MicroRNAs Are Indispensable for Reprogramming Mouse Embryonic Fibroblasts into Induced Stem Cell-Like Cells

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters.

Citation

Published Version
doi:10.1371/journal.pone.0039239

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:10436249

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
MicroRNAs Are Indispensable for Reprogramming Mouse Embryonic Fibroblasts into Induced Stem Cell-Like Cells

Byeong-Moo Kim1,2, Marc-Christian Thier3, Sangnam Oh1,2, Richard Sherwood2,4, Chryssa Kanellopoulou5, Frank Edenhofer3, Michael Y. Choi1,2*

1 Department of Medicine/GI Unit, Massachusetts General Hospital, Boston, Massachusetts, United States of America, 2 Harvard Medical School, Boston, Massachusetts, United States of America, 3 Institute of Reconstructive Neurobiology, Life and Brain Center, University of Bonn, Bonn, Germany, 4 Brigham and Women’s Hospital, Boston, Massachusetts, United States of America, 5 Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, United States of America

Abstract
MicroRNAs play a pivotal role in cellular maintenance, proliferation, and differentiation. They have also been implicated to play a key role in disease pathogenesis, and more recently, cellular reprogramming. Certain microRNA clusters can enhance or even directly induce reprogramming, while repressing key proteins involved in microRNA processing decreases reprogramming efficiency. Although microRNAs clearly play important roles in cellular reprogramming, it remains unknown whether microRNAs are absolutely necessary. We endeavored to answer this fundamental question by attempting to reprogram Dicer-null mouse embryonic fibroblasts (MEFs) that lack almost all functional microRNAs using a defined set of reprogramming factors. Transduction of reprogramming factors using either lentiviral or piggyBac transposon vector into two, independently derived lines of Dicer-null MEFs failed to produce cells resembling embryonic stem cells (ESCs). However, expression of human Dicer in the Dicer-null MEFs restored their reprogramming potential. Our study demonstrates for the first time that microRNAs are indispensable for dedifferentiation reprogramming.


Editor: Petras Dzeja, Mayo Clinic, United States of America

Received April 4, 2012; Accepted May 22, 2012; Published June 21, 2012

This is an open-access article, free of all copyright, and may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. The work is made available under the Creative Commons CC0 public domain dedication.

Funding: The research was supported by NIH grants to M.Y.C. (K08 DK078641). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.
* E-mail: mchoi@partners.org

Introduction
MicroRNAs (miRNAs) play a pivotal role in cellular maintenance, proliferation, and differentiation. They have also been implicated to play a key role in disease pathogenesis, and more recently, cellular reprogramming. Certain microRNA clusters can enhance or even directly induce reprogramming, while repressing key proteins involved in microRNA processing decreases reprogramming efficiency. Although microRNAs clearly play important roles in cellular reprogramming, it remains unknown whether microRNAs are absolutely necessary. We endeavored to answer this fundamental question by attempting to reprogram Dicer-null mouse embryonic fibroblasts (MEFs) that lack almost all functional microRNAs using a defined set of reprogramming factors. Transduction of reprogramming factors using either lentiviral or piggyBac transposon vector into two, independently derived lines of Dicer-null MEFs failed to produce cells resembling embryonic stem cells (ESCs). However, expression of human Dicer in the Dicer-null MEFs restored their reprogramming potential. Our study demonstrates for the first time that microRNAs are indispensable for dedifferentiation reprogramming.

