Journal of Reproduction and Development, Vol. 63, No 4, 2017

—Original Article—

miR-6539 is a novel mediator of somatic cell reprogramming that represses the translation of *Dnmt3b*

Fujia WU[1)]*, Li TAO[1)]*, Shuai GAO[1)]*, Likun REN[1)], Zhuqing WANG[1)], Shumin WANG[1)], Jianhui TIAN[1)] and Lei AN[1)]

[1)]*National Engineering Laboratory for Animal Breeding, Key Laboratory of Animal Genetics, Breeding and Reproduction of the Ministry of Agriculture, College of Animal Science and Technology, China Agricultural University, Beijing 100193, P. R. China*

Abstract. Global DNA hypomethylation has been shown to be involved in the pluripotency of induced pluripotent stem (iPS) cells. Relatedly, DNA methyltransferases (DNMTs) are believed to be a substantial barrier to genome-wide demethylation. There are two distinct stages of DNMT expression during iPS cell generation. In the earlier stage of reprogramming, the expression of DNMTs is repressed to overcome epigenetic barriers. During the late stage, the expression of DNMTs is upregulated to ensure iPS cells obtain the full pluripotency required for further development. This fact is strongly reminiscent of microRNAs (miRNAs), critical regulators of precise gene expression, may be central to coordinate the expression of DNMTs during reprogramming. Using a secondary inducible system, we found that miR-6539 had a unique expression dynamic during iPS cell generation that inversely correlated with DNMT3B protein levels. Enforced upregulation of miR-6539 during the early stage of reprogramming increased the efficiency of iPS cell generation, while enforced downregulation impaired efficiency. Further analysis showed that *Dnmt3b* mRNA is the likely target of miR-6539. Notably, miR-6539 repressed *Dnmt3b* translation via a target site located in the coding sequence. Our study has therefore identified miR-6539 as a novel mediator of somatic cell reprogramming and, to the best of our knowledge, is the first to demonstrate miRNA-mediated translation inhibition in somatic cell reprogramming via targeting the coding sequence. Our study contributes to understand the mechanisms that underlie the miRNA-mediated epigenetic remodeling that occurs during somatic cell reprogramming.
Key words: *Dnmt3b*, Induced pluripotent stem (iPS), miR-6539, Reprogramming, Translation

(**J. Reprod. Dev. 63:** 415–423, 2017)

Pluripotency strongly associates with global DNA hypomethylation in the inner cell mass (ICM) of blastocysts, their embryonic stem (ES) cell derivatives [1, 2], and induced pluripotent stem (iPS) cells [3–5]. Several studies have shown that global DNA hypomethylation is the most important contributor to the pluripotency of these cell types. This is largely through promoting transcription of genes important for maintaining self-renewal and repressing differentiation [6–8]. During the induced-reprogramming of somatic cells to generate iPS cells, demethylation is the key to establish the global DNA hypomethylation that is associated with pluripotency. This is similar to the process that naturally occurs during preimplantation development [9–11]. Prior work has also indicated that DNA demethylation is required for the process of mesenchymal-epithelial transition (MET), during which some of the first detectable changes in early reprogramming stage occur [12]. It is hypothesized that if DNA methylation is not fully removed during iPS cell generation, a significant proportion of cells remain trapped in a partially reprogrammed state [11].

DNA methyltransferases (DNMTs) have previously been considered a major barrier to reprogramming [10]. Treatment with 5-aza-2'-deoxycytidine (AZA, a non-specific inhibitor of DNMTs) facilitates the generation of pluripotent stem cells from somatic cells under different reprogramming systems [10, 11, 13]. Furthermore, silencing *Dnmt1* and *Dnmt3b* promotes somatic cell reprogramming by demethylating pluripotency-associated gene promoters [11, 14]. However, at later stage of reprogramming, upregulation of *Dnmt*s expression is essential to the developmental potential of fully pluripotent iPS cells [15]. This is supported by data showing that ES cells deficient in *Dnmt1* and/or *Dnmt3a/3b* have impaired pluripotency and differentiation potential [16, 17]. Together, these studies highlight the fact that DNMT expression is highly dynamic and must be precisely controlled during somatic cell reprogramming.

MicroRNAs (miRNAs) are critical regulators of gene expression that function by either targeting specific mRNAs for degradation or through suppressing translation. Remarkably, they have been virtually implicated in reprogramming, pluripotency and differentiation, by orchestrating the expression of key regulators of pluripotency and differentiation, including pluripotency factors, *Dnmts*, and apoptotic genes [18, 19]. In addition, the importance of miRNAs to the establishment and maintenance of pluripotency is further demonstrated by studies indicating that transfection of specific miRNA mimics,

Received: December 12, 2016
Accepted: May 12, 2017
Published online in J-STAGE: June 9, 2017

* F Wu, L Tao and S Gao contributed equally to this work.
Correspondence: J Tian (e-mail: tianjh@cau.edu.cn); L An (e-mail: anleim@cau.edu.cn)

or administration of miRNA inhibitors, can promote somatic cell reprogramming [20–24].

In many cases, specific mRNA can be targeted by more than one miRNA via both mRNA degradation by targeting sites in the 3' untranslated regions (UTRs) and translation inhibition by targeting sites in the coding sequences (CDSs) [25, 26]. This led us to postulate that miRNA functional redundancy may be involved in the regulation of *Dnmt*s during somatic cell reprogramming. To investigate this hypothesis, our study used QRT-PCR to screen miRNA expression patterns from our previously published miRNA dataset [27]. This identified miR-6539 as a miRNA with a unique expression dynamic during somatic cell reprogramming. Specifically, miR-6539 expression was significantly higher during the early phase of reprogramming and then consistently lowered until the formation of iPS cells. These dynamics correlated inversely with the protein levels of DNMT3B during reprograming, suggesting that *Dnmt3b* was a potential target of miR-6539. These data led us to speculate that miR-6539 is significantly involved in somatic cell reprogramming. To investigate this hypothesis, we utilized a secondary inducible system that used doxycycline (Dox)-controlled Tet-on inducible OSKM (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*). This system has previously been shown to be effective at evaluating reprogramming efficiency due to consistent proviral integration [28–30]. Using the system, we confirmed that miR-6539 was a novel mediator that repressed *Dnmt3b* expression during somatic cell reprograming. More importantly, our results indicated that miR-6539 facilitates *Dnmt3b* repression by inhibiting translation via a target site in the CDS. To the best of our knowledge, our study is the first to report miRNA-mediated translation inhibition by targeting the CDS during induced somatic cell reprogramming. These data contribute to the understanding of mechanisms that underlie miRNA-involved epigenetic remodeling during reprogramming.

Materials and Methods

Animals

All mice were housed in an animal facility at normal temperatures (20 ± 2°C), under a controlled light cycle (12 h light: 12 h dark), and with free access to water and food. All procedures were performed in accordance with protocols described in the China Agricultural University Guide for the Care and Use of Laboratory Animals.

