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Endometrial genome-wide DNA methylation patterns of Guanzhong dairy goats at days 5 and 15 of the gestation period

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ABSTRACT

Uterine receptivity for the embryo is established and maintained through a series of precise cellular and molecular events, such as DNA methylation. There have been no studies to elucidate entire genome DNA methylation changes associated with embryo receptivity development of the endometrium (RE). In the present study, there was development of a complete genome-wide DNA methylome maps of the RE using whole-genome bisulphite sequencing and bioinformatics analysis. As many as 163.06 Gb of sequencing data averaging 81.53 Gb per sample were obtained for genome bisulphite sequencing of endometrium samples. There were distinct genome-wide DNA methylation patterns in pre-receptive endometrium (PE; Day 5 of gestation) and RE (Day 15 of gestation). There were as many as 16,467 differentially methylated regions (DMRs); 21,391 DMRs were less methylated in RE samples compared with PE samples (P-values ≤ 0.05 and $|\log2$ (fold change)$| \geq 2$). Compared with PE samples, methylation ratios of *IGF2BP2*, *ACOX2*, *PTGDS*, *VEGFB* and *PTGDR2* genes were markedly less in RE samples (P-value ≤ 0.05 and $|\log2$ (fold change)$| \geq 2$). Conversely, in RE samples there was a markedly greater methylation ratio of *IGFBP3* and *IGF1R* genes. The results of KEGG analysis indicated that these genes were involved in the signalling pathways for insulin, mitogen-activated protein kinase, gonadotropin-releasing hormone, vascular endothelial growth factor and progesterone-mediated oocyte maturation, which participated in differential regulation of goat endometrial development during receptive and prereceptive phases. The results of previous and the present study indicate resulting proteins of *IGF2BP2*, *PTGDS*, *VEGFB*, *PGR*, *IGFBP3* and *IGF1R* gene expression may have important functions in regulating endometrial receptivity for the embryo.

1. Introduction

Epigenetic mechanisms regulate processes controlling implantation, placentation, organ formation and foetal growth (Horsthemke and Ludwig, 2005). Epigenetic gene regulation involves reversible alteration of genomic patterns and chromatin organisation through DNA methylation and histone modification (Esteller et al., 2002; Bhattacharjee et al., 2016; Huang and

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Abbreviations: DMRs, differentially methylated regions; RE, receptive endometrium; PE, prereceptive endometrium; TSS, transcription start site; GnRH, gonadotropin-releasing hormone; KEGG, genes and genomes; DMGs, differentially methylated genes; GO, gene ontology; TEs, transposable elements; *IGF2BP2*, insulin-like growth factor 2 mRNA binding protein 2; *ACOX2*, acyl-CoA oxidase 2; *PTGDS*, prostaglandin D2 synthase; *SMAD2*, SMAD family member 2; *VEGFB*, vascular endothelial growth factor B; *PGR*, progesterone receptor; *TLE3*, transducin-like enhancer of split 3

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Ovcharenko, 2017). The deoxyribonucleic acid methylation pattern parallels that for gene expression (Notley et al., 2017). In general, methylation inhibits the activation of initiation in an immediate vicinity of transcription start sites (TSS); however, this phenomenon depends on binding of specific transcription factors (Jones, 2012; Baubec and Schubeler, 2014). Methylation of coding region of genes possibly affects gene expression by stimulating transcription elongation and alternative splicing (Zhang et al., 2014; van den Dungen et al., 2016). Major forms of DNA methylation include addition of a methyl group to the fifth carbon of a cytosine base (5 mC) and adenine methylation, with CpG methylation being the most studied (Bird, 1992). Other forms of cytosine methylation consist of CHH and CHG, with H representing adenine (A), guanine (G) or thymine (T) (Suzuki and Bird, 2008; Jones, 2012). In animals, cytosine methylation almost consists exclusively of CpG methylation, as a result of small amounts of CHH and CHG methylation which may also be observed in embryonic stem cells (Law and Jacobsen, 2010). Maintenance of pregnancy is determined by dynamic changes in gene expression during maternal recognition of pregnancy and embryonic implantation. During sensitive periods of pregnancy establishment, aberrant endometrial gene expression can result in implantation failure and infertility. Rahnama et al. (2009) provided evidence that E-cadherin has an important function in uterine receptivity for the embryo, and expression of this gene is epigenetically regulated in endometrial epithelial carcinoma cells and suppressed by combined actions of DNA methyltransferase -1, -3A and -3B (Rahnama et al., 2009). Treatment with gonadotrophin-releasing hormone (GnRH) analogues may increase methylation of endometrial *Hoxa10* promoter and affect endometrial receptivity (Li et al., 2015).

