Gene Expression, Vol. 14, pp. 251–263
Printed in the USA.

1052-2166/09 $90.00 + .00
E-ISSN 1555-3884
www.cognizantcommunication.com

Gene Expression Pattern and Downregulation of DNA Methyltransferase 1 Using siRNA in Porcine Somatic Cells

ANGELICA M. GIRALDO, TODD D. VAUGHT, LIMIN FU, ALISON J. DUNCAN, AMY M. VANCE, MICHAEL MENDICINO, AND DAVID L. AYARES

Revivicor, Inc., Blacksburg, VA 24060, USA

DNA methylation plays a significant role in the expression of the genetic code and affects early growth and development through their influence on gene expression. Manipulation of the DNA methylation marks of differentiated cells will allow a better understanding of the different molecular processes associated with chromatin structure and gene expression. The objective of this study was to identify small interfering RNAs (siRNAs) with the ability to reduce DNA methyltransferase 1 (Dnmt1) mRNA and consequently decrease Dnmt1 protein as well as DNA methylation in porcine cells. Fibroblasts from four porcine fetuses were established and cultured in 5% CO_2 in air at 38°C. Optimal transfection conditions were evaluated using a FITC-labeled control siRNA. Four Dnmt1-specific siRNAs were evaluated upon transfection of each cell line. A nonsilencing siRNA was used as a negative control. The expression patterns of Dnmt1 were analyzed by Q-PCR. The combination of 1 μg of siRNA and a 1:6 siRNA to transfection reagent ratio produced the highest transient transfection rates without affecting cell viability. Downregulation of Dnmt1 varied between siRNAs. Transfection of porcine cells with highly effective siRNAs resulted in a drastic reduction of Dnmt1 mRNA and a slight decrease in protein production. However, this small reduction in the protein concentration induced significant genomic hypomethylation. These data suggest that although Dnmt1 mRNA abundance plays an important role during protein regulation, Dnmt1 enzyme is mainly posttranscriptionally regulated. Subsequent use of these cells for cloning, differentiation, and cancer studies will provide insight as to how methylation of the DNA affects genomic reprogramming.

Key words: DNA methyltransferases; DNA methylation; siRNA; Epigenetics; Gene expression

INTRODUCTION

DNA methylation plays a significant role in gene expression, which influences early growth and development (19). The principal function of this epigenetic modification is to regulate the repression of genes not required in specific cell types at specific stages of development without changing the DNA sequence (48). Moreover, methylation can be reversed in case of requirement for cellular differentiation via gene expression/repression regulation (52). A second but equally essential role of DNA methylation in mammals is to provide an imprinting mark that distinguishes between silenced and expressed parental alleles of certain genes (5).

Methylation of the DNA consists of the covalent addition of a methyl group to the number 5 carbon of the cytosine pyrimidine ring (14). This reaction is catalyzed by DNA methyltransferase (Dnmt) enzymes and is generally associated with transcriptional silencing due to the inability of transcription factors to bind to methylated DNA sequences (19). Dnmt1 enzyme is most likely responsible for maintaining the methylation states of sites during cell division. It is the affinity of Dnmt1 for the hemimethylated sites resulting from semiconservative replication that ensures methylation patterns are maintained once established (7).

Manipulation of the DNA methylation marks of differentiated cells should facilitate the understanding

Address correspondence to Angelica Giraldo, Revivicor, 1700 Kraft Dr., Suite 2400, Blacksburg, VA 24060, USA. Fax: 540 961 7958; E-mail: agiraldo@revivicor.com

of the different molecular processes associated with chromatin structure and gene expression. Rearrangement of the methylation pattern should improve our understanding of the differentiation-associated cellular changes, which may facilitate the manipulation of stem cell differentiation into a desired cell type, or conversely, the dedifferentiation of specific cell types into pluripotent stem cells (23,52). Additionally, removal of methylation groups abnormally localized in the promoter region of tumor suppressor genes may reestablish the normal expression of these genes and act as an anticancer therapy. Overmethylation causes increased chromatin condensation, thus reduction in the chromatin compaction of donor cells may increase the efficiency of somatic cell nuclear transfer (SCNT). It has been proposed that cells characterized by hypomethylated DNA should be more easily reprogrammed during NT than cells with highly compacted chromatin (2,11,33).

The DNA methylation status of somatic cells could be altered by regulating the activity of epigenetic-modifying enzymes. Genomic hypomethylation has been previously achieved by inducing downregulation of Dnmt1 (10,13,26,40). Different approaches to artificially inhibit Dnmt1 in somatic cells, including the addition of chemical inhibitors, gene knock-out and small interfering RNA (siRNA) have been employed. Enright et al. demonstrated that 5-aza-2′-deoxycytidine (5-AZA) can reduce the level of DNA methylation by depleting the cells of Dnmt1 enzyme activity (11). However, chemical inhibitors could target other proteins or enzymes and affect other biochemistry pathways in addition to the targeted system. Therefore, results obtained from a chemical additive must always be questioned as to whether the outcome observed was the result of an off-target effect. Moreover, 5-AZA has been shown to have cytotoxic effects (21). Gene knock-out models generated by interrupting the Dnmt1 gene have been reported in mice (13,25,27). For large animals, such as livestock species, knockout of Dnmt1 would require the application of low-efficiency homologous recombination technology combined with SCNT, making this endeavor very labor intensive, and costly to produce and maintain especially in large animals (31).

Recently, RNA interference (RNAi) has become a powerful and widely accepted tool for the analysis of gene function in many organisms including plant, invertebrate, and mammalian cells. The specificity of RNAi is comparable to gene knock-out experiments but much less time consuming and expensive. RNAi is based on double-stranded RNAs (dsRNAs) that trigger sequence-specific degradation of mRNA. A siRNA, consisting of an in vitro-synthesized 21-base pair oligonucleotide duplex, can mediate RNA interference, and gene knockdown, in a sequence-specific manner in cultured mammalian cells. Briefly, siRNA duplexes bind to a nuclease complex to form the RNA-induced silencing complex (RISC). The activated RISC targets the homologous transcript by base pairing interactions and cleaves the mRNA ~12 nucleotides from the 3′ terminus of the siRNA (17,34).

RNAi technology has been previously used to reduce the expression of Dnmt1 in human cancer cells, as well as murine and bovine fibroblasts (1,15,26,40). However, depletion of Dnmt1 protein and DNA methylation after treatment with Dnmt1-specific siRNAs was not always observed (1). Therefore, the objective of this study was to identify the ability of several siRNAs to downregulate Dnmt1 mRNA as well as Dnmt1 protein and DNA methylation in porcine fibroblast cells.

MATERIALS AND METHODS

siRNA Design and Transfections

Four short interfering RNAs, each targeting a different segment of the transcript sequence, were designed using the porcine Dnmt1 transcript sequence (GenBank accession number DQ060156). The oligonucleotides were designed using the HiPerformance Design Algorithm software and were synthetically produced by Qiagen, Inc. (Valencia, CA). A nonsilencing siRNA was included as a negative control (Table 1). Transfection efficiency and cell viability were determined in cells transfected with 3′ fluorescein-labeled nonsilencing siRNA and stained with propidium iodide (PI) (30 μg/ml). Transfections with Dnmt1-specific siRNA were performed using the lipofection conditions as determined in the optimization experiments.