Cell culture

Our study used a secondary inducible system, in which four doxycycline (Dox)-inducible transcription factors, *Oct4*, *Sox2*, *Klf4*, and *c-Myc*, were transduced into mouse embryonic fibroblasts (MEFs) isolated from ROSA26-M2rtTA transgenic mice. Secondary-inducible MEFs were produced from all-iPS cell mice through tetraploid complementation [28, 30].

MEFs and secondary MEFs were cultured in a fibroblast medium containing DMEM (Gibco, Life Technologies, Grand Island, NY, USA), supplemented with 10% FBS (Gibco, Life Technologies), 1 mM L-glutamine, 1% nonessential amino acids, and penicillin/streptomycin (Chemicon, Temecula, CA, USA). iPS cells were cultured on mitomycin C (MMC)-treated MEFs in ES medium, which contained DMEM supplemented with 15% FBS, 1 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 1% nonessential amino acids, and 1000 U/ml leukemia inhibitory factor (all from Chemicon, Temecula).

Vectors

DNA template encoding the precursor sequence of miR-6539 was amplified by PCR from genomic DNA isolated from a mouse liver. This was cloned into the *Bam*HI and *Eco*RI restriction sites of a PCDH5 plasmid to construct the overexpression vector for miR-6539. The wild-type (WT) *Dnmt3b* CDS fragment (including the predicted target site) was amplified from mouse cDNA, while the mutant (Mut) *Dnmt3b* CDS fragment was synthesized by Beijing Zixi Bio Tech, Beijing, China. The plasmids '*Dnmt3b* WT' and '*Dnmt3b* Mut' were generated by inserting the wild-type *Dnmt3b* CDS fragment and mutant *Dnmt3b* CDS fragment into the *Not*I and *Xho*I restriction sites of psiCHECK-2 plasmid, respectively. All plasmids created during this study were verified by sequencing. The primers used for vector construction are included in Table 1.

Generation of iPS cells and miR-6539-derived iPS cells

Secondary MEFs were seeded at 2×10^4 cells per well on a gelatin-coated 12-well plate. After ES cell-like colonies had developed (typically day 12 after addition of 1 μg/ml Dox), the media were replaced with ES cell medium for 2–3 days. Colonies were then mechanically isolated and digested to be cultured as ES cells on MMC–treated MEFs.

For miR-6539-derived iPS cells, secondary MEFs were transfected the day after seeding with 200 nM miR-6539 mimic by Lipofectamine 2000 (Invitrogen, Life Technologies, Carlsbad, CA, USA), according to the manufacturer's recommendations. Twenty-four hours after transfection with the miR-6539 mimic, culture medium was replaced with ES medium and supplemented with 1 μg/ml Dox. Media were changed daily. After ES cell-like colonies had developed (again, approximately day 12 after 1 μg/ml Dox addition), the medium was replaced with ES medium without Dox and cells were incubated for a further 2–3 days. Finally, colonies were mechanically isolated and digested for culturing on MMC-treated MEFs.

Alkaline phosphatase (AP) staining

AP staining was performed using an Alkaline Phosphatase Detection Kit (Sigma-Aldrich, St. Louis, MO, USA), following the instructions of the manufacturer. Cells were stained at day 8 after the addition of 1 μg/ml Dox when iPS colonies had developed.

Reverse Transcription PCR (RT-PCR)

Total RNA was extracted from cell pellets using TRIzol reagent (Invitrogen, Life Technologies). Each RNA sample was treated with DNase I (Thermo Scientific™) to remove any potentially contaminating genomic DNA and then used as a template for reverse transcription with an iScriptTM cDNA synthesis kit (Bio-rad, Hercules, CA, USA), according to the manufacturer's recommendations. The primers for ES cell marker genes are listed in Table 1.

Quantitative RT-PCR (QRT-PCR) to assess expression of miRNAs and marker genes

For miRNA quantification, the Bulge-LoopTM miRNA qPCR

Table 1. Primers used

Primer	Sequence (5′–3′)	Application
Pre-miR-6539-F	CGGGAATTCGAAGAATGTCCTTCACCTATTG	Cloning for miR-6539 overexpression vector
Pre-miR-6539-R	CCTGGATCCCCTATAGGGAGCTGTGAAAATC	Cloning for miR-6539 overexpression vector
Dnmt3b WT-F	GCACTCGAGGTTGTACCCAGCAATTCCTG	Cloning for *Dnmt3b* WT vector
Dnmt3b WT-R	TTAGCGGCCGCGATTGACGTTAGAGAGATCATTG	Cloning for *Dnmt3b* WT vector
Gapdh-F	TGCCCCCATGTTTGTGATG	QRT-PCR for *Gapdh*
Gapdh-R	TGTGGTCATGAGCCCTTCC	QRT-PCR for *Gapdh*
Oct4-F	TCTTTCCACCAGGCCCCCGGCTC	QRT-PCR for *Oct4*
Oct4-R	TGCGGGCGGACATGGGGAGATCC	QRT-PCR for *Oct4*
Sox2-F	TAGAGCTAGACTCCGGGCGATGA	QRT-PCR for *Sox2*
Sox2-R	TTGCCTTAAACAAGACCACGAAA	QRT-PCR for *Sox2*
Nanog-F	AGGGTCTGCTACTGAGATGCTCTG	QRT-PCR for *Nanog*
Nanog-R	CAACCACTGGTTTTTCTGCCACCG	QRT-PCR for *Nanog*
Dnmt3b-F	TTATCGTTAATGGGAACTTCAGTG	QRT-PCR for *Dnmt3b*
Dnmt3b-R	CATGTCCTGCGTGTAATTCAG	QRT-PCR for *Dnmt3b*
Me-Oct4-out-F	GAGGATTGGAGGTGTAATGGTTGTT	Methylation analysis of *Oct4*
Me-Oct4-out-R	CTACTAACCCATCACCCCCACCTA	Methylation analysis of *Oct4*
Me-Oct4-in-F	TGGGTTGAAATATTGGGTTTATTT	Methylation analysis of *Oct4*
Me-Oct4-in-R	CTAAAACCAAATATCCAACCATA	Methylation analysis of *Oct4*
Me-Nanog-out-F	AAGTATGGATTAATTTATTAAGGTAGTT	Methylation analysis of *Nanog*
Me-Nanog-out-R	AAAAAACCCACACTCATATCAATATA	Methylation analysis of *Nanog*
Me-Nanog-in-F	AAGTATGGATTAATTTATTAAGGTAGTT	Methylation analysis of *Nanog*
Me-Nanog-in-R	CAACCAAATAACCTATCTAAAAA	Methylation analysis of *Nanog*

Primer Set (RiboBio, Guangzhou, China) was used to detect miRNA expression with a SYBR Green-based PCR Master Mix (Bio-rad) and a Bio-rad CFX96 Real-Time PCR System. For marker gene quantification, a 1/10 dilution of cDNA was used as a template with a SYBR Green-based PCR Master Mix (Bio-rad) on a Bio-rad CFX96 Real-Time PCR System. The relative expression levels of miRNAs and mRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method and either U6 or glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as internal controls, respectively, using a previously reported protocol [23].

