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Discovery of DNA Methylation Status of Peg3, Cdkn1c and Gtl2 in Cloned and Natural Lambs*

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Abstract Current research has determined that many cloned animals have heterogeneous DNA methylation profiles. However, few studies have compared the methylation profiles of both naturally produced lambs and cloned lambs created using somatic cell nuclear transfer. The paucity of research in this area is because of insufficient resources to study limited cloned offspring, the ovine genome, and ovine genomic imprinting. In this study, to show the degree of reprogramming in cloned lambs, we cloned the putative differentially methylated regions (DMRs) of *Peg3* from sheep and analyzed the DNA methylation patterns in CpG islands and DMRs of the putative imprinted genes *Peg3*, *Cdkn1c* and *Gtl2* in cloned lambs. We have provided evidence that *Peg3* was highly methylated. The degree of methylation was 95.45% in the kidney and 88.18% in the lung of a natural sheep and 98.18% in the kidney and 87.27% in the lung for one cloned sheep. The bisulphite sequencing results for *Cdkn1c* show complete non-methylation (0%, 0.53%, 0.53%, 0.53%) in all samples. In addition, *Gtl2* was hypomethylated in all lambs, from a linear correlation analysis, there were some differences in the quantitative values from both groups (correlation $r^2 = 0.77$). These data show that the DNA methylation status of the three imprinted genes was similar in cloned and natural lambs.

Key words DNA methylation, *Peg3*, *Cdkn1c*, *Gtl2*, cloned lambs
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Somatic cell nuclear transfer (SCNT) has been successfully used for many mammalian species. However, the technique has a high failure rate because of the production of defects resulting in increased abortion rates; perinatal death; low pregnancy rates; and increased fetal and placental abnormalities, including large offspring syndrome. Until recently, abnormal epigenetic modifications such as DNA methylation, histone modifications, and chromatin reconstructions have been reported in several cloned species. Genomic imprinting and DNA methylation have an important impact on normal mammalian development, fetal growth, and adult behavior. Genomic imprinting, an important parent-of-origin phenomenon, occurs when a parental allele expression from a cluster of genes is triggered by differential epigenetic/chromatin modifications during mammalian embryonic development[1]. According to the Geneimprint database, over 200 imprinted (or predicted) genes in humans, approximately 147 genes in mice, and 16 genes in sheep have been reported. (http://www.geneimprint.com/). Imprinted genes have the allele-specific hallmark, which depends on whether they are

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the maternally or paternally inherited copy, and they may be created by various mechanisms, such as DNA methylation, histone modification, and RNA interference. These mechanisms are believed to reinforce the ideas that various elements interact to regulate the expression of imprinted genes in clusters [2-4]. For example, in mammals, imprinted genes are under the control of a single major cis-acting element, the imprinting control region (ICR) or the differential methylated region (DMR) [5]. DNA methylation is deemed the most common mechanism. Cytosine methylation of CpG dinucleotide is a post-synthetic DNA modification in which a methyl group is added to the carbon-5 of the cytosine in a CpG dinucleotide [6]. The distributions of these CpG sequences form an island within or near gene promoters or first exons regions of the genes[6-7]. Accordingly, in this study, we analyzed the DNA methylation status of a CpG island within gene promoters or first exons of *Peg3*, *Cdkn1c*, and *Gtl2* in cloned sheep.