Establishment, Culture, and Cryopreservation of Cell Lines

Primary cultures of fibroblasts from four 40-day-old porcine fetuses were established using explants. Briefly, pieces of minced skin were placed in 55-cm^2 culture dish containing Dulbecco's modified Eagle medium plus 20% fetal bovine serum (FBS) and cultured in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5°C. Culture medium was replaced every 3–4 days. Cells were passaged as needed using 0.05% trypsin with EDTA as a dissociation reagent. For cell freezing, the fibroblasts were resuspended in FBS and 10% dimethyl sulfoxide and cooled at 1.0°C/min until reaching −80°C prior to plunging and storage in liquid nitrogen. Approximately 1×10^6 cells were frozen in 0.5 ml of freezing medium per

TABLE 1
OLIGONUCLEOTIDE SEQUENCES

Oligonucleotide			Sequence 5′–3′	Start (bp)
siRNA1	Dnmt1 specific	Target binding site	ATGGAAGAAGATGATAAAGAA	739
		Forward	GGAAGAAGAUGAUAAAGAAdTdT	
		Reverse	UUCUUUAUCAUCUUCUUCCdAdT	
siRNA2		Target binding site	CTGGCTGAAATGAGACAGAAA	2728
		Forward	GGCUGAAAUGAGACAGAAAdTdT	
		Reverse	UUUCUGUCUCAUUUCAGCCdAdG	
siRNA3		Target binding site	CACTGTATGAGTGGAAATTTA	5120
		Forward	CUGUAUGAGUGGAAAUUUAdTdT	
		Reverse	UAAAUUUCCACUCUACAGdTdG	
siRNA4		Target binding site	CGGGAAGTGAATGGATATCTA	346
		Forward	GGAAGUGAAUGGAUAUCUAdTdT	
		Reverse	UAGAUAUCCAUUCACUUCCdCdG	
siRNA5	Nonsilencing	Target binding site	AATTCTCCGAACGTGTCACGT	
		Forward	UUCUCCGAACGUGUCACGUdTdT	
		Reverse	ACGUGACACGUUCGGAGAAdTdT	
Primer set 1	Dnmt1	Forward	CGGACTGCTCCCTACAGAAG	690
		Reverse	CGTTTGCCAGCTAGACCTTT	923
Primer set 2		Forward	TCGAAGGATGATGGGAAGAC	2599
		Reverse	ACCCACTCGATACTGGATGC	2841
Primer set 3		Forward	GAAACTGTTCTGTGAGGTTTGC	5006
		Reverse	TCCACTCATACAGTGGTAAATTTGAT	5134
Primer set 4		Forward	AGGAGGGCTACCTGGCTAAA	269
		Reverse	TGTGCCAAGTCTGGAAACAG	456
Primer set 5	Dnmt3a	Forward	GAATCGCTACAGGGCTTCTG	1299
		Reverse	CTGGATATGCTTCTGCGTGA	1613
Primer set 6	Dnmt3b	Forward	CTGGATATGCTTCTGCGTGA	88
		Reverse	GGCATCACTGGAGTGTCTGA	314
Primer set 7	Gapdh	Forward	TCGGAGTGAACGGATTTG	352
		Reverse	CCTGGAAGATGGTGATGG	570

Primers and siRNA specific for Dnmt1 were designed using the porcine sequence DQ060156. Dnmt3a, Dnmt3b, and Gapdh primers were designed using the sequence accession number NM_001097437, DQ830979, and AF017079, respectively. Primers flanking the binding site of every siRNA were used to analyze the gene expression of Dnmt1 after transfection.

cryovial. Cells were thawed by holding the cryovial for 10 s at room temperature followed by submersion in a 38°C water bath. Thawed cells were washed once in culture medium before being replated.

RNA Isolation and RT-PCR

Total RNA was isolated from fibroblasts using TRIZOL® (Invitrogen, Carlsbad, CA) as previously described by Chomczynski and Sacchi (6). Total RNA was coprecipitated with glycogen and residual genomic DNA was removed with DNase I. Reverse transcription (RT) was carried out in a total volume of 20 μl using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA) according to the manufacturer's instructions. The iScript RT Reaction Mix contained 4 μl of iScript reaction mix, 1 μl of reverse transcriptase, 4 μl of nuclease-free water, and 11 μl of mRNA. A reaction mix was formulated for the sample, a non-reverse transcriptase, and a non-template control reaction.

Real-Time PCR

PCR primers for the amplification of Dnmt1, Dnmt3a, Dnmt3b, and glyceraldehyde 3-phosphate dehydrogenase (Gapdh) were designed using the Primer 3.0 software (http://frodo.wi.mit.edu/). To avoid amplifying partially degraded molecules, primers for Dnmt1 were designed to amplify the region flanking the siRNA binding site (Table 1). Comple-

mentary DNA was amplified using SYBR Green PCR Master Mix in the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The real-time PCR mix (25 µl) consisted of 2 µl of cDNA, 12.5 µl of SYBR green master mix, 9.5 µl of nuclease-free water, and 0.5 µl each of forward and reverse primers (10 µmol) for each gene. A reaction mix was formulated for the sample, for a no-reverse transcriptase, and a no-template control reaction. The program used for the amplification of all genes consisted of a denaturing cycle of 3 min at 95°C; 40 cycles of PCR (95°C for 10 s, 55°C for 45 s, and 95°C for 1 min); a melting curve analysis consisting of 95°C for 1 min followed by 55°C for 1 min, a step cycle starting at 55°C for 10 s with a 0.5°C/s transition rate; and cooling at 4°C.

Final quantification was performed using a comparative C_T method (29). A mixture of RNA from somatic cells was used as calibrator for all the target genes. Gapdh was used as the endogenous control gene. Briefly, the signal of the reference gene Gapdh was used to normalize the target gene signals of each sample. The amount of target transcripts relative to the calibrator was calculated by $2^{-\Delta\Delta CT}$. Therefore, all target gene transcription was expressed as an *n*-fold difference relative to the calibrator.

PCR Validation

PCR products were analyzed by electrophoresis on an agarose gel and sequenced to confirm the amplification of the proper product. To determine if the primers amplified a single product in a quantitative manner, efficiency levels, correlation coefficients, and melting curves of cDNA at four different dilutions were analyzed by Q-PCR. All the target genes led to efficiencies between 90% and 97% and correlation coefficients of 0.98–1.00.

Methylated DNA Immunolabeling

Levels of Dnm1 enzyme and DNA methylation of fibroblasts were detected by immunolabeling with an anti-Dnmt1 and an anti-5-methylcytidine antibody (Serotec, Raleigh, NC). Dissociated cells were fixed and immunolabeled as previously described by Habib et al. (16). Briefly, samples were permeabilized by incubation in a PBS solution supplemented with 1% BSA and 0.1% Tween 20 (PBST-BSA). Fibroblasts were fixed with 0.25% paraformaldehyde in PBS for 10 min at 37°C followed by the addition of 88% methanol previously cooled at −20°C. After two washes with PBST-BSA at room temperature, samples were treated with 2N HCl at 37°C for 30 min followed by incubation in 0.1 M borate buffer (pH 8.5) for 5 min. Nonspecific binding sites were blocked with 1% BSA in PBS. Samples were incubated with a mouse anti-human DNA methyltransferase (2.5 µg/ml, Abcam, Cambridge, MA) or a mouse anti-5-methylcytidine antibody (1 µg/ml, Serotec) for 30 min at room temperature followed by labeling with a goat anti-mouse IgG conjugated with Alexa Fluor® 488 (40 µg/ml, Molecular Probes, Eugene, OR). Cells were counterstained with 30 µg/ml PI and 10 µg/ml RNase. Cells were then washed and resuspended in PBS. Appropriate staining controls for autofluorescene and nonspecific binding were included. Specificity of the primary antibodies for nuclear epitopes was determined with epifluorescence microscopy and levels of Dnmt1 and DNA methylation were analyzed by flow cytometry.

