns of these circRNAs closely resembled those of their respective host gene mRNAs. Recent studies suggest that circRNA expression is tissue- or cell type-specific, subject to specific regulation during development or disease progression, and is thought to play specific biological roles in these contexts [26]. In contrast, some circRNAs, such as human circTNFAIP3, are widely expressed across multiple tissues [27]. The four circRNAs examined in this study lacked tissue specificity, similar to circTNFAIP3.

The expression levels of many circRNAs are reportedly less than 10% of those of their host linear mRNAs [28–30]. However, for some circRNAs, expression is independent of that of their host linear mRNAs, and in certain cases, they have even been reported to be expressed at higher levels than their hosts [31–34]. In this study, bta_circ_8048 and bta_circ_1913 were found to be expressed at less than 10%, comparable to many previously examined circRNAs. Conversely, bta_circ_9746 and bta_circ_6638 were expressed at relatively high levels, exceeding 20% of their host linear mRNAs.

circRNAs may contain miRNA-binding sites within their nucleotide sequences and are thought to possess a “sponge” function, adsorbing and releasing miRNAs [35]. miRNAs are single-stranded RNA of 18–25 nucleotides that bind to the 3' untranslated region of the target mRNA to suppress translation, thereby playing diverse physiological roles, including apoptosis, cell proliferation, and development [36]. circRNAs are considered to regulate the expression of specific genes through their miRNA sponge activity and are believed to play an important role in the functional manifestation of various tissues. Based on the results of this study, miRNA binding sites were suggested within the sequences of the four circRNAs, indicating that they may exert physiological functions by acting as sponges for these miRNAs. Therefore, GO analysis was performed on the target mRNAs of the miRNAs presumed to be captured by each circRNA to infer the circRNA–miRNA–mRNA axis. For bta_circ_9746, terms related to sphingolipid synthesis and metabolism were detected in the Biological Process category, whereas terms related to transcription were enriched in the Molecular Function category. In the analyses of bta_circ_8048, bta_circ_1913, and bta_circ_6638, several terms were also enriched; however, their significance remains unclear.

In addition to the aforementioned findings, this study identified, for the first time, circRNAs derived from bovine trophoblast-specific genes. Among the circRNAs detected by circRNA-seq were bta_circ_2015, bta_circ_2421, and bta_circ_2423, which originated from PRP6, PAG2, and PAG4, respectively. Unfortunately, in this study, bta_circ_2015 and bta_circ_2421 could not be identified as circular RNAs. For bta_circ_2421, although a product retaining a single BSJ was obtained from sequence analysis, a product with two BSJs produced by rolling circle amplification was not confirmed. The expression levels of these two circRNAs may be low, and further detailed investigations are necessary in the future. In contrast, bta_circ_2423 was demonstrated to be an RNA with a circular structure through RT–PCR validation, sequence confirmation, and RNase R assays. In the RNase R assays, the amount of bta_circ_2423 in the RNase R-treated group was reduced to approximately half of that in the untreated group. However, since the host gene mRNA PAG4 was almost completely absent in the RNase R-treated group, it was determined that placental cDNA contained RNase R-resistant circular RNA. A similar phenomenon has been reported for circRNA expression in the goat endometrium; however, the underlying mechanism remains unclear [37].

It was found that bta_circ_2423, like its host gene PAG4 mRNA, is specifically expressed in the placenta of cows. To date, comprehensive analyses, such as circRNA-seq, have enabled the investigation of circRNA expression in the placentas of humans [18] and cattle [19]; however, trophoblast-specific circRNAs

have not yet been identified in these studies. In this study, we identified bta_circ_2423 for the first time as a circRNA derived from a trophoblast-specific gene that is specifically expressed in the bovine placenta.

Both PAG4 mRNA and bta_circ_2423 were continuously expressed in the placenta from early to late pregnancy, with their expression increasing in the middle and late stages compared to that in early pregnancy. Since it has been shown that PAG4 is expressed in the placenta from day 25 to day 250 of pregnancy [38], the PAG4 expression profile in this study was similar to that pattern. Taken together, the results of this study elucidate the expression dynamics of bta_circ_2423 during pregnancy.

In this study, we showed that bta_circ_2423 binds to three types of miRNAs, and that each of these miRNAs has approximately 500–1,600 candidate target genes. The target gene sets of each miRNA were predicted to possess functions not only in nonspecific physiological processes such as membrane transport, metabolism, and RNA splicing, but also in functions strongly associated with the placenta and pregnancy. The target gene sets of bta-miR-197 and bta-miR-3596 included multiple immune-related GO terms, such as bacterial response and interleukin-22 (IL-22)-mediated signaling pathway. The immune system in the placenta has two aspects, one of which is its defensive function against pathogens. In humans, intra-amniotic infection by bacteria is known to cause preterm birth and abnormalities in both the fetus and mother; thus, this defensive function is considered important [39]. Another aspect of placental immunity is immune tolerance toward semi-allogeneic fetuses [40]. IL-22 is a cytokine produced by CD^{4+} T cells, and in humans, the interaction between IL-22 and IL-4 production by decidual $CD4^{+}$ T cells is suggested to be involved in miscarriage [41]. Thus, bta_circ_2423 may also be involved in immune mechanisms during pregnancy through its miRNA sponge activity against bta-miR-197 and bta-miR-3596. However, in this study, we only identified the circular form of PAG4 RNA (bta_circ_2423), which is specifically expressed in the bovine placenta, and estimated its function. Therefore, it is necessary to examine the function of this circular form of PAG4 RNA in detail through *in vitro* experiments to clarify its role.

