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Genetic and epigenetic regulation of major histocompatibility complex class I gene expression in bovine trophoblast cells

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Problem: The regulatory mechanisms governing differential expression of classical major histocompatibility complex (MHC) class I (*MHC-Ia*) and non-classical MHC class I (*MHC-Ib*) genes are poorly understood.

Method of study: Quantitative reverse transcription- polymerase chain reaction (PCR) was used to compare the abundance of MHC-I transcripts and related transcription factors in peripheral blood mononuclear cells (PBMC) and placental trophoblast cells (PTC). Methylation of *MHC-I* CpG islands was detected by bisulfite treatment and next-generation sequencing. Demethylation of PBMC and PTC with 5′-aza-deoxycytidine was used to assess the role of methylation in gene regulation.

Results: *MHC-I* expression was higher in PBMC than PTC and was correlated with expression of *IRF1*, class II MHC transactivator (*CIITA*), and *STAT1*. The *MHC-Ia* genes and *BoLA-NC1* were devoid of CpG methylation in PBMC and PTC. In contrast, CpG sites in the gene body of *BoLA-NC2*, *-NC3*, and *-NC4* were highly methylated in PBMC but largely unmethylated in normal PTC and moderately methylated in somatic cell nuclear transfer PTC. In PBMC, demethylation resulted in upregulation of *MHC-Ib* by 2.8- to 6-fold, whereas *MHC-Ia* transcripts were elevated less than 2-fold.

Conclusion: DNA methylation regulates bovine *MHC-Ib* expression and is likely responsible for the different relative levels of *MHC-Ib* to *MHC-Ia* transcripts in PBMC and PTC.

KEYWORDS

bovine, DNA methylation, non-classical *MHC-I*, transcription

1 | INTRODUCTION

Major histocompatibility complex class I (MHC-I) molecules are cell surface glycoproteins that play a critical role in triggering an immune response. *MHC-I* genes are classified into classical (*MHC-Ia*) and non-classical (*MHC-Ib*) subtypes according to their sequence polymorphism, expression patterns, and gene structure.[1,2] Classical *MHC-I* genes are highly polymorphic and are ubiquitously expressed in most somatic cells. MHC-Ia proteins activate cluster of differentiation 8-positive (CD8$^+$) T lymphocytes by presenting peptide antigens derived from internal proteins. Activated T lymphocytes subsequently clear cells that display the same antigens from intracellular pathogens.[3-5] In contrast, non-classical *MHC-I* genes exhibit limited polymorphism and are expressed in a more tissue-restricted manner, such as in the placenta during pregnancy.[2,6]

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MHC-Ib proteins primarily function to inhibit immune responses.[7] For example, the human MHC-Ib protein human leukocyte antigen (HLA)-E inhibits natural killer cells.[8,9] During pregnancy, HLA-G inhibits both T cells and natural killer cells to provide an immunologically favorable environment at the maternal-fetal interface that protects the conceptus from the maternal immune system.[6,10,11] HLA-G is also thought to contribute to immune evasion of tumour cells,[9,12] and blocking HLA-G with a specific antibody may offer an innovative therapeutic strategy for cancer.[13]

In cattle, *MHC-I* genes are abundantly expressed in lymphocytes, but expression in placental trophoblast cells is very low, particularly during the first two trimesters of pregnancy.[14] Abnormally high expression of *MHC-I* in placental trophoblast is linked to a higher rate of miscarriage in somatic cell nuclear transfer (SCNT) pregnancies.[15] Moreover, *MHC-Ia* and *-Ib* genes are differentially expressed among various tissues in cattle. Microarray screening in bovine peripheral blood mononuclear cells (PBMC) showed that *MHC-Ia* accounted for more than 90% of total *MHC-I* transcripts, whereas in bovine placental trophoblast cells, (PTC) *MHC-Ia* and *-Ib* accounted for 22% to 66% and 34% to 79% of total transcripts, respectively.[2]

Because both *MHC-Ia* and *MHC-Ib* genes play important roles in the regulation of immune responses, it is important to know how *MHC-I* gene expression is regulated. Previous studies investigating human *MHC-I* gene regulation focused on functional promoter elements (reviewed in Ref. 16). Important *cis*-regulatory elements that regulate *MHC-I* expression include enhancer A,[17] interferon (IFN)-stimulated regulatory element,[18] and the SXY module,[19,20] which are bound by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), interferon regulatory factor 1 (IRF1) and MHC class II (MHC-II) enhanceosome A, respectively.[17-20] Regulation of *MHC-I* genes by cytokines and inflammatory factors, such as interferon gamma (IFN-γ),[21] transforming growth factor beta (TGF-β),[22] and tumour necrosis factor alpha (TNFα),[23] can partially explain differences in *MHC-I* expression patterns among tissues. However, the extremely different transcription patterns for *MHC-Ia* and *-Ib* genes in bovine PBMC and PTC suggests that other mechanisms, such as epigenetic regulation, may also be involved in controlling expression of *MHC-I* genes in cattle.

Methylation of cytosine residues at CpG dinucleotides is one of the best-studied epigenetic modifications in the mammalian genome and is known to have a prominent effect on gene expression.[24] DNA hypermethylation silences the expression of *HLA-A* and *HLA-B* in certain cancers,[25] and demethylation treatment induces the expression of *HLA-G*.[26] Suarez-Alvarez et al demonstrated that promoter methylation plays a very important role in regulating the expression of *MHC-I* in human embryonic stem cells.[27]

The objective of this study was to elucidate the regulatory mechanisms responsible for differential expression of *MHC-Ia* and *MHC-Ib* in bovine PBMC and PTC, with a focus on epigenetic mechanisms. Our working hypotheses were that: (i) bovine *MHC-Ia* and *MHC-Ib* genes are regulated by the same transcription factors, and (ii) the relative abundance of bovine *MHC-Ia* and *MHC-Ib* transcripts in PBMC and PTC is controlled by DNA methylation.

2 | MATERIALS AND METHODS

2.1 | Animals

The Utah State University Institutional Animal Care and Use Committee approved all procedures for the handling and treatment of cattle used in this study (protocols #1171 and #1506). Cattle were maintained at the Utah State University Animal Science Farm or the Caine Dairy Center in Wellsville, Utah.

2.2 | Isolation and culture of PBMC

Cattle blood was collected using vacutainer tubes containing acid citrate dextrose (BD, Franklin Lake, NJ, USA). Whole blood was centrifuged at 200 *g* for 30 minutes. The buffy coat layer was transferred to a new conical tube and resuspended in 10 mL phosphate-buffered saline (PBS). This suspension was overlaid on Ficoll-Hypaque density gradient (Accurate Chemical & Scientific, Westbury, NY) and centrifuged at 1400 *g* for 30 minutes to obtain the PBMC fraction. The PBMC were then washed three times with PBS. For the gene expression experiments, RNA from three cows was prepared as described below. For the DNA methylation study, DNA from four cows was isolated as described below.

