Comparative analysis on genome-wide DNA methylation in longissimus dorsi muscle between Small Tailed Han and Dorper×Small Tailed Han crossbred sheep

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Objective: The objective of this study was to compare the DNA methylation profile in the longissimus dorsi muscle between Small Tailed Han and Dorper×Small Tailed Han crossbred sheep which were known to exhibit significant difference in meat-production.

Methods: Six samples (three in each group) were subjected to the methylated DNA immuno-precipitation sequencing (MeDIP-seq) and subsequent bioinformatics analyses to detect differentially methylated regions (DMRs) between the two groups.

Results: 23.08 Gb clean data from six samples were generated and 808 DMRs were identified in gene body or their neighboring up/downstream regions. Compared with Small Tailed Han sheep, we observed a tendency toward a global loss of DNA methylation in these DMRs in the crossbred group. Gene ontology enrichment analysis found several gene sets which were hypomethylated in gene-body region, including nucleoside binding, motor activity, phospholipid binding and cell junction. Numerous genes were found to be differentially methylated between the two groups with several genes significantly differentially methylated, including transforming growth factor beta 3 (TGFB3), acyl-CoA synthetase long chain family member 1 (ACSL1), ryanodine receptor 1 (RYR1), acyl-CoA oxidase 2 (ACOX2), peroxisome proliferator activated receptor-gamma2 (PPARG2), netrin 1 (NTN1), ras and rab interactor 2 (RIN2), microtubule associated protein RP/EB family member 1 (MAPRE1), ADAM metallopeptidase with thrombospondin type 1 motif 2 (ADAMTS2), myomesin 1 (MYOM1), zinc finger, DHHC type containing 13 (ZDHHC13), and SH3 and PX domains 2B (SH3PXD2B). The real-time quantitative polymerase chain reaction validation showed that the 12 genes are differentially expressed between the two groups.

Conclusion: In the current study, a tendency to a global loss of DNA methylation in these DMRs in the crossbred group was found. Twelve genes, TGFB3, ACSL1, RYR1, ACOX2, PPARG2, NTN1, RIN2, MAPRE1, ADAMTS2, MYOM1, ZDHHC13, and SH3PXD2B, were found to be differentially methylated between the two groups by gene ontology enrichment analysis. There are differences in the expression of 12 genes, of which ACSL1, RIN2, and ADAMTS2 have a negative correlation with methylation levels and the data suggest that DNA methylation levels in DMRs of the 3 genes may have an influence on the expression. These results will serve as a valuable resource for DNA methylation investigations on screening candidate genes which might be related to meat production in sheep.

Keywords: DNA Methylation; Small Tailed Han Sheep; Dorper×Small Tailed Han Crossbred Sheep; Methylated DNA Immunoprecipitation Sequencing (MeDIP-seq); Longissimus Dorsi Muscle

INTRODUCTION

China is the largest mutton producer and consumer in the world. However, China does not have good indigenous commercial mutton sheep breeds, and hybridization of local breeds with known commercial mutton sheep breeds has been widely used.
The Small Tailed Han sheep is a Chinese indigenous breed that is famous for its precociousness and prolificacy [1]. The Dorper sheep, which is originated from South Africa, is well known for its hardiness, early maturity, and rapid growth [2]. Recently, the Dorper sheep has been imported into China as a meat sire breed to improve growth performance and carcass traits of the local Small Tailed Han sheep. The Dorper×Small Tailed Han crossbred sheep has therefore become a widely reared breed for mutton production in northern China. Compared with Small Tailed Han, the Dorper×Small Tailed Han crossbred sheep exhibited higher carcass weight, net meat weight, and greater dressing percentage. However, the underlying molecular mechanism for the growth and meat production differences remains unclear.

Currently, extensive genetic studies have identified many genetic polymorphisms affecting growth in sheep [3,4]. However, polymorphisms or quantitative trait loci cannot provide adequate explanations for them. Recently, DNA methylation, an important epigenetic mechanism in eukaryotes, has received considerable attention because of its potential effect on complex traits. It is believed that DNA methylation regulates many biological processes, including gene expression, genomic imprinting, and X chromosome inactivation [5-7]. Using the methylated DNA immunoprecipitation-sequencing (MeDIP-seq) technology, methylation studies have been extensively conducted in animals [8-10].