Through a comprehensive analysis of circRNAs expressed in the bovine placenta during early pregnancy, we identified, for the first time, a trophoblast-specific circular PAG RNA in cattle. Analysis of the circRNA–miRNA–mRNA network indicated that this circular PAG RNA may contribute to placental function through miRNA sponging activity. PAG is a protein highly expressed in the placenta of species belonging to Cetartiodactyla, and 21 PAG genes have been identified in cattle [13,42]. PAG is produced in trophoblast cells, but its function remains unknown and has only been implicated in the modulation of immune activity [43,44] and maintenance of pregnancy [45]. This study suggests that circular PAG4 RNA (bta_circ_2423) may interact with three miRNAs. Thus, in addition to the previously considered role of PAG as a protein, circular PAG RNA may perform novel physiological functions by regulating gene expression through the miRNA sponge activity.

Methods

Animals and sample collection

The present study utilized bovine tissues that were previously collected and preserved by Koshi *et al.*[46], Ohta *et al.*[47], and Baba *et al.*[48]. Tissue collection in these studies was conducted following approval by the Iwate University Laboratory Animal Care and Use Committee (A201043 and A201336) and in accordance with relevant guidelines and regulations, including the Guidelines for Proper Conduct of Animal Experiments by the Science Council of Japan. No new tissue samples were collected for the current study. Placental tissues around day 60 (days 54, 55, 64, 64, and 65, n = 5), middle (days 120, 150, and 156, n = 3), and late (days 224, 240, and 250, n = 3) of gestation and the endometrium during the estrous cycle were collected from Japanese Black cows, as previously reported [46–48]. The bovine placentome consists of a fetal cotyledon and maternal caruncle. In the present study, we used cotyledons as placental tissues. Bovine tissues, including the lung, heart, liver, spleen, testis, skin, and muscle, were obtained from three Holstein cattle, as previously reported [48].

RNA extraction

RNA was extracted from tissue samples using TRIzol Reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. The extracted RNA was DNase-treated using the TURBO DNase-free Kit (Thermo Fisher Scientific, Waltham, MA, USA) or RNase-Free DNase Set (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

circRNA sequencing and data analysis

RNA library preparation, circRNA sequencing, and data analysis were performed by Novogene Co. LTD (Beijing, China), as previously reported by Wang *et al.* [49]

For library preparation, pooled placental RNA from three cows around day 65 (days 64, 64, and 65) of gestation was used. Ribosomal RNA was removed by Ribo-zero rRNA Removal Kit (Epicentre Biotechnologies, Madison, WI, USA) from total RNA, and rRNA free residue was cleaned up by ethanol precipitation. Subsequently, linear RNA was digested with RNase R (Epicentre Biotechnologies). Sequencing libraries were generated using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) following the manufacturer's recommendations. Libraries were sequenced using an Illumina NovaSeq 6000 system (Illumina, San Diego, CA, USA). Raw reads were deposited in the DNA Data Bank of Japan (DDBJ) under the accession number PRJDB16827.

Clean reads were used for all downstream analyses. The reference genome and gene model annotation files were downloaded directly from the genome website browser. Paired-end clean reads were aligned to the reference genome using Spliced Transcripts Alignment to a Reference (STAR) software (Dobin et al., 2013). An upgraded computational pipeline (CIRCexplorer2) was applied to detect circRNAs and identify alternative (back-) back-splicing in back-spliced circRNAs [34,50]. The expression of circRNAs is usually represented by fragments that are mapped to the back-spliced exon–exon junction sites. In

addition to the raw fragment numbers, normalized RNA-seq fragments that were mapped to a specific back-spliced exon–exon junction by total mapped fragments (fragments mapped to back-spliced junction Per Million mapped fragments, FPM) were used to quantify circRNA expression.

RT–PCR and nucleotide sequence analysis

One to two half micrograms of total RNA was reverse transcribed into cDNA using SuperScript IV VILO Master Mix (Thermo Fisher Scientific) or High-Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Conventional RT-PCR was performed using cDNA synthesized using the SuperScript IV VILO Master Mix and TaKaRa Ex Taq (TaKaRa, Shiga, Japan). PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. The amplified products were subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced using a BigDye Terminator Cycle Sequencing Kit and an automated sequencer (Applied Biosystems). The primers used for conventional RT-PCR are listed in Supplementary Table S1. RT-qPCR was performed using cDNA synthesized using the High-Capacity Reverse Transcription kit, Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), and ABI7300 real-time PCR system (Applied Biosystems), as described previously [51]. For the quantitative analysis of bta_circ_2421 and bta_circ_2423, TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) with TaqMan MGB probes (bta_circ_2421, CTACATCATCAAGATCG; bta_circ_2423, CCTACATCCTCAAGATT) was used for quantitative analysis. The primers used are listed in Supplementary Table S2. The thermal cycling conditions included initial sample incubation at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. To quantify the mRNA copy number, a standard curve was generated for each gene using a serial dilution of synthetic oligonucleotides containing the corresponding circRNA and mRNA sequences (Supplementary Table S3). The dissociation curve for the detection of the SYBR Green-based objective amplicon was confirmed, and the circRNA and mRNA copy numbers were determined at each amplicon peak point. The ratio of the mRNA expression level of each gene to that of the gene encoding ribosomal protein L 27 (RPL27) and beta-actin was calculated to adjust for variations in RT–qPCR. To detect circRNAs using conventional RT–PCR and RT–qPCR, we used divergent primers designed to amplify sequences around the BSJ [52].

