

Aire Promotes the Self-Renewal of Embryonic Stem Cells Through Lin28

Gu Bin,¹ Zhang Jiarong,¹ Wang Shihao,¹ Song Xiuli,¹ Xu Cheng,¹ Chen Liangbiao,² and Zhang Ming¹

Abstract

Autoimmune regulator (Aire) is one of the most well-characterized molecules in autoimmunity, but its function outside the immune system is largely unknown. The recent discovery of *Aire* expression in stem cells and early embryonic cells and its function in the self-renewal of embryonic stem (ES) cells highlight the importance of Aire in these cells. In this study, we present evidence that Aire promotes the expression of the pluripotent factor Lin28 and the self-renewal of ES cells. We presented the first evidence that the *let-7* microRNA family contributed to the self-renewal promoting effect of Aire on ES cells. Moreover, we showed that *Aire* and *Lin28* are co-expressed in the genital ridge, oocytes, and cleavage-stage embryos, and the expression level of *Lin28* is correlated with the expression level of *Aire*. Although it is widely considered to be a promiscuous gene expression activator, these results indicated that Aire promotes the self-renewal of ES cells through a specific pathway (i.e., the activation of Lin28 and the inhibition of the *let-7* microRNA family). The correlation between *Aire* and *Lin28* expression in germ cells and early embryos indicated an *in vivo* function for Aire in totipotent and pluripotent stem cells. This study presents the first molecular pathway that incorporates Aire into the pluripotency network. Moreover, it presents the first evidence that microRNAs contribute to the regulatory function of Aire and highlights a novel function of Aire in stem cell biology and reproduction. These functions reveal novel perspectives for studying the molecular mechanisms behind the establishment and sustenance of pluripotent identity.

Introduction

SELF-RENEWAL, DEFINED AS THE sustenance of pluripotent differentiation potential while proliferating, is one of the most important properties of embryonic stem (ES) cells and the basis to acquire pluripotent stem cells for regenerative medicine [1]. The molecule mechanisms that promote the self-renewal of pluripotent stem cells have been subjected to intense study. A core of transcriptional circuits consisting of Oct4, Nanog, and Sox2 has been established to play central roles in the self-renewal process [2]. Recent data have extended this network to include much more transcriptional regulators [3]. Recent protein interaction studies have identified numerous interacting proteins with the core pluripotency factors [4]. A complex interacting network consisting of proteins from different functional categories such as transcriptional factors, chromatin structure modifiers, epigenetic modifiers, and RNA processors have been revealed as controlling the self-renewal of pluripotent stem cells [5]. In addition to the recent discovery of micro-RNAs as another important entity which regulates the self-renewal of pluripotent stem cells [6], the molecule network that sustains the

self-renewal of pluripotent stem cells is complex and consists of molecules that regulate cell behavior at different levels. The integration of novel players into this network would greatly promote the understanding of how the pluripotency network sustains the self-renewal.

Autoimmune regulator (Aire) is considered a core regulator in immune tolerance, because it is an omnipotent gene that could activate thousands of genes with different tissue specificities in medullary thymic epithelium cells (mTECs) [7,8]. Mutation of the *Aire* gene compromises immune tolerance and causes an autoimmune syndrome called autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy (APECED) [9,10]. However, we have recently identified the expression of *Aire* in ES cells and showed that the expression of *Aire* decreased with the differentiation of ES cells [11]. Data published by other groups and our unpublished observations have shown that *Aire* is expressed in blastomeres (from the 1-cell stage to blastocyst), induced pluripotent stem (iPS) cells, the embryonic genital ridge, and germ cells of both sexes [12]. Except for its restricted expression in mTECs and stromal cells of lymph nodes [13], *Aire* presented an expression profile that is very similar to the

¹The Institute of Genetics, College of Life Sciences, Zhejiang University, Hangzhou, China.

²The Institute of Genetics and Developmental Biology, Chinese Academic of Sciences, Beijing, China.

core pluripotency regulators such as *Oct4* and *Nanog*. We have also shown that *Aire* knockdown attenuated the self-renewal of mouse ES (mES) cells and the expression of *Oct4* and *Nanog* [11]. These data insinuated that the *Aire* gene is specific to pluripotent stem cells and actively participates in the pluripotency regulation network. Open chromatin state and global gene expression are considered specific characteristics for pluripotency, and general gene expression modulators, such as Wdr5 and TAF3, promote the self-renewal of ES cells [14–21]. Thus, it is worth investigating how an omnipotent expression activator such as *Aire* is integrated into the pluripotency regulation network and how it contributes to the self-renewal of ES cells.

Although the physiological role of *Aire* in autoimmunity is clear, the molecular mechanism of how *Aire* affects cellular behavior is not clear. Several models, including direct transcription regulation [22], chromatin modification [23], and activating genes by regulating developmental cell fate [24], have been proposed. Although there is supporting evidence for each model, a consensus has not been established. Moreover, *Aire* target genes are cell-type specific, with differing target profiles in mTECs, monocytes, and spermatogonias [7,25,26]. Therefore, pluripotent stem cell-specific target molecules and mechanisms could exist for *Aire* to regulate the gene expression and self-renewal of ES cells. Unraveling these targets and mechanisms would not only reveal new aspects of the pluripotency maintenance mechanism but also illuminate the deep implications of a general mechanism of *Aire*-dependent gene regulation.

Lin28 is a RNA binding protein that was originally characterized in a mutant screen of *Caenorhabditis elegans* as a development-regulating gene [27,28]. It has been proposed that Lin28 is an important mediator of the proliferation and self-renewal of ES cells [29,30]. More importantly, Lin28 is 1 of the 4 reprogramming factors initially used to produce human-induced pluripotent stem cells [31]. Unlike other core pluripotent regulators such as *Oct4* and *Nanog*, which perform their functions by regulating transcription in the cell nucleus [2], Lin28 acts at the posttranscriptional level through 2 major mechanisms. First, Lin28 inhibits the maturation of the *let-7* microRNA family by binding and modifying its precursors [32–34]. *Let-7* microRNAs target and inhibit the expression of pluripotency and proliferation promoters such as HMGA2 and oppose the effects of a family of ESC cell-cycle-regulating miRNAs that maintain self-renewal [35,36]. Since these effects promoted the differentiation of ES cells, the inhibition of *let-7* by Lin28 promotes the self-renewal of ES cells. Second, Lin28 promotes the self-renewal of ES cells by directly interacting with the mRNAs of pluripotency and cell-cycle regulators, including *Oct4* and *CDK4*, to promote their ribosome recruitment and translation [30,37,38]. Although Lin28 is an important regulator of pluripotency and one of the few clearly characterized nodes that link the pluripotency network to microRNAs, the upstream regulators of *Lin28* in pluripotent stem cells have not been determined. Delineating the regulators of *Lin28* in pluripotent stem cells would significantly improve the understanding of the pluripotency sustenance network. Since Lin28 has been implicated as an active player in the reprogramming process that drives tumorigenesis [31,39,40], the characterization of regulators of Lin28 may also shed insight into the transforming mechanisms.

In this study, we report that *Aire* promotes the expression of *Lin28* in ES cells. We demonstrate that *Aire* inhibits the expression of the *let-7* microRNA family by activating Lin28, which sustains the undifferentiated state and promotes the proliferation of mES cells. Moreover, we show that *Aire* and *Lin28* are co-expressed in germ cells and preimplantational mouse embryos, and the expression of *Lin28* in genital ridges and oocytes correlates with the expression of *Aire*. To our knowledge, this is the first report of a molecular pathway that links *Aire* to pluripotency and the first link between *Aire* and the microRNA world. These results establish *Aire* as a novel player in the pluripotency network, present a new regulatory mechanism for the core pluripotency and reprogramming factor Lin28, and indicate microRNAs as novel targets of *Aire*.

Materials and Methods

Ethics statement

All animal work was conducted according to relevant national and international guidelines.

Cell lines and cell culture

Mouse embryonic fibroblast (MEF) feeder cells were isolated from 13.5 dpc ICR mice embryos. MEFs were routinely cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in 5% CO₂. MEFs were inactivated by Mitomycin C and plated at a density of 4 × 10⁴ cells/cm² for ES culture.

The mES D3 cell line was purchased from ATCC and cultured on mitomycin-inactivated MEFs in DMEM supplemented with 15% fetal bovine serum (Invitrogen) and 1000 ng/mL LIF (Millipore) at 37°C in 5% CO₂. The pluripotency of the mES cells was routinely analyzed with an ALP staining Kit (Sigma-Aldrich) for SSEA-1 staining and teratoma formation. In addition, the karyotype was routinely checked.

Embryonic bodies were prepared by suspension culture in DMEM supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in 5% CO₂ and recovered after 6 days' differentiation for colony formation assay and real-time polymerase chain reaction (PCR) assay.

Animals and housing

The heterozygous *Aire*-knockout mice strain (C57BL6/J background; Jackson 004743) was purchased from Jackson Laboratories and bred in house [7]. Homozygous *Aire*-knockout mice were generated by breeding heterozygous pairs.

Antibodies

The following antibodies were used in this study: Rat anti-mouse *Aire* monoclonal antibody (eBioscience), rabbit anti-Lin28A monoclonal antibody (Cell Signaling), rabbit anti-mouse *Aire* polyclonal antibody (Huabio), rabbit anti-GAPDH polyclonal antibody (Huabio), rabbit anti-*Oct4* polyclonal antibody (Huabio), Alexa 488-conjugated goat anti-rat IgG (Invitrogen), and Alexa 555-conjugated goat anti-rabbit IgG (Invitrogen).

