Biochem Genet (2018) 56:639–649
https://doi.org/10.1007/s10528-018-9866-5

ORIGINAL ARTICLE

Polymorphic Imprinting of *SLC38A4* Gene in Bovine Placenta

Da Xu[1] · Cui Zhang[1] · Junliang Li[1] · Guannan Wang[1] · Weina Chen[1,2] · Dongjie Li[3] · Shijie Li[1]

Received: 27 March 2018 / Accepted: 18 May 2018 / Published online: 21 May 2018
© Springer Science+Business Media, LLC, part of Springer Nature 2018

Abstract Imprinted genes are characterized by monoallelic expression that is dependent on parental origin. Comparative analysis of imprinted genes between species is a powerful tool for understanding the biological significance of genomic imprinting. The *slc38a4* gene encodes a neutral amino acid transporter and is identified as imprinted in mice. In this study, the imprinting status of *SLC38A4* was assessed in bovine adult tissues and placenta using a polymorphism-based approach. Results indicate that *SLC38A4* is not imprinted in eight adult bovine tissues including heart, liver, spleen, lung, kidney, muscle, fat, and brain. It was interesting to note that *SLC38A4* showed polymorphic status in five heterogeneous placentas, with three exhibiting paternal monoallelic expression and two exhibiting biallelic expression. Monoallelic expression of imprinted genes is generally associated with allele-specific differentially methylation regions (DMRs) of CpG islands (CGIs)-encompassed promoter; therefore, the DNA methylation statuses of three CGIs in the *SLC38A4* promoter and exon 1 region were tested in three placentas (two exhibiting paternal monoallelic and one showing biallelic expression of *SLC38A4*) and their corresponding paternal sperms. Unexpectedly, extreme hypomethylation (< 3%) of the DNA was observed in all the three detected placentas and their corresponding paternal sperms. The absence of DMR in bovine *SLC38A4* promoter region implied that DNA methylation of these three CGIs does not directly or indirectly affect the

Da Xu and Cui Zhang contributed equally to this work and should be considered co-first authors.

✉ Shijie Li
lishijie20005@163.com

[1] Department of Biochemistry and Molecular Biology, College of Life Science, Hebei Agriculture University, Baoding 071001, China

[2] College of Chinese Medicine, Hebei University, Baoding 071000, China

[3] College of Life Science and Life Engineering, Hebei Science and Technology University, Shijiazhuang 050018, China

polymorphic imprinting of *SLC38A4* in bovine placenta. This suggested other epigenetic features other than DNA methylation are needed in regulating the imprinting of bovine SLC38A4, which is different from that of mouse with respect to a DMR existence at the mouse's slc38a4 promoter region. Although further work is needed, this first characterization of polymorphic imprinting status of *SLC38A4* in cattle placenta provides valuable information on investigating the genomic imprinting phenomenon itself.

Keywords Polymorphic imprinting · *SLC38A4* · Bovine placenta · Methylation · CGIs

Introduction

Genomic imprinting is an essential epigenetic phenomenon in mammalian cells that causes monoallelic expression of a small amount of genes with a parent-specific pattern (Barlow and Bartolomei 2014). Gene imprinting regulates embryonic and placental growth, and affects animal postnatal development and the occurrence of disease (Peters 2014). To date, about 150 chromosomal loci are identified to be imprinted in humans (Wei et al. 2014; Partida et al. 2017). Aberrant regulation of the imprinted loci is associated 12 congenital diseases (Soellner et al. 2017). Compared to humans and mice, few imprinted genes have been reported in cattle. Most imprinted genes are conserved in humans and mice (Peters 2014). Genomic imprinting evolution and biological significance have been explained by various theories, and comparative expression and imprinting status analysis among species are useful tools for learning the biological significance of genomic imprinting.

