
RESEARCH ARTICLE

Expression and imprinting of *DIO3* and *DIO3OS* genes in Holstein cattle

WENZHI YANG¹, DONGJIE LI², GUANGNAN WANG¹, XIHONG WU¹,
MINGYUE ZHANG¹, CUI ZHANG¹, YALI CUI¹ and SHIJIE LI^{1*}

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

²*College of Life Science and Life Engineering, Hebei Science and Technology University, Shijiazhuang 050018, China;*

^{1*} Corresponding author, lishijie20005@163.com

Abstract: *DIO3* and *DIO3OS* are two imprinted genes identified in the mouse and humans. The *DIO3* gene, which encodes for the type 3 deiodinase, is preferentially expressed from the paternal allele, while the *DIO3OS* transcript is transcribed in opposite orientation to *DIO3*, multiple non-coding alternatively splicing isoforms from maternal allele. In this study, the five splice variants of *DIO3OS* were identified in Holstein cattle and had complex, tissue-specific expression patterns observed in eight tissues, including heart, liver, spleen, lung, kidney, muscle, fat and brain. In the G+C rich region upstream from the cattle *DIO3* gene, there were three small conserved regions and some promoter elements similar to those observed in the mouse and humans. An allele-specific expression analysis-based SNP method revealed that *DIO3* and *DIO3OS* genes exhibited monoallelic expression in the eight tissues, indicating that *DIO3* and *DIO3OS* are imprinted in cattle.

Keywords: *DIO3*, *DIO3OS*, cattle, expression, imprinting

Introduction

In recent years, long non-coding RNAs (lncRNAs) have been found to transcribe across most of the mammalian genome (e.g. Carninci *et al.* 2005; e.g. Katayama *et al.* 2005; e.g. Huang *et al.* 2011). LncRNAs regulate gene expression via overlap with the target gene or promoter (e.g. Amaral and Mattick 2008), or by modification of the chromatin structure through epigenetic marks (e.g. Khalil *et al.* 2009). Some lncRNAs are located within regions of genomic imprinting, which are characterized by parent-origin-specific monoallelic expression. Some antisense imprinted lncRNAs are expressed when they are inherited from the opposite parental chromosome and overlap with the sense protein-coding genes. The majority of lncRNAs identified in the imprinted region have tissue-specific transcript isoforms (e.g. Numata *et al.* 2010).

DIO3 (type 3 deiodinase) and *DIO3OS* (*DIO3* opposite strand) genes, paired sense/antisense transcripts, lie at the distal end of the *GTL2/DIO3* imprinted cluster. *DIO3* and two other protein-coding genes (*DLK1* and *RTL1*) are preferentially paternally expressed in the *GTL2/DIO3* imprinted region of mouse and humans (e.g. Hagan *et al.* 2009). *DIO3OS* is an lncRNA transcribed antisense to *DIO3*, and partially overlaps the *DIO3* promoter region in mouse and humans (e.g. Hernandez 2005). Multiple *DIO3OS* transcripts are detected in both mouse and humans, with their exon-intron structure conserved (e.g. Hernandez *et al.* 2004). In rat, both *DIO3* and *DIO3OS* genes are expressed from the same alleles and exhibit a brain

region-specific imprinted expression pattern (e.g. Dietz *et al.* 2012). The cattle *DIO3* cDNA sequence has been identified (e.g. Connor *et al.* 2005), however, the *DIO3OS* transcripts and the imprinting status of *DIO3* and *DIO3OS* genes have not been reported. The objectives of this study were to determine how the *DIO3OS* gene alternatively splices and characterize *DIO3* and *DIO3OS* imprinting in cattle.

Materials and methods

Animals and tissues

Tissue samples from 32 adult female dairy cows (Holstein) were collected from a local abattoir for use in this study. Samples from heart, liver, spleen, lung, kidney, muscle, subcutaneous fat and brain were frozen in liquid nitrogen immediately after collection. All procedures involving the use of animals were approved by the Agriculture Research Animal Care Committee of Hebei Agriculture University.

