Silencing Histone Deacetylase–Specific Isoforms Enhances Expression of Pluripotency Genes in Bovine Fibroblasts

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Abstract

Histone deacetylases (HDACs) catalyze deacetylation of histones that results in altered transcriptional activity. Inhibitors of HDACs have been shown to induce transcriptional changes that contribute positively to reprogramming somatic cells either by nuclear transfer or inducing a pluripotent state. However, the exact molecular mechanisms whereby HDAC inhibitors function and the specificity of the HDAC isoforms in cell reprogramming are not yet fully understood. Herein, we report the ability of individual isoform-specific HDACs to modulate endogenous expression of pluripotency-associated genes in bovine somatic cells. This *in vitro* study showed that a transient selective depletion of HDACs resulted in elevated mRNA levels of Oct-4, Sox2, and Nanog. In particular, we found that inhibition of specific HDAC isoforms using small interfering (si) RNA significantly increased expression of Nanog, a key factor required for totipotency induced by somatic cell nuclear transfer and for maintaining pluripotency in embryonic and induced pluripotent stem cells. Our study suggests that this gene might be the most susceptible to HDAC activity inhibition. Moreover, a regulatory role of the class III HDAC, SIRT3, on an Oct4–Sox2–Nanog transcriptional network was revealed. We observed the upregulation of pluripotency-related genes by depletion of SIRT3. SIRT3 is localized to mitochondria and is associated with energy metabolism processes, suggesting metabolic changes may be linked to reprogramming in bovine fibroblasts. In conclusion, we show that targeting selective HDACs can potentially be useful to enhance reprogramming and that sirtuins may play a pivotal role in somatic cell reprogramming by upregulating an Oct4–Sox2–Nanog transcriptional network. Dedifferentiating donor somatic cells by upregulating developmentally important genes through specific knockdown of epigenetic targets, in particular HDACs, may provide a path to improving livestock cloning and the *in vitro* production of pluripotent cells.

Introduction

HISTONE DEACETYLASES (HDACs) CATALYZE deacetylation of histones, resulting in altered chromatin structure and epigenetic regulation of transcriptional activity. To date, 18 HDAC family members have been identified and classified into two categories: (1) Zinc-dependent (class I, class II, and class IV), and (2) nicotinamide adenine dinucleotide (NAD⁺)-dependent enzymes (class III, sirtuins) (Yang and Seto, 2008). HDACs have been shown to regulate many important biological processes, including cell proliferation, differentiation, and development. Emerging evidence from *in vitro* and *in vivo* studies indicate the usefulness of agents targeting HDACs in the treatment of diseases using models of cancer, diabetes, and neurodegenerative disease (for review, see Lawless et al.,

2009) and for reprogramming somatic cells to a pluripotent state (Huangfu et al., 2008b) or by somatic cell nuclear transfer (Enright et al., 2003; Shi et al., 2003). Examples of HDAC inhibitors (i) that have demonstrated usefulness for reprogramming include valproic acid (VPA), butyrate, trichostatin A (TSA), and Scriptaid. VPA, a class I and II inhibitor, enhances reprogramming efficiency (Huangfu et al., 2008a), facilitates the generation of protein-induced pluripotent stem cells (Rim et al., 2012; Zhou et al., 2009), enhances Oct-4 promoter activity (Teng et al. 2010), and reduces the number of factors required for reprogramming, importantly, the oncogenes *c-myc* and *Klf4* (Huangfu et al., 2008b). Butyrate is an HDACi that enhances the generation of human induced pluripotent stem cells (iPSCs) by increasing histone H3 acetylation, promoter DNA demethylation, and the expression of

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endogenous pluripotency-associated genes (Mali et al., 2010). In mice, butyrate increases the efficiency of iPSC generation by reducing the frequency of partially and/or unsuccessfully reprogrammed cells (Liang et al., 2010). Previous studies have also demonstrated that HDACi have a modest effect on the efficiency of reprogramming induced by somatic cell nuclear transfer (Kishigami et al., 2007). TSA has been reported to improve preimplantation development of bovine cloned embryos (Oh et al., 2012) and TSA and another HDACi, Scriptaid, significantly enhance the development of porcine somatic cell nuclear transfer (SCNT) embryos (Zhao et al., 2010). These examples of first-generation HDACi are typically characterized as broad (pan) or class-specific inhibitors that also have numerous off-target effects. Thus, the exact molecular mechanism, or mechanisms, whereby HDACi contribute to reprogramming is poorly understood.

