Knockdown of the Dnmt1s Transcript Using Small Interfering RNA in Primary Murine and Bovine Fibroblast Cells

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ABSTRACT RNA interference (RNAi) has rapidly developed into one of the most widely applied technologies in molecular and cellular research, and although young, is now an essential experimental tool. The versatility of RNAi, especially in mammalian species, lends to its potential applications in a wide array of fields. Without having to genetically manipulate the genome, the ability to selectively reduce the level of a specific transcript using small interfering RNA (siRNA) molecules has great appeal in studying reprogramming issues in somatic cell nuclear transfer (SCNT) embryos. In such embryos, the aberrant expression of the somatic isoform of Dnmt1 (Dnmt1s), the enzyme responsible for maintaining DNA methylation in all somatic cells, has been implicated as one factor in the improper reprogramming of the donor genome. In the present study, the ability to develop a method allowing for the knockdown, or reduction, of Dnmt1s in primary fibroblast cells, like those commonly used as karyoplast donors in SCNT studies, was investigated in primary murine and bovine fibroblast cells as well as in a compromised cell line (NIH/3T3). Two Dnmt1s-specific siRNA candidates were designed and tested. Using optimized conditions, these siRNAs were transiently transfected into the cells with total RNA and nuclear protein being collected. A 56.5% knockdown in Dnmt1s was achieved in the compromised and primary murine cells whereas Dnmt1s was reduced by 15.4% in the primary bovine cells. A reduction in Dnmt1s mRNA did not correspond to a reduction in protein as determined by immunodetection of Western blots. Overall, this study demonstrated the ability of siRNA to knockdown Dnmt1s mRNA in primary fibroblast donor cells. In order to substantially increase the efficiency while decreasing the anomalies seen in SCNT, novel techniques, like the one proposed, are needed to assist the oocyte's ability to reprogram a differentiated genome. *Mol. Reprod. Dev. 72: 311–319, 2005.* © 2005 Wiley-Liss, Inc.

Key Words: DNA methyltransferase; Dnmt1; siRNA; somatic cell nuclear transfer; SCNT; methylation; reprogramming

INTRODUCTION

Small interfering RNAs (siRNAs), duplexes of 21-nt RNA molecules which induce RNA interference (RNAi) in mammalian cells, have become a powerful tool used to trigger gene-specific silencing in numerous model organisms (Hannon, 2002; Scherr et al., 2003). Induction of the RNAi pathway by siRNA induces a cascade of events leading to the sequence-specific, post-transcriptional degradation of a targeted gene product. Discovered in 2001 (Caplen et al., 2001; Elbashir et al., 2001), siRNAs have the ability to reduce, and in some cases virtually eliminate, an mRNA sequence from a cellular host; something classically achieved previously only through genetic manipulations.

The incorporation of such a technique into a research project involves, but is not limited to, the following variables: (i) designing a functional siRNA targeted exclusively to the mRNA transcript of interest, (ii) synthesizing or constructing the siRNA, (iii) selecting and optimizing its delivery, and (iv) monitoring the knock-down efficiency and/or the cellular response to gene silencing (Elbashir et al., 2002). Research indicates the knock-down of a gene product is variable and may depend on the stability, efficacy, efficiency, and potency of each siRNA (McManus and Sharp, 2002; Gong and Ferrell, 2004; Reynolds et al., 2004).

When carried out in primary cells, siRNA studies are challenging as such cells are more refractory than many commonly used immortalized cell lines (Rubinson et al., 2003). The need to use primary cells in various research areas is desirable as they more closely resemble the situation in the living organism. Thus, their use for gene silencing experiments may accelerate and increase the value for applications such as target validation, gene discovery, or gene therapeutic approaches and may

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lead to new discoveries in gene-specific siRNA-based therapies.

As a whole, RNAi through the use of siRNAs in mammalian cells is an invaluable tool which has tremendous potential and versatility in a vast array of fields (Buckingham et al., 2004; Dorsett and Tuschl, 2004). A yet untapped area is in the field of somatic cell nuclear transfer (SCNT): the asexual process of generating offspring genetically identical to a single parental unit. Though successful, the low efficiencies (between 0% and 5% depending on species), persistent anomalies (Young et al., 1998; Farin et al., 2004), and perinatal death associated with the current technique of SCNT-derived offspring are thought to result from the lack of, or inadequate, epigenetic reprogramming of the donor genome (Rideout et al., 2001; Dean et al., 2003; Shi et al., 2003; Wrenzycki and Niemann, 2003). One such epigenetic modification which must be reprogrammed during embryogenesis, and thus also in SCNT-derived embryos, is the methylation status of the genome.