Embryo implantation is regulated by a complex reproductive process. A multitude of signalling molecules have important functions in embryo implantation, however, the mechanisms underlying the actions of these molecules remain to be elucidated (Silveira et al., 2017; Yen et al., 2017). In early pregnancy, successful embryo implantation necessitates establishment of uterine receptivity. Uterine receptivity is established and maintained through a series of precisely regulated cellular and molecular functions, such as presentation of pinopodes and expression of genes for and secretion of cell adhesion molecules, glycoproteins and cellular factors (Hirate et al., 2016). Abnormal expression of genes related to establishment of uterine receptivity for the embryo directly affects embryo implantation, leading to spontaneous abortion (Chegini et al., 2002; Quenby et al., 2007). The mechanism by which embryos attach to uterine epithelia at the beginning of implantation remains to be determined, but it possibly involves an initial adhesive interaction between apical membranes of trophoblast ectoderm and uterine epithelium (Denker, 1993). This interaction is considered unusual as epithelial cell types do not normally adhere to one another as a result of apical membrane interactions, and this effect is possible only when the uterine epithelial cells are in a specific functional state called receptivity (Psychoyos, 1986). The receptive period is hormonally controlled and is characterised by changes in the molecular milieu of uterine epithelial cells (Thie et al., 1996); these changes include a decrease in thickness of the cellular glycocalyx and surface charge (Niklaus et al., 2001) and replacement of microvilli with bulbous protrusions known as pinopodes (Kimber and Spanswick, 2000; Stavreus-Evers et al., 2001). Loss of apico-basal polarity without concurrent loss in epithelial cell adhesion molecules allows apical cell–cell interactions and attachment of uterine and trophoblast epithelial cells (Tinel et al., 2000). In establishment of uterine receptivity, gene expression is affected not only by heredity but also by epigenetic modifications, such as methylation, histone acetylation and actions of small-interfering RNAs (Salilew-Wondim et al., 2016). Scientific evidence is still needed, however, to elucidate whether methylation of genes parallels uterine receptivity during implantation and potentially affects establishment of this receptivity. Research should, therefore, be conducted to explore the molecular mechanisms of uterine receptivity for the embryo as a result of changes in methylation regulation.

In the present study, genome-wide DNA methylation profiles of goats in pre-receptive endometrium (PE: period preceding the time of endometrial signalling for uterine implantation) and receptive endometrium (RE: period of embryo receptivity development in the endometrium). Experiments were performed to assay the methylome distribution in the genome, to analyse differentially methylated regions (DMRs) and to identify differentially methylated genes (DMGs).

2. Materials and methods

2.1. Ethics statement

Dairy goats were maintained utilising the standards defined in Proclamation No. 5 of the Ministry of Agriculture, China. Sample collection procedures were approved of by the Institutional Animal Care and Use Ethics Committee of Northwest A&F University and performed in accordance with the ‘Guidelines for Experimental Animals’ of the Ministry of Science and Technology (Beijing, China). This study was approved by the Institutional Animal Care and Use Ethics Committee of Northwest A&F University.

2.2. Tissue collection and genomic DNA extraction

The experimental animals used in this study were of a well-known native breed of Chinese Guanzhong dairy goats. A total of ten healthy, 24 month-old multiparous dairy goats were treated to induce oestrous synchronisation. The initial day of mating was considered day 0 of pregnancy. Gestational days 5 and 15 are important time points for embryo implantation in goats (Igwebuike, 2009). Experimental goats were observed thrice daily to ascertain behavioural oestrous symptoms and were mated naturally twice during oestrus. The goats were euthanized following intravenous injection of barbiturates (30 mg/kg) at gestational days 5 (PE) and 15 (RE). Endometrial samples were acquired from anterior wall of uterine cavity of five dairy goats at gestational day 5 and another five animals at gestational day 15, in addition, considering the spatial differences in methylation patterns in uterine environments, tissues from 10 different locations on the anterior wall of uterine endometrium were collected to pool the DNA. All tissue samples were washed briefly with phosphate buffered saline and then immediately frozen in liquid nitrogen until DNA extraction.

Genomic DNA was extracted following standard procedures using a TIANamp Genomic DNA Kit (Tiangen, Beijing, China). RNA contamination was evaluated using RNase A (Tiangen, Beijing, China). Total DNA quantity and purity were analysed with Bioanalyser 2100 (Agilent, CA, USA). $OD_{260/280}$ ratios were > 1.8 and < 2.0 for all samples. Low-quality total DNAs from PE and RE samples were not used for further study.