RNase R treatment

RNase R treatment of total RNA was performed as described by Gu *et al*. [53]. Briefly, placental RNA isolated from approximately day 60 of gestation was incubated with or without 0.05 U/μL RNase R (Epicentre Biotechnologies, Madison, WI, USA) at 37°C for 45 min in a buffer containing 20 mM Tris-HCl (pH 8.0), 0.1 M KCl, and 0.11 mM $MgCl_2$. After incubation for 10 min at 70°C, cDNA was prepared using the High-Capacity Reverse Transcription kit (Applied Biosystems) in accordance with the manufacturer's instructions.

Construction of a competing endogenous RNA network of circRNAs-miRNAs-mRNAs

Target sites for miRNAs on circRNA nucleotide sequences were identified using the miRNA target prediction software miRWalk [54] and miRanda [55]. From the results of the two algorithms, miRNAs that were considered to bind to the same circRNAs were selected. Target genes for these miRNAs were searched using miRWalk, and the resulting gene set was subjected to Gene Ontology (GO) enrichment analysis using the PANTHER classification system [56], which was used to examine the enrichment of GO terms for Biological Process, Molecular Function, and Cellular Component.

Statistical analysis

The expression levels of bta_circ_2423 and PAG4 mRNA from early to late gestation were quantified using RT-qPCR. The data were subsequently analyzed using one-way ANOVA, followed by Tukey–Kramer test, employing JMP version 7 software (SAS Institute Inc., Cary, NC, USA). Results with p-values < 0.05 were considered significant.

Declarations

Competing interests

The authors declare no competing interests.

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Author Contribution

N.A. and M.S. designed the study, performed the experiments, drafted the initial manuscript, and critically reviewed the manuscript. T.A. performed the experiments. H.Y. and T.K. performed the experiments and critically reviewed the manuscript. T.I.O. critically reviewed and revised the manuscript. K.K. conceptualized and designed the study, coordinated and supervised the data collection, and critically reviewed and revised the manuscript.

Data Availability

The sequencing data supporting the findings of this study were deposited in the DNA Data Bank of Japan (DDBJ) under the accession number PRJDB16827.

References

1. Lasda, E. & Parker, R. Circular RNAs: diversity of form and function. *RNA* **20**, 1829–1842. https://doi.org/10.1261/rna.047126.114 (2014).
2. Li, X., Yang, L. & Chen, L. L. The Biogenesis, Functions, and Challenges of Circular RNAs. *Mol. Cell*. **71**, 428–442. https://doi.org/10.1016/J.MOLCEL.2018.06.034 (2018).
3. Abu, N. & Jamal, R. Circular RNAs as Promising Biomarkers: A Mini-Review. *Front. Physiol.* **7**, 355–355. https://doi.org/10.3389/FPHYS.2016.00355 (2016).
4. Haque, S., Harries, L. W., Haque, S. & Harries, L. W. Circular RNAs (circRNAs) in Health and Disease. *Genes* **8**, 353–353. https://doi.org/10.3390/genes8120353 (2017).
5. Greene, J. et al. Circular RNAs: Biogenesis, function and role in human diseases. *Front. Mol. Biosci.* **4**, 265463–265463. https://doi.org/10.3389/FMOLB.2017.00038/BIBTEX (2017).
6. Fan, B. et al. CircSTK3 drives the metastasis of colorectal cancer by regulating epithelial-mesenchymal transition. *iScience* **26**, 106170. https://doi.org/10.1016/j.isci.2023.106170 (2023).
7. Huang, Y., Liang, B. & Chen, X. Exosomal circular RNA circ_0074673 regulates the proliferation, migration, and angiogenesis of human umbilical vein endothelial cells via the microRNA-1200/MEOX2 axis. *Bioengineered* **12**, 6782–6792. https://doi.org/10.1080/21655979.2021.1967077 (2021).
8. Hu, C. et al. CircADAMTS16 Inhibits Differentiation and Promotes Proliferation of Bovine Adipocytes by Targeting miR-10167-3p. *Cells* 12 (2023). https://doi.org/10.3390/cells12081175
9. Liu, J. et al. Prolactin-Responsive Circular RNA circHIPK3 Promotes Proliferation of Mammary Epithelial Cells from Dairy Cow. *Genes (Basel)*. 11. https://doi.org/10.3390/genes11030336 (2020).
10. Abdelhady, A., Kelleher, A. M. & Spencer, T. E. Trophoblast differentiation in the bovine placenta. *J. Reprod. Dev.* **72**, 466–475. https://doi.org/10.1262/jrd.2025-121 (2026).
11. Davenport, K. M., Ortega, M. S., Johnson, G. A., Seo, H. & Spencer, T. E. Review: Implantation and placentation in ruminants. *Animal 17 Suppl.* (1), 100796. https://doi.org/10.1016/j.animal.2023.100796 (2023).
12. Johnson, G. A. et al. Understanding placentation in ruminants: a review focusing on cows and sheep. *Reprod. Fertil. Dev.* **36**, 93–111. https://doi.org/10.1071/rd23119 (2023).
13. Wallace, R. M., Pohler, K. G., Smith, M. F. & Green, J. A. Placental PAGs: gene origins, expression patterns, and use as markers of pregnancy. *Reproduction* **149**, R115–R126. https://doi.org/10.1530/rep-14-0485 (2015).
14. Byatt, J. C., Shimomura, K., Duello, T. M. & Bremel, R. D. Isolation and characterization of multiple forms of bovine placental lactogen from secretory granules of the fetal cotyledon. *Endocrinology* **119**, 1343–1350. https://doi.org/10.1210/endo-119-3-1343 (1986).
15. Zieler, C. G., Kessler, M. A. & Schuler, L. A. Characterization of a novel prolactin-related protein from bovine fetal placenta. *Endocrinology* **126**, 2377–2382. https://doi.org/10.1210/endo-126-5-2377 (1990).