Solute carrier family 38 member 4 (*slc38a4*), also named *ATA3*, is a member of the System A gene family and plays a major regulatory role in the sodium-dependent amino acid transport system (Sugawara et al. 2000). This gene is paternally expressed in all tested mice tissues (Mizuno et al. 2002); however, it shows complex isoform- and tissue-specific maternal expressions in swine (Bischoff et al. 2009). The first aim of this study was to assess the expression pattern and imprinting status of the *SLC38A4* in bovine adult tissues and placenta.

Genomic imprinting is controlled by imprinting control regions (ICRs) that possess differentially methylated regions (DMRs) derived from the germline, which retains the modification in somatic tissues (Reik and Walter 2001; Docherty et al. 2014). A maternally methylated DMR exists at the mouse *slc38a4* promoter region (Chotalia et al. 2009). The other objective of this study was to investigate the role of DNA methylation modifications in imprinting of the *slc38a4* gene by analyzing the DNA methylation status of three CpG rich regions around the bovine *SLC38A4* promoter.

Materials and Methods

Tissue Collection

Holsteins bovine tissue samples ($n=32$) used in this study were collected from a local abattoir. 30 bovine term placental samples (consisting of multiple sites), with corresponding maternal whole-blood samples and paternal sperms' samples being used for artificial insemination of normal pregnancies, were collected from a local cattle farm. Whatever the source, all collected samples were frozen in liquid nitrogen for later DNA and RNA extractions, respectively.

DNA Extraction and Single Nucleotide Polymorphism (SNP) Identification

Genomic DNA was extracted from liver and placental (including maternal blood and paternal sperms) samples using the DNA Extraction Kit (Sangon Biotech, Shanghai, China). The exonic SNP was determined by sequencing the PCR products directly. The primers SLC-Fg (5′-TCGTGGTTGGCATCATCT-3′) and SLC-Rg (5′-GGGGAGAGGGAAAAGAGTAT-3′) were designed based on the bovine *SLC38A4* sequence (GenBank, NM_001205943). The 25 μL PCR volumes contained 1 μL of DNA template, 1 μL of each primer (10 μM), 9.5 μL of ddH_2O and 12.5 μL of ES Tap Master Mix (CWBio, Beijing, China). The PCR was performed as follows: an initial denaturation at 94 °C for 4 min, followed by 30 cycles (at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s) and a final extension at 72 °C for 10 min. The 416 bp PCR products were purified using a UNIQ-10 column DNA gel extraction kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions and sequenced directly to be sent to Bio sequencing Corporation (BGI, Beijing, China).

RNA Preparation and Reverse Transcription

Total RNAs were isolated from the frozen tissues by a Trizol RNA extraction kit (Invitrogen, Carlsbad, CA, USA) following the instructions of the manufacture. To remove possible genomic contamination, total RNAs were treated with RNase-free DNase I. The quantity and quality of the RNA was assessed using NanoDrop ND-1000 spectrophotometer system.

Subsequently, Total RNA (2 μg) was reverse-transcribed to cDNA using a reverse transcription (RT) kit (Promega, Madison, WI) according to the manufacturer's protocol.

Expression Analysis

Expression of the *SLC38A4* gene in different tissues was detected by RT-PCR. Total RNA prepared from three individuals was reverse transcribed into cDNA for use as a template. The intron-spanning primers, SLC-F (5′-TACACATTTGAC ACCCCTCTC-3′) and SLC-R (5′-AATACATCATCACACACTGCTCTA-3′), were designed to analyze bovine *SLC38A4* gene expression by RT-PCR. The *GAPDH*, a

housekeeping gene, was amplified as the internal control with the primers Gap-F (5′-GCACAGTCAAGGCAGAGAAC-3′) and Gap-R (5′-GTGGCAGTGATGGTGGA-3′) designed according to the sequence (GenBank, BTU85042). The RT-PCR products were purified and directly sequenced.

Allelic Expression Analysis

Allelic expression was investigated by comparing the sequence results of PCR products from genomic DNA and cDNA of *SLC38A4*. Total RNA isolated from organ samples and placentas heterozygous individuals was reverse-transcribed into cDNA and used for the allelic transcription analysis of the bovine *SLC38A4* gene.