Upregulating key developmentally important genes to threshold levels is likely a minimal requirement for reprogramming. Indeed, a recent report by Radziszewska and colleagues (2013) demonstrated that a defined Oct4 level is critical for naïve pluripotency acquisition. The role of specific HDAC isoforms in regulating expression of developmentally important genes required to be activated for successful reprogramming has not been described. Although a variety of HDAC inhibitors have been identified, there are a limited number of isoform-selective inhibitors, and lack of selectivity is often referred to in the literature. The development of novel, isoform-selective inhibitors potentially may lead to improved efficacy of cell reprogramming and minimize or alleviate off-target effects. In an effort to begin to understand the influence of specific HDACs on the upregulation of pluripotency genes, a fundamental requirement for reprogramming by SCNT, or inducing a pluripotent state, we used HDAC isoform-specific small interfering (si) RNA to knock down the expression of specific HDACs and we reported their ability to modulate endogenous expression of pluripotency-related genes in bovine somatic cells. In the present study, we document that a transient selective depletion of HDACs resulted in elevated expression of Oct4, Sox2, and Nanog in bovine fetal fibroblasts. Reactivation of pluripotency genes prior to somatic cell nuclear transfer, or induction of a pluripotent state, may enhance reprogramming efficiency.

Materials and Methods

Establishment of the cell line

The primary fibroblast culture was established from a 50-day-old bovine fetus recovered from a local abattoir as previously described (Giraldo et al., 2008). Skin from the decapitated and eviscerated fetus was cut into 1-mm² pieces and washed three times in Dulbecco phosphate-buffered saline (dPBS; Gibco, Life Technologies, Grand Island, NY) containing 100 U/mL of penicillin and 100 µg/mL of streptomycin. The cell line was established by enzymatic dissociation. Briefly, the tissue pieces were placed in a conical tube with Dulbecco's modified Eagle medium with high glucose (DMEM; Gibco, Life Technologies, Grand Island, NY) containing 0.5% of collagenase type I in a 5% CO₂ atmosphere at 39°C for 3 h. DMEM with 10% calf serum (CS) was added to inactivate the collagenase, and the cell suspension was centrifuged at 350 × g for 5 min. Cells were resuspended in

DMEM supplemented with 10% CS, penicillin, and streptomycin and cultured in 25-cm² flasks under a 5% CO₂ atmosphere and 90% humidity at 39°C.

Maintenance and cryopreservation of the cell line

Cells were passaged upon reaching 90% confluence by enzymatic dissociation, counted using a hemocytometer, and reseeded in a T75 flask followed by culture in a multi-layered HYPERFlask® (Corning Life Sciences, Lowell, MA) until ≈ 70–80% confluent. Trypsin-dissociated cells were counted, resuspended in culture medium supplemented with 10% dimethyl sulfoxide (DMSO) at a concentration of 10⁶ cells/mL, and cooled at 1°C/min until reaching –80°C. Cryopreserved cells were stored in liquid nitrogen.

siRNA design and transfections

siRNAs were synthesized by Dharmacon of Thermo Fisher Scientific (Lafayette, CO, USA). All siRNAs were designed against the bovine sequences for the genes of interest; nucleotide sequences of siRNA are given in Table 1. Non-silencing and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) siRNAs were included as negative and positive controls, respectively. An extra negative control “mock” group (transfected only with transfection reagent, no siRNA addition) was also included. Cells were seeded in tissue culture 24-well plates (Corning Life Sciences, Lowell, MA, USA) 24 h prior to experiments at density of 5 × 10⁴ cells/well in 24-well plates. Cells were transfected with siRNA (50 nM or 75 nM) for 48 h using DharmaFECT 1 transfection reagent (Dharmacon) at a concentration of 0.6%, according to the manufacturer's protocol. The target mRNA knockdown was assessed 2, 5, and 7 days posttransfection.