DNA methyltransferase 1 (Dnmt1) is the enzyme responsible for maintaining DNA methylation patterns and may contribute to the aberrant methylation status and resulting gene expression failures inherent in SCNT-derived embryos (Bestor, 2000; Reik et al., 2001; Ratnam et al., 2002; Gao and Latham, 2004). As shown in the mouse, SCNT embryos, in contrast to fertilized embryos, aberrantly express the somatic form of Dnmt, Dnmt1s, which has precociously been found in the nuclei (Chung et al., 2003) and may contribute to the inadequate passive demethylation which occurs in such embryos (Bourc'his et al., 2001; Dean et al., 2001). Elevated levels of Dnmt1 can cause genomic hypermethylation, loss of imprinting, and embryonic lethality (Vertino et al., 1996; Biniszkiewicz et al., 2002; Etoh et al., 2004). Thus, the abundant and precocious expression of Dnmt1s may account for the hypermethylation and lack of proper demethylation observed in SCNT embryos. Therefore, developing a method to reduce the level of the somatically expressed Dnmt1s enzyme in a donor cell, such as a primary fibroblast cell, is essential for future studies looking into the effects Dnmt1s has on the efficiencies of SCNT.

Here, we investigated the siRNA-induced knockdown of Dnmt1s in primary murine and bovine fibroblast cells in addition to an easily transfectable compromised murine cell line. The condition in which to perform transient transfections was optimized for each cell type using a nonsilencing control siRNA. Using two siRNAs, specific for different regions on the Dnmt1 transcript, transient transfections were conducted using the said optimized conditions. We show that the siRNA technology was effective in reducing the expression level of Dnmt1s at the mRNA level in all cell lines examined, but no observable reduction at the protein level was observed across all groups and time points in this study. To our knowledge, this is the first report demonstrating the mRNA abundance of Dnmt1s can be altered in a primary fibroblast cell type.

MATERIALS AND METHODS

siRNA Design

siRNAs were synthetically produced corresponding to the Dnmt1s transcript. Target sights were selected by aligning the murine (Locus# NM_010066), bovine (Locus# NM_182651), and ovine (Locus# 29536010) cDNA sequences (found at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene) using CLUSTLW (Biology Workbench; http://workbench.sdsc.edu/). Two homologous regions between the aligned sequences were selected following standard siRNA design recommendations (Elbashir et al., 2002). A BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) was performed on the selected sequences to ensure only the specified gene is targeted for knockdown by the chosen siRNA. The siRNA duplexes were synthesized by Xeragon/Qiagen, Inc. (Valencia, CA) with 5′ phosphate, 3′ hydroxyl, and two 3′-TT overhangs on the sense and anti-sense strands. The following sequences were generated for Dnmt1s: sequence #1 sense 5′-CCAAGCAGGCAUCUCGGAA and antisense 5′-UUCCGAGAUGCCUGCUUGG; sequence #2 sense 5′-GAGAUGCCAUCACCCAAAA and antisense 5′-UUUUGGGUGAUGGCAUCUC. A nonsilencing (negative) control was used in all transfections: sense 5′-UUCUCCGAACGUGUCACGU and antisense 5′-ACGUGACACGUUCGGAGAA (Qiagen, cat.#1022077) and during optimization experiments a 3′-Fluorescein labeled nonsilencing (negative) control (Qiagen, cat.#1022079) was used.

Cell Lines and Media Composition

Primary cultures of fibroblast cells were isolated from murine (E13 outbred mice) and bovine (E30 Angus fetus) fetuses. As a positive control culture, the compromised NIH/3T3 cell line (ATCC #CRL-1658) was used. All cells were cultured at 37°C under 5% CO_2 in a humidified incubator. The mouse embryonic fibroblasts (MEF) and NIH/3T3 cells were cultured in high glucose DMEM (Hyclone, #SH30081.02), 10% FBS (Hyclone, #SH30071.03), 1% penicillin/streptomycin (Gibco, #15070-063), and 2 mM L-glutamine (Gibco, #25030-081). The bovine fetal fibroblasts (BFF) cells were cultured in medium containing DMEM/F12 (Gibco, #11320-033), 10% FBS, and 1% penicillin/streptomycin.

siRNA Transfection Optimization and Dnmt1s Knockdown

Transfection conditions were optimized for all three cell lines as described by the manufacturer of the RNAiFect transfection reagent (Qiagen, #301605). Frozen cell vials were thawed and seeded into a 75 cm^2 tissue culture flask (BD Falcon, #353136). Cells were grown to 75%–85% confluence and subcultured into 24-well culture plates (Costar, #3524) at a density of 3×10^4 cells per well. Transfection efficiency was evaluated using one of three siRNA concentrations (0.5, 1.0, and 1.5 μg/ per well) of a 3′-Fluorescein labeled nonsilencing (negative) control and four ratios of siRNA concentra-

tion to RNAiFect reagent (1:3, 1:6, 1:9, and 1:12). Medium containing the siRNA-transfection reagent complexes was added drop-wise into each well 36–48 hr after subculture and incubated undisturbed for 6 hr. For visualization, medium was aspirated and the cells were rinsed and overlaid with 1 ml DPBS. Transfection efficiency was determined using fluorescence microscopy with representative pictures taken of each treatment group.

