Biochemical Genetics (2019) 57:801–812
https://doi.org/10.1007/s10528-019-09925-1

ORIGINAL ARTICLE

Tissue-Specific Monoallelic Expression of Bovine *AXL* is Associated with DNA Methylation of Promoter DMR

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Received: 4 June 2018 / Accepted: 2 May 2019 / Published online: 9 May 2019

Abstract

The AXL protein is a receptor tyrosine kinase and is often implicated in proliferation, migration and therapy resistance in various cancers. The *AXL* gene in humans is maternally expressed and paternally imprinted with differentially methylated regions (DMR) surrounding the promoter region. However, the imprinting status and epigenetic regulation of *AXL* gene in cattle remain unclear. Therefore, we explored the molecular structure along with the patterns of allelic expression and DNA methylation of the bovine *AXL* gene. First, the complete cDNA sequence of bovine *AXL* was gathered by Sanger method, from transcripts obtained from RT-PCR, 5′ and 3′ -RACE. In silico BLAST alignments showed that the longest mRNA sequence of bovine *AXL* consists of 19 exons and encodes a protein of 887 amino acids. We further analyzed the allelic expression of bovine *AXL* by employing single-nucleotide polymorphism (SNP)-based sequencing method. A SNP site (GenBank Accession no: rs210020651) found in exon 7 allowed us to distinguish the two parental alleles. Monoallelic expression of *AXL* was observed in four adult bovine tissues (heart, liver, spleen and fat), while biallelic expression was found in the other adult tissues such as the lung, kidney, muscle, brain and placenta. To determine whether the DNA methylation played a role in the tissue-specific imprinting of bovine *AXL*, we performed bisulfite sequencing of two regions: region 1 was a CpG island (CGI) in *AXL* promoter, mapping to 643 bp upstream of the transcription start site of *AXL* 5′-v1 transcripts, while region two was homologous to the region of human *AXL* DMR, with 10 CpG sites overlapping the first translation start site (TSS1) of bovine *AXL*. In region 2, DNA from both monoallelic and biallelic expressed tissues were mostly found to be completely unmethylated. However, tissue-specific differential methylation patterns were found in monoallelic expressed tissues such as the heart and liver while hypomethylation was noted in the promoter CpG island in biallelic expressed tissues such as the lung. These observations demonstrated that the tissue-specific

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10528-019-09925-1) contains supplementary material, which is available to authorized users.

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monoallelic expression of bovine *AXL* is dependent on the DNA methylation of its promoter region.

Keywords Tissue-specific · DMR · *AXL* · Bovine

Introduction

Genomic imprinting refers to an epigenetic process that results in gene expression from either the maternal or the paternal allele, and is present in therian mammals (Barlow and Bartolomei 2014) and angiosperms (Rodrigues and Zilberman 2015). In humans, there are approximately 200 confirmed imprinted gene (Wei et al. 2014; Tucci et al. 2019), some of which play critical roles in regulating embryonic and placental growth and subsequent postnatal development (Peters 2014; Plasschaert and Bartolome 2014). More than 200 other loci predicted to be imprinted have been assigned by imputation strategy (Luedi et al. 2007). Some imprinted genes display tissue-specific and developmental stage-specific (Andergassen et al. 2017; Perez et al. 2015, 2016). In addition, dysregulation of the imprinted loci has been associated with 12 congenital diseases (Soellner et al. 2017). Imprinted loci are often found clustered in the genomes of mammals and are characterized by differentially methylated regions (DMRs) of DNA that confer parent-origin-specific transcription (Reik and Walter 2001; Docherty et al. 2014).

AXL (derived from the Greek word 'anexelekto') is a receptor tyrosine kinase belonging to the TAM (TYRO3, AXL and MERTK) subfamily, and was first identified as a transforming gene in human chronic myeloid leukemia (O'bryan et al. 1991). *AXL* is a putative tumor suppressor and dormancy regulator in prostate cancer (Axelrod et al. 2018). The methylation of *AXL* promoter is associated with lung function growth during adolescence.

