

Research Article

Maternal placenta modulates a deleterious fetal mutation[†]

Hongen Xu¹, Hubert Pausch², Heli Venhoranta³, Karolina Rutkowska⁴,
Christine Wurmser², Beate Rieblinger⁵, Tatiana Flisikowska⁵,
Dmitrij Frishman¹, Lech Zwierzchowski⁶, Ruedi Fries²,
Magnus Andersson³, Alexander Kind⁵, Angelika Schnieke⁵
and Krzysztof Flisikowski^{5,*}

¹Department of Bioinformatics, Wissenschaftszentrum Weihenstephan, Technische Universität München, Freising, Germany; ²Chair of Animal Breeding, Technische Universität München, Freising, Germany; ³Department of Production Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki, Saarentaus, Finland; ⁴Department of Animal Improvement, Institute of Genetics and Animal Breeding, Polish Academy of Sciences, Jastrzebiec, Poland; ⁵Chair of Livestock Biotechnology, Technische Universität München, Freising, Germany and ⁶Department of Molecular Biology, Institute of Genetics and Animal Breeding, Polish Academy of Sciences, Jastrzebiec, Poland

* **Correspondence:** Chair of Livestock Biotechnology, Technical University in Munich, Liesel-Beckmann-Straße 1, 85354 Freising, Germany. Tel: +49-8161-71-2036; Fax: +49-8161-71-2108; Email: flisikowski@wzw.tum.de

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Abstract

Intrauterine growth restriction (IUGR) is caused by dysregulation of placental metabolism. Paternally inherited IUGR mutations in the fetus influence maternal physiology via the placenta. However, it is not known whether the maternal placenta also affects the extent of IUGR in such fetuses. In cattle and other ruminants, maternal–fetal communication occurs primarily at the placentomes. We previously identified a 3' deletion in the noncoding MER1 repeat containing imprinted transcript 1 (*MIMT1*) gene that, when inherited from the sire, causes IUGR and late abortion in Ayshire cattle with variable levels of severity. Here, we compared the transcriptome and genomic imprinting in fetal and maternal placentome components of wild-type and *MIMT1*^{Del/WT} fetuses before IUGR became apparent, to identify key early events. Transcriptome analysis revealed fewer differentially expressed genes in maternal than fetal *MIMT1*^{Del/WT} placentome. *AST1*, within the PEG3 domain, was the only gene consistently reduced in IUGR in both fetal and maternal samples. Several genes showed an imprinting pattern associated with IUGR, of which only secernin 3 (*SCRN3*) and paternally expressed 3 (*PEG3*) were differentially imprinted in both placentome components. Loss of strictly monoallelic, allele-specific expression (~80:20) of *PEG3* in the maternal *MIMT1*^{Del/WT} placenta could be associated with incomplete penetrance of *MIMT1*^{Del}. Our data show that dysregulation of the PEG3 domain is involved in IUGR, but also reveal that maternal placental tissues may affect the penetrance of the paternally inherited IUGR mutation.

Summary Sentence

Aberrant expression of imprinted genes specifically in maternal placental tissues affects the penetrance of a paternally inherited IUGR mutation in cattle.

Key words: developmental biology, epigenetics, fetal development, genomic imprinting, imprinted genes, intrauterine growth restriction, placenta, PEG3, cattle.

Introduction

Growth of the developing fetus is determined by placental function. Placental weight correlates with birth weight and is a good indicator of the placenta's capacity to support fetal growth [1]. Intrauterine growth restriction (IUGR) is mainly caused by external and maternal factors leading to placental dysfunction [2], but can also result from abnormal expression of paternally inherited genes. Severe IUGR results in late abortion, stillbirth, and neonatal complications in humans [3] and domestic animals [4]. It is well known that maternal genotype influences fetal growth [5]. However, it is difficult to discern the effect of the fetal genome on maternal physiology, because of confounding effects of both maternal metabolism and physiology on fetal growth and the fetal inheritance of maternal genes [6]. Polymorphisms in fetal-imprinted genes are associated with aberrant placental gene expression and maternal placental hormone concentrations in women carrying fetuses with growth disturbances such as Beckwith–Wiedemann syndrome (BWS) [7]. BWS varies considerably in severity [8] and causative mutations exhibit variable penetrance. It has also been shown that normal female mice gestating pups carrying knockout mutations of imprinted insulin-like growth factor 2 P0 transcript (*P0-Igf2*) and cyclin-dependent kinase inhibitor 1C (*Cdkn1c*), *p57^{Kip2}* genes causing restricted fetal growth can develop hypertension during pregnancy, indicating that the fetal genome affects maternal physiology [9, 10]. These phenomena reflect the complex, reciprocal nature of fetal–maternal communication in animals and human placenta [11].

Placental morphology varies considerably between mammals [12], but several factors involved in placental response to maternal stress [2, 13] and fetal growth restriction are common between species, including humans [14–16]. Transcriptome analysis of isolated maternal or fetal placental tissues is a powerful means of

identifying cell–cell communication at the feto–maternal interface [17]. Samples of human and rodent placenta are however mixtures of maternal and fetal cells that cannot easily be disassociated, so comparative analysis of maternal and fetal sides is currently limited to cells cultured in vitro, which risks altering gene expression due to culture conditions and disrupted communication with adjacent cells [18, 19]. Ruminants such as cattle have the advantage that the principal site of fetal–maternal communication [20–22], the placentome, can readily be separated during the first months of gestation, enabling analysis of each side. Placentomes are small areas of intimate contact and comprise distinct fetal (cotyledon) and maternal (caruncle) components.

