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Comparative functional genomics of mammalian DNA methyltransferases

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Identifying molecular mechanisms regulating mammalian embryonic development has been an exciting quest for Erdogan Memili. He has a passion for uncovering mysteries behind molecular reprogramming events, including embryonic genome activation, at the onset of development. His current research focuses on identifying molecular determinants of high developmental competency and fertility in mammalian gametes and preimplantation embryos using functional genomics approaches. He has earned his PhD in Endocrinology and Reproductive Physiology from the University of Wisconsin-Madison, and had post-doctoral training in embryonic stem cells and epigenetics at the Harvard Medical School.

Abstract DNA methylation involves biochemical modification of DNA by addition of methyl groups onto CpG dinucleotides, and this epigenetic mechanism regulates gene expression in disease and development. Mammalian DNA methyltransferases, DNMT (DNMT1, DNMT3A and DNMT3B), together with the accessory protein DNMT3L establish specific DNA methylation patterns in the genome during gametogenesis, embryogenesis and somatic tissue development. The present study addresses the structural and functional conservation of the DNMT in humans, mice and cattle and the patterns of mRNA abundance of the different enzymes during embryogenesis to improve understanding of epigenetic regulation in early development. The findings showed a high degree of structural and functional conservation among the human, mouse, and bovine DNMT. The results also showed similar patterns of transcript abundance for all of the proteins at different stages of early embryo development. Remarkably, all of the DNMT with an important role in DNA methylation (DNMT1, DNMT3A, DNMT3B, and DNMT3L) show a greater degree of structural similarity between human and bovine than that between human and mouse. These results have important implications for the selection of an appropriate model for study of DNA methylation during early development in humans.

KEYWORDS: Bovine, embryogenesis, DNA methylation, epigenetics

Introduction

Regulation of gene expression without any actual modification of DNA sequence, or epigenetics, is a topic that has garnered increasing attention in the post-genomic era. Epigenetic regulation causes differential expression of genes depending on the type of tissue and stage of development. DNA methylation is a well-studied epigenetic process with a variety of key roles in gene repression (Bird, 2002; Meehan and Stancheva, 2001), control of cellular differentiation (Ehrlich, 2003a; Li, 2002), gene regulation during embryonic development (Ng and Bird, 1999; Okano et al.,

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1999; Reik et al., 2001), X chromosome inactivation, and genomic imprinting (Chow and Brown, 2003; Murphy and Jirtle, 2003). Other important roles of DNA methylation include silencing of endogenous retroviruses, suppression of homologous recombination, and protection from the mutagenic effects of the abundant transposable elements in mammalian genomes (Bestor, 2000; Yoder et al., 1997).

DNA methylation results from the activity of a family of enzymes called DNA methyltransferases (DNMT) that catalyse the addition of a methyl group to the cytosine residues at CpG dinucleotides (Bird, 1986). Four different DNA methyltransferases have been identified and their structure as well as functions have been extensively reviewed (Bestor, 2000; Hermann et al., 2004; Kumar et al., 1994). These DNA methyltransferases are widely conserved among different species. Mammalian DNMT contain at least three structural regions: the N-terminal regulatory domain, which is responsible for the localization of DNMT in the nucleus, the C-terminal catalytic domain, which is responsible for the methyltransferase activity, and the central linker, consisting of repeated glycyl lysine dipeptides (Araujo et al., 2001). The regulatory N-terminal domain contains a proliferating cell nuclear antigen-binding domain (PBD), a nuclear localization signal (NLS), a cysteine-rich zinc finger DNA-binding motif (ATRX), a polybromo homology domain (PHD), and a PWWP (Pro-Trp-Trp-Pro) tetrapeptide chromatin-binding domain (Bestor, 2000). The C-terminal DNMT catalytic domain contains 10 different characteristic sequence motifs, six of which are evolutionally conserved: I, IV, VI, VIII, IX and X (Turek-Plewa and Jagodzinski, 2005).

The first identified DNA methyltransferase, DNMT1, plays a key role in maintenance of DNA methylation by restoring the methylation pattern on newly synthesized hemi-methylated DNA strands during replication (Bestor et al., 1992; Pradhan et al., 1999). An interesting DNMT1 isoform lacking 118 amino acids from the N-terminal domain (DNMT1o) is exclusively active in oocytes and preimplantation embryos and is later replaced by the regular DNMT1 (Bestor, 2000). DNMT2, the smallest mammalian DNMT, contains only the methyltransferase motifs of the C-terminal domain, and although it is highly conserved, its biological function has been enigmatic (Dong et al., 2001; Yoder and Bestor, 1998). Some studies show that DNMT2 acts as a DNA methyltransferase (Kunert et al., 2003), while other studies have detected little DNA methylation activity. Recent research has demonstrated that DNMT2 methylates $tRNA^{Asp}$ in the cytoplasm (Goll et al., 2006; Rai et al., 2007). DNMT3a and DNMT3b are similar proteins that have been identified as de-novo DNA methyltransferases acting upon hemi-methylated and unmethylated DNA with equal efficiency during early embryonic development (Okano et al., 1998, 1999). The DNA cytosine-like 5-methyltransferase (DNMT3L) protein lacks the most important C-terminal methyltransferase motifs, but possesses an active nuclear localization signal sequence (NLS) and the ATRX zinc finger motif (identical to the ones in DNMT3A and DNMT3B enzymes), which enable nucleus translocation and DNA binding. DNMT3L has a plant homeodomain (PHD)-like motif that activates histone deacetylase 1 (HDAC1) (Deplus et al., 2002; Turek-Plewa and Jagodzinski, 2005) and has recently also been shown to recognize histone H3 tails that are unmethylated at lysine 4 (Ooi et al., 2007) and induce *de novo* DNA methylation by recruitment or activation of DNMT3A2. Thus, DNMT3L has a dual role in de-novo DNA methylation, interacting with unmethylated lysine 4 of histone H3 through its PHD-like domain, while interacting and activating DNMT3A through its carboxy-terminal domain (Jia et al., 2007).

Modulation of DNA methylation during early embryogenesis is a dynamic and developmentally regulated process. Genome-wide DNA demethylation, with the exception of methylation marks at imprinted genes, occurs during the first embryonic stages (Oswald et al., 2000; Reik et al., 2001). The paternal genome is significantly and actively demethylated within hours of fertilization, before the onset of DNA replication, whereas the maternal genome is demethylated after several cleavage divisions (Mayer et al., 2000). This demethylation is followed by de-novo DNA methylation, which establishes a new embryonic methylation pattern. The DNA of blastocysts is thus relatively undermethylated. The exact biological function of this dynamic reprogramming of DNA methylation in early development is unknown. Several studies support the hypothesis that DNA methylation is crucial for the establishment of gene expression during embryonic development (Eden and Cedar, 1994; Jones et al., 1998). However, recent data suggest that DNA methylation may only affect genes that are already silenced by other mechanisms in the embryo, indicating that DNA methylation could be a consequence rather than a cause of gene silencing during development (Bestor, 2000; Bird, 2002; Walsh and Bestor, 1999).

Genomic imprinting is the best example of epigenetic control of gene expression. It is established during gametogenesis and early embryonic development (Edwards, 2003). For a group of genes only the maternal copy is expressed while the paternal copy remains silenced through DNA methylation. For other genes, the paternal copy is the only transcriptionally active one, while the maternal allele is not expressed (Murphy and Jirtle, 2003; Swales and Spears, 2005). In humans, genomic imprinting errors have been associated with several disorders including Prader-Willi, Angelman, Beckwith-Wiedemann, and Russell—Silver syndromes, Wilm's tumour and retinoblastoma (Edwards and Ludwig, 2003; Prescott and Wilkie, 2007). Animal studies have also shown a direct link between genomic imprinting disorders and the use of assisted reproduction. The large offspring syndrome (LOS) is one of the best studied imprinting disorders in cattle linked with IVF and culture of embryos (Young et al., 1998). It consists of unusually large offspring that can also exhibit a number of organ defects caused by imprinting errors in the IGF2R gene (Lazzari et al., 2002; McEvoy et al., 2000).