Plasmid construction and lentivirus production

The full-length coding regions of mouse *Aire* and *Lin28* genes were isolated from mES cDNAs by PCR amplification and cloned into a pCS-UBC lentivirus vector plasmid, which was constructed in house, derived from the pCS plasmid (Addgene plasmid 12158) by enzymatic digestion [41]. Cloned gene expression was under the control of the human ubiquitin promoter (UBC). ShRNA coding DNA sequences were obtained from the RNAi Consortium (www.broadinstitute.org/rnai/trc) and synthesized by Sangon (Shanghai) (Table 1). A pLKO TRC-cloning vector (plasmid 10878) was obtained from Addgene [42].

For lentivirus packaging, plasmids were co-transfected with PsPAX (Addgene plasmid 12260), pMD2. G (Addgene plasmid 12259) packaging plasmids into HEK293T cells. Lentivirus was collected in an mES culture medium and used to infect mES cells after the supplementation of 4 μ g/mL Polybrene (Sigma).

Genital ridge isolation

Aire^{+/+} C57BL6/J female mice were superovulated by consecutive injections, which were spaced 44–48 h apart, containing 5 IU PMSG and 5 IU hCG and crossed with *Aire*^{+/+} C57BL6/J males. Virginal plugs were checked to confirm successful mating. Genital ridges were isolated from 12.5 dpc embryos under a dissecting microscope, and the genders of genital ridges were identified with morphology criteria.

Oocyte and early embryo isolation and immunofluorescent staining

For oocyte isolation, *Aire*^{+/+} and *Aire*^{-/-} female C57BL6/J mice were stimulated by an intraperitoneal injection of 7.5 IU of PMSG. After 46–48 h, germinal vesicle (GV) oocytes were isolated by puncturing follicles in M2 medium (Sigma). For preimplantational embryo isolation, *Aire*^{+/+} C57BL6/J female mice were superovulated by consecutive injections of 5 IU PMSG and 5 IU hCG (44–48 h between injections) and crossed with *Aire*^{+/+} C57BL6/J males. Twenty-four hours and 48 h after an injection of 5 IU hCG, successfully pregnant females were used for collecting zygotes (day 0.5) and 2-cell embryos (day 1.5), respectively.

GV oocytes, zygotes, and 2-cell embryos were fixed and permeabilized with Fix/Perm buffer from the Foxp3 staining buffer set (eBioscience) for 1 h at 37°C. The oocytes and embryos were blocked for 45 min at 37°C in a blocking buffer (Perm buffer; eBioscience) supplemented with 5% normal goat serum. After blocking, the oocytes and embryos were consecutively stained with the rat anti-mouse *Aire* monoclonal antibody (eBioscience), Alexa 488-conjugated goat

anti-rat IgG (Invitrogen), rabbit anti-Lin28A monoclonal antibody (Cell Signaling), and Alexa 555-conjugated goat anti-rabbit IgG (Invitrogen). The oocytes and embryos were then mounted in VECTASHIELD HardSet Mounting Medium with 4',6-diamidino-2-phenylindole. The samples were examined under an LSM 710 Confocal Microscope (Zeiss).

Colony formation assay

For the colony formation assay of ES cells, mES cells were seeded on 24-well cell culture plates on feeder cells at a density of 600 cells per well and grown for 4 days. Alkaline phosphatase (ALP) staining was performed with an ALP assay kit (Sigma). Macroscopic pictures were taken with a digital camera (Panasonic DMC-ZS1). The number of ALP-positive clones was counted under a phase-contrast microscope. The results are displayed as statistical averages of 3 independent experiments.

For colony formation assay of embryonic body (EB) cells differentiated for 6 days, embryonic bodies were collected and dissociated to single cells by trypsinization. The cells were then seeded on 24-well cell culture plates on feeder cells at a density of 3000 cells per well and grown for 8 days. ALP staining was performed with an ALP assay kit (Sigma). The number of ALP-positive clones was counted under a phase-contrast microscope. The results are displayed as statistical averages of 3 independent experiments.

Real-time reverse transcriptase-PCR

Total RNA was isolated with Trizol reagent, and cDNAs were synthesized with the ReverTra Ace[®] qPCR RT Kit (Toyobo). Tests were performed with SYBR Green reagents purchased from Toyobo and primers acquired from the Primer Bank (<http://pga.mgh.harvard.edu/primerbank/>), which are listed in Table 2.

Small RNAs were isolated with the mirVana[™] miRNA Isolation Kit (Ambion), according to the manufacturer's instructions. The small RNAs were tailed with *Escherichia coli* polyA polymerase (Ambion) and reverse transcribed using the ReverTra Ace[®] qPCR RT Kit (Toyobo) with a tagged polyA primer (5'-ATTCTAGAGCCGAGGCGCCGACA TGT(24)VN-3'). MicroRNA qPCR was performed using SYBR Green reagents (Toyobo) with specific forward primers acquired from the Invitrogen database (Table 3) and a general reverse primer (5'-ATTCTAGAGCCGAGGCGGC-3').

All real-time PCR analyses were performed on a RealPlex PCR apparatus (Eppendorf).

Western blotting

Western blotting was performed as previously described [43]. ES cells were harvested in lysis buffer consisting of

TABLE 1. SHRNA SEQUENCES USED IN THIS STUDY

Name	Sequence
scramble	CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG
shAire-1	CCGGCCAGTGGCAATTTGAAGAACAACACTCGAGTGTCTTCAAATTGCCACTGGTTTTTG
shAire-2	CCGGCCGAGACACCACCTTCTCTCTCGAGAAGAGAAGGGTGGTGTCTCGGTTTTTG
shLin28-1	CCGGCCCAGTAAGAATGCAACTTAACTCGAGTTAAGTTGCATTCTTACTGGGTTTTTG
shLin28-2	CCGGCACCTTAAAGAAGTCTGCCAACTCGAGTTGGCAGACTTCTTAAAGGTGTTTTTG

shRNA sequences

TABLE 2. PRIMERS FOR PROTEIN CODING GENES USED IN THIS STUDY

Name	Sense sequence	Anti-sense sequence
<i>Aire</i> FL	GGGGTACCATGGCAGGTGGGGATGGAATG	GCTCTAGATCAGGAAGAGAAGGGTGGTGTG
<i>Lin28</i> FL	GCGAATTCATGGGCTCGGTGTCCAACCAG	GGATCGATTCAATTCCTGGGCTTCTGGGAGCA
<i>Aire</i> qPCR	AGGTCAGCTTCAGAGAAAACCA	TCATTCCCAGCACTCAGTAGA
<i>Oct4</i> qPCR	CAGCCAGACCACCATCTGTC	GTCTCCGATTGTCATATCTCCTG
<i>Nanog</i> qPCR	TTGCTTACAAGGGTCTGCTACT	ACTGGTAGAAGAATCAGGGCT
<i>Lin28</i> qPCR	GGCATCTGTAAGTGGTTCAACG	CCCTCCTTGAGGCTTCGGA
<i>Gata6</i> qPCR	CATCACCATCACCCGACCTAC	GGCCCTGTAAGCTGTGGAG
<i>Brachyury</i> qPCR	GGATTACATCGTGAGAGTTGG	GTCACAGCTATGAACTGGGTC
<i>Gata4</i> qPCR	CAAACCTCAATGTGTCTCTTTGC	AGAGTAAAGCCTATCTCGCTGT
<i>Sox7</i> qPCR	TGGACACGTATCCCTACGGG	TCCTGACATGAGGACAGAGAAG
<i>Nestin</i> qPCR	CCCCTTGCCATAATACCCTTGA	GCCTCAGACATAGGTGGGATG
<i>Fgf5</i> qPCR	GAAGCGTCTCACTCCCGAAG	GAAGAAAACGTGCGCTACT

50 mM Tris-HCl pH 7.4, 1% NP-40, and 1% sodium dodecyl sulfate (SDS). Equal amounts of the protein lysate were separated by electrophoresis on a 12.5% Laemmli SDS-polyacrylamide gel and then transferred onto polyvinylidene fluoride membranes. The membranes were developed with an ECL kit purchased (GE Healthcare Life Sciences, Piscataway, NJ) after incubation with primary and secondary antibodies.

Immunofluorescence

Immunofluorescence was performed as previously described [43]. The subjects were observed, and images were acquired under an LSM 710 Confocal Microscope (Zeiss).

Quantifications and statistics

The fluorescence strength of the immunofluorescent stainings was measured by Magnification software (Orbicle). Intergroup differences were assessed by student's *t*-test using the StatView 5.0 program against control groups (SAS Institute Inc.); **P* < 0.05, ***P* < 0.01. The column charts were drawn by Origin 8.0 software (OriginLab).