16. Soares, M. J., Konno, T. & Alam, S. M. The prolactin family: effectors of pregnancy-dependent adaptations. *Trends Endocrinol. Metabolism*. **18**, 114–121. https://doi.org/10.1016/j.tem.2007.02.005 (2007).
17. Maass, P. G. et al. A map of human circular RNAs in clinically relevant tissues. *J. Mol. Med. (Berl)*. **95**, 1179–1189. https://doi.org/10.1007/s00109-017-1582-9 (2017).
18. Qian, Y. et al. Potential Significance of Circular RNA in Human Placental Tissue for Patients with Preeclampsia. *Cell. Physiol. Biochem.* **39**, 1380–1390. https://doi.org/10.1159/000447842 (2016).
19. Su, X. et al. CircRNA expression profile of bovine placentas in late gestation with aberrant SCNT fetus. *J. Clin. Lab. Anal.* **33**, 22918–22918. https://doi.org/10.1002/jcla.22918 (2019).
20. Ou, Y. et al. Circular RNA circ_0111277 attenuates human trophoblast cell invasion and migration by regulating miR-494/HTRA1/Notch-1 signal pathway in pre-eclampsia. *Cell. Death Dis.* **11**, 479. https://doi.org/10.1038/s41419-020-2679-6 (2020).
21. Gao, Y. et al. Identification and characterization of circular RNAs in Qinchuan cattle testis. *Royal Soc. open. Sci.* **5** https://doi.org/10.1098/RSOS.180413 (2018).
22. Zhang, C. et al. Circular RNA of cattle casein genes are highly expressed in bovine mammary gland. *J. Dairy Sci.* **99**, 4750–4760. https://doi.org/10.3168/JDS.2015-10381 (2016).
23. Liu, Y. et al. Direct detection of circRNA in real samples using reverse transcription-rolling circle amplification. *Anal. Chim. Acta*. **1101**, 169–175. https://doi.org/10.1016/J.ACA.2019.12.027 (2020).
24. Boss, M., Arenz, C. A. & Fast and Easy Method for Specific Detection of Circular RNA by Rolling-Circle Amplification. *ChemBioChem* 21, 793–796 (2020). https://doi.org/10.1002/cbic.201900514
25. Hossain, S. T., Malhotra, A. & Deutscher, M. P. How RNase R Degrades Structured RNA. *J. Biol. Chem.* **291**, 7877–7887. https://doi.org/10.1074/jbc.m116.717991 (2016).
26. Misir, S., Wu, N. & Yang, B. B. Specific expression and functions of circular RNAs. *Cell Death & Differentiation 2022 29:3* 29, 481–491 (2022). https://doi.org/10.1038/s41418-022-00948-7
27. Du, L. et al. A Previously Undiscovered Circular RNA, circTNFAIP3, and Its Role in Coronavirus Replication. *mBio* 12 (2021). https://doi.org/10.1128/MBIO.02984-21/ASSET/F3DA7B00-1615-442F-B4F9-5782D5A3AF43/ASSETS/IMAGES/MEDIUM/MBIO.02984-21-F009.GIF
28. Guo, J. U., Agarwal, V., Guo, H. & Bartel, D. P. Expanded identification and characterization of mammalian circular RNAs. *Genome Biol.* **15**, 1–14. https://doi.org/10.1186/S13059-014-0409-Z/FIGURES/6 (2014).
29. Jeck, W. R. et al. Circular RNAs are abundant, conserved, and associated with ALU repeats. *RNA* **19**, 141–157. https://doi.org/10.1261/RNA.035667.112 (2013).
30. Salzman, J., Chen, R. E., Olsen, M. N., Wang, P. L. & Brown, P. O. Cell-Type Specific Features of Circular RNA Expression. *PLoS Genet.* **9**, e1003777–e1003777. https://doi.org/10.1371/JOURNAL.PGEN.1003777 (2013).
31. Conn, S. J. et al. The RNA Binding Protein Quaking Regulates Formation of circRNAs. *Cell* **160**, 1125–1134. https://doi.org/10.1016/J.CELL.2015.02.014 (2015).