2.3. Whole-genome bisulphite sequencing (WGBS) and data processing

Each library was constructed by pooling 5 μg homogenised total DNA from PE and RE samples. Briefly, genomic DNA was fragmented into 100 to 300 bp by Sonication (Covaris, Beijing, China) and purified with MiniElute polymerase chain reaction (PCR) purification kit (Qiagen, Redwood, USA). Fragmented DNA was end-repaired, and a single “A” nucleotide was added to the 3′end of blunt fragments. Genomic fragments were subsequently ligated to methylated sequencing adapters. Fragments with adapters were bisulphite-converted using methylation-gold kit (ZYMO, Los Angeles, USA). Furthermore, converted DNA fragments were PCR-amplified (Lu et al., 2017) and sequenced using Illumina HiSeq 2500 by Gene Denovo Biotechnology Co. (Guangzhou, China). To obtain high-quality clean readings, raw reads were filtered using the following criteria: 1) remove reads containing more than 10% of unknown nucleotides (N); 2) remove low quality reads containing more than 40% of low quality (Q-value ≤ 20) bases.

2.4. Methylation level analysis

Obtained clean reads were mapped to species reference genome using BSMAP software (Li and Xi, 2009) (version: 2.90) by default. Then, a custom Perl script was used to determine amounts of methylated cytosine and calculate methylation based on methylated cytosine percentage in the entire genome, in each chromosome and in different regions of the genome for each sequence context (CG, CHG and CHH). To assess different methylation patterns in different genomic regions, methylation profiles at 5′-flanking 2 kb regions and gene sequences [or transposable elements (TEs)] were plotted based on average methylation for each 100 bp interval.

2.5. DMR analysis

The DMRs for each sequence context (CG, CHG and CHH) between two samples were identified according to the following stringent criteria: a) more than five methylated cytosine molecules in at least one sample; b) The total depth of sequencing for each methylation cytosine site is > 10, and the depth of support for methylation cytosine is > 4; c) region length measures between 40 bp and 10 kb; d) distance between adjacent methylated sites < 200 bp; e) fold change of average amount of methylation > 2; f) Pearson’s chi-square test (χ^2) value is $P \leq 0.05$. At adjacent 2 kb (upstream or downstream) or body regions of genes or TEs, putative DMRs overlapping were sorted for further study.

2.6. Enrichment analysis of DMR-related genes

The GO represents an international standardised gene functional classification system offering a dynamic-updated controlled vocabulary and a strictly defined concept to comprehensively describe properties of genes and their products in any organism. The GO features three ontologies, namely, molecular function, cellular component and biological process. The GO term serves as the basic unit of GO. Each GO term belongs to a type of ontology. The GO enrichment analysis provides all GO terms that are significantly enriched in DMGs compared with the genome background and filters DMGs corresponding to biological functions. Firstly, all DMGs were mapped to GO terms in the GO database (http://www.geneontology.org/). Gene numbers were then calculated for every term. A hypergeometric test was performed to detect significantly enriched GO terms in DMGs compared with the genome background. The following formula was used to calculate the P-value:

$$\mathrm{P} = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}.$$

In the present study, N represents the number of all genes with GO annotation; n corresponds to the number of DMGs in N; M stands for the number of all genes annotated to specific GO terms; m represents the number of DMGs in M. Fisher's Exact Test is $P \leq 0.05$ as threshold. The GO terms meeting this condition were defined as significantly enriched GO terms in DMGs. This analysis was used to determine the main biological functions that are regulated as a result of DMGs.

Gene products usually to regulate certain biological functions. Pathway-based analysis allows for further understanding of gene biological functions. The KEGG is a major and public pathway-related database (Kanehisa, 2008). Use of the pathway enrichment analysis allowed for identification of significantly enriched metabolic pathways or signal transduction pathways regulated by proteins resulting from DMGs compared with the entire genome background. The formula used for calculations is the same as that used in the GO analysis. In the equation, N represents the number of all genes with KEGG annotation, n corresponds to the number of DMGs in N, M stands for the number of all genes annotated to specific pathways, and m represents the number of DMGs in M. The Fisher's Exact Test was used with a $P \leq 0.05$ as the threshold value. Pathways meeting this condition were defined as significantly enriched pathways by the DMGs.