PLoS ONE | www.plosone.org 1 June 2012 | Volume 7 | Issue 6 | e39239

MicroRNAs (miRNAs) have emerged as a new category of genes that influence many cellular processes including proliferation and differentiation. miRNAs are small, noncoding, single stranded RNAs usually 22 nucleotides long that can base pair with target mRNAs in the open reading frame or 3’ untranslated region [1]. miRNAs downregulate target genes by inhibiting protein translation and destabilizing mRNAs via deadenylation [2]. To generate functional miRNAs along the canonical pathway, two serial RNA cleavage steps involving two RNase III-containing enzymes are necessary. First, the Microprocessor complex formed by the hairpin recognizing RNA binding protein, Dgcr8, and the RNase III enzyme, Drosha, cleaves the primary miRNA transcript (pri-miRNA) to form precursor miRNAs (pre-miRNAs) in the nucleus [3,4,5,6]. Next, Exportin-5 transports the pre-miRNA to the cytoplasm [7,8] where, a second RNase III-containing enzyme, Dicer, cleaves it to generate mature miRNAs in the cytoplasm [9,10]. After Dicer cleavage, the gene-silencing, guide strand is able to associate with miRNA-induced silencing complex, which assists in the contact between the miRNA and the target mRNA [11]. In addition to the canonical pathway, Dicer processes all miRNAs along the non-canonical biogenesis pathways except in a few rare examples [12,13,14,15], and thus, without Dicer, the cell lacks almost all mature miRNAs. Finally, Dicer has been found to process endogenous small interfering RNAs (siRNAs) in oocytes and ESCs [16,17,18]. However, whether endogenous siRNAs function or even exist in mammalian somatic cells including MEFs remains unknown [19].

Highlighting the importance of these pathways, targeted deletion of the Dicer gene in mice causes embryonic lethality at embryonic day (E) 7.5 [20,21]. In fact, the embryos probably start to arrest at a stage prior to E7.5 because the number of Dicer-null embryos is about 50% lower than expected from Mendelian ratios. Mice that lack Dicer may survive to E7.5 because of the presence of maternal Dicer protein in the cytoplasm [20]. Despite early embryonic lethality in these mutant mice, at least two research groups have been able to generate Dicer-null ESC lines [22,23]. Remarkably, these mutant mouse ESCs are viable and retain typical morphology of wild-type ESCs, forming oval-shaped colonies. They also express ESC specific markers, including Oct4, at levels comparable to wild-type ESCs. However, as compared to wild-type, Dicer-deficient ESCs proliferate much more slowly and do not exhibit pluripotent differentiation capability.

Similar to transcription factors, miRNAs have the ability to modulate the expression of several genes, and therefore, contribute significantly to cellular gene expression programs. This is likely the reason why miRNAs have potent functions not only in normal cellular processes and in diseased states, but also in forced reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) [24,25,26,27,28]. For instance, members of ESC-specific
cell cycle-regulating miRNAs enhance the efficiency of cellular reprogramming when Oct4, Sox2, and Klf4 are transduced, and can replace Myc in reprogramming mouse fibroblasts to iPSCs by acting downstream of Myc [27]. More recently, iPSCs were successfully generated by lentiviral expression of the miR302/367 cluster or transient transfection of miRNA mimics, miR200c, and clusters of miR302s, and miR369s, without any exogenous transcription factor expression [25,26]. Finally, repressing key miRNA processing molecules such as Drosha, Dicer, and Ago2 resulted in significant decrease in reprogramming efficiency [29]. The knockdown approach used in this study was reported to repress between 70–80% in reprogramming efficiency. Hence, although it was demonstrated that miRNAs as a whole are able to modulate reprogramming, it remains unclear whether they are in fact necessary for cellular reprogramming. In this study, we formally answer this question by attempting to reprogram Dicer-null MEFs that lack almost all functional miRNAs by transducing a set of defined transcription factors known to activate the dedifferentiation program. Although two different gene delivery methods were used on two independently derived Dicer-null MEFs, combinations of Oct4, Sox2, Klf4, cMyc, and Lin28 failed to generate cells that resemble Dicer-null ESCs. However, Dicer-null, induced stem cell-like cells were successfully produced when the human Dicer homologue was introduced in Dicer-null MEFs before the dedifferentiation step, suggesting that miRNAs are indispensable for cellular reprogramming.

