RESEARCH ARTICLE

Expression of DNA methyltransferases (DNMTs) at mRNA level in ovine endometrium during estrus cycle and early pregnancy

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Östrus siklusu ve erken gebelikte koyun endometriyumunda DNA metiltransferazların (DNMT) mRNA düzeyinde ekspresyonu

Öz

Amaç: Bu çalışmanın amacı, östrus döngüsü ve erken gebelik sırasında DNA metiltransferaz enzim genlerinin (DNMT1, DNMT3A ve DNMT3B) ekspresyonunun koyun endometriyumunda düzenlenip düzenlenmediğini açıklamaktır.

Gereç ve Yöntem: İnterkarunkular bölgeleri içeren endometriyal örnekler çiftleşmeyi takiben gebeliğin 12 (P12, n = 4), 16 (P16, n = 4) ve 22. (P22, n = 4) günlerinde ve östrus döngüsününe karşılık gelen 12. (C12, n = 4), 16. (C16, n = 4) ve 22. (C22, n = 4) günlerde toplam 24 koyundan toplandı. DNMT'lerin nispi mRNA ekspresyon düzeyleri gerçek zamanlı polimeraz zincir reaksiyonu (RT-qPCR) kullanılarak değerlendirildi.

Bulgular: Tüm DNMT'lerin ekspresyonları östrus döngüsü ve erken gebelik sırasında koyun endometriyumda tespit edildi. DNMT1 mRNA ekspresyonunun kararlı durum seviyeleri P16'da C16'dan daha yüksekti, ancak grupların geri kalanında değişmedi. Bununla birlikte, gebeliğin erken döneminde DNMT3A mRNA seviyesi P22 ve P16'ya kıyasla P12'de en yüksek ekspresyon seviyelerine sahipti (p<0.01). Öte yandan, DNMT3A mRNA seviyesi P16'da C16'dan daha düşüktü (p<0.01), ayrıca P22'de C22'ye kıyasla benzer bir düşüş bulundu (p<0.01). DNMT3B mRNA ekspresyon seviyesi ise hem östrus döngüsü ve hem de erken gebelik sırasında değişmedi.

Öneri: Östrus siklusu ve erken gebelik sırasında koyun endometriyumundaki gen ekspresyonunun kontrolü için DNA metilasyonu ile ilgili DNMT'ler gerekli olabileceği kanısına varıldı.

Anahtar kelimeler: DNMT1, DNMT3A, DNMT3B, koyun endometriyum, gen ekspresyonu

Abstract

Aim: To elucidate mRNA expression of DNA methyltransferase enzyme genes (DNMT1, DNMT3A, and DNMT3B) in ovine endometrium whether they are modulated during the estrus cycle and early pregnancy.

Materials and Methods: The endometrial samples including intercaruncular sites was obtained from a total of 24 ewes on days of 12 (P12, n = 4), 16 (P16, n = 4) and 22 (P22, n = 4) of pregnancy following mating and cyclic days of 12 (C12, n = 4), 16 (C16, n = 4) and 22 (C22, n = 4) of the estrous cycle. The relative mRNA level of DNMTs were evaluated through real-time quantitative RT-qPCR.

Results: Abundances of DNMTs including DNMT1, DNMT3A, and DNMT3B were detected during the estrous cycle and early pregnancy in the ovine endometrium by this study. DNMT1 mRNA steady-state level was greater in P16 than in C16 whereas they did not change within the rest of the groups. The level of DNMT3A mRNA, during early pregnancy, had the highest expression levels on P12 compared to P22 and P16 (p<0.01). However, the level of DNMT3A mRNA was lower in P16 than in C16 (p<0.01) and had a similar decrease in P22 compared to C22 (p<0.01). DNMT3B mRNA level did not differ (p>0.01) during the estrus cycle and early pregnancy.

Conclusion: DNA methylation related DNMTs may be required to control of gene expression in the ovine endometrium during the estrus cycle and early pregnancy.