Methylation Analysis

Three CGIs were found surrounding the *SLC38A4* promoter and exon 1 using the online SoftBerry program (http://www.softberry.com/). For each CGIs, primers were designed for nested or seminested PCR with the online software for methprimer (http://www.urogene.org/methprimer). Primer sequences are shown in Table 1.

DNA was extracted from three heterozygote placentas (6, 13 and 15) and their corresponding paternal sperms samples. Genomic DNA (800 ng) was bisulfate treated using the EZ DNA Methylation Kit (Zymo, Orange County, CA) following manufacturer's guidelines. These bisulfite-converted DNAs were used as templates for amplification of specific CGIs. One microliter of tenfold dilution of the first-round PCR product was the template of the second-round PCR. PCR products of second round were purified and cloned into pMD19-T vectors (Takara). At least twenty clones containing the amplified fragments were sequenced. The SNPs in each CGI were used to distinguish the two parental strands. In each individual, the percentage of overall mCpGs of both parental strands was calculated, respectively. If the difference of methylation levels between the two parental strands is over 50% in a CGI, then this region was defined as a DMR.

Results

Biallelic Expression of *SLC38A4* in Bovine Adult Tissues

A 683 bp fragment was amplified using primers SLC-F and SLC-R in eight tissues (heart, liver, spleen, lung, kidney, muscle, fat. and brain), indicated that *SLC38A4* was expressed in all detected tissues and showed a higher expression level in the liver than that in other tissues (Fig. 1a).

SNP site was searched in the coding sequence of the bovine *SLC38A4* gene in 32 individuals, and an A/G transition (GenBank, rs137028117) was identified in six individuals (Fig. 1b). Three out of six heterozygous individuals were used to analyze the allele expression of *SLC38A4*. Comparison of the sequencing results of RT-PCR and genomic DNA PCR products indicates that biallelic expression of *SLC38A4*

Table 1 Primers used for bisulfite sequencing

Region (CGIs)	Primer name	Sequence (5′–3′)	Annealing temperature First round/ Second round (°C)	Final product size (bp)	Number of CpGs
1	Forward	TTAGTTTTATTTGGGAGGATGTTTT	55/54	494	21
	Outside reverse	AACTTTCCCAACCTATACCCTTTAC			
	Inside reverse	ATCATTAACCTACAATAACCCACAC			
2	Outside forward	GGTTTTTTAATTTTTTGGGGTTTAG	53/55	298	24
	Inside forward	GTTAGGAGAAGTGATTGTGTTAAGG			
	Reverse	TTTTAAATCCTACATCCAATATCCC			
3	Outside forward	GATTTTTTGGAGGAATTGGTTTTA	55/53	338	24
	Outside reverse	CTCCTACCCCTCTAAAAATCTAAAC			
	Inside forward	TTTTTTTTGATAGTTGGGTTTTTTG			
	Inside reverse	CCCCTCTAAAAATCTAAACCCTTAT			

