Sodium Butyrate Promotes Generation of Human Induced Pluripotent Stem Cells Through Induction of the miR302/367 Cluster

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Small molecules (SM) can greatly enhance the efficiency of induced pluripotent stem (iPS) cell generation, but the mechanisms by which they act have not been fully explored. We show here that an SM cocktail (NaB, PD03259, and SB431542) significantly promotes iPS cell generation from human fibroblasts, and NaB is more potent than the other two common histone deacetylase inhibitors (valproic acid and Trichostatin A) in promoting cellular reprogramming. Our data indicate that the SM cocktail substantially upregulates the miR302/367 cluster expression by increasing the stability and transcriptional level of this microRNA (miRNA) cluster in a manner dependent on the four defined transcription factors (TFs). Among the four TFs, Oct4 in particular appears to be required for the induction of the miR302/367 cluster by the SM cocktail. We also found that NaB alone can enhance the TFs-dependent upregulation of the miR302/367 cluster. Using a promoter reporter assay, we show that the SM cocktail remarkably enhanced the transcriptional activity of the four TFs in the miR302/367 promoter. Notably, attenuation of miRNA302/367 using a miRZip impairs the ability of the SM cocktail in promoting reprogramming. Collectively, these findings suggest that the SM cocktail promotes reprogramming at least partly through the induction of the miR302/367 cluster expression. Further insights into this process may pave the way for the generation of iPS cells using only SM cocktails.

Introduction

HUMAN INDUCED PLURIPOTENT stem (iPS) cells, which exhibit properties similar to human embryonic stem (hES) cells, have been directly generated from human somatic cells by forced expression of defined transcription factors (TFs), including either the combinations of Oct4, Klf4, Sox2, and c-Myc, or Oct4, Sox2, Nanog, and Lin28 [1–4]. Although somatic cells from various tissues are reprogrammable by integrating or nonintegrating approaches, a major roadblock for using this new technology is the low reprogramming efficiency (from 0.01% to 0.2%) with slow kinetics (almost 4 weeks). Recently, progress has been made toward enhancing the efficiency of human iPS cell generation by using small molecules (SM). Butyrate, a small-chain fatty acid histone deacetylase (HDAC) inhibitor, greatly enhances the efficiency of generating murine and human iPS cells with TFs [5,6]. Inhibition of both transforming growth factor-beta (TGF- β) and mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinases (ERK) signaling pathways using SMs (SB431542 and PD0325901, respectively) enhances reprogramming of human fibroblasts with the four individual TFs [7]. Recently, we reported a simple approach for generating

fully reprogrammed human iPS cells by using a single polycistronic retroviral vector expressing four TFs, in combination with a cocktail consisting of these three SMs (NaB, SB431542, and PD0325901), which are inhibitors of HDACs, TGF- β , and MAPK/ERK [8]. Although these three SMs greatly enhance reprogramming efficiency with fast kinetics, the mechanism of how these SMs play a role in the reprogramming process remains elusive.

MicroRNAs (miRNAs), 18–24 nucleotide single-stranded RNAs, bind to partially complementary target sites in mRNA 3' untranslated regions, which results in the degradation of the target mRNAs, or translational repression of the encoded proteins [9]. In general, one miRNA may target multiple genes and one gene can be repressed by multiple miRNAs, which results in the formation of complex regulatory feedback networks [10]. Based on their specific modulation mechanism, miRNAs have been found to play important roles in regulating the self-renewal and differentiation of hES cells, and in regulating cellular reprogramming [11–16]. Some specific miRNAs can enhance the efficiency of TFs-mediated iPS cell generation [17]. Notably, direct overexpression of the miR302/367 cluster or with the other two mature miRNAs can reprogram somatic cells into iPS cells

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[14,15]. Interestingly, Ware et al. reported that butyrate promotes self-renewal and decreases differentiation of ES cells, which is evident by a slower decline in miR302/367 miRNAs [18]. The miR302/367 cluster is an ES cell-specific miRNA cluster and a target of Oct4 and Sox2 [19].