Keywords: DNMT1, DNMT3A, DNMT3B, ovine endometrium, gene expression

Introduction

Epigenetics attempts to elucidate how gene expression can be regulated without changing the genetic code. DNA methylation is one biological modification within epigenetic process that helps modify DNA gene expression by attaching a methyl group to a cytosine in the DNA code occurring globally over the genome (Iqbal et al 2011). Areas in the genome with many cytosine and guanine nucleotides, known as CpG islands, are generally not methylated, and are located near transcription start sites; however, it is speculated that genes are repressed when these islands become methylated (Vavouri et al 2011). Curradi et al. (2002) suggest that the lack of methylation is essential for transcription to operate. The methylation of these areas are responsible for inactive genes, such as the inactive X chromosome.

In mammals, DNA methyltransferases (DNMT) are a group of enzymes needed to transfer of methyl groups to the DNA. Four DNMT's that put the initial pattern of methyl groups in sites on the DNA sequence or they work as maintenance DNMT's that copy the methylation from a strand of DNA to a new strand through replication (Phillips, 2008). In mammals, global cytosine methylation patterns are independent of each other and are formed by three encoded DNA methyltransferases (DNMT1, DNMT3A, DNMT3B). DNA methyltransferases are generally divided into two classes that provide de novo methylation or methylation maintenance. The level of DNA methylation differs between tissues. However, in some cases, DNA methylation level is significantly affected by environmental conditions, rearing style, and nutrition (Kadayifci et al 2018, Law and Holland 2019).

The critical and balanced secretion of ovarian hormones and embryo-derived local factors causes modeling and functional changes in the endometrium required for a new cycle or to sustain an established pregnancy. Regulation of the gene expression in the endometrium has vital importance in the maternal recognition of the pregnancy and the process of embryo implantation into the uterus (Atli et al 2010, Salilew-Wondim et al 2012, Chen et al 2015). Also, gene expressions change in the endometrium during the estrous cycle and early pregnancy (Atli et al 2011, 2012) while impacting endometrial receptivity (Mazzoni et al 2020). Moreover, differentially expressed genes appeared with the regulation of estrogen and progesterone (P4) during pregnancy (Shimizu et al 2010). Therefore, the endometrium is subject to transcriptional changes to facilitate the achievement of successful pregnancy in the ovine endometrium (Brooks et al 2016). However, the role of underlying mechanisms regulating changes in the gene expression in the ovine endometrium has not been elucidated in detail.

Given that specific patterns of gene expression and repression are important in the control of mammalian reproduction, the aim was to elucidate mRNA expression of DNMTs including DNMT1, DNMT3A, and DNMT3B in ovine endometrium if they are modulated during the estrus cycle and early pregnancy.

Material and Methods

Animal diets and experimental design

Animals included in this study were fed following the nutritional necessities of the National Research Council (2007). Twenty-four multiparous Anatolian Merino ewes (n=24) were included; grouped into cyclic (n=12) and pregnant (n=12), randomly. Briefly, cycles of the ewes were synchronized with two cloprostenol (a synthetic analog of prostaglandin F2alpha; PGF2α, 125 mcg) injections 11 days apart. Immediately after the second injection, estrus was checked two times using teaser rams. Estrus of ewes was obtained through teaser ram at eight-hour intervals for five days following second injection and the ewes that showed estrus were recorded. Then, ewes were set free to finish their entire cycle and checked for new naturel estrus with teaser rams. In this new estrus, ewes in the pregnant group were mated (day 0) 2 times, 12 h difference, with a fertile ram. The estrus day in the cyclic group was accepted as day zero (day 0). To provide a similar effect of progesterone and understand the only effect of pregnancy or embryo on endometrial, cyclic ewes were treated with a natural progesterone implant via intravaginal on day 13 of the cycle for days 16 and 22 groups, and progesterone implants were kept until the ewes were slaughtered. Ewes were arranged to be slaughtered on days of 12 (n = 4; P12), 16 (n = 4; P16) or 22 (n = 4; P22) of gestation after mating, and on cyclic days of 12 (n = 4, C12), 16 (n = 4, C16) or 22 (n = 4, C22) of the estrous cycle. The pregnancy was confirmed in ewes by seeing he embryonic trophoblast in the uterine lumen for 12, 16, and 22 days of pregnancy (Spencer et al 2004). Intercaruncular tissues were collected rapidly and kept at -80°C until RNA isolation.