For Dnmt1s knockdown experiments, all cells were treated using the transfection conditions as determined in the optimization experiments described above. In these experiments fresh medium was applied 6 hr post-transfection and was preceded by three washes with culture medium to remove any remaining siRNA complexes. Transfected cells were allowed to incubate in standard culture conditions with RNA and/or protein being collected at 0, 24, 48, and 72 hr post-transfection. Transfections were carried out in triplicate.

Flow Cytometry

The NIH/3T3 and MEF cells were plated, transfected, and cultured as described above in the optimization step. Cells were exposed to the siRNA complexes for 6 hr, whereupon they were harvested and counted using trypan blue (Sigma #T8154). Prior to flow cytometry analysis, 100 μl of propidium iodide (Roche #1348639) was added to a 1 ml cell suspension containing 10^6 cells (final concentration, 50 μg/ml). Cells were analyzed using a MoFlo High-Performance Cell Sorter (DakoCytomation, Ft. Collins, CO).

RNA Isolation, cDNA Preparation, and Real Time RT-PCR

At the specified time points, culture medium was aspirated and cells were washed 1× with DPBS (Hyclone, SH30028.02). The cells were harvested and total RNA being isolated using the RNeasy Mini Kit (Qiagen, #74104) per the manufacturer's instructions. Samples were quantified using an RNA 6000 Nano Assay (Agilent Technologies) and the Agilent 2100 Bioanalyzer which also verifies the integrity of each isolated product. RNA samples were treated with DNase (Promega, #M6101) and 0.75 μg cDNA was prepared using Applied Biosystems High-Capacity cDNA Archive Kit (#4322171) with the addition of 1 U/μl of RNase Inhibitor (Ambion, #2694) per reaction. Reaction conditions for reverse transcription were 25°C for 10 min followed by a 120 min incubation at 37°C.

Real-time RT-PCR was performed using an ABI Prism 7900HT detection system (Applied Biosystems, Foster City, CA). All primers and fluorescent probes for exonic regions were purchased from Applied Biosciences (Foster City, CA). The following TaqMan Gene Expression Assay (ABI) ID numbers, with the corresponding gene locus ID in brackets, were used to detect the murine transcripts: DNA methyltransferase(cytosine-5)1–Mm00599763_m1 (NM_010066) and beta-glucuronidase (used as an endogenous control)–Mm00446953_m1 (NM_010368). To amplify the bovine Dnmt1s sequence, a Custom TaqMan Gene Expression Assay was developed using locus ID NM_182651 imported into ABI's File Builder Software (http://home.appliedbiosystems.com/support/software/filebuilder). Eukaryotic 18S rRNA (ABI, #4319413E) was used as an endogenous control for all bovine real time RT-PCR reactions. Amplification was carried out in duplicate using the TaqMan Universal PCR Master Mix (ABI, #4304437), at 50°C for 2 min (1 cycle), 95°C for 10 min (1 cycle), and 40 cycles at 95°C for 15 sec, and 60°C for 1 min with fluorescent probe and appropriate primer pairs.

Protein Isolation, Western Blotting, and Immunodetection

Nuclear extracts were isolated using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce, #78833) according to the supplier's directions at 0, 24, 48, and 72 hr post-transfection for each treatment (siRNA#1, siRNA#2, and nonsilencing control) by pooling cells from three wells of a 24-well plate. The concentration of each sample was determined using the Micro BCA Protein Assay (Pierce, #23235). SDS–PAGE was performed using the NuPAGE Bis–Tris Gel and Buffer system (Invitrogen; Carlsbad, CA) on 4%–12% gradient gels with proteins being transferred onto a PVDF membrane. Immunodetection was conducted to detect Dnmt1s and β-actin using commercially available antibodies (Dnmt1, Imagenex Corporation, San Diego, CA; β-actin, Santa Cruz Biotechnology, Inc., #sc-1616). Secondary antibodies were conjugated with HRP (Santa Cruz Biotechnology, Inc) and chemiluminescence detection was carried out using the ECL Plus Western Blotting Detection System (Amersham Biosciences, #RPN2132) and captured using a ChemiImager 4400 (Alpha Innotech Corp.; San Leandro, CA). The membranes were probed first for Dnmt1s then stripped for 35–55 min at 50°C in a solution containing 62.5 mM Tris (pH 6.7), 2% SDS, and 100 mM β-mercaptoethanol, and re-probed for β-actin.