Levels of Dnmt1 and DNA Methylation

Determination of the relative level of transfection efficiency, Dnmt1 protein, and methylated DNA was performed in a FACSAria (Becton Dickinson Immunocytometry Systems, San Jose, CA). Labeled cells were excited at 488 nm with a 15-mW argon laser. A 610-nm diachronic mirror (beam splitter) and a 610/20-nm band pass filter were used to detect PI levels. Differences in DNA content were used to determine phases of the cell cycle. Levels of transfection efficiency, DNA methylation, and Dnmt1 were detected with a 530/30-nm filter. A minimum of 10,000 events were analyzed per cell line and treatment. Relative levels of fluorescence were reported as arbitrary units of fluorescence (AUF).

Experimental Design

Experiment 1. Cell lines were synchronized in G_0/G_1 by contact inhibition, replated, cultured, and collected 48, 54, 60, 72, 96, and 120 h postseeding (PS) and used to determine the levels of Dnmt1 transcript, Dnmt1 protein, and DNA methylation. Three biological replicates per cell line and time PS were performed.

Experiment 2. Optimal transfection conditions were determined by transfecting a cell line with a 3′ fluorescein-labeled nonsilencing siRNA using different ratios of siRNA to transfection reagent (µg/µl: 1:3, 1:6, and 1:9), as well as siRNA concentrations (0.5, 1.0, and 1.5 µg). Cells were counterstained with PI to analyzed cell viability 24 h posttransfection (PT). Three biological replicates per siRNA to transfection ratio and siRNA concentration were performed.

Experiment 3. Cell lines were transfected with each Dnmt1-specific siRNA and a nonsilencing siRNA using the lipofection conditions as determined in Experiment 1. Cells were collected 6, 12, 24, 48, and 72 h PT and used to determine the abundance of Dnmt1–Dnmt3a and Dnmt3b transcripts, as well as the level of Dnmt1 protein and DNA methylation. Three biological replicates per cell line, siRNA, and time PT were performed.

Statistical Analysis

Data were analyzed using SigmaStat Statistical Software Version 3.5 (Systat Software, Richmond, CA). Variances of the relative levels of methylated DNA, Dnmt transcripts, and protein of all cell lines were calculated by ANOVA. Differences in Dnmt1 and methylation level content between siRNA, cell line, and time PT were statistically analyzed by Tukey's test. In this study $p < 0.05$ was considered significant.

RESULTS

Levels of Dnmt1 and DNA Methylation During Cell Cycle Progression

The specificity of the anti-Dnmt1 antibody was demonstrated by Western blot (WB) and immunolabeling analysis of porcine cells. Incubation of cells with anti-Dnmt1 antibody produced a band of ~180 kDa (WB analysis) and showed specificity for nuclear epitopes (immunolabeling analysis) in porcine cells as predicted (Fig. 1). Incubation of fibroblast cells with anti-5-methylcytidine antibody and Alexa Fluor 488 shows the specificity of this antibody for nuclear structures and its affinity for areas of highly compacted chromatin (Fig. 2).

RNA and flow cytometric analysis of cell lines PF1, PF2, PF3, and PF4 showed that the levels of Dnmt1 and DNA methylation varied significantly between cell lines. PF1 had the highest level of Dnmt1 transcript and methylated DNA. PF2, PF3, and PF4 had 32%, 73%, and 45% less Dnmt1 mRNA and 18%, 66%, and 51% less methylated DNA than PF1, respectively.

Levels of Dnmt1 transcript and DNA methylation changed with time in culture (Fig. 3). Gene expression of Dnmt1 in porcine fibroblasts was higher during the growing phase of culture (first 60 h PS) than in growth-arrested cells (≥ 72 h PS). Likewise, DNA methylation levels decreased significantly after cells reached 100% confluence.

Dnmt1 protein and methylated cytosine content at different stages of the cell cycle were quantified by flow cytometric analysis. Fibroblasts in G_0/G_1 have less methylated cytosines than cells in S and G_2/M phases (1811 ± 264, 3159 ± 520, and 3969 ± 610 AUF, respectively). The levels of Dnmt1 protein were lower during G_0/G_1 (1139 ± 93 AUF) and increased in G_2/M phase (1951 ± 112 AUF).

Optimization of siRNA Transfection

Using a FITC-labeled nonsilencing siRNA, the ratio between siRNA concentration and the liposome, and siRNA concentration were optimized in porcine fibroblast cells. Flow cytometric analysis of porcine fibroblasts 24 h PT demonstrated that the percentage of transfected cells, the level of transfection, and the percentage of dead cells increase significantly when cells were treated with higher concentrations of siRNA or siRNA/liposome ratio (Table 2). Cells transfected with 1.5 μg of labeled control siRNA and 1:9 siRNA/liposome ratio produced the highest percentage of transfected cells (86.3 ± 1.0) and concen-

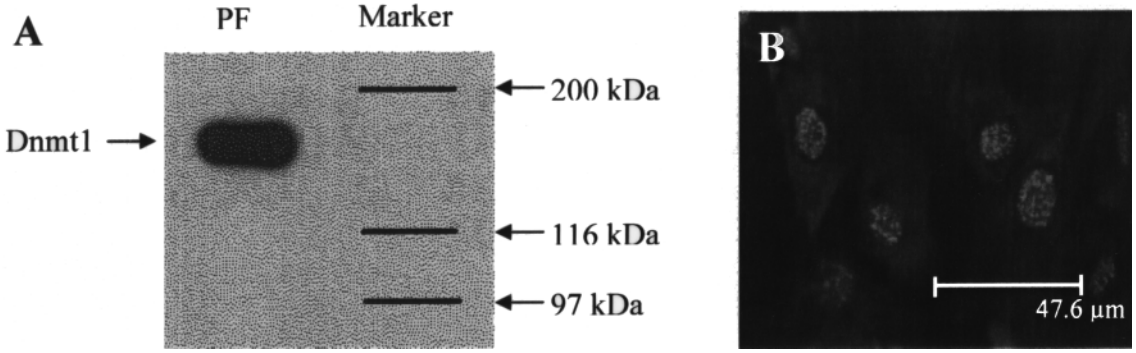

Figure 1. Specificity of the anti-Dnmt1 antibody in porcine fibroblast cells. Analysis of porcine fibroblasts (PF) by Western blot demonstrated that the antibody binds to a protein of the expected molecular weight for Dnmt1 (A). PF cells incubated with the anti-Dnmt1 and labeled with an Alexa Fluor 488 antibody showed, as predicted, the specificity of the antibody for nuclear epitopes (B).

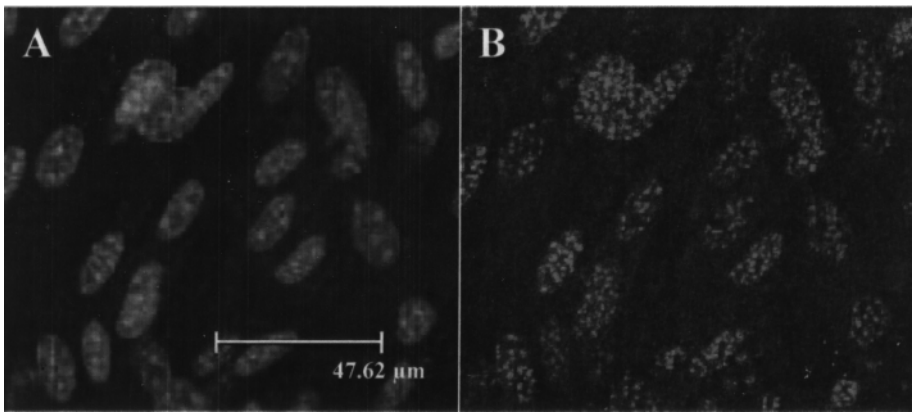

Figure 2. Porcine fibroblasts stained with propidium iodide (A). Cells incubated with anti-5-methylcytidine antibody and labeled with Alexa Fluor 488 (B). The staining pattern shows the specificity of the antibodies for nuclear structures and their affinity for areas of highly compacted chromatin.