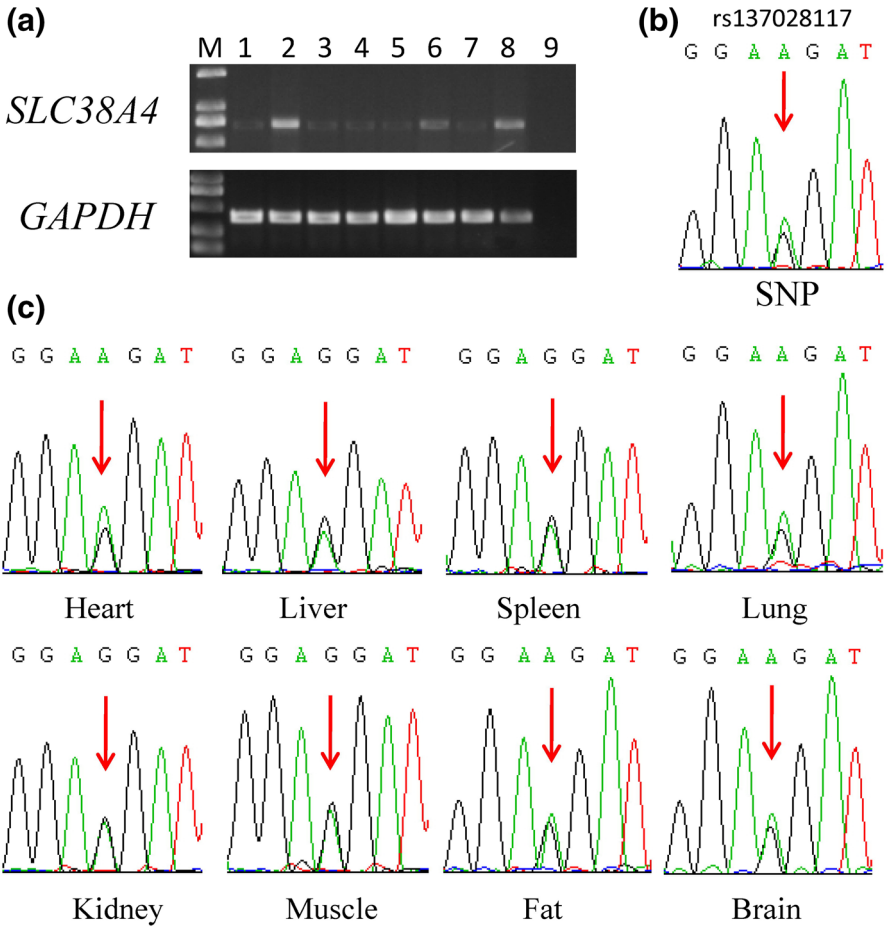

Fig. 1 Biallelic expression of SLC38A4 in adult bovine tissues. **a** Expression of the bovine *SLC38A4* transcripts obtained from eight adult tissues analyzed by RT-PCR. M, DL2000 (2000, 1000, 750, 500). Lanes 1–8: RT-PCR products of heart, liver, spleen, lung, kidney, muscle, fat. and brain, respectively. Lane 9: negative control. The sizes of RT-PCR products were 683 bp for *SLC38A4* and 375 bp for *GAPDH*. **b** SNP site of the *SLC38A4* gene (an A/G SNP). **c** Biallelic expression of *SLC38A4* in eight detected bovine tissues

occurs in all eight analyzed tissues (Fig. 1c). This suggests that *SLC38A4* is not imprinted in bovine adult tissues.

Polymorphic Imprinting of *SLC38A4* in Bovine Placenta

The approach used for detecting the SNP and expression of *SLC38A4* in adult tissues was also employed to investigate placentas (Fig. 2a). Five heterozygous placentas were identified from 30 samples based on the A/G SNP (GenBank, rs137028117) (Fig. 2b). Interestingly, the expression of *SLC38A4* in the five heterozygous