In this study, we aimed to survey the genome-wide DNA methylation pattern in the longissimus dorsi muscle (LDM) to identify methylated genes which contributed to the differences in growth and carcass traits between Small Tailed Han and Dorper×Small Tailed Han crossbred sheep. Our data revealed the DNA methylation of the two breeds, identified differentially methylated genes between breeds and genes related to meat production.

MATERIALS AND METHODS

Animal care
This study was approved by the Animal Ethics Committee of Jilin Academy of Agricultural Sciences. Sampling was carried out according to the “Guidelines on Ethical Treatment of Experimental Animals” (2006) established by the Ministry of Science and Technology, China.

Sample collection
Animals used in this study were from two breeds: Dorper×Small Tailed Han crossbred (F1, T) and Small Tailed Han sheep (C). The animals were all weaned at 4 months of age and raised from weaning to slaughter on a diet following the National Research Council (NRC) standard. The animals were raised in semi-confinement in the central region of Jilin province, China from March, 2015. The ewes in both groups were raised under same conditions on feed and water ad libitum and were humanely sacrificed at 280 days. Longissimus dorsi muscle tissues from six ewes (three in each group) were collected and snap frozen in liquid nitrogen until DNA or RNA extraction. DNA from these tissues was extracted with standard phenol chloroform method. Total RNA was isolated from each sample with RNAEx Total RNA Isolation Solution (GK3006, GENEray, Shanghai, China) according to the manufacturer’s instructions.

The methylated DNA immunoprecipitation-sequencing
MeDIP DNA libraries were prepared for a total of six samples (three in each group) following the protocol as previously described [11]. Briefly, DNA was fragmented to approximately 100 to 500 bp using a Bioruptor sonicator (Diagenode Inc., Denville, NJ, USA). Sequencing libraries were constructed with the Paired-End DNA Sample Prep kit (Illumina Inc., San Diego, CA, USA) following the manufacturer’s instructions. Adaptor-ligated DNA was immunoprecipitated by a monoclonal anti-methylcytidine antibody (Diagenode, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed to validate the quality of immunoprecipitated fragments. DNA fragments of 200 to 300 bp were excised from the gel and purified using a gel extraction kit (Qiagen, Inc., Valencia, CA, USA). The extracted fragments were quantified using the Agilent 2100 Analyzer (Agilent Technologies, Palo Alto, CA, USA). Enriched fragments were amplified by adaptor-mediated PCR. DNA libraries were subjected to paired-end sequencing with a 50 bp read length using the Illumina HiSeq 2000 platform (Illumina, USA).

Bioinformatics analysis
Raw sequencing data were first processed to filter out low-quality reads containing adaptors or low-quality bases. The clean data were then aligned to the *Ovis aries* reference genome (oviAri3) using the Burrows-Wheeler Alignment software [12]. Uniquely mapped reads were retained for subsequent analyses.

Genome-wide methylation peak scanning was conducted using the model-based analysis of ChiP-Seq (MACS) [13]. The distribution of peaks in different regions of the sheep genome in each sample, including the promoter, 5′-untranslated region (UTR), 3′-UTR, exons, introns, downstream (2 kbp), CpG islands, and repeats, were analyzed. A CGI was defined using the following three criteria: i) greater than 200 bp in length; ii) GC content ≥50%; and iii) CpG observed/expected ratio ≥0.6. Gene information was downloaded from the public File Transfer Protocol site of Ensembl (ftp://ftp.ensembl.org/pub/release-75/gtf/ovis_aries/Ovis_aries.Oar_v3.1.75.gtf.gz).

To identify differentially methylated region (DMRs) in the samples, their peaks were merged and the differences in the number of reads within those peaks between the two groups were analyzed using the t-test (p<0.01).