Results

Aire promoted the self-renewal of mES cells

We explored the function of *Aire* in ES cells by gene overexpression. As shown in Fig. 1A, the infection of mES cells with lentiviral vector encoding the mouse *Aire* gene significantly enhanced the expression of *Aire* mRNA. Wes-

tern blotting demonstrated that the *Aire* protein level significantly increased on gene overexpression in mES cells (Fig. 1B). ES cell self-renewal was examined with different assays. First, we examined the ability of mES cells to form ALP-positive colonies after *Aire* overexpression. As shown in Fig. 1C, *Aire* overexpression significantly enhanced the ability of mES cells to form ALP-positive colonies. Next, we examined the expression of 2 core pluripotent genes, *Oct4* and *Nanog*, in the context of *Aire* overexpression. Real-time reverse transcriptase PCR (RT-PCR) analysis revealed that *Aire* overexpression significantly enhanced the mRNA expression of the pluripotent marker *Nanog*, while the *Oct4* expression was not significantly altered (Fig. 1D). We further examined the *Oct4* protein levels in mES cells on *Aire* overexpression with western blotting. As shown in Fig. 1B, the level of *Oct4* protein in the mES cells significantly increased after *Aire* overexpression. Therefore, *Aire* overexpression enhanced the colony genicity of mES cells and promoted the transcriptional expression of some pluripotent genes (e.g., *Nanog*), while posttranscriptionally regulated other pluripotent genes (e.g., *Oct4*). We also examined the differentiation capacity of mES cells on *Aire* overexpression by examining the expression of several germ layer marker genes in embryonic bodies. Embryonic bodies were derived from vector-infected and *Aire* lentivirus-infected cells. As shown in Fig. 1E and consistent with our previous report, *Aire* expression was eliminated 6 days after differentiation [11], while in *Aire*-overexpressing cells, the expression level of *Aire* 6 days after differentiation is similar to that of undifferentiated cells. On *Aire* overexpression, the day 6 EBs possessed significantly lower expression of the trophoblast marker *Gata6* [44],

TABLE 3. PRIMERS FOR MICRORNAs

Name	Sense sequence	Anti-sense sequence
<i>mmu-let-7a</i>	GCGGTGAGGTAGTAGGTTGTATAGTT	ATTCTAGAGCCGAGGCGGC
<i>mmu-let-7b</i>	CCTGAGGTAGTAGGTTGTGTGGTT	
<i>mmu-let-7c</i>	CGCTGAGGTAGTAGGTTGTATGGTT	
<i>mmu-let-7d</i>	GCAGAGGTAGTAGGTTGCATAGTT	
<i>mmu-let-7e</i>	GGCTGAGGTAGGAGGTTGTATAGTT	
<i>mmu-let-7f</i>	GCCCTGAGGTAGTAGATTGTATAGTT	
<i>mmu-let-7g</i>	GGCCTGAGGTAGTAGTTGTACAGTT	
<i>mmu-let-7i</i>	GCTGAGGTAGTAGTTTGTGCTGTT	
<i>mmu-sno-U6</i>	TGCTCGCTTCGGCAGCACATATAC	

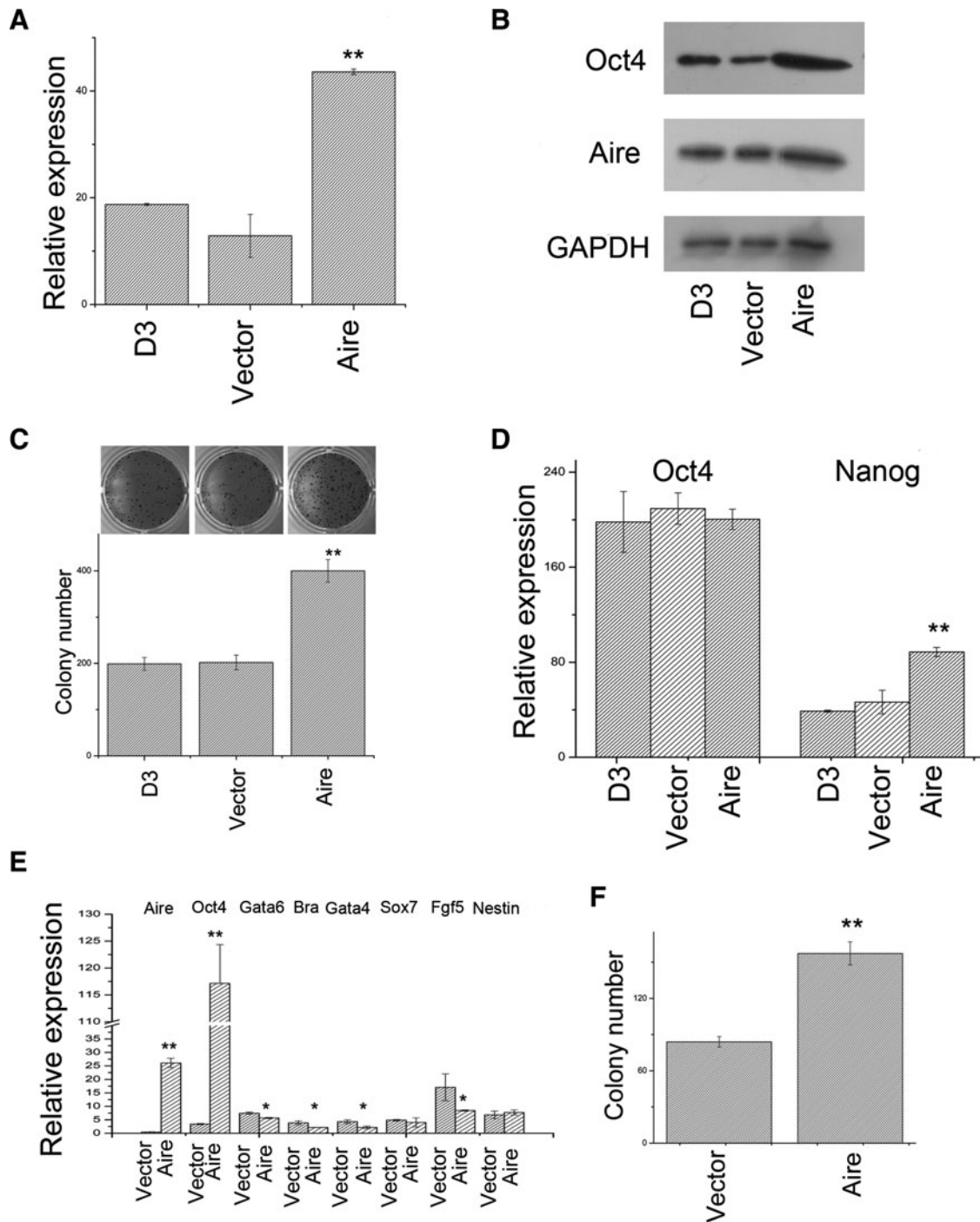


FIG. 1. Aire promoted the self-renewal of embryonic stem (ES) cells: **(A)** Real-time reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of *Aire* expression level in normal mouse ES (mES) cells (D3), vector-infected mES cells (Vector), and *Aire* encoding lentivirus-infected mES cells (Aire). The expression level is shown as relative to β -actin expression ($n=3$, $*P<0.05$, $**P<0.01$). **(B)** Western blotting analysis of GAPDH, Aire, and Oct4 protein expression in normal mES cells (D3), vector-infected mES cells (Vector), and *Aire* encoding lentivirus-infected mES cells (Aire). **(C)** Colony genicity of normal mES cells (D3), vector-infected mES cells (Vector), and *Aire* encoding lentivirus-infected mES cells (Aire). The upper panel shows macroscopic pictures of one representative well of each group. The lower panel shows statistics ($n=3$, $*P<0.05$, $**P<0.01$). **(D)** Real-time RT-PCR analysis of *Oct4* and *Nanog* expression levels in normal mES cells (D3), vector-transfected mES cells (Vector), and *Aire* gene-transfected mES cells (Aire). The target gene expression level is shown as relative to β -actin expression ($n=3$, $*P<0.05$, $**P<0.01$). **(E)** Real-time RT-PCR analysis of the expression levels of *Aire*, *Oct4*, and germ layer marker genes in day 6 embryonic bodies (EBs) derived from vector-infected mES cells (Vector), and *Aire* encoding lentivirus-infected mES cells (Aire). The expression levels are shown as relative to β -actin expression ($n=3$, $*P<0.05$, $**P<0.01$). **(F)** Colony genicity of day 6 EB cells derived from vector-infected mES cells (Vector), and *Aire* encoding lentivirus-infected mES cells (Aire) ($n=3$, $*P<0.05$, $**P<0.01$).

mesoderm marker *Brachyury* [45], and endoderm marker *Gata4* [46], while the expression level of ectoderm marker *Fgf5* differed with marginal significance [47] ($P=0.041$). The expression levels of ectoderm marker *Nestin* and primitive endoderm marker *Sox7* were not affected [48,49]. Since there is still a certain level of expression of these germ layer markers in EBs derived from *Aire*-overexpressing ES cells, these cells still possessed the ability to differentiate into the 3 germ layers and extraembryonic cells. We checked whether the pluripotent gene *Oct4* was properly shut down during EB differentiation after *Aire* overexpression. As shown in Fig. 1E, the expression of *Oct4* gene was significantly decreased after differentiation in vector-infected cells, but it is sustained at a high level in *Aire*-overexpressing cells. To further confirm that *Aire* promoted the self-renewal of ES cells in differentiation promoting conditions, we examined the ability of the cells from day 6 EBs to form ALP-positive colonies. As shown in Fig. 1F, the overexpression of *Aire* significantly enhanced the colony formation ability of d6 EB cells. These results indicated that *Aire* promoted the self-renewal proliferation of mES cells in conditions that encouraged self-renewal. Moreover, *Aire* quantitatively inhibited differentiation and promoted self-renewal in conditions that encouraged differentiation.

Aire promoted the expression of *Lin28* in mES cells

While dissecting the molecular mechanism by which *Aire* promoted the self-renewal of ES cells, we considered that *Aire*-induced posttranscriptional expression of *Oct4* was a clue and searched for molecules that posttranscriptionally regulate the expression of *Oct4*. Since *Lin28* has been proposed to promote the translation of *Oct4* by triggering its ribosome recruitment [37], we examined the expression of *Lin28* after *Aire* overexpression. As shown in Fig. 2A, the expression of *Lin28* was significantly enhanced after *Aire* overexpression. Western blotting analysis revealed that *Lin28* protein expression in the mES cell population increased significantly after *Aire* overexpression (Fig. 2B). Moreover, immunofluorescent analysis revealed that *Aire* overexpression significantly elevated the expression of *Lin28* in situ. Since we have already demonstrated that *Aire* knockdown attenuated the self-renewal ability of ES cells [11], we sought to determine whether it also attenuated the expression of *Lin28*. As shown in Fig. 2D, the expression of *Lin28* mRNA was significantly attenuated in ES cells by *Aire*

knockdown. The expression of *Lin28* protein was also attenuated by *Aire* knockdown, as shown in Fig. 2E. Immunofluorescence analysis determined that the *Lin28* expression was significantly decreased on *Aire* knockdown. These results indicated that *Aire* promotes the expression of *Lin28*, and since *Lin28* is vital for the self-renewal and reprogramming process [29–31], we speculated that the activation of *Lin28* expression contributed significantly to the self-renewal promoting effect of *Aire*.