Gao et al. 2018 *AXL* was originally identified to be expressed preferentially from the maternal allele, with polymorphic imprinting found in human white blood cells (Choufani et al. 2011). Additionally, a paternally methylated DMR containing 14 CpG sites was found in the promoter region of human *AXL* gene. In mouse embryos and placenta, *Axl* was found to be expressed from the maternal allele in a DNA methylation-dependent manner (Choufani et al. 2011). The objective of the present study was to investigate the patterns of allelic expression of *AXL* in the context of DNA methylation in bovine adult tissues and placenta.

Materials and Methods

Animal Tissues

Eight types of bovine tissues (brain, liver, lung, spleen, kidney, fat, heart and muscle) and term placentas were used in this study. Tissue samples were collected from

a local abattoir while term placentas were obtained from a cattle farm. The collected tissues were immediately snap-frozen and stored at − 80 °C.

RNA Isolation and cDNA Synthesis

For cloning the cDNA sequence of *AXL,* the total RNA was isolated and purified from frozen brain tissue using a RNA extraction kit (TianGen, Beijing, China) and following the manufacturer's instructions. The extraction was performed with three brain tissues collected from different cattle, and the RNA samples were finally pooled. A total of 1 μg of RNA was reverse transcribed to cDNA using a reverse transcription system (Promega, Madison, USA). The reaction was set up in a total volume of 20 μl that included 1 μl total RNA (1 μg), 4 μl 5 × GoScript™ Reaction Buffer, 13 μl Nuclease-free water, and 2 μl 10 × GoScript™ Enzyme Mix. The mixture was incubated at 42 °C for 60 min and then at 70 °C for 15 min.

Cloning of the cDNA Sequence of AXL

Primers of RT-v-F and RT-v-R were first designed to amplify the partial cDNA sequence of *AXL* as per the sequence specified by GeneBank accession no. XM_010814979. All the primers are provided in Supplementary Table 1. The RT-PCR was performed using FastPfu Fly DNA Polymerase PCR Kit (Transgen, Beijing, China), adhering to the routine PCR program with the annealing temperature of 55 °C and the extension time of 3 min. The 25 μl reaction mixture contained 1 μl cDNA, 0.5 μl each of primers RT-v-F and RT-v-R, 0.25 μl FastPfu Fly DNA Polymerase (5 u/1 μl), 5 μl 5 × FastPfu Fly Buffer, 2 μl 2.5 mm dNTP and 15.75 μl ddH_2O.

The method of RACE was applied to obtain the 5′ and 3′ ends of the *AXL* gene with SMARTer® RACE 5′/3′ kit (TaKaRa, Dalian, China). Two gene-specific primers for 5′ RACE (5′-GSPout and 5′-GSPin) and two gene-specific primers for 3′ RACE (3′-GSPout and 3′-GSPin) were designed according to the sequence obtained using the primers RT-v-F and RT-v-R. The amplification reactions of 5′ and 3′ ends were carried out with nested PCR, with the outer primers used to amplify the first-round and the inner primers used during the second round. The RACE products were purified using a gel recovery kit and were eventually linked to PMD19-T vector (TaKaRa, Dalian, China), and then sequenced by Sanger method. Finally, the sequencing results of the recombinant plasmids were analyzed.

SNP Identification

DNA was isolated from liver tissues and placentas of all samples stored at − 80 °C using a DNA Extraction Kit (TianGen, Beijing, China). With reference to the cloned sequence of bovine *AXL* (GenBank Accession no. XM_010814979), a pair of primers RT-1-F and RT-1-R were designed to identify SNPs. The 25 μl PCR reaction containing 1 μl DNA, 0.5 μl each of primers RT-1-F and RT-1-R, 0.25 μl *Taq* Plus DNA Polymerase (5 u/1 μl), 2.5 μl 10 × *Taq* Plus Buffer, 2 μl 2.5 mm dNTP and

18.25 µl ddH_2O. Cycling was performed under the following conditions: initial denaturation at 95 °C for 15 min, 35 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. The PCR products were purified and then sequenced. The genotype was identified by observing the sequencing chromatogram, and the heterozygous individuals were identified based on their double picks.

Allelic Expression Analysis

Tissue samples including placentas from three heterozygous individuals were used for the analysis of allelic expression of bovine *AXL*. For this, RNAs isolated from the samples were reverse transcribed using EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix kits (TransGen, Beijing, China), and the resulting cDNAs were in turn used as templates for subsequent RT-PCR reactions utilizing the primers RT-1-F and RT-1-R. The reaction volumes and procedures were similar to those used during the SNP identification analysis except that the DNA templates were replaced by cDNAs. Finally, the RT-PCR products were purified and sequenced.