However, the role of miRNAs in the regulation of SM-mediated reprogramming remains largely unknown. In our current study, we investigated how SMs promote reprogramming through the regulation of the miR302/367 cluster. Here, we report that an SM cocktail (consisting of NaB, SB431542, and PD0325901) increased the expression of the miR302/367 cluster by increasing miRNA stability and transcription in a manner dependent on reprogramming TFs. We show that Oct4 is a primary reprogramming TF required by the SM cocktail to upregulate expression of the miR302/367 cluster. Further, we found that NaB alone, but not SB431542 and PD0325901, was able to increase the expression of miR302/367 cluster and enhance transcription activity of reprogramming factors in the miR302/367 promoter. Lastly, we demonstrate that the miR302/367 cluster is essential for the SM cocktail and NaB to promote reprogramming by a lentivirus-mediated stable inhibition approach. Our findings provide new insights into the molecular mechanisms of cellular reprogramming instructed by SMs and miRNAs.

Materials and Methods

Cell culture

The primary human foreskin fibroblasts (HFFs) were purchased from Millipore and cultured in FibroGRO™-LS Complete Media. 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (high glucose) containing 10% fetal bovine serum (FBS). hES and human iPS cells were cultured on mouse embryonic fibroblast (MEF) feeder cells in conventional hES cells culture medium (DMEM/F12, 20% knockout serum replacement, 1% glutamax, 1% nonessential amino acids, 1% penicillin/streptomycin, 0.1 mM β -mercaptoethanol, and 20 ng/mL bFGF). All cell culture products were purchased from Invitrogen except where mentioned.

Construction of luciferase reporter and luciferase assay

To construct the miR302/367-Luc reporter, ~1kb promoter region of the miR302/367 cluster was amplified by PCR using specific primers (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/scd) and cloned into pGL3-Basic vector (Promega). 293T cells and HFFs were cultured in 24-well plate overnight, and then cotransfected with 100 ng of the luciferase reporter, 350 ng of pMig or pMig-OKSM, and 50 ng of CMV-LacZ by using Fugene HD (Roche). After 48 h of transfection, cells were lysed in 250 μ L of the passive lysis buffer (Promega) and assayed with a dual luciferase assay kit (Promega), as directed by the manufacturer. The luciferase activities were expressed as relative luciferase/LacZ activities, normalized to those of control transfections in each experiments.

Production of retrovirus and lentivirus

For retrovirus generation, 293T cells were plated at 8×10^5 cells per 60-mm dish, cultured overnight, and then transfected with 1.5 μ g of pCL-Ampho (IMGENEX) plus 2.5 μ g of pMig, pMig-OKSM, or pMig-miR302/367 by Fugene HD (Promega),

according to the manufacturer's instructions. For the preparation of the lentivirus, 293T cells were transfected with a mixture of DNA containing 2.5 μ g of lentiviral vectors and 2.5 μ g of the packaging mixture (Genecopoeia) by Fugene HD. Media containing retroviruses or lentiviruses was collected 24 h after transfection and filtered through a 0.45 μ m pore-size filter.

Reprogramming of HFFs

HFFs were seeded in a 12-well plate at 1×10^4 cells per well 1 day before transduction, and incubated with retroviruses (pMig vector, pMig-OKSM, and pMig-miR302/367) or lentiviruses (miRZip ctr or miR302i)-containing supernatant supplemented with 4 μ g/mL polybrene (Sigma), followed by centrifugation (900 g for 30 min). Four days postinfection, infected cells were split using 0.025% trypsin-EDTA and plated on MEF feeders. After 24 h, the medium was switched to hES cells culture medium and treated with 0.5 mM sodium butyrate (Sigma), 2 μ M SB431542 (Stemgent), and 0.5 μ M PD0325901 (Stemgent) or other combinations of chemical compounds indicated in Figs. 1 and 6. Medium was changed every other day until the induced colonies were counted based on hES cell colony morphology at day 20–23 postinfection.