Data Analysis

Real Time RT-PCR data were analyzed using the SDS software package supplied with the ABI Prism 7900HT in which the threshold cycle (C_T) and baseline settings are automatically determined for each investigated transcript. The C_T values were normalized to the expression level of the endogenous control, beta-glucuronidase (GUS) or 18s (target gene C_T—calibrator gene C_T), and the relative expression of Dnmt1s was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) based on the expression of the nonsilencing control group within the same time period. For statistical analysis, delta C_T values of nonsilencing control and treatment groups within the same time period were subjected to a one-tailed T-test ($P < 0.05$).

RESULTS

This study investigated the knockdown potential of siRNAs designed for two regions on the somatic isoform

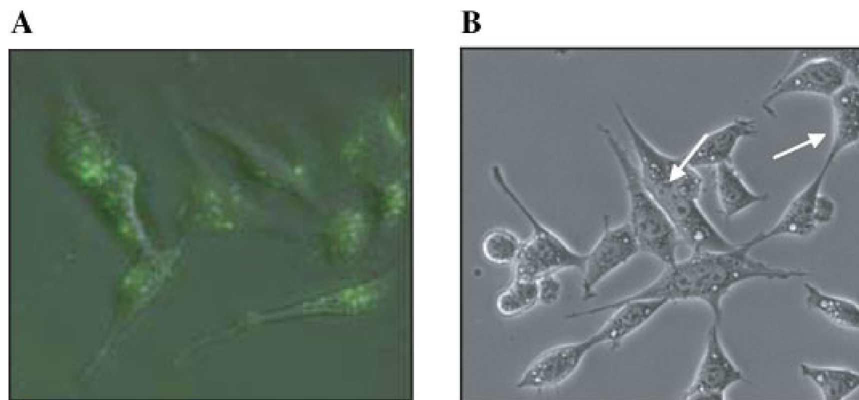

Fig. 1. Specific morphology traits of transfected cells. **A**: Peri-nuclear punctate staining of fluorescently labeled siRNA in MEF cells. **B**: Regardless of siRNA (Dnmt1 specific or a nonsilencing control), vacuole-like structures (arrows) were observed in all three treated cell lines. [See color version online at www.interscience.wiley.com.]

of the DNA Methyltransferase 1 (Dnmt1s) transcript between the murine, bovine, and ovine species. Based on homology and sequence recommendations set by the Tuschl lab (Elbashir et al., 2002), two candidate siRNAs were produced and tested in murine and bovine fetal fibroblast cells. Using a fluorescently labeled control siRNA, transient transfection conditions were optimized following the supplier's (Qiagen) recommendations for cell confluency, siRNA concentration, and the ratio between siRNA concentration and transfection reagent (siRNA:TR). Visual assessment of fluorescence 6 hr post-transfection within each well revealed a noticeable positive correlation between an increase in the siRNA:TR ratio and the amount of background fluorescence. In all treatment groups, diffuse fluorescence within the cells' cytosol could be discerned as reflected by a localized and prominent punctate fluorescence within the cell (Fig. 1A). Morphologically, some