All genes containing DMRs were subjected to gene ontology and Kyoto encyclopedia of genes and genomes pathway enrichment analyses using the database for annotation, visualization and integrated discovery (DAVID) web server (http://david.najas.info).
Table 1. Characteristics of the Dorper×Small Tailed Han crossbred (F1) and Small Tailed Han sheep

| Sample | Weaning weight (kg) | Final weight (kg) | Cooked meat percentage (%) | Shear force (kgf) | Carcass weight (kg) | Net meat weight (kg) | Dressing percentage (%) | Meat percentage (%) |
|--------|---------------------|------------------|---------------------------|------------------|-------------------|---------------------|------------------------|---------------------|
| C13    | 22.86±4.12          | 22.54±4.20       | 57.98±1.36                | 3.38±0.44        | 24.33±1.86        | 18.30±0.85          | 49.01±1.64             | 38.36±1.95          |
| C14    | 37651517            | 38.36±1.95       | 37221630                  | 0.82             | 2.7 ×             | 2.6 ×               | 53.98±1.36             | 43973672            |
| C7     | 10467652            | 95.89±8.81       | 40647652                  | 3.07±0.50        | 21829729          | 21456357            | 24.24±2.72             | 2.5 ×               |
| T10    | 40073504            | 56.57±9.38       | 38889408                  | 8.81             | 2.3 ×             | 35965704            | 54.22±2.30             | 2.4 ×               |
| T5     | 28575092            | 24.33±1.86       | 37221630                  | 0.44             | 2.5 ×             | 21829729            | 24.24±2.72             | 2.3 ×               |
| T8     | 35965704            | 24.24±2.72       | 24.33±1.86                | 0.85             | 2.4 ×             | 24290732            | 24.24±2.72             | 2.6 ×               |

* p<0.05.

Table 2. Summary of sequence read alignments to the reference genome and genes of MeDIP-Seq data for the Small Tailed Han (C) and Dorper×Small Tailed Han crossbred (T) libraries

| Sample | Total reads | Mapped reads | Unique mapped reads | Genome mean depth |
|--------|-------------|--------------|---------------------|-------------------|
| C13    | 35965704    | 33407122     | 21829729            | 2.5 ×             |
| C14    | 30590924    | 28575092     | 16517504            | 2.3 ×             |
| C7     | 40073504    | 37651517     | 21456357            | 2.7 ×             |
| T10    | 37651517    | 37651517     | 35965704            | 2.4 ×             |
| T5     | 43973672    | 41451188     | 24290732            | 2.6 ×             |
| T8     | 39585158    | 37221630     | 20727942            | 2.5 ×             |
The peaks of the two groups of samples were merged as candidates (DMRs). We used t-test to detect the DMRs between the two groups. The results showed that there were a total of 42 promoters of differentially methylated genes between the Small Tailed Han and the crossbred group, of which 34 were down methylated and 8 were up methylated in the LDM in the crossbred group compared to the Small Tailed Han (Table 3; Figure 5). In other words, the DNA methylation level in the LDM was decreased in the crossbred group compared to the Small Tailed Han group. In addition, 746 DMRs were detected in gene bodies or downstream of the gene, suggesting that more DMRs were in gene body regions rather than promoters, and the DMRs were more prone to be hypomethylated in the crossbred group, which indicated that global methylation loss might be related to the changes of meat quality.

**Functional enrichment analysis for genes with DMRs**

To examine the potential functions of the genes which showed differential methylation status, we performed an enrichment analysis for genes with DMRs in gene body and flanking regions (2 kb). DMRs on chromosome X were excluded from this analysis since DNA methylation concentrates on the X chromosome due to X chromosome inactivation. After multiple testing corrections, as shown in Table 4, all genes with DMR in their flanking region or gene-body were enriched in nucleoside binding ($p = 0.0048$), motor activity ($p = 0.0050$), ATP binding ($p = 0.0074$), dynein

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**Figure 1.** The distribution of the methylated DNA immunoprecipitation-sequencing (MeDIP-seq) reads in different CG density regions.

**Figure 2.** MeDIP-Seq reads distribution in 2 kb region upstream of the transcription start sites (TSS), 5’-untranslated regions (UTRs), coding DNA sequence (CDS), introns, 3’-UTRs, 2 kb region downstream of the TTS and repeats.
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Complex (p = 0.0085), vesicle-mediated transport (p = 0.0165) and cytoskeleton (p = 0.0167). Hypermethylated genes with DMRs in their promoters and gene-body were not significantly enriched in any pathways or gene ontology items which might be due to the relative small number of these genes. As shown in Table 4, gene-body hypo-methylated genes were significantly enriched for the molecular function of nucleoside binding (p = 0.0245), motor activity (p = 0.0248), phospholipid binding (p = 0.0385), and cell junction (p = 0.0395).