RNA extraction and reverse transcription

RNA isolated from three animals was used to analyze the structure and expression patterns of *DIO3OS* splicing variants. Total RNA was prepared from various tissues, including heart, liver, spleen, lung, kidney, muscle, subcutaneous fat and brain with Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. Genomic DNA was removed using DNase I. The quality and quantity of the remaining RNA were tested using ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). Total RNA (1 µg) was reverse transcribed using five units of Superscript RT enzyme (Promega, Madison, WI, USA) and oligo (dT) primers. A reaction without the RT enzyme was performed as a negative control to ensure no amplification from genomic

DNA in the subsequent RT-PCR. The RT protocol includes 65 °C for 5 min to denature the secondary RNA structure, 37 °C for 1 h and 70 °C for 15 min.

Cloning of cattle *DIO3OS* transcript variants

Putative *DIO3OS* sequence was identified by searching the expressed sequences in 10 kb of the cattle *DIO3* gene 5'-flanking region using the UCSC Genome Browser (<http://genome.ucsc.edu>). PCR primers for amplification of putative cattle *DIO3OS* transcripts were designed manually based on cattle *DIO3OS* exons in splice variant ESTs DY170744 (*DIO3OS*-VF, 5'-GGAACGTCGGGACTGG-3') and DN281584 (*DIO3OS*-VR, 5'-ATTTATTGGATTCTCGG-3'). The amplification conditions were as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, a final extension at 72 °C for 10 min. PCR reaction mixture (25µl) contained: 1 µl forward and reverse primers (10µM), 1 µl first-strand cDNA, 9.5 µl ddH₂O and 12.5 µl ES Taq MasterMix (CWBio, China). Template cDNA from each tissue was standardized by amplifying a 375-bp internal control of *GAPDH* (a house keeping gene, GenBank accession no. BTU85042). The *DIO3OS* amplified products were extracted from agarose gels, purified, cloned into PMD18-T vectors (TaKaRa, China) and sequenced using an ABI PRISM 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA).

DNA extraction and PCR amplification to identify SNP

Genomic DNA was extracted from 32 frozen liver tissue samples with a DNA Extraction Kit (Sangon, China) and screened for single nucleotide polymorphisms (SNPs). About 100 ng of genomic DNA was used as the template. *DIO3*-F

(5'-GTAGGGGTCTTTAGGTG-3') and *DIO3*-R (5'-AAGGCAGGAACAAGAC-3') primers were used to amplify a 537 bp *DIO3* DNA fragment. A 437 bp *DIO3OS* DNA fragment was obtained using the *DIO3OS*-F (5'-CCCACCCAGAGGAAAGG-3') and *DIO3OS*-R (5'-GCAGCGTTGGCTTTTG -3') primers. PCR reaction mixture (25µl) contained: 1µl forward and reverse primers (10µM), 1µl genomic DNA, 9.5µl ddH₂O and 12.5µl ES Tap MasterMix (CWBio, China). The amplification conditions were as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, a final extension at 72 °C for 10 min. PCR products were purified with a UNIQ-10 column DNA gel extraction kit (Sangon, China) and sequenced directly with an ABI PRISM 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA).

Allele-specific expression of cattle *DIO3* and *DIO3OS* genes

RNA from three heterozygous Holsteins was used to analyze imprinting status of cattle *DIO3* and *DIO3OS* genes. Total RNA was isolated from eight tissues (heart, liver, spleen, lung, kidney, muscle, subcutaneous fat and brain) and reverse transcribed into cDNA. Primers were designed to determine mRNA expression levels of *DIO3*, *DIO3OS* and *GAPDH* (as an internal control) based on gene specific sequences (GenBank accession no. BTU85042). Template cDNA from each tissue was standardized by amplifying a 375-bp internal control of *GAPDH* (a house keeping gene) with primers Gap-F (5'-GCACAGTCAAGGCAGAGAA C-3') and Gap-R (5'-GTGGCAGTGATGGCGTGGA-3'). *DIO3*-F, *DIO3*-R, *DIO3OS*-F, and *DIO3OS*-R primers were used to amplify a 537 bp *DIO3* fragment and a 437 bp *DIO3OS* fragment, respectively. The RT-PCR products contain the identified SNP

site. PCR products were electrophoresed on 1.5% agarose gel and scanned by a gel automatic imaging system (Pro logic 212 imaging system, Carestream, USA). Relative mRNA levels were expressed as the ratio of the intensity of the detected band to the intensity of the *GAPDH* band. All reactions were repeated three times to ensure repeatability. The RT-PCR products were purified and sequenced directly.