Paternally expressed gene 3 (*Peg3*) is an imprinted gene located on human chromosome 19q13.4 [8] and in the proximal region of mouse chromosome 7 [9]. It encodes a krüppel-type (C2H2) zinc-finger protein, most of which are thought to function as transcription factors[10-11]. *Peg3* is expressed predominantly adult tissues of the brain, ovaries, testes, and placenta[8]. The overall genomic structure of *Peg3* is well-conserved in most mammals [12], and the genomic distances between individual exons are conserved [13]. The recent isolation of the maternally expressed genes *Zim1* (zinc finger gene imprinted 1), *Zim2*, and *Zim3*[13-15] and the paternally expressed genes *Usp29* (ubiquitin-specific processing protease 29), *Zfp264* (Zinc finger protein gene 264) and APeg3 (antisense to paternally expressed gene 3) are located near Peg3 [14-18]. This suggests the presence of larger imprinted domains that follow the sequence of the telomere-*Zfp264-Zim3-Usp29-Peg3-Zim1-Zim2* centromere in the proximal region of mouse chromosome 7 or in other mammals. *Peg3* exon 1 is associated with CpG-rich regions, termed CpG islands. Some of these CpG islands are differentially methylated in both mice and humans, and a well-conserved region within the exon 1 was found in three species [19], which suggests that ovine *Peg3* exon1 may also be differentially methylated. To date, little is known about ovine *Peg3*.

Cyclin-dependent kinase inhibitor 1C (*Cdkn1c*), the genomic imprint of *p57Kip2* in humans, mice, and sheep, is only expressed from the maternal allele. It is expressed in all tissues at all stages of development and repressed in the paternal allele. Furthermore, the CpG sites are methylated exclusively on the paternal allele [20]. The gene maps to human chromosome 11p15.5 [21] and to mouse chromosome 7. On the paternal chromosome, DNA methylation at the ICR, which lies within the promoter of Kcnq1ot1, and the paternal transmission of the deletion led to bi-allelic expression of *Ascl2*, *Kcnq1*, *Cdkn1c*, *Slc22a18*, *Phlda2*, and *Osbpl5*[22]. One group examined the effect of paternal inheritance of the *Kcnq1ot1* deletion on the growth of heterozygotes compared to their wild-type littermates and observed a 10% ～20% decline in weight [22]. This is consistent with previous reports showing that *Cdkn1c* (and other five imprinting genes) may lead to the disruption of embryonic development[23]. *Gtl2* (gene trap locus 2), also named *Meg3* (maternally expressed gene 3), is maternally expressed non-coding RNAs that map to a region on mouse chromosome 12 [24], on human chromosome 14 (GI:55384), and on the far-end of ovine chromosome 18 [25]. The imprinted locus of *Gtl2* is well conserved in mice, humans, and sheep. The primary *Gtl2* transcripts are differently processed in various cell types during development. *Gtl2* transcript levels are present in parthenogenic embryos, but may be reduced, which is consistent with the pattern of inheritance of the *Gtl2* lacZ phenotype[26]. Similar to *H19* and *Igf2*, *Gtl2* and *Dlk* were co-expressed in the same tissues throughout development, though not after birth[27].

In view of this, we cloned the putative DMR of *Peg3* from sheep, predicted the CpG islands of ovine imprinted gene *Peg3*, *Cdkn1c* and *Gtl2*, and investigated the DNA methylation patterns in CpG islands and differentially methylated regions of three putative imprinted genes in cloned lambs.

1 Materials and methods

1.1 Production of cloned sheep and tissue collection

We obtained skin fibroblast cells from biopsies of an adult female Mongolian sheep ear. The fibroblasts were donors for nuclear transfer. Culture and passage of donor cells, oocyte collection and enucleation, nuclear transfer, oocyte activation, embryo culture, and embryo transfer were carried out, and five full-term female cloned sheep were obtained[28]. The procedures were approved by the ethical committee of Animal

Science at Inner Mongolia Agricultural University. Two deceased, three-day-old cloned lambs (named D1, D2) without abnormal histomorphology were selected. Five organs (livers, kidneys, hearts, muscles, and lungs) were collected from these two lambs. Two female control lambs (named N1, N2) that were naturally produced and had normal phenotypes and no a history of poor health or reproductive disorders were selected. These two matched controls were the same 3-day old, Mongolian sheep. The above all design idea in accordance with the previous[29].