We previously reported that a 3' deletion in the *MIMT1* (MER1 repeat containing imprinted transcript 1) gene was responsible for paternally inherited IUGR, late abortion, and stillbirth in Ayrshire cattle [23]. The stillborn calves are typically 50% undersized, but the *MIMT1^{Del}* mutation displays variable penetrance. Approximately 15% of *MIMT1^{Del/WT}* calves undergo full gestation and are healthy at birth despite reduced weight (authors' observations).

MIMT1 lies within the imprinted PEG3 (paternally expressed gene 3) domain and is transcribed as a long noncoding RNA (lncRNA) (Figure 1A). In mice, bidirectional promoter activity has been identified at the Peg3-DMR (differentially methylated region) located between *Peg3* and ubiquitin specific peptidase 2 (*Usp9*) and has been proposed as an imprinting control region (ICR) [24]. In mouse, there is no separate *MIMT1* gene, the first exon of murine *Usp29* is the same as the first exon in bovine and human *MIMT1*. In humans, *PEG3* and zinc finger imprinted 2 (*ZIM2*) have 5' exons in common and originate from a single transcript [25]. This suggests that the *MIMT1* transcript may regulate multiple genes at the 5' end of the domain. Interestingly, the survival of a proportion of *MIMT1^{Del/WT}* fetuses suggests that either *PEG3* expression

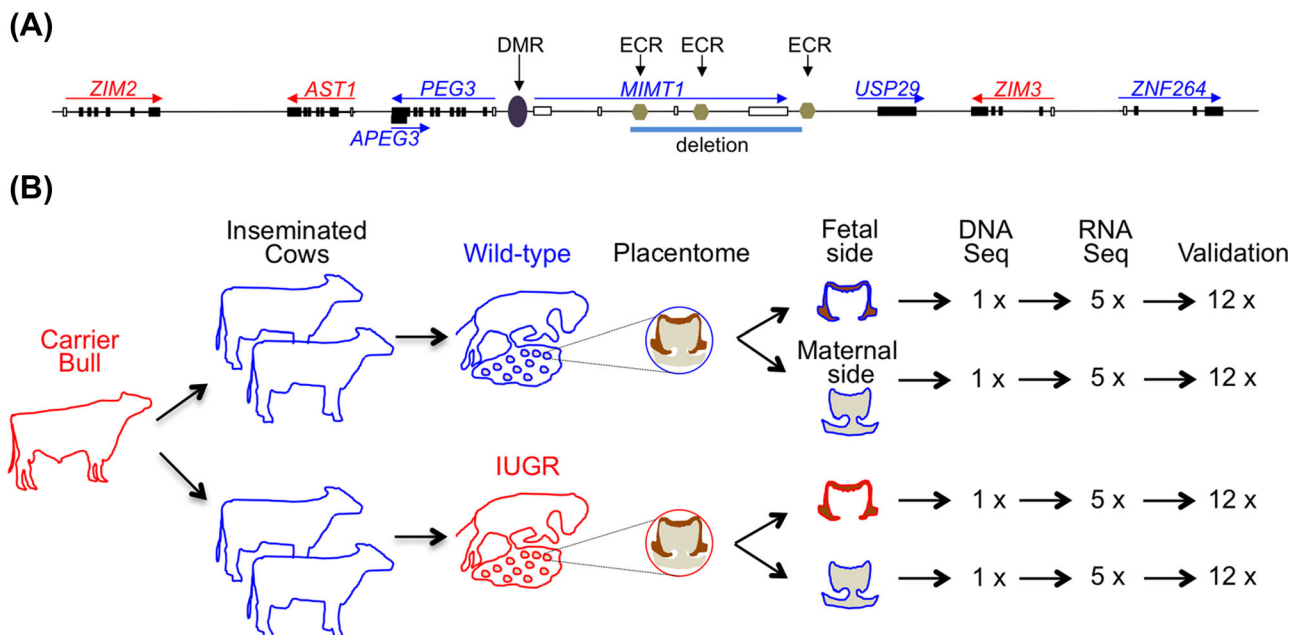


Figure 1. (A) Schematic presentation of the PEG3 domain in cattle. Paternally expressed genes are marked in blue and maternally expressed genes in red. (B) Experimental design used to study the effect of IUGR paternal origin genotype on fetal and maternal placentome components in cattle. “Validation” refers to qPCR and pyrosequencing.

can be regulated in the absence of *MIMT1* in fetal placenta, and/or maternal placenta plays a role in modifying the extent of growth restriction in *MIMT1^{Del/WT}* fetuses.

The aim of this study was to determine the impact of the fetal *MIMT1^{Del}* mutation on global gene expression in both fetal and maternal placenta. We examined placenta samples taken before growth restriction became apparent to identify early, possibly causative, changes that take place before the onset of global dysregulation of placental metabolism during IUGR.

Material and methods

Ethics statement

Tissue sampling and insemination were carried out by standard veterinary protocols according to the European Union Normative for Care and Use of Experimental Animals. All animal experiments were approved by the Animal Ethics Committee of the State Provincial Office of Southern Finland (ESAVI-2010-08583/YM-23).

Animals and tissue sampling

A total of 48 tissue samples were collected. That is, the maternal and fetal placenta sides from 12 cows gestating *MIMT1^{Del/WT}* fetuses and 12 cows gestating wild-type fetuses (referred to as wild-type). Samples were immediately frozen in liquid nitrogen and stored at -80°C for further analyses.