RNA Isolation and quantitative real-time reverse transcriptase polymerase chain reaction

Total RNA was extracted using TRIzol (Invitrogen, Life Technologies, Grand Island, NY, USA) and column-purified with PureLink™ RNA Mini Kit and DNase I treatment (Applied Biosystems/Ambion, Life Technologies, Grand Island, NY, USA). cDNA synthesis was performed with 500 ng of total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems). Endogenous mRNA levels for HDAC2, HDAC4, HDAC5, HDAC6, HDAC9, HDAC10, HDAC11, Dnmt1, Sirt3, and Sirt5 were measured with Applied Biosystems Taqman® Gene Expression Assays (Applied Biosystems). Reactions were performed in MicroAmp Optic 384-well Reaction Plates (Applied Biosystems) using the ABI Prism 7900 Sequence Detection System (Perkin Elmer, Boston, MA) with the condition of 2 min at 48°C, 10 min at 95°C, and then 40 cycles of 15 s at 95°C and 1 min at 60°C. Quantitative real-time polymerase chain reaction (qPCR) was performed in triplicate for each sample, and each run included a standard curve, nontemplate control, and negative reverse transcriptase (RT) control. Levels of gene expression were quantified relative to the level of GAPDH using a standard curve method.

Statistical analysis

The data were expressed as mean ± standard deviation (SD). Differences among the treatment groups were assessed

TABLE 1. SEQUENCES OF SI RNAs TARGETING HISTONE DEACETYLASES REPRESENTING ALL CLASSES OF HDAC FAMILY USED IN THE STUDY

Gene description	Gene ID		Sequence 5' to 3'
DNMT1, DNA (cytosine-5-)-methyltransferase 1	281119	Sense:	CCUAUAACGCCAAGAGCAAU
		Antisense:	PUUGCUCUUGGCGUUAUAGGUU
HDAC2, histone deacetylase 2	407223	Sense:	CAGUUAAGGUCAUGCUAUU
		Antisense:	PUUAGCAUGACCUUUAACUGUU
HDAC4, histone deacetylase 4	517559	Sense:	GAAAUUACGCUCCAGGCUAUU
		Antisense:	PUAGCCUGGAGCGUAAUUCUU
HDAC5, histone deacetylase 5	504242	Sense:	GUUCGAGGCUAAAGCAGAAUU
		Antisense:	PUUCUGCUUUAGCCUCGAACUU
HDAC6, histone deacetylase 6	513602	Sense:	GGUUCACAGCCUAGAAUAAUU
		Antisense:	PAUAUUCUAGGCUGUGAACC UU
HDAC9, histone deacetylase 9	535415	Sense:	GCGAAUUC AAGGUCGAAAAUU
		Antisense:	PUUUUCGACCUUGAAUUCGCUU
HDAC10, histone deacetylase 10	510654	Sense:	GGACGUGGCUGUUCGGUAUUU
		Antisense:	PAUACCGAACAGCCACGUCCUU
HDAC11, histone deacetylase 11	519899	Sense:	CAGACGGGAGGAACCAUAAUU
		Antisense:	PUUAUGGUUCCUCCCGUCUGUU
DNMT1, DNA (cytosine-5-)-methyltransferase 1	281119	Sense:	CCUAUAACGCCAAGAGCAAUU
		Antisense:	PUUGCUCUUGGCGUUAUAGGUU
SIRT3, sirtuin 3	614027	Sense:	ACAGCAUCCUCCAGCAGUAUU
		Antisense:	PUACUGCUGGAGGAUGCUGUUU
SIRT5, sirtuin 5	507347	Sense:	UCCAAUUUGUCCAGCCUUAUU
		Antisense:	PUAAGGCUGGACAAAUUGGAUU
Nonsilencing		Sense:	UUCUCCGAACGUGUCACGUTdT
		Antisense:	ACGUGACACGUUCGGAGAATdT
GAPDH, glyceraldehyde-3-phosphate dehydrogenase	281181	Sense:	UGAACCACGAGAAGUAUAAUU
		Antisense:	PUUAUACUUCUCGUGGUUCAUU

Nonsilencing and GAPDH siRNAs were included as negative and positive controls, respectively.

via *t*-test or one-way analysis of variance (ANOVA) followed by a post hoc Tukey test using the R software environment for statistical computing and graphics (<http://www.r-project.org>). A *p* value of ≤ 0.05 was considered significant.