1.2 Cloning putative DMR of Peg3 from sheep

We referenced the *Peg3* sequence of sheep (GI: 417531910) published in the GenBank database. Primers for sheep *Peg3* putative DMR were designed by Primer 5.0, *Peg3*-F 5′ AGTCTCTGGCTCTTTAG-AATACTG 3′ and *Peg3*-R 5′ AATATGAAGGGAA-CAACCAGGA 3′. The PCR programmer included a denaturation step of 5 min at 95℃, and then 30 cycles of 30 s at 98℃, 30 s at 62℃, 1 min at 72℃, with a final extension step for 10 min at 72℃. The purified PCR products were cloned into the pMD19-T vector for sequence analysis (TaKaRa, Dalian, China), and three positive clones were sequenced in both directions.

1.3 DNA isolation and bisulphite treatment

We isolated genomic DNA from all tissues (including the kidney, lung, liver, heart, and muscle tissues) of cloned lambs and control sheep with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Bisulphite treatment of genomic DNA was performed as previously described by us[29]. Briefly, 1 μg of genomic DNA was denatured with 3 mol/L NaOH for 30 min at 37℃. The DNA was then treated with a solution containing sodium bisulphite and hydroquinone (final concentrations of 3 mol/L and 0.5 mmol/L, respectively, at pH 5.0). The reaction mixture was overlaid with mineral oil and incubated in the dark at 50℃ for 14 h. DNA was washed with the DNA Clean-Up Wizard system (Promega) and suspended in 20 μl of water.

1.4 PCR amplification, cloning and sequencing

PCR amplification of the bisulphite-treated DNA were performed using the primers shown in Table 1, which were designed from the sequence of the DMR region of every imprinted gene, using the online software METHPRIMER (http://www.urogene.org/methprimer/). PCR was performed with a 2 × Premix Ex TaqTM Hot Start Version (TaKaRa) as follows: one cycle of 95℃ for 5 min; 30 cycles of 95℃ for 30 s, 55℃ for 1 min, 72℃ for 1 min, and 72℃ for 7 min (for *Peg3*). The Tm for *Cdkn1c* and *Gtl2* are 50℃ and 53℃, respectively. The PCR products were recovered using the Wizard SV Gel and PCR Clean-Up System (Promega), after electrophoresis in 1% agarose. The PCR products were cloned into the pMD19-T vector (TaKaRa), and at least 10 insert positive plasmid clones were sequenced by an ABI PRISM 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA) to ensure each line in bisulphite sequencing results represents a separate clone. The methylation patterns were analyzed in sequences derived from clones with ⩾98% cytosine conversions only. All PCR amplifications were repeated at least three times for each DMR for all tissues[29].

Table 1 PCR primers for bisulphite sequencing

Gene	Primer sets (5′ to 3′)	Product length/bp	CpG sites
Peg3	Forward: GTTTTAAATTTGTATGAGTTAGATAGTGAT	232	11
	Reverse: AATACTACTCCAAAACCAAAATCTC		
Cdkn1c	Forward: GATAGTTAGAGTATTGGTAATGGAG	188	19
	Reverse: CCAACTAAAAATTATAATCCCAAC		
Gtl2	Forward: GAATAGTTTTTAGGGATTTTTAGTG	161	13
	Reverse: CAAACCCAATTACTAACCTCTC		

2 Results

2.1 Cloning of sheep Peg3 CpG-rich regions

As shown in Figure 1, we obtained a 307 bp fragment of *Peg3* from sheep liver genomic DNA, and the nucleotide sequence of 5′ region in the first exon of the ovine *Peg3* is described in Figure 1a. The nucleotide sequence shows the highest similarity with

cattle, horse, human, and dog (Figure 1a), sharing 96.9%, 93.7%, 92%, and 88.1% homology with that of cattle (GI:50872144), horse (GI:194216094), human (GI:262072967), and dog (GI:73947372), respectively. In addition, sequences for five species were concatenated to construct the homology tree(Figure 1b). As expected, sheep and cattle clustered together to represent Artiodactyla, both sharing a common ancestor.