3. Results

3.1. Summary of methylome sequencing

A total of 163.06 Gb of sequencing data averaging 81.53 Gb per sample were obtained for WGBS of endometrium samples. In Table S1, there is reporting of a statistical summary for sequencing result variables. The WGBS yielded 495,915,242 (PE) and 534,532,428 (RE) unique clean data. Unique data ratios were 94.45% and 95.11% for both libraries (Table S1), respectively. Using the Bowtie program of the BSgenome1.38 package, an unique clean data per library were then mapped to *Capra hircus* reference genome. In the PE group, unique mapped data C- > T and G- > A totalled 154,405,284 and 154345416, followed by 171,533,942 and 171,693,636 in the RE group. For both libraries, there were mapping efficiencies of 66.60% (PE) and 68.60% (RE). In Tables S2 and S3, there are the data for distribution of WGBS reads in different chromosomal regions (chromosomes 1–29 and chromosome X). Distribution of WGBS reads in different genome regions represents a genome-wide methylation pattern. Amount of methylation was analysed in the promoter, exon and intron (Additional information 1 and 2), respectively. The extent of DNA methylation of CG, CHG and CHH contexts were less near the TSS and transcription end sites but gradually increased at other genomic locations. Promoters had relatively less methylation to ensure normal functions of promoter regions. By contrast, introns had relatively greater methylation to prevent abnormal transcription of the intron sequence.

3.2. Characterisation of DMR-related genes

Compared with the PE group, there was identification of 28313/34533 hyper/hypomethylated DMRs in the RE group, which were mainly distributed in Chromosomes 1 (111181451-111182480), 5 (103270921-103271441), 8 (67465261-67466060) and X (74381637-74382081 and 79601544-79602061). Methylation ratios of these regions in the RE group were 4.95, 6.25, 6.13, 5.93 and 5.73 greater than those of the PE group, respectively ($P \leq 0.05$; Table S4). In the present study, the methylation ratios of 8,543 CpG islands in the RE group were greater than those of the PE group ($P \leq 0.05$; Table S4). There was also DNA methylation in other regions except for in CpG islands. The CpG island shores represent a region adjacent to CpG islands (2 kb), where methylation closely parallels transcriptional activation. The results of the present study indicate methylation ratios of 11,596 CpG island shores in the RE group were greater compared with those of the PE group ($P \leq 0.05$). Although regions where there was an abundance were observed and methylated differently between the two libraries, there was a focus on genes meeting the designated criteria: P-value ≤ 0.05 and $|\log2 \text{ (fold change)}| \geq 2$ (Table S5). There were as many as 37,858 DMRs differentially methylated in PE and RE samples, of which 16,467 were methylated to a relatively greater, and 21,391 to a relatively lesser extent in RE compared with PE samples (P-value ≤ 0.05, and $|\log2 \text{ (fold change)}| \geq 2$). Compared with PE samples, methylation ratios of insulin-like growth factor 2 mRNA binding protein 2 (*IGF2BP2)*, acyl-CoA oxidase 2 (*ACOX2*), prostaglandin D2 synthase (*PTGDS*), SMAD family member 2 (*SMAD2*), vascular endothelial growth factor B (*VEGFB*) and prostaglandin D2 receptor 2 (*PTGDR2*) genes were markedly less in RE samples (P-value ≤ 0.05 and $|\log2 \text{ (fold change)}| \geq 2$). Conversely, RE samples had markedly greater methylation ratios of progesterone receptor (*PGR*), IGF binding protein 3 (*IGFBP3*), IGF1 receptor (*IGF1R*) and transducin-like enhancer of split 3 (*TLE3*) genes (Table 1). Other DMGs are shown in Table 1.

3.3. GO annotation and KEGG pathway of DMGs

In the present study, GO enrichments of DMGs were categorised into 319 functional groups with P-value ≤ 0.05. Of the 98 terms that had enrichment for molecular functions (Table S7), the most enriched GO terms included zinc ion binding (GO:0008270) with 1,229 annotated genes (Fig. 1), followed by ATP binding (GO:0005524), protein binding (GO:0005515), metal ion binding (GO:0046872) and calcium ion binding (GO:0005509). In the cellular compartment GO category, 77 terms were enriched. The GO terms enriched to the greatest extent comprised the cytoplasm (GO:0005737) with 1,971 annotated genes, followed by cytosol (GO:0005829), integral to plasma membrane (GO:0005887), nucleoplasm (GO:0005654) and Golgi membrane (GO:0000139). In biological processes, 144 GO terms were enriched and were related to various processes, such as proteolysis (GO:0006508), apoptotic process (GO:0006915), protein transport (GO:0015031), cell differentiation (GO:0030154), multicellular organismal development (GO:0007275) and signal transduction (GO:0007165). Interestingly, annotations for *IGF2BP2* (GO:0005515), *PGR* (GO:0005737), *VEGFB* (GO:0016020), *IGF1R* (GO:0008286) and *SMAD2* (GO:0035556) were included.