Aire promoted the self-renewal of mES cells by activating *Lin28*

With the aim of validating that *Lin28* activation contributed to the effect of *Aire* on mES cell self-renewal, we performed genetic rescue experiments. First, we knocked down *Lin28* expression in *Aire*-overexpressing mES cells. As shown in Fig. 3A and B, *Lin28* mRNA and protein levels were recovered by 2 shRNAs targeting *Lin28* to the level of vector-transfected mES cells. A colony genicity assay was performed to examine self-renewal ability. As shown in Fig. 3C, the colony genicity of the *Aire*-overexpressing cells was significantly attenuated after *Lin28* knockdown. A further confirmation was made by overexpressing *Lin28* in *Aire*-knockdown mES cells. As shown in Fig. 3D and E, after infection with lentiviral vectors encoding mouse *Lin28*, the *Lin28* expression in *Aire*-knockdown mES cells was similar to or a little higher than in the scrambled shRNA transgenic mES cells. The colony genicity was assayed to examine self-renewal ability. As shown in Fig. 3F, while the colony genicity of *Aire* knockdown mES cells was significantly lower than in scrambled shRNA transgenic cells, *Lin28* overexpression in these mES cells rescued colony genicity from *Aire* knockdown. These results indicated that the activation of *Lin28* expression significantly contributed to the self-renewal promoting effect of *Aire* in mES cells.

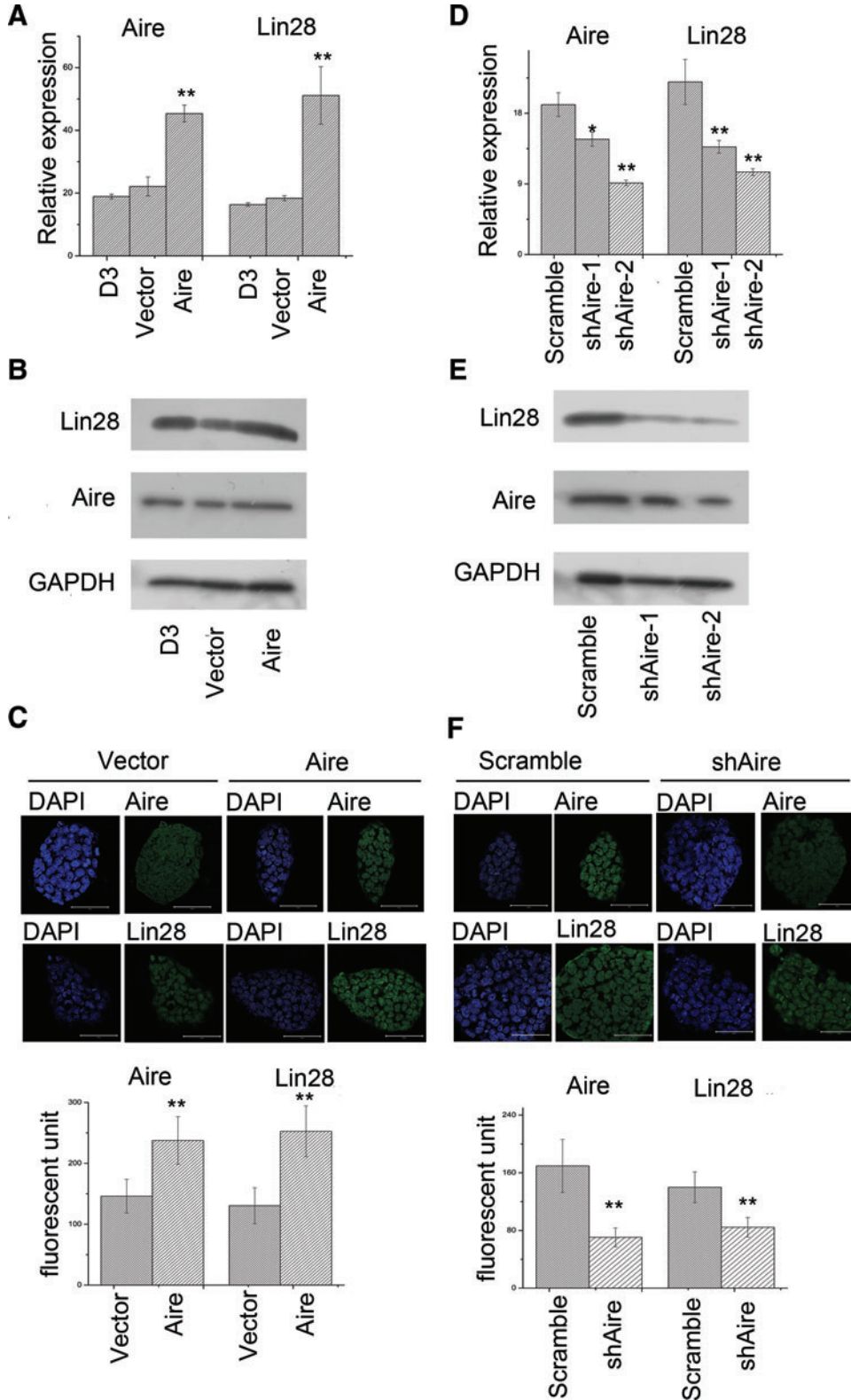
Aire regulated *let-7* micro RNAs

Previous reports demonstrated that *Lin28* inhibits the maturation of the differentiation-promoting *let-7* microRNA family and promotes ES cell self-renewal [29,32]. The inhibition of the *let-7* microRNA family by *Lin28* has also been considered vital for its function as a reprogramming factor and oncogene [31,39,40]. Since we have shown that *Aire* promoted *Lin28* expression in mES cells, we investigated

FIG. 2. *Aire* promoted the expression of *Lin28* in ES cells. **(A)** Real-time RT-PCR analysis of the *Aire* and *Lin28* expression in normal mES cells (D3), vector-infected mES cells (Vector), and *Aire* encoding lentivirus-infected mES cells (*Aire*). The expression level was shown as relative to β -actin expression ($n=3$, $*P<0.05$, $**P<0.01$). **(B)** Western blotting analysis of the expression of GAPDH, *Aire*, and *Lin28* protein in normal mES cells (D3), vector-infected mES cells (Vector), and *Aire* encoding lentivirus-infected mES cells (*Aire*). **(C)** Immunofluorescence analysis of the expression of *Aire* and *Lin28* in vector-infected mES cells (Vector), and *Aire* encoding lentivirus-infected mES cells (*Aire*). The upper panel displays figures of representative colonies (bar=50 μ m). The lower panel shows statistical analysis according to the same random fluorescent unit ($n=30$, $*P<0.05$, $**P<0.01$). **(D)** Real-time RT-PCR analysis of *Aire* and *Lin28* expression in scrambled shRNA-infected mES cells (scramble) and mES cells infected with lentivirus encoding 2 *Aire* shRNAs (sh*Aire*-1 and sh*Aire*-2). The expression level was shown as relative to β -actin expression ($n=3$, $*P<0.05$, $**P<0.01$). **(E)** Western blotting analysis of GAPDH, *Aire*, and *Lin28* protein expression in scrambled shRNA-infected mES cells (scramble) and mES cells infected with lentivirus encoding 2 *Aire* shRNAs (sh*Aire*-1 and sh*Aire*-2). **(F)** Immunofluorescence analysis of *Aire* and *Lin28* expression in scrambled shRNA-infected mES cells (scramble) and mES cells infected with lentivirus encoding sh*Aire*-2 shRNA (sh*Aire*). The upper panel shows figures of representative colonies (bar=50 μ m). The lower panel shows the statistical analysis according to the same random fluorescent unit ($n=30$, $*P<0.05$, $**P<0.01$). Color images available online at www.liebertpub.com/scd

whether Aire regulated the levels of mature *let-7* microRNAs. First, we examined the expression of *let-7* microRNAs in mES cells after *Aire* overexpression. As displayed in Fig. 4A, the expression of all 8 *let-7* family microRNAs was significantly lower in Aire-overexpressing mES cells compared with vector-infected cells. Subsequently, we examined the

level of *let-7* microRNAs in mES cells after *Aire* knockdown. As shown in Fig. 4B, the *Aire* knockdown in mES cells resulted in a significant increase of the *let-7* microRNA family except for the *let-7d* and *let-7i*, which also showed an increasing trend. To further confirm that Aire regulated the *let-7* microRNA family indirectly through Lin28, genetic rescue