DNA Methylation Analysis

A total of three different types of tissues from two heterozygous individuals (6, 29) were examined for *AXL* DNA methylation by bisulfite sequencing. Two regions were evaluated; 13 CpG sites in region 1 and 10 CpG sites in region 2, which fell within the 284 bp and 271 bp amplicons harbored in the *AXL* promoter region (chr18: 50675728–50676011; UCSC Genome Brower at http://genome.ucsc.edu/Bos_taurus_UTD_3.1.1/bosTau8) and the homologous region of human *AXL* DMR (chr18: 50677162–50677432), respectively. Methylated primers were designed with the help of an online software (http://www.urogene.org/methprimer). Genomic DNA (800 ng) was bisulfite treated using the EZ DNA Methylation kit (Zymo, Orange County, CA) following the manufacturer's guidelines and the resulting cytosine-converted DNA was used as a template for subsequent methylation assays. Two regions were amplified by nested PCR or semi-nested PCR for 40 cycles using FastPfu Fly DNA Polymerase. The second round of PCR was performed by using 1 µl of a tenfold dilution of the first-round PCR product as template, following which the end-products were purified and eventually cloned into pMD19-T vectors (Takara). Plasmid DNA was extracted from clones containing the amplicons with the help of a GeneElute Plasmid Miniprep kit (Sigma). Independent plasmid clones (11–17) were sequenced and the output data were analyzed using Methprimer software (http://www.urogene.org/methprimer/). The methylation percentage of mCpG/(mCpG + CpG) of each tissue was calculated. In addition, the methylation percentage of each clone was analyzed. In principle, a clone was considered to be either hypermethylated or hypomethylated, based on its methylation levels exceeding 50% or not, respectively (Imamura et al. 2005; Liu et al. 2008).

Results

Acquisition of Complete cDNA Sequence of Bovine AXL Gene

The full-length cDNA of bovine *AXL* was generated using adult kidney tissue and by employing the techniques of RT-PCR and RACE. The sequencing results have been uploaded to GenBank (Accession no. MG687368–MG687370 and MG734708–MG734712). Initially, two transcripts (RT-v1 and RT-v2) were obtained through RT-PCR. The 2124 bp RT-v1 transcript variant consisted of 18 exons (exon 2–19). When aligned with RT-v1 transcript, RT-v2 skipped a 1454 bp sequence containing 11 complete exons (exon 5–15), along with a partial sequence of 3′ end belonging to exon 4 and resulted in a 671 bp transcript. Additionally, nested primers designed for the procedures of 5′- and 3′-RACE resulted in four 5′ RACE (5′-v1, 5′-v2, 5′-v3 and 5′-v4) and two 3′ RACE (3′-v1 and 3′-v2) transcripts respectively (Fig. 1).

With four alternative promoters existing in bovine *AXL* gene, the 5′-v1 has the longest exon 1 consisting of 568 bp. Two polyadenylation sites were identified in exon 19, resulting in two 3′ ends. The complete and the longest transcript of bovine *AXL* consisting of 4547 bp was assembled by combining 5′-v1, RT-v1 and 3′-v2 and included 19 exons. The coding sequences (CDS) were predicted using the online software ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). Two AUG translation start sites (TSS) in the context of Kozak consensus sequence (CAGC**ATG**G) were found in exon 1 (TSS1) and exon 2 (TSS2), respectively. Two ORFs of 2661 bp and 2424 bp potentially encoded proteins of 887 and 808 amino acids (aa), and were termed AXL-1 and AXL-2, respectively. The protein encoded by human *AXL* gene consists of 894 amino acids, and is composed of two immunoglobulin-like motifs at the N-terminal, followed by two fibronectin type-III motifs. Analyses of the protein structures of AXL-1 and AXL-2 revealed that AXL-1 has the same motifs as the human AXL protein, while AXL-2 lacked an immunoglobulin-like motif at the N-terminal (Fig. 2).