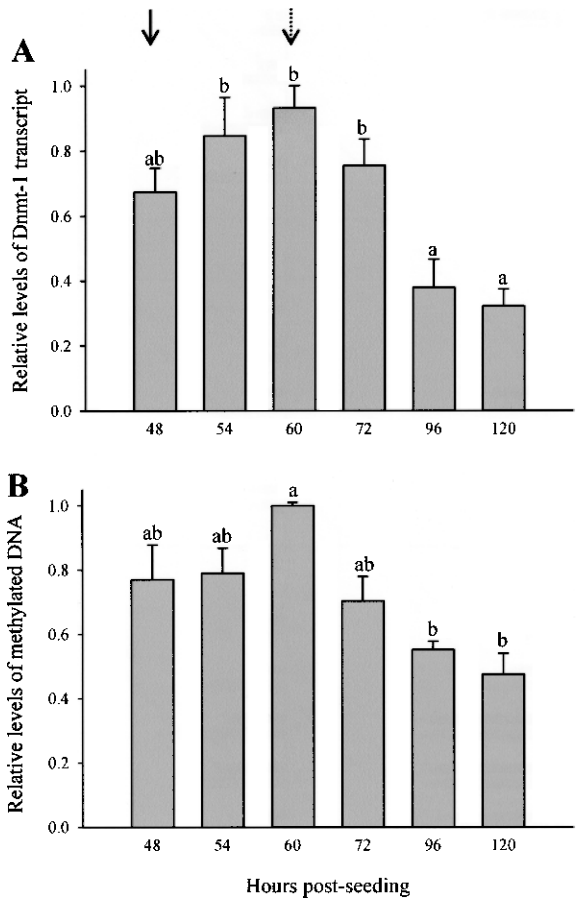

Figure 3. Relative level of Dnmt1 mRNA (A) and methylated DNA in porcine fibroblast cells (B). Different letters indicate significant difference in the level of gene expression of Dnmt1 or DNA methylation after seeding ($p < 0.05$). Solid and dotted arrow indicates that cells reached 80% and 100% confluence, respectively. Bars indicate SEM.

tration of intracytoplasmic FITC-siRNA (1679 ± 42 AUF). However, the percentage of dead cells (17.9 ± 0.1) was higher than in the other treatment groups. Fibroblasts transfected with 1.0 µg (2 nM) of siRNA and 1:6 siRNA/liposome ratio displayed a high transfection efficiency (large percentage of transfected cells with high concentration of intracellular siRNA) and satisfactory percentage of viable cells. Therefore, this siRNA concentration and ratio were used to transfect porcine fibroblast cells with Dnmt1-specific siRNA.

Downregulation of Dnmt1 Transcript by siRNA

Levels of Dnmt1 mRNA after lipofection varied between cell lines and siRNA used. Thus, cell lines seem to respond differently to siRNA treatment (Fig. 4). While the levels of Dnmt1 in cell lines PF1, PF2, and PF4 decreased after lipofection with at least one of the siRNAs used, PF3 appeared to be refractory to siRNA treatments.

All the siRNAs caused a decrease in the level of Dnmt1 in at least one of the four cell lines analyzed. However, some combinations of siRNA/cell line showed a higher reduction of Dnmt1. Lipofection of PF1, PF2, and PF4 with siRNA4, siRNA3, and siRNA2 produced a 77.2%, 43.7%, and 56.82% reduction in the level of Dnmt1 mRNA 6 h PT, respectively. Reduced amounts of Dnmt1 transcript were detected as early as 6 h PT and remained low for up to 48 h in some cell lines.

Levels of Dnmt1 transcript changed with siRNA treatment as well as with time in culture. Cell lines treated with nonsilencing and Dnmt1-specific siRNAs displayed lower amounts of Dnmt1 RNA after reaching 100% confluence. As described earlier, cells synchronized in G_0/G_1 by contact inhibition have less

TABLE 2
TRANSFECTION EFFICIENCY AND CELL VIABILITY OF PORCINE FIBROBLASTS TREATED WITH DIFFERENT FITC-LABELED CONTROL siRNA CONCENTRATIONS AND RATIO BETWEEN siRNA CONCENTRATION AND LIPOSOME

siRNA (μg)	siRNA/liposome (μg/μl)	Transfected Cells ± SEM (%)	Transfection Level ± SEM (AUF)	Dead Cells ± SEM (%)
0.5	1:3	42.1 ± 1.4^{a1}	670 ± 8^{a1}	2.0 ± 0.2^{a1}
	1:6	61.6 ± 3.1^{b1}	831 ± 16^{b1}	3.0 ± 0.2^{a1}
	1:9	68.9 ± 0.5^{b1}	1014 ± 2^{c1}	6.7 ± 1.0^{b1}
1.0	1:3	67.3 ± 1.3^{a2}	908 ± 29^{a2}	3.5 ± 0.1^{a1}
	1:6	84.1 ± 0.2^{b2}	1443 ± 16^{b2}	9.3 ± 0.7^{b2}
	1:9	77.8 ± 2.1^{ab1}	1310 ± 35^{c2}	18.2 ± 2.1^{c2}
1.5	1:3	74.3 ± 0.6^{a3}	1137 ± 18^{a3}	11.6 ± 1.3^{a2}
	1:6	$76.2 \pm 2.0^{a1,2}$	1343 ± 33^{b2}	13.5 ± 3.7^{a2}
	1:9	86.3 ± 1.0^{b2}	1679 ± 42^{c3}	17.9 ± 0.1^{a2}

AUF, arbitrary units of fluorescence.[a,b,c]Significant difference between siRNA/liposome ratio at every given siRNA concentration.[1,2,3]Significant difference between siRNA concentration at every given ratio.

Dnmt1 content than cells in the other phases of the cell cycle.

Levels of Dnmt3a and Dnmt3b RNA After siRNA Transfection

The level of Dnmt3a and Dnmt3b RNA did not change significantly between the cell lines analyzed. In porcine fibroblast cells, Dnmt3a transcript was approximately 10 times more abundant than Dnmt3b RNA. Cells treated with Dnmt1-specific siRNA had similar levels of Dnmt3a and Dnmt3b than cells transfected with the nonspecific siRNA (Fig. 5).

Levels of Dnmt1 Protein After siRNA Transfection

Several treatment combinations effectively downregulated the gene expression of Dnmt1; however, only cells with 50% or less of the normal level of this transcript had lower levels of Dnmt1 protein than untreated cells. Moreover, dramatic reductions in the level of Dnmt1 RNA resulted in only a discrete reduction of the protein. Lipofection of PF1, PF2, and PF4 with siRNA4, siRNA3, and siRNA2 led to a 18.8%, 16.0%, and 24.7% reduction in the level of Dnmt1 protein 6 h PT, respectively. The concentration of Dnmt1 remained low for up to 24 h PT. Cells treated with Dnmt1-specific and nonsilencing siRNAs displayed similar levels of Dnmt1 protein 48 h PT.

DNA Methylation Levels After Treatment With Dnmt1-Specific siRNA

Levels of methylated DNA changed with time in culture in siRNA-treated and control cells. Lower levels of methylation were detected after cells reached 100% confluence and were synchronized in G_0/G_1 phase of the cell cycle.

Cells exhibiting reduced levels of Dnmt1 protein after siRNA treatment were also characterized by hypomethylated DNA (Fig. 6). PF1/siRNA4 and PF4/siRNA2 had lower levels of methylated DNA after 6 (25.7% and 34.8%, respectively) and 12 h PT (37.1% and 24.5%, respectively) than cells transfected with the nonsilencing siRNA. After 24 h PT, the methylation levels of these cells were reestablished or slightly increased. When PF2 was treated with siRNA3, a hypomethylation pattern was detected for 48 h PT. The level of DNA methylation was reduced by a 27.8%, 39.6%, 31.9%, and 49.4% at 6, 12, 24, and 48 h PT, respectively.