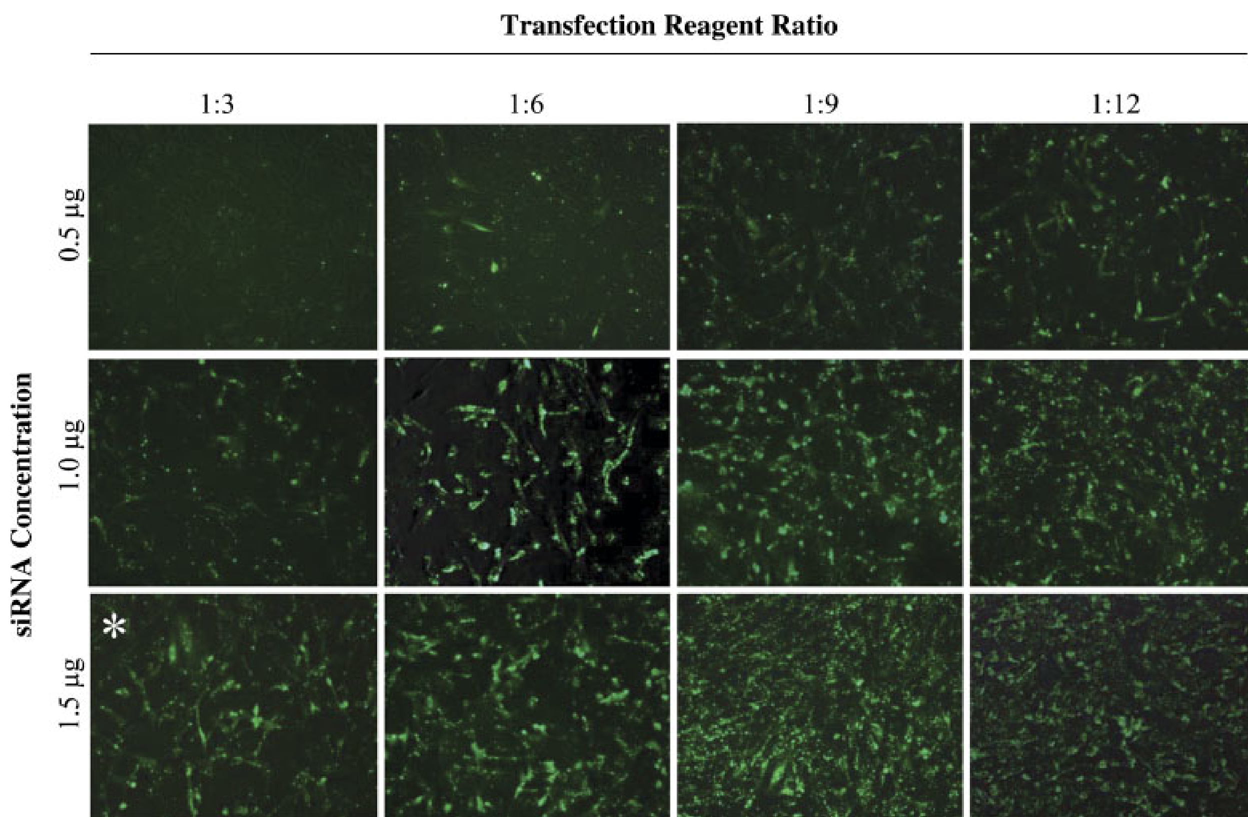

Fig. 2. Transfection optimization using a fluorescein labeled nonsilencing control siRNA. Merged phase contrast and fluorescent images for the BFF cell lines. Asterisk designates the treatment group used for Dnmt1 knockdown. [See color version online at www.interscience.wiley.com.]

cells had the appearance of vacuole-like structures within the cytoplasm (Fig. 1B). Cell viability was not severely affected, as indicated by an increase in culture confluency over time.

Using fluorescence microscopy and a 3×3 factorial allotment of treatments, the optimization experiments revealed a high number of transfected cells in the 1.5 μg siRNA/1:9; 1.0 μg siRNA/1:12; and 1.5 μg siRNA/1:12 groups in the murine cell populations (NIH/3T3 and MEF) with little or no transfected cells in the remaining six groups (pictures not shown). The BFF cells had a decrease in cell viability and proliferation and abnormal morphology in cells transfected with a siRNA:TR ratio of 1:6 or higher. Based on visual fluorescence levels in the transfected BFF cells, the 1.5 μg siRNA/1:3 group displayed the greatest transfection efficiency when taking into account the observed morphology (as described above) of the cells (Fig. 2C). Therefore, all subsequent siRNA transfections in the BFF cell line utilized this siRNA concentration and ratio.

In order to obtain a more accurate assessment of the transfection efficiency in the murine cultures, flow cytometry was performed on the transfected NIH/3T3 and MEF cell lines using the 1.5 μg siRNA/1:9, 1.0 μg siRNA/1:12, and 1.5 μg siRNA/1:12 treatment groups. To avoid counting the fluorescent punctate particles unincorporated within the cell and which failed to be washed away, an inclusive gate was set to exclusively count fluorescent events corresponding to the diameter of a control population of cells. The 1.5 μg siRNA/1:12 treatment group showed the highest level of transfection resulting in 99.6% (NIH/3T3) and 54.8% (MEF) fluorescent cells with less than a 2% death rate (Fig. 3). However, at these high levels of transfection reagent ratio (1:12), the cell morphology in these optimization groups (observed using fluorescence microscopy) was altered, this would have resulted in such cells being classified as nonviable cells for future studies. Thus, the two 1:12 treatment groups were not chosen to carry out further transfection studies in the murine cultures. Based on data from cell morphology, transfection optimization rates, and flow cytometry, the optimized treatment groups were determined to be 1.5 μg siRNA/1:9 (murine) and 1.5 μg siRNA/1:3 (bovine). All Dnmt1s knockdown experiments were carried out using these transfection conditions.

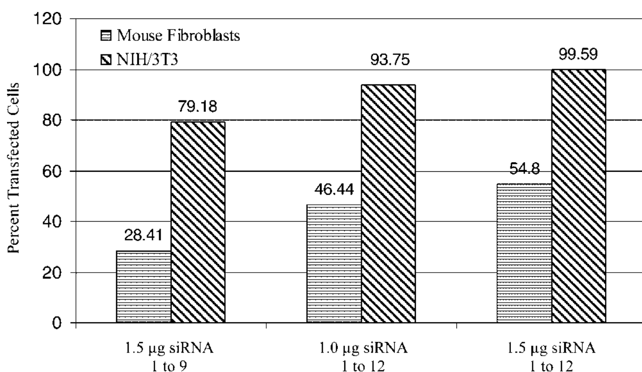

Fig. 3. Percentage of transfected NIH and MEF cells using flow cytometry on three optimization groups. Less than a 2% death rate was observed in all three treatment groups as determined using propidium iodide.