Bisulfite sequencing

Genomic DNA was extracted using a genomic DNA extraction kit (TIANGEN, Beijing, China) and the standard protocol. The extracted DNA was then treated with a Methylamp DNA Modification kit (Epigentek, New York, NY, USA) and used as a template to amplify sequences in the promoter regions of *Oct4* and *Nanog* using a two-round, nested PCR. Subsequent PCR products were cloned into vectors using a pEASY-T5 Zero cloning kit (TransGen Biotech, Beijing, China). For validation, five randomly selected clones were sequenced.

Western blot

Forty-eight hours after transfection with the miR-6539 overexpression vector and corresponding control vector, HEK293T cells were washed twice using PBS and lysed using RIPA buffer (CWBio, Beijing, China). Equivalent amounts of protein from each sample were separated on SDS-PAGE gels and then transferred to polyvinylidene fluoride (PVDF) membranes. These were then incubated overnight at 4°C in blocking solution with the primary antibodies anti-DNMT3B (1:1000; Abcam, Cambridge, UK; mouse monoclonal antibody to DNMT3B, Cat#: ab13604) or anti-β-actin (1:2000; Abcam, mouse monoclonal antibody to β-actin, Cat#: ab8226) (β-actin was selected as a loading control). ECL peroxidase-labeled anti-mouse antibody (1:5000, ZsBio, Beijing, China) was used as the secondary antibody. Finally, data were analyzed using ImageJ software (https://imagej.nih.gov/ij/).

Dual-luciferase reporter assay

HEK293T cells in 24-well plates were transfected with 400 ng miR-6539 overexpression vector and 400 ng psiCHECK-2 vector (Promega, Madison, WI, USA) using Lipofectamine 2000 (Invitrogen, Life Technologies). Forty-eight hours post-transfection, cell lysates were collected and the Firefly and Renilla luciferase signals measured using a dual-luciferase reporter assay system (Promega), according to the instructions of the manufacturer. Firefly reporter activity was used to normalize the Renilla luciferase signal.

Statistical analyses

All data are presented as the mean ± SD from three independent experiments. Differences between control and experimental groups were evaluated using two-tailed Student's *t*-tests, where *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

Results and Discussion

The expression of miR-6539 inversely correlated with Dnmt3b expression during somatic cell reprogramming

To explore the potential role of miRNAs in somatic cell reprogram-

ming, we filtered miRNAs from our previous miRNA expression dataset that were highly enriched in MEFs or iPS cells (Fig. 1A and B) [27]. For each potentially associating miRNA, the expression dynamics during reprogramming were evaluated using QRT-PCR. This revealed that most potential miRNAs were highly dynamic during reprogramming in a secondary inducible system. However, among this group of miRNAs, we noted that miR-6539 had a unique expression dynamic. During the early stage of reprogramming, miR-6539 was markedly higher, with a peak on day 4. After this peak, miR-6539 expression consistently decreased until the formation of iPS colonies (Fig. 1C). This dynamic was the inverse of DNMT3B protein levels during reprogramming, but not *Dnmt3b* mRNA levels (Figs. 1D–F). Previous studies have shown that there are two phases of DNMT expression during somatic cell reprogramming. During the early phase, DNMT levels are lower to facilitate global DNA demethylation as high DNMT abundance blocks the demethylation process [11]. Furthermore, treatment with DNMT inhibitors promotes iPS cell induction [10, 11, 13]. However, during the late phase, levels of DNMT3A and DNMT3B protein are elevated [23]. *Dnmt3a*- and *Dnmt3b*-deficient iPS cells also show restricted developmental potential [15], suggesting that DNMTs are required for full pluripotency.

Next, we used the miRNA target prediction software packages TargetScan, Miranda, and miRBase to identify potential binding sites for miR-6539. This analysis highlighted *Dnmt3b* as a likely target of miR-6539 during reprogramming via a target site in the CDS located within the C-terminal domain (exon 16 of transcript variant 1). Further analysis showed that this target site is common to all nine transcript variants of *Dnmt3b*. These data, together with the inverse correlation we identified between miR-6539 and DNMT3B protein levels, led us to hypothesize that miR-6539 is significantly involved in epigenetic remodeling during somatic cell reprogramming, likely by affecting the expression dynamics of DNMT3B.

miR-6539 facilitates DNA demethylation during the earlier phase of reprogramming and promotes iPS cell formation

To determine if miR-6539 facilitates DNA demethylation during the earlier phase of reprogramming, we detected the methylation status of *Oct4* and *Nanog* promoter regions after overexpressing miR-6539. Demethylation of these genes has previously been shown to be critical to pluripotency and important to reprogramming [31]. As shown in Fig. 2A, enforced upregulation of miR-6539 induced rapid demethylation of the *Oct4* and *Nanog* promoter regions during the early phase of reprogramming, compared to untransfected and mimic control groups.

Next, to further identify the role of miR-6539 in overcoming the epigenetic barrier to somatic cell reprogramming, we determined whether enforced upregulation of miR-6539 facilitated reprogramming efficiency (Fig. 2B). This showed that enforced upregulation of miR-6539 during the early phase of reprogramming significantly increased the number of alkaline phosphatase positive (AP^+) iPS colonies, compared to mimic control (Fig. 2C). These iPS colonies were also found to passage normally with a well-characterized morphology (Fig. 2D). In contrast, the efficiency of iPS cell generation was greatly decreased when miR-6539 inhibitor was introduced during somatic cell reprogramming (Fig. 2C). These results show that miR-6539 is involved in the induction of iPS cells.

Our study supports prior work by Guo *et al*. [23], and confirms a well-known relationship between miRNAs and target genes, by showing that a single miRNA can target many genes (miR-29b targets both *Dnmt3a* and *Dnmt3b*) and conversely, one gene may also be targeted by many miRNAs (for example, *Dnmt3b* is targeted by both miR-29b and miR-6539). This 'multiple-to-multiple' relationship between miRNAs and their target genes, previously shown to be involved in carcinogenesis [32–35], also has an essential role during somatic cell reprogramming.

Pluripotency characterization of miR-6539-derived iPS cells

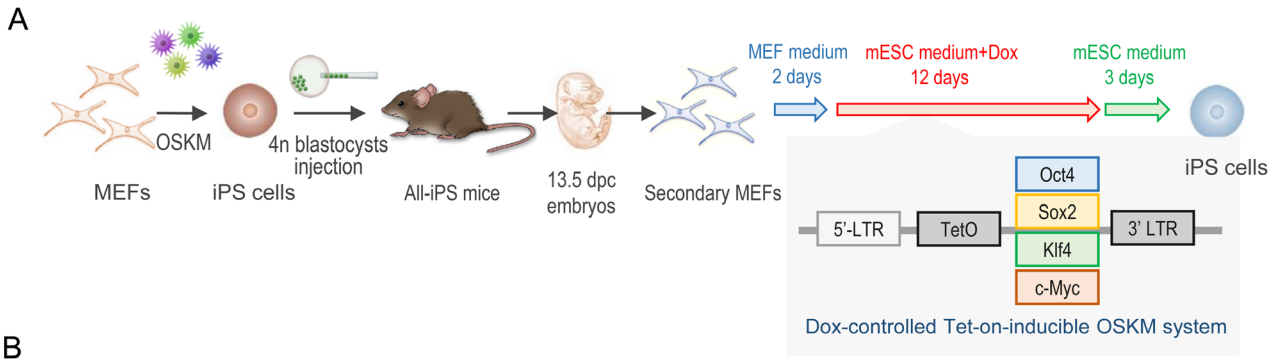
To examine the pluripotency of iPS cells after the introduction of miR-6539 (miR-6539-derived iPS cells), we first assessed the expression of several endogenous pluripotency markers (*Oct4*, *Sox2*, and *Nanog*), from three different miR-6539-derived iPS cell lines, using RT-PCR (Fig. 3A). Results from QRT-PCR further indicated that expression of these pluripotency markers in miR-6539-derived iPS cells was comparable to that in control iPS and R1 ES cells (Fig. 3B). Supporting previous studies, fully activated endogenous pluripotency genes, *Oct4* and *Nanog*, had hypomethylation in their promoter regions (Fig. 3C). In summary, these data show that the pluripotency-associated characteristics of miR-6539-derived iPS cells are comparable to those of control iPS and ES cells.