DNA and RNA extraction

Genomic DNA was extracted using the GenElute Mammalian Genomic DNA Kit (Sigma Aldrich), and total RNA extracted using the Direct-zol RNA Mini Prep Kit (Zymo Research) according to the manufacturer's instructions.

Next-generation sequencing (NGS) of the genome

Paired-end libraries were prepared using the paired-end TruSeq DNA sample prep kit (Illumina Inc., San Diego, CA, USA) and sequenced using a Illumina HiSeq 2500 instrument (Illumina Inc., San Diego, CA, USA) to produce 125 bp paired-end reads. The resulting reads were processed with the Illumina BaseCaller during the sequencing step. Alignment of reads to the University of Maryland bovine reference sequence (UMD3.1) [57] was performed with *BWA* (version 0.6.1-r104) [58] using default parameters. The resulting SAM files were converted into BAM files with *SAMtools* (version 0.1.18) [59]. Duplicate reads were identified and marked with the *MarkDuplicates* command of *Picard* (<http://picard.sourceforge.net/>).

Single nucleotide and short insertion and deletion polymorphisms were identified using the multisample approach implemented in *SAMtools*'s *mpileup* along with *BCFtools* [59]. *Beagle* (version 3.2.1) phasing was used to improve primary genotype calling by *SAMtools*. Functional effects of variants were predicted based on gene annotation of the UMD3.1 assembly of the bovine genome [60].

NGS RNA sequencing

Libraries for RNA sequencing of whole biopsies were prepared using the TruSeq Stranded mRNA LT Kit (Illumina). RNA integrity and fragment size were tested using the RNA6000 Nano kit (Agilent) on an Agilent Bioanalyzer 2100 (Agilent).

Libraries were sequenced with a HiSeq2500 ultra-high-throughput sequencing system (Illumina) to produce 100-base-paired end reads. An average of 54 million reads per sample

were generated and mapped to the University of Maryland bovine reference sequence (UMD3.1) using the 2-pass method of the *STAR* aligner with default parameters [61]. A total of 80% of these reads uniquely hit to the bovine reference genome. Duplicate reads were denoted with the *MarkDuplicates* command of *Picard* (<http://broadinstitute.github.io/picard>). Aligned reads (omitting duplicate reads) were assigned to gene sequences as defined in the 10.2.77 porcine gene set and counted with *featureCounts* [62]. Normalization of read counts and estimation of fold change was carried out using *DESeq2* [63]. Hierarchical clusters and heat maps for 20 genes with the most significantly different levels of expression were generated using the R package *pheatmap* (<https://CRAN.R-project.org/package=pheatmap>).

Variant calling based on *STAR* alignments was performed according to GATK [64] best practice recommendations for RNAseq [65, 66]. The GATK tool *SplitNCigarReads* was used to split reads into exons and remove false variants resulting from overhangs. This step included reassignment of the *STAR* alignment mapping qualities. GATK recalibration of base scores was based on the Ensembl release 85 variant database. Variant calling was carried out using GATK *HaplotypeCaller* with the *dontUseSoftClippedBases* option. GATK *VariantFiltration* was applied to clusters of at least three SNPs within a window of 35 bases between them, with the following parameters: Fisher strand value >30.0 and a quality by depth value <2.0 . The probability of allelic imbalance for each SNP was calculated based on the number of reference and alternate allele reads in heterozygous animals using a two-sided binomial test. *P* values were adjusted for FDR (*q* value) to take account of multiple testing.

Quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR)

A total of 500 ng of total RNA was used to synthesize complementary DNA (cDNA) using Superscript III reverse transcriptase (Invitrogen). Two-step quantitative PCR (qPCR) experiments were carried out using Fast SybrGreen MasterMix (Applied Biosystems) and run on an ABI 7500 thermocycler (Applied Biosystems). Primer specificity and capture temperature were determined by melt curve analysis. The relative expression difference between IUGR ($n = 12$) and wild-type ($n = 12$) in both tissues was calculated for each animal ($\Delta\Delta\text{CT}$). All cDNA samples were assayed in triplicate and relative expression levels normalized to endogenous glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression.

Allele quantification using pyrosequencing

Genomic DNA was used for allele quantification using heterozygous SNPs. An allele proportion of 50:50 at the DNA level for valid pyrosequencing assays was expected. A total of 200 ng total RNA was used to synthesize cDNA using Superscript IV reverse transcriptase (Invitrogen). Complementary DNA was amplified by RT-PCR, and allele expression for selected SNPs was analyzed by pyrosequencing using the Pyromark Q24 system (Qiagen). Pyrosequencing primers were designed using PyroMark Assay Design Software 2.0 (Qiagen). Allele expression differences at individual SNPs were evaluated using Student *t*-test.

Primers

The authors will provide all primer sequences used in this study on request.

Results

Experimental overview

The experimental outline is shown in Figure 1B. Growth retardation of *MIMT1^{Del/WT}* fetuses occurs in the second and last trimester of pregnancy. We previously observed major alterations to gene expression in fetal placenta of *MIMT1^{Del/WT}* fetuses [26]. Here, we explored the effect of the *MIMT1^{Del/WT}* genotype on the fetomaternal interaction in the placentome before onset of growth retardation. We collected fetal and maternal placenta of 12 wild-type and 12 *MIMT1^{Del/WT}* fetuses terminated at 94 ± 12 days of gestation, all were gestated in wild-type cows and all were fathered by the same *MIMT1^{Del/WT}* sire. Normal gestation in this *Bos taurus* breed is 280 ± 10 days. DNA and total RNA were isolated from fetal and maternal parts of the placentome, and the transcriptome and genomic imprinting were analyzed.