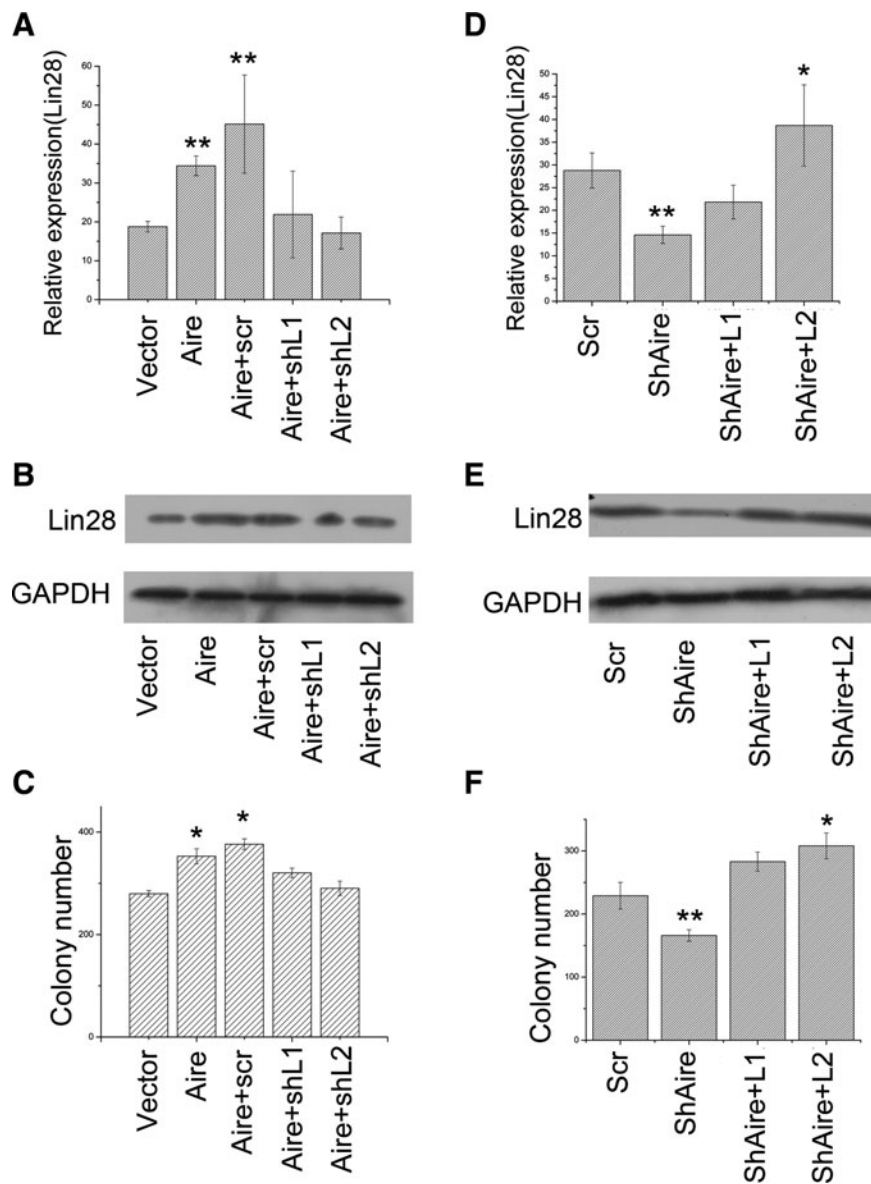


FIG. 3. Aire promoted the self-renewal of ES cells through Lin28: **(A)** Real-time RT-PCR analysis of the expression level of *Lin28* in vector-infected mES cells (Vector), *Aire* encoding lentivirus-infected mES cells (Aire), mES cells co-infected with *Aire*-encoding lentivirus and scrambled shRNA-encoding lentivirus (Aire+scr), and mES cells co-infected with *Aire* encoding lentivirus and 2 *Lin28* shRNAs encoding lentivirus (Aire+shL1 and Aire+shL2) cells. The expression levels are displayed as relative to β -actin expression ($n=3$, $*P<0.05$, $**P<0.01$). **(B)** Western blotting analysis of GAPDH and *Lin28* protein expression in vector-infected mES cells (Vector), *Aire* encoding lentivirus-infected mES cells (Aire), mES cells co-infected with *Aire* encoding lentivirus and scrambled shRNA encoding lentivirus (Aire+scr), or mES cells co-infected with *Aire* encoding lentivirus and one of 2 *Lin28* shRNA encoding lentiviruses mES cells (Aire+shL1 and Aire+shL2). **(C)** Colony genicity of vector-infected mES cells (Vector), *Aire* encoding lentivirus-infected mES cells (Aire), mES cells co-infected with *Aire* encoding lentivirus and scrambled shRNA encoding lentivirus (Aire+scr), mES cells co-infected with *Aire* encoding lentivirus, and 2 *Lin28* targeting shRNA encoding lentivirus (Aire+shL1 and Aire+shL2) cells ($n=3$, $*P<0.05$, $**P<0.01$). **(D)** Real-time RT-PCR analysis of the expression level of *Lin28* in scrambled shRNA encoding lentivirus-infected ES cells (scramble), shAire-2 encoding lentivirus-infected ES cells (shAire), ES cells co-infected with 2 lines of shAire-2 encoding lentivirus, and *Lin28* encoding lentivirus co-infected (shAire+L1 and shAire+L2). The expression level is shown relative to β -actin expression ($n=3$, $*P<0.05$, $**P<0.01$). **(E)** Western blotting analysis of the GAPDH and *Lin28* protein expression in scrambled shRNA encoding lentivirus-infected ES cells (scramble), shAire-2 encoding lentivirus-infected ES cells (shAire), and ES cells co-infected with 2 lines of shAire-2 encoding lentivirus along with *Lin28* encoding lentivirus (shAire+L1 and shAire+L2). **(F)** Colony genicity of scrambled shRNA encoding lentivirus-infected ES cells (scramble), shAire-2 encoding lentivirus-infected ES cells (shAire), and 2 lines of shAire-2 encoding lentivirus and *Lin28* encoding lentivirus co-infected (shAire+L1 and shAire+L2) ES cells ($n=3$, $*P<0.05$, $**P<0.01$).

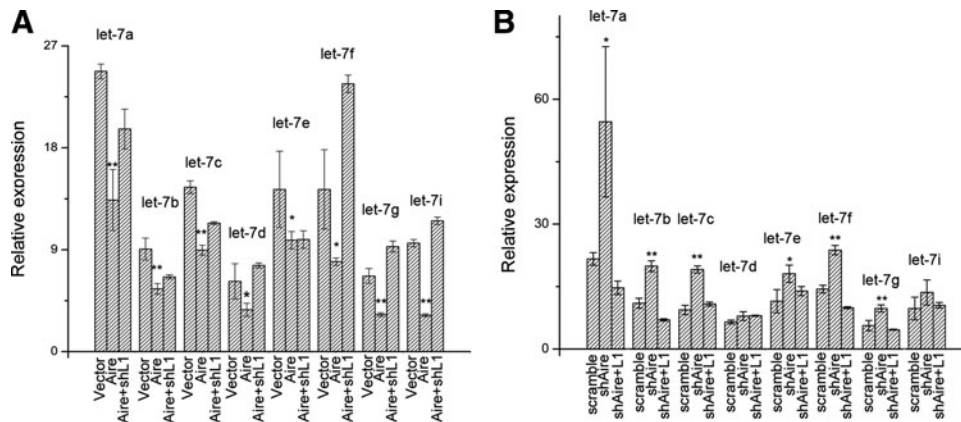


FIG. 4. Aire regulated the level of *let-7* group microRNA in ES cells. **(A)** Real-time RT-PCR analysis of *let-7* family microRNA expression in vector-infected mES cells (Vector), *Aire* encoding lentivirus-infected mES cells (Aire), and mES cells co-infected with *Aire* encoding lentivirus and a *Lin28* targeting shRNA encoding lentivirus (Aire + shL1). Target gene expression level is shown relative to sno-U6 small RNA ($n=3$, $*P<0.05$, $**P<0.01$) **(B)** Real-time RT-PCR analysis of *let-7* family microRNA expression in scrambled shRNA encoding lentivirus-infected ES cells (scramble), shAire-2 encoding lentivirus-infected ES cells (shAire), and mES cells co-infected with shAire-2 encoding lentivirus and a *Lin28* encoding lentivirus (shAire + L1). Target gene expression level is shown relative to sno-U6 small RNA ($n=3$, $*P<0.05$, $**P<0.01$).

experiments were performed. As shown in Fig. 4A, when *Lin28* was knocked down in *Aire* overexpressing mES cells, the level of most *let-7* microRNAs increased and approached the level in vector-infected cells. As depicted in Fig. 4B, when *Lin28* was overexpressed in *Aire* knockdown mES cells, the level of most *let-7* microRNAs decreased and approached the level in scramble shRNA-infected cells. These results indicated that *Aire* inhibited the maturation of the *let-7* microRNA family through *Lin28*, and the inhibition of the *let-7* microRNA family contributed to the self-renewal promoting effect of *Aire*.