For the demethylation study, which required that PBMC be cultured for several days, PBMC were isolated from three healthy Angus cows and resuspended in RPMI 1640 medium supplemented with 1 mmol/L L-glutamine (HyClone Laboratories, Logan, UT), 100 μg/mL penicillin (HyClone Laboratories), 100 μg/mL streptomycin (HyClone Laboratories), 0.125 μg/mL concanavalin A (GE Healthcare, Uppsala, Sweden), and 10% heat-inactivated fetal bovine serum (FBS, HyClone Laboratories). Cells were cultured with or without 100 μmol/L 5′-aza-2-deoxycytidine (Acros Organics, Geel, Belgium), which demethylates DNA, at 37°C in an atmosphere of 5% CO_2. After 3 days in culture, RNA was isolated and converted to cDNA for measurement of *MHC-I* expression by quantitative reverse transcription polymerase chain reaction (qRT-PCR).

2.3 | Isolation and culture of PTC

Day 35 PTC samples were collected from seven artificial insemination (AI) and five SCNT pregnancies. SCNT embryos were produced and transferred into recipient cows as previously described.[28,29] Pregnant cows were euthanized on day 35 ± 1 of gestation at a USDA-inspected slaughter facility following standard protocols. Trophoblast cells were collected from the placenta by carefully peeling the distinct tan-colored trophoblast layer away from the other layers of the placenta, snap frozen in liquid nitrogen, and maintained at −80°C until used for RNA and DNA isolation.

Trophoblast cells were also isolated from day 35 AI conceptuses for the demethylation experiment, which required culturing the cells for several days. To isolate PTC for cell culture, the trophoblast layer of the placenta was washed three times with PBS, cut into small pieces, and treated with 20 mL 0.25% trypsin/DNase solution (HyClone Laboratories, Logan, UT) at

37°C on a plate shaker for 20 minutes. Samples were then filtered through four layers of cheesecloth, and any undigested tissue was incubated in fresh trypsin/DNase solution for an additional 30 minutes. Next, dispersed cells were pooled and filtered through a 100 μm nylon cell strainer (BD science, Franklin Lake, NJ). The filtrate was overlaid on 40% Percoll (GE health care, Waukesha, WI) and centrifuged at 800 *g* for 10 minutes at room temperature. Cells at the Percoll interface were collected and washed three times with DMEM/F12 medium. Finally, cells were resuspended in DMEM/F12 medium with 10% FBS, 100 μg/mL penicillin and 100 μg/mL streptomycin and cultured with or without 100 μmol/L 5′-aza-2-deoxycytidine (Acros Organics, Geel, Belgium) at 37°C in an atmosphere of 5% CO_2. After 3 days in culture, RNA was isolated and converted to cDNA for measurement of *MHC-I* expression by qRT-PCR.

2.4 | Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA from PBMC, and day 35 AI and SCNT PTC were isolated using an Ambion PureLink RNA mini isolation kit (Life Technologies, Carlsbad, CA). First-strand cDNA from each sample was generated from 1 μg total RNA using the SuperScript VILO cDNA synthesis kit (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Two quantitative polymerase chain reaction (PCR) platforms were used in this work. The primers that were used for *MHC-I* and transcription factor genes with both qRT-PCR platforms are listed in Table 1. To establish the relative abundance of bovine *MHC-I* subtypes and the change after demethylation treatment, real-time PCR was performed on an Eppendorf Realplex Mastercycler (Eppendorf, Hamburg, Germany). Each 20 μL reaction included 25 ng diluted cDNA, 200 nmol of each primer, and 10 μL SYBR-Green master mix (Life Technologies, Carlsbad, CA). The cycling parameters were 94°C for 2 minutes of hot-start, followed by 40 cycles of 94°C for 15 seconds, 60°C for 20 seconds and 72°C for 30 seconds. To study the correlation between expression of bovine *MHC-I* and related transcription factors in PTC and PBMC, and the level of expression of DNA methyltransferases, qRT-PCR was performed using a Fluidigm 48.48 Dynamic Array chip with a Fluidigm BioMark high-throughput, quantitative PCR system.[30] Quantitative PCR data were analyzed using the formula for relative quantification described by Pfaffl.[31] The relative mRNA levels were normalized to *GAPDH* mRNA levels.

2.5 | DNA sequencing of MHC-I CpG islands

Genomic DNA from PBMC and PTC was extracted using the Purelink Genomic DNA Mini Kit (Life Technologies, Carlsbad, CA). Primers for amplifying the CpG island regions within each *MHC-I* gene are listed in Table 2. PCR reactions using Phusion Hot-Start II High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA) were performed at 98°C for 30 seconds, followed by 30 cycles of 98°C for 15 seconds, 62°C for 30 seconds and 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. PCR products were cloned into the pCR-Blunt vector (Life Technologies, Carlsbad, CA) and transformed into *E. coli* DH5α. Plasmids were then isolated and sequenced on an ABI PRISM 3730 DNA Analyzer (ABI, Foster City, CA).

TABLE 1 Primers for quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)	Efficiency
MHC-I	TTGTGGAGACCAGGCCTTCAG	GAGAACCAGGCCAACAATGATG	0.99
MHC-Ia	TTGTGGAGACCAGGCCTTCAG	AATGATGCCCATGGTGAGGAA	0.99
BoLA-NC1	GGATCAAGAGACGCGGATACAA	CCGCAGCCGTGCATCCACT	0.98
BoLA-NC2	GGGTGCGCTGATCCTCACT	CCACCCACCGCGCTGTA	0.95
BoLA-NC3	CCAAGGAAAGTCAACAGGAATC	AATCTCTGCCGTCGTAGGC	0.94
BoLA-NC4	AGCGATGACAAGAGATGCCAAGAA	CCGCACCGTCATAGGCGT	0.96
NFKB1	CTGCTCACCACCCTCCTC	GCACTTTGTTAAGAGTTAGCAAG	0.97
RELA	CCTTTCAATGGACCCACCGA	GAGAGATGGCGTAAAGGAATAG	0.95
IRF1	GATGCCTGTCTGTTTCGGA	TGGTGAGGGGTGGGAGCAT	0.97
CREB1	CAGACCACTGATGGACAGCAA	TGGGGAAGACGCCATAACAACT	0.99
RFX5	TGTATCTCTACCTTCAGCTCC	GGCAGGTGTCGGTATGCT	0.97
RFXAP	CTCAGGAAACGTCAAACTGGA	CACCACTTCTGGACTTCTTAGTAA	0.94
NFYC	TCCAAGTCCAGGGGCAGC	CTGGGCTTGACCTTGTGG	0.97
CIITA	CTGTGTCACCCGTTTCAGG	GAGATTGCCAAGGTCTTCCACA	0.98
STAT1	TCATTTGTGGTGGAAAGACAGC	GTGCCCAGAATGTTGAACTTC	0.97
DNMT1	TGCCTCAGTGCCTCCA	GCGTGGTTCGGAGGATCT	0.95
DNMT3A	ATGACGATGGCTATCAGTCCTA	TCTTCTTTGATGGCTGCCTG	0.95
DNMT3B	AGGACTGGAGTGTGCGTC	GAATCGCAGGGTATAACTTGG	0.96
GAPDH	GAGAAGGCTGGGGCTCACTTG	GCTGACAATCTTGAGGGTGTTG	0.99

BoLA, bovine leukocyte antigen; *CIITA*, class II MHC transactivator; MHC, major histocompatibility complex.