miR-6539 represses the translation process of Dnmt3b via the target site in the CDS

To understand the mechanisms that underlie the effect that miR-6539 expression had on somatic cell reprogramming, we investigated the potential miR-6539 target site in *Dnmt3b*. This site in the CDS was predicted by three separate target prediction programs (TargetScan, Miranda, and miRBase). Luciferase reporters were constructed that had either the wild-type (WT) or a mutant (Mut) target *Dnmt3b* sequence (Fig. 4A). Compared to the empty control vector, overexpression of miR-6539 significantly suppressed luciferase activity of the construct containing the wild-type target sequence of *Dnmt3b*. Moreover, this inhibitory effect was significantly attenuated when the luciferase construct containing the mutant target sequence of *Dnmt3b* was used (Fig. 4B). These results indicate that miR-6539 specifically suppressed *Dnmt3b* via targeting its CDS.

As CDS-targeting miRNAs typically lead to translation inhibition [36], we assessed the endogenous expression of *Dnmt3b* at both the mRNA and protein levels after overexpressing miR-6539. As expected, *Dnmt3b* mRNA levels were not significantly changed (Fig. 4C) but DNMT3B protein abundance was significantly decreased (Fig. 4D). Taken together, our results indicated that miR-6539-mediated suppression of *Dnmt3b* was via translation inhibition, rather than mRNA degradation, through a complementary interaction with the target site in the CDS.

There have been several studies showing that miRNAs are involved in somatic cell reprogramming [37–40]. However, miRNA-mediated translation inhibition through targeting the CDS has not previously been reported. There are also increasingly more miRNA target sites being discovered in the CDSs of mammalian transcripts [41, 42]; however, our study is the first to demonstrate that miRNA-induced translation inhibition is involved in epigenetic remodeling during



B

miRNA	Sequence	Location	RPKM in MEFs	RPKM in iPS cells	Fold change (iPS / MEFs)
miR-6539	GCACAGUGAUGAACUCUGAGGGCU	chr14:67859636-67859745[-]	11.228	296.505	26.41
miR-30e-3p	CUUUCAGUCGGAUGUUUACAGC	chr4:120772606-120772697[-]	6.293	953.180	151.48
miR-183-5p	UAUGGCACUGGUAGAAUUCACU	chr6: 30169668-30169737 [-]	6.416	47266.943	7367.04
miR-143-5p	GGUGCAGUGCUGCAUCUCUGG	chr18:61649196-61649258[-]	503.716	4.848	0.0096
miR-107-3p	AGCAGCAUUGUACAGGGCUAUCA	chr19:34820687-34820773[-]	1487.335	5.691	0.0386
miR-185-5p	UGGAGAGAAAGGCAGUUCCUGA	chr16:18327401-18327465[-]	642.277	39.549	0.0616

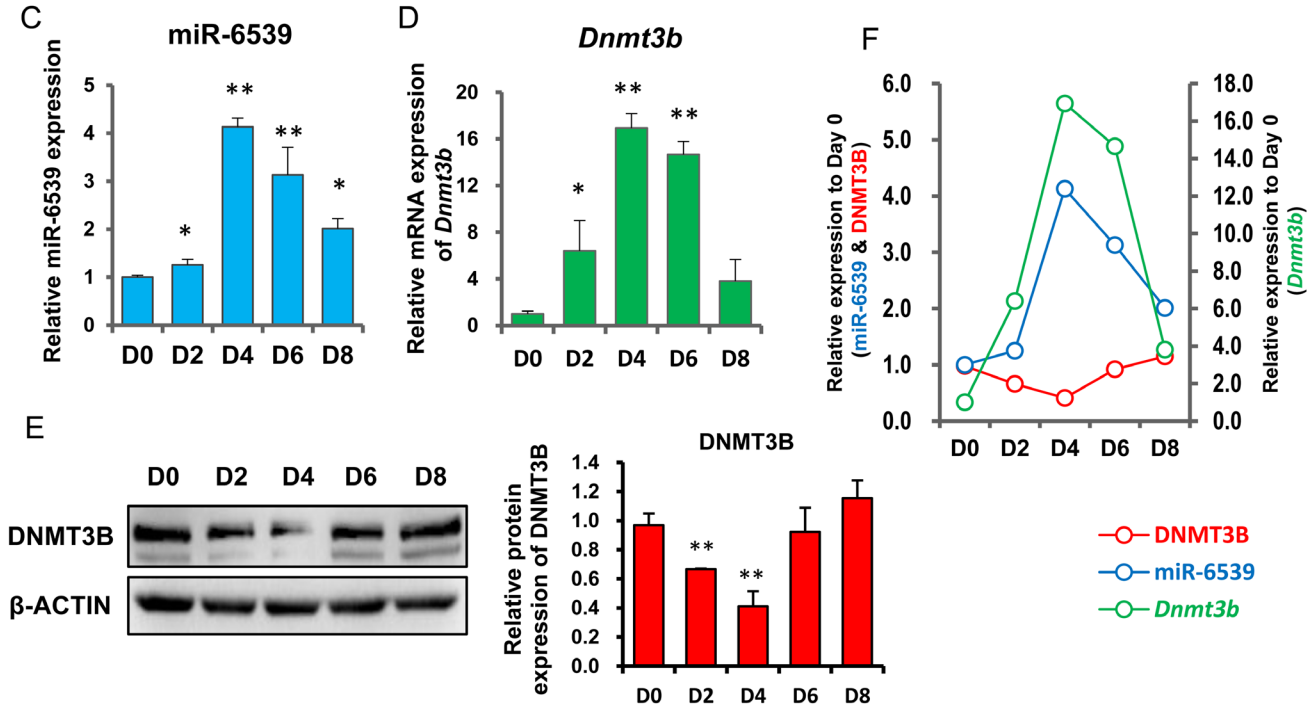
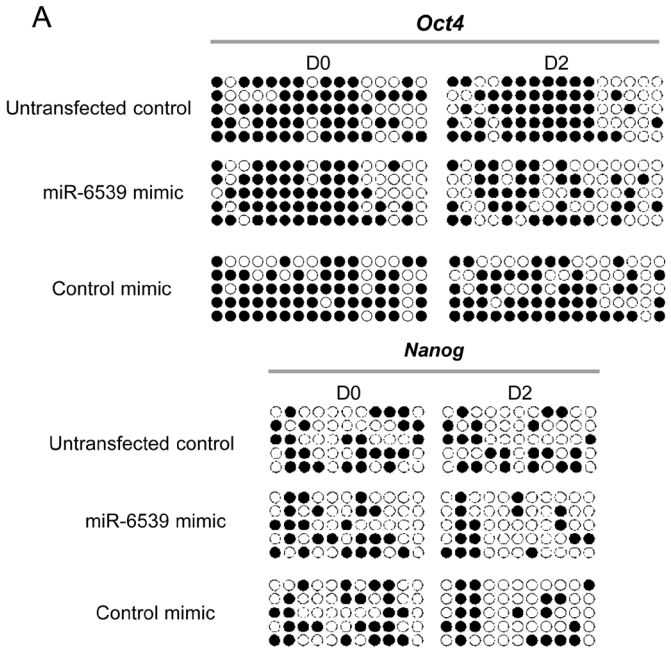


