

# Functional screen reveals essential roles of miR-27a/24 in differentiation of embryonic stem cells

Yanni Ma<sup>1,2,†</sup>, Nan Yao<sup>1,3,†</sup>, Guang Liu<sup>1,3,†</sup>, Lei Dong<sup>1,2</sup>, Yufang Liu<sup>1,3</sup>, Meili Zhang<sup>1,3</sup>, Fang Wang<sup>1,2</sup>, Bin Wang<sup>1,2</sup>, Xueju Wei<sup>1,2</sup>, He Dong<sup>1,2</sup>, Lanlan Wang<sup>1,2</sup>, Shaowei Ji<sup>1,3</sup>, Junwu Zhang<sup>1,2</sup>, Yangming Wang<sup>4</sup>, Yue Huang<sup>1,3,\*</sup> & Jia Yu<sup>1,2,\*\*</sup>

## Abstract

MicroRNAs play important roles in controlling the embryonic stem cell (ESC) state. Although much is known about microRNAs maintaining ESC state, microRNAs that are responsible for promoting ESC differentiation are less reported. Here, by screening 40 microRNAs pre-selected by their expression patterns and predicted targets in *Dgcr8*-null ESCs, we identify 14 novel differentiation-associated microRNAs. Among them, miR-27a and miR-24, restrained by c-Myc in ESC, exert their roles of silencing self-renewal through directly targeting several important pluripotency-associated factors, such as Oct4, Foxo1 and Smads. CRISPR/Cas9-mediated knockout of all miR-27/24 in ESCs leads to serious deficiency in ESC differentiation *in vitro* and *in vivo*. Moreover, depleting of them in mouse embryonic fibroblasts can evidently promote somatic cell reprogramming. Altogether, our findings uncover the essential role of miR-27 and miR-24 in ESC differentiation and also demonstrate novel microRNAs responsible for ESC differentiation.

**Keywords** c-Myc; differentiation; embryonic stem cells; iPSC generation; microRNA

**Subject Categories** RNA Biology; Stem Cells; Molecular Biology of Disease

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## Introduction

Embryonic stem cells (ESCs) retain unlimited self-renewal potential in tissue culture and their propagation is characterized by a short

cell cycle (White & Dalton, 2005). Under appropriate conditions *in vitro*, ESCs can be induced to differentiate into all somatic cell types. These lineage commitments involve the silencing of self-renewal program and the activation of lineage-specific programs (Jaenisch & Young, 2008; Silva & Smith, 2008). In contrast, reprogramming of somatic cells to induced pluripotent stem cells (iPSCs) is achieved by the re-establishment of the pluripotent state (Jaenisch & Young, 2008; Hochedlinger & Plath, 2009; Young, 2011).

Accumulating evidence reveals that microRNAs (miRNAs) play important roles in control of pluripotent stem cell state. ESCs lacking of key enzymes in the miRNA biogenesis pathway, such as Dicer or *Dgcr8*, show deficiency in self-renewal and differentiation (Kanellopoulou *et al*, 2005; Murchison *et al*, 2005; Wang *et al*, 2007). A number of miRNAs have been reported to participate in regulating ESC self-renewal and differentiation (Barroso-delJesus *et al*, 2008; Wang *et al*, 2008; Ren *et al*, 2009; Sengupta *et al*, 2009). For example, ES cell-specific cell cycle (ESCC)-regulating miRNAs (Wang *et al*, 2008), miR-520 cluster (Ren *et al*, 2009), miR-302-367 cluster (Barroso-delJesus *et al*, 2008) and miR-92b (Sengupta *et al*, 2009) are important for the maintenance of ESC self-renewal. Contrarily, miR-134, miR-296, miR-470, miR-145 and let-7 family are involved in silencing of self-renewal program and/or promoting differentiation of ESCs (Tay *et al*, 2008a,b; Xu *et al*, 2009; Melton *et al*, 2010). Introduction of the ESCC-like miRNAs and/or suppression of those lineage commitment-related miRNAs can promote the reprogramming of somatic cells to iPSCs (Judson *et al*, 2009, 2013; Anokye-Danso *et al*, 2011; Choi *et al*, 2011; Li *et al*, 2011; Subramanyam *et al*, 2011; Yang *et al*, 2011). ESCC miRNAs were identified by screening a comprehensive miRNA mimic library in *Dgcr8*-deficient ESC, which overcomes issues of redundancy and saturation that are inherent to the miRNA system (Wang *et al*,

1 State Key Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, School of Basic Medicine Peking Union Medical College, Beijing, China

2 Department of Biochemistry and Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, School of Basic Medicine Peking Union Medical College, Beijing, China

3 Department of Medical Genetics, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, School of Basic Medicine Peking Union Medical College, Beijing, China

4 Peking-Tsinghua Joint Center for Life Sciences, Institute of Molecular Medicine, Peking University, Beijing, China

\*Corresponding author. Tel: +86 10 69156462; E-mail: huangyue@pumc.edu.cn

\*\*Corresponding author. Tel: +86 10 69156423; E-mail: j-yu@ibms.pumc.edu.cn

†These authors contributed equally to this work

2008). However, miRNAs, which can suppress self-renewal and may contribute to lineage commitment of ESCs, have not been fully addressed. So, we aim to uncover novel miRNAs which can silence ESC self-renewal and to better understand the intricate regulatory mechanisms of ESC differentiation.

Here, we report the identification of a novel class of differentiation-associated miRNAs by functional screening a collection of 40 miRNAs pre-selected by their expression patterns and predicted targets. miR-27a-3p and miR-24-3p, two representatives of these miRNAs, whose expressions are relatively low and restrained by c-Myc in ESCs, directly target the critical pluripotency transcription factors (Oct4, Foxo1) and signal transducers (gp130, Smads) to suppress ESC self-renewal program. Moreover, CRISPR/Cas9 technology-mediated bi-allelic double knockout of two *miR-23~27~24* clusters in ESCs leads to serious defects in mesoderm differentiation of ESCs *in vitro* and *in vivo*. In addition, the efficiency of iPSC generation is improved evidently when miR-27a-3p or miR-24-3p is suppressed in mouse embryonic fibroblasts (MEFs).

## Results

### Bioinformatic prediction of miRNAs silencing ESC self-renewal

The expression profile of miRNAs in ESCs or during ESC differentiation could, to some extent, hint their roles in regulating ESC self-renewal or differentiation. We used a newly designed strategy that combined miRNA expression pattern and miRNA with predicted targets to be pluripotency factors to identify candidate miRNAs responsible for ESC differentiation (Fig 1A). Specifically, by analyzing the published small RNA sequencing data of ESCs and MEFs (Marson *et al*, 2008), we found that there were 44 miRNAs enriched in MEFs compared with ESCs (Supplementary Table S1). From the sequencing data of ESCs and day 5 embryoid body (EB) derivatives (Claudio *et al*, 2009), 53 miRNAs were found to be up-regulated during EB formation, which recapitulates early events of embryogenesis (Supplementary Table S2). Using TargetScan (Lewis *et al*, 2003), we identified 454 miRNAs (Supplementary Table S3) that were predicted to target nine pluripotency-associated transcription factors, including Oct4 (also known as Pou5f1), Sox2, Nanog, Klf4, c-Myc, Lin28a, Sall4, Rex1 and Stella (Heo *et al*, 2008; Ng & Surani, 2011). By intersecting the two lists, we selected 52 miRNA candidates, which were not only up-regulated in MEFs or during EB differentiation but also potentially targeting the key ESC identity factors (Fig 1B and C). Among them, there were respective 30 and 25 miRNAs enriched in MEFs or up-regulated during EB differentiation, 3 miRNAs which were up-regulated both in MEFs and during EB differentiation, 14 miRNAs (including let-7 family) which had been reported to play important roles in suppressing mouse ESC self-renewal (Fig 1C). We focused on 40 miRNAs, excluding 12 well-studied miRNAs from the predicted 52 candidate miRNAs, in controlling ESC pluripotency and let-7c was set as a control.

### Validation of the functions of candidate miRNAs in *Dgcr8*<sup>-/-</sup> ESCs

To uncover the roles of these miRNAs, we re-introduced miRNA mimics individually into *Dgcr8*<sup>-/-</sup> ESCs which exhibit a cell cycle

defect and are incapable to silence the self-renewal program upon differentiation induction.

We firstly investigated the colony-forming ability of transfected ESCs. Replating assays showed that 17 miRNAs decreased the capacity of ESCs to reform colonies. Of them, miR-9-5p, 200c-3p, 96-5p, 218-5p, 300-3p, 124-3p, 377-3p, 129-5p, 24-3p and 27a-3p, as well as let-7c, notably produced less and grossly differentiated colonies and possessed higher percentage of differentiated colonies than scramble control. There were 15 miRNAs which did not affect the colony-forming ability of ESCs, 8 miRNAs which enhanced the colony formation and yielded more compact and undifferentiated colonies (Fig 1D, Supplementary Fig S1 and Supplementary Table S4). Furthermore, we also detected the alkaline phosphatase (AP) activity in mimic-transfected ESCs without replating. To quantitatively analyze the AP activity, we scored the degree of AP staining on a scale from -3 to 3, with -3 being maximal loss of staining and with 3 being maximal boost of staining (Supplementary Fig S2 and Supplementary Table S5). We discovered that 21 miRNAs decreased the AP activity, 11 miRNAs mildly affected the AP activity and 8 miRNAs boosted the AP activity of ESCs (Fig 2A). Of them, miR-129-5p, 200c-3p, 218-5p, 145a-5p, 9-5p, 300-3p, 31-5p, 24-3p markedly caused loss of AP activity and yielded flat and differentiated cell colonies even when the cells were maintained in ESC culture conditions.