Alkaline phosphatase and immunofluorescence staining

Alkaline phosphatase staining was performed according to the manufacturer's instructions using the Alkaline Phosphatase Detection Kit (Stemgent). The cells were fixed in 4% paraformaldehyde for 20 min at room temperature, washed thrice with phosphate-buffered saline, and blocked for 30 min with 5% FBS containing 0.05% Triton X-100, followed by incubation with primary and secondary antibodies. The antibodies were diluted in 1% FBS containing 0.05% Triton X-100. The TRA-1-60 antibody was purchased from Stemgent.

Gene expression analysis by semiquantitative RT-PCR and real-time qPCR

All of the total RNAs were extracted using Quick-RNA™ MicroPrep Kit (Zymo Research).

For semiquantitative RT-PCR analysis of the primary miR302/367 cluster, first strand reverse transcription was performed with 200 ng RNA using SuperScript III First-Strand Synthesis SuperMix Kit (Invitrogen), according to the manufacturer's instructions. For semiquantitative RT-PCR analysis of individual mature miR302/367 miRNAs, 500 ng RNA was reverse-transcribed using NCode™ VILO™ miRNA cDNA Synthesis Kit (Invitrogen). For real-time qPCR analysis of individual mature miR302/367 miRNAs, reverse-transcription was performed on 10 ng RNA using TaqMan MicroRNA Reverse Transcription Kit with specific RT primers (Applied Biosystems) and real-time PCR's TaqMan primers and probes (Applied Biosystems). The list of primers is included in the Supplementary Table S1.

Results

NaB displays a distinctive ability to promote cellular reprogramming

We recently found that an SM cocktail consisting of NaB, PD03259, and SB431542 selectively promotes the generation

of fully reprogrammed iPS cells [8]. These SMs are inhibitors of HDACs, MAPK/ERK, and TGF- β signaling pathways, respectively. To further investigate which combination of our SM candidates would promote the reprogramming process with the highest efficiency, we infected primary HFFs with the polycistronic retroviral vector pMig-hOKSM. After 2–3 weeks of culture using standard reprogramming procedures, the group treated with a cocktail consisting of these three SM (NaB + SB431542 + PD0325901, 3-SM) had the greatest iPS cell colony number, when compared with any other group (Fig. 1A). Notably, iPS cell colonies only appeared noticeably in the groups treated with sodium butyrate alone or together

with PD0325901 and/or SB431542, but not in groups treated with SB431542 or PD0325901 alone, suggesting that NaB has a distinctive role in reprogramming (Fig. 1A).

To test if the inhibition of HDACs with other inhibitors has a similar effect as NaB in promoting reprogramming, we replaced NaB with two other HDAC inhibitors, valproic acid (VPA) and Trichostatin A (TSA), and performed cellular reprogramming. We found that replacing NaB with VPA and TSA significantly decreased the reprogramming efficiency (Fig. 1B), indicating that the inhibition of HDACs is unlikely the primary mechanism by which NaB promotes reprogramming. Similarly, we assessed the reprogramming ability