32. Rybak-Wolf, A. et al. Circular RNAs in the Mammalian Brain Are Highly Abundant, Conserved, and Dynamically Expressed. *Mol. Cell.* **58**, 870–885. https://doi.org/10.1016/j.molcel.2015.03.027 (2015).
33. You, X. et al. Neural circular RNAs are derived from synaptic genes and regulated by development and plasticity. *Nature Neuroscience 2015 18:4* 18, 603–610 (2015). https://doi.org/10.1038/nn.3975
34. Zhang, X. O. et al. Diverse alternative back-splicing and alternative splicing landscape of circular RNAs. *Genome Res.* **26**, 1277–1287. https://doi.org/10.1101/GR.202895.115 (2016).
35. Ma, B. et al. Mechanisms of circRNA/lncRNA-miRNA interactions and applications in disease and drug research. *Biomed. Pharmacother*. **162**, 114672. https://doi.org/10.1016/j.biopha.2023.114672 (2023).
36. Bartel, D. P. & MicroRNAs Genomics, Biogenesis, Mechanism, and Function. *Cell* **116**, 281–297. https://doi.org/10.1016/S0092-8674(04)00045-5 (2004).
37. Song, Y. et al. Analyses of circRNA profiling during the development from pre-receptive to receptive phases in the goat endometrium. *J. Anim. Sci. Biotechnol.* **10**, 34. https://doi.org/10.1186/s40104-019-0339-4 (2019).
38. Green, J. A. et al. Pregnancy-Associated Bovine and Ovine Glycoproteins Exhibit Spatially and Temporally Distinct Expression Patterns During Pregnancy 1. *Biol. Reprod.* **62**, 1624–1631 (2000).
39. Šket, T., Ramuta, T., Starčič Erjavec, M. & Kreft, M. E. The Role of Innate Immune System in the Human Amniotic Membrane and Human Amniotic Fluid in Protection Against Intra-Amniotic Infections and Inflammation. *Front. Immunol.* **12**, 735324. https://doi.org/10.3389/fimmu.2021.735324 (2021).
40. Ander, S. E., Diamond, M. S. & Coyne, C. B. Immune responses at the maternal-fetal interface. *Sci. Immunol.* **4** https://doi.org/10.1126/sciimmunol.aat6114 (2019).
41. Logiodice, F. et al. Decidual Interleukin-22-Producing CD4 + T Cells (Th17/Th0/IL-22 + and Th17/Th2/IL-22+, Th2/IL-22+, Th0/IL-22+), Which Also Produce IL-4, Are Involved in the Success of Pregnancy. *Int. J. Mol. Sci.* **20** https://doi.org/10.3390/ijms20020428 (2019).
42. Touzard, E. et al. Specific expression patterns and cell distribution of ancient and modern PAG in bovine placenta during pregnancy. *Reproduction* **146**, 347–362. https://doi.org/10.1530/rep-13-0143 (2013).
43. Dosogne, H. et al. Pregnancy-associated glycoprotein and decreased polymorphonuclear leukocyte function in early post-partum dairy cows. *Vet. Immunol. Immunopathol.* **67**, 47–54. https://doi.org/10.1016/s0165-2427(98)00215-3 (1999).
44. Dosogne, H., Massart-Leën, A. M. & Burvenich, C. Immunological aspects of pregnancy-associated glycoproteins. *Adv. Exp. Med. Biol.* **480**, 295–305. https://doi.org/10.1007/0-306-46832-8_34 (2000).
45. Thompson, I. M. et al. Effects of lactation and pregnancy on metabolic and hormonal responses and expression of selected conceptus and endometrial genes of Holstein dairy cattle. *J. Dairy. Sci.* **95**, 5645–5656. https://doi.org/10.3168/jds.2011-5113 (2012).

46. Koshi, K., Ushizawa, K., Kizaki, K., Takahashi, T. & Hashizume, K. Expression of endogenous retrovirus-like transcripts in bovine trophoblastic cells. *Placenta* **32**, 493–499. https://doi.org/10.1016/j.placenta.2011.04.002 (2011).
47. Ohta, T. et al. Expression profiles of perforin, granzyme B and granulysin genes during the estrous cycle and gestation in the bovine endometrium. *Anim. Sci. J. = Nihon chikusan Gakkaihō*. **85**, 763–769. https://doi.org/10.1111/asj.12209 (2014).
48. Baba, K., Nakaya, Y., Shojima, T. & Muroi, Y. Identification of novel endogenous betaretroviruses which are transcribed in the bovine placenta. *Journal of …* 85, 1237–1245 (2011). https://doi.org/10.1128/JVI.01234-10.
49. Wang, D., Luo, Y., Wang, G. & Yang, Q. Circular RNA expression profiles and bioinformatics analysis in ovarian endometriosis. *Mol. Genet. Genom. Med.* **7**, e00756–e00756. https://doi.org/10.1002/MGG3.756 (2019).
50. Dong, R., Ma, X. K., Chen, L. L. & Yang, L. Genome-Wide Annotation of circRNAs and Their Alternative Back-Splicing/Splicing with CIRCexplorer Pipeline. *Methods Mol. Biol.* **1870**, 137–149. https://doi.org/10.1007/978-1-4939-8808-2_10 (2019).
51. Kikuchi, M. et al. Newly identified interferon tau-responsive Hes family BHLH transcription factor 4 and cytidine/uridine monophosphate kinase 2 genes in peripheral blood granulocytes during early pregnancy in cows. *Domest. Anim. Endocrinol.* **68**, 64–72. https://doi.org/10.1016/j.domaniend.2019.01.006 (2019).
52. Pandey, P. R. et al. Methods for analysis of circular RNAs. *Wiley Interdisciplinary Reviews: RNA*. **11**, e1566–e1566. https://doi.org/10.1002/WRNA.1566 (2020).
53. Gu, X. et al. Circular RNA hsa_circ_101555 promotes hepatocellular carcinoma cell proliferation and migration by sponging miR-145-5p and regulating CDCA3 expression. *Cell Death & Disease 2021 12:4* 12, 1–18 (2021). https://doi.org/10.1038/s41419-021-03626-7
54. Sticht, C., De La Torre, C., Parveen, A., Gretz, N. & miRWalk An online resource for prediction of microRNA binding sites. *PLOS ONE*. **13**, e0206239–e0206239. https://doi.org/10.1371/JOURNAL.PONE.0206239 (2018).
55. John, B. et al. Human MicroRNA Targets. *PLoS Biol.* **2**, e363–e363. https://doi.org/10.1371/JOURNAL.PBIO.0020363 (2004).
56. Mi, H., Muruganujan, A., Casagrande, J. T. & Thomas, P. D. Large-scale gene function analysis with the PANTHER classification system. *Nat. Protoc.* **8**, 1551–1566. https://doi.org/10.1038/nprot.2013.092 (2013).