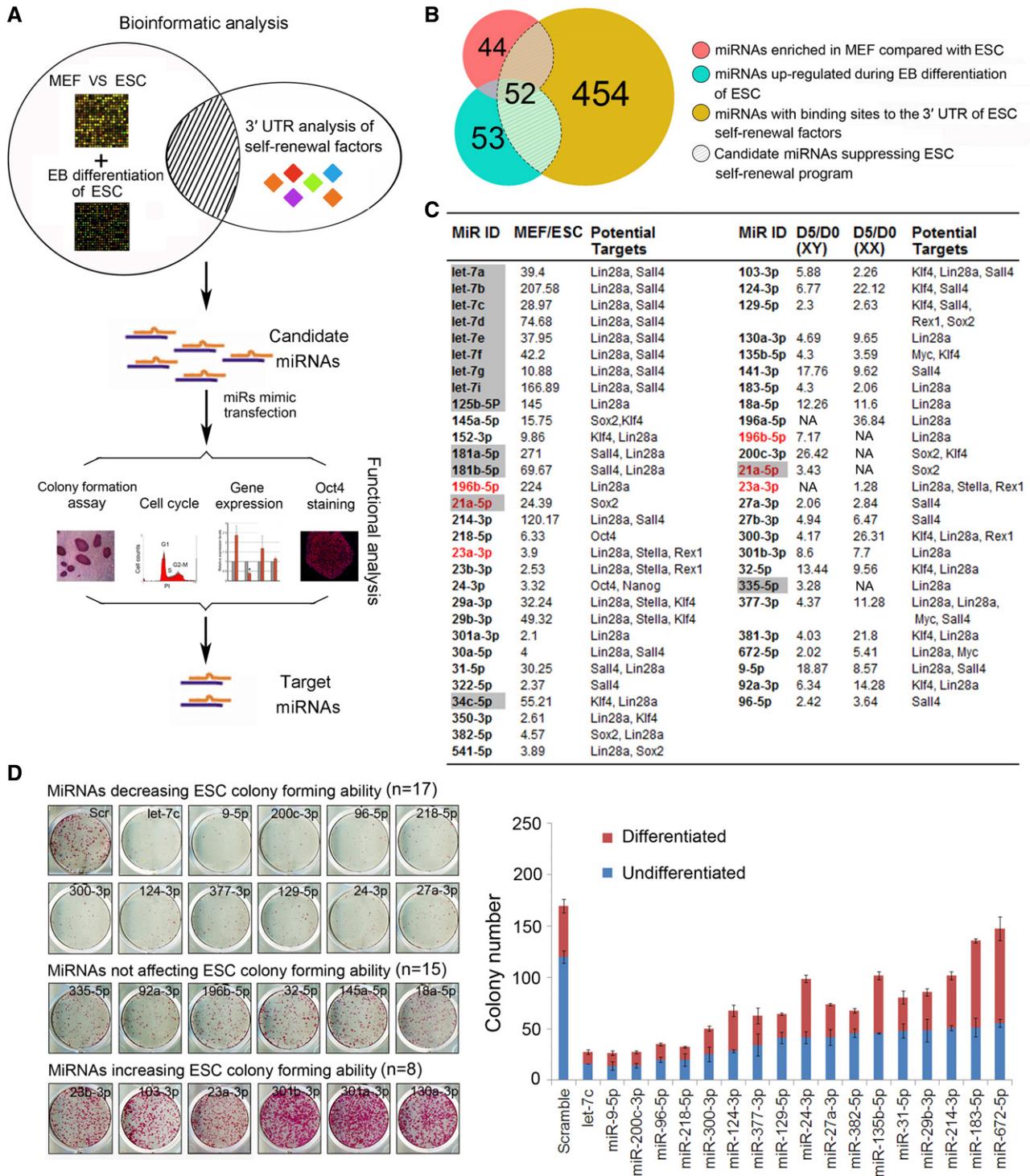
Another feature of self-renewing ESCs is their shortened G1 phase in cell cycle compared to differentiated cells (White & Dalton, 2005). Propidium iodide staining followed by flow cytometry analysis was performed in miRNA mimic-treated ESCs and revealed that 18 miRNAs could increase the fraction of cells in G1 phases, 12 miRNAs could reduce the fraction of cells in G1 phases and 10 miRNAs mildly affected cell cycle (Supplementary Fig S3A and B, and Supplementary Table S6). Similar to let-7c, miR-9-5p, 24-3p, 124-3p, 96-5p, 27a-3p, etc., led to G1 phase accumulation significantly (Fig 2B).

We further analyzed the expression profiles of critical pluripotency factors and several differentiation markers through q-PCR. The results showed that most of the miRNAs decreasing AP activity in ESCs suppressed the expression of multiple pluripotency factors and obviously promoted cell differentiation to a certain extent. Conversely, some miRNAs played opposite roles in regulating ESC pluripotency (Fig 2C and Supplementary Table S7).

The master transcription regulator of pluripotency maintenance, Oct4, was simultaneously analyzed in miRNA mimic-treated ESCs using immunofluorescence (Fig 2D). The results of Oct4 staining were consistent with that of AP staining. miR-218-5p, 200c-3p, 129-5p, 135b-5p, 24-3p, 9-5p, 32-5p and 27a-3p notably decreased Oct4 expression. Conversely, several miRNAs, such as miR-30a-5p, 541-5p, 152-3p and 141-3p, improved Oct4 expression (Fig 2D and Supplementary Fig S3C).

### Grading and scoring of screening results

Systematically analyzing the data from colony formation assay, AP staining, cell cycle, gene expression pattern and Oct4 staining mentioned above, we ranked the miRNAs mainly based on their ability to silence ESC self-renewal (Fig 2E, Supplementary Fig S4A and Supplementary Table S8). The top 15 miRNAs were considered to be important differentiation-associated miRNAs, which can silence ESC self-renewal evidently. Most of them can inhibit the expression of pluripotency factors, decrease AP activity and also



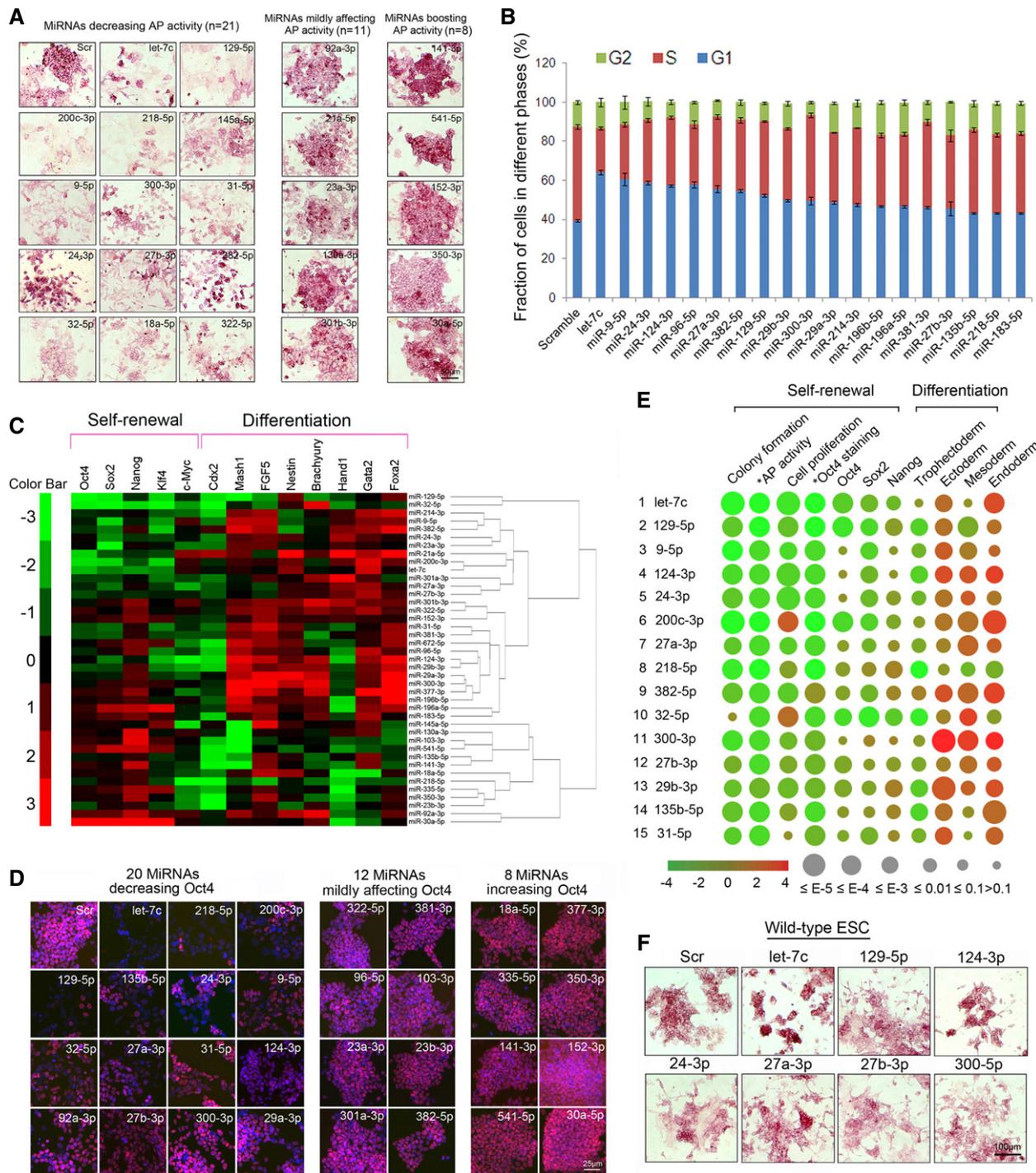
**Figure 1. Screening of target miRNAs silencing ESC self-renewal.**

**A** Screening strategy. miRNAs potentially targeting important ESC pluripotency factors meanwhile enriched in MEFs or up-regulated during EB differentiation were selected as candidate miRNAs. Candidate miRNAs silencing ESC self-renewal were identified by the function analyses in *Dgcr8*<sup>-/-</sup> ESCs.

**B** Distribution of candidate miRNAs. There were 44 miRNAs enriched in MEFs (red region), 53 miRNAs up-regulated during EB differentiation (blue region) and 454 miRNAs with binding sites to the 3' UTR of pluripotency factors (yellow region). The gray region represents 52 selected candidate miRNAs in silencing self-renewal.

**C** List of candidate miRNAs. Left panel shows candidate miRNAs enriched in MEFs comparing with ESCs. Right panel shows candidate miRNAs up-regulated during EB differentiation. Gray highlights the known miRNAs suppressing ESC self-renewal. Red marks the same miRNAs present in both panels. XY and XX indicate ESCs derived from male or female mice respectively. NA means D5/D0 was extremely high as the expression value in D0 was zero.

**D** Colony formation assay of miRNA mimic-transfected *Dgcr8*<sup>-/-</sup> ESCs. There were 17 miRNAs that decreased the colony-forming ability, 15 miRNAs that did not affect colony formation and 8 miRNAs enhanced colony formation of ESCs. Representative pictures are shown. Full data are available in Supplementary Fig S1.



**Figure 2. Functional analyses of candidate miRNAs in controlling ESC self-renewal.**

**A** Alkaline phosphatase staining of ESCs after miRNA mimic transfection. There were 21 miRNAs that decreased the AP activity, 11 miRNAs that mildly affected the AP activity and 8 miRNAs boosted the AP activity of ESCs. Representative pictures are shown. Full data are available in Supplementary Fig S2.

**B** Cell cycle distribution in representative miRNAs evoking G1 phase accumulation in miRNA mimic-transfected *Dgcr8*<sup>-/-</sup> ESCs. Error bars indicate SD (n = 3). Full data are shown in Supplementary Fig S3A and B.

**C** The heatmap representation of gene expression pattern in miRNA mimic-transfected *Dgcr8*<sup>-/-</sup> ESCs.

**D** Immunofluorescence staining of Oct4 in miRNA mimic-transfected ESCs. 20 miRNAs decreased Oct4 expression and produced small and grossly differentiated cell colonies, 12 miRNAs mildly affected Oct4 expression, and 8 miRNAs improved Oct4 expression and yielded compact and undifferentiated cell colonies. Representative pictures are shown. Full data are available in Supplementary Fig S3C.