TABLE 2 Primers for major histocompatibility complex *(MHC)-I* CpG island DNA sequencing and 454 methylation sequencing

Category	Primer name	Sequence (5′-3′)
CpG island sequencing	MHC-IF	KATCRGGGCAAAGTCCCAG
	MHC-IR	CGCAGCARCGTGTCCTTYCC
Methylation sequencing round 1	F1F	GACGGCTTGCGGCTACAGGAGGGATTAGGGTAAAGTTTTA
	F1R	GGGCAGCGGACTGTTCTTTCCCTCCAAACCCCRCACTCACC
	F2F	GACGGCTTGCGGCTACANGTTATGRGGTYGYGAATTTTTT
	F2R	GGGCAGCGGACTGTTCTAACTACRTATCRTCCACRTAACC
	F3F	GACGGCTTGCGGCTACATAGGTTTTTATTTTWTGAGKTATTTT
	F3R	GGGCAGCGGACTGTTCTCRCRATAATTAAACYCAAACTA
	F4F	GACGGCTTGCGGCTACAATTAGAGYGAGGTYGGTGAGYG
	F4R	GGGCAGCGGACTGTTCTAAACCCCATTTTYCTCTCYTC
Methylation sequencing round 2	MID-PBMC-F	CGTATCGCCTCCCTCGCGCCATCAGACGAGTGCGTGACGGCTTGCGGCTACA
	MID-PBMC-R	CTATGCGCCTTGCCAGCCCGCTCAGACGAGTGCGTGGGCAGCGGACTGTTCT
	MID-PTC-F	CGTATCGCCTCCCTCGCGCCATCAGAGACGCACTCGACGGCTTGCGGCTACA
	MID-PTC-R	CTATGCGCCTTGCCAGCCCGCTCAGAGACGCACTCGGGCAGCGGACTGTTCT
	MID-SCNT-F	CGTATCGCCTCCCTCGCGCCATCAGATCAGACACGGACGGCTTGCGGCTACA
	MID-SCNT-R	CTATGCGCCTTGCCAGCCCGCTCAGATCAGACACGGGGCAGCGGACTGTTCT

PBMC, peripheral blood mononuclear cells; PTC, placental trophoblast cells; SCNT somatic cell nuclear transfer.

2.6 | Bisulfite-sequencing of MHC-I CpG islands

To determine the methylation status of the *MHC-I* CpG island regions in PBMC and PTC, 1 μg genomic DNA from each sample was bisulfite-treated using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA) according to the manufacturer's recommended protocol. Because methylation-specific PCR is inefficient for reactions generating products longer than 500 bps, the MHC-I genetic region containing the CpG islands was split into four fragments. Specific primers for each genomic DNA fragment were designed using Methprimer[32] and primer sequences are provided in Table 2. Roche 454 sequencing was performed as previously described,[33] with minor modifications. Two PCR steps were performed to produce amplicons for 454 sequencing. First, primer pairs with *MHC-I*-specific sequence and flanking adaptor sequence were used to amplify CpG island fragments from bisulfite-treated genomic DNA; the cycling parameters were 94°C for 2 minutes, followed by 35 cycles of 94°C for 15 seconds, 52-57°C for 30 seconds and 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. Sequencing adaptors and molecular ID tags (454 Life Sciences) were then added in a second round of PCR using primers that hybridized to the adaptors added in the first PCR. After the second PCR, equal amounts of each amplicon were mixed and then sequenced on a 454 GS FLX+DNA sequencer (Roche, Indianapolis, IN). Sequencing reads were analyzed using DNASTAR Lasergene software (Madison, WI).

2.7 | Statistical analysis

Quantitative PCR data were analyzed using the relative quantification method described by Pfaffl.[31] For each sample, the percentage of each *MHC-I* gene, or gene subset in the case of *MHC-Ia*, was based on the relative abundance compared to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), the internal control. The abundance was calculated using the equation: $R_{MHC\text{-}X} = \text{Efficiency}_{MHC\text{-}X}{}^{-CT(MHC\text{-}X)}/\text{Efficiency}_{GAPDH}{}^{-CT(GAPDH)}$. The percentage of *MHC-X* in each sample was obtained using the formula: $MHC\text{-}X\ \% = R_{MHC\text{-}x}/(R_{MHC\text{-}Ia} + R_{NC1} + R_{NC2} + R_{NC3} + R_{NC4})$.

All statistical analyses were performed using R software. One-way ANOVA with a Bonferroni post-hoc test for multiple group comparisons was performed to determine significance. A probability of $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Expression of *MHC-I* is regulated by transcription factors

Expression of all *MHC-I* subtypes was significantly higher in PBMC than in PTC from AI or SCNT pregnancies ($P < .001$; Table 3). Expression of *MHC-I* subtypes was also significantly higher in SCNT PTC than in AI PTC. The transcription factor genes interferon regulatory factor 1 (*IRF1*) and signal transducer and activator of transcription 1 (*STAT1*), and the transcriptional coactivator gene class II MHC transactivator (*CIITA*) showed a similar expression pattern to *MHC-I*. Expression of nuclear factor kappa B subunit 1 (*NFKB1*; previously known as *NFκB p50*), RELA proto-oncogene (*RELA*; previously known as *NFκB p65*) and regulatory factor X5 (*RFX5*) was significantly higher in PBMC compared to PTC, while expression of these transcription factors did not differ in AI and SCNT PTC. No significant difference in the expression of transcription factor genes cyclic-AMP responsive element binding protein 1 (*CREB1*), regulatory factor X-associated protein (*RFXAP*) and nuclear transcription factor Y subunit gamma (*NFYC*) was observed among the groups (Figure 1 and Table 3). Expression of

TABLE 3 Statistical analysis of gene expression

Gene	Mean			One-way ANOVA		
	AI PTC	SCNT PTC	PBMC	AI PTC vs SCNT PTC	AI PTC vs PBMC	SCNT PTC vs PBMC
MHC-Ia	1.00 ± 1.05	9.52 ± 6.76	148.63 ± 14.8	**	***	***
BoLA-NC1	1.00 ± 0.50	18.43 ± 32.91	124.01 ± 58.68	***	***	***
BoLA-NC2	1.00 ± 1.21	4.60 ± 1.93	73.06 ± 8.41	*	***	**
BoLA-NC3	1.00 ± 1.08	36.07 ± 17.44	65.21 ± 13.71	***	***	*
BoLA-NC4	1.00 ± 1.62	9.98 ± 5.79	50.26 ± 20.11	**	***	*
IRF1	1.00 ± 0.48	20.14 ± 13.28	729.61 ± 268.30	***	***	***
STAT1	1.00 ± 0.27	3.49 ± 1.45	10.71 ± 1.89	*	***	***
CIITA	1.00 ± 0.91	12.53 ± 7.61	228.62 ± 90.65	*	***	***
NFKB1	1.00 ± 0.22	2.47 ± 0.66	15.86 ± 5.67	ns	***	***
RELA	1.00 ± 0.43	1.64 ± 0.64	3.97 ± 0.59	ns	***	***
RFX5	1.00 ± 0.17	1.06 ± 0.66	2.12 ± 0.27	ns	*	*
CREB1	1.00 ± 0.32	1.53 ± 0.67	1.53 ± 0.383	ns	ns	ns
RFXAP	1.00 ± 0.42	1.41 ± 0.81	0.25 ± 0.04	ns	ns	ns
NFYC	1.00 ± 0.27	0.95 ± 0.46	0.58 ± 0.08	ns	ns	ns

