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—Original Article—

The *Dnmt3b* Splice Variant is Specifically Expressed in *In Vitro*-manipulated Blastocysts and Their Derivative ES Cells

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Abstract. Manipulation of preimplantation embryos *in vitro*, such as *in vitro* fertilization (IVF), *in vitro* culture (IVC), intracytoplasmic sperm injection (ICSI), somatic cell nuclear transfer (SCNT) and other assisted reproduction technologies (ART), has contributed to the development of infertility treatment and new animal reproduction methods. However, such embryos often exhibit abnormal DNA methylation patterns in imprinted genes and centromeric satellite repeats. These DNA methylation patterns are established and maintained by three DNA methyltransferases: Dnmt1, Dnmt3a and Dnmt3b. Dnmt3b is responsible for the creation of methylation patterns during the early stage of embryogenesis and consists of many alternative splice variants that affect methylation activity; nevertheless, the roles of these variants have not yet been identified. In this study, we found an alternatively spliced variant of *Dnmt3b* lacking exon 6 (*Dnmt3bΔ6*) that is specific to mouse IVC embryos. *Dnmt3bΔ6* also showed prominent expression in embryonic stem (ES) cells derived from *in vitro* manipulated embryos. Interestingly, IVC blastocysts were hypomethylated in centromeric satellite repeat regions that could be susceptible to methylation by Dnmt3b. *In vitro* methylation activity assays showed that Dnmt3bΔ6 had lower activity than normal Dnmt3b. Our findings suggest that Dnmt3bΔ6 could induce a hypomethylation status especially in *in vitro* manipulated embryos.
Key words: DNA methylation, Dnmt3b, *In vitro* culture

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The 5th position cytosine residues in CpG sequences are often methylated in vertebrate genomic DNA [1]. DNA methylation plays an essential role in the normal development of mammalian embryos by regulating gene expression through genomic imprinting, X chromosome inactivation and genomic stability [2–6]. In vertebrates, two types of DNA methyltransferase activity have been reported, the *de novo* and maintenance types. In mice, *de novo*-type DNA methylation activity creates gene-specific methylation patterns during the implantation stage of embryogenesis, while maintenance-type activity ensures clonal transmission of lineage-specific methylation patterns during replication. Dnmt1 is responsible for the latter activity. On the other hand, two DNA methyltransferases, Dnmt3a and Dnmt3b, are responsible for creation of methylation patterns during the early stages of embryogenesis [7, 8] and have been shown to possess *de novo*-type DNA methylation activity *in vitro* [9–12]. Recent studies have shown that Dnmts function in cooperation with each other to facilitate DNA methylation in both humans and mice [13–15].

In mice, Dnmt3b is the major *de novo* DNA methyltransferase in E (embryonic stage) 4.5–7.5 embryos, and its expression is downregulated after midgestation [8, 16]. Disruption of Dnmt3b results in embryonic lethality at E13.5 and hypomethylation of centromeric minor satellite repeats [8]. In humans, DNMT3B mutations have been shown to cause ICF (immunodeficiency, chromosomal instability, and facial anomalies) syndrome [8, 17, 18]. Dnmt3b contains a C-terminal catalytic domain and a N-terminal regulatory domain including the PWWP domain and consists of more than 16 alternative splice variants [7, 19–22]. Among these variants, both Dnmt3b1 and Dnmt3b2 contain all of the highly conserved motifs (I, IV, VI, VIII, IX and X) in their catalytic domains. In contrast, Dnmt3b3 and Dnmt3b6, lacking motifs VII and VIII and nine amino acids of motif IX [19], are catalytically inactive both *in vitro* [9] and *in vivo* [23]. On the other hand, the splicing variant of the regulatory domain, human DNMT3BΔ5 (the same variant as mouse Dnmt3bΔ6), which lacks exon 5 (exon 6) adjacent to the PWWP domain, was recently reported to be upregulated in cancer cell lines that often show hypomethylation in centromeric repeated sequences [24]. Gopalakrishnan *et al*. indicated that this splicing region is responsible for DNA binding activity [24]; however, the role of this region has not yet been fully investigated. Interestingly, forced expression of DNMT3BΔ5 results in hypomethylation of centromeric and pericentromeric repeated sequences [24]. Similarly, forced expression of human specific DNMT3B4, which lacks a catalytic domain, induced DNA demethylation on satellite 2 in pericentromeric DNA [25]. These reports indicate that Dnmt3b variants have complicated roles in maintenance of DNA methylation status.