RNA isolation and cDNA synthesis

Intercaruncular tissues were for RNA isolation as defined previously by (Hitit et al 2018). Briefly, 50 mg of tissues using 800 μl mL TRIzol® were crushed and 275 μl chloroform was added to obtain phase separation by centrifugation at 13000 RPM for 15 minutes. After that, 500 μl isopropanol was poured, and immediately supernatant phase was collected for precipitation of RNA. Subsequently, the supernatant was thrown, and the pellet was washed using 75 % ethanol. The pellet of RNA was dilueted with 50 μl of and saved in the freezer. RNA quality and concenration were assessed using spectrophotometer (Colibri Microvolume Spectrometer) observing the absorbance from 260/A280. Two μg RNA was

converted to cDNA using commercial kit (I-Script, BioRAD).

Gene expression

The gene expression profiles of DNMTs enzymes were carried out using the quantitative polymerase chain reaction (qPCR) with specific primers of interest (Table 1). qPCR was set as shown: 10 µl iTaq™ universal SYBR® Green (BioRad), 1 µL cDNA, 10 pMol each primer, and dH2O to a final volume of 10 µL (Hitit et al 2020). PCR conditions were arranged as shown: initial denaturation, 94°C for 8 min, then 42 denaturation cycles, annealing, and further amplification at 94°C for 40 s, 59°C for 30 s, then 72°C for 2.5 minutes, respectively. The analysis of melting was set up as shown: 94°C for the 60s, and the fluorescence was measured at 1°C increment starting from 52°C up to 95°C by qPCR (Bio-Rad CFX96; Bio-Rad, USA). SDHA was employed as a reference gene for qPCR analysis and relative expression to normalize the gene expression data. The analysis was performed in duplicate for each gene.

Protein-protein interaction and Gene ontology network analysis

The protein association of DNMTs genes was established through the STRING (version 11.5, http://string-db.org), and finally shown in Cytoscape (version 3.9.1). Interaction of gene network with a confidence score ≥ 0.4 was established using Cytoscape. The evidence for interactions including databases, experiments, text mining, and co-expression, is limited to "Ovis aries". GO enrichment was clarified using Cytoscape by plug-in of ClueGO V2.5.7 (Bindea et al 2009). GO was elucidated using a cellular molecular function, component, and biological processes. Two-sided hypergeometric tests for P-value to 0.05 was set and multiple test correction was evaluated by Bonferroni step down adjustment. Kappa's score was 0.4. for the threshold.

Statistical analysis

PCR (Ct) data were measured for relative expression (Hitit et al 2020). The method of $2^{-\Delta\Delta Ct}$ was employed to calculate relative expression (Livak and Schmittgen 2001). qPCR normalized data were analyzed through t-test and ANOVA with Tukey's post hoc test. $p < 0.05$ value was set as significance.

Results

DNMT1 mRNA levels were demonstrated on days 12, 16, and 22 during the estrus cycle and early pregnancy in Figure 1. When assessed during early pregnancy, DNMT1 mRNA was demonstrated to be similar in P12, P16, and P22, compared to each other ($p > 0.05$) while the level of DNMT1 mRNA was found to be greater in P16 than in C16 ($p < 0.01$). However the level of DNMT1 mRNA did not change between C12 vs P12 and C22 vs P22 ($p > 0.05$), also cyclic groups were not differed ($p > 0.05$).