**Table 3.** Numbers of genes showing differential methylation in different gene regions

| Small Tailed Han vs crossbred | Differentially methylated gene |
|------------------------------|--------------------------------|
|                              | Upstream 2 kb | 5'-UTR | CDS | Intron | 3'-UTR | Downstream 2 kb |
| Hyper-methylation            | 26            | 2      | 90  | 87     | 4      | 3               |
| Hypo-methylation             | 64            | 2      | 181 | 305    | 9      | 15              |

UTR, untranslated region; CDS, coding DNA sequence.

**Figure 3.** The distribution of CpGs numbers in peaks.

**Figure 4.** Peak distribution in different components of the genome.
duction, we selected genes with DMRs in their flanking regions and gene bodies which are also known to be associated with sheep meat production. As shown in Table 5 [4,14-19], seven methylated genes identified in the present study are previously reported to be involved in carcass traits in animals.

Validation of MeDIP-seq results by bisulphite sequencing PCR
To validate MeDIP-seq data, the methylation level of transforming growth factor beta 3 (TGFB3) promoter region was examined by bisulphite sequencing PCR (BSP). As shown in Figure 6, the methylated region and unmethylated region showed good consistency between the MeDIP-seq results and BSP results. To further confirm the reliability of DMRs analysis, the methylation level of four randomly selected DMRs in gene acyl-CoA oxidase 2 (ACOX2) intron, acyl-CoA synthetase long chain family member 1 (ACSL1) CDS region, and upstream regions of ras and rab interactor 2 (RIN2) and zinc finger, DHHC type containing 13 (ZDHHC13) were analyzed by BSP. DMRs in upstream region of RIN2 showed significant difference between the two groups (p<0.05, t-test). Although no significant difference, the BSP results of other three DMRs showed similar methylation changes as MeDIP-seq data (Figure 7).

Validation of differentially expressed for differentially methylated genes
To validation of differentially expressed for differentially methylated genes between groups, we confirmed twelve differentially methylated genes in MeDIP-seq by real-time quantitative PCR, including TGFB3, ACSL1, ryanodine receptor 1 (RYR1), ACOX2, peroxisome proliferator activated receptor-gamma2 (PPARG2), netrin 1 (NTN1), RIN2, microtubule associated protein RP/EB family member 1 (MAPRE1), ADAM metallopeptidase with thrombospondin type 1 motif 2 (ADAMTS2), myomesin 1 (MYOM1), ZDHHC13, and SH3 and PX domains 2B (SH3PX2DB). The results of real-time quantitative PCR demonstrated these genes are differentially expressed, thus validating the MeDIP-seq data (Figure 8). There were differences in the expression of each gene between the two populations. Two genes (TGFB3, ACSL1) are significantly different (p<0.05). Seven genes (RYR1, ACOX2, PPARG2, MAPRE1, ADAMTS2, MYOM1, and ZDHHC13) are significantly different (p<0.01). The others showed no significant difference. The methylation levels between the two groups are shown in Figure 9. TGFB3 and ACSL1 were significantly different

| Table 4. Gene ontology analysis of all DMR related genes and gene-body hypomethylated genes |
|-----------------------------------------------|---------------------------------|-----------------|-----------------|-----------------|
| Category                                      | Term               | Description     | Gene count | p value          |
| All DMR related genes                         |                   |                 |             |                 |
| Molecular function                            | G0:0001882        | Nucleoside binding | 94         | 0.0048           |
| Molecular function                            | G0:0003774        | Motor activity   | 18          | 0.0050           |
| Molecular function                            | G0:0032559        | Adenyl ribonucleotide binding | 89 | 0.0061           |
| Molecular function                            | G0:0001883        | Purine nucleoside binding | 94 | 0.0073           |
| Molecular function                            | G0:0005524        | ATP binding      | 89          | 0.0074           |
| Cellular component                            | G0:0030286        | Dynein complex   | 9           | 0.0085           |
| Molecular function                            | G0:0030554        | Adenyl nucleotid e binding | 94 | 0.0121           |
| Biological process                            | G0:0016192        | Vesicle-mediated transport | 45 | 0.0165           |
| Cellular component                            | G0:0005856        | Cytoskeleton     | 84          | 0.0167           |
| Gene-body hypomethylated genes                |                   |                 |             |                 |
| Molecular function                            | G0:0001882        | Nucleoside binding | 63         | 0.0245           |
| Molecular function                            | G0:0003774        | Motor activity   | 13          | 0.0248           |
| Molecular function                            | G0:0001883        | Purine nucleoside binding | 63 | 0.0303           |
| Molecular function                            | G0:0032559        | Adenyl ribonucleotide binding | 60 | 0.0371           |
| Molecular function                            | G0:0005543        | Phospholipid binding | 14         | 0.0385           |
| Cellular component                            | G0:0030054        | Cell junction    | 30          | 0.0395           |
| Molecular function                            | G0:0030554        | Adenyl nucleotide binding | 63 | 0.0396           |