As is generally known, *in vitro* embryo manipulation technologies such as IVC, ICSI and SCNT have contributed to the

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development of infertility treatments and animal reproduction techniques. However, it has been reported that *in vitro* manipulated embryos often show abnormal DNA methylation patterns in differentially methylated regions (DMRs) of imprinted genes [26–29]. Moreover, a growing number of reports suggest that embryo manipulation *in vitro* increases the risk of diseases caused by aberrant DNA methylation patterns, such as Angelman syndrome (AS) and Beckwith–Wiedemann syndrome (BWS) [30–34]. Nevertheless, the factor(s) that causes DNA methylation instability in *in vitro* manipulated embryos has not yet been identified. Hence, we initiated experiments on the basis of the expectation that Dnmt3bΔ6 is responsible for hypomethylation in *in vitro* manipulated embryos.

Materials and Methods

Animals

C57BL/6J mice (B6, JAX mice), CD1 mice (Charles River, Yokohama, Japan) and F344 rats (CLEA Japan, Tokyo, Japan) were used in this study. All animal experiments were conducted according to the guidelines of the Animal Care and Experimentation Committee of Gunma University, Showa Campus, Japan.

Embryo collection, culture and transfer

C57BL/6J females were superovulated by injection of 5 units of pregnant mare serum (PMSG, ASKA Pharmaceutical, Tokyo, Japan) followed by injection of 5 units of human chorionic gonadotropin 48 h later (hCG, ASKA Pharmaceutical). Females were bred to C57BL/6J males overnight. Approximately 24 h after hCG, the females were sacrificed, and fertilized embryos were collected from the oviducts. These embryos were cultured as IVC blastocysts to the blastocyst stage (114 h after hCG) in M16 medium. In contrast, the *in vivo*-developed blastocysts were collected from uteri 96 h after hCG. The methods used to produce parthenogenetic embryos [35] and SCNT embryos have been described previously [36]. Some of the IVC and *in vivo*-developed blastocysts were transferred to the uterine horns of pseudopregnant recipient females (CD1) 2.5 days postcoitus (dpc), and these embryos were harvested after 7 days (9.5 dpc).

Cell counting of blastocysts

Inner cell mass (ICM) and trophectoderm (TE) cell numbers of blastocysts were counted as previously reported [37].

Generation of ES cell lines

The methods used for generation of ES cell lines have been previously described [35, 38]. Briefly, blastocysts were transferred into gelatinized tissue culture wells (2–3 blastocysts per well of a 4-well multiplate, Nunc, Rochester, NY, USA) and cultured for 7 days in ES medium and DMEM (Gibco, Gland Island, NY, USA) containing 17.5% Knockout SR (Gibco) following standard procedures [39, 40]. After 7 days, ICM outgrowths were harvested in trypsin/EDTA (0.25%/1 mM, Gibco), disaggregated by mouth pipetting and plated onto gelatinized tissue culture wells in ES medium (passage 1). Clones morphologically resembling ES cells were then picked and disaggregated a second time. The cells were then expanded and passaged prior to freezing or use.

DNA isolation and methylation analysis

DNA was isolated from about 10 blastocysts in each pool. Bisulfite treatment was carried out using an EpiTect Bisulfite Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. PCR amplification of major and minor satellite DNA was performed for each set of primers:

major satellite,
5'-AAATCTAGAAATGTTTATTGTAGGA-3' and
5'-TTCGGATCCTAAAATATATATTTCTCAT-3';
minor satellite,
5'-TATGGAAAATGATAAAAATTATATTG-3' and
5'-ATTATAACTCATTAATATACACTATTC-3'.