Results

Optimization of siRNA transfection conditions for fetal bovine fibroblasts

siRNAs targeting HDACs representing all classes of the HDAC family were used in the study. Several factors were considered to optimize siRNA treatment. First, to determine the most suitable transfection reagent and its concentration, the viability of cells treated with DharmaFECT Transfection Reagents (DharmaFECT 1, DharmaFECT 2, and DharmaFECT 3) at the concentrations of 0.2%, 0.6%, and 1.2% was evaluated (data not shown). To reduce off-target effects and maintain high silencing potency and efficiency of siRNA transfection, four oligonucleotides were designed, synthesized, and tested at the concentrations of 25 nM, 50 nM, and 75 nM. The ability of siRNA to silence expression of a target gene was determined by qPCR. Figure 1 shows the changes in mRNA expression after 48 h of treatment, with the most potent siRNAs specific to target genes in comparison to nonsilencing siRNA transfection. The results demonstrated significant 31–81% suppression in relative gene expression detected as compared to the control level ($p < 0.05$). Therefore, the subsequent experiments were performed using optimized conditions (0.6% DharmaFECT 1, either 50 nM or 75 nM siRNA concentration).

Effect of targeting HDACs with siRNA on Oct-4 mRNA expression in fetal bovine fibroblasts

Once appropriate siRNA transfection conditions were established and validated, we next attempted knockdown of the HDACs to investigate the effect of targeted gene silencing on pluripotency gene expression at the mRNA level. As a proof-of-concept for the approach, we first analyzed expression of Oct-4. Figure 2 shows the effect of silencing a single HDAC isoform on Oct-4 expression following gene knockdown. The siRNA treatments resulted in the effective increase of Oct-4 mRNA level in a time-dependent manner. Significant upregulation of Oct-4 was observed after knockdown of HDAC4, HDAC6, HDAC10, SIRT3, and SIRT5. HDAC6, HDAC10, SIRT3, and SIRT5 treatments resulted in significantly enhanced Oct-4 expression on day 2. The effect was abrogated by day 7. In contrast, HDAC4 siRNA treatment induced Oct-4 expression that remained elevated up to day 7 after transfection.

Changes in pluripotency genes expression

The reprogramming of somatic cells is generally a slow, stochastic process that requires multiple cell divisions (Chan et al., 2009; Hanna et al., 2009). To assess the temporal patterns of gene knockdown with siRNA and the induction of pluripotent gene expression, we measured expression levels of target and pluripotent genes 2 and 7 days posttransfection. Figure 3 demonstrates sustained knockdown of HDACs 2, 4, and 9 and DNMT1 and SIRT3 throughout the study period. qPCR analysis of Oct-4, Nanog, and Sox2 gene expression

FIG. 1. The changes in mRNA expression after 48h of treatment with the siRNA specific to the target gene. A 31–81% decrease of mRNA abundance was observed in comparison to nonsilencing siRNA as assessed by qPCR. Data are presented as mean $\pm$ SD. (*) $p < 0.05$; (***) $p < 0.001$.

was performed on day 7. Figure 4 demonstrates that silencing of HDACs resulted in increased expression of pluripotency-related genes. qPCR analysis showed that all treatments elevated the expression of Nanog in the bovine fetal fibroblasts. The most striking effect was observed after silencing Sirtuin 3; SIRT3 siRNA alone and in combinatorial treatment with HDAC4 siRNA significantly increased the expression of Oct-4, Nanog, and Sox-2. Collectively, these findings highlight the importance of specific HDAC inactivation in the regulation of pluripotency genes.

Discussion

In the present study, the roles of individual HDAC isoenzymes and their ability to modulate endogenous expression of pluripotency-associated genes were examined

using RNA interference. Herein we show that silencing selective HDACs upregulates expression of pluripotency genes in bovine fetal fibroblasts. Our data extended the findings showing that inhibiting activity of specific HDACs facilitates upregulation of key developmental genes, a basic requirement for somatic cell reprogramming. Elucidation of this mechanism can open new opportunities to improve efficiency in generating pluripotent cells or livestock cloning.

Our study further reveals that sirtuins function to regulate the Oct4–Sox2–Nanog transcriptional network. Down-regulation of both class III HDACs, SIRT3 and SIRT5, resulted in elevated Oct-4 mRNA level. Moreover, knocking down SIRT3 in combination with HDAC4 depletion sustained elevated Oct-4 expression until day 7 of the study and upregulated expression of Nanog and Sox-2.

FIG. 2. The effect of silencing histone deacetylases on Oct-4 expression during 7-day study course. Data are presented as mean ± SD. (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$.

Sirtuins are a class III NAD^+ -dependent HDAC. The involvement of sirtuins in early embryonic development and somatic cell reprogramming has previously been documented. SIRT1 regulates Nanog expression in mouse embryonic stem cells (ESCs) (Han et al., 2008), and its expression is downregulated during human (h) ESC differentiation (Calvanese et al., 2010). SIRT1 overexpression was shown to stimulate iPSC formation, in part, through Nanog enhancement, with the most potent action in the initial phase of reprogramming (Lee et al., 2012). Whether SIRT3 is involved in reprogramming is not known, and to our knowledge the role of SIRT3 in the reprogramming process has not been documented.