Mean values were calculated using *GAPDH* as an internal control. The fold change in gene expression for each PTC and peripheral blood mononuclear cells (PBMC) sample was calculated with respect to the mean value for the artificial insemination (AI) PTC samples (AI PTC n = 5; somatic cell nuclear transfer [SCNT] PTC n = 5; PBMC n = 3). Statistical comparisons between groups were performed using one-way ANOVA with the Bonferroni post-hoc test. ns, not significant; BoLA, bovine leukocyte antigen; *CIITA*, class II MHC transactivator; PTC, placental trophoblast cells.

*$P < .05$.

**$P < .01$.

***$P < .001$.

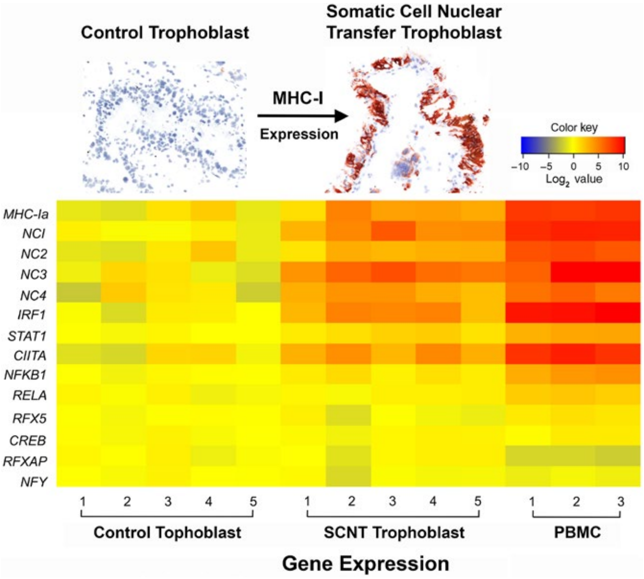

FIGURE 1 qRT-PCR analysis of bovine major histocompatibility complex (*MHC)-I* and transcription factor gene expression in peripheral blood mononuclear cells (PBMC) and placental trophoblast cells (PTC). Data were normalized to *GAPDH*. The heatmap shows the fold change of gene expression relative to the average for the artificial insemination (AI) PTC samples

MHC-I subtypes in PBMC and PTC from AI and SCNT pregnancies was positively correlated with elevated expression of *IRF1*, *CIITA*, *STAT1*, *NFKB1*, and *RELA* (Table 4).

3.2 | Expression of *MHC-Ia* and *-Ib* in bovine PBMC and PTC

Major histocompatibility complex class I gene expression was assessed using qRT-PCR. The majority of *MHC-I* transcripts in PBMC were from *MHC-Ia* loci (94.8%), whereas only 5.2% of *MHC-I* transcripts were from *MHC-Ib* (*NC1-NC4*) loci, confirming a previous report by Davies, et al.[2] Non-classical *MHC-I* contributed a significantly larger proportion of the overall *MHC-I* transcript pool in PTC than PBMC, 33.8% and 25.1% in AI and SCNT PTC groups, respectively (Figure 2 and Table 5).

3.3 | Methylation of CpG islands in *MHC-Ia* and *MHC-Ib* genes

The DNA sequences of six *MHC-I* reference alleles were retrieved from GenBank (NW_001494163). In silico analysis (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) identified a CpG island of approximately 1500 bp spanning from approximately 300 bp upstream of the transcription start site to the third exon in each bovine *MHC-I* gene (Figure 3A). Methylation status of these CpG islands was determined by bisulfite conversion followed by sequencing on a 454 GS FLX+ sequencer. Information about the sequencing reads from four PBMC samples is provided in Table 6, and information about the sequencing reads from two AI PTC and two SCNT PCT samples is presented in Table 7. The methylation status of the CpG islands for two *MHC-Ia* and four *MHC-Ib* alleles per PBMC or PTC sample are

TABLE 4 Pearson correlation coefficients between major histocompatibility complex (MHC)-I expression and transcription factor expression

	Transcription Factors								
Gene	*IRF1*	*STAT1*	*CIITA*	*NFKB1*	*RELA*	*CREB1*	*RFX5*	*RFXAP*	*NFYC*
MHC-Ia	0.96**	0.96**	0.90**	0.94**	0.93**	0.51	0.80**	0.50	0.45
BoLA-NC1	0.95**	0.98**	0.95**	0.96**	0.96**	0.60*	0.84**	0.60*	0.41
BoLA-NC2	0.88**	0.94**	0.91**	0.91**	0.96**	0.55	0.84**	0.55	0.40
BoLA-NC3	0.87**	0.88**	0.85**	0.94**	0.80**	0.66*	0.74**	0.67*	0.29
BoLA-NC4	0.78**	0.90**	0.98**	0.84**	0.88**	0.52	0.77**	0.52	0.43

*Correlation is significant at the 0.05 level (2-tailed).

**Correlation is significant at the 0.01 level (2-tailed). BoLA, bovine leukocyte antigen; *CIITA*, class II MHC transactivator.

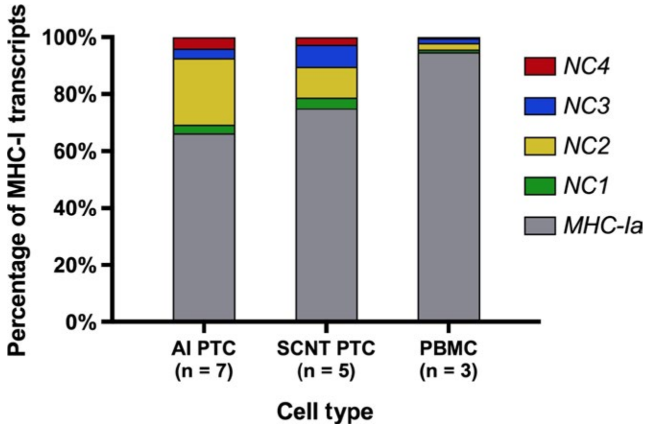

FIGURE 2 Major histocompatibility complex (*MHC)-Ia* and *MHC-Ib* transcript percentages in placental trophoblast cells (PTC) and peripheral blood mononuclear cells (PBMC). Expression of *MHC-Ia* and *MHC-Ib* (*BoLA-NC1*, *-NC2*, *-NC3*, *-NC4*) was detected by qRT-PCR. Average percentages are shown

summarized in Figures 3 and 4, respectively. The results indicated that the *MHC-Ia* genes and the bovine leukocyte antigen non-classical 1 (*BoLA-NC1*) *MHC-Ib* gene were completely unmethylated in all three cell types. Exons two and three and the intervening intron of the *BoLA-NC2*, *-NC3*, and *-NC4 MHC-Ib* genes were methylated to varying degrees in the three types of samples. Methylation in this region was the highest in PBMC, the lowest in AI PTC, and intermediate in SCNT PTC.