The amplification consisted of a total of 21 cycles at 94 C for 10 sec, 55 C for 30 sec and 72 C for 60 sec for the major satellite primers and a total of 26 cycles at 94 C for 10 sec, 58 C for 30 sec and 72 C for 60 sec for the minor satellite primers using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). PCR products derived from 3 independent pools were subcloned into a TA cloning vector (pCR 2.1, Invitrogen). Positive clones for each sample were sequenced using the BigDye terminator method (ABI PRISM 3100, Applied Biosystems).

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was purified from embryos and nonhuman tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was obtained from human tissue using Human Total RNA Master Panel II (BD Biosciences, San Jose, CA, USA). Purified RNA from 50 blastocysts or 1 μg of purified RNA from postimplantation embryos and tissues was reverse transcribed using Superscript II (TaKaRa, Otsu, Japan) and Oligo(dT)$_{12–18}$ (TaKaRa) in a total volume of 20 μl. The mRNA expression level of each *Dnmt3bs* was determined by PCR using LA Taq HS DNA polymerase (Takara) and the primer sets shown in Table 1. PCR products were separated electrophoretically on a 2.5% agarose gel. For densitometry, the Quantity One software (Bio-Rad, Hercules, CA, USA) was used according to the manufacturer's instructions, and the expression ratios of –exon 6/+exon 6 (–exon 5/+exon 5) were calculated. Quantitative real-time RT-PCR for *Dnmt3b* was also carried out as previously reported [38].

Identification of Dnmt3bΔ6 splice variants

Mouse *Dnmt3b* cDNA was amplified from SCNT ES cells using primers S5 and AS3 (Table 1). PCR products were subcloned into the TA cloning vector (pCR 2.1; Invitrogen). RT-PCR primers for the *Dnmt3b* splice sites (Table 1) were used to identify splice variants. To confirm the variant sequences, the clones were sequenced using the BigDye terminator method (ABI PRISM 3100, Applied Biosystems).

Purification of recombinant Dnmt3b

Recombinant His-tagged Dnmt3b protein was produced in sf9 cells using the baculovirus expression system (Invitrogen) as described previously [41]. The ATG coding initiation methionines

Table 1. Sequences of the primers used for PCR amplification

Gene name		Sequence	Product size	Target
Mouse				
Dnmt3b	S1	gggaacttcagtgaccagtcctc	565 bp	Exon 6
	AS1	ccacctgtgtggtatcca		
	S2	ctggagagtcactggaggaccagctgaagc	290 bp	Exon 11
	AS2	ctctcctcatcctcccctcggtccttc		
	S3	acaaccgtccattcttctgg	391 bp	Catalytic region
	AS3	acgtccgtgtagtgagcaggga		
	S4	ctttcaggaaacaatgaaggga	2716 bp	Full length
	AS4	gctgaggtacagtgatgtga		
	S5	aaagcccggcttctcggaga	2002 bp	Dnmt3bΔ6
	AS3	acgtccgtgtagtgagcaggga		variants
Gapdh	S	aatgcatcctgcaccaccaa	106 bp	
	AS	gtggcagtgatggcatggac		
Rat				
Dnmt3b	S	gtgaagcggatgatggagat	458 bp	Exon 6
	AS	ctttccccacacaaggtcac		
Gapdh	S	aatgcatcctgcaccaccaa	106 bp	
	AS	gtggcagtgatggcatggac		
Human				
DNMT3B	S	ccaggactcgttcagaaagc	237 bp	Exon 5
	AS	cgtctgtgaggtcgatggta		
GAPDH	S	aatgcctcctgcaccaccaa	106 bp	
	AS	gtggcagtgatggcatggac		

of mouse *Dnmt3b1Δ6* and *Dnmt3b1* were directly ligated using a BamH1 linker without any spacer sequence and then subcloned into the BamH1 site of the multicloning site of the pFAST-BACHTb vector (Invitrogen). Recombinant baculoviruses were constructed according to the manufacturer's instructions, and the viruses were amplified by three rounds of infection to obtain a high-titer baculovirus stock. Sf9 cells were maintained in Grace's medium containing 10% (v/v) fetal bovine serum at 27 C. The recombinant baculoviruses harboring Dnmt3b1Δ6 and Dnmt3b1 were infected into 5×10^8 sf9 cells at an M.O.I. (multiplicity of infection) of 2. After infection, the cells were incubated for 16 or 66 h and then harvested. Purification of His-Dnmt3b was carried out as described previously [41].