Fig. 1. Generation of iPS cells using a secondary inducible system and the expression dynamics of miR-6539 and *Dnmt3b*. (A) A schematic diagram illustrating the process for generating iPS cells using a secondary inducible system. (B) Expression profiles of candidate miRNAs that were enriched in MEFs or iPS cells. (C) Expression dynamics of miR-6539 during somatic cell reprogramming, assessed using QRT-PCR. (D) Expression dynamics of *Dnmt3b* mRNA during somatic cell reprogramming, assessed using QRT-PCR. (E) Expression dynamics of DNMT3B protein during somatic cell reprogramming, assessed by western blot. The right panel shows the relative protein level of DNMT3B, based on the quantification of signal intensity from three independent experiments. (F) The correlation between miR-6539 expression and *Dnmt3b* mRNA or protein levels. * ($P < 0.05$) and ** ($P < 0.01$) indicate expression levels that are significantly different from day 0.

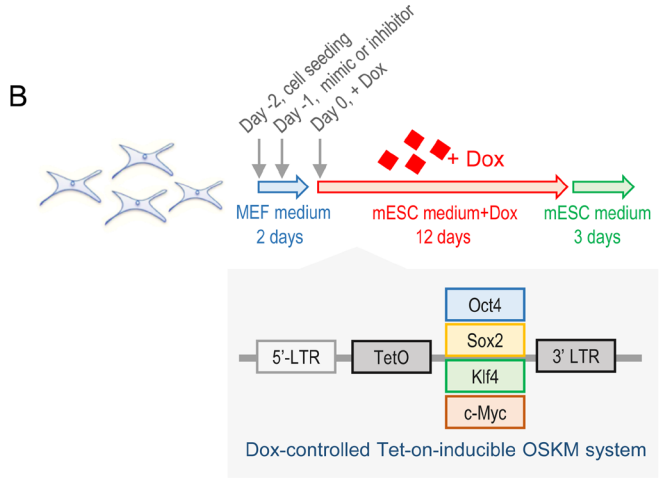


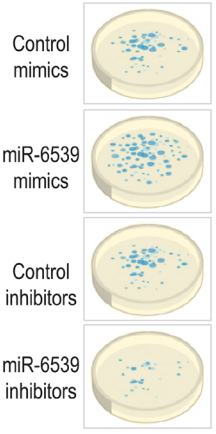
Percentage of methylated CpGs in *Oct4* promoter region

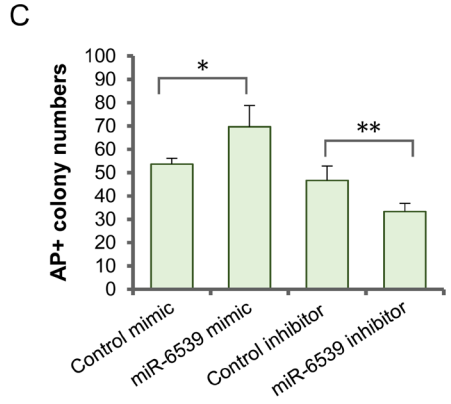
Oct4 promoter	D0	D2
Untransfected control	70.0%	63.8%
miR-6539 mimic	67.5%	48.8%
control mimic	72.5%	61.3%

Percentage of methylated CpGs in *Nanog* promoter region

Oct4 promoter	D0	D2
Untransfected control	45.5%	38.2%
miR-6539 mimic	41.8%	29.1%
control mimic	43.6%	36.4%







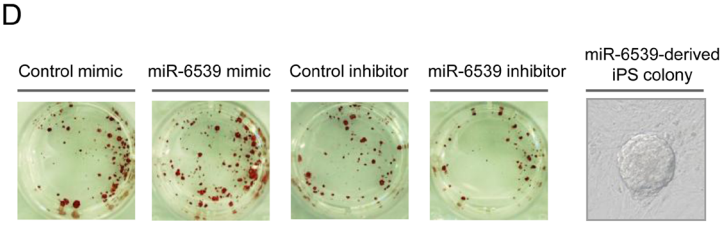
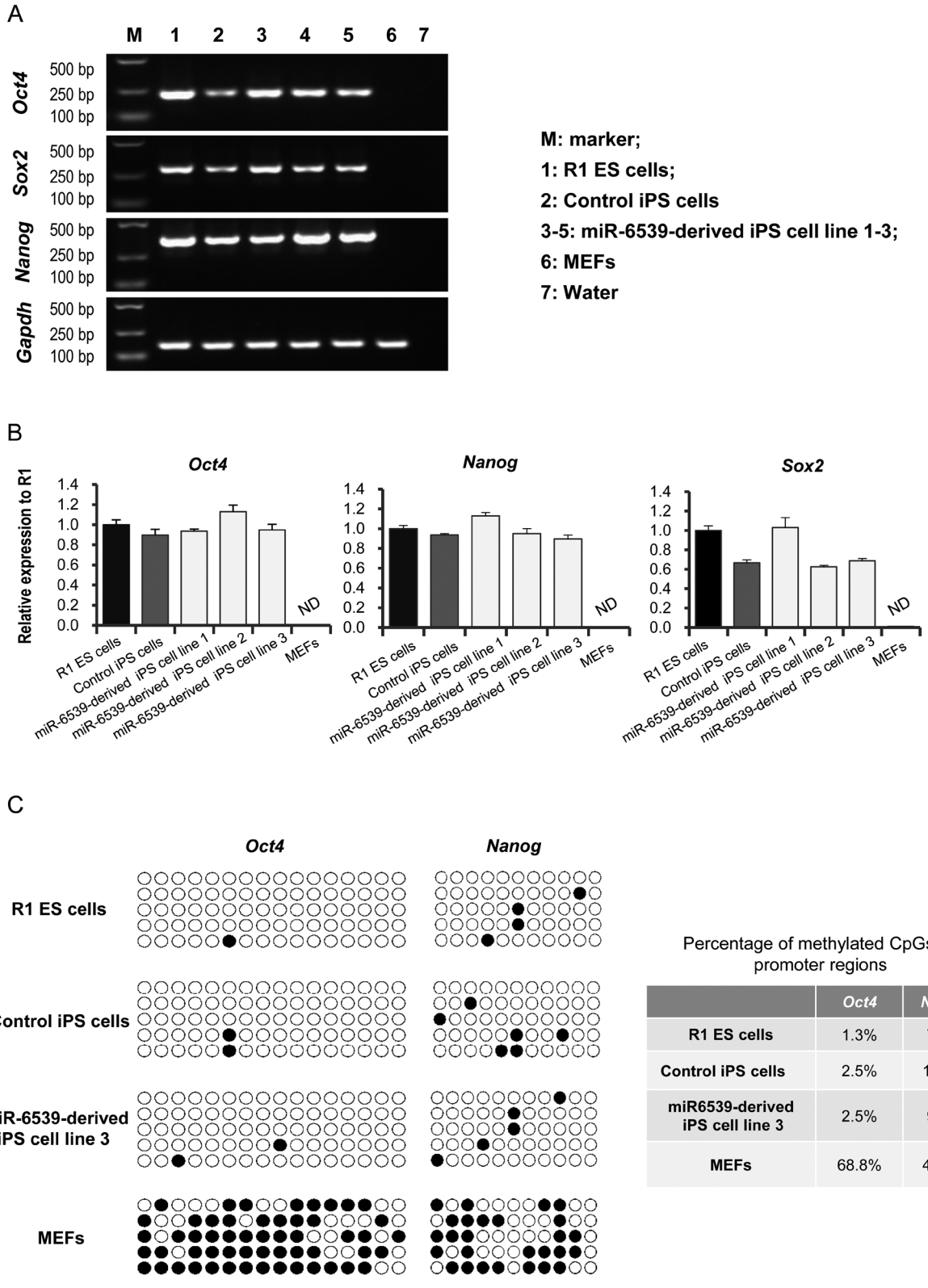