**E** Ranking of the miRNAs based on their ability to silence ESC self-renewal program. The color of the circles represents the fold change of these subjects compared to scramble control; the size of the circles represents the magnitude of P-values as determined by Student's t-test. Full list is available in Supplementary Fig S4A.

**F** AP staining of V6.5 ESCs after mimic transfection (full data are available in Supplementary Fig S4B).

repress cell proliferation, but there were several miRNAs which can inhibit ESC self-renewal but had no influence or inverse effect on cell proliferation, hinting their distinct molecular mechanisms in regulating ESC pluripotency. Moreover, up-regulation of several lineage markers was observed in these miRNA mimics treated ESCs.

### Evaluation of the roles of the differentiation-associated miRNAs in wild-type ESCs

We further evaluated the roles of the top 14 miRNAs by transfecting miRNA mimics to wild-type V6.5 ESCs. Among them, seven miRNAs (let-7c, miR-300-5p, 24-3p, 27a-3p, 124-3p, 27b-3p and 129-5p) can decrease the AP activity and produced smaller or flat cell colonies although not as dramatically as that in *Dgcr8*<sup>-/-</sup> ESCs (Fig 2F). However, the other seven miRNAs had no influence on the AP activity of V6.5 ESCs (Supplementary Fig S4B). These results agree with recent studies that the ESC-specific or enriched miRNAs can antagonize the effects of these differentiation-associated miRNAs in wild-type ESCs (Melton *et al*, 2010; Wang *et al*, 2013b). This does not indicate that these miRNAs are nonfunctional in ESCs and just suggests that it cannot be observed in wild-type ESC through miRNA mimic over-expression. Maybe, knockout of these miRNAs in mouse can reflect their physiologic roles better. Interestingly, two of the seven effective miRNAs, miR-27a-3p and miR-24-3p, are the products from the same *miR-23a~27a~24-2* cluster (Lee *et al*, 2004; Zhou *et al*, 2007) and possess similar properties to inhibit self-renewal of ESCs. Moreover, we previously studied their synergistic roles in promoting erythropoiesis in human (Ma *et al*, 2013; Wang *et al*, 2014). Thus, we decided to further investigate the roles and molecular mechanisms of them in controlling ESC pluripotency. Beyond this, miR-23a-3p, 27a-3p and 24-3p will be simplified as miR-23a, 27a and 24, respectively.

### The expression pattern of *miR-23a~27a~24-2* cluster during ESC differentiation and in mouse adult tissues

To better understand the role of *miR-23a~27a~24-2* cluster in promoting ESC differentiation, we investigated the expression pattern of the cluster during ESC differentiation *in vitro*. All the three members of the miRNA cluster (Fig 3A) were up-regulated during EB formation (Fig 3B) and retinoic acid (RA)-induced differentiation (Fig 3C), suggesting their roles in promoting ESC differentiation. Similarly, the expression level of primary *miR-23a~27a~24-2* transcript was consistent with that of mature miRNAs (Fig 3D and E). In addition, *miR-23a~27a~24-2* cluster is abundantly expressed in MEFs, in contrast to its low expression in ESCs. In somatic tissues, these miRNAs are expressed ubiquitously and relatively at higher levels in heart, intestine, kidney, lung, muscle, stomach and uterus, compared to their low expression level in wild-type ESCs (Fig 3F), suggesting that they might function in the generation and/or maintenance of differentiation states of these tissues during mouse development.

### miR-27a and miR-24 suppress the self-renewal of ESCs

To further confirm the roles of *miR-23a~27a~24-2* in ESCs, we detected the protein level of the three master transcription factors,

Oct4, Sox2 and Nanog in miRNA-transfected *Dgcr8*<sup>-/-</sup> and V6.5 ESCs. miR-24 significantly induced the down-regulation of the three pluripotency factors in *Dgcr8*<sup>-/-</sup> ESCs and the effect was comparable to that of let-7c. By contrast, the effectiveness of miR-23a and miR-27a was weaker. The suppression on the pluripotency-associated factors in wild-type ESCs was not obvious in contrast to *Dgcr8*<sup>-/-</sup> ESCs (Fig 3G). In addition, decrease of Oct4 and Nanog, accompanied by elevation of differentiation markers (Foxa2, Gata2 and Nestin), as evidenced by immunofluorescence was observed in let-7c, miR-27a and miR-24 over-expressed ESCs (Fig 3H and I). The results indicate that miR-27a and miR-24 are critical negative regulators of ESC self-renewal and can induce spontaneous differentiation of ESC.

### miR-27a and miR-24 directly target pluripotency factors and signal transducers of ESC self-renewal networks

We further dissected the mechanism by which they function to repress self-renewal and promote differentiation in ESCs. The important pluripotency factors including Oct4, Nanog, Foxo1 (Zhang *et al*, 2011), Sall4, HIF2 $\alpha$  and several signal transducers maintaining ESC self-renewal, such as gp130 (also known as IL6st), LIFR, Smad3 and Smad4, were predicted to have the binding sites of miR-27a or miR-24 in their 3' UTRs, and c-Myc was predicted to have two binding sites of miR-24 in its protein coding sequence (CDS) (Fig 4A). So we firstly examined the protein levels of these factors in the miR-27a or miR-24 over-expressed V6.5 ESCs. We observed an apparent decrease in Foxo1, gp130 and Smad3 expression in the presence of miR-27a mimics. Expressions of c-Myc, Nanog, Oct4 and Smad4 were all decreased by over-expression of miR-24 in ESCs (Fig 4B). Next, we asked whether these genes can be directly targeted by miR-27a or miR-24. We constructed luciferase reporters that harbor the 3' UTRs or CDS of these genes. Since miR-24 has been reported to be able to down-regulate c-Myc by binding to its seedless 3' UTR in human cells (Lal *et al*, 2009), the reporter harboring the entire 3' UTR of c-Myc was also engineered. Reporter assays in 293T cells revealed that miR-27a significantly reduced the luciferase activities of Foxo1, gp130 and Smad3. Meanwhile, miR-24 significantly reduced the luciferase activities of Oct4 and Smad4 but not that of c-Myc and Nanog (Fig 4C). The discrepancy with the previous report (Lal *et al*, 2009) may be due to the non-conservative 3' UTR of c-Myc among species. Further luciferase assay of the mutant reporters (Supplementary Fig S5A) indicated that the repression of miR-27a and miR-24 on their targets was dependent on the miRNA binding sites (Fig 4D). We also demonstrated miR-27a and miR-24 can down-regulate the protein levels of their targets in *Dgcr8*<sup>-/-</sup> ESCs (Supplementary Fig S5B).

### Validation of the binding of the miRNAs to their targets in ESCs

To provide direct evidence for functional requirements of miR-27a and miR-24 in regulating their targets, we determined the binding of the miRNAs to the 3' UTRs of these targets by ribonucleoprotein immunoprecipitation (RNP-IP) (Hassan *et al*, 2010b). *Dgcr8*-null ESCs were transfected with miRNA mimics and then were immunoprecipitated by Ago2 antibody. RNAs isolated from Ago2-IP were subjected to RT-PCR for detecting the presence of target mRNAs and miRNAs in the Ago2 complex (Fig 4E). The presence of

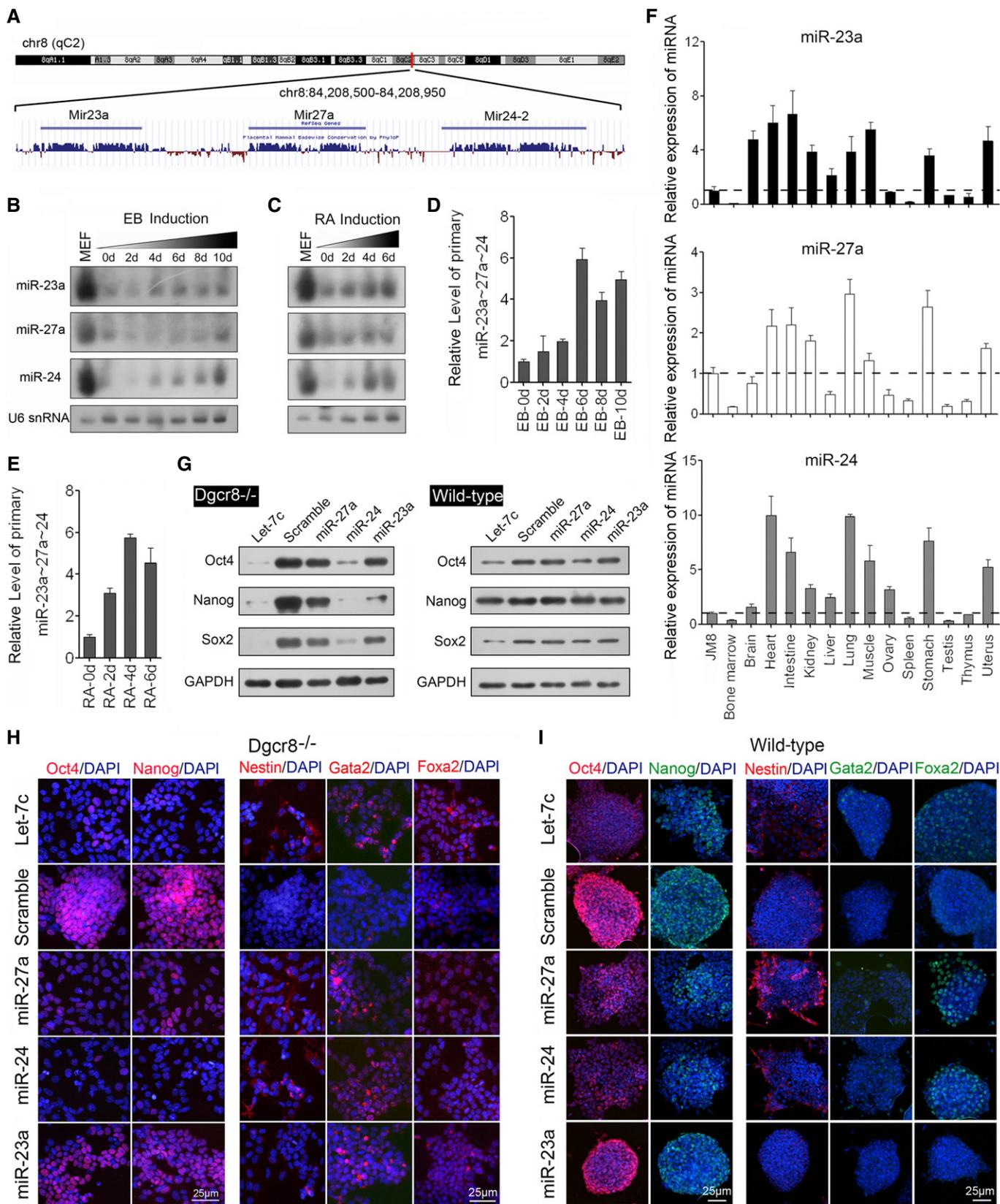


Figure 3.