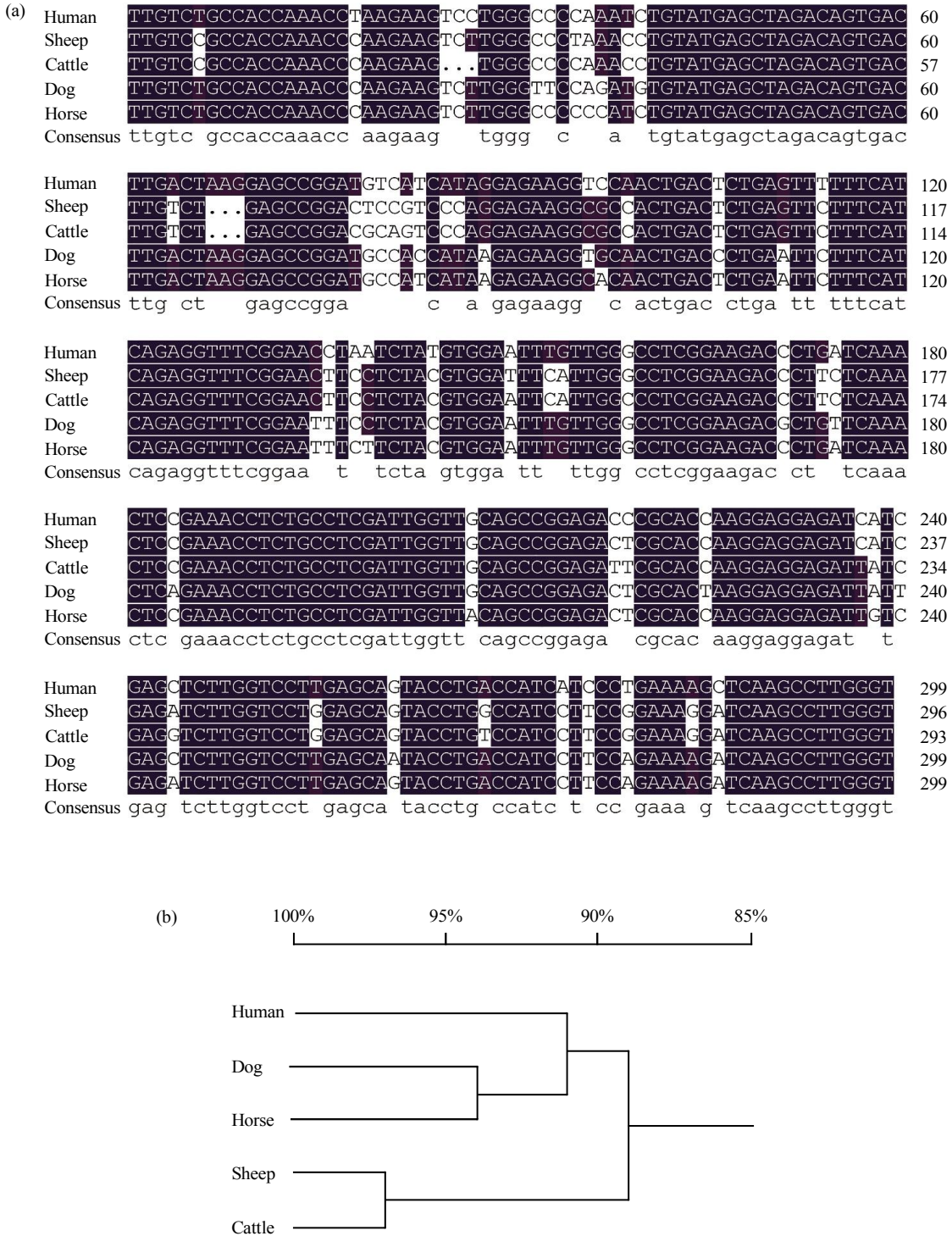

Fig. 1　The features of ovine 5′ region in first exon of Peg3

(a) The alignment of ovine 5′ region in the first exon of *Peg3* among sheep, cattle, horse, human, and dog. (b) Homology tree of the ovine 5′ region in the first exon of *Peg3* from 5 different animals (including human).