Real time RT-PCR was used to quantitatively determine the knockdown achieved using siRNAs designed for two independent regions on the Dnmt1s transcript in the murine and bovine cells. Cells were transfected in triplicate with either siRNA#1, siRNA#2, or a nonsilencing control siRNA in a 24-well plate format as in the optimization experiments. Real time RT-PCR was run using cDNA generated from mRNA extracted 24 and 48 hr post-transfection from all three siRNA groups. In order to compare results between siRNA treatments, C_T values were normalized to either beta-glucuronidase or 18s rRNA and were compared to a nonsilencing control within the same time period. Results are expressed in percent knockdown (%KD) of Dnmt1s with a 100% expression level being assigned to the nonsilencing control group. Results indicate a significant knockdown was achieved in all three cell lines ($P < 0.05$) (Fig. 4). A 56.5% reduction (or knockdown) in Dnmt1s occurred in both murine cultures (NIH/3T3 and MEF) whereas a 15.4% reduction was observed in the BFF cells as compared to the nonsilencing control within the same time period. However, this reduction occurred 24 hr earlier in the NIH/3T3 and BFF transfections than those carried out in the MEF cells (Fig. 4). A significant difference in the knockdown capability between siRNA#1 and siRNA#2 was only seen in the 48 hr NIH/3T3 transfected cells ($P < 0.05$). Although not significant, transfections with siRNA#1 in the NIH/3T3 culture and siRNA#2 in the MEF culture resulted in an overall greater Dnmt1s knockdown. Using these designed siRNAs, the real time RT-PCR results indicate murine Dnmt1s can be reduced by >50% not only in a compromised cell line but in a less easily transfected primary culture as well. These same siRNAs did not reduce the expression of Dnmt1s to the same degree in bovine cells.

In order to determine whether mRNA knockdown correlated with protein knockdown, nuclear protein extracts were generated from all three treatment groups (siRNA#1, siRNA#2, and nonsilencing control) at 24, 48, and 72 hr post-transfection. Western blotting and immunoprobing for Dnmt1s and β-actin (Fig. 5) revealed the presence of Dnmt1s across all treatments and time periods along with a proteolytic cleavage fragment at 145 kDa. The presence of a very faint β-actin band at 43 kDa suggests minimal cytoplasmic protein contamination in the nuclear protein lysates. These results indicate the reduction of Dnmt1s protein was not observed at these time points and under these conditions in all three cell types.

DISCUSSION

This study attempted to decrease the amount of somatically expressed maintenance methyltransferase (Dnmt1s) using siRNAs designed based on sequence homology between three phylogenetically divergent

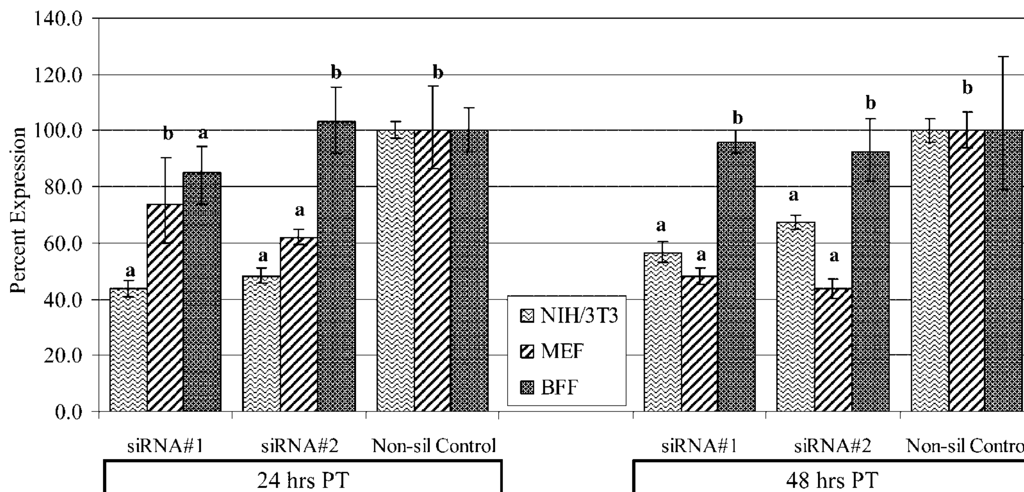

Fig. 4. Real time RT-PCR results reflecting the percent expression of Dnmt1 following siRNA transfections. Cell lines and treatment groups include: NIH/3T3, MEF, and BFF cells transfected with either siRNA#1 or siRNA#2 or a nonsilencing control siRNA. Groups with different letters are significantly different ($P < 0.05$).

animals (ovine, bovine, and murine). Using siRNAs designed against two different sites on the Dnmt1s transcript, our results indicate that the steady-state level of Dnmt1s mRNA was depleted in both a compromised and primary murine cell line. Although a significant knockdown of Dnmt1s was achieved using these same siRNAs in primary bovine cells, it was not to the same degree as seen in the murine cultures. However, this is the first report of Dnmt1s being knocked-down in primary fibroblast cell types of any species.