In vivo relationships between Aire and Lin28

The embryonic genital ridge is one of the most important sources of pluripotent embryonic germ cells [50,51]. Since *Aire* is expressed in germ cells and *Lin28* possesses the ability to sustain a pluripotent state [26,29], we examined whether *Aire* regulates *Lin28* in germ cells. *Aire* and *Lin28* expression was examined with real-time RT-PCR in genital ridges isolated from 12.5 dpc embryos of both sexes. As shown in Fig. 5A, *Aire* and *Lin28* were expressed in the genital ridges of both sexes at a much higher level than in ES cells. Moreover, *Aire* expression in the female genital ridges was significantly higher than in the male ones. Concomitantly, *Lin28* expression levels in female genital ridges were significantly higher than in the male ones. This positive correlation between *Aire* and *Lin28* in genital ridges indicated a positive effect of *Aire* on *Lin28* in embryonic germ cells. Subsequently, we compared the *Aire* and *Lin28* expression by immunofluorescent staining in oocytes in wild-type (Wt) and *Aire*-knockout (Mut) mice. As shown in Fig. 5B, *Aire* was present in oocytes from wild-type mice but was absent in oocytes from *Aire*-knockout mice. This finding implied that *Aire* was maternally stored in the oocytes of normal mice, while maternal proteins in *Aire*-knockout mice were depleted. A direct observation revealed a weaker *Lin28* staining in oocytes from *Aire*-knockout mice relative to wild-type mice, and statistical analysis revealed a significantly lower fluorescent strength of

Lin28 staining in *Aire*-knockout oocytes than in wild-type mice. However, *Lin28* was still present in *Aire*-knockout oocytes, which suggests that *Aire* was not exclusively required for the expression of *Lin28* but quantitatively regulated the expression of *Lin28* in oocytes. Since *Aire* and *Lin28* were stored in oocytes as maternal proteins, we examined the presence of *Aire* and *Lin28* in totipotent mouse blastomeres before the major maternal embryo transition. As shown in Fig. 5C, *Aire* and *Lin28* were observed in the cytoplasm of fertilized mouse eggs and 2-cell embryos. Interestingly, *Aire* and *Lin28* demonstrated foci-shaped colocalization in the early embryos, which suggested functional interactions. These results indicated that *Aire* and *Lin28* are co-expressed in germ cells, totipotent cells, and pluripotent stem cells. Moreover, *Aire* quantitatively regulated the expression of *Lin28*.

Discussion

A novel player in the pluripotency network

The molecular network that defines and sustains pluripotency has been intensely studied. There had been a widely accepted network centered on a self-regulated circuit, comprising Oct4, Nanog, and Sox2, which served as a major sustaining force of the pluripotent state of stem cells [2]. However, recent studies have revealed many novel sides of the pluripotency network. Molecular entities such as histone modifiers [20,52], chromatin structure remodelers [53,54], general transcription factors [21], and microRNAs [6] have been shown to critically regulate pluripotency maintenance and reprogramming. These results indicated that the pluripotency network is a multifaceted network that comprises regulators functioning at various levels of regulation. The discovery of novel players in this network would greatly improve the understanding of pluripotency. Since open chromatin structure and promiscuous transcription have been proposed to be a basic property of the pluripotent state [14], it is expected that the regulator of these properties

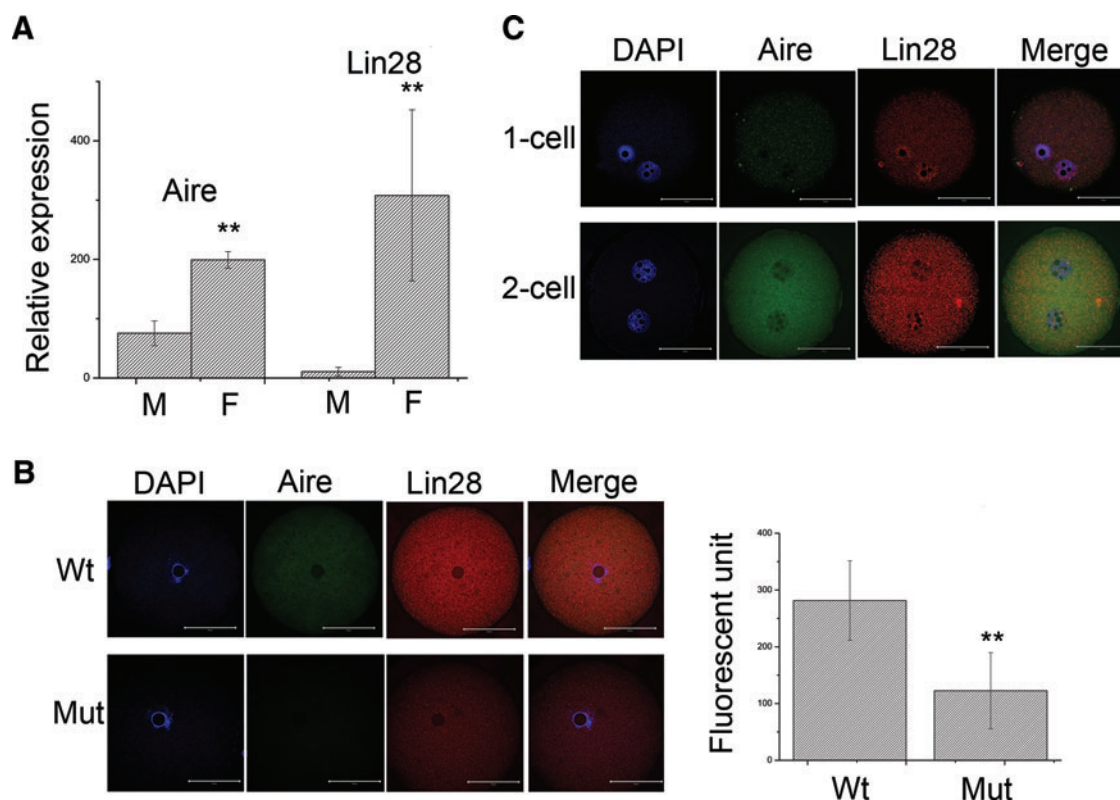


FIG. 5. Aire and Lin28 in germ cells and early embryos. **(A)** Real-time RT-PCR analysis of *Aire* and *Lin28* expression in the 12.5 dpc male genital ridge (M) and the 12.5 dpc female genital ridge (F). Target gene expression level is shown relative to β -actin expression ($n=3$, $*P<0.05$, $**P<0.01$). **(B)** Immunofluorescence analysis of Aire and Lin28 expression in germinal vesicle (GV) stage oocytes from wild-type (Wt) and *Aire*-knockout (Mut) mice. The left panel shows staining of a representative oocyte. The right panel shows statistical analysis according to the same random fluorescent unit ($n=9$, $*P<0.05$, $**P<0.01$). **(C)** Immunofluorescence analysis of Aire and Lin28 expression in 1-cell-stage and 2-cell-stage mouse embryos. Embryos from both stages expressed Aire and Lin28. Color images available online at www.liebertpub.com/scd

should function in the pluripotency network. In this study, we have demonstrated that the promiscuous transcription regulator Aire is a novel player in the pluripotency network. Along with our previous observation that Aire promoted the expression of tissue-specific genes in ES cells [11], a link between the pluripotency networks, open chromatin, and promiscuous transcription is now established. We also highlight the functional significance of the first phase of the biphasic *Aire* expression pattern in development: pre-implantational totipotent cells and pluripotent stem cells [12]. Moreover, we have unexpectedly observed that despite being a promiscuous transcription regulator, Aire is integrated into the pluripotency network through a specific transmitter, Lin28. This finding suggests that promoting promiscuous transcription and pluripotency maintenance are 2 parallel functions of the *Aire* gene. Our data indicated that the promiscuous transcription regulator Aire specifically promoted the expression of *Lin28* at the transcriptional level. Although many different mechanisms including direct transcription initiation activation through CBP interaction [22], transcription elongation sustenance through p-TEFb interaction [55], and epigenetic activation have been proposed to be responsible for the transcription activating effect of Aire [23], but most of them seemed not to be specific to ES cells. It is possible that this specific effect is related to the specific transcriptional network of ES cells, but more confirmation is

needed. The parallel function of Aire in ES cells for promiscuous transcription promotion and pluripotency sustenance also begs several questions. Why has evolution integrated these 2 seemingly independent functions into a single gene? How are these 2 functions differentially conducted and regulated? Do these 2 functions still co-exist in adult cells, similar to mTECs, and, consequently, support the developmental model of Aire function in the thymus? Since Lin28 is a major mediator of reprogramming of differentiated cells into induced pluripotent stem cells [31], the results suggested the potential of Aire as a new component of the cocktail used by regenerative medicine researchers to reprogram adult cells. Since Aire is in the center of a multi-molecular network consisting of chromatin remodelers, transcription regulators, and RNA processors [56], another important contribution of this work to the understanding of pluripotency network is that the integration of Aire into the pluripotency network would significantly expand the network and emphasize more new players.

Aire interacts with microRNAs

Protein coding genes and microRNAs are 2 major clades of functional molecules in biological systems. MicroRNAs regulate a wide range of biological processes, including but not excluded to cell proliferation [57], stem cell self-renewal

[6], embryonic development [58], and immune response [59]. Specifically, it had been proposed that pluripotent stem cells possess characteristic microRNA expression profiles, and microRNAs (e.g., mir-294 and mir-295) promote the self-renewal of pluripotent stem cells and cell reprogramming [6, 60]. Although long held as a promiscuous activator of the expression of protein coding genes, there is no evidence of a functional relationship between Aire and microRNAs. In this work, we presented the first evidence that Aire could regulate the level of the *let-7* microRNA family through the regulation of the microRNA binding protein Lin28. As we have shown, the regulation of *let-7* miRNAs by Aire is functionally transmitted to the pluripotency maintenance effect on ES cells. These results added a new level to the complexity of the regulation mechanism of Aire on its targets and could help explain some observations in the field. For example, Gillard *et al.* have shown that the pluripotency marker genes *Oct4* and *Sox2* were expressed in normal mouse mTECs, while expression was abrogated in *Aire*-knockout mTECs [24]. We have also observed that *Aire* knockdown in mES cells resulted in attenuated expression of *Oct4* and *Nanog* [11,24]. Since *Oct4*, *Sox2*, and *Nanog* were not among the canonical Aire-dependent tissue-specific genes, our discovery might indicate that the regulation of miRNAs, similar to the *let-7* family, potentially contributes to these observations. Whether Aire also regulated miRNAs other than the *let-7* family and in cell types other than ES cells remains to be determined. miRNAs have been linked to autoimmune diseases, and circulating miRNAs function in immunological processes [59,61]. Thus, establishing the relationship between Aire and miRNAs provides a new perspective for understanding the function and regulatory mechanisms of Aire. Further investigation would potentially reveal the major regulatory nature of Aire on immunotolerance and other processes.