3.4 | Expression of DNA methyltransferase (DNMT) isoforms

DNA methylation is catalyzed and maintained by three DNA methyltransferases: DNMT1, DNMT3A, and DNMT3B.[34] Expression of the three DNA methyltransferase genes in PBMC, AI, and SCNT PTC were measured by qRT-PCR (Figure 5). Both *DNMT1* and *DNMT3A* were expressed at a similar level among all groups. However, expression of *DNMT3B* was significantly lower in PBMC than that in either AI or SCNT PTC. Expression of *DNMT3B* in SCNT PTC was lower than in AI PTC, but the difference was not statistically significant.

3.5 | Demethylation of *MHC-Ib* genes in PBMC

To test whether bovine *MHC-I* expression can be affected by demethylation, PBMC and AI PTC were cultured in medium containing 100 μmol/L 5′-aza-2-deoxycytindine for 3 days to reduce overall methylation, and these cells were compared to cells cultured without

TABLE 5 Analysis of MHC-I subtype percentage

	Percentage			One way ANOVA		
Gene	AI PTC	SCNT PTC	PBMC	AI PTC vs SCNT PTC	AI PTC vs PBMC	SCNT PTC vs PBMC
BoLA-NC1	3.02 ± 1.33%	3.87 ± 1.09%	0.96 ± 0.04%	ns	ns	*
BoLA-NC2	23.37 ± 8.72%	10.72 ± 2.89%	2.25 ± 0.86%	*	**	*
BoLA-NC3	3.48 ± 3.03%	7.85 ± 0.96%	1.62 ± 0.79%	ns	*	**
BoLA-NC4	3.95 ± 4.33%	2.62 ± 0.74%	0.38 ± 0.16%	ns	**	**
MHC-Ia	66.14 ± 9.72%	74.93 ± 4.49%	94.78 ± 1.44%	ns	***	**

For each sample, the percentage of each major histocompatibility complex *(MHC)-I* gene, or gene subset in the case of *MHC-Ia*, was calculated from the relative abundance compared to *GAPDH*, the internal control, as described in the methods section.[31] The percentage of each subtype in each cell type is the mean value for all of the animals in the group (AI PTC n = 7; somatic cell nuclear transfer [SCNT] PTC n = 5; PBMC n = 3). Statistical comparisons between groups were performed using one-way ANOVA with the Bonferroni post-hoc test for multiple comparisons. ns, not significant; BoLA, bovine leukocyte antigen; PBMC, peripheral blood mononuclear cells; PTC, placental trophoblast cells.

*$P < .05$.

**$P < .01$.

***$P < .001$.

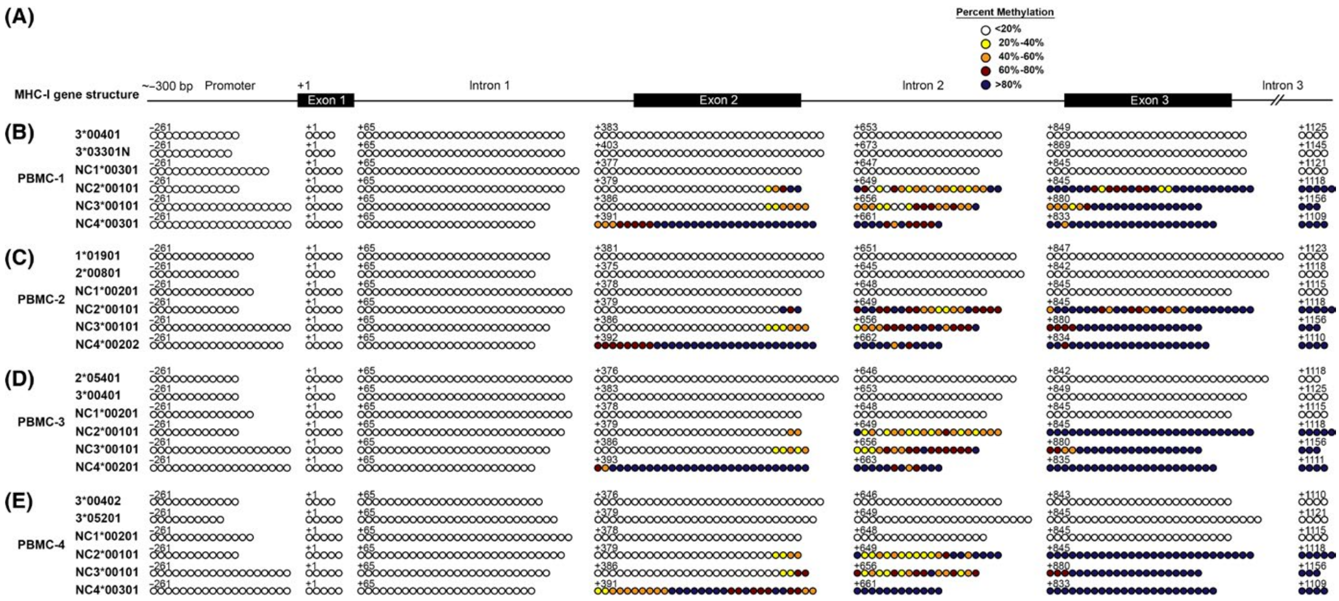

FIGURE 3 Methylation profile of CpG islands in major histocompatibility complex (*MHC*)-*Ia* and *MHC-Ib* genes of peripheral blood mononuclear cells (PBMC). (A) Genomic organization of bovine *MHC-I* CpG islands, from -300 bp upstream of the transcription start site through the third exon, which is about 1500 Kb. (B-E) Methylation status of the CpG sites of each *MHC-I* allele in four PBMC samples. Each horizontal line of circles represents the methylation status of an individual allele. Different colors of circles denote variation in the methylation level. The numbers above each line of circles stand for the position in the genomic sequence relative to the first base of the start codon

5′-aza-2-deoxycytindine. Expression of each *MHC-I* gene was analyzed by qRT-PCR. The results indicated that *MHC-Ib* but not *MHC-Ia* expression was significantly upregulated in PBMC after demethylation treatment. Expression of the *BoLA-NC1*, *-NC2*, *-NC3*, and *-NC4* genes in PBMC was increased 2.6-, 5.8-, 6.2- and 6.8-fold, respectively. However, in PTC *MHC-Ia* and *MHC-Ib* were only upregulated 1.5- to 2.5-fold (Figure 6A). After demethylation, the percentage of *MHC-Ib* in the overall pool of *MHC-I* transcripts in PBMC increased to 41.3%, similar to PTC before (40.2%) and after (33.7%) treatment (Figure 6B).