Results

Conservation of the cattle *DIO3* 5'-flanking region

In the mouse and humans, 1.5 kb of the G+C rich (80% of the sequence) region was found to be upstream of the *DIO3* gene and 83% homologous between the two species. A finer comparative analysis shows there are three small and more highly conserved regions designated A, B and C (240, 420, and 290 bp, respectively) with 86%, 92%, and 96% homology between mouse and humans. Analysis of the 5'-flanking region of cattle *DIO3* gene (Genbank accession no. NM_001010993.3) shows that the G+C-rich region spans about 2.2 kb and is 81% and 78% homology to those of mouse and humans, respectively (Fig 1). The three small and more highly conserved regions A, B and C were also observed in the cattle *DIO3* 5'-flanking region, with 88%, 87%, and 80% homology to those of mouse, and 88%, 89%, and 82% homology to those of humans (Fig 1). Analysis of the cattle basal promoter regions (240 bp of the 5'-flanking region) shows some similarly conserved promoter elements to those in mouse and humans (Fig 1), including the TATA, CAAT and GC boxes.

Identification and structure of cattle *DIO3OS*

The Putative *DIO3OS* sequence was identified by searching the expressed sequences in 10 kb of the cattle *DIO3* gene 5'-flanking region using the UCSC Genome Browser (<http://genome.ucsc.edu>). 18 ESTs, including 10 spliced (Genbank accession no. DN280164, DN281584, BM106149, CO892913, DY048675, DY195561, BM254689, DT813312, DY179744 and CR850372) and 8 unspliced transcripts (Genbank accession no. AW657862, BE755453, BM105526, AW655553, BM288146, DT815882, BF705934 and EH170095) were found. RT-PCR was used to amplify the transcripts from different tissues of three individuals. Five alternatively spliced transcripts of 792, 644, 949, 680 and 734 bp were identified. These transcripts were named from *DIO3OS-v1* to *DIO3OS-v5* (Fig 2) and submitted to NCBI (GenBank accession no. from KU169889 to KU169893). Most of these splice variants contain five exons. When aligned with *DIO3OS-v1*, *DIO3OS-v2* lacks exon 4, and 291-bp-long intron 4 turns to an exon in *DIO3OS-v3* splice. *DIO3OS-v4* has a shorter exon 1 and 2. A shorter exon 3 with 54 missing bp at the 5' end was observed in the *DIO3OS-v5* splice. The cattle *DIO3OS* cDNAs contain multiple small open reading frames (ORFs). However, none of the ATGs were consistent with Kozak consensus sequence. The longest open reading frame is potentially composed of 157 amino acids, which are encoded by exon 1, 2, 3 and part of exon 4 in the *DIO3OS-v3* splice.

Expression patterns of cattle DIO3OS splicing variants

RT-PCR analysis show a complex *DIO3OS* expression pattern of transcripts in multiple tissues (Fig 3). The splice variants in the order from largest to smallest were *DIO3OS-v3*, *DIO3OS-v1*, *DIO3OS-v5*, *DIO3OS-v4* and *DIO3OS-v2*. The *DIO3OS-v1* splice variant was expressed in five types of tissues: liver, spleen, kidney, skeletal muscle and subcutaneous fat. The *DIO3OS-v2* variant was detected only in liver. The *DIO3OS-v3* variant was expressed in four tissues: heart, liver, skeletal

muscle and subcutaneous fat. The *DIO3OS*-v3 variant was expressed in three tissues: liver, skeletal muscle and subcutaneous fat. The *DIO3OS*-v5 variant was expressed in skeletal muscle and subcutaneous fat. None of the five *DIO3OS* splices was detected in lung and brain tissue.