In the last decade, several reports have shown a higher risk of genomic imprinting disorders for children conceived by means of assisted reproductive technologies, namely IVF and intracytoplasmic sperm injection (ICSI) (Allen and Reardon, 2005; Bertelsmann et al., 2008; Georgiou et al., 2006; Horsthemke and Ludwig, 2005; Maher, 2005; Maher et al., 2003; Niemitz and Feinberg, 2004; Paoloni-Giacobino, 2006). It is important to consider that the increased frequency of imprinting disorders among children conceived by different assisted reproduction techniques cannot solely be attributed to the reproductive technologies since patients requesting assisted reproduction have a lower fertility rate

and tend to be of advanced age, which could contribute to the frequency of the disorders (Edwards and Ludwig, 2003).

The present study focuses on the structural and functional conservation among DNA methyltransferases of human, mouse, and bovine as means of better understanding the role of these enzymes in epigenetic regulation during early mammalian embryonic development.

Materials and methods

The nucleotide and protein reference sequences for mouse, human, and bovine DNMT (DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L) were obtained from National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/ accessed 17/06/09) (see **Table 1**).

Structural analyses of DNMT

Pairwise visual sequence comparisons were done using dot matrix alignment of the protein reference sequences. The comparisons were generated for each DNMT for mouse versus human, mouse versus cow, and human versus cow using the on-line dottup tool from the European Molecular Biology Open Software Suite EMBOSS using a word size of 10 (http://emboss.bioinformatics.nl/cgi-bin/emboss/dottup accessed 17/06/09). A solid diagonal line indicates sequence similarity. A break in the line with a shift indicates an insertion or a deletion in one of the sequences. A gap indicates low similarity.

Pairwise sequence similarity was computed for the mouse, human, and bovine sequences using the GAP program (Huang, 1991) with Blosum62 as the scoring matrix and the following alignment parameters: match 11, mismatch 4, gap-open penalty 10, and gap-extension penalty 2. The program is available from Michigan Tech University (http://genome.cs.mtu.edu/align/align.html accessed 17/06/09) Multiple sequence alignments for mouse, human and bovine DNMT were performed using both ClustalW (Chenna et al., 2003) and T-Coffee (Notredame et al., 2000) available on-line from the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw accessed 17/06/09) using the Gonnet scoring matrix, a gap-open penalty of 10, and gap-extension penalty of 0.5. High level views of multiple sequence alignments and conserved domains were generated using in-house software that is part of the MSAVIS package (Lindeman et al., 2007).

Phylogenetic trees for the DNMT were generated using the sequences listed in **Table 2**, with chicken used as the out-group for drawing the trees for all proteins except for DNMT3L, where opossum was used as the out-group. Multiple sequence alignments were generated using T-Coffee with the parameters listed above. Phylogenetic trees were generated using both neighbour joining and maximum likelihood methods implemented in the Phylip program (Felsenstein, 2005). Phylip programs used to generate the trees were PROTDIST, NEIGHBOR, PROMLK, SEQBOOT, and CONSENSE. The distance matrix for neighbour joining was computed using the JTT model (Jones et al., 1992). The molecular clock assumption was tested using a procedure described by Tuimala (Tuimala, 2006). Because the assumption of a molecular clock was rejected with $P = 0.05$ for DNMT1 and DNMT3b, but not for DNMT2 and DNMT3a, PROML (no molecular clock) was used for all phylogenetic analyses. The ML program PROML was run with the iterative search option (s) and with the global search (g) option for subtree pruning and regrafting to improve the quality of the tree. The consensus trees were inferred from 250 bootstrap replications for the ML and NJ methods. Trees generated by the two methods were similar and ML trees are shown in the results, since this method is generally

Table 1 Mouse, human and cow DNA methyltransferase (DNMT) isoforms included in the study.

Isoform	*Species*	*Chr*	*Gene ID*	*mRNA*	*Protein*	*No. of amino acids*
DNMT1	Mouse	9 5.0 cM	13433	NM_010066.3	NP_034196.3	1619
	Human	19p13.2	1786	NM_001379.1	NP_001370.1	1616
	Cow	7q15	281119	NM_182651.1	NP_872592.1	1611
DNMT2	Mouse	2 A1	13434	NM_010067.2	NP_034197.2	415
	Human	10p15.1	1787	NM_004412.3	NP_004403.1	391
	Cow	13	353353	NM_181812.1	NP_861528.1	391
DNMT3a	Mouse	12 A2-A3	13435	NM_007872.4	NP_031898.1	908
	Human	2p23	1788	NM_022552.3	NP_072046.2	912
	Cow	11	359716	XM_867643.2	XP_872736.1	909
DNMT3b	Mouse	2 A2-A3	13436	NM_001003961.1	NP_001003961.1	859
	Human	20q11.2	1789	NM_006892.3	NP_008823.1	853
	Cow	13	31074162	AY244710	AAP20552.1	826
DNMT3L	Mouse	10 C1	54427	NM_001081695.1	NP_001075164.1	421
	Human	21q22.3	29947	NM_013369.2	NP_037501.2	387
	Cow	1	613785	XM_864897.2	XP_869990.2	417

Chr = chromosome.

Table 2 Percent identity scores for DNA methyltransferases (DNMT) in human, mouse and cow.

	DNMT1	*DNMT2*	*DNMT3a*	*DNMT3b*	*DNMT3L*
Human vs mouse	77	75	95	81	57
Mouse vs cow	75	76	97	75	60
Human vs cow	88	85	96	84	72

Percent identity scores were obtained from pairwise sequence alignments as the number of identities in the alignment divided by the number of residues compared (gap positions are excluded).

considered to be more accurate and the branches in the ML trees had higher bootstrap-values than those in the NJ trees. Trees were drawn using the online Interactive Tree of Life (iTOL) tool (Letunic and Bork, 2007).

Functional analyses of DNMT

Protein sequences were analysed using the conserved domain database (CDD) at the NCBI website [http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi (accessed 17/11/09)]. The program imports multiple sequence alignments from SMART (Simple Modular Architecture Research Tool), Pfam (Pfam-A seed alignments from the Protein families database of alignments and HMM), and COG (Clusters of Orthologous Groups of proteins), which are used to compare the amino acid sequences in the query protein to sequences with known domains (Marchler-Bauer et al., 2005).

Determination of DNMT transcript abundance in bovine oocytes and preimplantation embryos

In-vitro maturation, fertilization and culture of embryos (IVM/IVF/IVC)

Oocytes were collected from 2 to 8 mm follicles of bovine ovaries obtained from a local slaughterhouse. Only oocytes containing several layers of cumulus cells and homogenous cytoplasm were selected. Oocytes were washed three times in Tyrode's lactate (Murphy and Jirtle)-HEPES before transferring into maturation media. The maturation medium used was Tissue Culture Medium (TCM) 199 (Gibco/Invitrogen, USA) supplemented with 0.2 mmol/l pyruvate, 0.5 μg/ml FSH (Sioux Biochemicals, Sioux City, IA, USA), 5 μg/ml LH (Sioux Biochemicals), 10% fetal calf serum (FCS, Gibco/Invitrogen), 100 IU/ml penicillin and 100 mg/ml streptomycin (Gibco/Invitrogen). Ten oocytes in each 50 μl maturation drop were covered with mineral oil and incubated for 24 h at 39°C in a humidified incubator with 5% CO_2 (Sagirkaya et al., 2006). After 24 h, mature oocytes were washed twice with TL-HEPES. Mature oocytes were randomly selected for either RNA isolation or fertilization. Pools of 100 oocytes were frozen at −80°C in RLT lysis buffer (Qiagen Valencia, CA, USA) until RNA isolation.

For fertilization, groups of 10 oocytes washed with TL-HEPES were transferred into 44 μl drops of fertilization medium (glucose-free modified Tyrode's medium supplemented with 0.2 mmol/l pyruvate, 6 mg/ml fatty acid-free bovine serum albumin, 100 IU/ml penicillin and 100 mg/ml streptomycin). Percoll gradient was used for separation of live spermatozoa in frozen−thawed semen (Parrish et al., 1995). Briefly, sperm were thawed at 36°C for 1 min, and then carefully layered on top of the Percoll gradient system. Sperm were diluted in L-HEPES to 5.0×10^7 cells/ml and 2 μl of diluted spermatozoa was added to the 44 μl fertilization drops, which produced a final sperm concentration of 2.0×10^6 cell/ml. Fertilization was completed by adding 2 μl of 5 μg/ml heparin, and 2 μl PHE solution (20 mmol/l penicillamine, 10 mmol/l hypotaurine, 1 mmol/l epinephrine) and co-culture of oocytes and spermatozoa for 18 h in the incubator (Leibfried and Bavister, 1982).