FIG. 1. Effects of SMs on reprogramming efficiency. **(A)** Reprogramming efficiency after treatment with SMs. Primary HFFs were transduced with polycistronic retroviruses containing four TFs (pMig-OKSM), seeded at a density of 10,000 transduced cells per well of 12-well plate, and then treated with different combinations of SMs (NaB: 0.5 mM, SB431542: 2 μ M, PD0325901: 0.5 μ M). Emerging colonies were stained with FITC-labeled TRA-1-60-specific antibody. Values are mean $\pm$ SD. * $P < 0.05$; ** $P < 0.01$. **(B–D)** Effect of different inhibitors of HDACs and signaling pathways of MAPK/ERK and TGF- β on reprogramming efficiency. HFFs were infected with OKSM-expressing retroviruses, seeded at a density of 10,000 transduced cells per well of a 12-well plate, then treated with the following combinations of SMs: SB431542 and PD0325901 with three different HDACs inhibitors **(B)**; NaB and SB431542 with three individual MAPK/ERK inhibitors **(C)**; NaB and PD0325901 with three unique TGF- β inhibitors **(D)**. True iPS cell colonies were counted by immunofluorescence staining with FITC-labeled TRA-1-60-specific antibody. Values are mean $\pm$ SD. * $P < 0.05$; ** $P < 0.01$; N.S., not significant ($P > 0.05$). iPS, induced pluripotent stem; HDAC, histone deacetylase; TFs, transcription factor; TGF- β , transforming growth factor-beta; MAPK/ERK, mitogen-activated protein kinases/extracellular signal-regulated kinases; HFFs, human foreskin fibroblasts. Color images available online at www.liebertpub.com/scd

of other inhibitors of MAPK/ERK and TGF- β signaling pathways, which had similar roles to PD0325901, and SB431542, respectively. Our results showed that replacement with the other two MAPK/ERK signaling pathway inhibitors (i.e., PD98059 and U0126) and TGF- β signaling pathway inhibitors (i.e., A83-01 and ALK5 inhibitor) did not decrease reprogramming efficiency (Fig. 1C, D and Supplementary Fig. S1). These data suggest that the enhancement of reprogramming by PD0325901 and SB431542 is likely mediated through the inhibition of MAPK/ERK and TGF- β signaling pathways.

3-SM cocktail induced expression of the miR302/367 cluster dependent on four TFs

Our data (Fig. 1A) indicate that the 3-SM cocktail could provide a promising tool to identify new regulators with the potential to affect the reprogramming process. Therefore, we employed the 3-SM cocktail to identify candidate miRNAs that are involved in the reprogramming process. By profiling all known human miRNAs, we identified that the miRNAs from the miR302/367 cluster were induced at least 5-fold in the group treated with 3-SM cocktail than the control group (data not shown). Because the miR302/367 cluster is specifically expressed in hES cells, is regulated by reprogramming factors Oct4, Sox2, and Nanog [19,20], and is directly involved in reprogramming [14–16], we chose this cluster for further analysis. First, we decided to confirm the results from the array by using the TaqMan real-time stem-loop RT-PCR method, a reliable technique for quantifying the levels of mature miRNAs [21]. Consistent with previous reports [22], the results show that forced expression of four reprogramming TFs upregulated the transcriptional level of miR302/367 miRNAs (Fig. 2A–E). The expression of miRNAs from the miR302/367 cluster was noticeably upregulated in a time-dependent manner in HFFs infected with pMig-OKSM after the 3-SM cocktail treatment, when compared with the control group without treatment (Fig. 2A–E). These data suggest that OKSM TFs were required for 3-SM cocktail to upregulate mature miRNAs from the miR302/367 cluster.

Next, we asked which one of the four TF(s) is required to facilitate the 3-SM cocktail to induce mature miR302/367 miRNAs. To address this question, we infected HFFs with various combinations of retroviruses expressing the four individual TFs and treated them with the 3-SM cocktail. Consistent with our data (Fig. 2A–E) and previous studies [5,6], our qPCR analysis showed that coexpression of four TFs alone upregulated mature miR302/367 miRNAs. To test the role of each TF in the upregulation of the miR302/367 miRNAs, we took turns omitting each of the four TFs from the TF mixture. Our results showed that all four of the TFs are required for the 3-SM cocktail to maximally induce ma-

ture miR302/367 miRNAs. Interestingly, however, single Oct4 could also partially increase the expression levels of four mature miR302/367 miRNAs after treatment with the 3-SM cocktail, when compared with the other three TFs (Fig. 2F–J), suggesting that Oct4 is a primary regulator of the miR302/367 cluster. Together, our data indicate that the induction of mature miR302/367 miRNAs by the 3-SM cocktail is dependent on the four TFs, mainly Oct4.