Fig. 2. Effect of miR-6539 overexpression or inhibition on iPS cell formation. (A) Effects of enforced upregulation of miR-6539 on DNA methylation patterns at the promoter regions of *Oct4* and *Nanog* during the early phase of reprogramming. Open and closed circles indicate unmethylated and methylated CpG dinucleotides, respectively. Tables show the percentages of methylated CpGs in the promoter regions of *Oct4* and *Nanog*. (B) A schematic outlining the strategy for upregulating or downregulating miR-6539 during the early stage of reprogramming. (C) Quantification and (D) representative images of AP+ colonies after transfection with miR-6539 mimic or inhibitor. * $P < 0.05$, ** $P < 0.01$.



Percentage of methylated CpGs in promoter regions

	Oct4	*Nanog*
R1 ES cells	1.3%	7.3%
Control iPS cells	2.5%	10.9%
miR6539-derived iPS cell line 3	2.5%	9.1%
MEFs	68.8%	49.1%

Fig. 3. Pluripotency analyses of miR-6539-derived iPS cells. (A) Expression of pluripotent transcription factors (*Oct4*, *Nanog*, and *Sox2*) in miR-6539-derived iPS cell lines, assessed using RT-PCR. (B) QRT-PCR analysis of *Oct4, Nanog*, and *Sox2* mRNA levels in miR-6539-derived iPS cell lines, R1 ES cells, and control iPS cells and MEFs. (C) Bisulfite sequencing of the promoter regions of *Oct4* and *Nanog* in miR-6539-derived iPS cells, R1 ES cells, control iPS cells, and MEFs. The table shows the percentages of methylated CpGs in the promoter regions of *Oct4* and *Nanog* in different cell types.

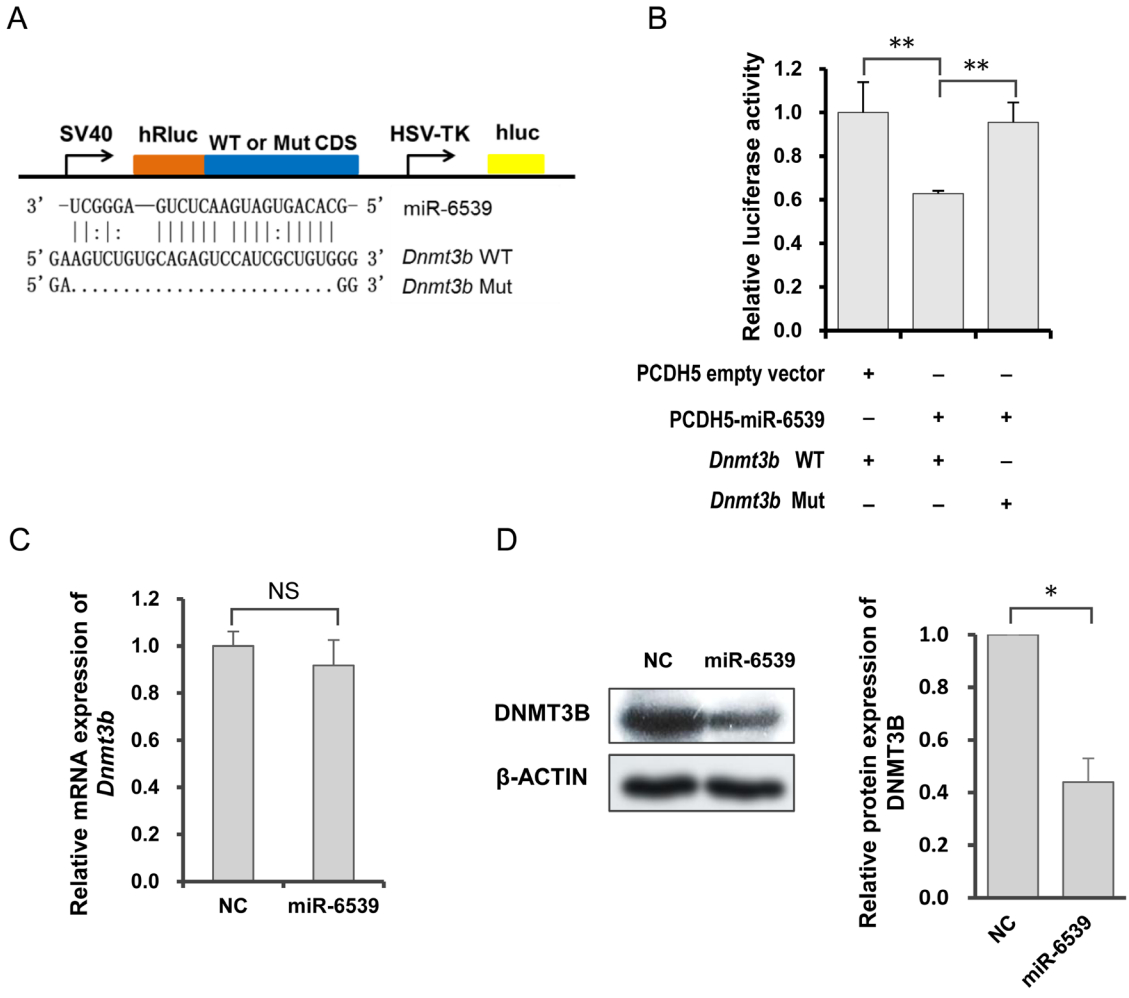


Fig. 4. miR-6539 represses the translation of *Dnmt3b* via a target site located in the CDS. (A) A diagram showing the complementary interaction between miR-6539 and the target site in the CDS of *Dnmt3b*. (B) Relative luciferase activity after transfection with luciferase reporter vectors containing WT or Mut *Dnmt3b* CDS. (C) QRT-PCR analysis of the effect that miR-6539 overexpression had on *Dnmt3b* mRNA expression level. (D) Western blot analysis of the effect that miR-6539 overexpression had on DNMT3B protein level. β-actin was used as loading control. * $P < 0.05$.

induced somatic cell reprogramming.