Gene expression changes in fetal and maternal placentome in intrauterine growth restriction fetuses

The transcriptomes of 20 samples (fetal and maternal placenta samples from 5 cows carrying wild-type fetuses and 5 carrying *MIMT1^{Del/WT}* fetuses) were sequenced to a depth of about 54 million reads per sample to determine transcript levels in fetal and maternal placentome in normal and *MIMT1^{Del/WT}* fetuses. Expression of ~20,000 genes was analyzed for all tissue samples.

Genes were ranked by expression \log_2 fold difference and adjusted *P* value. As expected, the maternal placentome was less affected by fetal genotype than the fetal side: 128 and 308 genes were differentially expressed in maternal and fetal placentome, respectively. Hierarchical cluster analysis revealed that both tissues formed relatively homogenous groups (Figure 2C and D). Of the top 50 genes (SI Appendix, Supplementary Tables S1 and S2, $P < 0.005$), 11 were generally expressed at higher level (Figure 2A) and 11 generally at lower level (Figure 2B) in samples of both fetal and maternal placentome of *MIMT1^{Del/WT}* fetuses. One gene, *AST1* (artiodactyl-specific transcript 1), showed the most marked difference in expression between wild-type and *MIMT1^{Del/WT}* fetuses in both placentome sides, and was consistently downregulated in fetal (5.63-fold less; FDR (false discovery rate) = 6.01×10^{-20}), and maternal samples (5.37-fold less; FDR = 0.003). This accords with our previous detection of decreased *AST1* expression in fetal placentome using a microarray [26]. *AST1* lies within the PEG3 domain and is transcribed as a lncRNA with unknown function. Other genes with at least two-fold decreased expression and *P* values < 0.005 were thrombospondin 1 (*THBS1*), RAS and EF-hand domain containing (*RASEF*), phosphoglycerate dehydrogenase (*PHGDH*) in fetal placentome and Ras association domain family member 6 (*RASSF6*), glutamate decarboxylase 1 (*GAD1*), baculoviral IAP repeat containing 5 (*BIRC5*) in maternal placentome of *MIMT1^{Del/WT}* fetuses. Genes with the greatest increase in expression (> 2 -fold) and known involvement in prenatal development and fetal growth restriction were mesoderm specific transcript (*MEST*), carcinoembryonic antigen related cell adhesion molecule 1 (*CEACAM1*), and coagulation factor V (*F5*) in fetal placentome [27–29]; and insulin like growth factor 2 (*IGF2*), natriuretic peptide receptor 2 (*NPR2*), and T-box 3 (*TBX3*) in maternal placentome [30, 31]. For example, *MEST* showed two-fold higher expression in most fetal and maternal *MIMT1^{Del/WT}* samples, suggesting it had undergone loss of imprinting, a finding also observed in human IUGR placenta [32]. *IGF2* was expressed higher in maternal placentome of *MIMT1^{Del/WT}* than wild-type fetuses, but

no RNAseq reads mapped to *IGF2* in fetal placentome. *IGF2* is a major determinant of placental and fetal growth [10, 33], and our data suggest that the effect is related to the maternal component in bovine placenta.

Expression analysis of *AST1*

AST1 expression data suggested that this gene plays a role in aberrant placental physiology during IUGR, warranting more detailed analysis. The current assembly of the bovine reference genome (UMD3.1, UCSC Genome Browser) indicates that *AST1* has seven exons [34]. Analyzing raw RNA sequencing data by number of mapped reads, we found that most sequencing reads aligned to *AST1* exons 3, 5, 6, and 7. The number of mapped reads was approximately 10 times higher in fetal than maternal placentome in wild-type fetuses (Figure 3A and B), and was significantly reduced in both fetal ($P = 2.6 \times 10^{-24}$) and maternal ($P = 1.6 \times 10^{-7}$) placentome of *MIMT1^{Del/WT}* fetuses (Figure 3C). This was also confirmed by qPCR analysis using primers specific for *AST1* exons 5 and 7 in fetal ($P = 1.8 \times 10^{-6}$) and maternal ($P = 6.6 \times 10^{-7}$) placentome (Figure 3D). Very weak *AST1* expression was detected by RNA sequencing (8 ± 3 of mapped reads to each of exons 5, 6, 7) and by qPCR using primers specific for exons 5 and 7 in fetal placentome of *MIMT1^{Del/WT}* fetuses (Figure 3C and D), and none was detected by RNA sequencing or qPCR analyses of maternal placentome of *MIMT1^{Del/WT}* fetuses (Figure 3C and D). This is consistent with our microarray study on fetal placentome [26], but not with our later study, where *AST1* expression was not detected in maternal normal and IUGR samples [35]. This could be explained by the ability of different methods to detect less abundant and multiple RNA-splicing events [36]. Here, we found that *AST1* exon 2 is not expressed in maternal placentome. Using RT-PCR primers specific for exons 3 and 7, we obtained a 583 bp PCR product from fetal and maternal placentome homologous to GenBank *AST1* RNA sequence (NR_038119.1) (Figure 3E).