Figures

Figure 1

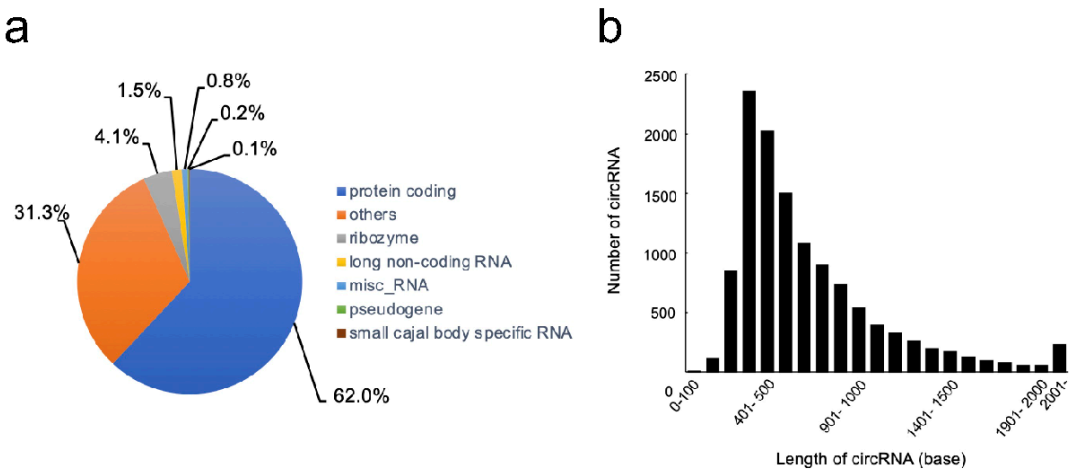

Figure 1

General characteristics of the circRNAs in bovine placenta by circRNA-seq analysis. (a) Classification of mapped reads into known types of RNAs. The clean reads were aligned to the reference genome, and the RNAs were quantified based on annotation using the union model. (b) Length distribution of circRNAs. The length distribution of circRNAs from all samples is shown in 100 base intervals and >2001.