2.2 Identification and characterization of CpG island of ovine Peg3, Cdkn1c and Gtl2

As predicted, *Peg3*, *Cdkn1c* and *Gtl2* gene from sheep also have conserved CpG islands similar to other species (Figure 2), as analyzed by the online software METHPRIMER (http://www.urogene.org). As shown in Figure 2a, we obtained a CpG island in ovine 5′ region in the first exon of *Peg3* (Figure 2a). The CpG islands in *Cdkn1c* and *Gtl2* were evident(Figure 2b, c).

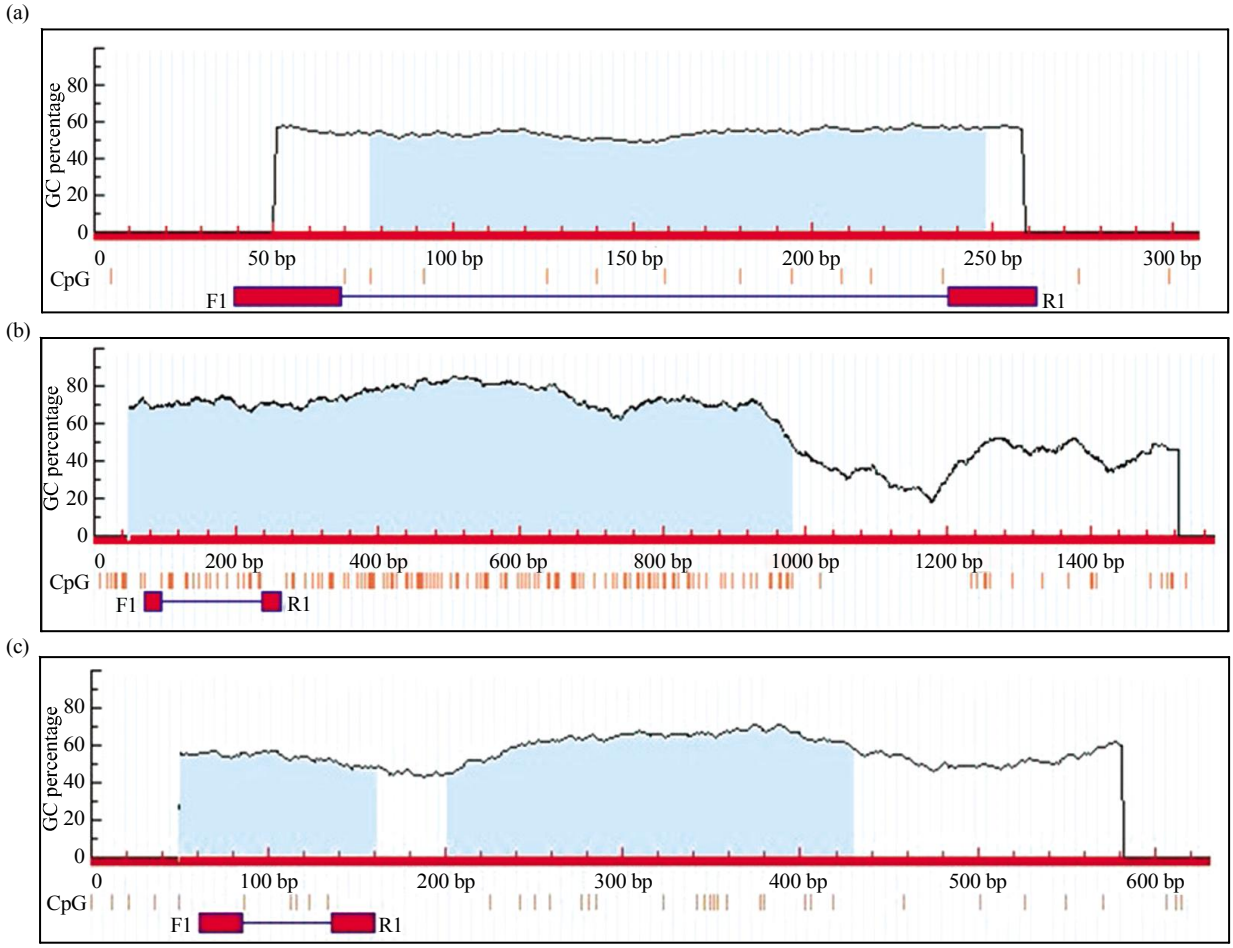

Fig. 2 Predicted CpG islands

(a) CpG island of ovine 5' region in first exon of *Peg3* predicted by Methprimer. (b) CpG island of ovine *Cdkn1c* predicted by Methprimer. (c) CpG islands of ovine *Gtl2* predicted by Methprimer.

2.3 Methylation status of Peg3, Cdkn1c, and Gtl2 in cloned lambs

Eleven CpG sites of the imprinted gene *Peg3* putative DMR were analyzed. The hypermethylated strands were revealed in all groups (Figure 3a). The degree of methylation was 95.45% in the kidney and 88.18% in the lung of a natural sheep (N1) and 98.18% in the kidney and 87.27% in the lung for one cloned sheep (D2) (Table 2). The bisulphite sequencing results for *Cdkn1c* show complete non-methylation (0%, 0.53%, 0.53%, 0.53%) in all samples (Figure 3b), and there were not significant differences between N1 and D2 (Table 2). In one word， we found that the degree of methylation of *Peg3* were hypermethylated and of *Cdkn1c* were non-methylation in livers, hearts, muscles, kidney, lung of both natural sheep and cloned sheep (in this paper part of data not published, Table 2). Likewise we analyzed 13 CpG sites of imprinted gene *Gtl2* in its promoter CpG island, the results showed hypomethylation in five tissues (Figure 3c, Table 2). We analyzed the methylation status of the CpG islands of *Gtl2* between the control groups and the cloned