RNA-sequencing-based allelic expression analysis in placentome of intrauterine growth restriction fetuses

We next studied the impact of the fetal *MIMT1^{Del/WT}* genotype on genomic imprinting in each placentome component. We first sequenced the genomes of the *MIMT1^{Del/WT}* carrier bull, one wild-type and one *MIMT1^{Del/WT}* sample to identify placental genomic heterozygous variants that could be used to detect allele-specific expression (ASE). Minimum-quality criteria were established to minimize potential false positive RNA variants, as previously published [37]; these were ≥ 10 RNAseq mapped reads to the reference genome and the presence of monoallelic expression in fetal and (or) maternal samples. Comparison of heterozygous genomic single-nucleotide polymorphism (SNPs) in the two fetuses with the carrier bull's genome sequence revealed ~16k suitable heterozygous SNPs. Of these, 1444 SNPs showed unequal ASE, and 132 SNPs showed monoallelic expression in either fetal or maternal placentome of wild-type and *MIMT1^{Del/WT}* fetuses. We identified 37 strong candidate-imprinted genes that exhibited monoallelic maternal- or paternal-specific expression in the bovine placentome components. Fifteen of these, including *ERO1A* (endoplasmic reticulum oxidoreductase 1 alpha), *FN1* (fibronectin 1), *SCRN3* (secernin 3), *MKI67* (marker of proliferation Ki-67), and *PAG21* (pregnancy-associated glycoprotein 21) showed a different ASE pattern between wild-type fetal and maternal placentome (SI Appendix, Supplementary Table S3). In fetal placentome, *PEG3*, *SCRN3*, *EIF4G1* (eukaryotic translation initiation factor 4 gamma 1), and *RCSD1* (RCSD domain containing 1) showed different ASE

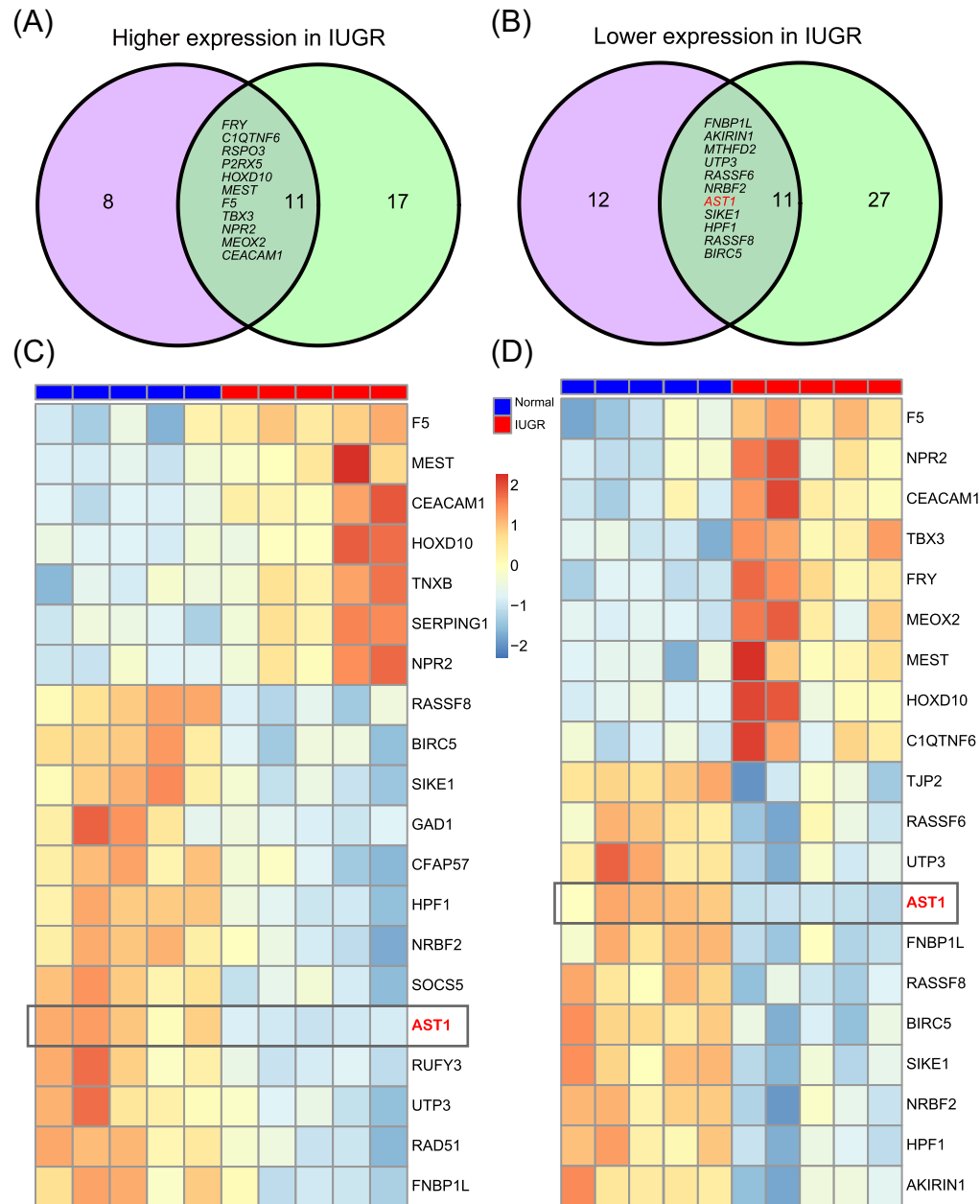


Figure 2. Differentially expressed genes in wild-type and *MIMT1*^{Del/WT} fetal and maternal placentome: (A) overlap of top 50 upregulated genes between fetal and maternal IUGR placentome samples; (B) overlap of top 50 downregulated genes between fetal and maternal IUGR placentome samples; (C) heatmap of the top 20 genes showing transcriptome pattern specific for wild-type and IUGR in fetal placentome samples; (D) heatmap of the top 20 genes showing transcriptome pattern specific for wild-type and *MIMT1*^{Del/WT} in maternal placentome samples. Genes are ranked by normalized expression differences between the two groups and *P* value. *AST1* is outlined in red.