After 18 h, cumulus cells were removed from oocytes by vortexing in a 1.5 ml Eppendorf tube for 3 min. Presumptive zygotes were washed three times by TL-HEPES and transferred into 50 μl culture drops of synthetic oviduct fluid under mineral oil (25 zygotes per drop). At 48 h post-insemination (hpi), cleavage rate (proportion of zygotes that reached the 2-cell stage) was recorded and 2-cell embryos were randomly selected for RNA isolation or further development. Pools of 100 2-cell-stage embryos were frozen at −80°C in RLT lysis buffer until RNA isolation. Embryos selected for further development were kept under the same cultured conditions.

At 96 hpi, the proportion of embryos reaching the 8-cell stage was recorded and 8-cell-stage embryos were randomly selected for RNA isolation or further development. Pools of 100 8-cell embryos were frozen at −80°C on RLT lysis buffer until RNA isolation. Embryos selected for further development were kept under the same cultured conditions. Five μl of FCS was added into each culture drop.

Isolation of RNA

Total RNA was isolated from pools of 100 oocytes, 100 2-cell embryos, 100 8-cell embryos and 10 blastocysts using an RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. Quality of total RNA was estimated using the Bioanalyser 2100 RNA 6000 picochip kit (Agilent, Palo Alto, CA, USA). RNA quantity and purity was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total RNA from all groups was normalized to 4 ng and used for cDNA synthesis using SuperScript III Platinum Two Step quantitative real-time polymerase chain reaction (qRT-PCR) kit according to the manufacturer's protocol. Cycling temperatures and times were 25°C for 10 min, 42°C for 50 min, and 85°C for 5 min.

Real time PCR

Primers were designed using Primer Premier 5 software (Premier Biosoft International, Palo Alto, CA, USA). All primers were designed to span exon−intron boundaries to differentiate genomic DNA amplification (Tables 3 and 4). Complementary DNA was generated using the SuperScript III Platinum

Table 3 DNA methyltransferase (DNMT) sequences used for phylogenetic trees.

Isoform	*Taxonomy*[a]	*Database*	*Accession*	*Status*
DNMT1	*Gallus gallus*	NCBI	NP_996835.1	Refseq
	Monodelphis domestica	NCBI	NP_001028141.1	Refseq
	Bos taurus	NCBI	NP_872592.1	Refseq
	Homo sapiens	NCBI	NP_001370.1	Refseq
	Pan troglodytes	UCSD	chr19.11.012.a	Predicted Build 2, v1
	Macaca mulatta	NCBI	XP_001104704.1	Predicted[1]
	Mus musculus	NCBI	NP_034196.3	Refseq
	Rattus norvegicus	NCBI	NP_445806.1	Refseq
	Canis familiaris	UCSD	chr20.54.038.a	Predicted v 2.0 May 2005
DNMT2	*Gallus gallus*	NCBI	NP_001020002.1	Curated
	Monodelphis domestica	NCBI	XP_001377353.1	Predicted
	Bos taurus	NCBI	NP_861528.1	Refseq
	Homo sapiens	NCBI	NP_004403.1	Refseq
	Pan troglodytes	NCBI	XP_001151907.1	Predicted
	Macaca mulatto	NCBI	hmm23493[b]	Predicted ab initio Build 1.1
	Mus musculus	NCBI	NP_034197.2	Refseq
	Rattus norvegicus	NCBI	NP_001026813.1	Refseq
	Canis familaris	NCBI	XP_848593.1	Predicted
DNMT3a	*Gallus gallus*	NCBI	NP_001020003.1	Refseq
	Monodelphis domestica	NCBI	XP_001380132.1	Predicted
	Bos taurus	NCBI	XP_872736.1	Predicted
	Homo sapiens	NCBI	NP_072046.2	Refseq
	Pan troglodytes	NCBI	XP_001148246.1	Predicted
	Macaca mulatto	NCBI	XP_001083234.1	Predicted
	Mus musculus	NCBI	NP_031898.1	Refseq
	Rattus norvegicus	NCBI	NP_001003958.1	Refseq
	Canis familaris	NCBI	XP_540110.2	Predicted
DNMT3b	*Gallus gallus*	NCBI	NP_001019999.1	Refseq
	Monodelphis domestica	NCBI	XP_001362485.1	Predicted
	Bos taurus	NCBI	AAP20552.1	From mRNA
	Homo sapiens	NCBI	NP_008823.1	Refseq
	Pan troglodytes	NCBI	XP_514580.2	Predicted
	Macaca mulatta	NCBI	XP_001107249.1	Predicted
	Mus musculus	NCBI	NP_001003961.1	Refseq
	Rattus norvegicus	NCBI	NP_001003959.1	Refseq
	Canis familaris	NCBI	hmm47423	Predicted ab initio Build 2.1
DNMT3L	*Gallus gallus*	NCBI	NA	NA
	Monodelphis domestica	NCBI	XP_001377724.1|	Predicted
	Bos taurus	NCBI	XP_869990.2	Predicted
	Homo sapiens	NCBI	NP_037501.2	Refseq
	Pan troglodytes	NCBI	XP_525483.2	Predicted
	Macaca mulatta	NCBI	XP_001118368.1	Predicted
	Mus musculus	NCBI	NP_001075164.1	Refseq
	Rattus norvegicus	NCBI	NP_001003964.1	Refseq
	Canis familaris	NCBI	XP_849972.1	Predicted ab initio Build 2.1

NA = not available; NCBI = National Centre for Biotechnology Information; UCSD = University of California at San Diego.
[a] Eight mammalian species were included in the phylogenetic trees, with chicken (*Gallus gallus*) as the outgroup for analysis. For DNMT3L, opossum was the outgroup due to lack of a chicken sequence.
[b] Gene has frameshifts or/and premature stops.

Two-Step qRT-PCR Kit (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. The samples were incubated for 10 min at 25°C, 50 min at 42°C and at 85°C for 5 min. Then 2 IU of *E. coli* Rnase H was

Table 4 Primer sequences used for gene expression analysis by real time PCR.

Gene	*Primer sequences and positions (5′—3′)*	*Fragment size (bp)*	*Accession Number*
DNMT1_F	AATGGGCAGATGTTCCATGC (2356—2376)	298	NM_182651.1
DNMT1_R	CCTCCGTCGGCTGAGTTTT (2653—2672)		
DNMT3A_F	CTGGCTCTTTGAGAATGTGGTG (2372—2394)	236	XM_867643
DNMT3A_R	TCACTTTGCTGAACTTGGCTATT (2607—2630)		
DNMT3B_F	GGGAAGGAGTTTGGAATAGGAG (698—720)	417	NM_181813
DNMT3B_R	CTCTGGTTGCTTGTTGTTAGGTT (1114—1137)		
GAPDH_F	TGCTGGTGCTGAGTATGTGGT (333—354)	295	XM_865742
GAPDH_R	AGTCTTCTGGGTGGCAGTGAT (627—648)		

added to each tube and incubated at 37°C for 20 min. qRT-PCR was performed to assess transcripts of DNMT1, DNMT3a, and DNMT3b relative to the housekeeping gene GAPDH. Quantitative assessment of RNA amplification was detected by SYBR GreenER qPCR SuperMixes for iCycler (Invitrogen Life Technologies, 11761—100). cDNA 5 μl was used for quantitative real-time PCR reactions according to the iCycler iQ Real-Time PCR instrument (Bio-Rad, USA). The primer concentration was adjusted to 10 μmol/l. The cycling parameters were 50°C for 2 min, 95°C for 8 min 30 s for denaturation, 40 cycles of 15 s at 95°C and 30 s at 60°C and 30 s at 72°C for amplification and extension respectively. The melting curve was performed starting at 55°C with 0.5°C increase for 10 s in 80 cycles. Expression values were calculated using the relative standard curve method. Standard curves were generated using 10-fold serial dilutions for GAPDH and all target genes by measuring the cycle number at which exponential amplification occurred. Results from different groups were analysed by one-way analysis of variance (ANOVA) by SAS 9.1 (SAS Institute Inc., Carey, NC, USA). Relative expression software tool (REST) was used to compare all samples of each group. The mathematical model used in REST software is based on the PCR efficiencies and the crossing point deviation between samples (Pfaffl et al., 2002).