DNA methylation activity assay

The DNA methylation activity was determined as described elsewhere [9]. In brief, 50 ng (about 0.5 pmol) of purified Dnmt3b, 0.1 mg of dGdC and 133 pmol (2.0 μCi and 5.3 μM) of [^{3}H]-*S*-adenosyl-L-methionine (AdoMet) (15Ci/mmol, GE Healthcare, Uppsala, Sweden) were added to 25 μl of the reaction buffer (2.7 M glycerol, 5 mM EDTA, 0.2 mM DTT, 40–160 mM NaCl and 20 mM Tris-Hcl at pH 7.4). After a 1-h incubation, the reaction was terminated with 1.5 mM nonradioactive AdoMet. The mixtures were then incubated with 0.1 μg of proteinase K (Nacalai Tesque, Kyoto, Japan) at 50 C for 20 min, and the level of radioactivity was then determined as described previously [42].

Statistical analysis

QUMA (QUantification tool for Methylation Analysis, http://quma.cdb.riken.jp/) was used to statistically analyze the bisulfite sequencing of CpG methylation. The entire set of CpG sites was evaluated with the Mann-Whitney *U*-test. The Student's *t*-test was used for gene expression analysis and cell number analysis. Data are shown as means and standard deviations (SD). A P-value of <0.05 was considered significant.

Results and Discussion

Dnmt3bΔ6 expression in embryos and ES cell lines

A lot of reports have suggested that *in vitro* manipulation causes aberrant DNA methylation patterns (mostly hypomethylation) in the DMRs of imprinted genes in preimplantation embryos, whereas there have been few reports about the methylation patterns in the satellite repeat regions in these embryos. Therefore, we first exam-

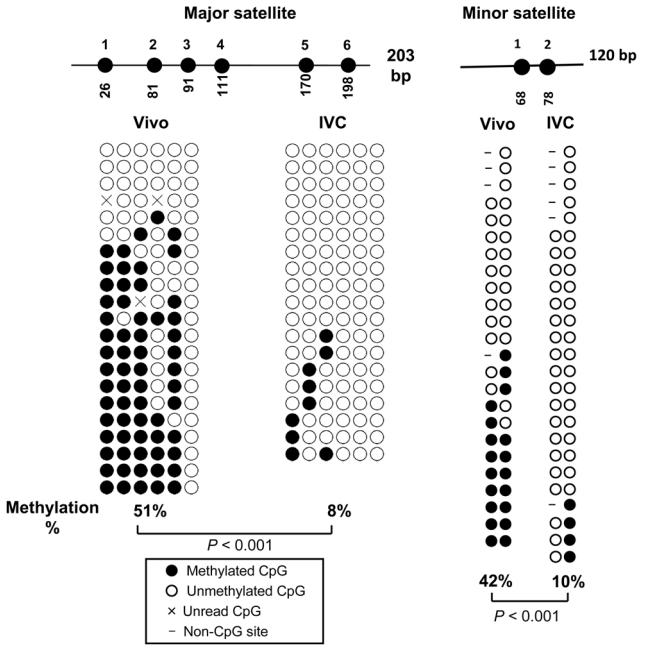

Fig. 1. DNA methylation status of centromeric repeat regions in blastocysts. Hypomethylation of major and minor satellite repeats was observed in *in vitro*-cultured blastocysts (IVC) relative to *in vivo*-developed blastocysts (Vivo).