between wild-type and *MIMT1*^{Del/WT} samples. In maternal placentome, *HBS1L* (HBS1 like translational GTPase), *RB1CC1* (RB1 inducible coiled-coil 1), *ABCA5* (ATP binding cassette subfamily A member 5), *SCRN3* and *MKI67* showed different allele expression between wild-type and *MIMT1*^{Del/WT}. RNA-sequencing-based allelic expression analysis revealed *SCRN3* as the only gene that showed alterations in allele expression affected by the *MIMT1*^{Del/WT} genotype in fetal and maternal placentome (SI Appendix, Supplementary Table S3). RNAseq read counts in fetal placentome were as follows: biallelic in wild-type (*C/T* allele ratio = 21/6) and maternal allele

expression in fetal (*C/T* allele ratio = 0/15; opposite monoallelic expression in wild-type (*C/T* allele ratio = 48/0), and IUGR (*C/T* allele ratio = 0/28) maternal placentome was observed. *SCRN3* expression has also been detected in mouse and human placenta [38, 39], but its function is unknown.

We next analyzed RNA variants that mapped to the PEG3 domain and identified 21 SNPs, mainly in exon 10 of the *PEG3* gene (SI Appendix, Supplementary Figure S1). Of these, 12 SNPs showed homozygous genotypes specific for wild-type and *MIMT1*^{Del/WT} fetal placentome. A representative SNP, rs110770973 (18:64275283;