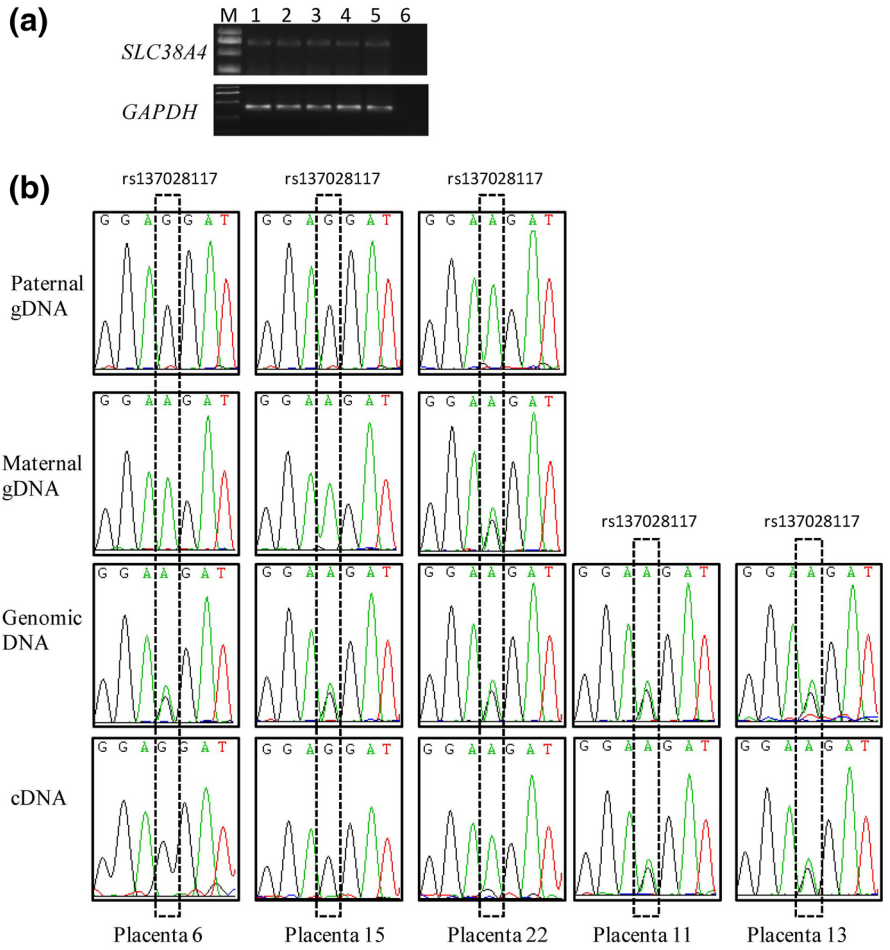

Fig. 2 Polymorphic imprinting of *SLC38A4* in bovine placentas. **a** Expression of the bovine *SLC38A4* in heterozygous placentas by RT-PCR. M, DL2000 (2000, 1000, 750, 500). Lanes 1–5 are RT-PCR products obtained from heterozygous placentas (placenta 6, 11, 13, 15, and 22), respectively. Lane 6 is negative control. The sizes of RT-PCR products were 683 bp for *SLC38A4* and 375 bp for *GAPDH*. **b** Sequencing results of SNP (rs137028117) in the *SLC38A4* gene. Polymorphic imprinting expression was deduced by comparing genotypes of gDNA, cDNA and parental gDNA in five heterozygous placentas

placentas (placenta 6, 11, 13, 15, and 22) was different. Monoallelic expression occurred in placenta 6, 15 and 22, while biallelic expression occurred in placenta 11 and 13 (Fig. 2c). To distinguish between paternal and maternal expressions in monoallelic expressed placentas (samples 6, 15, and 22), the parental genotype was analyzed by amplifying the genomic DNA of the corresponding maternal blood and paternal sperms samples. In placenta 6 and 15, the homozygous GG and AA genotypes were identified in the corresponding paternal and maternal genomic DNAs, respectively, thus indicating that *SLC38A4* exhibits paternal expression (allele G) in the placenta. For placenta 22, the genotypes of his paternal sperm and maternal

blood sample were the homozygous AA and heterozygous AG, respectively, so the A in the offspring cDNA originated from the sire. These results indicate that *SLC38A4* is polymorphically imprinted in cattle.

DNA Methylation is not Involved in Regulating Polymorphic Imprinting of *SLC38A4* in Placenta

CpG plot analysis (http://www.ebi.ac.uk/Tools/emboss/cpgplot/) shows that there are three CGIs in the region surrounding the bovine *SLC38A4* 5′ promoter and exon 1. To analyze whether methylation of the *SLC38A4* promoter region regulates imprinting expression in bovine placenta, the DNA methylation status of three CGIs were tested in three placentas and their corresponding paternal sperms. *SLC38A4* expression exhibited paternal imprinting in two placentas (6 and 15), while biallelic expression was detected in placenta 13. The three CGIs selected for bisulfite sequencing were located on the bovine *SLC38A4* gene from −234 to +260 bp, from +339 to +636 bp, and from +645 to +982 bp, and their 21, 24, and 24 CpGs were analyzed, respectively (Fig. 3).