Identification of SNP in cattle DIO3 and DIO3OS genes

To determine the allele-specific expression of the *DIO3* and *DIO3OS* genes, the SNPs were first detected by direct sequencing of PCR products from genomic DNA. Despite several primer pairs designed to search the SNP in cattle *DIO3OS* gene, no SNP site was found in the five most common exons of *DIO3OS* (data not show). A primer pair (*DIO3OS*-F and *DIO3OS*-R) was designed according to another unspliced transcript (Genbank accession no. EH170095, Fig 2) with a longer exon 2 (Fig 2). One SNP, the c.313 A>T transversion (Genbank accession no. EH170095, Fig 4A), was found in the 437 bp fragment of *DIO3OS* amplified from genomic DNA. A 537 bp *DIO3* fragment was amplified from genomic DNA and an informative SNP site, c. 1891 G>A transition (Fig 4A), was found in cattle *DIO3* sequence (Genbank accession no. NM_001010993.3). This SNP converts the Ala (GCG) codon to Thr (ACG).

Allele-specific expression of cattle DIO3 and DIO3OS genes

Heterozygous animals were used for allele-specific expression analysis of *DIO3* and *DIO3OS* genes. Allele-specific expression of the *DIO3* and *DIO3OS* genes was determined by comparing sequencing chromatograms of the base at heterozygosis sites between genomic DNA PCR and RT-PCR products of the same samples. A 537 bp *DIO3* fragment and a 437 bp *DIO3os* fragment were amplified from cDNA using the primers *DIO3*-F and *DIO3*-R, *DIO3os*-F and *DIO3os*-R, respectively (Fig 4B). The RT-PCR product sequencing results obtained from different tissues revealed that

only one parental allele (G in *DIO3* and A in *DIO3OS*) at the SNP site was expressed in heart, liver, spleen, lung, kidney, muscle, fat and brain tissues, suggesting that *DIO3* and *DIO3OS* are imprinted in cattle (Fig 4C).

Discussion

The *DIO3* gene encodes for the type 3 deiodinase and plays an important role in maintaining appropriate levels of thyroid hormone (TH) in the fetus and adult (e.g. St Germain 1997). The cattle *DIO3* cDNA sequences have been isolated previously (e.g. Connor *et al.* 2005), and the coding and 3'-untranslated regions are contained in a single exon similar to those of mouse and humans (e.g. Hernandez *et al.* 2004). A finer comparative sequence analysis shows that gene structure and promoter elements of the cattle *DIO3* gene are essentially identical to those of the mouse and humans.

Five *DIO3OS* cDNAs with the 5'-end within the CpG rich region are identified from different cattle tissues. Cattle *DIO3OS* presents at least five exons and also has a conserved polyadenylation site containing a *Bam*HI site, which has been found in mouse and humans. Similar to mouse and humans, cattle *DIO3OS* exon 1 is highly conserved (83 and 84% homology to those of mouse and humans, respectively) and includes sequences for pre-microRNA bta-mir1247. Its homologous sequences of mouse and humans encode mmir1247 and has-mir1247, respectively. Multiple small open reading frames (ORFs) are found in cattle *DIO3OS* cDNA, however, none of them includes a ATGs with high homology with the Kozak consensus sequence (e.g. Kozak 1987). These findings suggest that *DIO3OS* is a non-coding gene.

Genomic imprinting is a parent-dependent epigenetic marking, which induces gene monoallele expression in a parent origin-specific manner. The first long non-coding RNAs (lncRNAs) were identified in imprinting regions (e.g. Autuoro *et al.*