In summary, we have shown that miR-6539 is a novel mediator of somatic cell reprogramming that represses the translation of *Dnmt3b*. Upregulating miR-6539 during the early stage of somatic cell reprogramming may therefore be essential for reprogramming through demethylation of pluripotent genes. In addition, as miR-29b can target both *Dnmt3a* and *Dnmt3b* via sites in their 3' UTRs, it can be concluded that the 'multiple-to-multiple' relationship between miRNAs and target genes is also critical for induced somatic cell reprogramming. Importantly, this is the first report to show that miRNA-induced translation inhibition is involved in epigenetic remodeling during somatic cell reprogramming. This study provides a new insight into the mechanisms of miRNA-mediated epigenetic remodeling during somatic cell reprogramming.

Acknowledgments

This work was supported by grants from the National High-Tech R&D Program (2013AA102506) and the National Natural Science Foundation of China (No. 31672426 and 31472092).

References

1. **Leitch HG, McEwen KR, Turp A, Encheva V, Carroll T, Grabole N, Mansfield W, Nashun B, Knezovich JG, Smith A, Surani MA, Hajkova P.** Naive pluripotency is associated with global DNA hypomethylation. *Nat Struct Mol Biol* 2013; **20**: 311–316. [Medline] [CrossRef]
2. **Borgel J, Guibert S, Li Y, Chiba H, Schübeler D, Sasaki H, Forné T, Weber M.** Targets and dynamics of promoter DNA methylation during early mouse development. *Nat Genet* 2010; **42**: 1093–1100. [Medline] [CrossRef]
3. **Hochedlinger K, Plath K.** Epigenetic reprogramming and induced pluripotency. *Development* 2009; **136**: 509–523. [Medline] [CrossRef]
4. **Doi A, Park IH, Wen B, Murakami P, Aryee MJ, Irizarry R, Herb B, Ladd-Acosta C, Rho J, Loewer S, Miller J, Schlaeger T, Daley GQ, Feinberg AP.** Differential methyla-

tion of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. *Nat Genet* 2009; **41**: 1350–1353. [Medline] [CrossRef]