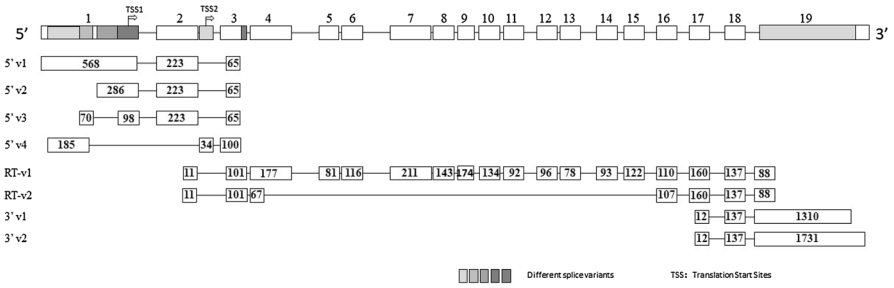

Fig. 1 Map illustrating the structure and splice variants of the bovine *AXL* gene. White boxes represent the exons with the numbers inside them describing their lengths. Straight lines represent the spliced introns. With a total of 19 exons on top, the different colors (light and dark gray) represent the different splice variants

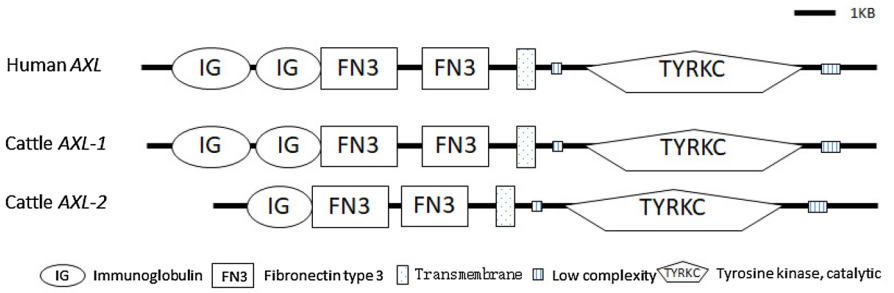

Fig. 2 Protein structure of human AXL and prediction of protein structure of bovine AXL-1 and AXL-2

Tissue-Specific Allelic Expression of Bovine AXL Gene

To analyze the allelic expression of bovine *AXL* gene, a transversion A > T SNP (GenBank Accession no. rs210020651) was first obtained by direct sequencing of an amplicon of 581 bp. The A > T SNP was a synonymous mutation, with both cases coding for leucine. Tissues including the placentas from three heterozygous individuals were used to examine *AXL* expression.

Using a primer pair spanning across intron 7, a RT-PCR product of 285 bp was generated from heterozygous animals and subsequently sequenced. Comparing the sequence chromatograms of genomic DNA and cDNA amplification products at the SNP locus revealed the monoallelic (T) expression of the *AXL* gene in heart, liver, spleen, and fat tissues, while biallelic (A/T) expression was found in lung, kidney, muscle, and brain tissues. Additionally, biallelic (A/T) expression of *AXL* was found in all heterozygous placentas (Fig. 3). These results indicated that the imprinting of bovine *AXL* gene is tissue-specific.

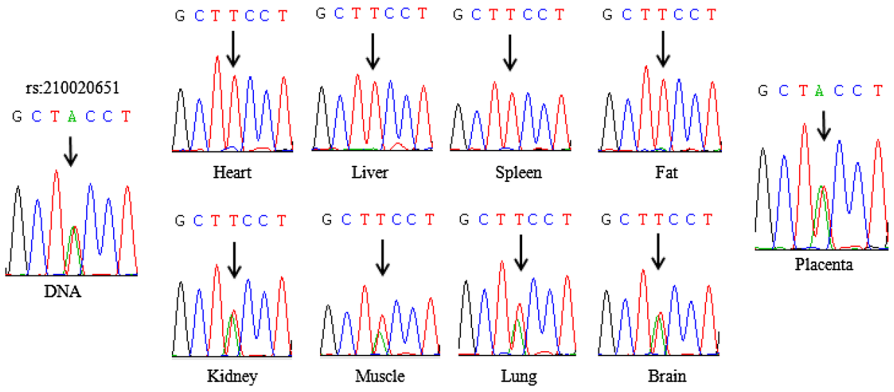

Fig. 3 Sequencing chromatograms for *AXL* in heterozygous bovine tissues. The point of SNP is indicated with an arrow. Heart, liver, spleen and fat tissues exhibited a monoallelic (T) expression while, lung, kidney, muscle, brain and placenta revealed a biallelic (A/T) expression