NaB exhibits unique capacity to upregulate the miR302/367 cluster

We showed that NaB displays a unique ability to promote reprogramming (Fig. 1A) and that the 3-SM cocktail can upregulate the miR302/367 cluster (Fig. 2). Therefore, we asked whether or not NaB is a chief SM in the 3-SM cocktail for inducing the expression of miR302/367 cluster. We treated OKSM-infected HFFs with different combinations of SM. Our data showed that mature miR302/367 miRNAs expression was largely upregulated by the combinations containing NaB, and by NaB alone (Fig. 3). Although the other two SMs (SB431542 and PD0325901) further enhanced the expression of miR302b, miR302a, and miR367, either of these two SMs alone or a combination of these two SMs failed to upregulate miRNAs in the miR302/367 cluster (Fig. 3). These results suggest that the expression levels of mature miR302/367 miRNAs upregulated by the combinations of SMs were correlated with their ability in promoting somatic cells reprogramming, as shown in Fig. 1A.

SMs increase miRNA stability and transcriptional level of the miR302/367

Because NaB has a superior effect on reprogramming efficiency when compared with VPA or TSA, we asked if NaB is more competent than VPA and TSA in upregulating the expression of the pri-miR302/367 cluster. To address this question, we first performed semiquantitative PCR analysis of the pri-miR302/367 expression in OKSM-transduced HFFs treated with the 3-SM cocktail for an increasing period of time (Fig. 4A). The results showed that the 3-SM cocktail increased expression of the primary miR302/367 cluster in a time-dependent manner. Next, we treated OKSM-transduced HFFs with the two inhibitors (SB431542 and PD0325901), together with NaB, VPA, or TSA. Consistent with the data in Fig. 3, our semiquantitative PCR analysis showed that NaB alone, or combined with SB and PD, greatly increased the expression of pri-miR302/367, when compared with VPA or TSA (Fig. 4B). Since the miR302/367 cluster has shown the ability to enhance reprogramming [14–16,23], our data suggest that the different contributions of HDAC inhibitors on reprogramming efficiency may be

FIG. 2. Expression of mature miR302/367 cluster miRNAs is induced by the 3-SM cocktail and is dependent on TFs. (A–E) qPCR analysis of mature miR302/367 cluster miRNAs in HFFs after treatment with the 3-SM cocktail. HFFs were infected with retroviruses containing pMig-OKSM or pMig only, and then treated with the 3-SM cocktail for 12, 24, 48, and 72 h. Each miRNA was quantified by TaqMan qPCR analysis. Values are mean $\pm$ SD. ** P < 0.01. (F–J) qPCR analysis of mature miR302/367 cluster miRNAs in HFFs expressing different combination of TFs. HFFs were transduced with retroviruses expressing different combinations of four TFs or containing pMig vector only (controls), and then treated with 3-SM cocktail. miRNA transcripts were measured by TaqMan qPCR analysis. Values are mean $\pm$ SD. * P < 0.05; ** P < 0.01. SM, small molecule; miRNA, microRNA. Color images available online at www.liebertpub.com/scd

FIG. 3. Expression of mature miR302/367 cluster miRNAs after treatment with different combinations of SMs. (A–E) HFFs were infected with retroviruses expressing OKSM or pMig vector only (controls), and then treated with individual or different combinations of three SMs: NaB, PD0325901 (PD), and SB431542 (SB). TaqMan qPCR was used to examine expression of the miR302b (A), miR302c (B), miR302a (C), miR302d (D), and miR367 (E). Values are mean $\pm$ SD. ** $P < 0.01$; N.S., not significant ($P > 0.05$). Color images available online at www.liebertpub.com/scd

partially due to their ability to modulate miR302/367 expression.