Results from the KEGG pathway analysis allow for further understanding of biological functions of DMGs. Overall, DMGs were enriched in 82 KEGG pathways, thus meeting the criterion of P-value ≤ 0.05 (Fig. 2; Table S8). These findings indicate that the biological functions regulated by these pathways may be important in development of endometrial receptivity for the embryo. The KEGG pathways have the most DMGs consisting of focal adhesion with 731 DMGs, followed by extracellular matrix (ECM)–receptor interaction (562 DMGs), cancer (387 DMGs), mitogen-activatedprotein kinase (MAPK) signalling (291 DMGs), insulin signalling (154 DMGs), GnRH signalling (110 DMGs), VEGF signalling (95 DMGs) and progesterone-mediated oocyte maturation (87 DMGs).

4. Discussion

Successful implantation is determined by morphological changes induced by receptive state in the uterus for the embryo. A RE and a normal and functional embryo at blastocyst developmental stage are prerequisites of successful implantation, which is characterised by synchronisation of functions between maternal and embryonic tissues (Simón et al., 2000). There are no previous studies

Table 1

Differentially methylated genes in promoter and gene body regions (Receptive endometrium vs. prereceptive endometrium).

Chromosome	Strand	start	end	Gene name	Methylation ratio (RE)	Methylation ratio (PE)	log2 (RE/PE)	*P*-value	Gene annotation
22	+	42659469	42660268	ACOX2	0.50	10.95	−4.46	0.00	acyl-CoA oxidase 2
29	–	35985579	35986779	PTGDR2	1.11	5.17	−2.22	0.00	prostaglandin D2 receptor 2
1	+	79780279	79781478	IGF2BP2	1.69	7.12	−2.07	0.00	insulin-like growth factor 2 mRNA binding protein 2
X	+	111789133	111790332	EIF1AX	6.25	1.56	2	0.01	eukaryotic translation initiation factor 1A, X-linked
4	+	73655119	73655346	IGFBP3	17.65	2.94	2.58	0.01	insulin-like growth factor binding protein 3
10	+	78123330	78124529	COX16	11.30	0.66	4.09	0.00	cytochrome c oxidase assembly protein COX16 homolog, mitochondrial
24	–	47502516	47502830	SMAD2	0.00	5.63	- Infinite	0.04	SMAD family member 2
10	–	16649194	16649727	TLE3	16.31	0.00	Infinite	0.00	transducin-like enhancer of split 3
24	+	50278378	50278740	MAPK4	18.33	0.00	Infinite	0.00	mitogen-activated protein kinase 4
4	–	86536833	86537246	GPR37	4.96	0.00	Infinite	0.02	G protein-coupled receptor 37
7	+	16359737	16359862	MAP2 K2	23.08	0.00	Infinite	0.00	mitogen-activated protein kinase kinase 2
26	+	21180082	21180234	PPRC1	13.89	0.00	Infinite	0.03	peroxisome proliferator-activated receptor gamma, coactivator-related 1
15	+	6852377	6853077	PGR	4.95	0.00	Infinite	0.03	progesterone receptor
11	–	103796018	103796817	PTGDS	0.00	3.97	Infinite	0.01	prostaglandin D2 synthase 21 kDa (brain)
29	+	40294718	40294911	VEGFB	0.00	14.89	- Infinite	0.01	vascular endothelial growth factor B
21	+	6095546	6095691	IGF1R	15.38	0.00	Infinite	0.02	insulin-like growth factor 1 receptor

Note: 2 digits after the decimal point are used to show *P*-value.