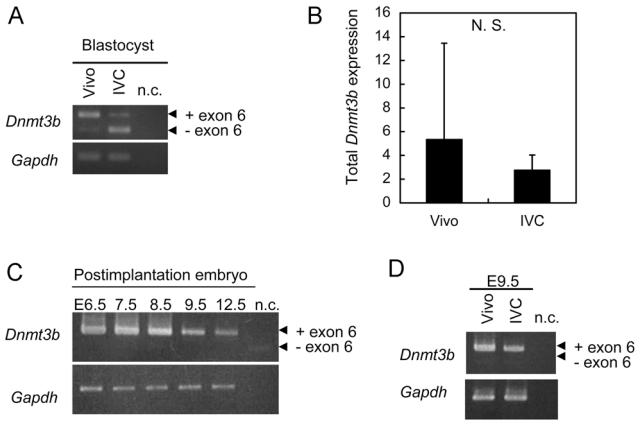

Fig. 2. *Dnmt3bΔ6* (exon 6 splice form) expression in mouse embryos. A: *Dnmt3b* containing exon 6 was the major transcript in blastocysts developed *in vivo* (Vivo), whereas both the spliced and unspliced forms were present in *in vitro*-cultured blastocysts (IVC). n.c., negative control. B: Total *Dnmt3b* expression was not significantly different (N. S.) between Vivo and IVC blastocysts. C: *Dnmt3bΔ6* was detected at very low levels in embryos postimplantation. D: *Dnmt3bΔ6* expression was not detectable in E9.5 fetuses derived from IVC embryos, indicating that the spliced form is expressed in undifferentiated cells and is then repressed during differentiation. n.c., negative control.

Table 2. Means of inner cell mass (ICM), trophectoderm (TE) and total cell number of blastocysts

	Cell number (mean ± SD)			
Blastocysts	Total	ICM	TE	
In vivo (96 h)	43.3 ± 7.4	12.1 ± 2.8	31.2 ± 6.3	(N = 11)
IVC (114 h)	40.0 ± 8.6	12.0 ± 4.4	28.0 ± 6.1	(N = 10)

ined the DNA methylation statuses of these regions, and we found that IVC blastocysts are aberrantly hypomethylated relative to control *in vivo*-derived blastocysts (Fig. 1). Dnmt3b is the primary *de novo* DNA methyltransferase that methylates satellite repeats, especially the centromeric minor satellite repeats [8]. We hypothesized that the abnormal hypomethylation could be caused by the altered expression of specific *Dnmt3b* splicing variants in *in vitro*-manipulated embryos. To determine whether distinct *Dnmt3b* splicing variants were specifically expressed in preimplantation embryos (blastocyst stage), we prepared IVC blastocysts (114 h after hCG) and *in vivo*-developed blastocysts (96 h after hCG). At these times, ICM, TE and total cell numbers were not significantly different between IVC and *in vivo* developed blastocysts, indicating that both embryos were at the same developmental stage (Table 2). RT-PCR using a primer set that recognizes exon 6 splicing variants showed that *Dnmt3bΔ6* was more highly expressed in IVC blastocysts than *in vivo*-developed blastocyst controls (Fig. 2A), although the total Dnmt3b expression levels were not significantly different (Fig. 2B). In general, Dnmt3b is the major *de novo* DNA methyltransferase in E4.5–7.5 embryos, and its expression is down-regulated after midgestation [8, 16]. Our data agree with the findings of these reports (Fig. 2C). In addition, *Dnmt3bΔ6* expression was not detected in *in vivo* embryos during postimplantation development (Fig. 2C). Similarly, *Dnmt3bΔ6* expression was not detectable in E9.5 embryos that were produced by transplantation of IVC blastocysts into pseudopregnant females (Fig. 2D). These results indicate that *Dnmt3bΔ6* is specifically expressed in preimplantation blastocysts. High levels of *Dnmt3bΔ6* were also detected in undifferentiated ES cells generated from *in vitro* manipulated embryos, such as those generated by IVC (Fig. 3A), by parthenogenetic methods or from SCNT blastocysts (Fig. 3B). Thus, *Dnmt3bΔ6* is specifically expressed in undifferentiated cell types such as preimplantation embryos manipulated *in vitro* and their derivative ES cells.