| Table 1. The gene primers used for qPCR			
Oligonucleotide	Nucleotide Sequence	Product Size (bp)	Accession
DNMT1 F	5'- GACGGCTTCTTCAGCACAA -3'	155	NM_001009473.1
DNMT1 R	5'- TCCAGGATGTTGCCGAACAG -3'		
DNMT3A F	5'- AAAGAACAGAAGGAGACCAAC -3'	155	XM_004007223.1
DNMT3A R	5'- TTGCGCTTGCTGATGTAG -3'		
DNMT3B F	5'- GGAGTTCAATAGGACAGCAAAG-3'	136	XM_004014480.1
DNMT3B R	5'- TAGCTCAGTGCACCACAA-3'		
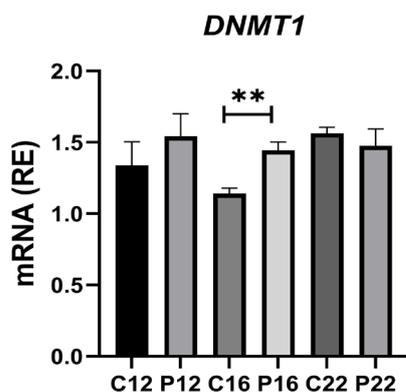
Figure 1. Expression of DNMT1 mRNA during estrus cycle and early pregnancy. Cyclic days (C12, C16, and C22) and pregnant (P12, P16, and P22). RE: relative expression. **: shows significance (p<0,01)
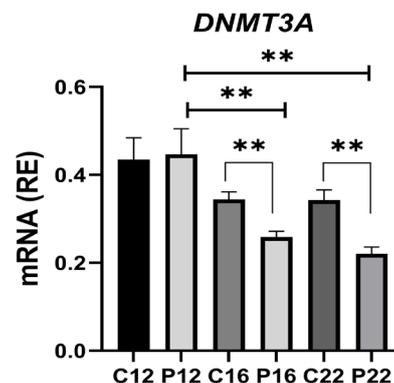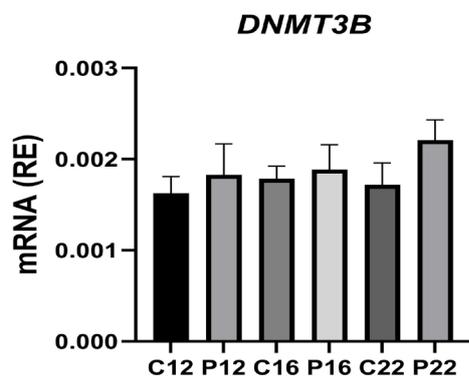

Figure 2. Expression of DNMT3A mRNA during estrus cycle and early pregnancy. Cyclic days (C12, C16, and C22) and pregnant (P12, P16, and P22). RE: relative expression. **: shows significance (p<0,01)

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DNMT3B

Figure 3. Expression of DNMT3B mRNA during estrus cycle and early pregnancy. Cyclic days (C12, C16, and C22) and pregnant (P12, P16, and P22). RE: relative expression.
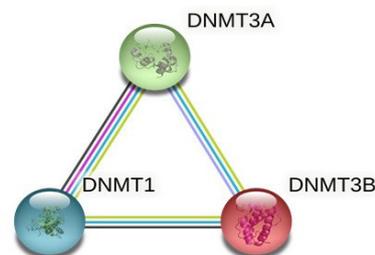

Figure 4. Protein-protein interaction network analysis of DNMTs. The PPI network consisting of 3 nodes and 3 edges was constructed through the target genes

Table 2. Molecular function (Gene ontology) and pathway of DNMTs (DNMT1, DNMT3A, and DNMT3B)

Term ID	Term description	Observed gene count	Strength	False discovery rate
GO:0003886	DNA (cytosine-5-)-methyltransferase activity	2	3.84	0.00024
Pathway ID	**Term description**	**Observed gene count**	**Strength**	**False discovery rate**
oas00270	Cysteine and methionine metabolism	3	2.66	4.01E-06
oas05206	MicroRNAs in cancer	3	2.2	4.27E-05
oas01100	Metabolic pathways	3	1.22	0.0245

DNMT3A mRNA levels during the estrus cycle and early pregnancy on days 12, 16, and 22 are shown in Figure 2. The level of DNMT3A mRNA had the highest expression levels in P12 compared to P22 and P16 during early pregnancy ($p<0.01$) while mRNA DNMT3A showed similar expression levels between P16 and P22 ($p>0.05$). On the other hand, the level of DNMT3A mRNA was lower in P16 than in C16 ($p<0.01$) and also had a similar decrease in P22 compared to C22 ($p<0.01$).

DNMT3B mRNA on days 12, 16, and 22 during estrus cycle and early pregnancy are shown in Figure 3. During the early pregnancy, DNMT3B mRNA was similar among pregnancy days ($p>0.05$), also in the estrous cycle, it has similar levels in C12, C16, and C22 ($p>0.05$). Furthermore, the mRNA expression levels of DNMT3B did not differ between C12 and P12, C16 and P16, and C22 and P22 ($p>0.05$).