Complementary mRNA abundance data for bovine DNMT in oocytes and 8-cell embryos was obtained from a bovine microarray experiment conducted earlier (Misirlioglu et al., 2006). Expression data for DNMT from mouse was collected from two separate studies (Ratnam et al., 2002; Vassena et al., 2005). Human DNMT expression data was obtained from Huntriss et al. (2004) and the patterns of expression were compared with those of bovine DNMT in order to establish the dynamics of expression of the different enzymes in oocytes and embryogenesis.

Results

Structural analyses of DNMT

Human DNMT2 (TRDMT1) has three isoforms. In this study, human isoform a and the corresponding isoform for the other two species was used for alignments. DNMT3A has two isoforms in mouse, three in humans and three in bovines. Isoforms corresponding to human isoform a were used, for the other two species (mouse isoform 1, and bovine isoform 2). DNMT3B has four isoforms in humans and mouse. Golding and Westhusin (2003) also report four isoforms for bovine DNMT3B (Golding and Westhusin, 2003) and designate Locus AY244710 as corresponding to human isoform 1. For the present study, isoform 1 was used for all species. Complete information regarding the chromosome, gene, accession numbers, protein lengths, and isoforms used is summarized in **Table 1**.

All DNMT showed a high degree of structural conservation at the protein level. The protein pairwise sequence alignments for all DNMT from the three species (mouse versus human, mouse versus cow and cow versus human) produced similarity scores; however, human and bovine proteins produced higher similarity scores. These results are summarized in **Table 2**. Dottup graphs, which are visual representations of the pairwise alignments, are summarized in **Figure 1** showing a solid diagonal line for similar sequences between both species and gaps for low similarities. As in the pairwise alignments, the human and cow comparison produced the higher similarities. The relatively low similarity of human and mouse DNMT3L when compared with human and bovine is particularly striking.

Figure 2 shows the phylogenetic trees generated for DNMT from eight mammals, opossum, and chicken. Chicken was used as the outgroup for drawing all trees except DNMT3L where opossum was used as the outgroup. Rat and mouse are shown to be more distantly related to human than cow for all enzymes with the exception of DNMT2. The extraordinary degree of conservation of DNMT3A results in a very shallow tree indicating that this enzyme is essential for survival and/or development. Recent results have shown that the C-terminal domains of both DNMT3A and DNMT3L interact forming a dimer. The complexed C-terminal domains of Dnmt3a and Dnmt3L further dimerize through a DNMT3A—DNMT3A interaction, forming a tetrameric complex with two active sites. Both interfaces (DNMT3A—DNMT3L and DNMT3A—DNMT3A) are essential for the de-novo methylation activity of DNMT3A (Jia et al., 2007). Both sequence alignment and phylogenetic results indicate that bovine and human DNMT3L show much greater similarity than mouse and human DNMT3L.

Functional analyses of DNMT

All the known mammalian DNMTases have a common structure consisting of a catalytic C-terminal cytosine-C5 specific DNA methylase domain. This domain is found in both prokaryotes and eukaryotes and six of the 10 conserved motifs from prokaryotes are also conserved in vertebrates. In addition, with the exception of DNMT2, all enzymes contain a

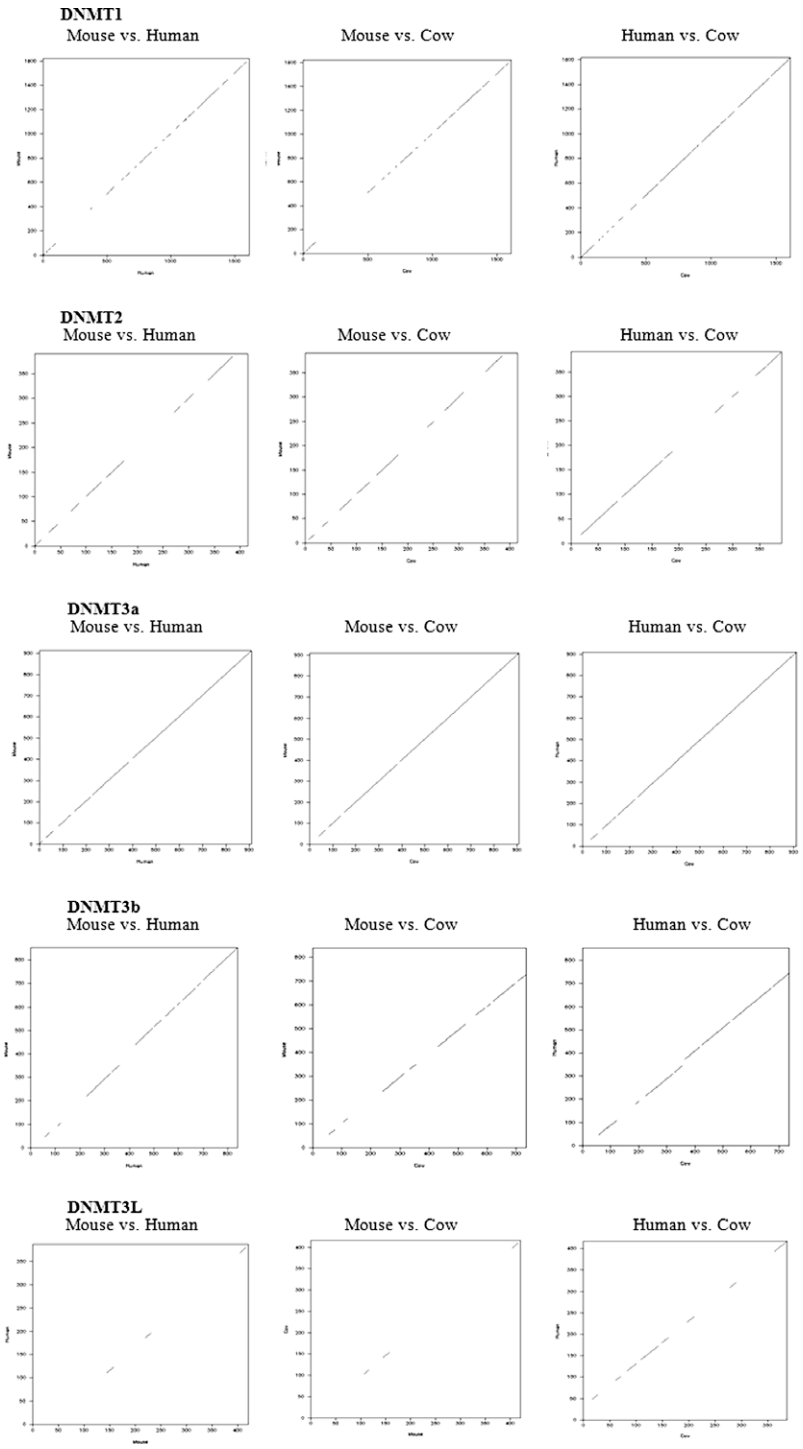

Figure 1 Dottup analysis of sequence similarities of the DNA methyltransferases DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L among mouse, human and bovine (window size: 10). A solid diagonal line indicates sequence similarity, breaks in the line indicate low similarity and a shift in the line indicates an insertion or a deletion. On each of the figures the species named first is the one on the *y* axis, and the second species is the one on the *x* axis.

large N-terminal domain that has been identified as having regulatory functions. **Figure 3** provides a high level view of the multiple sequence alignment of the DNMT enzymes with conserved domains from eight mammalian species (seven placental mammals and one marsupial), for which genome sequencing is complete.