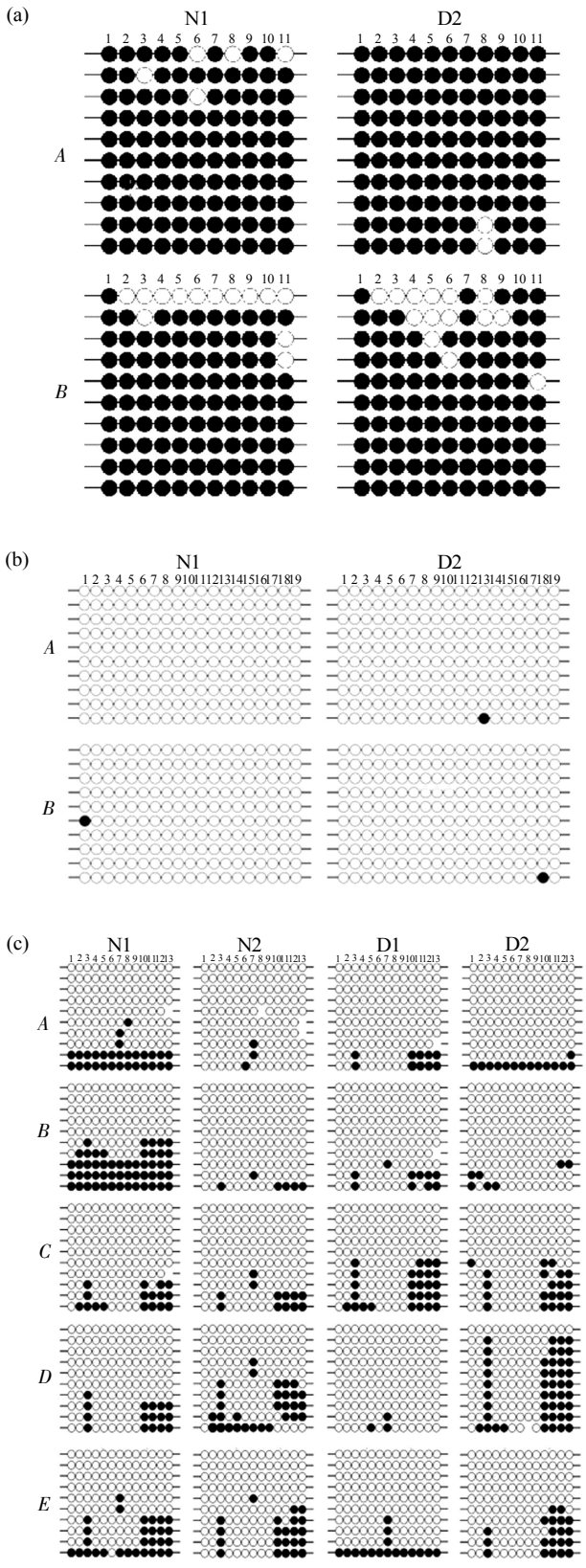

Fig. 3　DNA methylation patterns in lambs

(a) DNA methylation patterns of *Peg3* in two lambs. (b) DNA methylation profiles of *Cdkn1c* of two lambs. (c) DNA methylation profiles of *Gtl2* promoter of four lambs. Five tissues in four lambs were analyzed, including two naturally produced lambs (N1 and N2) and two cloned lambs that died after birth (D1 and D2). *A*, *B*, *C*, *D*, and *E* represent the tissue of the kidney, the lung, the liver, the heart, and the muscle, respectively. Open and closed circles represent unmethylated and methylated CpGs, respectively.

Table 2 Methylation status of Peg3, Cdkn1c, and Gtl2 in cloned lambs

	Peg3		*Cdkn1c*		*Gtl2*			
	N1	D2	N1	D2	N1	N2	D1	D2
A	95.45%	98.18%	0%	0.53%	22.31%	2.31%	7.69%	8.46%
B	88.18%	87.27%	0.53%	0.53%	40.00%	4.62%	7.69%	5.38%
C	–	–	–	–	13.08%	9.23%	20.00%	16.15%
D	–	–	–	–	12.31%	21.54%	2.31%	35.38%
E	–	–	–	–	22.31%	16.92%	10.00%	15.38%

The two cloned sheep, D1 and D2, and two control sheep called N1 and N2; *A*, *B*, *C*, *D* and *E* represent the tissue of the kidney, the lung, the liver, the heart and muscle, respectively.

lambs. From a linear correlation analysis, there were some differences in the quantitative values from both groups (correlation r^2= 0.77; Figure 4). We hypothesized that there would be differences in a region with correlation $r^2 < 0.85$. While this assumption does not provide a genome-wide representation, it will be helpful in understanding the interaction between the epigenetic, transcriptional, and mutational status in the development of cloned sheep.