SIRT3 is a member of sirtuin family preferentially localized to mitochondria and normally associated with energy metabolism processes. SIRT3 controls adenosine triphosphate (ATP) levels (Ahn et al., 2008) and reactive oxygen species (ROS) production (Kim et al., 2010). iPSCs rely on glycolysis followed by lactic acid fermentation to generate ATP. Previous studies show that promoting glycolysis may enhance reprogramming in human somatic cells (Zhu et al., 2010). Loss of SIRT3 increases glucose uptake and lactate production that results in the shift of cellular metabolism toward increased glycolysis (Kim et al., 2010; Finley et al., 2011). In fact, the increase in glycolytic intermediates that results from SIRT3

FIG. 3. The expression of genes targeted with siRNA. Data are presented as mean $\pm$ SD. (**) $p < 0.01$; (***) $p < 0.001$.

deletion is similar to that documented in hypoxia-induced metabolic reprogramming (Finley et al., 2011). Thus, it can be postulated that upregulation of pluripotency-related genes might result from metabolic changes in bovine fibroblasts.

Interestingly, we demonstrated that all HDAC isoforms analyzed in the study significantly increased expression of Nanog, suggesting that this gene may be especially susceptible to HDAC activity. Previous reports show that the *Nanog* gene is regulated by epigenetic mechanisms involving DNA

FIG. 4. Silencing HDACs resulted in overexpression of pluripotency-related genes on the day of nuclear transfer (day 7). Data are presented as mean $\pm$ SD. (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$.

methylation and histone modifications (Hattori et al., 2007). Chromatin immunoprecipitation assay revealed that histone H3 and H4 are highly acetylated at the locus in ESCs (Hattori et al., 2007), and *Nanog* promoter acetylation of histone H3 increases during reprogramming Moon et al., 2011). Moreover, SAHA, a histone 1 and 3 deacetylase inhibitor with an half-maximal inhibitory concentration (IC50) of ≈ 10 nM, upregulates *Nanog* in mouse embryonic fibroblasts by histone H3 Lys9 and Lys14 acetylation (Pandian et al., 2011). The growing body of evidence highlights *Nanog* as a transcriptional factor critically involved in Oct4–Sox2–*Nanog* networks regulating pluripotency (Chambers et al., 2003; Theunissen et al., 2011; Zaehres et al., 2005). In mouse fibroblasts, *Nanog* forces expression of Oct-4 during the course of reprogramming and improves generating iPSCs (Moon et al., 2013). *Nanog* is thought to be one of the critical players in bovine preimplantation development. Its transcripts have been localized both in the ICM and TE of the bovine blastocyst (Degrelle et al., 2005). *Nanog* plays an important role for maintaining pluripotency in cattle (Muñoz et al., 2008), and is a key factor for induction of pluripotency in bovine adult fibroblasts (BAFs). The induction and maintenance of pluripotency in BAFs requires the ectopic expression of *Nanog* in addition to POU5F1, SOX2, KLF4, and c-MYC; lack of *Nanog* in a cocktail of transcription factors results in significantly lower reprogramming efficiency (Sumer et al., 2011). Hence, the observed effects of selective HDAC isoform depletion on *Nanog* expression may allow delving deeper into understanding the complexity of epigenetics in reprogramming and open new avenues for more efficient cloning efficiency in cattle.

In conclusion, we have shown that targeting selective HDAC can potentially be useful to reprogramming somatic cells. We also showed that sirtuins, class III HDACs, can play a pivotal role in somatic cell reprogramming.

Acknowledgments

This work was funded by National Institutes of Health (NIH) Small Business Technology Transfer (STTR) grant 1 R41 GM088891. The authors thank Dr. David Burke and Ms. Susan Newman for technical advice. This project also used Genomics, Cell Biology, and Imaging core facilities that are supported in part by Center of Biomedical Research Excellence (COBRE) (NIH P20-RR021945) and NORC (Nutrition Obesity Research Center) (NIH 1P30-DK072476) the Center's grants from the National Institutes of Health.

Author Disclosure Statement

All authors, with the exception of Dr. Kenneth Bondioli, acknowledge financial interests in the form of stock and/or stock options in NuPotential, Inc.

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