Determination of DNMT transcript abundance in bovine oocytes and preimplantation embryos

The bioanalyser assessment showed RNA degradation for 2-cell and 8-cell embryos consistent with the physiological maternal mRNA degradation occurring at these stages. At

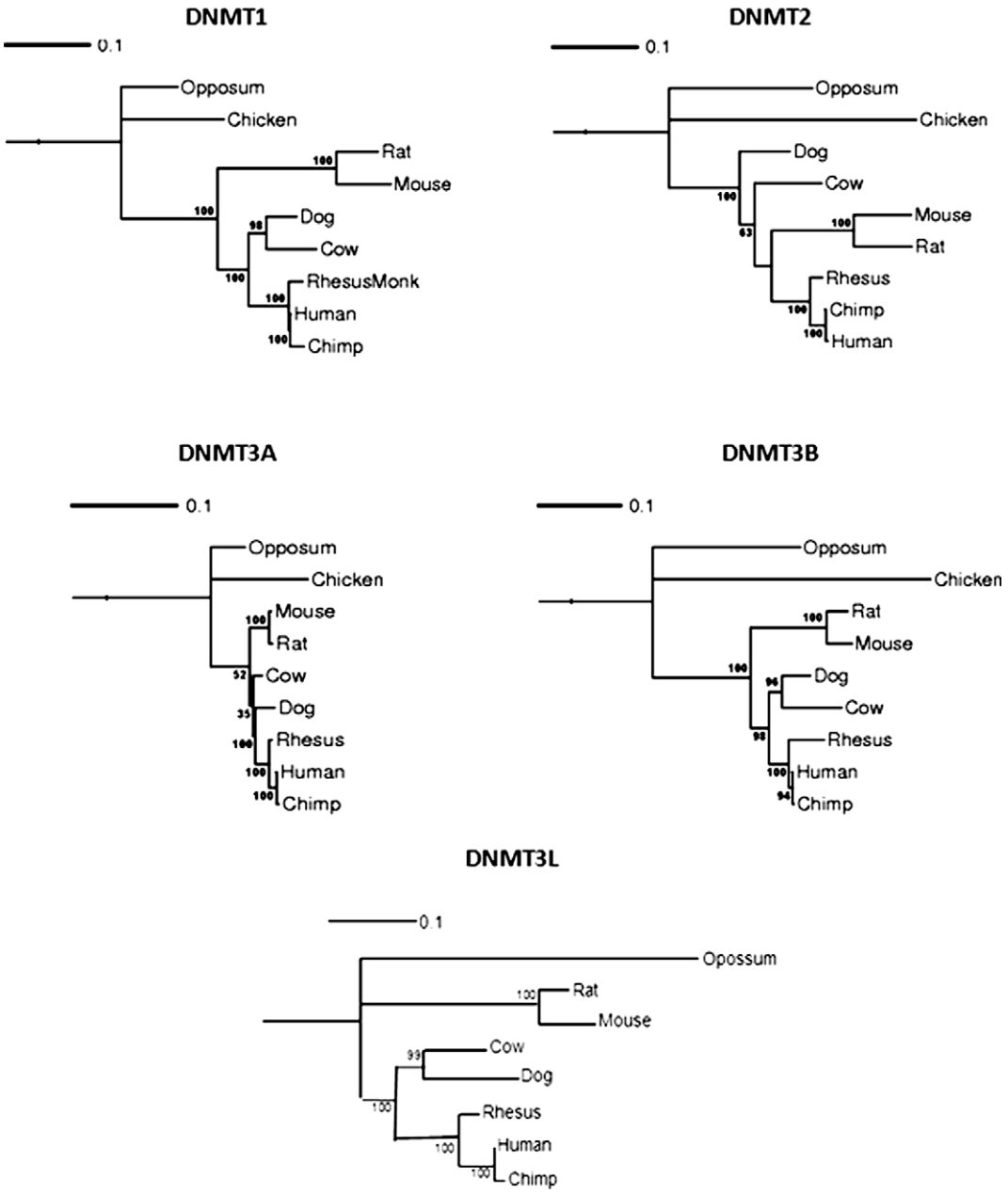

Figure 2 Phylogenetic trees of DNA methyltransferases (DNMT). Multiple sequence alignment was done using T-Coffee and the trees were generated using the Phylip maximum likelihood program with no molecular clock. Chicken was the outgroup for generating the trees. Bootstrap-values out of 250 replicates are shown for branches involving mammals.

the blastocyst stage, total RNA integrity was high as the 28s:18s ribosomal RNA band ratio was >1.9.

No differences in the levels of DNMT1 mRNA were found among metaphase II (MII), 2-cell, and 8-cell groups (**Figure 4**). However, the mRNA level of DNMT1 in the blastocyst group was significantly lower compared with the other groups ($P < 0.05$). The transcript levels of DNMT3a were similar between the MII and 8-cell groups, but they were significantly higher in the 2-cell and blastocyst groups compared with the oocytes ($P < 0.05$). The transcript levels of DNMT3b were similar among all the four groups.

Discussion

The predominant isoform of DNMT1 in somatic cells has 1619, 1616, and 1611 amino acids in mouse, human, and bovine species respectively. A shorter isoform of DNMT1, called DNMT1o, is found specifically in growing oocytes and during early preimplantation development (Ratnam et al., 2002). DNMT1o lacks the N-terminal 114 amino acid residues, since its translation initiation lies on exon 4 instead of exon 1. DNMT1o displays an increased in-vivo stability against degradation and stable ooplasmic stores of DNMT1o are available in the oocytes and early embryos.