Results from the GO functional analysis of PPI (Figure 4) on expressed genes (DNMT1, DNMT3A, and DNMT3B) of the DNMT family were constructed and mRNA of DNMT1, DNMT3A, and DNMT3B were consistently enriched in (GO:0003886) DNA (cytosine-5-)-methyltransferase activity (Table 2) while also involved in (oas00270) cysteine and methionine metabolism, (oas05206) microRNAs in cancer, and (oas01100) metabolic pathways (Table 2).

Discussion

The present study showed the presence of the mRNA expression of DNA methyltransferase enzymes in the ovine endometrium during the estrus cycle and early pregnancy. The intercaruncular side is an active part of the ovine endometrium to produce intrauterine secretions which is needed for embryonic growth therefore, we assessed DNMT1, DNMT3A, and DNMT3B mRNA expressions in the active site of the ovine endometrium.

The endometrium, which is exposed to hormonal and cyclical changes, is regulated by many the endometrial transcriptome genes (Talbi et al 2006). In the light of these data, the existence of a critical regulation in the endometrium in specific genes has been clarified for genes with some physiological effects. Cycle-dependent transformation of the endometrium in humans has been epigenetically associated with the expression of chromatin modification enzymes (Munro et al 2010; Zelenko et al 2012). However, as described in the GO terms and pathway, aberrant DNMT expression is likely to be implicated in endometrial cancer (Wang and Li 2017, Mahajan et al 2020), which may explain why stringent control of gene expression is also required.

In recent reports, it has been indicated that gene expressions in the endometrium are epigenetically modulated by mechanisms, for instance DNA methylation, small non-coding RNAs, and histone modifications (Fürst et al 2012, Hitit et al 2015a, Yang et al 2021) without changing the sequence of genes. DNA methylation, regarded as an epigenetic factor, regulates gene expression and DNA methylation requires three essential DNA methyl transferase enzymes (DNMT1, DNMT3A, and DNMT3B). While the control of gene expression in the endometrium has not been extensively studied, our previous study revealed the epigenetic contribution to the transcriptional regulation of the equine endometrium, in which critical patterns of DNA methylation enzymes are constitutively expressed (Hitit et al 2015b).

In the current study, in contrast to equine, except for DNMT1 and DNMT3A, only DNMT3B was found to be constitutively expressed in ovine endometrium. This may be consistent with that of DNMT3B mRNA because it is major de novo DNA methyltransferase and has shown to be expressed and functional during the early stage of embryonic development (Gagliardi et al 2018). Although lack of DNMT3B leads to impaired decidualization and embryo implantation it does not change global DNA methylation (Long et al 2021), thus explaining the stable expression of DNMT3B mRNA during early pregnancy in ovine endometrium.

The endometrial DNA methylome is regulated cycle-dependent and linked with gene expression through P4 and estrogen. DNMT1 mRNA was found to increase during the pre-receptive phase of pregnancy in mice endometrium (Ding et al 2012). In line with that receptivity, in bovine endometrium, DNTM1 mRNA was found to be higher in an presence of embryo than in to no embryo (Ponsuksili et al 2012). DNMT1 was reported to be increased explicitly at the implantation site from day 4 through 8 of pregnancy in mice (Gao et al 2012). In our study, concerning early pregnancy, although it did not change in P12 and P22, DNMT1 mRNA was upregulated in P16 against C16 whereby embryo implantation is established. However, upon DNMT3A was knocked down, it was shown not to be required for embryo

implantation (Li et al 2020). In our study, during early pregnancy, this is consistent with the downregulation of DNMT3A in P16 and P22. Also, we showed that DNMT3 mRNA was lower in P16 and P22 versus their cycle group C16 and C22. Accordingly, DNMT3A was downregulated in the secretory phase of cycle upon compared to the proliferative phase in human endometrium (Vincent et al 2011).

Conclusion

Considering the mRNA expression of DNMT1, DNMT3A, and DNMT3B and network analysis, they seemed to have critical role in ovine endometrium. It could be suggested that DNMTs may transcriptionally modulate control of gene expression during the estrus cycle and early pregnancy

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Conflict of Interest

The authors did not report any conflict of interest or financial support.

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Ethical Approval

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