**Figure 3. The essential role of miR-27a and miR-24 in ESC differentiation.**

- A The genomic location of *miR-23a-27a-24-2* cluster.  
 B, C Northern blot analysis of the expression of miR-23a, miR-27a and miR-24 during EB formation (B) and RA-induced differentiation of V6.5 ESCs (C). The heatmap is representative of the results of quantitative analysis.  
 D, E Expression of primary *miR-23a-27a-24-2* transcript during EB formation (D) and RA-induced differentiation (E). Error bars indicate SD ( $n = 3$ ).  
 F Q-PCR analysis of miR-23a, miR-27a and miR-24 expression in ESCs (JM8A3) and a variety of C57BL/6j adult tissues. Data are shown as mean + SD ( $n = 3$ ).  
 G Relative protein levels of Oct4, Sox2 and Nanog in miRNA mimic-transfected *Dgcr8*<sup>-/-</sup> ESCs and wild-type ESCs.  
 H, I Oct4, Nanog, Foxa2, Gata2 and Nestin immunofluorescence staining of mimic-transfected *Dgcr8*<sup>-/-</sup> (H) and wild-type ESCs (I).  
 Source data are available online for this figure.

miR-27a and miR-24 in RNAs isolated from the input and Ago2-IP in miRNA mimics over-expressed ESCs confirmed that miR-27a and miR-24 were successfully over-expressed and also can be bound by Ago2 complex. The presence of the target mRNAs in RNAs isolated from the Ago2-IP in miRNA mimics over-expressed ESCs confirmed that these targets were bound by Ago2 complex and the binding was dependent on the introduction of miRNAs. The absence of the target mRNAs and miRNAs in the IgG group ruled out the possibility of artifacts due to immunoprecipitation or DNA contamination (Fig 4F and G).

#### c-Myc restrains the expression of *miR-23a-27a-24-2* cluster in ESCs

c-Myc has been reported to transcriptionally repress miR-23a and 23b expression in two human cell lines (Gao *et al*, 2009). So we checked whether the transcription of *miR-23a-27a-24-2* cluster was repressed by c-Myc in ESCs. siRNA-based suppression of c-Myc in ESCs significantly up-regulated the expression of primary transcript of the cluster and also the mature miRNA level (Fig 4H and I). We next found that five putative c-Myc binding sites scattered within the proximal ~2 kb promoter region of *miR-23a-27a-24-2* cluster (Chr8: 84206504-84208504) (Fig 4J). ChIP-PCR showed that c-Myc could bind to the -15 site but not the other four sites (Fig 4K). Reporter assay revealed that c-Myc can repress the activity of the promoter and the repression was dependent on the -15 binding site (Fig 4L and M, and Supplementary Fig S5C). Analysis of publicly available c-Myc ChIP-Seq data (Chen *et al*, 2008) showed that c-Myc can also bind to multiple sites around *miR-23a-27a-24-2* cluster (Supplementary Fig S5D), which suggested that c-Myc might regulate expression of the miRNA cluster not only through the -15 bp proximal site but also through multiple binding sites. In addition, c-Myc can modulate the expression of the aforementioned target genes of miR-27a and miR-24 and the effect was mediated, at least in part, by miR-27a and miR-24 (Supplementary Fig S5E).

#### Inhibition of miR-27a and miR-24 promotes somatic cell reprogramming

miR-27a and miR-24 were abundant in MEFs indicating their roles for keeping the differentiated state of fibroblast in addition to their ability to repress ESC self-renewal. We hypothesized that inhibition of these two miRNAs in MEFs would enhance reprogramming of MEFs to iPSCs. To test this hypothesis, we directly transfected miR-27a or miR-24 inhibitors into MEFs harboring *Oct4-GFP* transgene at day 2 and day 6 after transduction with retroviral vectors expressing Oct4, Sox2 and Klf4 (OSK) and

scored reprogramming based on GFP expression in iPSC colonies (Fig 5A). The endogenous miRNAs were successfully inhibited by the miRNA inhibitors during this process (Fig 5B). As shown in Fig 5C, miR-27a and miR-24 inhibitions increased the number of AP-positive cell colonies. GFP<sup>+</sup> colonies were also counted and showed that miR-27a and miR-24 inhibition enhanced reprogramming by about twofold (Fig 5D).

Several iPSC clones were picked up and cultured to test their pluripotency. All iPSC clones were still GFP positive after 15 passages (Fig 5E). The endogenous Oct4, Sox2, Nanog and Klf4 were reactivated meanwhile the exogenous Oct4, Sox2 and Klf4 were silenced (Fig 5F and Supplementary Fig S6A). All iPSC clones showed hypomethylated promoters of endogenous *Oct4* and *Nanog*, identical to that of ESCs and totally different from their origin MEFs (Fig 5G and Supplementary Fig S6B). Immunostaining confirmed that Oct4, Nanog and SSEA1 were also activated in all iPSC clones (Fig 5H and Supplementary Fig S6C). Furthermore, all iPSC clones showed efficient EB formation *in vitro* and produce teratomas *in vivo* (Fig 5I and J, and Supplementary Fig S6D and E). Taken together, these results demonstrate that suppression of miR-27a or miR-24 enhances OSK-induced somatic cell reprogramming.

#### CRISPR/Cas9-mediated bi-allelic double knockout of *miR-23-27-24* clusters in ESCs

In consideration of important roles of miR-27a and miR-24 in ESC differentiation and somatic cell reprogramming, we are curious about whether miR-27a and miR-24 are indispensable for ESC differentiation. Five miRNAs (miR-23a, 23b, 27a, 27b, 24) constitute mouse *miR-23-27-24* clusters, which locate on two chromosomes as *miR-23a-27a-24-2* and *miR-23b-27b-24-1* cluster separately (Fig 6A). Their analogous functions in ESCs and similar sequence at the seed region suggest that the members may be functional compensative or redundant. So we generated *miR-23a-27a-24-2* cluster (*miR-23a* cluster) and/or *miR-23b-27b-24-1* cluster (*miR-23b* cluster) bi-allelic knockout V6.5 ESC clones using the newly developed powerful CRISPR/Cas9 genome editing system. Of 288 ESC clones that were screened using the PCR assay, 29 clones carried deletions of *miR-23a* cluster (both heterozygosity and homozygosity), 32 clones had deletions of *miR-23b* cluster and one clone carried deletion of all four alleles of the two clusters (Fig 6B). The ESC clone carrying all four alleles deletions and one ESC clone having *miR-23a* cluster homologous knockout were selected for further study and named as DKO and KO ESC clones separately. PCR assay showed that both of the two clusters were bi-allelic knocked out in DKO ESC clone, while *miR-23a* cluster alone was ablated in KO ESC clone (Fig 6C). Further sequencing of the

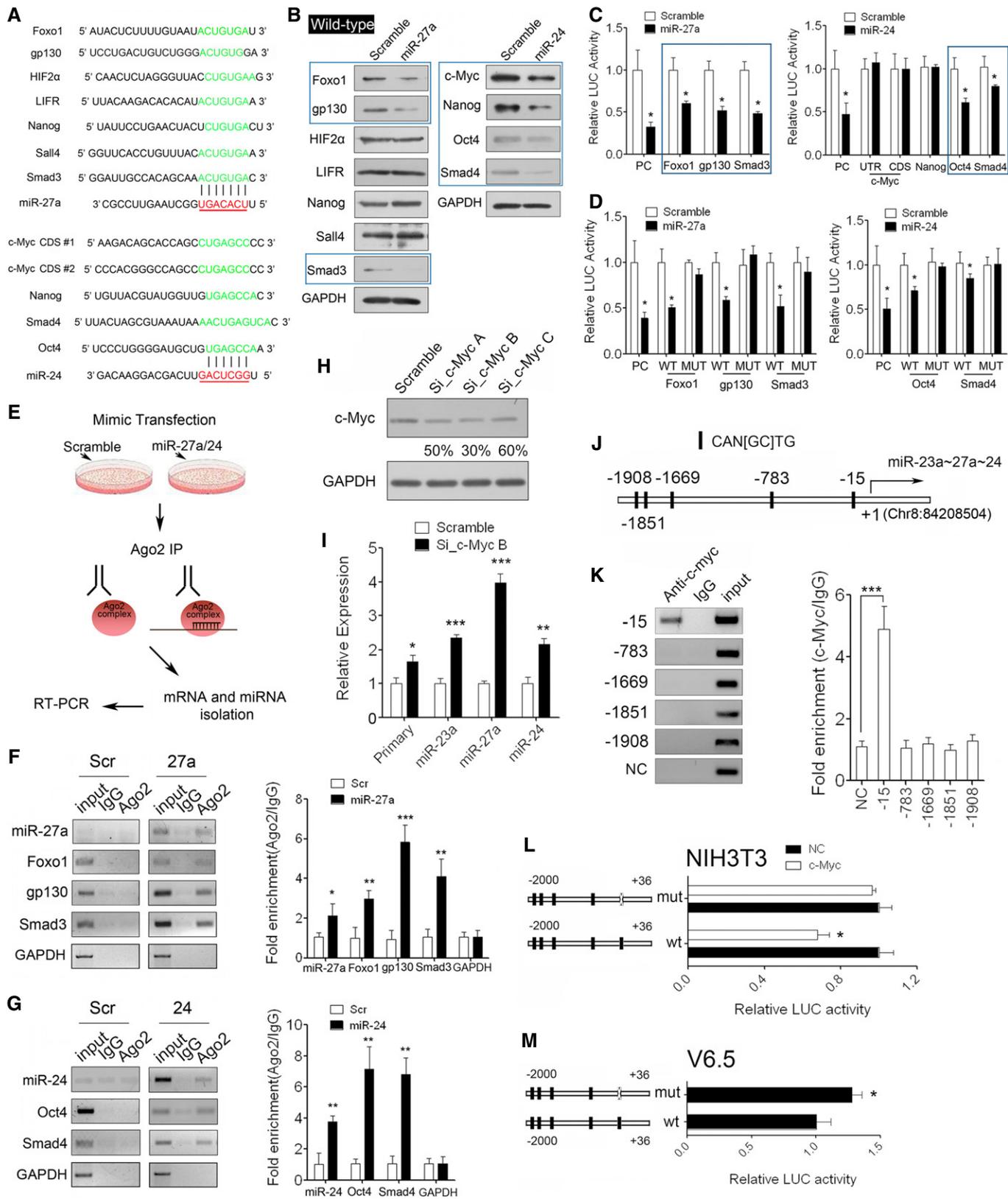


Figure 4.