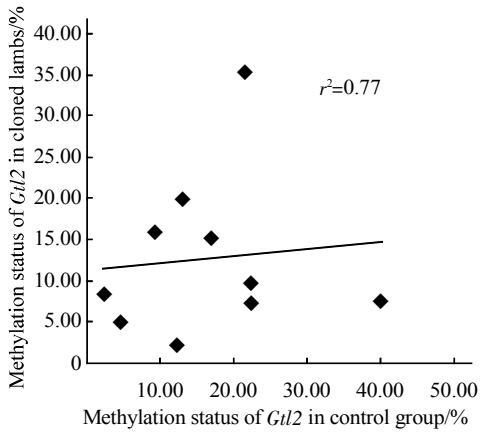

Fig. 4 Correlation analysis of methylation data for normal and cloned lambs

The scatterplot depicts the results from the methylation status in the CpG islands of *Gtl2*.

3 Discussion

In the current study, we purified a fragment of DNA of ovine *Peg3*. As expected from our previous findings on the DMR of *Peg3* and comparisons with DNA homologs of related animals, the fragment showed high similarity to the DNA of cattle, dogs and horses. We also surveyed the methylation status of the CpG islands of the *Peg3*, *Cdkn1c* and *Gtl2* imprinted domains in cloned sheep. Strangely, the methylation status of *Peg3* was shown to be hypermethylated in the CpG islands for both cloned and normal sheep. *Peg3* expression is silenced in various tumor types, including gliomas, choriocarcinomas, and ovarian tumors[30–31]. This silencing was found to be a result of DNA methylation [32]. Similarly, hypermethylation of cytosines at the PEG3-DMR was confirmed in ovarian tumor-derived DNA [33]. Although we did not analyze the *Peg3* expression in the sheep tissues, we have observed a pattern where approximately all the bisulfite clones are methylated. While this study only analyzed a limited number of samples, the CpG island had a highly consistent methylation pattern that we believe that the observations from these tissues are most likely meaningful.