DMR, differently methylated regions.
between the two groups (p<0.05) and RYR1, ACOX2, PPARG2, MAPRE1, ADAMTS2, MYOM1 and ZDHHC13 were highly significantly different (p<0.01). Of which, ACSL1, RIN2, and ADAMTS2 were found have a negative correlation between the expression levels of genes and methylation levels in DMRs.

**DISCUSSION**

Although global DNA methylation surveys have been performed on LDM tissues of sheep [20], the present study is the first to systematically compare the genome-wide LDM methylation profiles...
of Dorper×Small Tailed Han crossbred and Small Tailed Han sheep. We aimed to identify methylated genes associated with meat production.

Reads distribution analysis of our study found that uniquely mapped reads were enriched in the repeats and the gene body regions, which was consistent with previous reports in sheep using the RRBS [20], suggesting that MeDIP-seq is a cost-effective approach for analyses of the sheep DNA methylome. A similar methylation pattern was also observed in other animals and plants, such as chicken [21], bovine [22], pig [10], and Arabidopsis thaliana [23]. It is most likely that this methylation pattern is a mechanism that is conserved among different species.

Compared with Small Tailed Han sheep, we observed a tendency toward a global loss of DNA methylation in the crossbred group. In other words, the DNA methylation level in the LDM was decreased in the crossbred group compared to the Small Tailed Han group. Gene ontology enrichment analysis for genes hypomethylated in the gene-body regions found several important gene sets, such as motor activity. Currently, the relationship between gene-body hypomethylation and gene expression is still unclear. Previous studies on breast cancer showed that global DNA hypomethylation is coupled to gene silencing [24]. Therefore, it might be possible that gene-body hypomethylation in the crossbred group caused down regulation of certain genes and lower motor activity, leading to lower shear values and better meat tenderness. Consistent with this result, previous study reported that broilers reared in low stocking density showed greater motor activity and higher shear values [25].

**Figure 7.** Bisulphite sequencing PCR (BSP) validation for differential methylated regions (DMRs). For the BSP, the y axes were the mean methylation percentage of three sequencing samples for each group in the DMR. For the methylated DNA immunoprecipitation-sequencing (MeDIP-seq), the y axes indicated mean normalized methylation level in the DMR. The group C and group T is the Small Tailed Han and Dorper×Small Tailed Han crossbred sheep. The star (*) indicated the significant difference (p<0.05, t-test). Subfigures a-d were the DMRs in gene ACOX2 intron region (chr19:42869604-42870470), ACSL1 CDS region (chr26:13938540-13939276), RIN2 upstream region (chr13:38607690-38608423), and ZDHHC13 upstream region (chr21:25146996-25148088), respectively.
This study compared methylation differences and expression differences of 12 genes, including \( \text{TGFB3}, \text{ACSL1}, \text{Ryr1}, \text{ACOX2}, \text{PPARG2}, \text{NTN1}, \text{RIN2}, \text{MAPRE1}, \text{ADAMTS2}, \text{MYOM1}, \text{ZDHHC13}, \) and \( \text{SH3PX2B}. \) Three genes, whose expressions are negatively correlated with DNA methylation are \( \text{ACSL1}, \text{RIN2}, \) and \( \text{ADAMTS2}. \) The result suggest that the differential expression of these three genes between two groups may be caused by the differences of DNA methylation, and other differential expression

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**Figure 8.** Relative expression levels of 12 differently methylated genes. Data represent means±standard error of the mean (n = 3). * p≤0.05; ** p≤0.01. The Group C and Group T is the Small Tailed Han and Dorper×Small Tailed Han crossbred sheep, respectively.