Figure 2

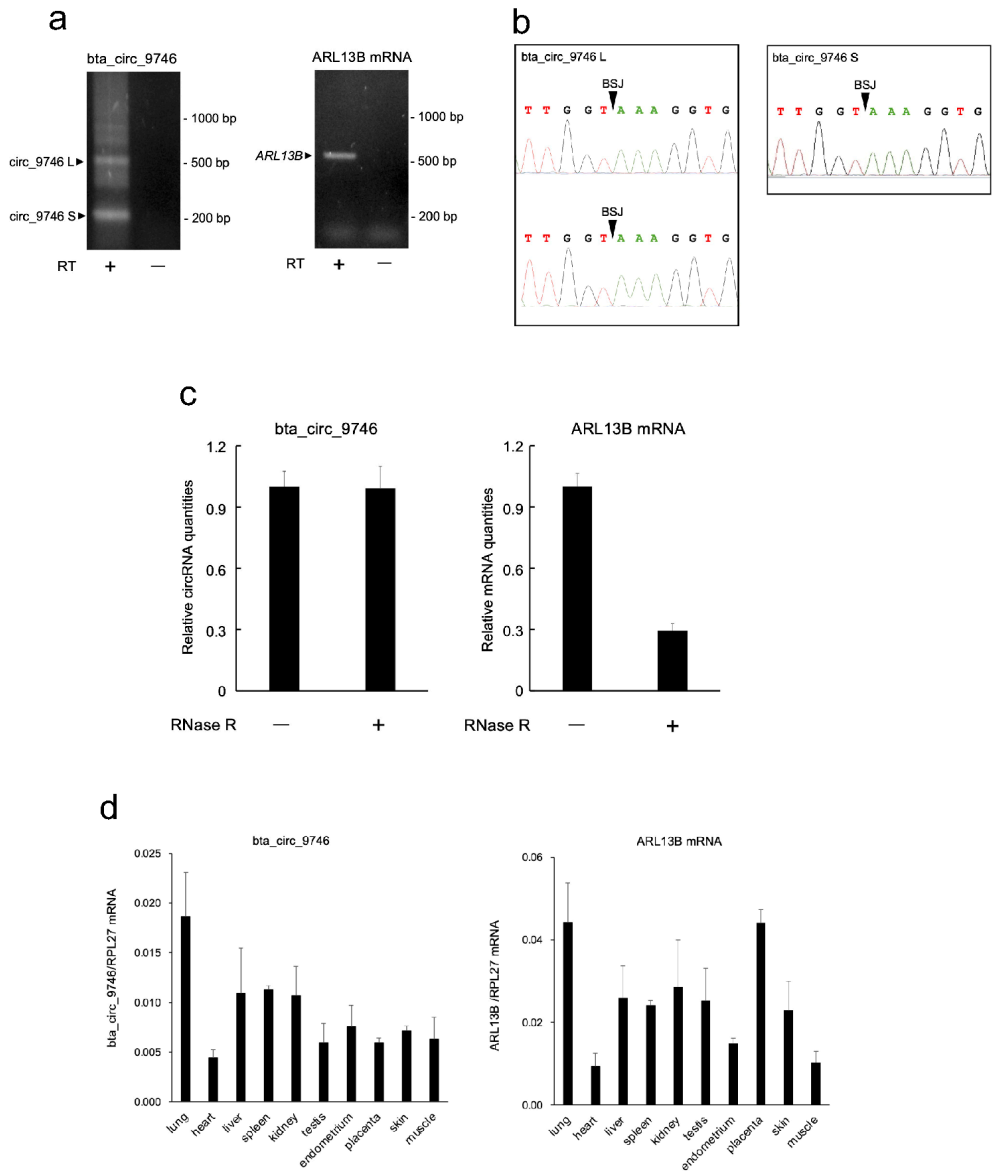

Figure 2

Identification of bta_circ_9746. (a) cDNA was synthesized from bovine placental total RNA with (RT+) or without (RT −) SuperScript IV reverse transcriptase. The expression of circ_9746 (left) and its host gene mRNA, ARL13B (right), was measured using conventional PCR. The circ_9746 L and S in panel (left) are indicate long- and short-form PCR products of circ_9746 RT-PCR, respectively. (b) RT-PCR products for circ_9746 L (left) and S (right) were subcloned into a sequencing vector, and sequencing analysis was

performed using Sanger sequencing. BSJ sites of circRNAs are indicated by black arrowheads on the DNA sequence chromatograms. (c) Total RNA isolated from the bovine placenta was treated with or without RNase R, and cDNA was synthesized. The quantities of circ_9746 (left) and ARL13B (right) in cDNA synthesized from placental RNA treated with or without RNase R (RNase R + or -) were measured using RT-qPCR. The circRNA and mRNA levels are indicated relative to the quantities of RNase R-untreated control (RNase R -). The values are expressed as mean ± SE (n=5). (d) Expression of bta_circ_9746 (left) and ARL13B (right) in various bovine tissues, including the lung, heart, liver, spleen, kidney, testis, endometrium, placenta, skin, and muscle, was analyzed using RT-qPCR. Cotyledonary tissue was collected around Day 60 of gestation as a bovine placental sample. The values are shown as the mean ± SE (placenta, n=5; other samples, n=3).

Figure 3

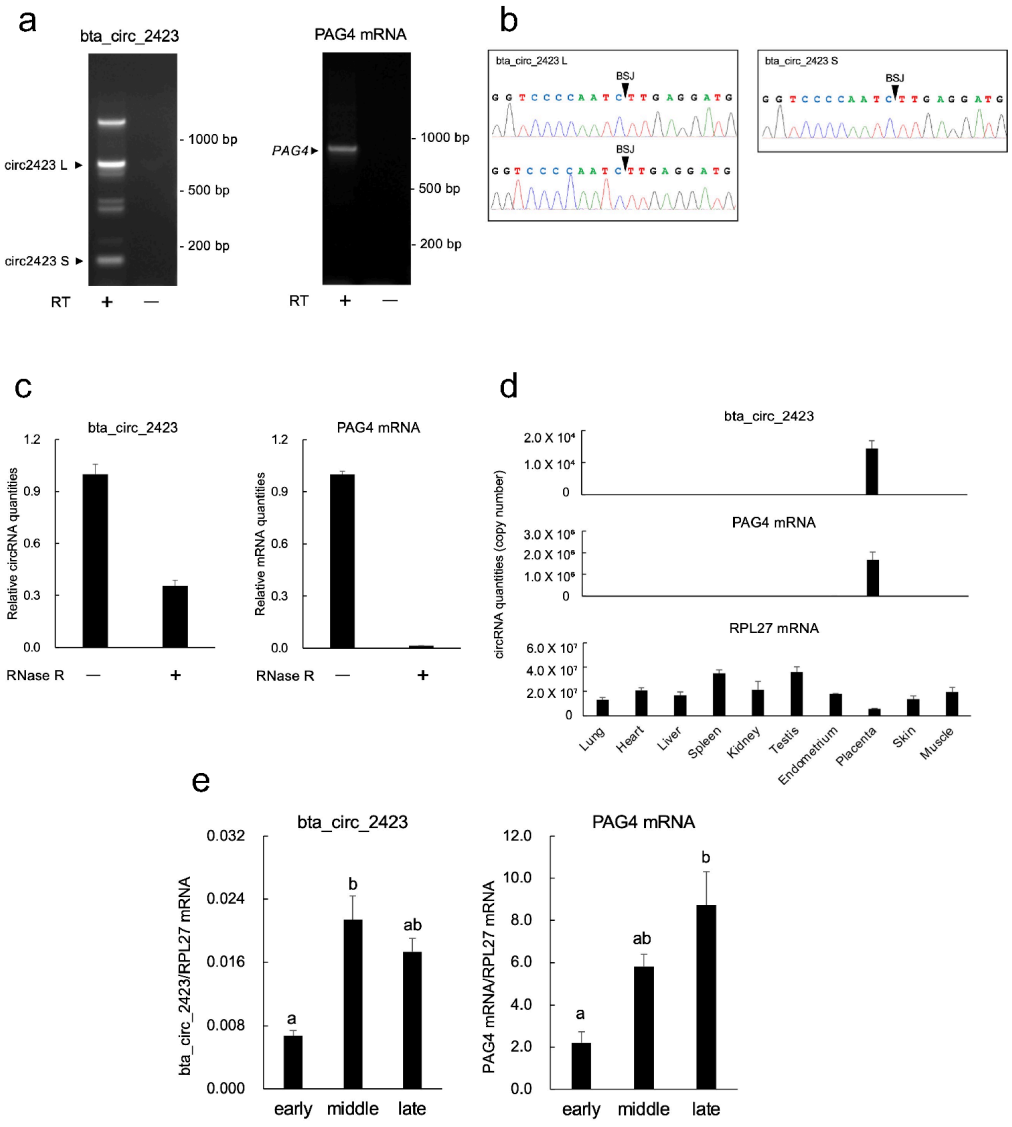

Figure 3

Identification of bta_circ_2423. (a) cDNA was synthesized from bovine placental total RNA with (RT+) or without (RT −) SuperScript IV reverse transcriptase. The expression of circ_2423 (left) and its host gene mRNA, PAG4 (right), was measured using conventional PCR. The circ_2423 L and S in panel (left) are indicate long and short form PCR products of circ_2423 RT-PCR, respectively. (b) RT-PCR products for circ_2423 L (left) and S (right) were subcloned into a sequencing vector, and sequencing analysis was