**Figure 4. miR-27a and miR-24 directly target critical pluripotency-associated factors and are restrained by c-Myc in ESCs.**

- A Computer prediction of the binding of miR-27a and miR-24 on the 3' UTR or CDS of the indicated pluripotency-associated genes.
- B Western blot analysis of the expression of target genes in miR-27a or miR-24 over-expressing V6.5 ESCs. Candidate target genes whose expression was decreased are framed.
- C The relative luciferase activity of the reporter constructs co-transfected with scramble or miR-27a and miR-24 mimics. Error bars indicate SD ( $n = 3$ );  $*P < 0.05$ . Candidate target genes whose luciferase activity was decreased are framed. PC group was a positive reporter control which harbored reverse complementary sequence of miR-27a or miR-24.
- D The relative luciferase activity of the wild-type 3' UTR or mutated 3' UTR reporter co-transfected with scramble or miR-27a and miR-24 mimics. Data are shown as mean + SD ( $n = 3$ );  $*P < 0.05$ .
- E The schema of Ago2 immunoprecipitation.
- F, G RT-PCR and qRT-PCR analysis of *in vivo* binding of the miRNAs with their targets. Error bars indicate SD ( $n = 3$ );  $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ .
- H Western blot analysis of c-Myc expression in siRNA-treated V6.5 ESCs.
- I Q-PCR analysis of primary-23a~27a~24-2, miR-23a, miR-27a and miR-24 expression in c-Myc knocked-down ESCs. Error bars indicate SD ( $n = 3$ );  $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ .
- J The five putative c-Myc binding sites scattered within the promoter region of mouse *miR-23a~27a~24-2* cluster.
- K CHIP-PCR and ChIP-q-PCR analysis of c-Myc binding to the putative sites in the promoter. Error bars indicate SD ( $n = 3$ );  $***P < 0.001$ .
- L Relative luciferase activity of wild-type and -15 binding site mutated promoter in NIH3T3 cells co-transfected with c-Myc or the blank vector (NC). Error bars indicate SD ( $n = 3$ );  $*P < 0.05$ .
- M Relative luciferase activity of wild-type and -15 binding site mutated promoter in V6.5 ESCs. Error bars indicate SD ( $n = 3$ );  $*P < 0.05$ .
- Source data are available online for this figure.

genomic PCR products confirmed the knockouts (Supplementary Fig S7A). All mature miR-23, miR-27 and miR-24 were undetectable in DKO ESC clone, while in KO ESC clone, mature miR-23b and miR-27b were normally expressed and miR-24 was reduced (Fig 6D). Both KO and DKO ESCs were morphologically normal and expressed ESC-specific markers (Fig 6E–G). Analyses of the potential off-targets regions showed that none of the 19 predicted sites were truly modified by CRISPR/Cas9 in both DKO and KO ESC clones (Supplementary Fig S7B–D).

**miR-23~27~24 clusters are required for ESC differentiation**

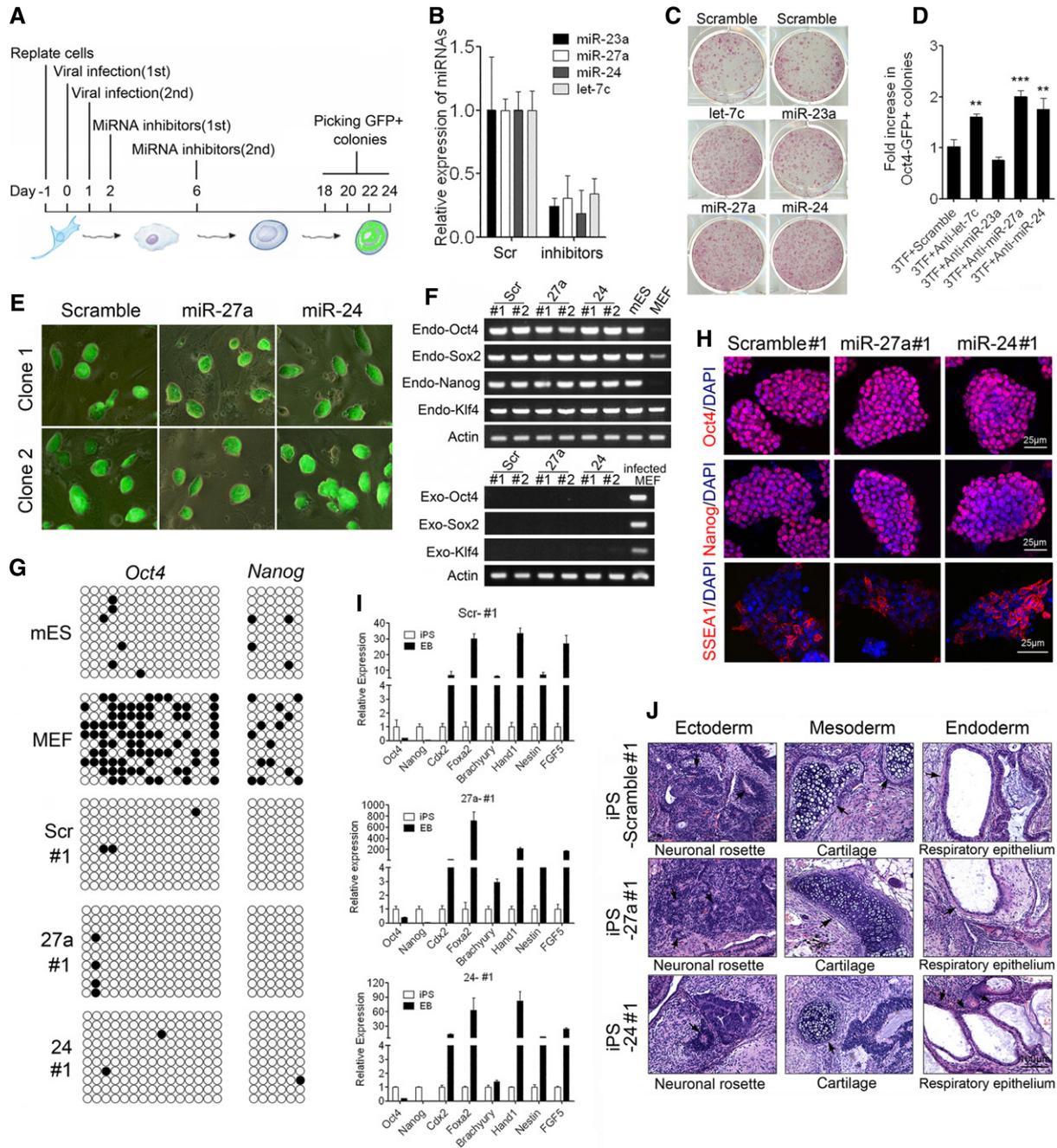
To assess the differentiation capacity of the knockout ESCs, we cultured them as EBs. As shown in Fig 7A, the up-regulation of Brachyury and Hand1 was delayed and was significantly lower than wild-type levels in EBs derived from DKO cells. By contrast, the expression of Nestin and Foxa2 was increased obviously, suggesting that the suppression of mesoderm lineages induced by *miR-23~27~24* cluster knockout might trigger the differentiation of other lineages. Meanwhile, the decline of Oct4 during EB formation was also delayed in the DKO cells. In addition, the repression of Brachyury and Hand1, as well as the promotion on the other lineage markers, can also be observed in EBs derived from KO cells, although the effect was weaker than that in DKO cells. These results indicate that *miR-23~27~24* clusters are required for silence of ESC self-renewal program and formation of early mesoderm.

To further demonstrate their indispensable roles in an exact lineage differentiation, we conducted cardiac differentiation of ESCs since mature miR-23/24/27 miRNAs were enriched in adult mouse hearts (Fig 3F). On day 8 of differentiation (2 days after EB plating), about 40% of the plated wild-type ESC-derived EBs already showed spontaneous contraction. The percentage continued to increase to 80–90% at day 12 and this proportion was kept up till the final time point we examined. In contrast, none of DKO ESC-derived EBs with spontaneous contraction was observed during the whole differentiation period (Fig 7B). The morphology of DKO ESC-derived EBs was also significantly different from that of wild-type ESCs and showed many bulgy bubbles. KO ESC-derived EBs

showed distinctly decreased beating incidence and the beating clusters were usually smaller than those in wild-type EBs (Fig 7B and Supplementary Videos S1 and S2). These data correlated with the mRNA and protein expression level of the cardiac marker genes. Real-time PCR revealed markedly lower expression of Nkx2.5, Tbx5,  $\alpha$ -MHC,  $\beta$ -MHC and cTnT in differentiating DKO and KO cultures compared with wild-type ESC derivations. The peak expression of these cardiac markers was also delayed in DKO ESCs during differentiation (Fig 7C). Immunofluorescence analysis of differentiation cultures revealed that V6.5 ESCs and KO ESCs-differentiated cells had well-organized actinin, ANP and troponin I expression, which was more in V6.5 ESC cultures than that of KO ESC cultures. Instead, DKO ESCs could not differentiate and form typical myofilament, with few expression of the three cardiac markers (Fig 7D). These data support *miR-23~27~24* clusters are absolutely required for cardiac lineage differentiation of ESC *in vitro* and the roles of *miR-23a* cluster could be compensated by *miR-23b* cluster partially.

Teratomas normally consist of a heterogeneous mix of differentiated cell types and usually are used to test the pluripotency of stem cells *in vivo*. In accordance with the defects in EB and cardiac differentiation, the teratomas produced with *miR-23~27~24* clusters knockout ESCs grew slowly and were significantly lighter than that derived from wild-type ESCs (Fig 7E and F). Some large undifferentiated regions indicative with Oct4 expression were detected in DKO and KO ESC-derived teratomas, which were occasional in the wild-type derivations (Fig 7G and Supplementary Fig S8A). Moreover, teratomas derived from DKO ESCs largely appeared ectoderm structures especially neuronal rosette, while mesoderm cells such as adipocyte, cartilage and muscle cells were distinctly less than that of wild-type ESCs (Fig 7H and Supplementary Fig S8B). Our results indicate that *miR-23a* and *miR-23b* cluster are indispensable for ESC differentiation especially the mesoderm differentiation *in vivo*.