Results and Discussion

**Dicer^+/− Mouse Embryonic Fibroblasts Lacking miRNAs are Viable Despite Suppressed Proliferation**

To test whether miRNAs are necessary for reprogramming a somatic cell type into induced stem cell-like cells, we first generated MEFs that lack almost all miRNAs. We utilized two different Dicer-null MEF lines from two independently generated mutant mouse lines that have different Dicer exons flanked by loxP sites [22,30]. When we crossed Dicer<sup>+/+</sup> mice, resulting pups were Dicer<sup>f/+</sup> to Dicer<sup>f/+</sup> in a 1:2:1 ratio. Dicer<sup>f/+</sup> MEFs harvested from E13.5 embryos proliferated normally and had morphology resembling wild-type MEFs (Fig 1A). However, once Cre recombinase was delivered by infecting cells with recombinant adenovirus (Adeno) encoding Cre, Dicer<sup>f/+</sup> MEFs lost both functional Dicer alleles to become Dicer<sup>D/D</sup> (Dicer-null) MEFs (Fig 1B). Adeno-Cre virus was able to consistently infect greater than 90% of MEFs at a multiplicity of infection (MOI) of 100, judged by co-expression of a GFP reporter (Fig 1A). To confirm that infection with Adeno-Cre virus led to deletion of the Dicer gene and prevention of Dicer protein expression, we performed immunoblot for Dicer protein. By 6 days post-induction (dpi) with Cre, Dicer protein was completely depleted (Fig 1C). We also confirmed reduction in the levels of select mature miRNAs in Dicer<sup>A/A</sup> MEFs. Reverse transcription-quantitative polymerase chain reaction (qRT-PCR) revealed that the levels of most mature miRNAs tested were nearly 98% depleted by 6 dpi (Fig 1D). Dicer-null MEFs had a typical cellular morphology comparable to wild-type MEFs. However, Dicer-null MEFs demonstrated a proliferation delay, while Dicer<sup>A/A</sup> MEFs retained normal proliferation rate resembling wild-type MEFs (Fig 1E). These results were in line with published phenotype of Dicer-null MEFs from independently generated conditional Dicer knockout mice [31].

![Figure 1. Dicer-null MEFs are viable despite suppressed proliferation. (A) Adeno-Cre virus was able to infect MEFs at high efficiency, judged by co-expression of GFP signal. Dicer<sup>A/A</sup> MEF generated from Dicer<sup>f/+</sup> MEF by Cre excision of Dicer gene had typical cellular morphology similar to wild-type (Dicer<sup>+/+</sup>) MEF. Boxed areas represent magnified view. (B) PCR analysis of genomic DNA to demonstrate Cre excision of Dicer gene to generate Dicer<sup>A/A</sup> (floxed DNA band) MEF from Dicer<sup>f/+</sup> MEF (floxed DNA band). (C) Dicer immunoblot confirmed that Cre induction led to deletion of Dicer.](http://www.plosone.org/资产/10.1371/journal.pone.0039239.s001)
gene and prevention of Dicer protein expression. By 6 days post induction (dpi) with Cre, Dicer protein was completely depleted. (D) qRT-PCR of select miRNAs confirmed reduction. The levels of mature forms of miRNA in Dicer<sup>−/−</sup> MEFs, the levels of most mature miRNAs tested were almost completely depleted by 6 days after Cre induction. Each value is represented relative to an assigned 0 dpi value of 1.0 for that miRNA. Data are presented as mean ±/− SD. (E) Dicer<sup>−/−</sup> MEFs demonstrated a proliferation delay. In contrast, Dicer<sup>+/−</sup> MEFs retained normal proliferation rate resembling Dicer<sup>+/+</sup> MEFs. All values are represented relative to an assigned Dicer<sup>+/+</sup> MEF value of 1.0 at 0 dpi. Data are presented as mean ±/− SD. doi:10.1371/journal.pone.0039239.g001