Previous studies have shown that the miR302/367 cluster is subject to transcriptional regulation by TFs such as Oct4 and Sox2, and is transcribed by RNA polymerase II and contains a 5' cap and a polyadenylated tail [19]. Thus, there are two possible mechanisms by which the 3-SM cocktail upregulates the miR302/367 cluster: (1) the 3-SM cocktail upregulates the pri-miR302/367 at the transcriptional level, and (2) the 3-SM cocktail increases the stability of the primary and/or mature miR302/367 miRNA.

To determine whether the 3-SM cocktail could upregulate the pri-miR302/367 promoter, we constructed a luciferase reporter driven by the proximal promoter region of the miR302/367 cluster (Fig. 4C) and performed a luciferase reporter assay by transfecting this reporter (miR302/367-Luc)

into 293T, HFFs, and HFFs stable lines expressing OKSM TFs (or vector only), respectively. Consistent with the previous study [19], our data show that the expression of OKSM activates the miR302/367 promoter and the 3-SM cocktail and NaB enhanced transactivity of the reprogramming TFs in the miR302/367 promoter (Fig. 4D).

To investigate the second possible mechanism, we transduced HFFs with miR302/367-expressing retroviruses and then treated these cells with the 3-SM cocktail, followed by an addition of actinomycin D, a RNA synthesis inhibitor. By qPCR analysis, we found that the 3-SM cocktail significantly increased the stability of the pri-miR302/367 (Fig. 5A), mature miR302b (Fig. 5B), and miR367 (Fig. 5C and Supplementary Fig. S2). Together, these data indicate that the 3-SM cocktail increases expression of mature miRNAs from the miR302/367 cluster by increasing their miRNA stability.

FIG. 4. SMs upregulate the pri-miR302/367 promoter in TFs-dependent manner. (A) Semi-quantitative PCR analysis of primary miR302/367 after treatment with the 3-SM cocktail for increasing times. HFFs were transduced with pMig-OKSM retroviruses, and then treated with or without the 3-SM cocktail for 3, 6, 12, and 24 h. Transcriptional level of pri-miR302/367 was examined by gene-specific, semi-quantitative RT-PCR. Density of amplified DNA bands was quantified by ImageJ (NIH) and normalized by GAPDH. (B) qPCR analysis of pri-miR302/367 after treatment with three HDAC inhibitors. OKSM-transduced HFFs were treated with a NaB alone, a mix of SB and PD, and NaB, VPA, or TSA in combination of a mixture containing SB and PD. As controls, the following two HFFs were included: cells infected with retroviruses containing pMig vector only, OKSM-transduced cells without treatment. Semi-quantitative RT-PCR was performed using gene-specific primers. Relative expression of pri-miR302/367 was quantified by Image J (NIH) and normalized by GAPDH expression. (C) Diagram of the miR302/367 promoter-driven luciferase reporter (miR302/367-Luc). The miR302/367 promoter contains TATA-box, two separate Sox2 binding sites, and a binary Oct4 response element. (D) Relative activity of the miR302/367-Luc after treatment with the 3-SM cocktail or NaB. 293T cells (left panel) or HFFs (right panel) were transfected with the miR302/367-Luc with either empty vector pMig or pMig-OKSM, and then treated with or without 3-SM cocktail or NaB for 48 h before luciferase assay. pCMV-LacZ was included in each transfection as an internal control to normalize luciferase activity. Values are mean $\pm$ SD. ** $P < 0.01$. VPA, valproic acid; TSA, trichostatin A. Color images available online at www.liebertpub.com/scd

FIG. 5. The stability of the miR302/367 cluster after treatment with the 3-SM cocktail. HFFs were transduced with retroviruses expressing the miR302/367 cluster, and then treated with or without 3-SM cocktail for increasing times before addition of Actinomycin D (RNA synthesis inhibitor) for indicated times. QPCR was performed to measure transcripts of pri-miR302/367 (A), mature miR302b (B), and mature miR367 (C) after treatment with the 3-SM cocktail. Values are mean $\pm$ SD. ** $P < 0.01$. Color images available online at www.liebertpub.com/scd