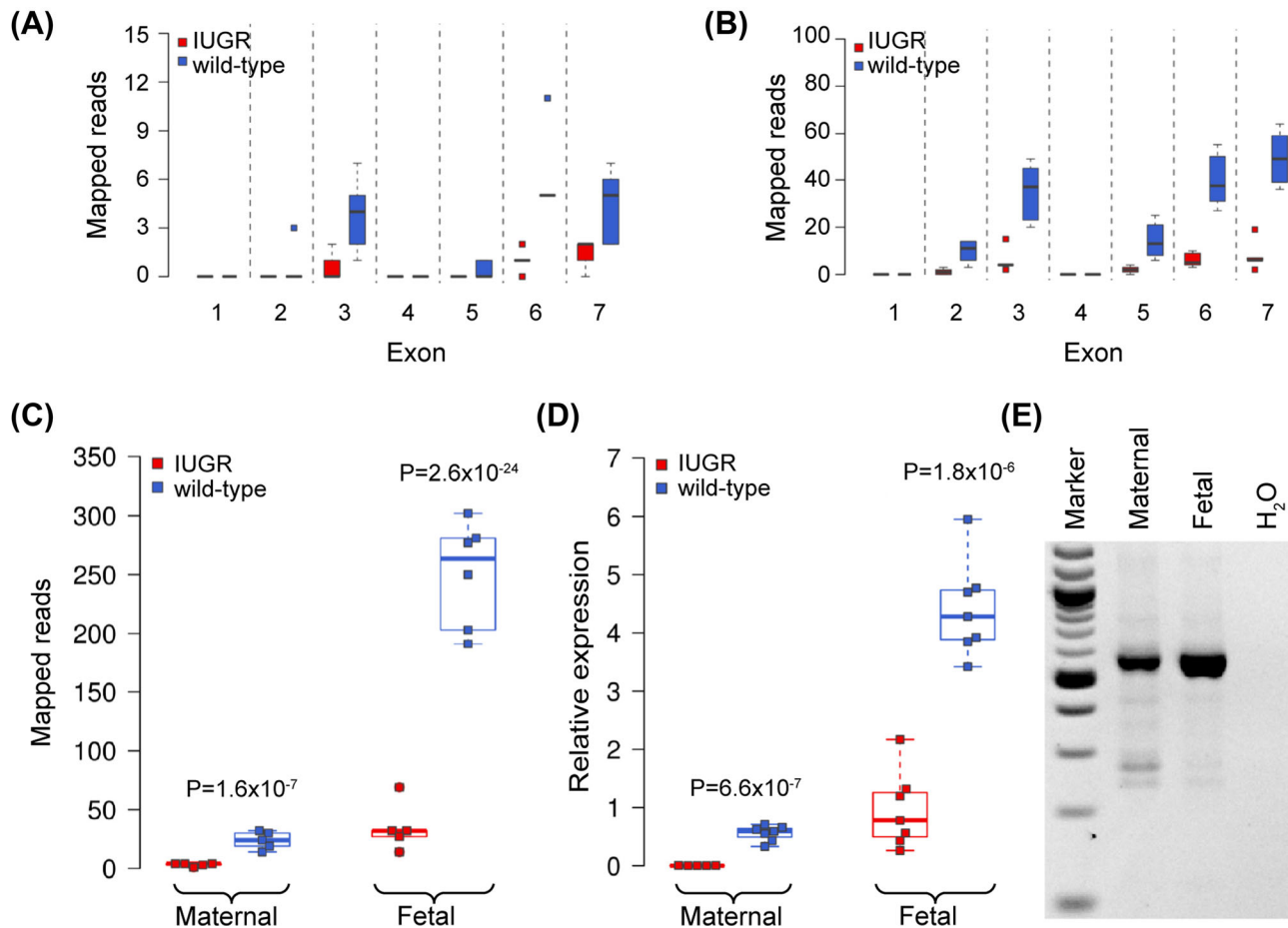


Figure 3. *AST1* gene expression in wild-type and IUGR fetal and maternal placentome: (A) RNAseq mapped read counts per exon between wild-type and *MIMT1^{Del/WT}* samples in maternal placentome; (B) RNAseq mapped read count per exon between wild-type and *MIMT1^{Del/WT}* samples in fetal placentome ($n = 6$ per group); (C) total number of RNAseq mapped reads to all exons in the *AST1* gene; (D) qPCR using primers specific for exons 5 and 7 of *AST1* in wild-type and *MIMT1^{Del/WT}* fetal and maternal placentome ($n = 12$ per group). Primers located in two different exons provide control for DNA contamination in the samples. Quantitative PCR measurements were normalized to *GAPDH* reference gene; (E) 583 bp RT-PCR product amplified using primers hybridizing to exons 3 and 7, for *AST1* isoform specific detection in fetal and maternal placentome. The RT-PCR fragment is homologous to GenBank *AST1* RNA sequence (NR.038119.1). *AST1* gene structure based on bovine reference sequence (UMD3.1) in the UCSC Genome Browser.

c.2586C>T; p.Asp862), was used for closer analysis that confirmed its ASE related to fetal genotype, i.e., paternal allele expression in wild-type and maternal expression in *MIMT1^{Del/WT}* fetal placentome (Figure 4A). As in wild-type fetal placentome, this SNP showed paternal-specific allele expression in maternal placentome and very few RNAseq reads in *MIMT1^{Del/WT}* maternal placentome, which precluded proper genotype calls. Validation of ASE for the rs110770973 SNP by pyrosequencing confirmed monoallelic expression of the C allele (100%) in fetal placentome, and leaky ASE ($82\% \pm 4\%$) in maternal placenta in *MIMT1^{Del/WT}* fetuses (Figure 4B). We also sequenced the entire *AST1* gene in all our samples, but no informative genomic variants for allele expression analysis were identified.

Discussion

Normal placental function is critical for fetal health, and alterations to placental gene expression can affect fetal growth [40]. For practical reasons, transcriptome studies have mainly been applied to samples of whole placenta. However, the placenta is a heterogeneous

combination of cell types of fetal and maternal origin. Bulk analysis may not detect or distinguish expression changes in different components, as are likely when a deleterious fetal mutation such as *MIMT1^{Del}* influences placental function. Our earlier study on *PEG3* gene expression suggested that the fetal *MIMT1^{Del/WT}* genotype primarily affects the fetal part of the placentome [35].

We examined the impact of fetal *MIMT1^{Del}* on global gene expression in both fetal and maternal placentome to identify transcriptome changes leading to IUGR. This approach revealed *AST1* to be significantly downregulated in both sides in cattle gestating *MIMT1^{Del/WT}* fetuses. Bovine *AST1* is located in the PEG3 domain at a locus orthologous to *Zim2* (zinc finger, imprinted 1) in mouse and *ZIM2* in human. It has been suggested that mouse *Peg3* negatively regulates *Zim1* [41]. We however observed decreased expression of both *PEG3* and *AST1*, suggesting these genes have a different relationship in cattle, reflecting interspecies differences in *PEG3* regulation [42]. Our finding that *AST1* downregulation occurs in early-stage IUGR is intriguing. The function of this gene and its place in PEG3 domain regulation is so far unknown, but certainly worthy of further investigation.

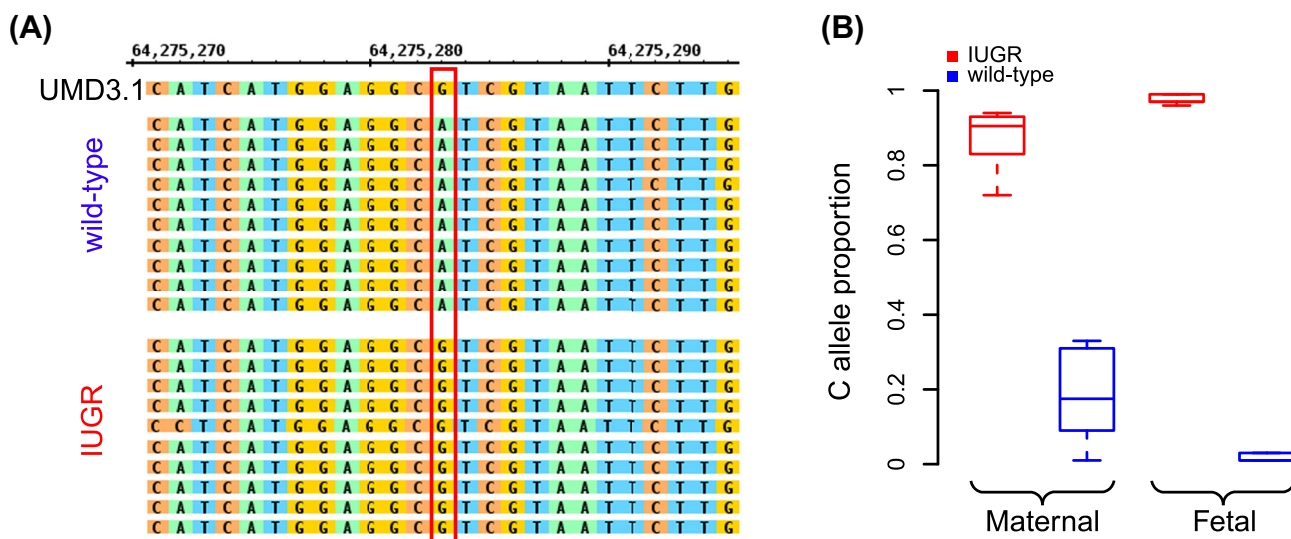


Figure 4. Allele-specific expression of *PEG3* in wild-type and IUGR fetal and maternal placentome: (A) a fragment of aligned *PEG3* exon 10 sequencing reads of wild-type (top) and IUGR fetus (bottom). Allele calls for the C/T rs64275283 SNP are marked; (B) allele expression ratio for the *PEG3* C/T rs64275283 SNP (wild-type, n = 6; IUGR, n = 7) measured by pyrosequencing.