4 | DISCUSSION

In this study, we analyzed of the expression of mRNA for transcription factors associated with *MHC-I* expression in bovine PTC and PBMC. Our results indicated that in comparison with PTC, PBMC express relatively high levels of mRNA for *MHC-I* transcription factors *IRF1*, *CIITA*, *NFKB1* and *RELA*, and the cell signaling gene *STAT1* (Table 3). In other studies, these transcription factors have been shown to induce *MHC-I* expression in humans and other mammals.[16-20] The ability of these transcription factors to induce MHC-I expression in bovine cells was recently confirmed by transfection of plasmids encoding these transcription factors into bovine fibroblast cells (Shi and Davies, manuscript in preparation). The transcription factors *IRF1* and *CIITA* appear to be particularly important for driving of *MHC-I* expression (Table 3 and Figure 1). The relatively low expression of these transcription factors in PTC is associated with a low level of *MHC-I* expression. In contrast, in PBMC, these transcription factors and the *MHC-I* genes are expressed at a very high level. Our study also suggests that DNA methylation suppresses expression of three *MHC-Ib* genes in PBMC but not in PTC. We found that *MHC-Ia* and *BoLA-NC1* were free of methylation at CpG sites in PBMC and PTC, whereas in PBMC, the *MHC-Ib* genes *BoLA-NC2*, *-NC3*, and *-NC4* were highly methylated in their second intron, and second and third exons (Figures 3 and 4). Accordingly, demethylation resulted in marked upregulation of *MHC-Ib* in bovine PBMC (Figure 6). Our study helps explain the abnormally high *MHC-I* expression in PTC from SCNT conceptuses and the differential expression of *MHC-Ib* in PTC and PBMC.[2,15,28] These findings shed light on how the bovine conceptus regulates trophoblast *MHC-I* expression to protect itself from immunologically mediated rejection and how the immunological cross-talk between the placenta and the uterine immune system differs in normal and SCNT pregnancies.

The core promoters of the bovine *MHC-Ia* and *MHC-Ib* genes have a high level of homology, and include all of the conserved transcription factor binding sites found in humans and other mammals.[35,36] In this study, we tested the expression of several transcription factor genes (*IRF1*, *CIITA*, *NFKB1*, *RELA*, *CREB1*, *RFX5*, *RFXAP*, and *NFYC*) and the cell signaling gene *STAT1* in three different types of cells. The expression patterns for all of the *MHC-I* subtypes were positively correlated with those of *IRF1*, *STAT1*, *CIITA*, *NFKB1*, *RELA*, and *RFX5* with the highest expression in PBMC and the lowest expression in AI PTC (Tables 3 and 4). *IRF1* and *CIITA* are both IFN-γ stimulated genes[37,38] and *STAT1* is involved in the IFN-γ signaling pathway,[38] suggesting that bovine *MHC-I* genes, like their human homolog, may be regulated by IFN-γ.

Although bovine *MHC-Ia* and *MHC-Ib* genes have a high level of homology in their promoter sequences, the relative levels of *MHC-Ia*

TABLE 6 Number of 454 sequencing reads for peripheral blood mononuclear cells (PBMC)

		Number of reads for each genomic region						
Sample	**Allele**	**Promoter**	**Leading peptide**	**Intron 1**	**Exon 2**	**Intron 2**	**Exon 3**	**Intron 3**
PBMC-1	*BoLA-3*00401*	8	14	6	7	7	4	4
	*BoLA-3*03301*	5	10	5	2	2	3	3
	*BoLA-NC1*00301*	2	12	10	4	4	7	7
	*BoLA-NC2*00101*	2	9	7	11	11	17	17
	*BoLA-NC3*00101*	7	10	3	11	11	5	5
	*BoLA-NC4*00301*	6	9	3	22	22	16	16
PBMC-2	*BoLA-2*00801*	3	5	2	4	4	5	5
	*BoLA-1*01901*	4	11	7	4	4	6	6
	*BoLA-NC1*00201*	8	14	6	2	2	4	4
	*BoLA-NC2*00101*	2	5	3	5	5	5	5
	*BoLA-NC3*00101*	6	8	2	17	17	10	10
	*BoLA-NC4*00202*	5	9	4	26	26	23	23
PBMC-3	*BoLA-3*00401*	9	17	8	6	6	9	9
	*BoLA-2*05401*	6	11	5	3	3	10	10
	*BoLA-NC1*00201*	3	8	3	3	3	8	8
	*BoLA-NC2*00101*	3	5	2	3	3	12	12
	*BoLA-NC3*00101*	12	18	6	4	4	2	2
	*BoLA-NC4*00201*	4	7	3	26	26	18	18
PBMC-4	*BoLA-3*00402*	4	10	6	5	5	8	8
	*BoLA-3*05201*	8	17	9	8	8	18	18
	*BoLA-NC1*00201*	14	25	11	3	3	9	9
	*BoLA-NC2*00101*	3	7	4	7	7	21	21
	*BoLA-NC3*00101*	14	24	10	3	3	8	8
	*BoLA-NC4*00301*	3	10	7	21	21	25	25

BoLA, bovine leukocyte antigen.

and *MHC-Ib* in the total pool of *MHC-I* transcripts are distinct in different cell types. In this study, the proportion of *MHC-Ib* in AI and SCNT PTC was 4.5- and 2.8-fold greater than that found in PBMC, respectively. This is reasonable, given the distinct functions of the two *MHC-I* subtypes and the different physiological roles of PBMC and PTC. In both humans and cattle, MHC-Ia proteins present peptide antigens to activate cytotoxic T cells that kill cells displaying antigens derived from intracellular pathogens, while the primary function of MHC-Ib molecules is regulation of the immune system. PBMC are important for immune surveillance and the high amounts of *MHC-Ia* expressed by these cells enable them to efficiently present peptide antigens to cytotoxic T lymphocytes. However, PTC are located at the maternal-fetal interface, which requires an immunologically favorable environment to foster fetal development. Thus, higher expression of *MHC-Ib* in PTC likely facilitates protection of the fetus from immune-mediated miscarriage.