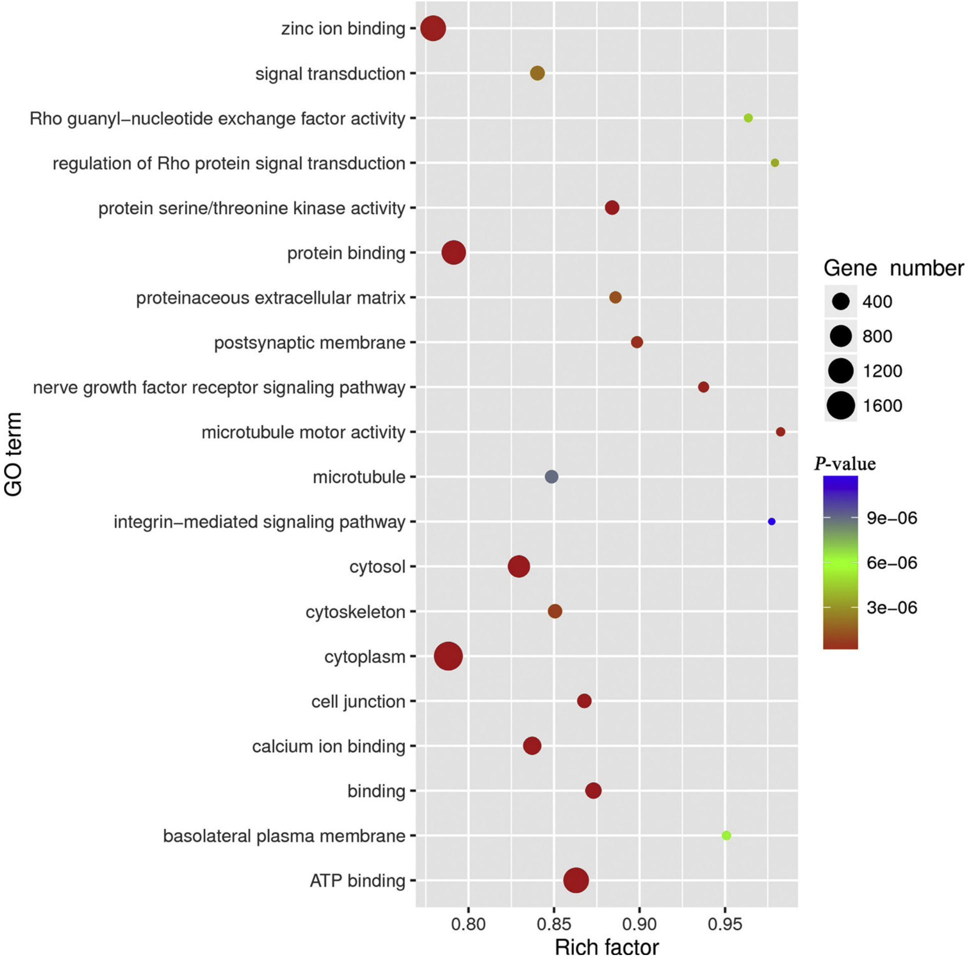

Fig. 1. Gene number of different GO terms in GO enrichment.
Rich factor: Ratio of the number of GO difference genes to the total number of GO genes; the larger the rich factor value, the greater the GO enrichment

on genome-wide methylation differences of PE and RE in dairy goats. In the present study, there were analyses of genome-wide DNA methylation profiles in the PE and RE with use of the WGBS. Results indicate 16,467 DMRs were methylated to a greater extent, and 21,391 DMRs were less methylated in RE samples compared with those of PE samples. Embryo implantation is regulated by embryonic signals results from various proteins, cytokines and growth factors that systematically modulate maternal anatomy, endocrinology, immunology and physiology so that there is an environment conducive to foetal development and survival (Jauniaux et al., 2000). Inadequate uterine receptivity for the embryo, however, results in two-thirds of the implantation embryo failures (Simon et al., 1998). Abnormal expression of genes related to establishment of uterine embryonic receptivity directly affects embryo implantation and can result in spontaneous abortion (Wang and Dey, 2006; Huang et al., 2017). Aberrant methylation at the *HOXA10* gene site may be responsible for aberrant gene expressions in the endometrium of women with endometriosis (Wu et al., 2005). The expression of the *HOXA10* gene can result in regulation of a variety of downstream genes, including cell adhesion molecules, signal transduction factors and metabolic mediators, and affects both endometrial stromal cell proliferation and epithelial cell morphogenesis (Zanatta et al., 2010; Qu et al., 2018).