2014), with the antisense lncRNA transcripts usually reciprocally imprinted to the sense protein-coding gene (e.g. Peters and Robson 2008; e.g. O'Neill 2005). Imprinted lncRNAs employ diverse mechanisms to regulate the parent origin-specific expression of target genes, including by promoting long-range intra-chromosomal interactions, or via transcriptional occlusion mechanisms (e.g. Kanduri 2016). Multiple sense/antisense pairs of coding and non-coding RNAs with significant overlap are found within imprinted gene clusters (e.g. Katayana *et al.* 2005; e.g. O'Neill 2005). *DIO3* and *DIO3OS* are two transcripts reciprocally imprinted and located in the *GTL2/DIO3* imprinted cluster. *DIO3OS/DIO3OS* partially overlaps the *DIO3/DIO3* promoter region in mouse and humans (Hernandez *et al.* 2005). However there was no significant overlapping observed in the cattle *DIO3/DIO3OS* locus in this study. This characteristic was similar to the rat *DIO3/DIO3OS* genes (e.g. Dietz *et al.* 2012).

In this study, we found that both *DIO3* and *DIO3OS* are monoallelically expressed in the eight detected tissues, including heart, liver, spleen, lung, kidney, muscle, fat and brain, indicating that *DIO3* and *DIO3OS* are imprinted in the cattle. Three other lncRNAs were identified as imprinted in cattle *GTL2/DIO3* in our previous studies (e.g. Zhang *et al.* 2012; e.g. Hou *et al.* 2011; e.g. Su *et al.* 2011). However, many imprinted genes exhibit tissue-specific allelic expression patterns (e.g. Arnaud *et al.* 2003; e.g. Plagge and Kelsey 2006). In the mouse fetus, *DIO3* is preferentially expressed from the paternal allele (e.g. Hernandez *et al.* 2002; e.g. Tsai *et al.* 2002), whereas biallelic *DIO3* expression was observed in the developing eye, testes, and cerebellum and postnatal brain neocortex (e.g. Martinez *et al.* 2014). Uniquely, both rat *DIO3* and *DIO3OS* transcripts exhibit brain region-specific imprinted expression patterns, and occur on the same alleles (e.g. Dietz *et al.* 2012).

In the mouse and humans, three DMRs have been determined in the *DLK1/GTL2* imprinted domain, the *DLK1*-DMR, the intergenic (IG) DMR and the *GTL2*-DMR. The IG-DMR is associated with proper imprinting of linked genes on the maternal chromosome and the *GTL2*-DMR may be implicated in imprinting on both parental chromosomes (e.g. Takada *et al.* 2000; e.g. Carr *et al.* 2007). However, methylation of the IG-DMR and the *GTL2*-DMR did not involve in tissue-specific allelic expression patterns of mouse *DIO3*, suggesting the existence of unidentified epigenetic modifications determining tissue-specific *DIO3* imprinting (e.g. Martinez *et al.* 2014).

Acknowledgement

This study was supported by the National Natural Science Foundation of China (31372312).

References

- Amaral P. P. and Mattick J. S. 2008 Noncoding RNA in development. *Mamm Genome* **19**, 454–492.
- Arnaud P., Monk D., Hitchins M., Gordon E., Dean W., Beechey C. V. *et al.* 2003 Conserved methylation imprints in the human and mouse *GRB10* genes with divergent allelic expression suggests differential reading of the same mark. *Hum Mol Genet* **12**, 1005-1019.
- Autuoro J. M., Pirnie S. P. and Carmichael G. G. 2014 Long noncoding RNAs in imprinting and X chromosome inactivation. *Biomolecules* **4**, 76-100.
- Carninci P., Kasukawa T., Katayama S., Gough J., Frith M. C, Maeda N. *et al.* 2005 The