**Dicer<sup>−/−</sup> Mouse Embryonic Fibroblasts Lacking miRNAs Fail to Reprogram**

We attempted to reprogram Dicer<sup>−/−</sup> MEFs using defined sets of transcription factors known to dedifferentiate various somatic cells into iPSCs [32,33,34]. We used two combinations of transcription factors, Oct4, Sox2, Klf4, cMyc, and Lin28 (5 TFs), and Oct4, Sox2, Klf4, and cMyc (4 TFs). Transducing 5 TFs increases reprogramming efficiency by twofold compared to 4 TFs [34]. In addition, we tested two different gene delivery methods to reprogram MEFs, piggyBac transposon carrying 2A peptide-linked reprogramming factors and a polycistronic doxycycline-inducible lentiviral system [34,35]. PiggyBac transposon vector transfection efficiencies in control and Dicer<sup>−/−</sup> MEFs were 30–40% and 25–35% respectively. As mentioned, we also used two different MEF lines produced from two independently generated mutant mice with floxel-Dicer alleles [22,30]. Despite these variations and optimizations, Dicer<sup>−/−</sup> MEFs could not be reprogrammed into induced stem cell-like cells when either 4 TFs or 5 TFs were transduced 6 days after induction with Cre (Fig 2A), when all mature miRNAs were depleted. We use the term “induced stem cell-like cells” instead of iPSCs because the reprogrammed cells without miRNAs would not be pluripotent. Instead, they would resemble Dicer-null ESCs known to have severe proliferation and differentiation defects [22,23]. Typically, iPSC colonies appear after 2 weeks of expression with 4 TFs; since the Dicer-null ESCs proliferate poorly, we decided to extend our reprogramming duration longer. However, we did not detect any reprogrammed cells even after 4 weeks post transduction of defined reprogramming factors.

Meanwhile, we were able to consistently and reliably reprogram control MEFs with genotypes Dicer<sup>+/+</sup>, Dicer<sup>+/−</sup>, and Dicer<sup>−/−</sup> into iPSCs using either 4 TFs or 5 TFs with the overall reprogramming efficiency between 0.3% and 0.5% (Supp Table S1), in line with published reports [34,35]. These iPSCs grew in colonies in the presence of leukemia inhibitory factor, stained for alkaline phosphatase (Fig 2C), expressed all stem cell markers tested (Fig 2F, G), acquired stem cell methylation pattern in Oct4 and Nanog promoters (Fig 2H), and differentiated normally to all three germ layers during teratoma assays (Data not shown). Control MEFs required 2 weeks of culturing after delivering 4 TFs or 5 TFs to generate iPSCs, consistent with published results [34,35].

**Dicer<sup>+/+</sup> Mouse Embryonic Fibroblasts That Still have Residual miRNAs Can Reprogram**

Although it was clearly evident that MEFs lacking miRNAs could not be reprogrammed we identified one condition in which reprogramming was possible even after Cre excision of relevant Dicer exons. When we transduced 4 TFs or 5 TFs one day after expressing Cre recombinase (Fig 2B), a few Dicer<sup>+/+</sup> colonies, confirmed by genomic PCR, formed after 2 weeks with the overall reprogramming efficiency of less than 0.3% (Fig 2C, D; Supp Table S1). These induced stem cell-like cells reprogrammed from Dicer<sup>+/+</sup> MEFs grew in colonies, acquired cellular morphology similar to ESCs, expanded indefinitely, and stained for alkaline phosphatase (Fig 2C). They also expressed all ESC markers tested by RT-PCR (Fig 2F) and immunofluorescence (Fig 2G), and acquired methylation patterns of Nanog and Oct4 promoters that were similar to control ESCs and iPSCs (Fig 2H). However, there were several phenotypic features that were dissimilar to control ESCs and iPSCs. Dicer<sup>+/+</sup> induced stem cell-like cells proliferated slower than control ES and iPSCs. Furthermore, these cells failed to differentiate into endoderm with Activin and other growth factors in culture, a condition that regularly yields more than 95% endoderm from ESCs (Data not shown) [36]. Finally, these cells could not give rise to any recognizable teratoma with germ layers upon subcutaneous injection into severe combined immunodeficiency (SCID) mice, demonstrating their severe differentiation defect. These mutant phenotypes were reminiscent of Dicer-null ESCs which also had severe proliferation and differentiation defects [22]. We were able to generate induced stem cell-like cells only when the combination of 4 TFs or 5 TFs were delivered just one day after Cre induction, but not if reprogramming factors were transduced 6 days after Cre induction. We believe that reprogramming Dicer<sup>+/+</sup> MEFs was possible only when reprogramming factors were introduced one day after Cre induction because residual Dicer protein and mature miRNAs are still present up to 3 days after deletion of Dicer gene (Fig 1D, 2E). By day 6 after Cre induction, residual Dicer protein and miRNAs are almost completely absent, inhibiting cellular reprogramming (Fig 1D, 2E). Likewise, the effect of residual Dicer protein has been recognized previously in vivo. Residual maternal Dicer protein in the absence of Dicer gene may allow prolonged survival of mouse and zebrafish Dicer mutant embryos [20,37].