In general, gene expression can be affected by various factors, such as nutrition, endocrine secretion, heredity and epigenetic modification (DNA methylation/demethylation). The relationship between gene expression and DNA methylation of eukaryotic genes can be divided into three types (Tanaka et al., 1983; Vedel et al., 1983; Ponnaluri et al., 2017): (1) a parallel relationship in which DNA methylation status coincides with extent of gene expression; (2) an antiparallel relationship in which hypomethylation leads to activation of gene expression, and hypermethylation where there is silencing of gene expression; (3) gene expression does not correlate with methylation status. Ponsuksili et al. (2012) suggested that interaction of time point and endometrial receptivity for the embryo has a marked effect on global DNA methylation, leading to divergent trends of greater or lesser DNA methylation in the group with relatively greater or lesser receptivity for the embryo, respectively (Ponsuksili et al., 2012). The DNA methylation regulates promoter regions of genes related to uterine receptivity for the emrbyo; such genes include cadherin 1, *PGR*, *Esr1*, leukaemia inhibitory factor, *IGF2* and *IGF2* receptor (Gao et al., 2012). The results from the WGBS analysis in the present study indicated there

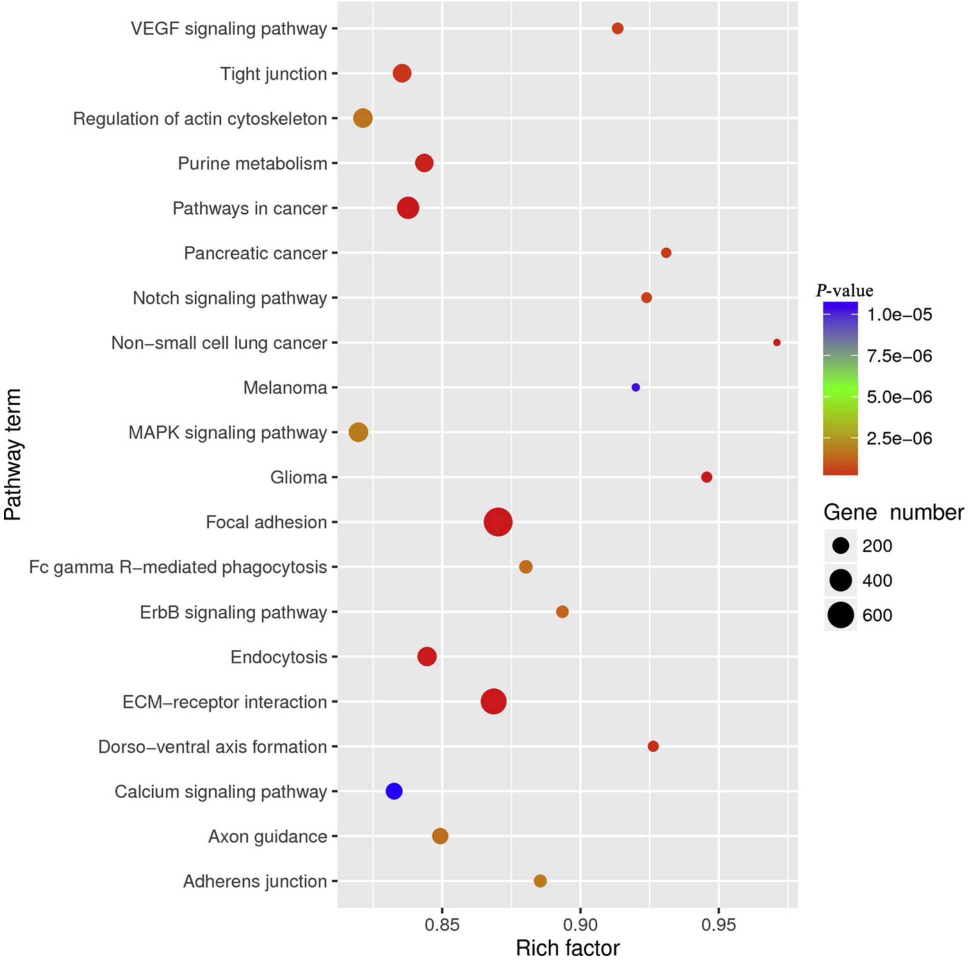

Fig. 2. Gene number of different pathway terms in KEGG pathway analysis.
Rich factor: The ratio of the number of KEGG differential genes to the total number of KEGG genes; the larger the rich factor value, the greater the KEGG enrichment