The miR302/367 cluster is the mediator for SMs to enhance TFs-mediated cellular reprogramming

To address the key question of whether or not miRNAs from the miR302/367 cluster are essential for the 3-SM cocktail in enhancing HFF reprogramming, we first blocked the function of miR302b in OKSM-transduced HFFs by using a miR302b-specific inhibitor (miRZip), because miR302b exhibited the highest expression during reprogramming (Supplementary Fig. S3). We performed standard reprogramming experiments with and without the 3-SM cocktail (Fig. 6A) or NaB (Fig. 6B), and our data show that inhibition of mature miR302b decreased the total number of TRA-1-60⁺ colonies by 30%, when compared with the miRZip control group (Fig. 6A, B). These data clearly indicate that the inhibition of miRNAs from the miR302/367 cluster attenuated the ability of the 3-SM cocktail or NaB to enhance the reprogramming efficiency. Further, we compared the effects of overexpression of the miR302/367 with the 3-SM cocktail on the reprogramming efficiency (Fig. 6C). Our data showed that the miR302/367 partially substituted for the ability of the 3-SM cocktail in enhancing the reprogramming process, suggesting that other miRNAs or molecules may also play roles in the reprogramming process facilitated by the 3-SM cocktail. Nevertheless, these data demonstrated that the miR302/367 cluster is a crucial mediator for the 3-SM cocktail to accelerate the reprogramming process in HFFs.

Discussion

Recent studies mainly focus on screening SMs and confirming their roles in reprogramming efficiency and only few studies have investigated the molecular mechanisms of how SMs play roles in the reprogramming process. For example, Mali et al. reported that butyrate enhances histone H3 acetylation and promotes DNA demethylation and the expression of endogenous pluripotency-associated genes to enhance reprogramming efficiency [6]. Liang et al. recently showed that butyrate facilitates reprogramming at an early stage only in the presence of exogenous c-Myc and upregulates ES cell-enriched genes during reprogramming [5]. Although, these studies provide insights in the actions of butyrate on reprogramming the underlying mechanisms by which it does this remain largely unknown. Our data clearly indicate that inhibition of HDACs is unlikely a primary mechanism for butyrate's action on reprogramming (Fig. 1B), because VPA and TSA, the two other potent HDAC inhibitors, are less effective than NaB by at least 10-fold in terms of their ability to promote reprogramming. By contrast, other analogous inhibitors have similar effects with PD0325901 and SB431542 on reprogramming (Fig. 1C, D), which suggests that these two SM promote reprogramming mainly through the inhibition of the MAPK/ERK and TGF- β signaling pathways.

Recent studies mainly investigated the transcription-factor profiles and did not provide a complete view of the action of butyrate on reprogramming [6]. Since miRNAs recognize multiple targets and have a tremendous ability and diversity to regulate the whole gene expression network, they could robustly control the reprogramming process. For example, overexpression of the miR302/367 cluster promotes cellular reprogramming [11,14–17,22,23]. It has been shown that the miR302/367 cluster is highly expressed in both mouse and

FIG. 6. The miR302/367 cluster is essential for the 3-SM cocktail and NaB to enhance reprogramming. (A, B) Reprogramming efficiency of HFFs after inhibition of miR302b using lentiviral-based miRZip. HFFs were infected with lentiviruses expressing miRZip that targets miR302b, and then transduced with OKSM-expressing retroviruses. Infected cells were seeded on feeder cells in 12-well plate at a density of 20,000 transduced cells per well and cultured in medium with the 3-SM cocktail (A) or NaB (B). iPS cell colonies were identified by staining with FITC-labeled anti-TRA-1-60 antibody. Values are mean $\pm$ SD. ** $P < 0.01$; N.S., not significant ($P > 0.05$). (C) Reprogramming efficiency of OKSM-transduced HFFs with miR302/367 overexpression or treated with the 3-SM cocktail. HFFs were transduced with OKSM-expressing retroviruses, and then infected with retroviruses expressing the miR302/367 cluster or pMig vector (control) only, or treated with the 3-SM cocktail. Cells were then seeded at a density of 20,000 transduced cells per well of a 12-well plate. TRA-1-60⁺ iPS cell colonies were identified by corresponding antibody. Values are mean $\pm$ SD. ** $P < 0.01$. Color images available online at www.liebertpub.com/scd