**Human Dicer Expression in Dicer<sup>+/+</sup> Mouse Embryonic Fibroblasts Allows Generation of iPSCs**

To verify that the inability to reprogram Dicer<sup>+/+</sup> MEFs was truly due to lack of functional Dicer protein and miRNAs, and not from an unrecognized mutation or variability in our assays, we attempted to rescue the capacity to reprogram by reintroducing Dicer gene into Dicer-null MEFs. We overexpressed reprogramming factors after integrating human Dicer cDNA into the genome of Dicer<sup>+/+</sup> MEFs using a piggyBac expression vector. Human Dicer protein has 93% sequence identity with its mouse homologue, and shares the key enzymatic function through the conserved ribonuclease III C terminal domain. Even when we transduced reprogramming factors 6 days after Cre induction to delete mouse Dicer exons, we were able to generate iPSCs from MEFs that expressed the human Dicer homologue (Fig 3A). These iPSCs lacked mouse Dicer, but instead expressed the human Dicer gene, as confirmed by RT-PCR (Fig 3B). Within 2 weeks after transducing reprogramming factors, Dicer<sup>+/+</sup> MEFs expressing human Dicer differentiated into iPSCs that grew in colonies. Reprogrammed iPSCs expressing human Dicer displayed typical ESC morphology, stained for alkaline phosphatase, and expressed stem cell markers (Fig 3C, D, E). Their promoters for stem cell genes Oct4 and Nanog became demethylated, resembling wild-type ESCs (Fig 3F). Upon subcutaneous injection into SCID mice, these cells formed teratomas that showed differentiation into all three germ layers (Fig 3G). Finally, to confirm that human Dicer has a robust enzymatic activity and can cleave mouse pre-miRNAs into mature miRNAs, we performed qPCR for a panel of mature mouse miRNAs in human Dicer expressing iPSCs. As expected, these cells expressing human Dicer had comparable levels of mature miRNAs to that of wild-type ESCs (Fig 3H).

Our results indicate that miRNAs are essential for reprogramming since Dicer-null MEFs could not give rise to induced stem...
MicroRNAs Are Indispensable for Reprogramming

A Day: -2 -1 0 1 2 3 4 5 6 7 Reprogram period 21-35

Seed Dcr f/f MEF
Adeno-Cre Infection TFs Seed Analysis

B Day: -2 -1 0 1 2 Reprogram period 16

Seed Dcr f/f MEF
Adeno-Cre Infection TFs Seed Analysis

D f/f iPSC Δ/Δ-1dpi
floxed (350bp) flox (224bp)

E Control 1dpi Cre 3dpi Cre 6dpi Cre
Dicer
β-Actin

F MEF ESC f/f iPSC Δ/Δ Δ/Δ 1dpi (-1 RT)
ERas Nanog NAT1 Cripto Oct4 Zfp42(Rex1) Sox2