**Figure 9.** Differential methylated modification of genes. There were significant differences in the methylation levels of each gene between two groups. Data represent means±standard error of the mean (n = 3). ** p≤0.01. The Group C and Group T is the Small Tailed Han and Dorper×Small Tailed Han crossbred sheep, respectively.
genes between the two groups may be due to reasons other than DNA methylation [26].

The expression regulation of ACSL1, RIN2, and ADAMTS2 may be affected by their methylation, and then affect the biological function of meat performance. ACSLs could transform fatty acid to ester acyl coenzyme A. The result indicates that ACSLs not only play a key role in the synthesis of triglycerides, phospholipids and cholesterol, but also affect the metabolism of fatty acid. ACSL1 can be used as a candidate gene for affecting fatty acids in bovine skeletal muscle, and can affect the composition of fat in beef [19]. RIN2 can connect three GTPases, R-Ras, Rab5, and Rac1, to promote endothelial cell adhesion through the regulation of integrin internalization and Rac1 activation [27], but there were no reports of an effect on animal production. ADAMTS2 has an important effect on the biosynthesis of collagen, it is presumed that the expression of the gene will affect the deposition of intramuscular fat. The recent study of Korean cattle showed that the expression levels of ADAMTS2 and ADAMTS4 have a significant impact in fat content of muscle tissue [17]. Thus further confirming that ADAMTS2 can indirectly regulate the deposition of intramuscular fat by affecting collagen synthesis.

DMR related genes are genes known to be involved in meat production. Current data also identified some DMR related genes which were reported to be involved in meat production. Most of these genes are from studies on the association between phenotypes and DNA polymorphisms. For example, two genes (ADAMTS2 and NTN1) have been reported to be related with post-weaning weight gain in sheep in a genome-wide association studies [4]. RYR1 is well known as a meat quality gene and T allele is associated with reduced meat quality [28]. RYR1 polymorphisms were also related to daily weight gain in pig as reported by Kadarmideen et al [14].

Differential mythelations in intron of ACOX2, MYOM1, SH3-PXD2B, and NTN1, and exon of RYR1, and upstream of MAPRE1 were also identified in our results. However, further studies are needed to confirm their regulatory roles in the higher carcass weight observed in the crossbred group.

Furthermore, TGFb3 with promoter hyper-methylation was detected in the crossbred group and has been reported to be related with growth in chicken [16]. Transforming growth factor β3 is known as a cytokine that is involved in cell differentiation, embryogenesis, and development [29]. Differential expression and methylation of this gene have been related to leg muscle development in chicken [30]. Similar to RYR1, PPARG2 is another gene with exon hypo-methylation. Polymorphisms of PPARG2 gene were reported to be associated with meat quality and production in cattle [15]. PPARG2 encodes PPAR-gamma protein which is a regulartor of adipocyte differentiation. In addition, PPARG2 has been implicated in numerous diseases including obesity [31]. Further investigations are needed to confirm the contribution from these genes to the difference between the crossbred group and Small Tailed Han group.

In summary, we provided a comprehensive analysis of genome-wide DNA methylation patterns in LDM of Dorper×Small Tailed Han crossbred and Small Tailed Han sheep. We identified remarkable DNA methylation changes between the two groups, such as a tendency toward hypomethylation in gene bodies in the LDM of the crossbred sheep. Furthermore, we identified numerous genes which might be potentially involved in the difference between the two groups. Several genes were highlighted due to their known association with growth and production, including TGFb3, ACSL1, RYR1, ACOX2, NTN1, RIN2, MAPRE1, ADAMTS2, MYOM1, ZDHHC13, and SH3PXD2B. There are differences in the expression of 9 genes, of which ACSL1, RIN2, and ADAMTS2 may be due to DNA methylation. These results will serve as a valuable resource for DNA methylation investigations on screening candidate genes which might be related to meat production in sheep.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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