The reason for designing siRNAs from homologous regions in the murine, bovine, and ovine Dnmt1s sequences was based in part on the concept of finding a universal siRNA to knockdown Dnmt1s in both primary murine and bovine cells. Obtaining a universal siRNA would allow comparative studies to be performed across species, which is of interest when performing cross species nuclear transfer studies (Arat et al., 2003). Successfully knocking-down Dnmt1s in both bovine and murine cells using a universally designed siRNA could be used to test the effects of genomic methylation and its correlation to the expression of selected genes as in our previously established cross species nuclear transfer model (Arat et al., 2003). However, the results obtained in this study suggest a universal siRNA is not equally effective within and between species. Thus, specific siRNAs should be designed and tested in order to obtain a maximal level of knockdown in differing cell types.

The efficiencies of transfecting two siRNAs simultaneously were not evaluated, but may be a strategy to use when assessing the knockdown of specific transcripts across different species. In addition to this, the co-transfection of two or more siRNA duplexes targeting different sites on the same mRNA transcript has been used to enhance gene silencing (Ji et al., 2003). However, when using a single specific siRNA our observations would support designing the siRNA specific to the species in question to obtain maximal knockdown levels. The efficiency of an siRNA-induced knockdown can depend on positional effects, and shifting siRNAs by only one or more nucleotides may significantly influence their silencing efficiency (Holen et al., 2002; Harborth

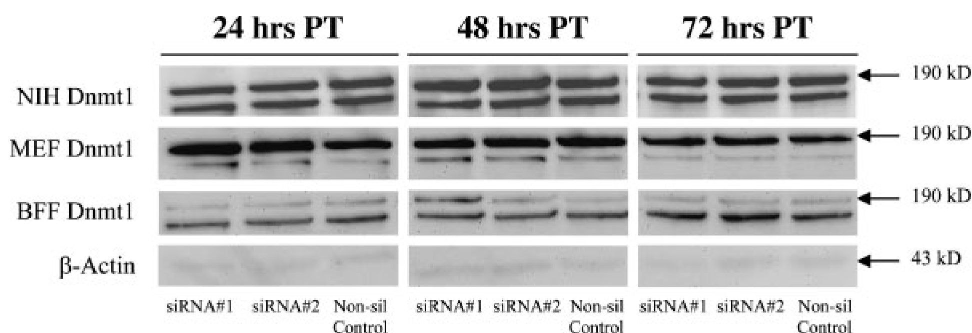

Fig. 5. Western blot analysis depicting Dnmt1 band at 190 kDa and its proteolytic cleavage fragment at 145 kDa for all three cell lines. Time points of 24, 48, and 72 hr post-transfection (PT) are shown for cells transfected with siRNA#1, siRNA#2, and the nonsilencing control siRNA. β-actin band appeared at 43 kDa.

et al., 2003; Hu et al., 2004). Such positional effects may explain why there was only a 15.4% significant knockdown in the primary bovine cells when using these same siRNAs. Because the bovine and murine Dnmt1 sequences are not 100% identical, the secondary and tertiary structures of the target mRNA may slightly differ and may also affect the location of associated proteins at any given region on the transcript. Similar to problems associated with antisense and ribozyme approaches, for an siRNA to be effective, site accessibility is essential for proper base pairing to occur (Scherr and Rossi, 1998; Scherr et al., 2001). The selection of siRNA target regions on an mRNA transcript is currently a trial-and-error process; thus, alternate regions on the bovine Dnmt1 transcript might produce different results.

In RNAi studies differences in siRNA effectiveness, transfection efficiencies, cell type, and culture state along with protein stability are all factors affecting the efficiency of an siRNA-induced protein knockdown (McManus and Sharp, 2002). An initial concern in this study was the transfection potential of our primary cells. The NIH/3T3 cell line, derived from NIH Swiss mouse embryos (Jainchill et al., 1969), was used as a positive control as it possesses morphological characteristics best suited for transfection assays. In this study, based on flow cytometry data, the transfection efficiency of the primary murine cells was 45%–50% lower than that observed in the compromised NIH/3T3 cells. Thus, a clear difference in the transfection capability of our two murine cell cultures existed. Surprisingly, this difference did not carry over into the observed level of Dnmt1s mRNA knockdown: within 48 hr of being transfected the greatest decrease in Dnmt1s mRNA expression was 56.5% for both murine cell cultures. Notably, the specific siRNA resulting in the greatest level of knockdown differed between the two murine cell lines. Studies indicate that regardless of optimized transfection conditions, there are cell-type-dependent global effects in regards to transfection efficiency, and cell-type-independent positional effects in regards to knockdown efficiency (Harborth et al., 2003).