Identification of Dnmt3bΔ6 splice variants

At least 16 *Dnmt3b* variants have been described in mice [22]; therefore, we performed PCR analysis using a primer set that amplified exon 6-deleted forms, and the PCR products were subcloned into a TA cloning vector. Each clone was characterized by

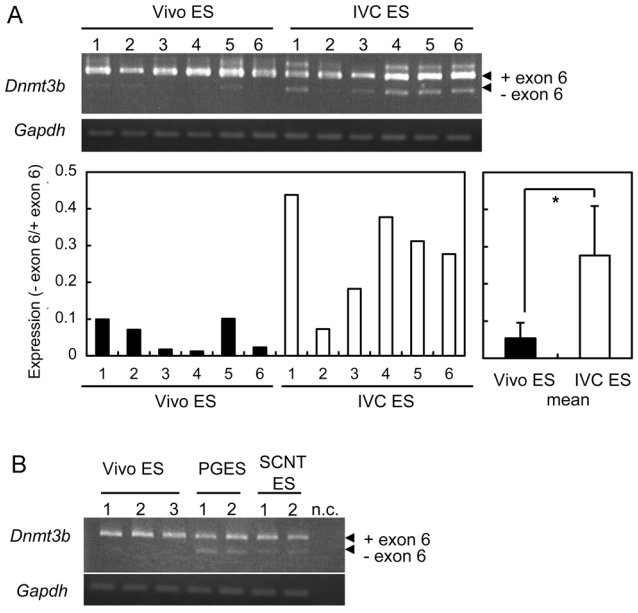

Fig. 3. *Dnmt3bΔ6* (exon 6 splice form) expression in mouse ES cells. A: *Dnmt3bΔ6* was more highly expressed in IVC embryo-derived ES cells (IVC ES) than in ES cells generated from *in vivo*-developed blastocysts (Vivo ES). * P<0.05. B: *Dnmt3bΔ6* was more highly expressed in ES cells that had been generated from *in vitro*-manipulated embryos, such as those generated by parthenogenesis (PG) and somatic cell nuclear transfer (SCNT). n.c., negative control.

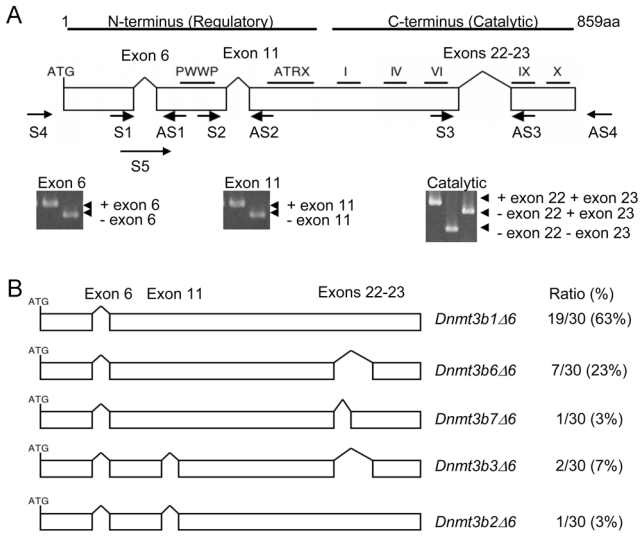

Fig. 4. Identification of mouse *Dnmt3bΔ6* splice variants. A: *Upper panel*: map of *Dnmt3b* mRNA showing the positions of conserved domains. *Arrows*: locations of the RT-PCR primers used in this study. *Lower panel*: RT-PCR analysis of cloned alternative splicing variants identified those lacking exons 6, 11 and 22–23. B: Organization of the *Dnmt3bΔ6* mRNA species present in SCNT ES cells. The PCR products amplified from *Dnmt3bΔ6* cDNA were subcloned into a TA cloning vector, and each clone was characterized by sequencing. The most common form of *Dnmt3b1Δ6* identified was *Dnmt3b1Δ6* (63%), while the second most common form was *Dnmt3b6Δ6* (23%).

RT-PCR and sequencing to identify individual variants. From these analyses, five variants lacking exon 6 were identified. The most prevalent form identified was *Dnmt3b1Δ6* (63%), which only lacked exon 6, and the second most common form was *Dnmt3b6Δ6* (23%), which lacked exon 6 and exons 22–23 of the catalytic domain (Fig. 4A and B).