G Dcr+/+ iPSC Dcr+/+ 1dpi
SSEA1 Nanog Merge

H Oct4 Nanog
MEF ESC Dcr+/+ iPSC DcrΔ/Δ 1dpi
cell-like cells. Human Dicer rescued the ability to generate mature miRNAs in Dicer-/- MEFs, and restored their reprogramming potential. The dramatic proliferation delay in Dicer-null MEF likely contributes to inhibiting cellular reprogramming as it has been demonstrated that an accelerated kinetics of iPSC formation is directly proportional to the increase in cell proliferation [38]. However, besides promoting proliferation, miRNAs likely have other functions that are essential for reprogramming since it is known that they regulate numerous genes and exert multiple cellular effects. Furthermore, we were able to reprogram Dicer-/- MEFs one day after Cre induction when the cells already had a significant growth delay (Fig 1E), suggesting that the impaired proliferation is not the only variable in preventing reprogramming. Identifying specific miRNAs that enable reprogramming would give clues about their mechanism of action. Although the mechanism of action still needs clarification, our results indicate for the first time that miRNAs are indispensable for dedifferentiation reprogramming.

Materials and Methods

Ethics Statement

Animals were handled according to relevant national and international guidelines under the protocol number 2010N000120, approved by the Massachusetts General Hospital’s Subcommittee on Research Animal Care. The committee approved the experiments conducted in this study.

Conditional Dicer Knockout Mice and Cell Culture

MEFs were prepared from E13.5 wild-type, Dicer-/-, and Dicer-/-embryos and cultured in DMEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% nonessential amino acids and 0.1 mM 2-mercaptoethanol (Invitrogen). A germline-competent mouse ESC line (W4) and mouse iPSC lines were cultured on reduced serum nonessential amino acids

Reprogramming MEFs Using Lentiviral Transduction

The lentiviral constructs Stemca OSKM and the tet-activator Fuv-m2RTTA (Addgene) were used to ectopically induce reprogramming genes. To generate viral particles, 5.8 x10^6 293T cells per 10 cm dish were transfected with respective vector and the packaging plasmids pSAX2 (Addgene) and pMS2.G (Addgene) in a ratio of 2:1:1. 48 hours after transfection the supernatant comprising the viruses was collected, mixed in a ratio of 2 (Stemca) : 1 (m2RTTA) and filtered through a 0.45 μm cellulose acetate filter. Finally, polybrene (Millipore) was added at a final concentration of 4 μg/ml to increase infection efficiencies. One day before transduction, MEFs were passaged onto six well plates to reach a density of 60–80% on the day of transduction (1–1.2 x10^5 cells/well). For transduction, the culture medium was replaced with MEFser replacement (Invitrogen). Medium was refreshed every other day. On day 14, colonies were either stained using the alkaline phosphatase detection kit (Chemicon) and counted, or picked and further expanded.

Cell Proliferation Assay

Cell proliferation was performed using cell proliferation assay kit (Promega) according to the manufacturer’s instructions. After plating MEFs, cells were treated with Adeno-Cre virus and analyzed 1, 3 and 5 days after infection. At the indicated time points, medium was replaced with MTS media and incubated at 37°C for 3 hours. Absorbance was recorded at 490 nm.