DNA Methylation Regulates the Tissue-Specific Imprinted Expression of Bovine AXL Gene

To determine whether the tissue-specific allele expression of bovine *AXL* is dependent on DNA methylation, we analyzed two potential differentially methylated regions around the *AXL* promoter and exon 1 (see Fig. 3), in both monoallelic expressed tissues (heart and liver) and biallelic expressed tissue (lung) through bisulfite sequencing. All positions were relative to the first base (+1) of translation initiation site 1 (TSS1) of *AXL*. Region 1 (− 1431 bp to − 1147 bp) is located within the CpG island of the putative promoter of *AXL*, with the 284 bp amplified product revealing 13 CG sites. Region 2 (− 166 bp to + 105 bp) was selected based on the homology with a similar human *AXL* DMR, and is located on exon 1 of bovine *AXL* with 10 CpG sites.

Results of the pyrosequencing analysis showed that region 2 exhibited hypomethylation (< 9%) in all of the tissues studied either for monoallelic or biallelic expression of *AXL*, and therefore, disagrees with the presence of a DMR in this region. However, in case of region 1, there was a difference noted in methylation status between the monoallelic and biallelic expressed tissues. Hypomethylation was seen in the two biallelic expressed lung tissues. However in *AXL* biallelic expressed heart and liver tissues, both hypomethylated strand (< 50%) and hypermethylated strand (˃50%) were observed in region 1 (Fig. 4). Although the two parental alleles were indistinguishable, it is now evident that a DMR is present in region 1.

The transcription factor binding sites in region 1 were predicted by using the online PROMO website (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) and it was further learnt that several important ones existed at 13 CpG sites (data not showed). For example, the binding sites of Sp1 (Specificity protein 1) and AP2 (Activator protein 2) were found to be present in the 5th and 13th CpG loci respectively. Taken together, the results indicate that region 1 has an important role in the transcription of the bovine *AXL* gene and its DNA methylation is involved in the regulation of its tissue-specific imprinted expression.

Discussion

AXL, a gene encoding a receptor tyrosine kinase was first cloned from human myeloid leukemia cells (O'bryan et al. 1991). *AXL* is a putative tumor suppressor and its expression and DNA methylation were found to be associated with diverse cancers (Rankin et al. 2010; Wang et al. 2013; Dengler et al. 2017; Axelrod et al. 2018) and numerous immune-related health conditions (Ozanne and Constancia 2007; Perera et al. 2009; Wang et al. 2015; Jung et al. 2017). Since a significantly higher level of expression of *AXL* has been reported in many tumor tissues and thereby implicated its role in promoting cell proliferation, migration and invasion, the AXL protein can be a potential therapeutic target for antibody-based therapies (Leconet et al. 2014; Bansal et al. 2015; Martinelli et al. 2015). Further, the methylation status of certain CpG loci related to *AXL* are known to change with prenatal exposure to tobacco smoke (Breton et al. 2009; 2011), while its higher methylation at birth was reported to be associated with a higher