DISCUSSION

RNAi technology has been used successfully to silence specific genes in plants, invertebrates and mammals. However, for a siRNA to cause sequence-specific gene silencing, technical challenges need to be overcome (47). The challenges include developing efficient ways of designing, identifying, and delivering effective siRNAs to cells, improving the accuracy with which susceptible sites in the target RNA molecules can be identified, and obtaining inducible tissue- and cell-specific regulation of siRNA without causing nonspecific or toxic effects to the cells (30).

Because the effectiveness of siRNA treatments varies between cell types and species, the optimization of a protocol to transfect siRNA into porcine fibroblast cells is essential to achieve significant downregulation of specific genes (1,18). Under the transfection conditions used in this study, the percentage of transfected cells, transfection efficiency, and cell viability were dependent upon siRNA and liposome concentration. Transfection of porcine cells with elevated concentrations of siRNA and liposome caused high transfection efficiency but lower cell via-

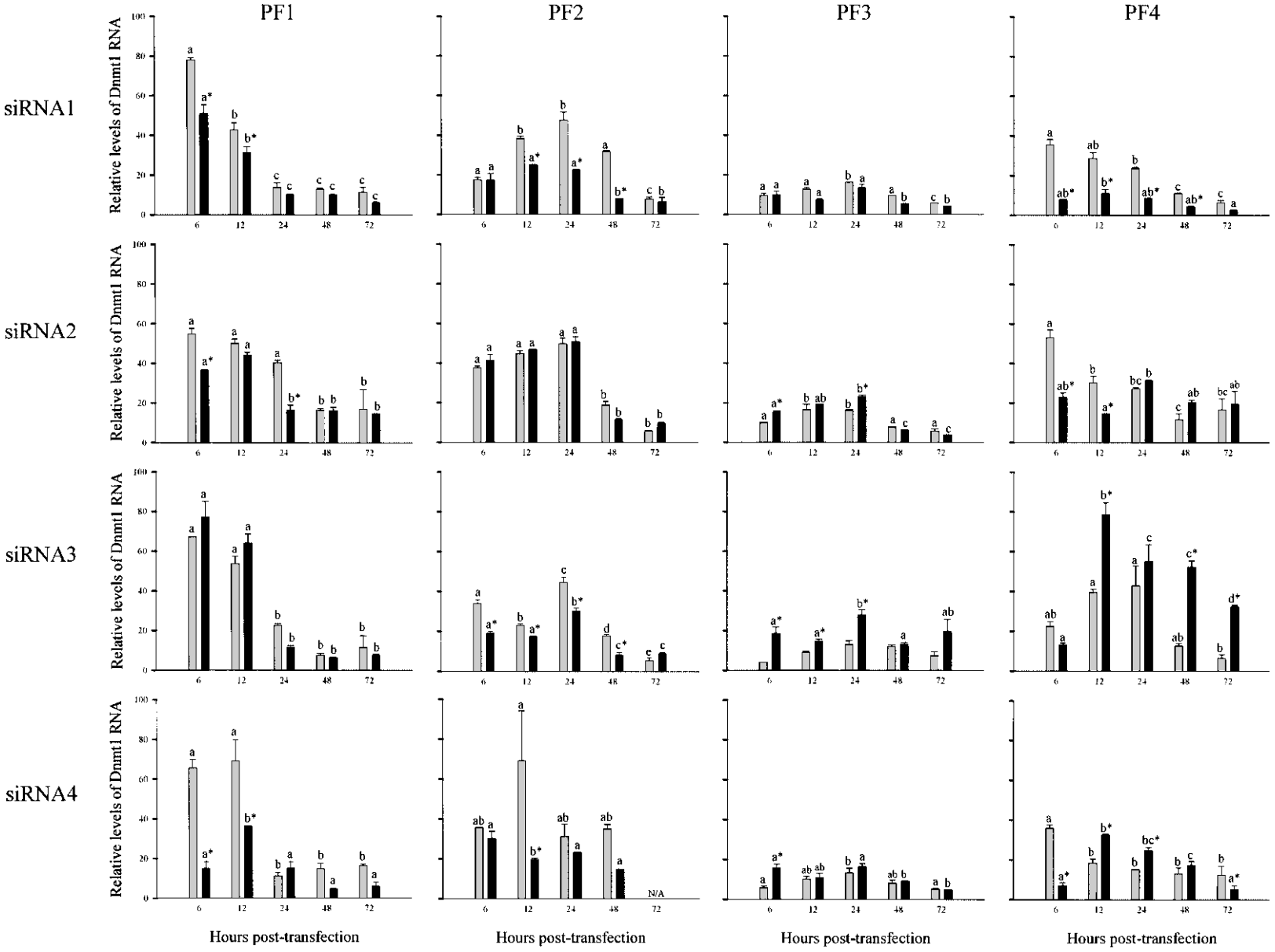

Figure 4. Gene expression of Dnmt1 in PF1, PF2, PF3, and PF4 transfected with siRNA1, siRNA2, siRNA3, or siRNA4. Gray and black columns represent the level of gene expression of Dnmt1 in control and siRNA-treated cells, respectively. Different letters indicate significant difference in the expression level of Dnmt1 over time. An asterisk indicates significant difference in the level of Dnmt1 between cells transfected with specific or nonsilencing siRNA at every given time point ($p < 0.05$). Bars indicate SEM.

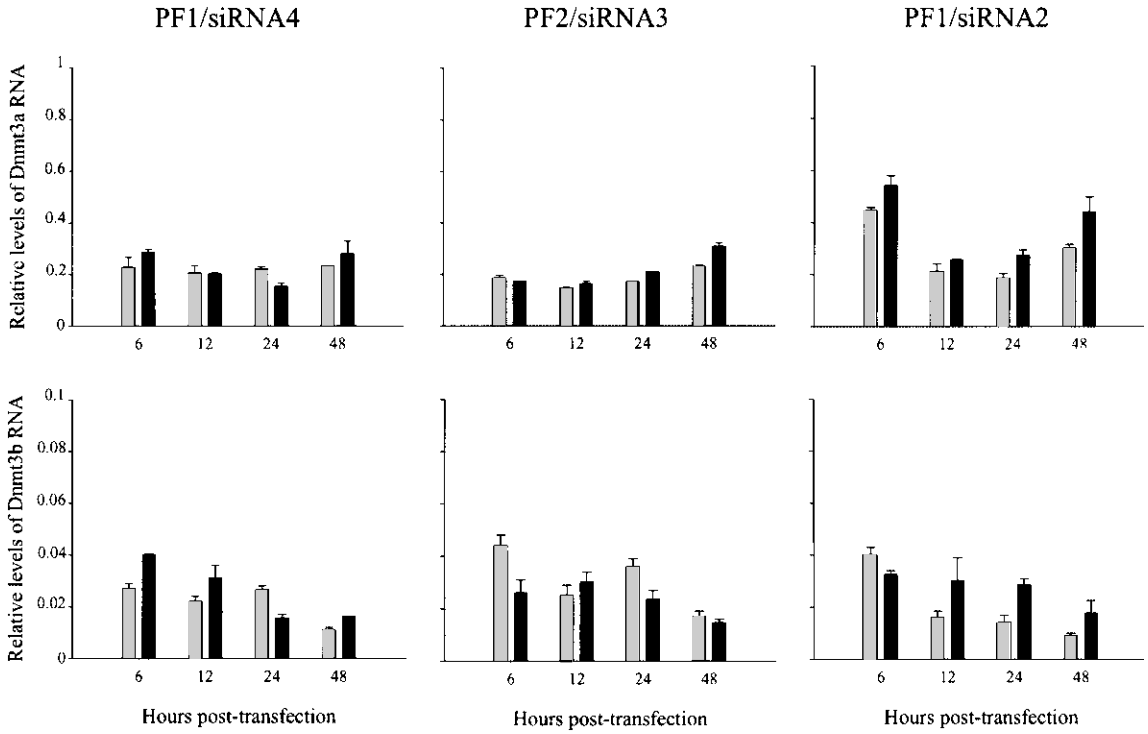

Figure 5. Relative levels of Dnmt3a and Dnmt3b RNA of PF1, PF2, and PF4 transfected with siRNA4, siRNA3, and siRNA2, respectively. Gray and black columns represent the relative level of the transcript in control and siRNA-treated cells, respectively. Different letters indicate significant difference in the level of the transcript between cells transfected with specific or nonsilencing siRNA at every given time point ($p < 0.05$). Bars indicate SEM.