DNA methylation activity of purified Dnmt3bΔ6

To determine the DNA methylation activity of Dnmt3b1Δ6 *in vitro*, sf9 cells were infected with recombinant baculoviruses encoding Dnmt3b1 and Dnmt3b1Δ6, and recombinant Dnmt3b1 and Dnmt3b1Δ6 were then purified (Fig. 5A and B). Dnmt3b1Δ6 demonstrated DNA methylation activity, although it showed lower activity than the control Dnmt3b1 (Fig. 5C). The second major variant, Dnmt3b6Δ6, which lacks the catalytic domain, was not examined; however, Dnmt3b6Δ6 is not expected to possess methylation activity, as all the other mouse Dnmt3b variants without the catalytic domain (e.g., Dnmt3b3) do not possess methylation activity. Thus, we concluded that there would be insufficient methylation activity in cells, such as IVC blastocysts, in which the dominant Dnmt3b form is Dnmt3bΔ6.

Tissue- and species-specific expression of Dnmt3bΔ6

According to the NCBI (National Center for Biotechnology Information) database, the exon 6 region is highly conserved among mice, rats and humans. To determine the expression pattern of *Dnmt3bΔ6* in these species, RT-PCR was performed using exon 6-specific primer sets. In mice, only low levels of *Dnmt3bΔ6* expression were observed in all tissues except for skeletal muscle (Fig. 6A). A very low level of *Dnmt3bΔ6* expression was also seen in rat tissues (Fig. 6B). Human *DNMT3B* lacks one noncoding exon at its 5'-end; therefore, the region equivalent to exon 6 in mouse *Dnmt3b* is exon 5 in human *DNMT3B*. Interestingly, the highest levels of human *DNMT3B* lacking exon 5 (*DNMT3BΔ5*) expression were observed in both the adult and fetal brain (Fig. 6 C). DNMT3B is reported to be necessary for nerve growth factor-mediated differentiation [43], and the DNMT3B mutations that occur in ICF syndrome lead to altered epigenetic modifications and aberrant expression of genes regulating neurogenesis [44]. This variant is thus proposed to be necessary for the development and maintenance of neural function in humans. In contrast, only a low level of *Dnmt3bΔ6* expression was observed in mouse and rat brains. The reason for the difference between human and rodent *Dnmt3b* expression is presently unclear. Further studies will be required to elucidate the specific roles of Dnmt3bΔ6 in neural development.

Dnmt3bΔ6 and centromeric hypomethylation

In vitro manipulated embryos often exhibit abnormal methylation patterns in genomic imprinting regions and centromeric repeats. In this study, we have shown the first evidence that *Dnmt3bΔ6*, which is highly expressed in *in vitro* manipulated

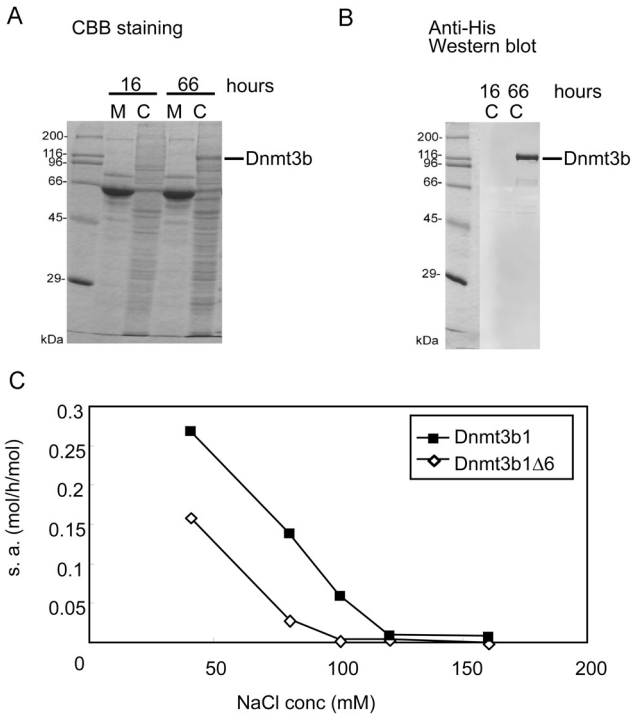

Fig. 5. *De novo* methylation activity of purified Dnmt3bΔ6. A: Recombinant baculoviruses encoding Dnmt3bΔ6 were generated and used to infect sf9 cells. Samples obtained 16 and 66 h after infection were subjected to SDS-PAGE and then stained with CBB. M, medium fraction; C, cell fraction. B: Purified protein was blotted with an anti-His antibody. C: The DNA methylation activity [specific activity (s.a.) in mol/h/mol] of Dnmt3b1Δ6 and Dnmt3b1 was titrated with NaCl.