5. **Hochedlinger K, Jaenisch R.** Induced pluripotency and epigenetic reprogramming. *Cold Spring Harb Perspect Biol* 2015; **7**: a019448. [Medline] [CrossRef]
6. **Do JT, Schöler HR.** Regulatory circuits underlying pluripotency and reprogramming. *Trends Pharmacol Sci* 2009; **30**: 296–302. [Medline] [CrossRef]
7. **Berdasco M, Esteller M.** DNA methylation in stem cell renewal and multipotency. *Stem Cell Res Ther* 2011; **2**: 42. [Medline] [CrossRef]
8. **Huang K, Fan G.** DNA methylation in cell differentiation and reprogramming: an emerging systematic view. *Regen Med* 2010; **5**: 531–544. [Medline] [CrossRef]
9. **Bhutani N, Brady JJ, Damian M, Sacco A, Corbel SY, Blau HM.** Reprogramming towards pluripotency requires AID-dependent DNA demethylation. *Nature* 2010; **463**: 1042–1047. [Medline] [CrossRef]
10. **Huangfu D, Maehr R, Guo W, Eijkelenboom A, Snitow M, Chen AE, Melton DA.** Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nat Biotechnol* 2008; **26**: 795–797. [Medline] [CrossRef]
11. **Mikkelsen TS, Hanna J, Zhang X, Ku M, Wernig M, Schorderet P, Bernstein BE, Jaenisch R, Lander ES, Meissner A.** Dissecting direct reprogramming through integrative genomic analysis. *Nature* 2008; **454**: 49–55. [Medline] [CrossRef]
12. **Li R, Liang J, Ni S, Zhou T, Qing X, Li H, He W, Chen J, Li F, Zhuang Q, Qin B, Xu J, Li W, Yang J, Gan Y, Qin D, Feng S, Song H, Yang D, Zhang B, Zeng L, Lai L, Esteban MA, Pei D.** A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts. *Cell Stem Cell* 2010; **7**: 51–63. [Medline] [CrossRef]
13. **Shi Y, Desponts C, Do JT, Hahm HS, Schöler HR, Ding S.** Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds. *Cell Stem Cell* 2008; **3**: 568–574. [Medline] [CrossRef]
14. **Wongtrakoongate P, Li J, Andrews PW.** DNMT3B inhibits the re-expression of genes associated with induced pluripotency. *Exp Cell Res* 2014; **321**: 231–239. [Medline] [CrossRef]
15. **Pawlak M, Jaenisch R.** De novo DNA methylation by Dnmt3a and Dnmt3b is dispensable for nuclear reprogramming of somatic cells to a pluripotent state. *Genes Dev* 2011; **25**: 1035–1040. [Medline] [CrossRef]
16. **Tsumura A, Hayakawa T, Kumaki Y, Takebayashi S, Sakaue M, Matsuoka C, Shimotohno K, Ishikawa F, Li E, Ueda HR, Nakayama J, Okano M.** Maintenance of self-renewal ability of mouse embryonic stem cells in the absence of DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b. *Genes Cells* 2006; **11**: 805–814. [Medline] [CrossRef]
17. **Jackson M, Krassowska A, Gilbert N, Chevassut T, Forrester L, Ansell J, Ramsahoye B.** Severe global DNA hypomethylation blocks differentiation and induces histone hyperacetylation in embryonic stem cells. *Mol Cell Biol* 2004; **24**: 8862–8871. [Medline] [CrossRef]
18. **Choi E, Choi E, Hwang K-C.** MicroRNAs as novel regulators of stem cell fate. *World J Stem Cells* 2013; **5**: 172–187. [Medline] [CrossRef]
19. **Mallanna SK, Rizzino A.** Emerging roles of microRNAs in the control of embryonic stem cells and the generation of induced pluripotent stem cells. *Dev Biol* 2010; **344**: 16–25. [Medline] [CrossRef]
20. **Ye D, Wang G, Liu Y, Huang W, Wu M, Zhu S, Jia W, Deng AM, Liu H, Kang J.** MiR-138 promotes induced pluripotent stem cell generation through the regulation of the p53 signaling. *Stem Cells* 2012; **30**: 1645–1654. [Medline] [CrossRef]
21. **Liao B, Bao X, Liu L, Feng S, Zovoilis A, Liu W, Xue Y, Cai J, Guo X, Qin B, Zhang R, Wu J, Lai L, Teng M, Niu L, Zhang B, Esteban MA, Pei D.** MicroRNA cluster 302367 enhances somatic cell reprogramming by accelerating a mesenchymal-to-epithelial transition. *J Biol Chem* 2011; **286**: 17359–17364. [Medline] [CrossRef]
22. **Anokye-Danso F, Trivedi CM, Juhr D, Gupta M, Cui Z, Tian Y, Zhang Y, Yang W, Gruber PJ, Epstein JA, Morrisey EE.** Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. *Cell Stem Cell* 2011; **8**: 376–388. [Medline] [CrossRef]
23. **Guo X, Liu Q, Wang G, Zhu S, Gao L, Hong W, Chen Y, Wu M, Liu H, Jiang C, Kang J.** microRNA-29b is a novel mediator of Sox2 function in the regulation of somatic cell reprogramming. *Cell Res* 2013; **23**: 142–156. [Medline] [CrossRef]
24. **Melton C, Judson RL, Blelloch R.** Opposing microRNA families regulate self-renewal in mouse embryonic stem cells. *Nature* 2010; **463**: 621–626. [Medline] [CrossRef]
25. **Hausser J, Syed AP, Bilen B, Zavolan M.** Analysis of CDS-located miRNA target sites suggests that they can effectively inhibit translation. *Genome Res* 2013; **23**: 604–615. [Medline] [CrossRef]
26. **Calin GA, Croce CM.** MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006; **6**: 857–866. [Medline] [CrossRef]
27. **Chang G, Gao S, Hou X, Xu Z, Liu Y, Kang L, Tao Y, Liu W, Huang B, Kou X, Chen J, An L, Miao K, Di K, Wang Z, Tan K, Cheng T, Cai T, Gao S, Tian J.** High-throughput sequencing reveals the disruption of methylation of imprinted gene in induced pluripotent stem cells. *Cell Res* 2014; **24**: 293–306. [Medline] [CrossRef]
28. **Wernig M, Lengner CJ, Hanna J, Lodato MA, Steine E, Foreman R, Staerk J, Markoulaki S, Jaenisch R.** A drug-inducible transgenic system for direct reprogramming of multiple somatic cell types. *Nat Biotechnol* 2008; **26**: 916–924. [Medline] [CrossRef]
29. **Kang L, Wang J, Zhang Y, Kou Z, Gao S.** iPS cells can support full-term development of tetraploid blastocyst-complemented embryos. *Cell Stem Cell* 2009; **5**: 135–138. [Medline] [CrossRef]
30. **Brambrink T, Foreman R, Welstead GG, Lengner CJ, Wernig M, Suh H, Jaenisch R.** Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. *Cell Stem Cell* 2008; **2**: 151–159. [Medline] [CrossRef]
31. **Gao Y, Chen J, Li K, Wu T, Huang B, Liu W, Kou X, Zhang Y, Huang H, Jiang Y, Yao C, Liu X, Lu Z, Xu Z, Kang L, Chen J, Wang H, Cai T, Gao S.** Replacement of Oct4 by Tet1 during iPSC induction reveals an important role of DNA methylation and hydroxymethylation in reprogramming. *Cell Stem Cell* 2013; **12**: 453–469. [Medline] [CrossRef]
32. **Creighton CJ, Hernandez-Herrera A, Jacobsen A, Levine DA, Mankoo P, Schultz N, Du Y, Zhang Y, Larsson E, Sheridan R, Xiao W, Spellman PT, Getz G, Wheeler DA, Perou CM, Gibbs RA, Sander C, Hayes DN, Gunaratne PH. Cancer Genome Atlas Research Network**. Integrated analyses of microRNAs demonstrate their widespread influence on gene expression in high-grade serous ovarian carcinoma. *PLoS ONE* 2012; **7**: e34546. [Medline] [CrossRef]
33. **Kim Y-K, Yu J, Han TS, Park S-Y, Namkoong B, Kim DH, Hur K, Yoo M-W, Lee H-J, Yang H-K, Kim VN.** Functional links between clustered microRNAs: suppression of cell-cycle inhibitors by microRNA clusters in gastric cancer. *Nucleic Acids Res* 2009; **37**: 1672–1681. [Medline] [CrossRef]
34. **Duhachek-Muggy S, Zolkiewska A.** ADAM12-L is a direct target of the miR-29 and miR-200 families in breast cancer. *BMC Cancer* 2015; **15**: 93. [Medline] [CrossRef]
35. **Pan W, Zhu S, Yuan M, Cui H, Wang L, Luo X, Li J, Zhou H, Tang Y, Shen N.** MicroRNA-21 and microRNA-148a contribute to DNA hypomethylation in lupus CD4+ T cells by directly and indirectly targeting DNA methyltransferase 1. *J Immunol* 2010; **184**: 6773–6781. [Medline] [CrossRef]
36. **Tay Y, Zhang J, Thomson AM, Lim B, Rigoutsos I.** MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. *Nature* 2008; **455**: 1124–1128. [Medline] [CrossRef]
37. **Subramanyam D, Lamouille S, Judson RL, Liu JY, Bucay N, Derynck R, Blelloch R.** Multiple targets of miR-302 and miR-372 promote reprogramming of human fibroblasts to induced pluripotent stem cells. *Nat Biotechnol* 2011; **29**: 443–448. [Medline] [CrossRef]
38. **Pfaff N, Fiedler J, Holzmann A, Schambach A, Moritz T, Cantz T, Thum T.** miRNA screening reveals a new miRNA family stimulating iPS cell generation via regulation of Meox2. *EMBO Rep* 2011; **12**: 1153–1159. [Medline] [CrossRef]
39. **Judson RL, Babiarz JE, Venere M, Blelloch R.** Embryonic stem cell-specific microRNAs promote induced pluripotency. *Nat Biotechnol* 2009; **27**: 459–461. [Medline] [CrossRef]
40. **Wang G, Guo X, Hong W, Liu Q, Wei T, Lu C, Gao L, Ye D, Zhou Y, Chen J, Wang J, Wu M, Liu H, Kang J.** Critical regulation of miR-200/ZEB2 pathway in Oct4/Sox2-induced mesenchymal-to-epithelial transition and induced pluripotent stem cell generation. *Proc Natl Acad Sci USA* 2013; **110**: 2858–2863. [Medline] [CrossRef]
41. **Forman JJ, Legesse-Miller A, Coller HA.** A search for conserved sequences in coding regions reveals that the let-7 microRNA targets Dicer within its coding sequence. *Proc Natl Acad Sci USA* 2008; **105**: 14879–14884. [Medline] [CrossRef]
42. **Takagi S, Nakajima M, Kida K, Yamaura Y, Fukami T, Yokoi T.** MicroRNAs regulate human hepatocyte nuclear factor 4α, modulating the expression of metabolic enzymes and cell cycle. *J Biol Chem* 2010; **285**: 4415–4422. [Medline] [CrossRef]