performed using Sanger sequencing. BSJ sites of the circRNAs are indicated by black arrowheads on the DNA sequence chromatograms. (c) Total RNA isolated from the bovine placenta was treated with or without RNase R, and cDNA was then synthesized. The quantities of circ_2423 (left) and PAG4 (right) in cDNA synthesized from placental RNA treated with or without RNase R (RNase R + or -) were measured using RT-qPCR. The circRNA and mRNA levels are indicated relative to the quantities of the RNase R-untreated control (RNase R -). Values are expressed as mean ± SE (n=5). (d) Expression of bta_circ_2423 (upper), PAG4 (middle), and RPLL27 (lower) in various bovine tissues, as shown in Figure 2 (d). The values are shown as the mean ± SE (placenta, n=5; other samples, n=3). (e) Expression of bta_circ_2423 (left) and PAG4 (right) in bovine cotyledonary tissues during early, middle, and late gestation, as measured by RT-qPCR.

Figure 4

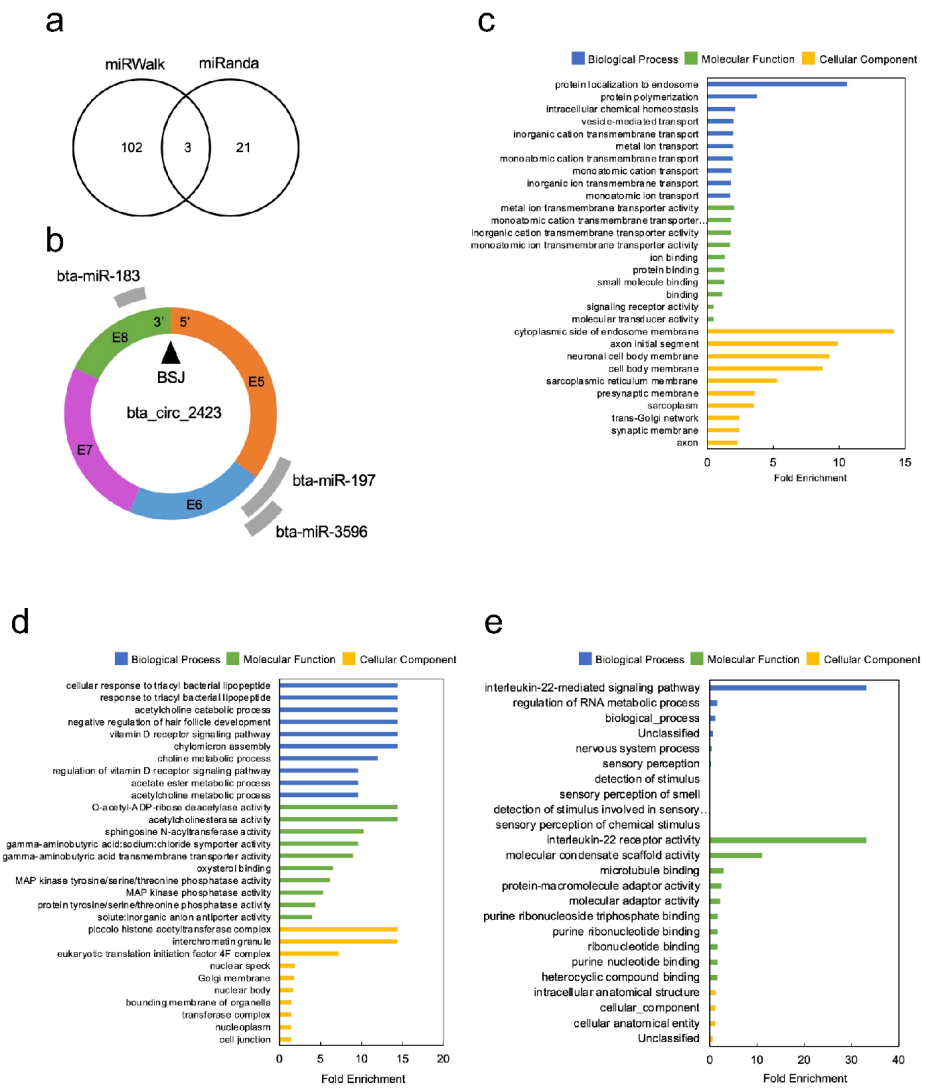

Figure 4

Prediction of miRNAs interacting with bta_circ_2423 and circRNA–miRNA–mRNA interaction network. (a) Screening of miRNAs that interact with bta_circ_2423 using the miRWalk and miRanda databases. Venn diagram revealing the overlap of miRNA predicted by the miRWalk and miRanda. (b) The predicted binding site for bta-miR-183, one of the three miRNAs commonly identified in (a), is shown schematically

within the bta_circ_2423 sequence. (c-d) After searching for mRNAs regulated by bta-miR-183 (c), bta-miR-197 (d), and bta-miR-3596 (e) using miRWalk, GO analysis was performed on these mRNAs.

Supplementary Files

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