It seems likely that the principal mechanism of IUGR in the bovine *MIMT1^{Del}* model is mediated via *PEG3* and its downstream target genes. Other genes identified as differentially expressed in bovine placenta of *MIMT1^{WT/Del}* fetuses such as *F5*, *CEACAM1*, and *RASSF8* (Ras association domain family member 8) were also differentially expressed in early placenta of *Peg3*-deficient mice [43]. *PEG3* is expressed in all placenta tissues in mouse and human [44, 45], and *Peg3*-deficient mice show fetal growth restriction and small but histologically normal placentae [27]. Similarly, *MIMT1^{WT/Del}* bovine fetuses have decreased placental *PEG3* expression, and have smaller but normal placentae [23]. *PEG3* is a well-known regulator of Wnt signaling, which plays an important role in placenta development [46], but also controls the transcription of groups of placenta-specific genes [43]. Disruption of *Peg3* function in mice also leads to aberrant expression of placenta-specific genes, for example in fetal brain [43], an effect similar to that we observe in *MIMT1^{WT/Del}* bovine fetuses [47].

Together with our previous data [26, 35], our findings suggest that the *MIMT1* transcript is a regulator of genes at the 5' end of the *PEG3* domain. The region of *MIMT1* defined by the *MIMT1^{Del}* deletion contains evolutionarily conserved regions, which we propose act as cis-regulatory elements for *PEG3* and other imprinted genes in the domain, as previously reported in mouse and human [48, 49]. Further studies of mice and humans are needed to elucidate the functions of *AST1* and *ZIM1* in prenatal development.

Although there is only limited invasion of fetal cells into the maternal side of synepitheliochorial placenta, they can influence the maternal component of the placentome and thus maternal physiology, and molecules of fetal origin have been detected in maternal blood circulation in cattle [50]. Moreover, embryo transfer experiments in domestic animals have shown that the fetal genome influences placental concentrations of hormones and growth factors [51, 52]. Fetal growth is associated with placenta size [53], and is controlled by parent-specific monoallelic expression [54]. We identified 37 genes showing monoallelic expression in the bovine

placentome, three of which were located in three imprinted clusters: *MEST/COPG2*, *H19/IGF2* and particularly the *PEG3/USP29* domain, regulation of which was affected by the *MIMT1^{Del/WT}* fetal genotype. Most of these genes (n = 26) have not previously been described as placental-imprinted in cattle or other mammals [37, 55, 56], possibly indicating that the imprinting status of these genes is restricted to the placentome in cattle. This is consistent with other studies that have reported that species-specific allele expression of distinct imprinted genes is responsible for the evolutionary diversity of placenta types [55, 56]. It is unlikely that we identified all SNPs that would exhibit ASE, but the genes that were analyzed, particularly *PEG3*, clearly indicated differential allele expression in fetal and maternal placentome. This is an important finding because parental-specific monoallelic expression in placenta balances fetal growth. Abnormal monoallelic maternal expression of *PEG3* in fetal *MIMT1^{Del/WT}* samples could explain the restricted growth of fetuses, because maternally expressed genes act as negative regulators of prenatal development. We suggest that the phenomenon whereby ~15% of *MIMT1^{Del/WT}* calves complete full gestation could be associated with leaky (~80:20) rather than strictly monoallelic *PEG3* allele expression in the maternal placenta of those fetuses.

Our study revealed that few genes are affected in both placentome components by fetal genotype, showing that maternal and fetal placental cells respond differently to the fetal IUGR genotype. Caution is therefore warranted when analyzing gene expression changes in nondissected samples of placenta. To some extent, single-cell transcriptomics can improve the detection of allele-specific gene expression in placenta [17].

In conclusion, we have expanded the list of candidate-imprinted genes in bovine placenta and demonstrated that abnormal monoallelic *PEG3* expression leads to IUGR. The nonprotein coding *MIMT1* and *AST1* genes probably regulate the parental-allele specificity of *PEG3* expression. Our unique genetic IUGR model has also revealed that maternal placenta modulates the penetrance of mutations in the fetal genome.

Supplementary data

Supplementary data are available at [BIOLRE](https://doi.org/10.1002/biolre.1249) online.

Supplementary Figure S1. Genotype distribution at polymorphic loci in the PEG3 gene obtained by RNAseq data analysis of wild-type ($n = 10$) and IUGR ($n = 10$) fetal and maternal placentome.

Supplementary Table S1. Genes showing differential expression between wild-type and IUGR in fetal placentome (excel file).

Supplementary Table S2. Genes showing differential expression between wild-type and IUGR in maternal placentome (excel file).

Supplementary Table S3. Genes showing monoallelic expression in fetal and (or) maternal placentome in cattle (excel file).

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