-
- transcriptional landscape of the mammalian genome. *Science* **309**, 1559–1563.
- Carr M. S., Yevtodiyenko A., Schmidt C. L. and Schmidt J. V. 2007 Allele-specific histone modifications regulate expression of the *Dkl1-Gtl2* imprinted domain. *Genomics* **89**, 280–290.
- Connor E. E., Laiakis E. C., Fernandes V. M., Williams J. L., Capuco A.V. 2005 Molecular cloning, expression and radiation hybrid mapping of the cattle deiodinase type II (*DIO2*) and deiodinase type III (*DIO3*) genes. *Anim Genet* **36**, 240-3.
- Dietz W. H., Masterson K., Sittig L. J., Redei E. E. and Herzing L.B. 2012 Imprinting and expression of *Dio3os* mirrors *Dio3* in rat. *Front Genet* **3**, 279-279.
- Hagan J. P., O'Neill B. L., Stewart C. L., Kozlov S. V. and Croce C. M. 2009 At least ten genes define the imprinted *Dkl1-Dio3* cluster on mouse chromosome 12qF1. *PLoS One* **4**, e4352.
- Hernandez A. 2005 Structure and function of the type 3 deiodinase gene. *Thyroid* **15**, 865–874.
- Hernandez A., Fiering S., Martinez E., Galton V. A. and St Germain D. 2002 The gene locus encoding iodothyronine deiodinase type 3 (*Dio3*) is imprinted in the fetus and expresses antisense transcripts. *Endocrinology* **143**, 4483–4486.
- Hernandez A., Martinez M. E., Croteau W. and St Germain D. L. 2004 Complex organization and structure of sense and antisense transcripts expressed from the *DIO3* imprinted locus. *Genomics* **83**, 413–424.
- Hou X. H., Li D. J., Su H., Hu J. Q., Li N. and Li S. J. 2011 Molecular cloning, expression, and imprinting status of maternally expressed gene 8 (*MEG8*) in dairy cattle. *Russian*

-
- Journal of Genetics* **47**, 994–8.
- Huang R., Jaritz M., Guenzl P., Vlatkovic I., Sommer A., Tamir I. M. *et al.* 2011 An RNA-seq strategy to detect the complete coding and non-coding transcriptome including full-length imprinted macro ncRNAs. *PLoS ONE* **6**, e27288.
- Kanduri C. 2016 Long noncoding RNAs: Lessons from genomic imprinting. *Biochim Biophys Acta* **1859**,102-11.
- Katayama S., Tomaru Y., Kasukawa T., Waki K., Nakanishi M., Nakamura M. *et al.* 2005 Antisense transcription in the mammalian genome. *Science* **309**, 1564–1566.
- Kozak M. 1987 At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. *J Mol Biol* **196**, 947–950.
- Martinez M. E., Charalambous M., Saferali A., Fiering S., Naumova A. K., St Germain D. *et al.* 2014 Genomic imprinting variations in the mouse type 3 deiodinase gene between tissues and brain regions. *Mol Endocrinol* **28**, 1875-86.
- Numata K., Kohama C., Abe K. and Kiyosawa H. 2010 Highly parallel SNP genotyping reveals high-resolution landscape of mono-allelic *Ube3a* expression associated with locus-wide antisense transcription. *Nucleic Acids Res* **39**, 2649-2657.
- O'Neill M. J. 2005 The influence of non-coding RNAs on allele-specific gene expression in mammals. *Hum Mol. Genet* **14**, 113–120.
- Peters J. and Robson J. E. 2008 Imprinted noncoding RNAs. *Mamm Genome* **19**, 493–502.
- Plagge A. and Kelsey G. 2006 Imprinting the Gnas locus. *Cytogenet Genome Res* **113**, 178-187.
- Su H., Li D., Hou X., Tan B., Hu J., Zhang C. *et al.* 2011 Molecular structure of cattle *Gtl2*

-
- gene and DNA methylation status of *Dlk1-Gtl2* imprinted domain in cloned cattles. *Animal Reproduction Science* **127**, 23–30.
- St Germain D. L. and Galton V. A. 1997 The deiodinase family of selenoproteins. *Thyroid* **7**, 655–668.
- Takada S., Tevendale M., Baker J., Georgiades P., Campbell E., Freeman T. *et al.* 2000 Delta-like and *Gtl2* are reciprocally expressed, differentially methylated linked imprinted genes on mouse chromosome 12. *Curr. Biol* **10**, 1135-1138.
- Tsai C., Lin S.P., Ito M., Takagi N., Takada S. and Ferguson-Smith A. C. 2002 Genomic imprinting contributes to thyroid hormone metabolism in the mouse embryo. *Curr Biol* **12**, 1221-1226.
- Zhang K., Li D., Wang M., Wu G., Shi Y. and Li S. 2014 The differential expression of alternatively spliced transcripts and imprinting status of *MEG9* gene in cows. *Anim Genet* **45**, 660-4.