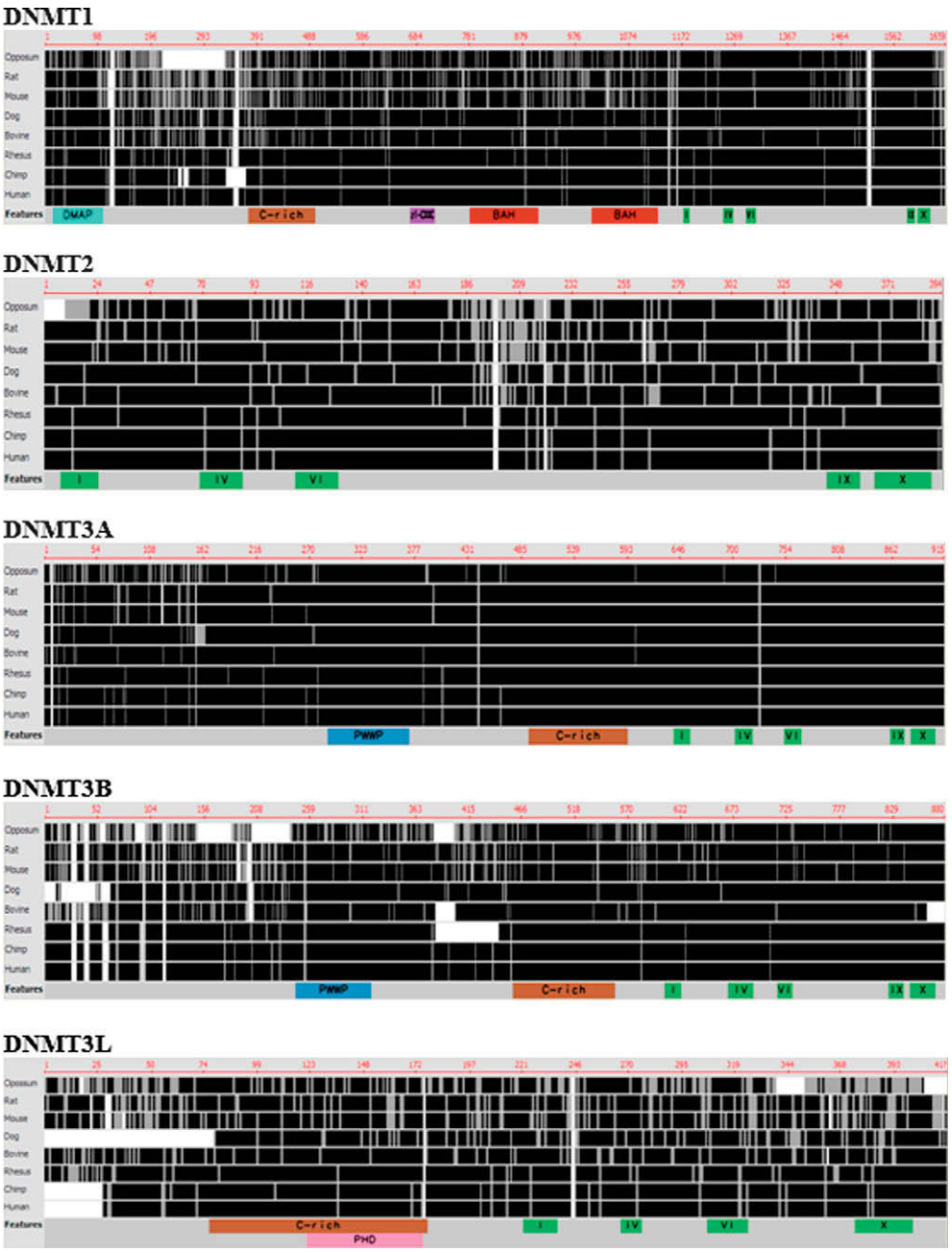

Figure 3 Multiple sequence alignment and functional domains of DNA methyltransferases (DNMT) from opossum, rat, mouse, dog, bovine, rhesus macaque, chimpanzee, and human. The visualization was produced using MSAVIS (Lindeman et al., 2007). Conserved domains (different colour tags at the bottom) were identified using the coding sequence tool at National Centre for Biotechnology Information. Positions in black represent identical amino acids for that column in all the species; positions in grey represent amino acids that differ. White indicates a gap.

Pairwise comparisons obtained for all of the DNMT proteins for mouse, bovine, and human species showed a higher sequence similarity between human and bovine than between mouse and the other two species. The almost complete conservation of DNMT3A among the three organisms is particularly noteworthy. Multiple sequence alignments produced using both T-Coffee and ClustalW gave similar alignments. However, the T-Coffee alignments had fewer gaps and maintained the structure of conserved domains.

T-Coffee alignments were used as input for Phylip to build the phylogenetic trees. Golding and Westhusin reported a high level of sequence conservation for DNMT2 among species (Golding and Westhusin, 2003). In the present study, conservation of DNMT2 sequences among the studied species was comparable to that of DNMT1. However, the phylogenetic tree of DNMT2 showed a different branching compared with that observed for the other DNA methyltransferases, with mouse and rat proteins being closer to

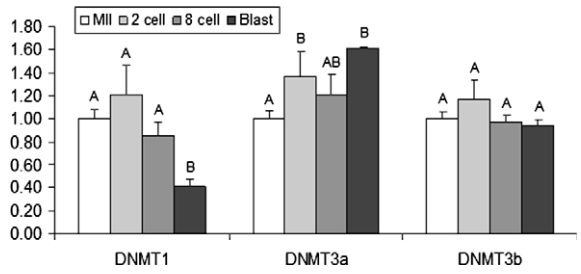

Figure 4 Real time polymerase chain reaction for analysis of the DNA methyltransferases DNMT1, DNMT3a, and DNMT3b mRNA abundance in bovine oocytes and embryos. Bars represent relative expression values of 2-cell embryos, 8-cell embryos, and blastocysts (Blast) to the expression in metaphase II (MII) oocytes. Different letters represent statistically significant differences ($P < 0.05$). Bars labelled (**A** and **B**) correspond to values not significantly different from both (**A** and **B**).

the proteins of primates. The function of DNMT2 in DNA methylation has not been resolved.

It is sometimes difficult to evaluate structural conservation across a wide range of mammals including the newly sequenced genomes because of the large number of predicted protein isoforms. This is particularly true for DNMT1, for which there are 11 predicted isoforms in the chimpanzee, all of which having strong sequence similarities to human DNMT1, but showing deletions or insertions with respect to human isoform a. Thus for all sequences, the isoform that most closely aligns with the human isoform listed in **Table 1** was used in the analyses. For some predicted proteins, manual annotation has been used to correct apparent missed exons and missed translation start sites. For example, in the predicted DNMT1 protein for rhesus monkey, there is a large deletion with respect to the chimp and human proteins. The first part of this deletion aligns with the rhesus genome within the predicted gene on chromosome 19. A second part of the deletion matches an unassembled contig. Likewise, some of the predicted proteins, such as DNMT1 in dog, use a different translation start site than the reference sequences in other species, although the more typical translation start site is present. In these cases, the protein has been re-annotated to use the canonical translation start site.

As expected, all of the DNMT have high levels of conservation of the conserved motifs in the C-terminal domain. DNMT1 has an N-terminal domain that binds a DNA methyltransferase 1 associated protein 1 (DMAP-1) transcriptional co-repressor (Rountree et al., 2000), a CXXC zinc finger domain containing eight conserved cysteine residues that bind to zinc, and two bromo adjacent homology (BAH) domains. The BAH domain is thought to mediate protein–protein interaction and to play a role in transcriptional silencing and remodelling of chromatin (Callebaut et al., 1999). Both DNMT3A and DNMT3B contain a PWWP domain that is essential for DNMT binding to chromatin (Turek-Plewa and Jagodzinski, 2005) and that is almost completely conserved in all species shown.

According to Carlson et al. (1992), DNMT1 is expressed approximately 50,000 fold higher in oocytes as compared with somatic cells. The splice variant DNMT1o lacks the first 114 N-terminal amino acids and is expressed in mouse oocytes. Another study shows that mouse DNMT1o is expressed in oocytes and zygotes and is later replaced by the complete variant (Ratnam et al., 2002). The Affymetrix Bovine GeneChips do not include particular probe sets for DNMT1o; therefore, it was not possible to differentiate this variant in a previous microarray experiment, which showed a six-fold decrease in DNMT1 transcript abundance in 8-cell embryos compared with that in MII oocytes (Misirlioglu et al., 2006). However, the sole analysis of mRNA levels for DNMT1 in pre-implantation embryos may underestimate protein concentrations, which are known to be very high in both mature oocytes and embryos (Bestor, 2000). In the present study, no significant difference was found in the levels of DNMT1 transcripts in the MII oocytes, 2-, and 8-cell embryos. However, the levels of DNMT1 transcripts were more than 2.5 times lower at the blastocyst stage as compared with the other developmental stages tested.

Examination of Dnmt2 mRNA in mouse determined a very low transcript abundance during earlier embryonic development increasing significantly between the 8-cell and morulae/blastocyst stages (Vassena et al., 2005). This pattern of expression contrasted with that of rhesus monkey and human. In rhesus monkey, DNMT2 mRNA was detected in similar levels from the germinal vesicle (GV) oocyte stage to the morulae stage with a slight decrease at the blastocyst stage (Vassena et al., 2005). In human, DNMT2 mRNA was variably detected in MII oocytes and blastocysts (Huntriss et al., 2004). Golding and Westhusin reported the presence of DNMT2 mRNA in all bovine tissues, being particularly abundant in adult testis and ovaries. They also detected bovine DNMT2 mRNA at all embryonic stages from the 2-cell to the blastocyst stage (Golding and Westhusin, 2003). In a microarray study, DNMT2 transcripts were 10 times higher in bovine oocytes compared with 8-cell embryos (Misirlioglu et al., 2006). In another study, similar levels of DNMT2 transcripts were found in bovine fibroblasts and blastocysts (Memili et al., unpublished data). These different patterns of DNMT2 mRNA abundance during early embryonic stages could suggest species-specific differences in this enzyme function, which has been heavily debated in recent years. The available data suggest that DNMT2 has weak methyltransferase activity on unmodified DNA and RNA. By contrast, the enzyme activity on a $tRNA^{Asp}$ template seems comparatively strong (Goll et al., 2006), which might indicate DNMT2 participation in complex nucleic acid modification pathways.