bility. Previous gene silencing experiments in mammalian cells have used siRNAs at varying concentrations, typically ranging from 1 to 200 nM (32,49). However, cells treated with high concentrations of siRNA sequences display abnormal gene expression of nontargeted genes and cell death (32). Additionally, Sledz et al. reported that some interferon-stimulated genes are induced in a concentration-dependent manner (38). Although RNAi mechanism itself is independent of the interferon system, transfection of 21-bp siRNAs does trigger an interferon response through protein kinase R (PKR), a double-stranded RNA-dependent serine-threonine protein kinase, resulting in a generalized suppression of protein syn-

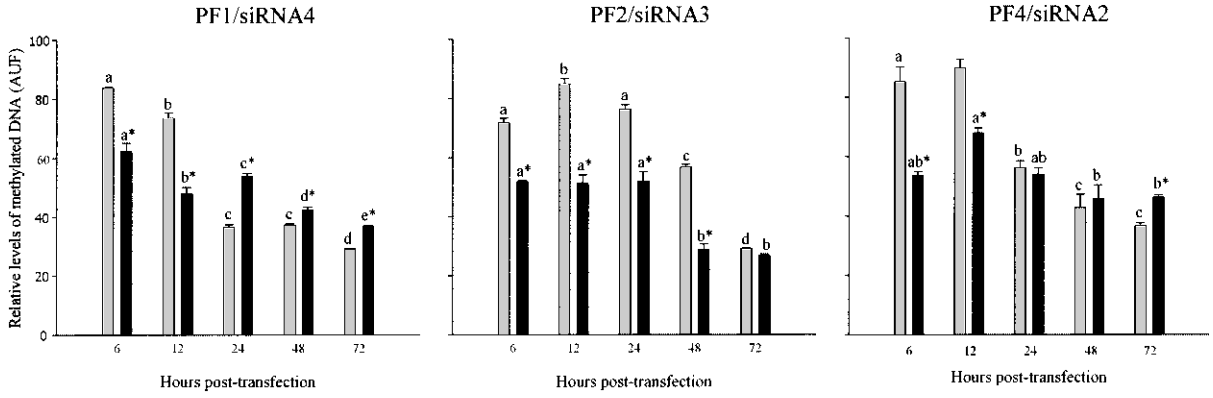

Figure 6. Relative levels of DNA methylation of PF1, PF2, and PF4 transfected with siRNA4, siRNA3, and siRNA2, respectively. Gray and black columns represent the DNA methylation level of control and siRNA-treated cells, respectively. Different letters indicate significant difference in the level of methylated DNA over time. An asterisk indicates significant difference in level of DNA methylation between cells transfected with specific or nonsilencing siRNA at every given time point ($p < 0.05$). Bars indicate SEM. AUF, arbitrary units of fluorescence.

thesis, followed by cell death via apoptotic and nonapoptotic pathways (4,53).

Moreover, high concentrations of liposomal delivery reagents, used for transfection of siRNA, can alter nonspecific gene expression and cause lipid-mediated toxicity (12,30,47). Thus, these studies argue that elevated concentrations of siRNA and liposome may have broad and complicating effects beyond the selective silencing of target genes when introduced into cells (4). These considerations underscore the importance of determining the optimal transfection conditions, so that high transfection efficiency and low cell mortality can be achieve, using minimal siRNA and liposome concentrations to avoid nonspecific effects and cell mortality.

Studies indicate that regardless of optimized transfection conditions, only a limited fraction of siRNAs appear capable of producing an effective reduction of specific gene expression in mammalian cells (44). In this study, the gene expression level of Dnmt1 varied between the siRNAs tested and porcine fibroblast cell lines used. The biological activity of the siRNAs tested in this experiment may be influenced by local characteristics of the target RNA and cellular resistance to siRNA (22,24,30,51).

The low activity of some siRNAs may be due to nonaccessibility of mRNA for cleavage, caused by higher order structures or protein coverage (8). Additionally, the GC content of a particular target gene region might affect its general accessibility to siRNA indirectly through the effect on protein coverage or mRNA unwinding (20). Thus, the use of different Dnmt1-specific siRNAs, targeting different regions of the mRNA, increases the probability of finding a highly effective siRNA.

Additionally, siRNA resistance as a mechanism to counteract RNAi-mediated gene silencing has been previously described in mammalian cells. dsRNA induces the homology-dependent degradation of cognate mRNA in the cytoplasm via RNAi, but it is also a target for adenosine-to-inosine RNA editing by adenosine deaminases acting on RNA (ADARs) (37). Gene silencing by siRNA is significantly more effective in transgenic mouse fibroblasts homozygous for the ADAR null mutation than in wild-type cells. This observation was further supported by the suppression of RNAi in fibroblast cells overexpressing functional ADAR, but not in cells overexpressing mutant ADAR lacking double-stranded RNA-binding domains. The results provide convincing evidence that ADAR is a cellular factor that limits the efficacy of siRNA in mammalian cells (51).

Although differences in siRNA effectiveness between species and cell types have been previously reported, possible differences in the gene expression level of the targeted gene between cell lines of the same origin have not been analyzed (1,18). Our results indicate that some porcine cell lines appear to be responsive to certain siRNAs but resistant to others. Differences in the endogenous concentration of ADAR between species or cell types, as well as individual cell lines, may explain the different gene expression level of Dnmt1 after treatment with the Dnmt1-specific siRNAs in the four cell lines analyzed in this study. The ability to accurately predict RNA secondary structures, as well as greater understanding of the siRNA resistance process, will reveal new mechanistic insights that can be exploited for the design of highly effective siRNAs.

Somatic cells require a minimum basal level of Dnmt1 to stabilize the genome, regulate replication, and methylate imprinted genes. Complete depletion of this transcript would result in cell death (3,45,50). Therefore, the objective of this study was to partially downregulate the level of Dnmt1, in a transient manner, rather than induce a total reduction of Dnmt1.

Of essential consideration in any knockdown experiment is the protein's half-life, its abundance, and the regulation of its expression (9). In differentiated cells the apparent half-life of the Dnmt1 mRNA is 5 h (28). Thus, the effect of siRNAs specific for Dnmt1 should be observed shortly after transfection. In this study, downregulation of Dnmt1 transcript was detected 6 h PT and remained low for up to 48 h PT before gradually increasing, in what appeared to be cell expansion dependent as a consequence of dilution of the siRNA with every division.