Morphologically, within 6 hr of transfection there was a discernable difference between the transfected and nontransfected groups in all three cell types as noted by vacuole-like structures within the cells cytoplasm. The possibility that these morphological changes resulted from the knockdown of Dnmt1s can be ruled out, as these vacuole-like structures were seen with both the custom and nonsilencing control siRNA. It is plausible that the vacuole-like structures observed in the cytoplasm may result from the cells' internalization of the siRNA-transfection reagent. Most nonviral vectors exploit the endocytic pathway for uptake and subsequent processing within the cell (Roth and Sundaram, 2004); thus, the vacuole-like structures may be the siRNA complexes housed within endosomal or lysosomal compartments (Hope et al., 1998).

Of essential consideration in any knockdown experiment is the protein's half-life, its abundance, and the regulation of its expression (Elbashir et al., 2002). The level of Dnmt1s expression changes significantly during the cell cycle, with high levels being expressed in the S phase and low levels in G1/G0 (Szyf et al., 1985; Adams, 1990). Notably, when cells enter into G1/G0 by the stimulus to differentiate or during low serum conditions, Dnmt1s mRNA and protein levels decrease, while the transcription of *Dnmt1* remains relatively active like that of a cycling cell (Teubner and Schulz, 1995; Liu et al., 1996). In essence, Dnmt1s is a post-transcriptionally regulated enzyme (Szyf et al., 1991; Liu et al., 1996). Although a significant knockdown of Dnmt1s mRNA was achieved in all three cultures, no reduction in protein was observed up to 72 hr following siRNA transfection. Thus, a 50%–60% decrease in Dnmt1s transcripts was not sufficient to decrease the protein levels of Dnmt1s within the transfected cells. This indicates the Dnmt1s-specific siRNAs were not transfected at an adequate concentration to overcome the steady-state level of Dnmt1s transcripts to result in a decrease in the protein level. In studies showing the successful knockdown of Dnmt1s protein in human colorectal cancer cells, transfections were carried out every day and the cultures were split every other day (Robert et al., 2003; Ting et al., 2004). In contrast, in our attempt to transiently knockdown Dnmt1s, the cells were subjected to a single round of transfection, and due to our timepoints, were never sub-cultured.

The objective of this study was to test our ability to knockdown the level of Dnmt1s in primary murine and bovine fibroblast cells, which are the common cell type used in SCNT studies. With the use of both antisense and small inhibitory RNA (siRNA) molecules, studies have shown the cellular level of Dnmt1s has been knocked-down in transformed and/or compromised cultures. Such studies have resulted in lower maintenance methyltransferase activity (Ting et al., 2004), decreased cell proliferation (Fournel et al., 1999), and global and gene-specific demethylation. However, not all Dnmt1s knockdown studies have reported a loss in DNA methylation patterns (Rhee et al., 2000; Ting et al., 2004). Such studies have been performed in transformed or compromised cell lines whose genomes may consist of, but are not limited to, chromatin deletions, duplications, and/or mutations. Therefore, knockdown studies using siRNA, like those described above, must be confirmed in primary cells and eventually in animals.

Although the antisense or siRNA knockdown of Dnmt1s can result in DNA demethylation (Fournel et al., 1999; Leu et al., 2003; Milutinovic et al., 2003; Robert et al., 2003; Kawasaki and Taira, 2004), this was not the underlying goal of our study. Our primary interest was in the elimination of Dnmt1s mRNA from within a primary culture of cells. The results presented here indicate that on an average a knockdown in Dnmt1s mRNA was achieved to a similar degree not only in a murine compromised cell line but also in a more difficult to transfect primary culture. However, these same siRNAs did not produce a Dnmt1s knockdown to the same degree when used in primary bovine cells, thus

indicating one limitation of identifying an interspecies universal siRNA duplex. We cannot discount the fact, however, that a significant level of Dnmt1s knockdown was achieved.

RNAi has rapidly developed into one of the most widely applied technologies in molecular and cellular research, and although young, is now an essential experimental tool. The versatility of RNAi leads to a number of exciting potential applications in a wide array of fields, such as in SCNT. Although over ten species have successfully been produced, cloning by SCNT is highly inefficient with the average number of live births ranging from less than 1% to more than 5% depending on the species (Wilmut et al., 2002). Although an effort to increase this low efficiency has actively been pursued using various reagents (Gibbons et al., 2002) along with altering the methods of activation and fusion, no substantial breakthroughs have been made.

In order to substantially increase the efficiency while decreasing the observed anomalies seen in SCNT, it is imperative that novel methods, such as the use of RNAi strategies, be incorporated to assist in the oocyte's ability to properly reprogram a differentiated genome. Until this occurs, the current field of SCNT is best suited for fundamental studies in the discovery of reprogramming molecules essential to cell de-differentiation and plasticity. Undoubtedly, the extent to which RNAi can be utilized has yet to be realized.

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