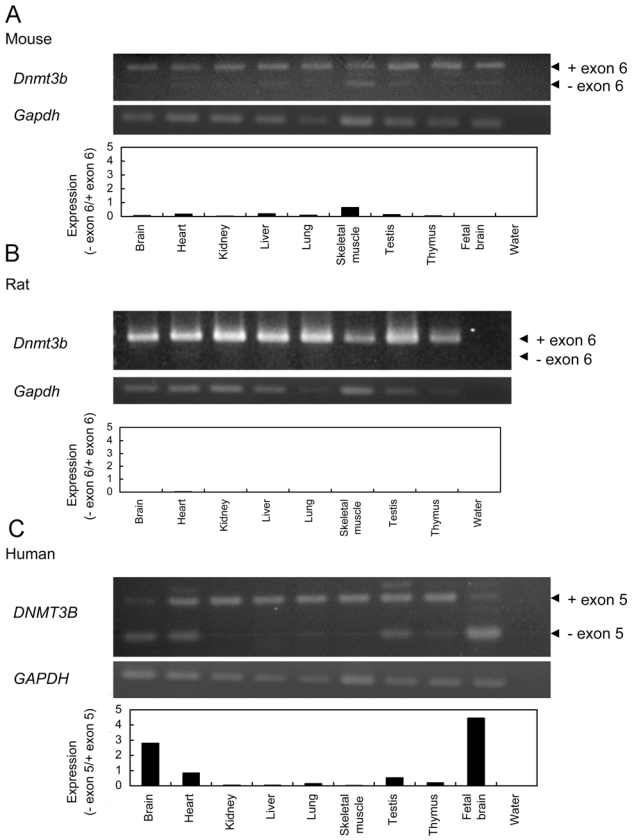

Fig. 6. *Dnmt3bΔ6* (*DNMT3BΔ5*) expression in various tissues. *Dnmt3b* containing exon 6 was the abundantly expressed variant in mouse tissues (A) and in rat tissues (B). C: In humans, *DNMT3B* lacking exon 5 was the predominant form in adult and fetal brains.

embryos, may be involved in abnormal DNA methylation. *Dnmt3bΔ6* (*DNMT3BΔ5*) is also overexpressed in human cancer cell lines [24] that often exhibit global hypomethylation, where it primarily affects repetitive regions of the genome, such as the centromeric and pericentromeric regions [45]. Notably, ectopic overexpression of DNMT3B3Δ5 resulted in repetitive element hypomethylation in cancer cells [24]. Thus, Dnmt3bΔ6 could be involved in DNA hypomethylation, especially in the centromeric and pericentromeric satellite repeat region in early embryos. After fertilization, the embryonic genome, including the centromeric satellite repeats, becomes demethylated, and these methylation levels are maintained during preimplantation development [46, 47]. Even blastocysts that develop *in vivo* exhibit low levels of *Dnmt3bΔ6* expression, implying that Dnmt3bΔ6 is essential for demethylation or maintenance of the methylation status of centromeric satellite repeats. Consistent with this hypothesis, excessive expression of *Dnmt3bΔ6* in IVC embryos may induce abnormal DNA methylation. Thus, the expression pattern of Dnmt3bΔ6 and other Dnmt3b variants seems to be precisely regulated at the preimplantation stage. Although we do not have information about how *Dnmt3b* splicing is regulated, small nuclear ribonucleoprotein particles (snRNPs) and their mediator SF2/ASF [48, 49] are likely candidates.

In vitro embryo manipulation technologies have contributed to the development of infertility treatments and new animal reproduction strategies. However, *in vitro* embryo manipulation increases the risk of epigenetic abnormalities, a crucial problem that remains to be solved. Our findings regarding Dnmt3bΔ6 expression may provide clues as to why epigenetic abnormalities occur in embryos manipulated *in vitro*.

Acknowledgments

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