The porcine cell lines analyzed in this study displayed significantly different levels of Dnmt1, suggesting that, as proposed by Liu et al. (28), Dnmt1 transcript is produced in excessive amounts to guarantee the fidelity of the methylation pattern during normal cell division. However, only a minimum concentration of Dnmt1 may be sufficient to preserve the methylation patterns characteristic of somatic cells (39). Interestingly, PF3, which displayed the lowest endogenous level of Dnmt1, was refractory to all the siRNAs tested, while the remaining cell lines underwent significant reduction of Dnmt1. These results may indicate that cells with high native levels of Dnmt1 can undergo a significant reduction of Dnmt1 without affecting the steady-state level of Dnmt1 transcript or Dnmt1 protein.

The activity of Dnmt1 shows marked changes as cells pass through the cell division cycle, with high levels being expressed in the G_2/M phase and low levels in G_0/G_1 (21,48). Cells synchronized in G_0/G_1 either by differentiation, low serum conditions, or

contact inhibition display reduced levels of Dnmt1 mRNA and Dnmt1 protein, while the transcription of Dnmt1 was shown to remain relatively active in cycling cells (28,42). These results demonstrated that the steady-state level of Dnmt1 mRNA is tightly regulated with the proliferative state of the cell (41). In siRNA experiments, caution should be taken to analyze cells at the appropriate growth phase because a reduction in the level of Dnmt1 due to synchronization of cells in G_2/M phase can be confused with downregulation of Dnmt1 mediated by direct effects of the siRNA (53).

Previous studies have reported a reduction in the level of Dnmt1 protein after inducing downregulation of the transcript in transformed, immortal, and cancer cell lines (13,26,40). However, not all the Dnmt1 mRNA knockdown studies have reported reduction of the protein or DNA methylation (1,35,43). The results of this study indicate that discrete downregulation of Dnmt1 mRNA may not overcome the steady-state level of Dnmt1 transcript necessary to cause a decrease in the protein level. Moreover, a drastic reduction of Dnmt1 mRNA resulted in only a slight decrease in protein production. However, this small reduction in the protein concentration induced significant hypomethylation of the genome. These data suggest that although Dnmt1 mRNA abundance plays an important role during protein regulation, the Dnmt1 enzyme is mainly posttranscriptionally regulated.

Additionally, studies by Rhee et al. suggest that the de novo Dnmts may be involved in the maintenance of methylation levels in somatic cells (36). Thus, Dnmt3a and Dnmt3b may compensate for the lack of Dnmt1 and perpetuate the methylation levels after Dnmt1 siRNA treatment. However, in this study the levels of Dnmt3a and Dnmt3b did not increase when the RNA levels of Dnmt1 decreased.

The use of alternative strategies may be essential for the efficient reduction of DNA methylation in porcine fibroblast cells. Multiple rounds of siRNA treatments or the use of a mixture of siRNAs against the same target sequence may decrease the expression of Dnmt1 and the subsequent methylation levels.

In conclusion, the efficiency of RNAi treatments depends on a variety of factors including siRNA sequence, cytoplasmic concentration of siRNA, accessibility of the siRNA to the targeted RNA, and cell line transfected. The use of highly effective Dnmt1-specific siRNAs resulted in a drastic reduction of the Dnmt1 transcript and a modest loss of the Dnmt1 enzyme. Moreover, porcine fibroblast cells displaying slightly lower levels of Dnmt1 protein had significantly reduced levels of hypomethylated DNA, suggesting that this enzyme is mainly posttranscriptionally regulated. Subsequent use of these cells for SCNT, differentiation, and cancer studies will provide insight as to how methylation of the DNA affects reprogramming.

REFERENCES

1. Adams, A. M.; Pratt, S. L.; Stice, S. L. Knockdown of the Dnmt1s transcript using small interfering RNA in primary murine and bovine fibroblast cells. Mol. Reprod. Dev. 72:311–319; 2005.
2. Bestor, T. H. The DNA methyltransferases of mammals. Hum. Mol. Genet. 9:2395–2402; 2000.
3. Biniszkiewicz, D.; Gribnau, J.; Ramsahoye, B.; Gaudet, F.; Eggan, K.; Humpherys, D.; Mastrangelo, M. A.; Jun, Z.; Walter, J.; Jaenisch, R. Dnmt1 overexpression causes genomic hypermethylation, loss of imprinting, and embryonic lethality. Mol. Cell. Biol. 22: 2124–2135; 2002.
4. Bridge, A. J.; Pebernard, S.; Ducraux, A.; Nicoulaz, A. L.; Iggo, R. Induction of an interferon response by RNAi vectors in mammalian cells. Nat. Genet. 34: 263–264; 2003.
5. Cezar, G. G. Epigenetic reprogramming of cloned animals. Cloning Stem Cells 5:165–180; 2003.
6. Chomczynski, P.; Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156–159; 1987.
7. Chung, Y. G.; Ratnam, S.; Chaillet, J. R.; Latham, K. E. Abnormal regulation of DNA methyltransferase expression in cloned mouse embryos. Biol. Reprod. 69:146–153; 2003.
8. Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411:494–498; 2001.
9. Elbashir, S. M.; Harborth, J.; Weber, K.; Tuschl, T. Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods 26:199–213; 2002.
10. Enright, B. P.; Kubota, C.; Yang, X.; Tian, X. C. Epigenetic characteristics and development of embryos cloned from donor cells treated by trichostatin A or 5-aza-2′-deoxycytidine. Biol. Reprod. 69:896–901; 2003.
11. Enright, B. P.; Sung, L. Y.; Chang, C. C.; Yang, X.; Tian, X. C. Methylation and acetylation characteristics of cloned bovine embryos from donor cells treated with 5-aza-2′-deoxycytidine. Biol. Reprod. 72:944–948; 2005.
12. Fedorov, Y.; King, A.; Anderson, E.; Karpilow, J.; Ilsley, D.; Marshall, W.; Khvorova, A. Different delivery methods-different expression profiles. Nat. Methods 2: 241; 2005.
13. Gaudet, F.; Hodgson, J. G.; Eden, A.; Jackson-Grusby,