Cdkn1c is one of the most studied imprinted genes in animals. It is involved in embryonic growth control and is likely to have a role in the etiology of Beckwith-Wiedemann syndrome in humans [22, 34]. *Cdkn1c* is faithfully imprinted in all mouse tissues in which it is expressed from early embryonic development through adulthood [22]. In this report, we provide evidence that the bisulphite sequencing results for *Cdkn1c* showed complete non-methylation. This may be due to a special methylation mechanism that exists in sheep. Future studies are required to determine and characterize this special mechanism.

The *Gtl2* gene lies within the *Dlk1-Dio3* imprinted cluster and close to the *Dlk1* gene. It is known that there are at least ten imprinted genes in the *Dlk1-Dio3* imprinted cluster [35]. The *Dlk1-Gtl2* imprinted domain has been extensively studied in mice and human due to its relation with embryonic

development and postnatal growth [36]. DNA methylation is one modification known to play a key role in the regulation of the *Dlk1-Gtl2* imprinted domain[37]. In cloned bovines, the *Gtl2* DMR exhibited hypermethylation, which was similar to controls [38]. This is in stark contrast to our previous report showing that *Gtl2* was hypomethylated in normal and cloned ovines. We speculate that the different findings from the DNA methylation studies in SCNT mammals are due to the use of different species and tissues and the examination of different genes or genomic regions[39-40].

In summary, in the present study three DMRs of imprinted genes were identified. There were different methylation levels in all tissues of cloned sheep and normal sheep, and these data show that the DNA methylation status of the three imprinted genes was similar in cloned and natural lambs. However, we believe that more studies are needed to determine if other mechanisms, such as histone acetylation and microRNA, play an important role or interact with methylation, resulting in the improper reprogramming and the low efficiency of SCNT.

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Peg3, Cdkn1c 和 Gtl2 基因在克隆绵羊和自然分娩绵羊中的 DNA 甲基化水平检测 *

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摘要　尽管研究证明很多克隆动物存在 DNA 甲基化异常的情况，却很少有研究比较克隆绵羊与自然分娩绵羊之间的甲基化情况，可能是由于克隆绵羊的获得、绵羊基因组、绵羊基因组印记等因素的限制．本研究中，为了证明克隆绵羊重编程的状况，克隆了 *Peg3* 基因的差异甲基化区域(differential methylated region，DMR)，并且分析了 *Peg3*、*Cdkn1c*、*Gtl2* 在克隆绵羊和自然分娩绵羊不同组织中的甲基化水平．研究发现，在克隆绵羊和自然分娩绵羊中 *Peg3* 呈现为超甲基化水平，在克隆绵羊的肾脏和肺脏中 DNA 甲基化水平为 95.45%、81.18%，相对于正常分娩的绵羊组织中的 98.18%、87.27%无显著性差异，而 *Cdkn1c* 在两组实验动物中的肾脏和肺脏中表现为非甲基化水平，分别为 0%、0.53%、0.53%和 0.53%，*Gtl2* 则是低甲基化水平，并且克隆绵羊与正常分娩绵羊之间的 DNA 甲基化水平无显著性差异(r^2 = 0.77)．这些结果表明，*Peg3*、*Cdkn1c*、*Gtl2* 三个印记基因在克隆绵羊和自然分娩绵羊组织中呈现类似甲基化水平，无显著性差异．

关键词　DNA 甲基化，*Peg3*，*Cdkn1c*，*Gtl2*，克隆绵羊
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