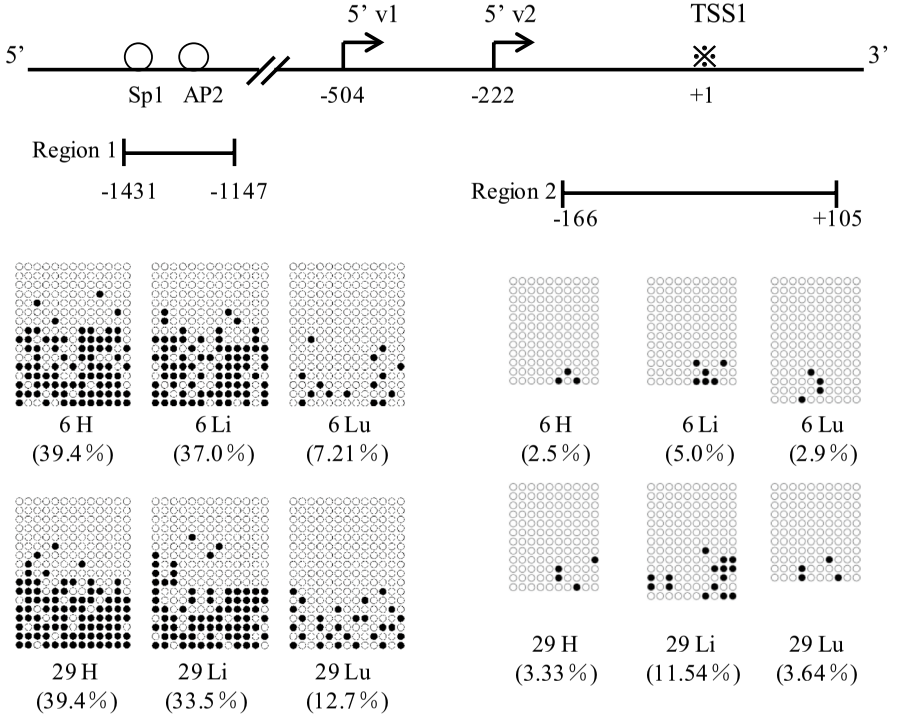

Fig. 4 DNA methylation of bovine *AXL* gene in monoallelic and biallelic expression tissues. 6 and 29 represent the two heterozygous individuals used for the study. Filled and open circles indicate methylated and unmethylated residues, respectively. The percentages of methylated CpG sites of the heterozygous heart (H), liver (Li) and lung (Lu) are indicated to the bottom of their respective pictures. TSS1 represents translation start site 1; Sp1 and AP2 indicate the binding sites of Specificity protein 1 and Activator protein 2, respectively

risk of childhood asthma symptoms (Gao et al. 2017) and with pubertal pulmonary dysfunction during adolescence (Gao et al. 2018).

The human *AXL* gene is located on chromosome 19 and contains 20 exons spanning a region of 44 kb (Schulz et al. 1993). The alternative splicing of exon 10 generates six isoforms of *AXL* mRNA. In the present study, we examined the multiple isoforms of bovine *AXL* gene. The longest transcript variant of 4547 bp contains 19 exons distributed across a sequence of 34,319 bp. Consistent with the human *AXL* gene, there were no TATA and CAAT boxes present in the 5′ upstream region of bovine *AXL* gene. Two transcription start sites were harbored in exon 1, and encoded two proteins of 887 aa and 807 aa. The protein of 887 aa exhibits a high similarity (90%) with that of human Axl, with the presence of a unique extracellular structure consisting of two immunoglobulin-like domains and two fibronectin type-III domains, in addition to a distinctive intracellular kinase domain (O'bryan et al. 1991). In the case of the protein of 807 aa, whether the lack of an immunoglobulin-like domain influences its function as an inducer of epithelial mesenchymal transition needs further research.

AXL gene is a maternally expressed, paternally imprinted gene in humans that harbors a methylated DMR in its promoter region. In mouse embryos and placenta, *Axl* is expressed from the maternal allele and is dependent on modifications through DNA methylation (Choufani et al. 2011). In this study, the examination of allelic expression of the bovine *AXL* gene in tissues such as the heart, liver, spleen, lung, kidney, muscle, fat and brain along with placenta of cattle revealed its tissue-specific imprinting status. In bovine heart, liver, spleen and fat tissues, the *AXL* gene showed a monoallelic expression while a biallelic expression was observed in lung, kidney, muscle and the placenta. In the course of investigating whether DNA methylation is associated with the tissue-specific imprinting of the *AXL* gene, we identified a DMR in the CpG island of its promoter region. This was consistent with the DNA methylation status in mice, although no DMR was found in the syntenic region of human *AXL*. Thus, despite the relative location of DMR being different in human, mouse and cattle, the imprinted expression of *AXL* is validated to be dependent on DNA methylation.

Acknowledgements This study was funded by Hebei province Natural Science Foundation of China (Grant No. C2016204092) and The National Natural Science Foundation of China (Grant No. 31372312).

Compliance with Ethical Standards

Conflicts of interest The authors declare that they have no conflict of interest.

Informed Consent Informed consent was obtained from the owner of the animal. All institutional and national guidelines for the care and use of laboratory animals were followed, and all animal experiments were approved by the Agriculture Research Animal Care Committee of Hebei Agriculture University.

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