The observation that the percentage of *MHC-Ia* and *MHC-Ib* transcripts differed between PBMC and PTC led us to determine the DNA methylation patterns for these genes. We found that the *BoLA-NC2*, *-NC3*, and *-NC4* genes in PBMC were hypermethylated within the gene body, but that these genes were only slightly or moderately methylated in the same region in PTC. In contrast, the *BoLA-NC1* and *MHC-Ia* genes were unmethylated in all of the samples that were examined. This may be explained by the genomic location of the *BoLA-NC1* gene, which is located near the *MHC-Ia* genes and far away from the other *MHC-Ib* genes.[1]

DNA methylation in promoter regions is a repressive epigenetic mark that down-regulates gene expression.[39,40] However, the *BoLA-NC2*, *-NC3*, and *-NC4* genes were methylated in their gene body but not the promoter region. A review by Jones stated that gene body CpG methylation is not generally associated with transcription repression because transcription elongation is not sensitive to DNA methylation in mammals, but also points out that cytosine methylation does inhibit transcription elongation in *Neurospora crassa*.[41] In contrast, Lorincz et al reported that methylation in the intragenic region represses expression by inhibiting the elongation process of transcription in murine cells.[42] Deaton et al demonstrated that intragenic DNA methylation represses the GATA-binding protein three (*GATA3*) gene in cluster of differentiation four positive (CD4$^+$), IFN-γ$^+$, interleukin 4-positive (IL-4$^+$) T cells, and this repression is potentially responsible

TABLE 7 Number of 454 sequencing reads for placental trophoblast cells (PTC)

		Number of reads for each genomic region						
Sample	**Allele**	**Promoter**	**Leading peptide**	**Intron 1**	**Exon 2**	**Intron 2**	**Exon 3**	**Intron 3**
AI PTC-1	*BoLA-3*00402*	11	18	7	11	11	7	7
	*BoLA-5*07201*	6	8	2	3	3	2	2
	*BoLA-NC1*00501*	2	19	17	9	9	12	12
	*BoLA-NC2*00103*	2	4	2	23	23	13	13
	*BoLA-NC3*00101*	10	12	2	7	7	5	5
	*BoLA-NC4*00301*	3	6	3	23	23	6	6
AI PTC-2	*BoLA-2*05401*	8	12	4	2	2	6	6
	*BoLA-3*00801*	9	17	8	3	3	10	10
	*BoLA-NC1*00201*	2	6	4	2	2	3	3
	*BoLA-NC2*00101*	3	5	2	3	3	4	4
	*BoLA-NC3*00101*	6	9	3	4	4	3	3
	*BoLA-NC4*00301*	3	7	4	8	8	10	10
SCNT PTC-1	*BoLA-2*00601*	7	11	4	2	2	6	6
	*BoLA-6*01402*	5	10	5	2	2	11	11
	*BoLA-NC1*00201*	4	22	18	2	2	13	13
	*BoLA-NC2*00101*	2	5	3	7	7	7	7
	*BoLA-NC3*00101*	15	30	30	6	6	4	4
	*BoLA-NC4*00201*	4	13	9	8	8	10	10
SCNT PTC-2	*BoLA-2*00601*	10	14	4	5	5	8	8
	*BoLA-6*01402*	6	8	2	5	5	5	5
	*BoLA-NC1*00201*	9	14	5	5	5	5	5
	*BoLA-NC2*00101*	2	4	2	5	5	4	4
	*BoLA-NC3*00101*	4	9	5	7	7	8	8
	*BoLA-NC4*00201*	3	8	5	8	8	7	7

AI, artificial insemination; BoLA, bovine leukocyte antigen; SCNT, somatic cell nuclear transfer.

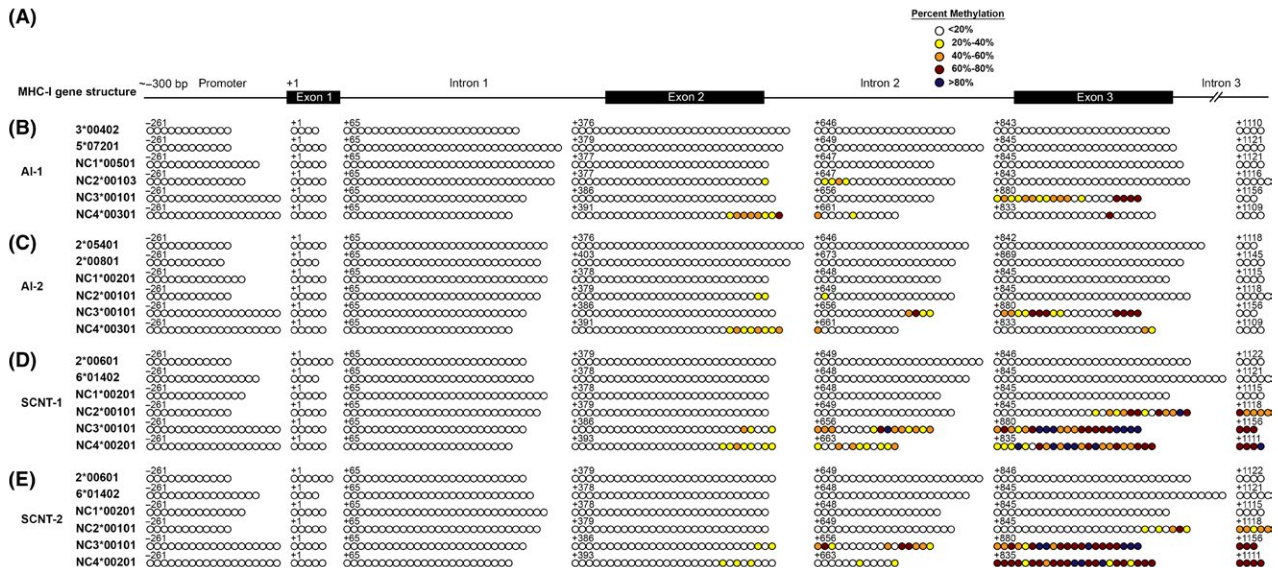

FIGURE 4 Methylation profile of CpG islands in major histocompatibility complex (*MHC)-Ia* and *MHC-Ib* genes of placental trophoblast cells (PTC). (A) Genomic organization of bovine *MHC-I* CpG islands. (B-C) Methylation status of the CpG sites of each *MHC-I* allele in two artificial insemination (AI) PTC samples and (D-E) in two somatic cell nuclear transfer (SCNT) PTC samples

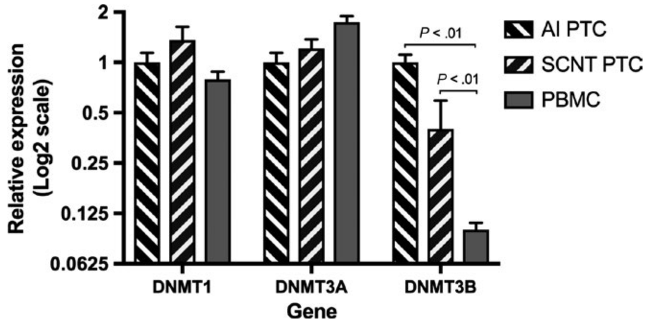

FIGURE 5 qRT-PCR analysis of *DNMT* expression in placental trophoblast cells (PTC) and peripheral blood mononuclear cells (PBMC). Data are presented as the fold change (mean±SEM) of gene expression in other groups relative to that of the artificial insemination (AI) PTC group (AI PTC n = 5; somatic cell nuclear transfer (SCNT) PTC n = 5; PBMC n = 3). Statistical analyses were performed using one-way ANOVA