RT-PCR and Western Blot Analysis

Total RNA was extracted by using Trizol reagent (Invitrogen). After taining, one microgram of total RNA was reverse-transcribed using an oligo(dT) adaptor primer by SuperScriptII (Invitrogen) according to the manufacturer’s instructions [39]. Quantitative RT-PCR was performed using Platinum SYBR Green qPCR superMix (Invitrogen) on the CFX96 Real-Time System (Bio-Rad). Serial dilutions of each RT-PCR product were used to generate a standard curve. Expression of individual transcripts was normalized to Gapdh expression. Protein blots were analyzed using antibodies to Dicer (1:1000, Abcam) and β-actin (1:2000, Abcam).
**Figure 3. Human Dicer expression in Dicer-null MEFs allows generation of iPSCs.** (A) Timeline of reprogramming Dicer-null MEFs rescued with human Dicer. Once human Dicer cDNA integrated into the Dicer MEF genome, reprogramming became possible even when factors were transduced 6 days after Cre induction. (B) Rescued iPSCs (ResDcr) lacked mouse Dicer (mDcr), but instead expressed human Dicer (hDcr) gene, verified by RT-PCR. (C, D, E) Dicer−/− MEFs expressing human Dicer can reprogram to become iPSCs. Rescued iPSCs expressing human Dicer showed ESC morphology and stained for alkaline phosphatase (boxed areas represent magnified view) (C), and expressed stem cell markers tested by RT-PCR (D) and immunofluorescence (E). (F) Rescued iPSC promoters for stem cell genes Oct4 and Nanog became demethylated, resembling wild-type ESCs. (G) Upon subcutaneous injection into SCID mice, rescued iPSCs formed teratomas that showed differentiation into all three germ layers. (H) Human Dicer can cleave mouse pre-miRNAs into mature miRNAs. qPCR for a panel of mature miRNAs in rescued iPSCs (ResDcr) lacking mouse Dicer demonstrated comparable expression levels to that of wild-type ESCs (W4). In contrast, mature miRNAs were completely depleted in Dicer-deficient mouse ESCs. Each value is represented relative to an assigned W4 value of 1.0 for that miRNA. Data are presented as mean ±/− SD. doi:10.1371/journal.pone.0039239.g003

**Bisulfite Genomic Sequencing Assay**

Genomic DNA was isolated and then treated for bisulfite sequencing with EpiTect Bisulfite Sequencing kit (Qiagen). The treated DNA was then used to amplify sequences of interest. The resulting fragments were cloned using the TOPO TA Cloning Kit (Invitrogen) and sequenced with promoter fragment amplification primers for Oct4 (forward: GGTTTTTTTAGAGGTGTGGAATTT, reverse: TCCAAAGCCTAATCCATCCAC) and Nanog (forward: GATTTTGAGTGGATTAATTTG-GAAATT, reverse: ACCAAAAACCCCCACCTGATAATCAA-TATA) [34].

**Immunocytochemistry and Immunofluorescence Assay**

Alkaline phosphatase staining was performed according to the manufacturer’s instructions using the Alkaline Phosphatase Detection Kit (Vector Lab). For immunofluorescence assay, cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature (RT) and washed with PBS. They were then incubated in blocking buffer (0.3% Triton X-100, 10% normal goat serum in PBS) for 30 minutes at RT, and incubated with primary antibody overnight at 4°C in blocking buffer. Afterward, cells were washed with PBS and incubated with secondary antibody in blocking buffer for 45–60 min at RT. Primary antibodies were mouse anti-Oct4 (1:400, Abcam), mouse anti-SSEA1 (1:400, Developmental Studies Hybridoma Bank at the University of Iowa), and rabbit anti-Nanog (1:500, Abcam). Secondary antibodies were Alexa Fluor 488, 555 donkey anti-mouse or rabbit IgG (1:500, Invitrogen). Nuclei were detected by DAPI (Sigma-Aldrich) staining.

**Teratoma Formation**

Approximately 1×10⁶ hDicer rescued DicerΔ/Δ iPSCs, stem cell-like cells (DicerΔ/Δ), and control iPSCs were injected subcutaneously into dorsal flanks of recipient SCID mice. Tumors were isolated 4–6 weeks later and subjected to histological analysis.

**Supporting Information**

**Table S1 Reprogramming efficiencies of various MEFs.** The overall reprogramming efficiencies were between 0.1% and 0.5% except for DicerΔ/Δ MEFs which could not be reprogrammed into induced stem cell-like cells when either 4 TEs or 3 TEs were transduced 6 days after induction with Cre (DicerΔ/Δ-lsdp). (TIF)

**Acknowledgments**

We thank David M. Livingston and Klaus Rajewsky for providing Dicer−/− mouse lines and Gustavo Mostoslavsky for Stemcca OSKM vector.

**Author Contributions**

Conceived and designed the experiments: BMK MCT FE MYC. Performed the experiments: BMK MCT RS SO. Analyzed the data: BMK MCT SO RS GK FE MYC. Contributed reagents/materials/analysis tools: GK FE MYC. Wrote the paper: BMK MYC.

**References**