There was a SNP site in each studied region: an A/T SNP (GeneBank, rs211032768) in CGI 1; an A/C SNP (GeneBank, rs207735752) in CGI 2; and an A/G SNP (GeneBank, rs209305091) in CGI 3. These sites were used to distinguish

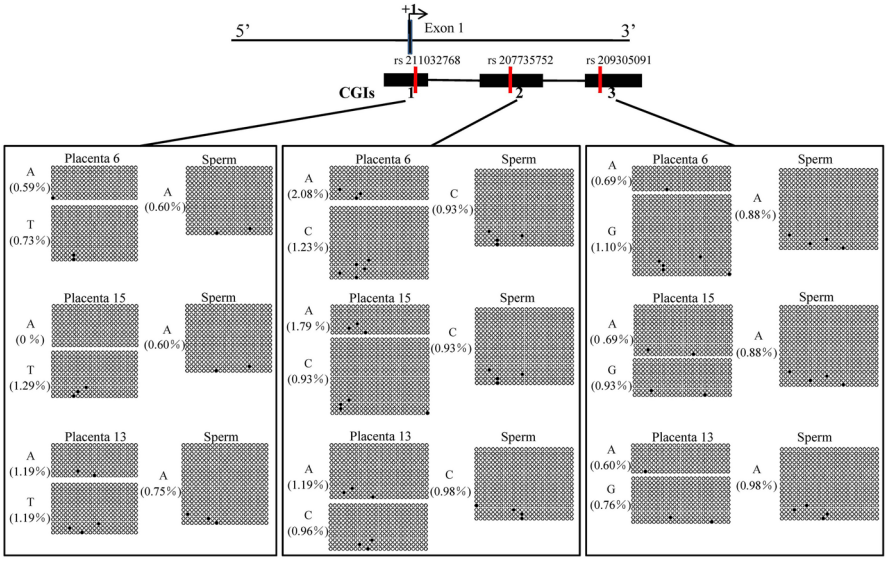

Fig. 3 Methylation profiles of three CGIs in bovine *SLC38A4* in three placentas and their corresponding paternal sperms. The locations of three CGIs are shown surrounding promoter and exon 1. The SNPs in three CGIs are indicated by red bar. Methylated and unmethylated residues are indicated by filled and open circles, respectively. The percentages of methylated CpG sites of the three heterozygous placentas and their corresponding sperms are shown at the left side of the methylation pictures (Color figure online)

two parental allele strands. Extreme hypomethylation (< 3%) was observed at the three CGIs in the three detected placenta and their corresponding paternal sperms. Furthermore, no significant difference was observed between the paternal and maternal strands. These results suggest that DNA methylation of these three regions does not appear to directly or indirectly affect the allelic transcription of bovine *SLC38A4*.

Discussion

In human, the *SLC38A4* gene encodes a neutral amino acid transporter and has a significant impact on the fetus and placental development (Desforges et al. 2006; Li et al. 2012). The *slc38a4* gene has different allelic expression patterns in mouse highly expressed placenta and liver, where paternal expression is observed in the placenta and biallelic expression is observed in the liver (Smith et al. 2003). The imprinted expression of *Slc38a4* can be disturbed by environmental endocrine disruptors in mice yolk sac (Kang et al. 2011). In this study, we report the biallelic expression of *SLC38A4* in adult bovine tissues, which is consistent with the previous observation that *SLC38A4* is not imprinted in the fetus (Zaitoun and Khatib 2006). *SLC38A4* shows a complex isoform- and tissue-specific maternal expression in swine (Bischoff et al. 2009). In short, the imprinting patterns of *SLC38A4* are different between mice, swine, and bovine.