L.; Dausman, J.; Gray, J. W.; Leonhardt, H.; Jaenisch, R. Induction of tumors in mice by genomic hypomethylation. Science 300:489–492; 2003.
14. Geiman, T. M.; Robertson, K. D. Chromatin remodeling, histone modifications, and DNA methylation—how does it all fit together? J. Cell. Biochem. 87:117–125; 2002.
15. Giraldo, A. M.; Lynn, J. W.; Purpera, M. N.; Godke, R. A.; Bondioli, K. R. Inhibition of DNA methyltransferase 1 expression in boine fibroblast cells for nuclear transfer. Reprod. Fertil. Dev. 20:95–96; 2008.
16. Habib, M.; Fares, F.; Bourgeois, C. A.; Bella, C.; Bernardino, J.; Hernandez-Blazquez, F.; de Capoa, A.; Niveleau, A. DNA global hypomethylation in EBV-transformed interphase nuclei. Exp. Cell Res. 249:46–53; 1999.
17. Haraguchi, S.; Saga, Y.; Naito, K.; Inoue, H.; Seto, A. Specific gene silencing in the pre-implantation stage mouse embryo by an siRNA expression vector system. Mol. Reprod. Dev. 68:17–24; 2004.
18. Harborth, J.; Elbashir, S. M.; Vandenburgh, K.; Manninga, H.; Scaringe, S. A.; Weber, K.; Tuschl, T. Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. Antisense Nucleic Acid Drug Dev. 13:83–105; 2003.
19. Hiendleder, S.; Mund, C.; Reichenbach, H. D.; Wenigerkind, H.; Brem, G.; Zakhartchenko, V.; Lyko, F.; Wolf, E. Tissue-specific elevated genomic cytosine methylation levels are associated with an overgrowth phenotype of bovine fetuses derived by in vitro techniques. Biol. Reprod. 71:217–223; 2004.
20. Holen, T.; Amarzguioui, M.; Wiiger, M. T.; Babaie, E.; Prydz, H. Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. Nucleic Acids Res. 30:1757–1766; 2002.
21. Juttermann, R.; Li, E.; Jaenisch, R. Toxicity of 5-aza-2′-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. Proc. Natl. Acad. Sci. USA 91:11797–11801; 1994.
22. Kawasaki, H.; Suyama, E.; Iyo, M.; Taira, K. siRNAs generated by recombinant human Dicer induce specific and significant but target site-independent gene silencing in human cells. Nucleic Acids Res. 31:981–987; 2003.
23. Koukalova, B.; Fojtova, M.; Lim, K. Y.; Fulnecek, J.; Leitch, A. R.; Kovarik, A. Dedifferentiation of tobacco cells is associated with ribosomal RNA gene hypomethylation, increased transcription, and chromatin alterations. Plant Physiol. 139:275–286; 2005.
24. Kretschmer-Kazemi, F. R.; Sczakiel, G. The activity of siRNA in mammalian cells is related to structural target accessibility: A comparison with antisense oligonucleotides. Nucleic Acids Res. 31:4417–4424; 2003.
25. Lei, H.; Oh, S. P.; Okano, M.; Juttermann, R.; Goss, K. A.; Jaenisch, R.; Li, E. De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. Development 122:3195–3205; 1996.
26. Leu, Y. W.; Rahmatpanah, F.; Shi, H.; Wei, S. H.; Liu, J. C.; Yan, P. S.; Huang, T. H. Double RNA interference of Dnmt3b and Dnmt1 enhances DNA demethylation and gene reactivation. Cancer Res. 63:6110–6115; 2003.
27. Li, E.; Bestor, T. H.; Jaenisch, R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69:915–926; 1992.
28. Liu, Y.; Sun, L.; Jost, J. In differentiating mouse myoblasts DNA methyltransferase is posttranscriptionally and posttranslationally regulated. Nucleic Acids Res. 24:2718–2722; 1996.
29. Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402–408; 2001.
30. Mittal, V. Improving the efficiency of RNA interference in mammals. Nat. Rev. Genet. 5:355–365; 2004.
31. Okano, M.; Bell, D. W.; Haber, D. A.; Li, E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99:247–257; 1999.
32. Persengiev, S. P.; Zhu, X.; Green, M. R. Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). RNA 10:12–18; 2004.
33. Ratnam, S.; Mertineit, C.; Ding, F.; Howell, C. Y.; Clarke, H. J.; Bestor, T. H.; Chaillet, J. R.; Trasler, J. M. Dynamics of Dnmt1 methyltransferase expression and intracellular localization during oogenesis and preimplantation development. Dev. Biol. 245:304–314; 2002.
34. Reich, S. J.; Fosnot, J.; Kuroki, A.; Tang, W.; Yang, X.; Maguire, A. M.; Bennett, J.; Tolentino, M. J. Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. Mol. Vis. 9:210–216; 2003.
35. Rhee, I.; Jair, K. W.; Yen, R. W.; Lengauer, C.; Herman, J. G.; Kinzler, K. W.; Vogelstein, B.; Baylin, S. B.; Schuebel, K. E. CpG methylation is maintained in human cancer cells lacking Dnmt1. Nature 404:1003–1007; 2000.
36. Rhee, I.; Bachman, K. E.; Park, B. H.; Jair, K. W.; Yen, R. W.; Schuebel, K. E.; Cui, H.; Feinberg, A. P.; Lengauer, C.; Kinzler, K. W.; Baylin, S. B.; Vogelstein, B. Dnmt1 and Dnmt3b cooperate to silence genes in human cancer cells. Nature 416:552–556; 2002.
37. Scadden, A. D.; Smith, C. W. RNAi is antagonized by A → I hyper-editing. EMBO Rep. 2:1107–1111; 2001.
38. Sledz, C. A.; Holko, M.; de Veer, M. J.; Silverman, R. H.; Williams, B. R. Activation of the interferon system by short-interfering RNAs. Nat. Cell. Biol. 5:834–839; 2003.
39. Spada, F.; Haemmer, A.; Kuch, D.; Rothbauer, U.; Schermelleh, L.; Kremmer, E.; Carell, T.; Langst, G.; Leonhardt, H. Dnmt1 but not its interaction with the replication machinery is required for maintenance of DNA methylation in human cells. J. Cell Biol. 176: 565–571; 2007.
40. Suzuki, M.; Sunaga, N.; Shames, D. S.; Toyooka, S.;

Gazdar, A. F.; Minna, J. D. RNA interference-mediated knockdown of DNA methyltransferase 1 leads to promoter demethylation and gene re-expression in human lung and breast cancer cells. Cancer Res. 64: 3137–3143; 2004.
41. Szyf, M.; Bozovic, V.; Tanigawa, G. Growth regulation of mouse DNA methyltransferase gene expression. J. Biol. Chem. 266:10027–10030; 1991.
42. Teubner, B.; Schulz, W. A. Regulation of DNA methyltransferase during differentiation of F9 mouse embryonal carcinoma cells. J. Cell. Physiol. 165:284–290; 1995.
43. Ting, A. H.; Jair, K. W.; Suzuki, H.; Yen, R. W.; Baylin, S. B.; Schuebel, K. E. CpG island hypermethylation is maintained in human colorectal cancer cells after RNAi-mediated depletion of Dnmt1. Nat. Genet. 36:582–584; 2004.
44. Ui-Tei, K.; Naito, Y.; Takahashi, F.; Haraguchi, T.; Ohki-Hamazaki, H.; Juni, A.; Ueda, R.; Saigo, K. Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucleic Acids Res. 32:936–948; 2004.
45. Unterberger, A.; Andrews, S. D.; Weaver, I. C.; Szyf, M. DNA methyltransferase 1 knockdown activates a replication stress checkpoint. Mol. Cell. Biol. 26: 7575–7586; 2006.
46. Vogel, M. C.; Papadopoulos, T.; Muller-Hermelink, H. K.; Drahovsky, D.; Pfeifer, G. P. Intracellular distribution of DNA methyltransferase during the cell cycle. FEBS Lett. 236:9–13; 1998.
47. Walters, D. K.; Jelinek, D. F. The effectiveness of double-stranded short inhibitory RNAs (siRNAs) may depend on the method of transfection. Antisense Nucleic Acid Drug Dev. 12:411–418; 2002.
48. Wolffe, A. P.; Matzke, M. A. Epigenetics: Regulation through repression. Science 286:481–486; 1999.
49. Wu, H.; Hait, W. N.; Yang, J. M. Small interfering RNA-induced suppression of MDR1 (P-glycoprotein) restores sensitivity to multidrug-resistant cells. Cancer Res. 6:1515–1519; 2003.
50. Xu, G. L.; Bestor, T. H.; Bourc'his, D.; Hsieh, C. L.; Tommerup, N.; Bugge, M.; Hulten, M.; Qu, X.; Russo, J. J.; Viegas-Pequignot, E. Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature 402:187–191; 1999.
51. Yang, W.; Wang, Q.; Howell, K. L.; Lee, J. T.; Cho, D. S.; Murray, J. M.; Nishikura, K. ADAR1 RNA deaminase limits short interfering RNA efficacy in mammalian cells. J. Biol. Chem. 280:3946–3953; 2005.
52. Yeo, S.; Jeong, S.; Kim, J.; Han, J. S.; Han, Y. M.; Kang, Y. K. Characterization of DNA methylation change in stem cell marker genes during differentiation of human embryonic stem cells. Biochem. Biophys. Res. Commun. 359:536–542; 2007.
53. Zheng, Z. M.; Tang, S.; Tao, M. Development of resistance to RNAi in mammalian cells. Ann. NY Acad. Sci. 1058:105–118; 2005.