for the co-existence of Th1 and Th2 characteristics in this special subpopulation of CD4 + T cells.[43] Huang et al showed expression of the insulin-like growth factor two (*IGF2*) gene was negatively regulated by intragenic DNA methylation in cattle.[44] In this study, we observed that the methylated *BoLA-NC2*, *-NC3*, and *-NC4* genes in PBMC contributed less to the overall *MHC-I* transcript abundance than their unmethylated counterparts did in PTC. Furthermore, demethylation treatment of PBMC with 5′-aza-deoxycytidine resulted in an upregulation of the *BoLA-NC2*, *-NC3*, and *-NC4* genes by 5- to 6-fold, but only slightly increased *MHC-Ia* and *BoLA-NC1* expression. The increased expression level of these *MHC-I* genes is inversely correlated with their methylation status, which supports the concept that DNA methylation in the intragenic region of the *BoLA-NC2*, *-NC3*, and *-NC4* genes alters the transcription of these genes. The argument that Jones made in his review that in mammals gene body methylation does not inhibit transcription was based on methylation changes in cancer cells where an increase in intragenic methylation of genes such as insulin-like growth factor two receptor (*Igf2r*), apolipoprotein E (*APOE*), and myogenic differentiation 1 (*Myod1*) was associated with increased expression.[41] However, it is highly possible that there are a large number of genes repressed by intragenic DNA methylation, such as the *GATA3* gene in humans and the *IGF2* gene in cattle, as well as the *BoLA-NC2*, *-NC3* and *-NC4* genes characterized in this study.

The pattern of expression of the *BoLA-NC1* gene was similar to that of the other *MHC-Ib* subtypes, even though this gene was unmethylated in all tissues examined, suggesting that *BoLA-NC1* may be regulated by other mechanisms. The co-activator CIITA acts as a platform for recruiting histone acetyltransferases to enhance *MHC-I* transcription[45] or recruiting histone deacetylases to inhibit the transactivation of CIITA,[46] it is possible that histone deacetylase-associated CIITA binds to the *BoLA-NC1* promoter and interferes with its transcription in PBMC. This form of regulation may also influence expression of other *MHC-I* genes and needs to be investigated in future studies. It is also possible that *BoLA-NC1* is regulated by microRNAs, possibly encoded by the pseudogenes that surround the locus.[1] This is known to be the case for the human *MHC-Ib* gene *HLA-G* which has been reported to be downregulated by microRNAs miR-148a, miR-152 and miR-133a binding to its 3′ untranslated region (UTR).[47,48] Other regulatory mechanisms for *BoLA-NC1* as well as other *MHC-I* subtypes warrant further investigation.

During embryogenesis, DNA is almost completely demethylated at fertilization,[49] and then gradually remethylated as development progresses.[50] DNA methyltransferases are responsible for transferring methyl groups to DNA, with DNMT1 predominantly methylating hemimethylated CpG dinucleotides to maintain methylation status after DNA replication and DNMT3A/3B responsible for de novo methylation. *DNMT3B* was differently expressed among cells, with higher expression in AI and SCNT PTC than in PBMC. This result suggests that the PTC derived from day 34 embryos are undergoing a rigorous remethylation process. However, *MHC-Ib* expression in PTC does not decrease as pregnancy progresses, suggesting that the *MHC-Ib* genes in PTC remain unmethylated. We also observed a difference in *MHC-Ib*

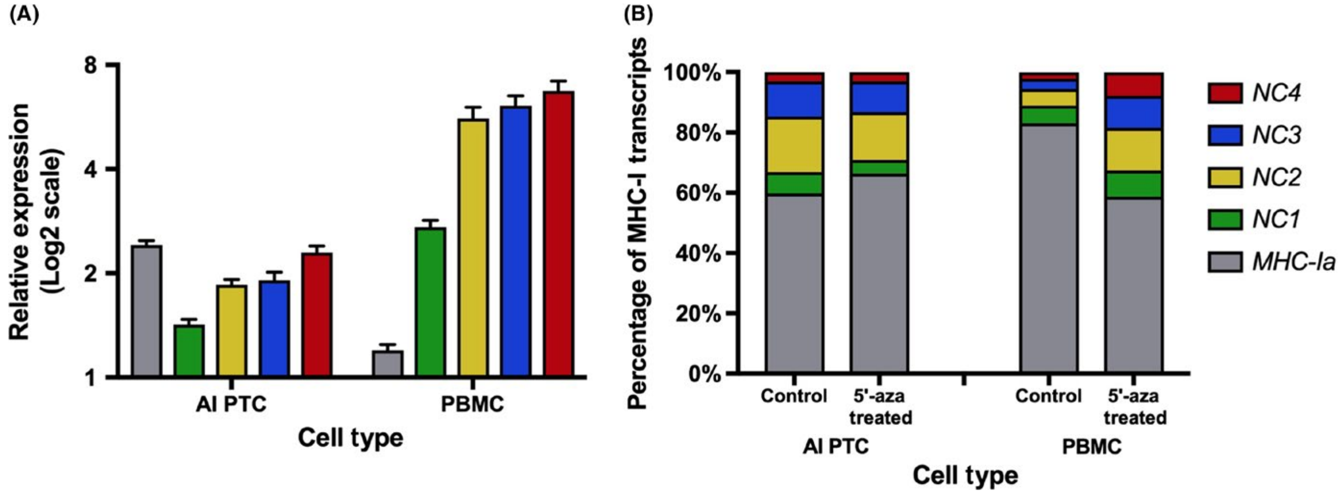

FIGURE 6 Change in major histocompatibility complex (*MHC)-I* transcription following demethylation treatment of peripheral blood mononuclear cells (PBMC) and placental trophoblast cells (PTC). Bovine PBMC and PTC were treated with 5′-aza-deoxycytidine for 3 days (n = 3 per group). (A) Fold change (mean ± SEM) of expression in the treated cells compared to cells cultured without 5′-aza-deoxycytidine. (B) Percentage of *MHC-I* subtypes in the overall pool of *MHC-I* transcripts

methylation between AI PTC and SCNT PTC, with the latter higher than the former. Dean et al reported that DNA methylation in an in vitro fertilized bovine embryo is reduced up to the eight cell stage, and then increased by de novo methylation beginning at the 16 cell stage.[51] However, DNA demethylation occurred only at the one-cell stage in SCNT embryos.[51] As *DNMT* genes are expressed at a similar level in both types of PTC, the higher DNA methylation level of the *BoLA-NC2*, *-NC3*, and *-NC4* genes in SCNT PTC is probably caused by insufficient DNA demethylation during embryogenesis.

In conclusion, our study suggests that bovine *MHC-I* expression is regulated by both genetic and epigenetic factors. Expression of the *MHC-I* genes was highly correlated with the expression of transcription factors *IRF1*, *CIITA*, *NFKB1*, *RELA*, *RFX5* and *STAT1*, with *IRF1* and *CIITA* exhibiting particularly high relative expression levels in both SCNT PTC and PBMC compared to AI PTC from day 35 conceptuses that are essentially negative for expression of MHC-I proteins.[15,28] In addition, our data indicate that expression of bovine *MHC-Ib* genes (*BoLA-NC2*, *-NC3*, and *-NC4*) is regulated by intragenic DNA methylation. Although we cannot rule out other epigenetic regulatory mechanisms for *MHC-I*, DNA methylation appears to be an important mechanism for regulating tissue-specific expression of *MHC-Ib* genes.

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