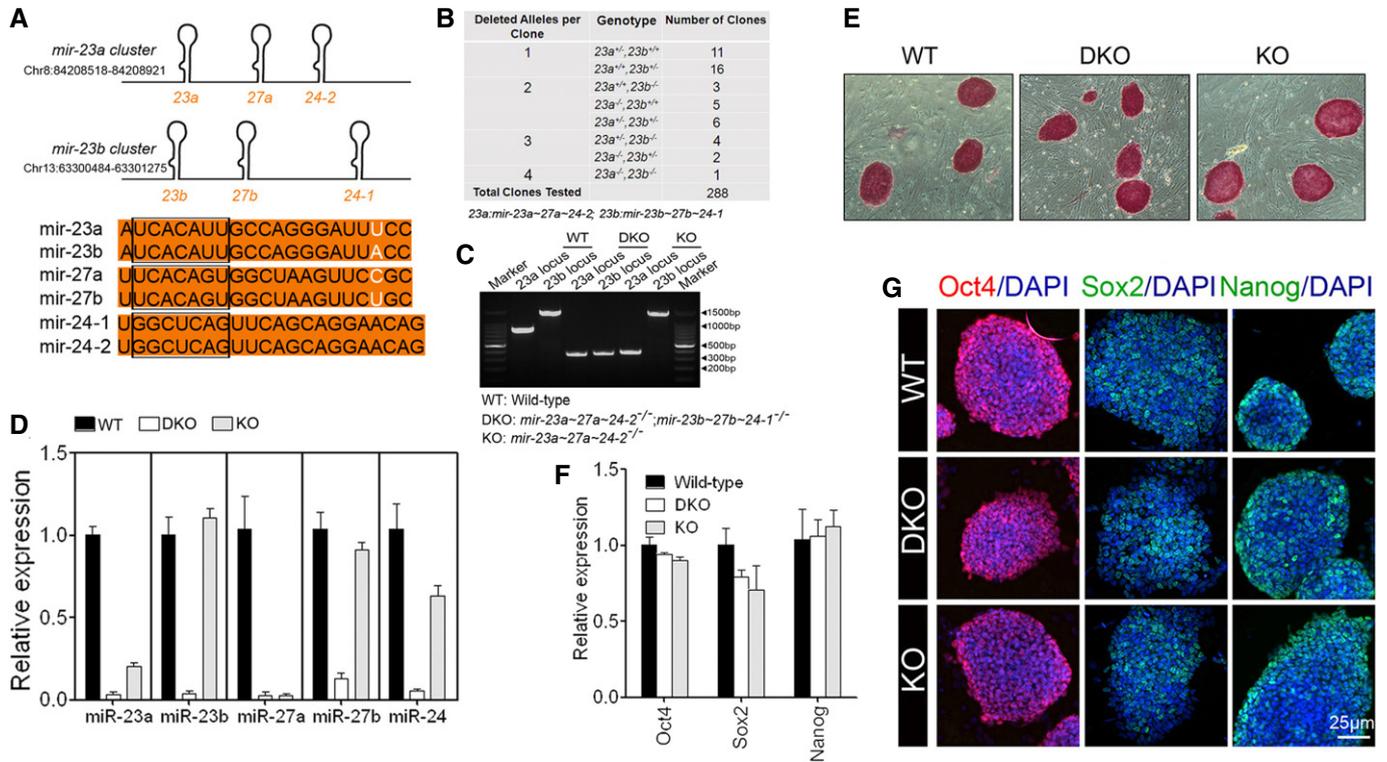
Further, we speculated the potential mechanism of *miR-23~27~24* clusters in regulating mesoderm differentiation preliminarily through comprehensive analysis of their targets. We predicted the potential targets of miR-23, 27, 24 and overlapped them with FunGenES Database (Schulz *et al*, 2009), which generated clusters



**Figure 5. Inhibition of miR-27a or miR-24 promotes somatic cell reprogramming to iPSC.**

A The schema of reprogramming MEFs to iPSCs by the three transcription factors Oct4, Sox2 and Klf4 (OSK).  
 B Validation of the effect of miRNA inhibition in MEFs by q-PCR. Error bars indicate SD (n = 3).  
 C AP staining of the iPSC colonies in reprogramming with OSK combined with scramble control, inhibitors of miR-23a, miR-27a, miR-24 and let-7c respectively.  
 D Fold increase in Oct4-GFP-positive cell colonies in the cells in (C). Data are shown as mean + SD (n = 5); \*\*P < 0.01; \*\*\*P < 0.001.  
 E Morphology of typical Oct4-GFP-positive iPSC colonies that were reprogrammed by OSK combined with miRNA inhibitors.  
 F Detection of endogenous and exogenous *Oct4*, *Sox2*, *Klf4* and *Nanog* expression in iPSCs, ESCs and MEFs by RT-PCR.  
 G Bisulfite genomic sequencing of the promoter regions of *Oct4* and *Nanog* in iPSCs, ESCs and MEFs. Open circles indicated unmethylated CpG dinucleotides, while closed circles indicated methylated CpGs.  
 H Immunofluorescence staining of Oct4, Nanog and SSEA1 of the iPSC colonies.  
 I Q-PCR analysis of the expression of self-renewal (*Oct4*, *Sox2*, *Nanog*) and differentiation (*Cdx2*, *Foxa2*, *Brachyury*, *Hand1*, *Nestin*, *Fgf5*) relative markers in EBs derived from the iPSCs. Error bars indicate SD (n = 3).  
 J Hematoxylin and eosin staining of teratoma derived from the iPSC colonies. Neuronal rosette (ectoderm), cartilage (mesoderm) and respiratory epithelium (endoderm) are marked with black arrows.

Source data are available online for this figure.



**Figure 6. Bi-allelic double knockout of *miR-23-27-24* clusters in ESCs by CRISPR/Cas9.**

A Genomic location and sequence alignment of mouse *miR-23-27-24* cluster miRNAs. Black box indicates the seed region.  
 B Summary of CRISPR/Cas9-mediated gene targeting in ESCs.  
 C Genomic PCR of the specific fragments spanning *miR-23-27-24* clusters. WT indicates wild-type ESCs; DKO indicates *miR-23a-27a-24-2<sup>-/-</sup>*; *miR-23b-27b-24-1<sup>-/-</sup>* ESCs; KO indicates *miR-23a-27a-24-2<sup>-/-</sup>* ESCs.  
 D Real-time PCR analysis of mature *miR-23a/b*, *miR-27a/b* and *miR-24* expression in wild-type, DKO and KO ESCs. Data are shown as mean + SD (n = 3).  
 E AP staining of wild-type and *miR-23-27-24* cluster knockout ESCs.  
 F Real-time PCR analysis of *Oct4*, *Sox2* and *Nanog* expression in wild-type and *miR-23-27-24* cluster knockout ESCs. Data are shown as mean + SD (n = 3).  
 G *Oct4*, *Sox2* and *Nanog* immunofluorescence staining of wild-type and single/double knockout ESCs.  
 Source data are available online for this figure.

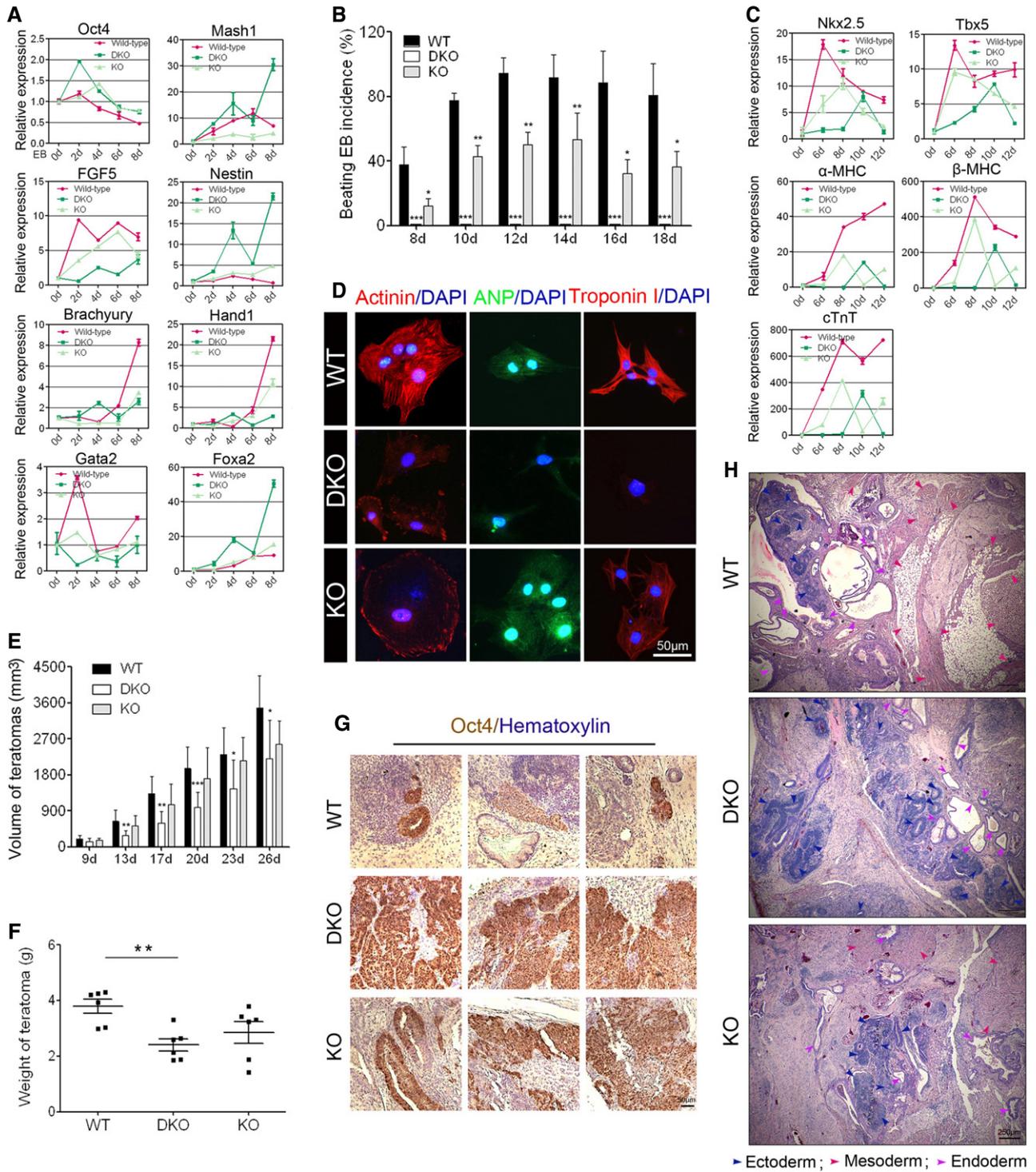
of transcripts that behave the same way in ESCs under the sixty-seven experimental conditions. As a result, the predicted targets were greatly enriched in the clustered transcripts, suggesting that the potential targets of *miR-23*, *27*, *24* are closely related to ESC state and differentiation (Supplementary Fig S9A). We also compared the enrichment of the targets in the individual clusters and found that the potential targets were more enriched in several clusters (Supplementary Table S12), which behave the similar way during ESC differentiation. All of them decreased during mesoderm differentiation and exhibited high expression level under neuronal and pancreatic differentiation (Supplementary Fig S9B and C). This indicates that *miR-23*, *27*, *24* regulate mesoderm differentiation of ESCs possibly through down-regulating the positive regulators of ectoderm or endoderm differentiation, meanwhile down-regulating the negative regulators of mesoderm differentiation.