hES cells but not somatic cells [19,20]. Interestingly, Jouneau et al. recently showed that members of the miR290–295, miR17–92, and miR302/367 clusters are differentially expressed in mouse ES cells and epiblast stem cells in a primed pluripotent state [24]. Notably, expression of miRNAs from the miR302/367 cluster is significantly lower in mouse ES cells than epiblast stem cells [24]. Thus, it suggests that the miR302/367 cluster could differentially regulate reprogramming and stemness.

Previous and recent studies indicate that the miR302/367 cluster is a target of Oct4 and Sox2, and its proximal promoter region occupies binary consensus binding motifs for Oct4 and the two separate binding sites for Sox2 [19]. In a luciferase reporter assay, Oct4 shows a stronger transcriptional activity than that of Sox2 in the promoter of the miR302/367 cluster [19]. Our current findings indicate that SMs PD0325901 and SB431542 have a limited effect on the expression of the miR302/367 cluster, but NaB significantly upregulates the expression of the miR302/367 cluster by enhancing the transcriptional activity of the four reprogramming factors in the promoter. Therefore, it is tempting to examine whether deletion of the Oct4 binding site would abolish the ability of the 3-SM cocktail to enhance the transactivation of the four reprogramming TFs in the miR302/367 promoter. In future studies, it would be worthwhile to investigate whether NaB and the 3-SM cocktail enhanced Oct4 transcriptional activity in the promoter of miR302/367. Noteworthy, iPS cells have been generated without Sox2 from melanocytes [25] and with Oct4 alone from adult neural stem cells [26]. It will be interesting to determine whether or not the miR302/367 is critical for iPS cell generation from these cells. Intriguingly, Ding's group generated human iPS cells from epidermal Keratinocytes and cell types other than HFFs using Oct4 plus

an SM cocktail consisting of 4 to 6 inhibitors [27]. Notably, Shimada et al. recently showed that a combination of five SMs including A-83-01, Chir99021, PD0325901, Sodium butyrate, and Y27632 accelerate the generation of human iPS cell from adipose-derived stem cells under physiological hypoxia [28]. Therefore, it is worthwhile to assess whether hypoxia can accelerate iPS cell generation from HFFs using OSKM and the 3-SM cocktail.

In summary, we identify for the first time the miR302/367 cluster as an important regulator that mediates the ability of the 3-SM cocktail in promoting reprogramming, thus providing direct evidence that the 3-SM cocktail enhances somatic cell reprogramming via miRNAs. Although the downstream regulation of the miR302/367 in the reprogramming process remains elusive, we uncovered a new mechanism in which SMs, such as NaB, enhance the transcriptional activity of the four reprogramming TFs in the miR302/367 promoter and increased miRNA stability, leading to the upregulation of the miR302/367. As a result, an elevated level of miRNA302/367 correlates with four TFs to promote cellular reprogramming (Fig. 7).

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Author Disclosure Statement

The authors declare that they have no conflict of interest.

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FIG. 7. Action model for the 3-SM cocktail to enhance cellular reprogramming. The 3-SM cocktail enhances the transcriptional activity of the four reprogrammed factors (largely via NaB), leading to upregulation of the miR302/367 whose stability is further sustained by the 3-SM cocktail. As a result, elevated level of the miRNA302/367 will enhance TFs-mediated cellular reprogramming. Color images available online at www.liebertpub.com/scd

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