Received 11 June 2016; accepted 14 October 2016

Unedited version published online: 18 October 2016

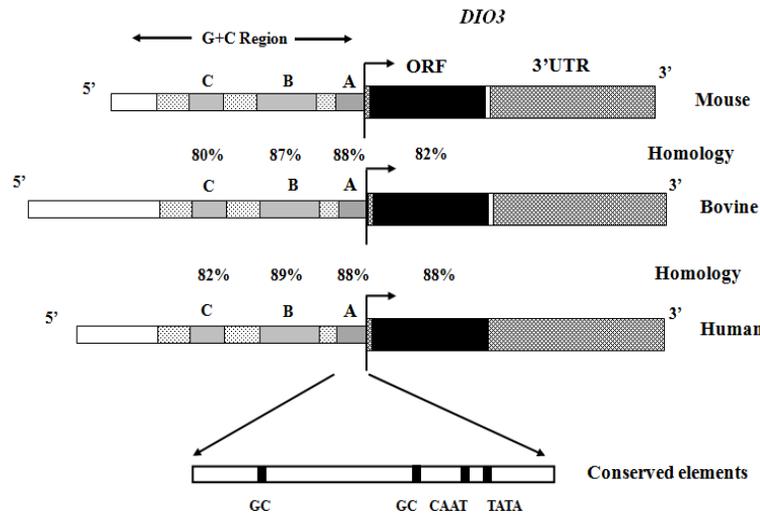


Fig.1 Homology of *DIO3* genes and promoter regions between cattle and mouse, and cattle and humans.

The *DIO3* exon is shown as a thick bar and the open reading frame (ORF) is shown in black. Highly conserved sequences within approximately 1.7 kb of 5' flanking region (designated A, B, and C) are shown in thinner, gray bars. Homology of A, B, and C between cattle and mouse, cattle and humans are showed respectively.

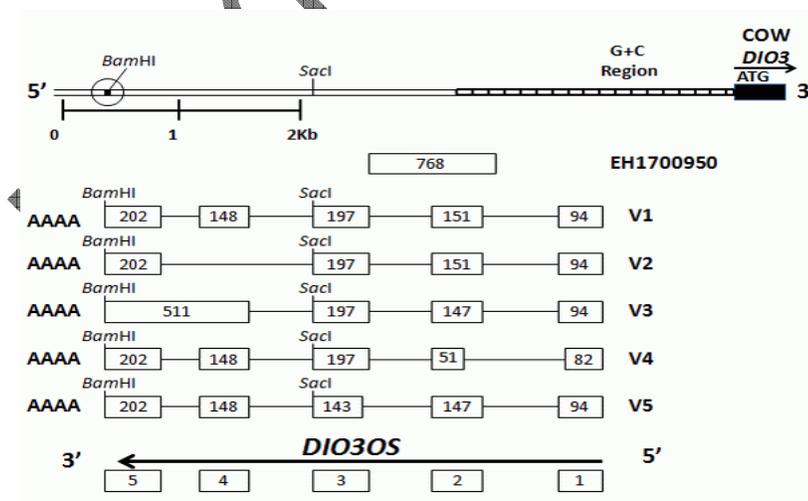


Fig.2 Partial structure and splice variants of the cattle *DIO3OS* gene.

The exon–intron structures, compared to the genomic sequence, five alternative splice variants for the cattle *DIO3OS* gene are shown. Exons are depicted as thick bars and introns as thin bars. The first cDNA (accession numbers EH1700950) was found in GenBank and used for allele-specific expression analysis of cattle *DIO3OS* gene, while the other 5 (numbered from v1 to v5) were generated in these studies from RNA of different cattle tissues by RT-PCR methods. The five exons most consistently observed in the *DIO3OS* gene are designated at the bottom as 1 through 5. *DIO3* transcriptional (arrow) and translational start sites (ATG) are indicated. The short region containing the polyadenylation site conserved in mouse, human and cattle is circled in the genomic diagram.