The previously reported pattern of Dnmt3b expression in mouse oocytes and embryos was reciprocal to that of Dnmt3a with low abundance in oocytes and early embryos, and then a sharp increase in abundance at the blastocyst stage (Vassena et al., 2005). In human, DNMT3B was detected continuously from the MII stage oocyte to the blastocyst stage (Huntriss et al., 2004). In bovines, a similar pattern of expression was reported for DNMT3a and DNMT3b with an average three-fold increase in mRNA abundance in the 8-cell embryos compared with the oocytes (Misirlioglu et al., 2006). In contrast, the present study did not find significant differences in DNMT3b abundance in the developmental stages studied and only significantly lower DNMT3a transcripts in MII oocytes compared with 2-cell embryos and blastocysts. These differences might be due to variation

in the stage of cell cycle at which the 8-cell embryos were collected in both studies. In addition, because the embryos were pooled, it is possible that there were differences in the numbers of viable embryos in the pools for both studies. Relative mRNA expression values are summarized in **Figure 4**.

Although DNMT3a and DNMT3b have high structural and functional similarities, it has been proposed that they have distinct genomic targets and functions (Okano et al., 1999). In humans, mutations in the DNMT3b gene cause the immunodeficiency, centromeric instability and facial anomalies syndrome known as ICF. Patients with ICF have hypomethylated DNA and abnormalities localized mostly to the juxtacentromeric heterochromatin of chromosomes 1 and 16 (Ehrlich, 2003b). From this syndrome, it is clear that de-novo DNA methylation through DNMT3b has an important role in late immune function and facial phenotype. The role of Dnmt3a and DNMT3b is paramount during embryonic development, yet again at this level functional differences between both enzymes are evident, since Dnmt3a deficient mice develop to term and appear normal at birth, while Dnmt3b deficient mice die in utero (Ueda et al., 2006).

One of the functional differences between DNMT3a and DNMT3b could be their interaction with the enzyme-like protein Dnmt3L, which lacks the catalytic domain common to the DNA methyltransferases. It has been reported that DNMT3L stimulates de-novo methylation by interacting with DNMT3a to (Chedin et al., 2002). Therefore, DNMT3L acts as a stimulatory factor for DNA methylation by Dnmt3a. This is particularly true for de-novo methylation of imprinted genes in mammalian germ cells (Jia et al., 2007). Homozygous Dnmt3L mutant male and female mice are viable, but infertile (Bourc'his et al., 2001; Hata et al., 2002, 2006). Furthermore, DNMT3L deficient oocytes showed aberrant methylation of the imprinted genes small nuclear ribonucleoprotein polypeptide N (Snrpn), paternally expressed 3 (Peg3) and insulin-like growth factor 2 receptor (Igf2r) (Lucifero et al., 2007). The level of interactions between the different DNMT has not been fully established yet, since depletion of either DNMT3L or DNMT1o in growing oocytes results in an increase in DNMT3B, suggesting a potential compensation mechanism by this enzyme, since an interaction between DNMT3L and DNMT3A is crucial for de-novo methylation.

The high degree of structural and functional conservation among the different DNMT, not only within mammals but in all eukaryotes, highlights the importance of DNA methylation patterns in the regulation of gene expression, particularly at the onset of development during gametogenesis and embryogenesis. Understanding the complex interactions between these enzymes and their roles could shed light on the role of epigenetics in human reproduction and disease.

Because of the importance of DNMT, it is essential to identify appropriate models for study of DNA methylation in humans. This study confirms a high degree of conservation in the protein sequences and functional domains among the studied species. Although mouse is routinely used as a model for mammalian embryonic development, the present results showed that bovine and human DNMT all have much higher similarities than the mouse and human DNMT. This difference is especially striking for DNMT3L that has recently been shown to have dual roles in de-novo methylation of DNA. These results have important implications for selection of an appropriate model for study of DNA methylation during embryogenesis in humans. The greater similarities of the DNMT in human and bovine when compared with human and mouse could argue for the bovine model. Additionally, the fact that thousands of cattle embryos are produced each year around the world by IVF using gametes from animals without impaired reproductive status, could contribute to the understanding on the real role of assisted reproduction treatments on epigenetic disorders.

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References

Allen, C., Reardon, W., 2005. Assisted reproduction technology and defects of genomic imprinting. Br. J. Obstet. Gynaecol. 112, 1589—1594.

Araujo, F.D., Croteau, S., Slack, A.D., et al., 2001. The DNMT1 target recognition domain resides in the N terminus. J. Biol. Chem. 276, 6930—6936.

Bertelsmann, H., de Carvalho Gomes, H., Mund, M., et al., 2008. The risk of malformation following assisted reproduction. Dtsch. Arztebl. Int. 105, 11—17.

Bestor, T.H., 2000. The DNA methyltransferases of mammals. Hum. Mol. Genet. 9, 2395—2402.

Bestor, T.H., Gundersen, G., Kolsto, A.B., et al., 1992. CpG islands in mammalian gene promoters are inherently resistant to de novo methylation. Genet. Anal. Tech. Appl. 9, 48—53.

Bird, A., 2002. DNA methylation patterns and epigenetic memory. Genes Dev. 16, 6—21.

Bird, A.P., 1986. CpG-rich islands and the function of DNA methylation. Nature 321, 209—213.

Bourc'his, D., Xu, G.L., Lin, C.S., et al., 2001. Dnmt3L and the establishment of maternal genomic imprints. Science 294, 2536—2539.

Callebaut, I., Courvalin, J.C., Mornon, J.P., 1999. The BAH (bromo-adjacent homology) domain: a link between DNA methylation, replication and transcriptional regulation. FEBS Lett. 446, 189—193.

Carlson, L.L., Page, A.W., Bestor, T.H., 1992. Properties and localization of DNA methyltransferase in preimplantation mouse embryos: implications for genomic imprinting. Genes Dev. 6, 2536—2541.

Chedin, F., Lieber, M.R., Hsieh, C.L., 2002. The DNA methyltransferase-like protein DNMT3L stimulates de novo methylation by Dnmt3a. Proc. Natl. Acad. Sci. USA 99, 16916—16921.

Chenna, R., Koike, Sugawara.H., Lopez, T., et al., 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. 31, 3497—3500.

Chow, J.C., Brown, C.J., 2003. Forming facultative heterochromatin: silencing of an X chromosome in mammalian females. Cell. Mol. Life Sci. CMLS 60, 2586—2603.

Deplus, R., Brenner, C., Burgers, W.A., et al., 2002. Dnmt3L is a transcriptional repressor that recruits histone deacetylase. Nucleic Acids Res. 30, 3831—3838.

Dong, A., Yoder, J.A., Zhang, X., et al., 2001. Structure of human DNMT2, an enigmatic DNA methyltransferase homolog that