## Discussion

The understanding of the regulatory mechanisms of ESC or iPSC differentiation is of crucial importance for their use in regenerative

medicine. Several differentiation-promoting miRNAs have been reported to target the ESC self-renewal network or participate in ESC differentiation (Tay et al, 2008a,b; Xu et al, 2009; Melton et al, 2010; Tarantino et al, 2010). Here, we uncovered a new class of miRNAs involved in silencing self-renewal by screening a selective collection of 40 miRNAs in *Dgcr8<sup>-/-</sup>* ESCs. We employed a variety of methods to synthetically judge their roles in controlling ESC pluripotency, lineage specification and cell cycle progression. During our manuscript preparation, Wang and colleagues reported a screen in *Dgcr8<sup>-/-</sup>* ESCs and uncovered 32 miRNAs that suppress the ESC self-renewal simply by testing the AP activity of ESCs (Wang et al, 2013b). Although they analyzed more miRNAs, our study provided more comprehensive information of each miRNA in regulating ESC pluripotency. Among 32 miRNAs they identified, the effect of several miRNAs (such as *miR-145* and *miR-18*) on differentiation was not significant in our multi-index evaluation system. We also provided the roles of another 4 miRNAs including the high ranking *miR-124* in regulating ESC pluripotency, which were not found in their study (Supplementary Table S9).

Our newly identified 14 differentiation-associated miRNAs may distinctly function due to their diverse expression patterns. Of them,



**Figure 7. miR-23~27~24 clusters are required for ESC differentiation.**

A Real-time PCR analysis of Oct4 and several lineage differentiation markers expression during EB formation of wild-type and knockout ESCs. Data are shown as mean  $\pm$  SD ( $n = 3$ ).

B Beating EB incidence during cardiac differentiation of wild-type and knockout ESCs. Data are shown as mean  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

C Real-time PCR analysis of several cardiac markers expression during cardiac differentiation of wild-type and knockout ESCs. Data are shown as mean  $\pm$  SD ( $n = 3$ ).

D Actinin, ANP and troponin I immunofluorescence staining of differentiation cultures derived from wild-type and knockout ESCs.

E Volumes of teratomas at different days after injection. Data are shown as mean  $\pm$  SD ( $n = 4$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

F Weight of teratomas derived from wild-type and knockout ESCs. Data are shown as mean  $\pm$  SD. \*\* $P < 0.01$ .

G Oct4 immunohistochemistry staining of teratomas derived from wild-type and knockout ESCs. Representative Oct4-positive regions are shown.

H Hematoxylin and eosin staining of teratomas derived from wild-type and knockout ESCs. Ectoderm, mesoderm and endoderm are marked with different color arrows.



state (Fig 8). Depleting them in MEFs can promote somatic cell reprogramming and knockout of *miR-23~27~24* clusters in ESCs leads to serious deficiency of ESC differentiation. Our study also suggested that *miR-23b~27b~24* cluster may resemble the roles of *miR-23a~27a~24* cluster in regulating ESC self-renewal and differentiation.

## Materials and Methods

### Bioinformatic analysis

miRNAs enriched in MEFs were chosen according to the criteria “the expression in MEFs was > 100, ratio of MEF/ESC was > 2” based on the published solexa sequencing data of 18–30 nucleotide transcripts in ESCs and MEFs (GSE11724) (Marson *et al*, 2008). miRNAs up-regulated during EB differentiation of ESCs were selected based on the rule of “ratio of D5/D0 in both the two ESC lines was > 2 or the expression value in D5 was > 0.1 and higher than that in D0 in both the two cell lines when the value in D0 was 0” based on the published sequencing data (Claudio *et al*, 2009). miRNAs potentially targeting to nine ESC pluripotency factors (Oct4, Sox2, Nanog, Klf4, c-Myc, Lin28a, Sall4, Rex1 and Stella) were predicted through TargetScanMouse 6.2 ([http://www.targetscan.org/mmu\\_61/](http://www.targetscan.org/mmu_61/)), which was also used to predict the targets of miR-27a and miR-24. The secondary structure of primary transcript of *miR-23a~27a~24-2* cluster was predicted by RNA structure software (<http://rna.urmc.rochester.edu/RNAstructureWeb/>). The transcription element search system (<http://www.cbil.upenn.edu/cgi-bin/tess>) was used to analyze the *miR-23a~27a~24-2* promoter sequence and predict the binding sites of c-Myc. Publicly available ChIP-Seq data for c-Myc binding in ES cells (GES11431) (Chen *et al*, 2008) were used to analyze the multiple binding sites for c-Myc across *miR-23a~27a~24-2* locus. FunGenES Database (<http://biit.cs.ut.ee/fungenes/>) (Schulz *et al*, 2009) was used to overlap with the predicted targets of miR-23, 27, 24 to speculate the potential mechanism of *miR-23~27~24* clusters in regulating mesoderm lineage commitment.

### Cell culture and differentiation

Mouse *Dgcr8*<sup>-/-</sup> ESCs were kindly provided by Prof. Dr. Yangming Wang who generated the cell line at University of California in 2007 (Wang *et al*, 2007). Mouse *Dgcr8*<sup>-/-</sup> ESCs and wild-type V6.5 ESCs were cultured on gelatin-coated plates in ESC medium, consisting of 15% fetal bovine serum (PAA), 2 mM GlutaMAX (GIBCO), 0.1 mM MEM Non-Essential Amino Acids Solution (GIBCO), 0.1 mM 2-mercaptoethanol (GIBCO), 50 U/ml penicillin, 50 µg/ml streptomycin (GIBCO) and 1,000 U leukemia inhibitory factor (LIF, Millipore). JM8A3 ESCs were cultured on feeder cells as described in Pettitt's paper (Pettitt *et al*, 2009). ESCs were passaged every other day. NIH3T3 cells and HEK293T cells were cultured in DMEM supplemented with 10% FBS.

For EB formation, V6.5 ESCs were dissociated with 0.05% trypsin-EDTA (GIBCO) and suspended in DMEM (Invitrogen) supplemented with the above components except for LIF on petri dish (Greiner) as a concentration of  $1.5 \times 10^5$  cells/ml. For retinoic acid (RA)-induced differentiation, V6.5 ESCs were plated on gelatin-coated 100-mm dish (Corning) as a density of  $8 \times 10^4$  cells/plate and cultured on differentiation medium containing 500 nM RA

(Sigma) in place of LIF. For cardiac differentiation, ESCs were suspended in DMEM supplemented with the above components except for LIF on low-adherent 96-well plate (Greiner) with concentration of 600 cells/well (day 0 of differentiation). At day 6 of differentiation, the EBs were transferred onto gelatin-coated 96-well plate. From day 6 until day 18, differentiation medium was replaced every second day. Starting on day 8 of differentiation (2 days after plating), each EB outgrowth was examined daily for spontaneously beating areas. EBs were also collected on days 0, 6, 8, 10 and 12 of differentiation for quantitative analysis of expression of cardiac markers. At day 18 of differentiation, a few EBs were trypsinized and replated on gelatin-coated glass coverslips at low density for immunofluorescence analyses. For *in vivo* differentiation of ESCs,  $3 \times 10^6$  ESCs were injected into a SCID Beige Mouse by subcutaneous injection. Volume of teratomas was measured every few days. Twenty-seven days after injection, teratomas were picked, weighed and fixed in PBS containing 4% formaldehyde and embedded in paraffin. Sections were stained with Oct4 antibody and also with hematoxylin and eosin.

### Oligonucleotides and transfection

A custom mouse miRIDIAN miRNA Mimic Library was obtained from Dharmacon (Thermo Fisher). One miRNA mimics were used for each individual miRNA. For miRNA mimic transfection,  $1 \times 10^5$  *Dgcr8*<sup>-/-</sup> ESCs or  $3 \times 10^4$  V6.5 ESCs were plated in gelatinized 6-well plates per well (or one-fifth of cells were plated on 24-well plates per well) on day 0 in ESC medium. On day 1, miRNA mimics were transfected at a concentration of 50 or 100 nM for *Dgcr8*<sup>-/-</sup> or V6.5 ESCs respectively using DharmaFECT 1 (Dharmacon, ThermoFisher) (Melton *et al*, 2010). For siRNA transfection,  $6 \times 10^4$  V6.5 ESCs were plated in gelatinized 12-well plates per well. c-Myc siRNAs (OriGene) were transfected at a concentration of 100 nM using DharmaFECT 1. For the rescue experiments, c-Myc siRNAs and the miRNA inhibitors were co-transfected at a concentration of 50 nM, respectively, using DharmaFECT 1. The transfected cells were cultured in ESC medium for 3 days and then were collected for further analysis. For evaluation of the screened miRNAs' functions in wild-type ESCs, transfected V6.5 ESCs were cultured in ESC medium without LIF for 3 days.

### Alkaline phosphatase staining and colony formation assay

Alkaline phosphatase activity was detected using Leukocyte Alkaline Phosphatase Kit (Sigma) following the manufacturer's protocol. Briefly, cells were fixed with 4% paraformaldehyde, washed twice by TBST, then stained for 30 min at RT, finally washed by TBST and suspended in PBS containing 20% glycerol for storage.

ESCs were transfected with miRNA mimics and 3 days later the cells were trypsinized and replated at 2,000 cells per well on gelatin-coated 12-well plate. The cells were cultured for 5 days before staining with Alkaline Phosphatase Kit and then undifferentiated and differentiated colonies were counted.

### Cell cycle analysis

ESCs were harvested and washed once at 4°C in PBS containing 0.5% BSA then added ice-cold 70% ethanol. The fixed cells were

immediately stored at  $-20^{\circ}\text{C}$  for at least 24 h. Cells were washed twice in ice-cold PBS to remove ethanol and then resuspended in PBS with 25  $\mu\text{g}/\text{ml}$  RNase A and 50  $\mu\text{g}/\text{ml}$  propidium iodide at  $37^{\circ}\text{C}$  for 1 h. Flow cytometry was performed using a Beckman Coulter and analyzed with ModFit.

### Immunofluorescence and immunoblot

Cells were fixed with 4% paraformaldehyde, then permeabilized with 0.5% Triton X-100 for 10 min and blocked with 3% BSA for 1 h at room temperature (RT). Cells were incubated with primary antibody overnight at  $4^{\circ}\text{C}$ , washed three times with PBS then incubated with secondary antibody for 1 h at RT. Afterwards, nuclei were labeled by DAPI and visualized by Olympus BX51. Whole-cell lysates were used for immunoblot analysis as previously described (Yu *et al*, 2008). The following antibodies were used for immunofluorescence (IF) or immunoblot (IB): Oct4 (sc-5279, Santa Cruz), Sox2 (2748S, Cell signaling), Nanog (AB5731, Millipore), Foxa2 (3231, Epitomics), Gata2 (11103, Proteintech), Nestin (ab6142, Abcam) for both IF and IB; SSEA1 (sc-21702, Santa Cruz), actinin (MAB1682, Millipore), ANP (AB5490, Millipore) and tropomyosin I (MAB3152, Millipore) only for IF; Brachyury (ab20680, Abcam), Hand1 (ab86009-100, Abcam), Foxo1 (1874-1, Epitomics), gp130 [06-291 (Millipore) for Fig 4B and Supplementary Fig S5B; 21175-1-AP (Proteintech) for Supplementary Fig S5D], HIF2 $\alpha$  (MAB3472, Millipore), LIFR (ab101228, Abcam), Sall4 (DR1052, Millipore), Smad3 (BS1798, Bioworld), c-Myc (9402S, Cell signaling), Smad4 (BS2050, Bioworld) and GAPDH (sc-365062, Santa Cruz) only for IB. Oct4 (sc-5279, Santa Cruz) was also used for immunohistochemistry. Results of immunoblots were quantified using ImageJ software.