were many biological processes, cellular compartments and molecular functions that differed between the PE and RE, for example, zinc ion binding (GO:0008270), cytoplasm (GO:0005737), proteolysis (GO:0006508) and apoptotic process (GO:0006915). In the endometrial tissue, preparation for embryo implantation allows reception of blastocyst as a result of regulation of immune cell functions, as well as secretion of cytokines, growth factors, chemokines and adhesion molecules (Dimitriadis et al., 2005; van Mourik et al., 2009). The RE samples had markedly lesser methylation ratios of *IGF2BP2*, *ACOX2*, *PTGDS*, *SMAD2*, *VEGFB* and *PTGDR2* genes. Conversely, *PGR*, *IGFBP3*, *IGF1R* and *TLE3* genes had markedly greater methylation ratios in RE samples. The *IGF2BP2*, *ACOX2*, *PTGDS* and *IGFBP3* potentially regulate the development of the endometrium, embryo implantation and early placental development (Oliveira et al., 2010; Tiirikka et al., 2010; Catalano et al., 2011; Zhang et al., 2017). The pattern of methylation changes of these genes (*IGF2BP2*, *ACOX2*, *PTGDS*, *BEGFB*, *PTGDR2*, *IGFBP3*, etc.) indicate these genes could be involved in the development of the endometrium and embryo implantation. Results indicate that the proteins produced as a result of these DMGs may have functions in uterine embryo receptivity.

The endometrium is a dynamic tissue that undergoes cycles of proliferation, differentiation, breakdown and repair (Jabbour et al., 2006). The knowledge gained from assessing KEGG pathways with enriched DMGs is a valuable resource for investigating specific processes, functions and pathways, facilitating identification of DMGs involved in development of RE in goats during the ‘window of implantation’. In the present study the expression of most DMGs was related to focal adhesion with 731 DMGs in this category. Some essential elements of embryo implantation include unique cell adhesion of trophoblast to endometrial epithelial cells and subsequent trophoblastic invasion of maternal tissue (Pandey et al., 2009). Samborski et al. (2013) reported that genes related to cell adhesion processes in related categories were overrepresented in genes with greater expression at day 14 of the pregnant endometrium and in genes with relatively greater expression at day 14 of the oestrous cyclic endometrium in pigs (Samborski et al., 2013). These results relate to important processes for the development of RE in the prereceptive phase: the folded endometrial epithelial bilayer and capacity of the endometrium to acquire adhesive properties that allow embryo adhesion and its subsequent invasion. Additionally, in the present study, the results of the KEGG pathway analysis indicated there was an enriched insulin signalling (154 DMGs), MAPK signalling (291 DMGs), GnRH signalling (110 DMGs), and VEGF signalling (95 DMGs) pathway as well as progesterone-mediated oocyte maturation (87 DMGs), meeting the criterion of P-value ≤ 0.05. From results of the present study, there was also involvement of *IGF2BP2*, *ACOX2*, *PTGDS*, *VEGFB*, *PTGDR2*, *PGR*, *IGFBP3* and *IGF1R* in these KEGG pathways. The *IGF-1* molecule mediates growth-promoting activity of hormones, induces endothelial cell migration and participates in regulation of angiogenesis (Xie et al., 2016). Downregulated *IGF-1* gene expression may be associated with a reduction in *VEGF* gene expression in endometrial glands to inhibit angiogenesis of the endometrium before embryo implantation and to reduce endometrial receptivity, leading to infertility in ectopic pregnancy patients (Chen et al., 2016). A prerequisite for development of uterine receptivity for embryonic implantation involves continuous actions on the endometrium by progesterone which, after a species-specific interval, downregulates PGR gene expression in the epithelium and stimulates production of progestamedins (de Ruijter-Villani and Stout, 2015). Results of the present study provide valuable information for future studies of molecular mechanisms underlying the development of RE in goats.

5. Conclusion

The WGBS assessments in the present study provided evidence for there being 16,467 DMRs that were hyper-methylated, and 21,391 DMRs that were hypo-methylated in the RE samples compared with those of PE samples. The DMGs were enriched with 319 GO functional groups (zinc ion binding (GO:0008270), ATP binding (GO:0005524), cytoplasm (GO:0005737), apoptotic process (GO:0006915), protein transport (GO:0015031) and cell differentiation (GO:0030154)) and 82 KEGG pathways ($P \leq 0.05$). Results from a previous and the present study indicate focal adhesion, insulin signalling pathway, GnRH signalling pathway, VEGF signalling pathway, ECM–receptor interaction, MAPK signalling pathway, progesterone-mediated oocyte maturation and pathways in cancer cells may have important functions in development of endometrial receptivity for the embryo. There was also identification of some DMGs (*IGF2BP2*, *PTGDS*, *VEGFB*, *PGR*, *IGFBP3* and *IGF1R*) that are associated with uterine receptivity for embryos. Results from the present study, however, require further experimental validation.

Declaration of Competing Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality in conducting the experiment and publishing the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anireprosci.2019.106124.

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