displays denaturant-resistant binding to DNA. Nucleic Acids Res. 29, 439–448.
Eden, S., Cedar, H., 1994. Role of DNA methylation in the regulation of transcription. Curr. Opin. Genet. Dev. 4, 255–259.
Edwards, R.G., 2003. Aspects of the molecular regulation of early mammalian development. Reprod. Biomed. Online 6, 97–113.
Edwards, R.G., Ludwig, M., 2003. Are major defects in children conceived in vitro due to innate problems in patients or to induced genetic damage? Reprod. Biomed. Online 7, 131–138.
Ehrlich, M., 2003a. Expression of various genes is controlled by DNA methylation during mammalian development. J. Cell. Biochem. 88, 899–910.
Ehrlich, M., 2003b. The ICF syndrome, a DNA methyltransferase 3B deficiency and immunodeficiency disease. Clin. Immunol. 109, 17–28.
Felsenstein, J., 2005. PHYLIP (Phylogeny Inference Package) version 3.6, Distributed by the author, Department of Genome Sciences, University of Washington, Seattle, Washington.
Georgiou, I., Syrrou, M., Pardalidis, N., et al., 2006. Genetic and epigenetic risks of intracytoplasmic sperm injection method. Asian J. Androl. 8, 643–673.
Golding, M.C., Westhusin, M.E., 2003. Analysis of DNA (cytosine 5) methyltransferase mRNA sequence and expression in bovine preimplantation embryos, fetal and adult tissues. Gene Expr. Patterns 3, 551–558.
Goll, M.G., Kirpekar, F., Maggert, K.A., et al., 2006. Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. Science 311, 395–398.
Hata, K., Kusumi, M., Yokomine, T., et al., 2006. Meiotic and epigenetic aberrations in Dnmt3L-deficient male germ cells. Mol. Reprod. Dev. 73, 116–122.
Hata, K., Okano, M., Lei, H., et al., 2002. Dnmt3L co-operates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. Development 129, 1983–1993.
Hermann, A., Gowher, H., Jeltsch, A., 2004. Biochemistry and biology of mammalian DNA methyltransferases. Cell. Mol. Life Sci. 61, 2571–2587.
Horsthemke, B., Ludwig, M., 2005. Assisted reproduction: the epigenetic perspective. Hum. Reprod. Update 11, 473–482.
Huang, X., 1991. A time-efficient, linear-space local similarity algorithm. Adv. Appl. Math. 12, 337–357.
Huntriss, J., Hinkins, M., Oliver, B., et al., 2004. Expression of mRNAs for DNA methyltransferases and methyl-CpG-binding proteins in the human female germ line, preimplantation embryos, and embryonic stem cells. Mol. Reprod. Dev. 67, 323–336.
Jia, D., Jurkowska, R.Z., Zhang, X., et al., 2007. Structure of Dnmt3a bound to Dnmt3L suggests a model for de novo DNA methylation. Nature 449, 248–251.
Jones, D.T., Taylor, W.R., Thornton, J.M., 1992. The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. 8, 275–282.
Jones, P.L., Veenstra, G.J., Wade, P.A., et al., 1998. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat. Genet. 19, 187–191.
Kumar, S., Cheng, X., Klimasauskas, S., et al., 1994. The DNA (cytosine-5) methyltransferases. Nucleic Acids Res. 22, 1–10.
Kunert, N., Marhold, J., Stanke, J., et al., 2003. A Dnmt2-like protein mediates DNA methylation in *Drosophila*. Development 130, 5083–5090.
Lazzari, G., Wrenzycki, C., Herrmann, D., et al., 2002. Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome. Biol. Reprod. 67, 767–775.
Leibfried, M.L., Bavister, B.D., 1982. Effects of epinephrine and hypotaurine on in-vitro fertilization in the golden hamster. J. Reprod. Fertil. 66, 87–93.
Letunic, I., Bork, P., 2007. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. Bioinformatics 23, 127–128.
Li, E., 2002. Chromatin modification and epigenetic reprogramming in mammalian development. Nat. Rev. Genet. 3, 662–673.
Lindeman, A., Bridges, B., Jankun-Kelly, T., 2007. Effective Display of Conserved Domains on a Multiple Sequence Alignment. IEEE Information Visualization. Available from: <http://vgtc.org/wpmu/infovis07/>.
Lucifero, D., La Salle, S., Bourc'his, D., et al., 2007. Coordinate regulation of DNA methyltransferase expression during oogenesis. BMC Dev. Biol. 7, 36.
Maher, E.R., 2005. Imprinting and assisted reproductive technology. Hum. Mol. Genet. 14, R133–R138.
Maher, E.R., Afnan, M., Barratt, C.L., 2003. Epigenetic risks related to assisted reproductive technologies: epigenetics, imprinting, ART and icebergs? Hum. Reprod. 18, 2508–2511.
Marchler-Bauer, A., Anderson, J.B., Cherukuri, P.F., et al., 2005. CDD: a Conserved Domain Database for protein classification. Nucleic Acids Res. 33, D192–D196.
Mayer, W., Niveleau, A., Walter, J., et al., 2000. Demethylation of the zygotic paternal genome. Nature 403, 501–502.
McEvoy, T.G., Sinclair, K.D., Young, L.E., et al., 2000. Large offspring syndrome and other consequences of ruminant embryo culture in vitro: relevance to blastocyst culture in human ART. Hum. Fertil. 3, 238–246.
Meehan, R., Stancheva, I., 2001. DNA methylation and control of gene expression in vertebrate development. Essays Biochem. 37, 59–70.
Misirlioglu, M., Page, G.P., Sagirkaya, H., et al., 2006. Dynamics of global transcriptome in bovine matured oocytes and preimplantation embryos. Proc. Natl. Acad. Sci. USA 103, 18905–18910.
Murphy, S.K., Jirtle, R.L., 2003. Imprinting evolution and the price of silence. BioEssays 25, 577–588.
Ng, H.H., Bird, A., 1999. DNA methylation and chromatin modification. Curr. Opin. Genet. Dev. 9, 158–163.
Niemitz, E.L., Feinberg, A.P., 2004. Epigenetics and assisted reproductive technology: a call for investigation. Am. J. Hum. Genet. 74, 599–609.
Notredame, C., Higgins, D.G., Heringa, J., 2000. T-Coffee: a novel method for fast and accurate multiple sequence alignment. J. Mol. Biol. 302, 205–217.
Okano, M., Bell, D.W., Haber, D.A., et al., 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99, 247–257.
Okano, M., Xie, S., Li, E., 1998. Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells. Nucleic Acids Res. 26, 2536–2540.
Ooi, S.K., Qiu, C., Bernstein, E., et al., 2007. DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. Nature 448, 714–717.
Oswald, J., Engemann, S., Lane, N., et al., 2000. Active demethylation of the paternal genome in the mouse zygote. Curr. Biol. 10, 475–478.
Paoloni-Giacobino, A., 2006. Implications of reproductive technologies for birth and developmental outcomes: imprinting defects and beyond. Expert Rev. Mol. Med. 8, 1–14.
Parrish, J.J., Krogenaes, A., Susko-Parrish, J.L., 1995. Effect of bovine sperm separation by either swim-up or Percoll method on success of in vitro fertilization and early embryonic development. Theriogenology 44, 859–869.
Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30, e36.

Pradhan, S., Bacolla, A., Wells, R.D., et al., 1999. Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of de novo and maintenance methylation. J. Biol. Chem. 274, 33002—33010.

Prescott, K.R., Wilkie, A.O., 2007. Genetic aspects of birth defects: new understandings of old problems. Arch. Dis. Child. Fetal Neonatal Ed. 92, F308—F314.

Rai, K., Chidester, S., Zavala, C.V., et al., 2007. Dnmt2 functions in the cytoplasm to promote liver, brain, and retina development in zebrafish. Genes Dev. 21, 261—266.

Ratnam, S., Mertineit, C., Ding, F., et al., 2002. Dynamics of Dnmt1 methyltransferase expression and intracellular localization during oogenesis and preimplantation development. Dev. Biol. 245, 304—314.

Reik, W., Dean, W., Walter, J., 2001. Epigenetic reprogramming in mammalian development. Science 293, 1089—1093.

Rountree, M.R., Bachman, K.E., Baylin, S.B., 2000. DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. Nat. Genet. 25, 269—277.

Sagirkaya, H., Misirlioglu, M., Kaya, A., et al., 2006. Developmental and molecular correlates of bovine preimplantation embryos. Reproduction 131, 895—904.

Swales, A.K., Spears, N., 2005. Genomic imprinting and reproduction. Reproduction 130, 389—399.

Tuimala, J., 2006. A Primer to Phylogenetic Analysis using the PHYLIP Package. CSC — Scientific Computing Ltd.

Turek-Plewa, J., Jagodzinski, P.P., 2005. The role of mammalian DNA methyltransferases in the regulation of gene expression. Cell. Mol. Biol. Lett. 10, 631—647.

Ueda, Y., Okano, M., Williams, C., et al., 2006. Roles for Dnmt3b in mammalian development: a mouse model for the ICF syndrome. Development 133, 1183—1192.

Vassena, R., Dee Schramm, R., Latham, K.E., 2005. Species-dependent expression patterns of DNA methyltransferase genes in mammalian oocytes and preimplantation embryos. Mol. Reprod. Dev. 72, 430—436.

Walsh, C.P., Bestor, T.H., 1999. Cytosine methylation and mammalian development. Genes Dev. 13, 26—34.

Yoder, J.A., Bestor, T.H., 1998. A candidate mammalian DNA methyltransferase related to pmt1p of fission yeast. Hum. Mol. Genet. 7, 279—284.

Yoder, J.A., Soman, N.S., Verdine, G.L., et al., 1997. DNA (cytosine-5)-methyltransferases in mouse cells and tissues. Studies with a mechanism-based probe. J. Mol. Biol. 270, 385—395.

Young, L.E., Sinclair, K.D., Wilmut, I., 1998. Large offspring syndrome in cattle and sheep. Rev. Reprod. 3, 155—163.

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