### RNA isolation, Northern blot and quantitative RT-PCR

Total RNA was extracted from the harvested cells using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. Northern blot analysis was performed as previously described (Yu *et al*, 2006). The probes were listed in Supplementary Table S11. cDNA was synthesized via M-MLV reverse transcriptase (Invitrogen). q-PCRs were performed using the SYBR Premix Ex Taq kit (Takara) as previously described (Ma *et al*, 2010). miRNA q-PCR was performed using the following Taqman probes (Applied Biosystems): miR-27a (TM408), miR-23a (TM399), miR-24 (TM402) and RNU6B (TM1093). The oligonucleotides used for PCR were listed in Supplementary Table S11. All the PCRs were performed in triplicate.

### Constructs and reporter assay

The reverse complementary sequence of miR-27a and miR-24 was inserted into the pMIR-reporter (Promega) to generate a reporter system (pMIR-27a, pMIR-24) that can detect mature miRNA expression and were performed as positive controls in 293T cells. The 3' UTR or CDS containing the miRNA binding sites of the potential target genes were PCR-amplified and cloned into pMIR-reporter downstream of the firefly luciferase gene to generate the corresponding reporters. Mutations at the miRNA binding site in these mRNA sequences were created using bridging PCR. For c-Myc overexpression, the c-Myc ORF was amplified and cloned into pcDNA6/

V5-His B. The DNA fragment of 2 kb upstream of the transcriptional start site of *miR-23a~27a~24-2* cluster was PCR-amplified and cloned into pGL3-basic upstream of the firefly luciferase gene to generate the pGL3-23a-promoter reporter. Mutations at the c-Myc binding site in the promoter sequence were created using bridging PCR. The primers were listed in Supplementary Table S11.

For the miRNA target analysis, 293T cells were co-transfected with 0.4  $\mu\text{g}$  reporter construct, 0.02  $\mu\text{g}$  pRL-TK vector, and 5 pmol miRNA mimic or scramble controls per well of 24-well plate. For the functional analysis of the *miR-23a~27a~24-2* promoter, NIH3T3 and V6.5 ESCs were co-transfected with 0.5  $\mu\text{g}$  pGL3-basic or pGL3-23a promoter constructs, 0.02  $\mu\text{g}$  pRL-TK vector and different amounts of pCMV6-c-Myc or empty pCMV6 vector per well of 24-well plate. Cells were harvested 48 h post-transfection and assayed with a Dual Luciferase Assay (Promega) according to the manufacturer's instructions. All transfection assays were performed in triplicate.

### Ago2 RNP-IP

Ago2 RNP-IP was performed as described previously (Hassan *et al*, 2010b) with minor modifications. Protein A agarose beads (Roche) were rinsed twice with wash buffer (50 mM Tris-HCl at pH 7.5, 50 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.05% NP-40, 1.5 mM DTT) and incubated with monoclonal anti-Ago2 (ab57113, Abcam) or mouse IgG (ab37355, Abcam) overnight at  $4^{\circ}\text{C}$ . Beads were then washed three times with wash buffer to remove the unbound IgGs and washed twice in lysis buffer (10 mM Tris-HCl at pH 7.5, 100 mM NaCl, 2.5 mM MgCl<sub>2</sub>, 0.5% NP-40). miRNA mimic-transfected *Dgcr8*<sup>-/-</sup> ESCs were harvested and rinsed twice in PBS and were lysed on ice for 5 min in a fresh lysis buffer supplemented with protease inhibitors and RNasin. The cell lysates were centrifuged at 10,000 g at  $4^{\circ}\text{C}$  for 8 min, and the supernatants were subjected to preclearance by incubation with protein A beads at  $4^{\circ}\text{C}$  for 60 min. An aliquot of lysate after preclearance was reserved for total RNA analysis. The remaining lysates proceeded to bind with either Ago2- or IgG-bound beads at  $4^{\circ}\text{C}$  for 2 h, and the beads were washed twice with wash buffer, then the beads were subjected to DNase treatment by incubating with 0.04 U/ml DNase I (Invitrogen) at  $37^{\circ}\text{C}$  for 20 min. The beads were then washed twice with wash buffer and proceeded to Proteinase K treatment by incubating with 20 mg/ml proteinase K in wash buffer containing 20% SDS at  $55^{\circ}\text{C}$  for 15 min. RNAs that co-immunoprecipitated were eluted twice with wash buffer and extracted by phenol-chloroform, then precipitated with ethanol. Total RNA from cell lysates was isolated using the same procedure. RNAs isolated from RNP-IP were subjected to cDNA synthesis using oligo(dT) or miRNA-specific RT primers and amplified by gene-specific or miRNA-specific primers. The primers are listed in Supplementary Table S11.

### Chromatin immunoprecipitation (ChIP) assay

ChIP-PCR was performed as previously described (Ma *et al*, 2013). V6.5 ESCs were cultured and collected for c-Myc ChIP experiments. Anti-c-Myc (9402S, Cell signaling) and Rabbit IgG (sc-66931, Santa Cruz) were used. The negative genomic locus (chr8:84207127-84207294), which does not include c-Myc binding site, was amplified as a negative control. The primers used for the ChIP-PCR were listed in Supplementary Table S11.

### Generation and characterization of iPSCs

*Oct4-GFP* MEFs (Lengner *et al*, 2007) were plated on gelatin-coated 6-well plates at  $1 \times 10^5$  cells per well (marked as day  $-1$ ). Retrovirus was added twice at day 0 and day 1, and then miRNA inhibitors were transfected at day 2 and day 6 at a concentration of 60 nM. MEFs were firstly cultured in DMEM supplemented with 10% FBS and changed to ESC medium at day 4 and finally changed to N2B27 2i medium at day 9. N2B27 medium is a 1:1 mixture of DMEM/F12 (Invitrogen) supplemented with N2 (Invitrogen) and Neurobasal (Invitrogen) medium supplemented with B27 (Invitrogen). N2B27-2i medium comprised of 1 mM Mek inhibitor PD0325901, 3 mM GSK3 inhibitor CHIR99021 and 1,000 U/ml LIF in N2B27 medium.

To distinguish the endogenous and exogenous Oct4, Sox2 and Klf4, the forward endogenous primers were designed to be located in the 5' UTR region of the endogenous gene, whereas the reverse exogenous primers were designed to be located in the vector. q-PCR was used to detect the expression of endogenous and exogenous Oct4, Sox2 and Klf4 in the iPSCs.

Bisulfite sequencing was performed using the Epitect Bisulfite Sequencing Kit (Qiagen) according to the manufacturer's instruction. PCR primers were listed in Supplementary Table S11. Amplified products were cloned into pGEM-T (Promega). Ten randomly selected clones were sequenced with the T7 and SP6 primers.

For teratoma formation assay,  $1.5 \times 10^6$  cells were injected into a SCID Beige Mouse by subcutaneous injection. Four to five weeks after injection, teratoma was picked and fixed in PBS containing 4% formaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin.

### CRISPR/Cas9-mediated miR-23~27~24 cluster knockout in mESC

sgRNA-specifying oligo sequences spanning *miR-23a~27a~24-2* or *miR-23b~27b~24-1* cluster were chosen to minimize likelihood of off-target cleavage based on publicly available online tools (<http://crispr.mit.edu/>). The annealed oligos were cloned into pX330 to construct the recombinant pX330-miR-23a-L, pX330-miR-23a-R, pX330-miR-23b-L and pX330-miR-23b-R. The four pX330-sgRNA plasmids and a puromycin-selection plasmid were co-electroporated into V6.5 ESCs. After 48 h of puromycin-selection, electroporated cells were trypsinized and replated, ESC colonies were picked up on day 7. Genomic DNA was extracted from the cells, and the knockout colonies were defined as having PCR amplification of reduced band as the specific primers spanned the deletions. Further, the PCR products were sequenced to confirm the knockouts. Meanwhile, some potential off-target regions (score > 0.5 and mismatch  $\leq 4$ ) predicted through CRISPR analysis tool online (<http://crispr.mit.edu/>) were amplified using High-Fidelity DNA Polymerase (New England Biolabs) and sequenced. The potential off-target regions were listed in Supplementary Table S10. The oligos and primers used in CRISPR/Cas9 were listed in Supplementary Table S11.

### Statistics

Student's *t*-test (two-tailed) was performed to analyze the data.  $P < 0.05$  were considered statistically significant as indicated by asterisks ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ). Scoring of each miRNA on silencing self-renewal was sum of the respective score of

miRNAs on colony formation, cell cycle progression, AP activity, Oct4 staining and the expression of Oct4, Sox2 and Nanog. Score on colony formation assay was the logarithm of the ratio of the colony number relative to scramble control, with base 2. Score on AP staining was in the range of  $-3$  to  $3$ , with  $-3$  being maximal loss of staining and  $3$  being maximal boost of staining. Score on cell proliferation was the logarithm of the ratio of G1 fractions (%) relative to scramble control, with base 1.1. Taking 1.1 as base was to make the grade in the range of  $-6$  to  $6$ . Score on Oct4 staining was in the range of  $-3$  to  $3$ , with  $-3$  being maximal loss of staining and  $3$  being maximal boost of staining. Score on Oct4 expression was the logarithm of Oct4 mRNA level relative to scramble control, with base 2. Score on Sox2 expression was the logarithm of Sox2 mRNA level relative to scramble control, with base 4. Score on Nanog expression was the logarithm of Nanog mRNA level relative to scramble control, with base 4. Taking 4 as base was to reduce the proportion of Sox2 and Nanog expression in the total Score.

**Supplementary information** for this article is available online:

<http://emboj.embopress.org>

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### Author contributions

JY and YH conceived the project; JY, YH and Y-NM designed the experiments; Y-NM, NY and GL carried out the majority of the experiments; LD conducted the bioinformatics analysis; Y-FL helped to conduct CRISPR/Cas9 mediated miRNA knockout in ESCs. M-LZ helped to conduct the teratoma formation assay. FW and HD performed the luciferase reporter assays; BW conducted the Western blot analysis; X-JW and L-LW performed the q-PCR analysis; S-WJ prepared MEFs. J-WZ and Y-MW provided valuable reagents and advice; all authors discussed the results; JY, YH and Y-NM wrote the manuscript.

### Conflict of interest

The authors declare that they have no conflict of interest.

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