Polymorphic imprinting refers to that the imprinting of a gene is variable between individuals. This epigenetic phenomenon has been observed in the human *5-HT2A* and *IGF2* genes (Bunzel et al. 1998; Sakatani et al. 2001). These variations in gene expression are considered a cause of phenotypic heterogeneity in human disease (Weinstein 2001). In this study, we obtained evidence for monoallelic (paternal) expression of *SLC38A4* in bovine placenta; however, biallelic expression of *SLC38A4* was also observed in other placenta, indicating that *SLC38A4* imprinting in the bovine population is polymorphic. Although the number of known genes demonstrating polymorphic imprinting is increasing, the molecular mechanisms underlying this phenomenon are largely uncharacterized. Polymorphic imprinting of the mouse *Ppp1r9a* and *Kvlqt1* genes are strain dependent, as differences in genetic background seem to affect the imprinting regulation of the two genes (Jiang et al. 1998). Polymorphic imprinting of the human *WT1* gene in placenta samples may be caused by alterations of the key sequence essential for *WT1* imprinting, which leads to an alteration of cis-acting factors.

SLC38A4 has a maternally methylated germline DMR in the promoter region of mice (Chotalia et al. 2009). In the cumulus cells-derived cloned mouse, the *SLC38A4* DMR showed maternal allele-specific methylation in donor cells that showed paternal expression, but not in brain of cloned mouse with biallelically *SLC38A4* expression. In the present study, there was no DMR found in the bovine *SLC38A4* promoter region in the placenta with imprinting expression of *SLC38A4*. These results indicate that polymorphic imprinting of bovine *SLC38A4* is not regulated by the methylation status of CGIs at the promoter, and that an epigenetic modification

other than DNA methylation might be needed for establishing *SLC38A4* imprinting in bovine placenta.

Acknowledgements This study was funded by Hebei province Natural Science Foundation of China (C2016204092) and The National Natural Science Foundation of China (31372312).

Compliance with Ethical Standards

Conflict of interest The authors are responsible for the results of the data enumerated in this experiment and have no conflict of interest.

Informed Consent All laboratory animals were obtained the consent to their owners. All protocols involving the use of animals were approved by the Agriculture Research Animal Care Committee of Hebei Agriculture University. The information about guidelines was obtained from each participant included in the study.

References

Barlow DP, Bartolomei MS (2014) Genomic imprinting in mammals. Cold Spring Harb Perspect Biol 6:a018382. https://doi.org/10.1101/cshperspect.a018382

Bischoff SR, Tsai S, Hardison N, Motsinger-Reif AA, Freking BA, Nonneman D, Rohrer G, Piedrahita JA (2009) Characterization of conserved and nonconserved imprinted genes in swine. Biol Reprod 81(5):906–920. https://doi.org/10.1095/biolreprod.109.078139

Bunzel R, Blümcke I, Cichon S, Normann S, Schramm J, Propping P, Nöthen MM (1998) Polymorphic imprinting of the serotonin-2A (*5-HT2A*) receptor gene in human adult brain. Brain Res Mol Brain Res 59(1):90–92. https://doi.org/10.1016/S0169-328X(98)00146-6

Chotalia M, Smallwood SA, Ruf N, Dawson C, Lucifero D, Frontera M, James K, Dean W, Kelsey G (2009) Transcription is required for establishment of germline methylation marks at imprinted genes. Genes Dev 23(1):105–117. https://doi.org/10.1101/gad.495809

Desforges M, Lacey HA, Glazier JD, Greenwood SL, Mynett KJ, Speake PF, Sibley CP (2006) SNAT4 isoform of system A amino acid transporter is expressed in human placenta. Am J Physiol Cell Physiol 290(1):305–312. https://doi.org/10.1152/ajpcell.00258.2005

Docherty LE, Rezwan FI, Poole RL, Jagoe H, Lake H, Lockett GA, Arshad H, Wilson DI, Holloway JW, Temple IK, Mackay DJ (2014) Genome-wide DNA methylation analysis of patients with imprinting disorders identifies differentially methylated regions associated with novel candidate imprinted genes. J Med Genet 51(4):229–238. https://doi.org/10.1136/jmedgenet-2013-102116