Implications into the extra-thymus function of Aire

Except for the mTECs, germ cells and early blastomeres were the other major expression entities of *Aire* [12]. Although Aire had been linked to the early wave of apoptosis during male germ cells development [26], no other clear functional relationship had been established between Aire and these cell types. Interestingly, except for blastocyst inner cell mass, germ cells and early blastomeres are important cellular sources for pluripotent stem cells, including embryonic germ cells, testis-derived pluripotent stem cells, and blastomere-derived pluripotent stem cells [62–64]. Moreover, core pluripotent genes, such as *Oct4* and *Nanog*, are expressed in germ cells and early blastomeres, and *Oct4* protein have been shown to be maternally stored in oocytes, which played critical roles in preimplantational development [65,66]. It is possible that the Aire-Lin28 pathway contributed to the potential to become pluripotent stem cells of these cell types, based on the similar expression pattern between Aire and pluripotent factors, the promotional effect of Aire on ES cell self-renewal, and the consistent regulatory relationship between Aire and Lin28 in ES cells, germ cells, and blastomeres. It will be interesting to see whether *Aire* is expressed in new pluripotent stem cell types, such as gonad-derived pluripotent stem cells or very small embryonic like stem cells, and explore the potential usage of Aire as a marker that

isolates new pluripotent cell types [63,64,67]. Interestingly, homozygous *Aire*-knockout mice suffered a high incidence of infertility [68]. Although autoimmune defects have been considered a major cause of this phenotype [7], other possibilities, including a germ cell developmental defect, defects in oocyte genomic reprogramming during fertilization, and defects in preimplantational development, have not been strictly excluded. Since Lin28 has been implicated in germ cell development [69], cellular reprogramming, and self-renewal of pluripotent stem cells, our discovery of the quantitative regulatory effect of Aire on Lin28 indicated that the effect of Aire on germ cell development and early development could contribute to the partial infertility phenotype in *Aire*-knockout mice. Extending this hypothesis, as a fraction of women suffering APECED suffer from infertility, the findings provide new insights into the development of diagnoses and therapeutics for this type of infertility.

Conclusion

In conclusion, our findings integrated Aire as a novel participant of the pluripotent network through Lin28 and presented the first functional interaction between Aire and microRNAs. These findings would shed deep implications on both the structure and function of the pluripotent networks and the molecule networks that Aire regulate gene expression and cellular behaviors.

Acknowledgment

The authors thank Dr. Inder Verma, Dr. David Root, and Dr. Didier Trono for their kind provision of plasmids. This work is supported by grants from the National Natural Science Foundation (31071201/C0704) and (30971625/C060604).

Author Disclosure Statement

No competing financial interests exist.

References

- Shenghui H, D Nakada and SJ Morrison. (2009). Mechanisms of stem cell self-renewal. *Annu Rev Cell Develop Biol* 25:377–406.
- Boyer LA, TI Lee, MF Cole, SE Johnstone, SS Levine, JP Zuckerman, MG Guenther, RM Kumar, HL Murray, et al. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* 122:947–956.
- Kim J, J Chu, X Shen, J Wang and SH Orkin. (2008). An extended transcriptional network for pluripotency of embryonic stem cells. *Cell* 132:1049–1061.
- van den Berg DLC, T Snoek, NP Mullin, A Yates, K Bezarostsi, J Demmers, I Chambers and RA Poot. (2010). An oct4-centered protein interaction network in embryonic stem cells. *Cell Stem Cell* 6:369–381.
- Pardo M, B Lang, L Yu, H Prosser, A Bradley, MM Babu and J Choudhary. (2010). An expanded oct4 interaction network: implications for stem cell biology, development, and disease. *Cell Stem Cell* 6:382–395.
- Houbaviy HB, MF Murray and PA Sharp. (2003). Embryonic stem cell-specific microRNAs. *Develop Cell* 5:351–358.
- Anderson MS, ES Venzani, L Klein, Z Chen, SP Berzins, SJ Turley, H von Boehmer, R Bronson, A Dierich, C Benoist and

- D Mathis. (2002). Projection of an immunological self shadow within the thymus by the aire protein. *Science* 298:1395–1401.
8. Mathis D and C Benoist. (2009). Aire. *Annu Rev Immunol* 27:287–312.
 9. Ramsey C, O Winqvist, L Puhakka, M Halonen, A Moro, O Kämpe, P Eskelin, M Pelto-Huikko and L Peltonen. (2002). Aire deficient mice develop multiple features of APECED phenotype and show altered immune response. *Hum Mol Genet* 11:397–409.
 10. Aaltonen J, P Bjorses, J Perheentupa, N Horelli-Kuitunen, A Palotie, L Peltonen, YS Lee, F Francis, HenningSteffen, et al. (1997). An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. *Nat Genet* 17:399–403.
 11. Gu B, J Zhang, Q Chen, B Tao, W Wang, Y Zhou, L Chen, Y Liu and M Zhang. (2010). Aire regulates the expression of differentiation-associated genes and self-renewal of embryonic stem cells. *Biochem Biophys Res Commun* 394:418–423.
 12. Nishikawa Y, F Hirota, M Yano, H Kitajima, J-i Miyazaki, H Kawamoto, Y Mouri and M Matsumoto. (2010). Biphasic aire expression in early embryos and in medullary thymic epithelial cells before end-stage terminal differentiation. *J Exp Med* 207:963–971.
 13. Hubert F-X, SA Kinkel, KE Webster, P Cannon, PE Crewther, AI Proietto, L Wu, WR Heath and HS Scott. (2008). A specific anti-*aire* antibody reveals *aire* expression is restricted to medullary thymic epithelial cells and not expressed in periphery. *J Immunol* 180:3824–3832.
 14. Gaspar-Maia A, A Alajem, E Meshorer and M Ramalho-Santos. (2011). Open chromatin in pluripotency and reprogramming. *Nat Rev Mol Cell Biol* 12:36–47.
 15. Efroni S, R Duttagupta, J Cheng, H Dehghani, DJ Hoepfner, C Dash, DP Bazett-Jones, S Le Grice, RDG McKay, et al. (2008). Global transcription in pluripotent embryonic stem cells. *Cell Stem Cell* 2:437–447.
 16. Meshorer E, D Yellajoshula, E George, PJ Scambler, DT Brown and T Misteli. (2006). Hyperdynamic Plasticity of chromatin proteins in pluripotent embryonic stem cells. *Develop Cell* 10:105–116.
 17. Guenther MG, SS Levine, LA Boyer, R Jaenisch and RA Young. (2007). A chromatin landmark and transcription initiation at most promoters in human cells. *Cell* 130:77–88.
 18. Gu B, J Zhang, W Wang, L Mo, Y Zhou, L Chen, Y Liu and M Zhang. (2010). Global expression of cell surface proteins in embryonic stem cells. *PLoS ONE* 5:e15795.
 19. Gu B, J Zhang, Y Wu, X Zhang, Z Tan, Y Lin, X Huang, L Chen, K Yao and M Zhang. (2011). Proteomic analyses reveal common promiscuous patterns of cell surface proteins on human embryonic stem cells and sperms. *PLoS ONE* 6:e19386.
 20. Ang Y-S, S-Y Tsai, D-F Lee, J Monk, J Su, K Ratnakumar, J Ding, Y Ge, H Darr, et al. (2011). *Wdr5* mediates self-renewal and reprogramming via the embryonic stem cell core transcriptional network. *Cell* 145:183–197.
 21. Liu Z, DR Scannell, MB Eisen and R Tjian. (2011). Control of embryonic stem cell lineage commitment by core promoter factor, TAF3. *Cell* 146:720–731.
 22. Pitkänen J, A Rebane, J Rowell, A Murumägi, P Ströbel, K Möll, M Saare, J Heikkilä, V Doucas, A Marx and P Peterson. (2005). Cooperative activation of transcription by autoimmune regulator AIRE and CBP. *Biochem Biophys Res Commun* 333:944–953.
 23. Org T, A Rebane, K Kisand, M Laan, U Haljasorg, R Andreson and P Peterson. (2009). AIRE activated tissue specific genes have histone modifications associated with inactive chromatin. *Hum Mol Genet* 18:4699–4710.
 24. Gillard GO, J Dooley, M Erickson, L Peltonen and AG Farr. (2007). Aire-dependent alterations in medullary thymic epithelium indicate a role for aire in thymic epithelial differentiation. *J Immunol* 178:3007–3015.
 25. Sillanpää N, CG Magureanu, A Murumägi, A Reinikainen, A West, A Manninen, M Lahti, A Ranki, K Saksela, et al. (2004). Autoimmune regulator induced changes in the gene expression profile of human monocyte-dendritic cell-lineage. *Mol Immunol* 41:1185–1198.
 26. Schaller CE, CL Wang, G Beck-Engeser, L Goss, HS Scott, MS Anderson and M Wabl. (2008). Expression of *aire* and the early wave of apoptosis in spermatogenesis. *J Immunol* 180:1338–1343.
 27. Ambros V and H Horvitz. (1984). Heterochronic mutants of the nematode *Caenorhabditis elegans*. *Science* 226:409–416.
 28. Moss EG, RC Lee and V Ambros. (1997). The cold shock domain protein LIN-28 controls developmental timing in *C. elegans* and is regulated by the *lin-4* RNA. *Cell* 88:637–646.
 29. Viswanathan SR and GQ Daley. (2010). Lin28: a microRNA regulator with a macro role. *Cell* 140:445–449.
 30. Xu B, K Zhang and Y Huang. (2009). Lin28 modulates cell growth and associates with a subset of cell cycle regulator mRNAs in mouse embryonic stem cells. *RNA* 15:357–361.
 31. Yu J, MA Vodyanik, K Smuga-Otto, J Antosiewicz-Bourget, JL Frane, S Tian, J Nie, GA Jonsdottir, V Ruotti, et al. (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318:1917–1920.
 32. Hagan JP, E Piskounova and RI Gregory. (2009). Lin28 recruits the TUTase Zcchc11 to inhibit let-7 maturation in mouse embryonic stem cells. *Nat Struct Mol Biol* 16:1021–1025.
 33. Viswanathan SR, GQ Daley and RI Gregory. (2008). Selective blockade of microRNA processing by Lin28. *Science* 320:97–100.
 34. Heo I, C Joo, Y-K Kim, M Ha, M-J Yoon, J Cho, K-H Yeom, J Han and VN Kim. (2009). TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. *Cell* 138:696–708.
 35. Nishino J, I Kim, K Chada and SJ Morrison. (2008). *Hmga2* promotes neural stem cell self-renewal in young but not old mice by reducing p16^{Ink4a} and p19^{Arf} expression. *Cell* 135:227–239.
 36. Melton C, RL Judson and R Blelloch. (2010). Opposing microRNA families regulate self-renewal in mouse embryonic stem cells. *Nature* 463:621–626.
 37. Qiu C, Y Ma, J Wang, S Peng and Y Huang. (2010). Lin28-mediated post-transcriptional regulation of Oct4 expression in human embryonic stem cells. *Nucleic Acids Res* 38:1240–1248.
 38. Jin J, W Jing, X-X Lei, C Feng, S Peng, K Boris-Lawrie and Y Huang. (2011). Evidence that Lin28 stimulates translation by recruiting RNA helicase A to polysomes. *Nucleic Acids Res* 39: 3724–3734.
 39. Büssing I, FJ Slack and H Großhans. (2008). let-7 microRNAs in development, stem cells and cancer. *Trends Mol Med* 14:400–409.
 40. Viswanathan SR, JT Powers, W Einhorn, Y Hoshida, TL Ng, S Toffanin, M O’Sullivan, J Lu, LA Phillips, et al. (2009). Lin28 promotes transformation and is associated with advanced human malignancies. *Nat Genet* 41:843–848.
 41. Miyoshi H, U Blömer, M Takahashi, FH Gage and IM Verma. (1998). Development of a self-inactivating lentivirus vector. *J Virol* 72:8150–8157.
 42. Moffat J, DA Grueneberg, X Yang, SY Kim, AM Kloepfer, G Hinkle, B Piqani, TM Eisenhaure, B Luo, et al. (2006). A

- lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell* 124:1283–1298.
43. Huang Y, B Gu, R Wu, J Zhang, Y Li and M Zhang. (2007). Development of a rabbit monoclonal antibody group against Smads and immunocytochemical study of human and mouse embryonic stem cells. *Stem Cells and Development* 26:387–391.
 44. Hay DC, L Sutherland, J Clark and T Burdon. (2004). Oct-4 knockdown induces similar patterns of endoderm and trophoblast differentiation markers in human and mouse embryonic stem cells. *Stem Cells* 22:225–235.
 45. Kitaguchi T, K Mizugishi, M Hatayama, J Aruga and K Mikoshiba. (2002). *Xenopus* Brachyury regulates mesodermal expression of *Zic3*, a gene controlling left–right asymmetry. *Develop Growth Diff* 44:55–61.
 46. Rojas A, W Schachterle, S-M Xu, F Martín and BL Black. (2010). Direct transcriptional regulation of *Gata4* during early endoderm specification is controlled by *FoxA2* binding to an intrinsic enhancer. *Develop Biol* 346:346–355.
 47. Pelton TA, S Sharma, TC Schulz, J Rathjen and PD Rathjen. (2002). Transient pluripotent cell populations during primitive ectoderm formation: correlation of *in vivo* and *in vitro* pluripotent cell development. *J Cell Sci* 115:329–339.
 48. Maye P, S Becker, H Siemen, J Thorne, N Byrd, J Carpentino and L Grabel. (2004). Hedgehog signaling is required for the differentiation of ES cells into neuroectoderm. *Develop Biol* 265:276–290.
 49. Frankenberger S, F Gerbe, S Bessonard, C Belville, P Pouchin, O Bardot and C Chazaud. (2011). Primitive endoderm differentiates via a three-step mechanism involving *nanog* and RTK signaling. *Develop Cell* 21:1005–1013.
 50. Leitch HG, K Blair, W Mansfield, H Ayetey, P Humphreys, J Nichols, MA Surani and A Smith. (2010). Embryonic germ cells from mice and rats exhibit properties consistent with a generic pluripotent ground state. *Development* 137:2279–2287.
 51. Shambloott MJ, J Axelman, S Wang, EM Bugg, JW Littlefield, PJ Donovan, PD Blumenthal, GR Huggins and JD Gearhart. (1998). Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc Natl Acad Sci* 95:13726–13731.
 52. Adamo A, B Sese, S Boue, J Castano, I Paramonov, MJ Barrero and JCI Belmonte. (2011). LSD1 regulates the balance between self-renewal and differentiation in human embryonic stem cells. *Nat Cell Biol* 13:652–659.
 53. Yan Z, Z Wang, L Sharova, AA Sharov, C Ling, Y Piao, K Aiba, R Matoba, W Wang and MSH Ko. (2008). BAF250B-associated SWI/SNF chromatin-remodeling complex is required to maintain undifferentiated mouse embryonic stem cells. *Stem Cells* 26:1155–1165.
 54. Schaniel C, Y-S Ang, K Ratnakumar, C Cormier, T James, E Bernstein, IR Lemischka and PJ Paddison. (2009). *Smarcc1/Baf155* Couples self-renewal gene repression with changes in chromatin structure in mouse embryonic stem cells. *Stem Cells* 27:2979–2991.
 55. Oven I, N Brdickova, J Kohoutek, T Vaupotic, M Narat and BM Peterlin. (2007). AIRE recruits P-TEFb for transcriptional elongation of target genes in medullary thymic epithelial cells. *Mol Cell Biol* 27:8815–8823.
 56. Abramson J, M Giraud, C Benoist and D Mathis. (2010). Aire's partners in the molecular control of immunological tolerance. *Cell* 140:123–135.
 57. Wang Y, S Baskerville, A Shenoy, JE Babiarz, L Baehner and R Blelloch. (2008). Embryonic stem cell-specific microRNAs regulate the G1-S transition and promote rapid proliferation. *Nat Genet* 40:1478–1483.
 58. Plasterk RHA. (2006). Micro RNAs in animal development. *Cell* 124:877–881.
 59. Contreras J and DS Rao. (2011). MicroRNAs in inflammation and immune responses. *Leukemia* 26: 404–413.
 60. Judson RL, JE Babiarz, M Venere and R Blelloch. (2009). Embryonic stem cell-specific microRNAs promote induced pluripotency. *Nat Biotech* 27:459–461.
 61. Siegel S, J Mackenzie, G Chaplin, N Jablonski and L Griffiths. (2012). Circulating microRNAs involved in multiple sclerosis. *Mol Biol Rep* 39:6219–6125.
 62. Tesar PJ. (2005). Derivation of germ-line-competent embryonic stem cell lines from preblastocyst mouse embryos. *Proc Natl Acad Sci U S A* 102:8239–8244.
 63. Conrad S, M Renninger, J Hennenlotter, T Wiesner, L Just, M Bonin, W Aicher, H-J Bühring, U Mattheus, et al. (2008). Generation of pluripotent stem cells from adult human testis. *Nature* 456:344–349.
 64. Kanatsu-Shinohara M, K Inoue, J Lee, M Yoshimoto, N Ogonuki, H Miki, S Baba, T Kato, Y Kazuki, et al. (2004). Generation of pluripotent stem cells from neonatal mouse testis. *Cell* 119:1001–1012.
 65. Hayashi K, SMC de Sousa Lopes and MA Surani. (2007). Germ cell specification in mice. *Science* 316:394–396.
 66. Foygel K, B Choi, S Jun, DE Leong, A Lee, CC Wong, E Zuo, M Eckart, RA Reijo Pera, WH Wong and MWM Yao. (2008). A novel and critical role for *oct4* as a regulator of the maternal-embryonic transition. *PLoS ONE* 3:e4109.
 67. Ratajczak M, E Zuba-Surma, B Machalinski, J Ratajczak and M Kucia. (2008). Very small embryonic-like (VSEL) stem cells: purification from adult organs, characterization, and biological significance. *Stem Cell Rev Rep* 4:89–99.
 68. Jiang W, MS Anderson, R Bronson, D Mathis and C Benoist. (2005). Modifier loci condition autoimmunity provoked by Aire deficiency. *J Exp Med* 202:805–815.
 69. West JA, SR Viswanathan, A Yabuuchi, K Cunniff, A Takeuchi, I-H Park, JE Sero, H Zhu, A Perez-Atayde, et al. (2009). A role for *Lin28* in primordial germ-cell development and germ-cell malignancy. *Nature* 460:909–913.

Address correspondence to:

Prof. Zhang Ming
The Institute of Genetics
College of Life Sciences
Zhejiang University
Hangzhou 310058
China

E-mail: zhangming_ls@zju.edu.cn

Dr. Chen Liangbiao
The Institute of Genetics and Developmental Biology
Chinese Academic of Sciences
Beijing 100101
China

E-mail: lbchen@genetics.ac.cn

Received for publication February 23, 2012

Accepted after revision April 27, 2012

Prepublished on Liebert Instant Online April 27, 2012