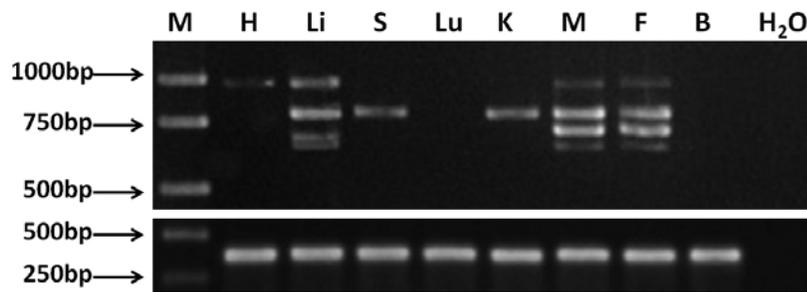


Fig.3 Expression patterns of the cattle *DIO3OS* splices in eight tissues analyzed by RT-PCR. The amplified fragments from big to small were 949 bp *DIO3OS*-v3, 792 bp *DIO3OS*-v1, 734 bp *DIO3OS*-v5, 680 bp *DIO3OS*-v4 and 644 bp *DIO3OS*-v2, respectively. Amplification of bp *GAPDH* from each tissue was used for confirmation of RNA integrity suitable for RT-PCR. M, DL-2000 marker (TaKaRa); H, heart; Li, liver; S, spleen; Lu, lung; K, kidney; M, muscle; F, subcutaneous fat; B, brain; H₂O, negative control.

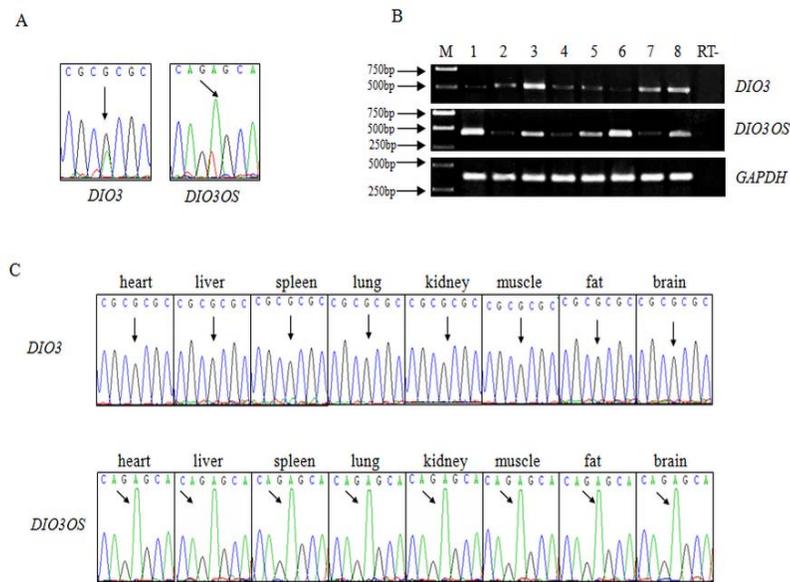


Fig.4 Identification of SNP and allele-specific expression of *DIO3* and *DIO3OS* identified by direct sequencing method.

(A) Single nucleotide polymorphism (SNP) site of the *DIO3* and *DIO3OS* gene. Sequence analysis of genomic DNA shows a c.1891 G>A SNP in *DIO3*, and a c.313 A>T SNP in *DIO3OS*. (B) Photographs of representative gels from RT-PCR analysis of 537 bp for *DIO3*, and 437 bp for *DIO3OS*, and 367 bp for *GAPDH*. M, DL-2000 marker (TaKaRa); lanes 1-8 are RT-PCR obtained from heart, liver, spleen, kidney, lung, skeletal muscles, subcutaneous fat and brain; RT-, negative control. (C) Allele-specific expression of *DIO3* and *DIO3OS*. Both *DIO3* and *DIO3OS* showed monoallelic expressions in eight detected tissues of heart, liver, spleen, kidney, lung, skeletal muscles, subcutaneous fat and brain.