Jiang S, Hemann MA, Lee MP, Feinberg AP (1998) Strain-dependent developmental relaxation of imprinting of an endogenous mouse gene, *Kvlqt1*. Genomics 53(3):395–399. https://doi.org/10.1006/geno.1998.5511

Kang ER, Iqbal K, Tran DA, Rivas GE, Singh P, Pfeifer GP, Szabó PE (2011) Effects of endocrine disruptors on imprinted gene expression in the mouse embryo. Epigenetics 6(7):937–950

Li Z, Lai G, Deng L, Han Y, Zheng D, Song W (2012) Association of *SLC38A*4 and system A with abnormal fetal birth weight. Exp Ther Med 3(2):309–313. https://doi.org/10.3892/etm.2011.392

Mizuno Y, Sotomaru Y, Katsuzawa Y, Kono T, Meguro M, Oshimura M, Kawai J, Tomaru Y, Kiyosawa H, Nikaido I, Amanuma H, Hayashizaki Y, Okazaki Y (2002) Asb4, Ata3, and Dcn are novel imprinted genes identified by high-throughput screening using RIKEN cDNA microarray. Biochem Biophys Res Commun 290(5):1499–1505. https://doi.org/10.1006/bbrc.2002.6370

Partida GC, Laurin C, Ring SM, Gaunt TR, Relton CL, Smith GD, Evans DM (2017) Imprinted loci may be more widespread in humans than previously appreciated and enable limited assignment of parental allelic transmissions in unrelated individuals. bioRxiv. https://doi.org/10.1101/161471

Peters J (2014) The role of genomic imprinting in biology and disease: an expanding view. Nat Rev Genet 15(8):517–530. https://doi.org/10.1038/nrg3766

Reik W, Walter J (2001) Genomic imprinting: parental influence on the genome. Nat Rev Genet 2(1):21–32. https://doi.org/10.1038/35047554

Sakatani T, Wei M, Katoh M, Okita C, Wada D, Mitsuya K, Meguro M, Ikeguchi M, Ito H, Tycko B, Oshimura M (2001) Epigenetic heterogeneity at imprinted loci in normal populations. Biochem Biophys Res Commun 283(5):1124–1130. https://doi.org/10.1006/bbrc.2001.4916

Smith RJ, Dean W, Konfortova G, Kelsey G (2003) Identification of novel imprinted genes in a genome-wide screen for maternal methylation. Genome Res 13(4):558–569. https://doi.org/10.1101/gr.781503

Soellner L, Begemann M, Mackay DJ, Grønskov K, Tümer Z, Maher ER, Temple IK, Monk D, Riccio A, Linglart A, Netchine I, Eggermann T (2017) Recent advances in imprinting disorders. Clin Genet 91(1):3–13. https://doi.org/10.1111/cge.12827

Sugawara M, Nakanishi T, Fei YJ, Martindale RG, Ganapathy ME, Leibach FH, Ganapathy V (2000) Structure and function of ATA3, a new subtype of amino acid transport system A, primarily expressed in the liver and skeletal muscle. Biochem Biophys Acta 1509(1–2):7–13. https://doi.org/10.1016/S0005-2736(00)00349-7

Wei Y, Su J, Liu H, Lv J, Wang F, Yan H, Wen Y, Liu H, Wu Q, Zhang Y (2014) MetaImprint: an information repository of mammalian imprinted genes. Development 141(12):2516–2523. https://doi.org/10.1242/dev.105320

Weinstein LS (2001) The role of tissue-specific imprinting as a source of phenotypic heterogeneity in human disease. Biol Psychiatry 50(12):927–931. https://doi.org/10.1016/S0006-3223(01)01295-1

Zaitoun I, Khatib H (2006) Assessment of genomic imprinting of *SLC38A4*, *NNAT*, *NAP1L5*, and *H19* in cattle. BMC Genet 7(1):49–59. https://doi.org/10.1186/1471-2156-7-49