Original Paper

SC1 Promotes MiR124-3p Expression to Maintain the Self-Renewal of Mouse Embryonic Stem Cells by Inhibiting the MEK/ERK Pathway

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Key Words

SC1 (pluripotin) • Self-renewal • MiR124-3p • MEK/ERK signalling pathway • Mouse embryonic stem (ES) cells

Abstract

Background/Aims: Self-renewal is one of the most important features of embryonic stem (ES) cells. SC1 is a small molecule modulator that effectively maintains the self-renewal of mouse ES cells in the absence of leukemia inhibitory factor (LIF), serum and feeder cells. However, the mechanism by which SC1 maintains the undifferentiated state of mouse ES cells remains unclear. **Methods:** In this study, microarray and small RNA deep-sequencing experiments were performed on mouse ES cells treated with or without SC1 to identify the key genes and microRNAs that contributed to self-renewal. **Results:** SC1 regulates the expressions of pluripotency and differentiation factors, and antagonizes the retinoic acid (RA)-induced differentiation in the presence or absence of LIF. SC1 inhibits the MEK/ERK pathway through Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis and pathway reporting experiments. Small RNA deep-sequencing revealed that SC1 significantly modulates the expression of multiple microRNAs with crucial functions in ES cells. The expression of miR124-3p is upregulated in SC1-treated ES cells, which significantly inhibits the MEK/ERK pathway by targeting Grb2, Sos2 and Egr1. **Conclusion:** SC1 enhances the self-renewal capacity of mouse ES cells by modulating the expression of key regulatory genes and pluripotency-associated microRNAs. SC1 significantly upregulates miR124-3p expression to further inhibit the MEK/ERK pathway by targeting Grb2, Sos2 and Egr1.

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Introduction

Mouse embryonic stem (ES) cells have been successfully isolated from cultured mouse blastocysts *in vitro* for nearly four decades[1, 2]. Cultured ES cells have three characteristics *in vitro*: self-renewal, pluripotency and limitless proliferation[3, 4]. The self-renewal of mouse ES cells maintained by activating pluripotent-related signalling pathways or inhibiting differentiation-related signalling pathways[5-7]. Some small molecule modulators maintain the undifferentiated and pluripotent state of mouse ES cells by affecting different signalling pathways. For example, CHIR99021 and BIO activate the canonical Wnt/ β -catenin pathway by inhibiting Gsk3 to maintain self-renewal and pluripotency[8, 9], and PD0325901 inhibits the fibroblast growth factor-4 (Fgf4)-stimulated MEK/ERK pathway to enhance Nanog expression and restrict differentiation[10-12]. Other small molecules also modulate the stem cell pluripotent state including the AMP-dependent protein kinase (AMPK) activator AICAR, the Activin/BMP/TGF- β pathway inhibitor SB431542 and the IP3 kinase inhibitor LY294002[13-15]. Currently, small molecules are widely used as tools to modulate signalling pathways and understand stem cell biology, because they are standardized, easily synthesized, simple to use and lack genetic defects [16, 17].

SC1 (pluripotin) is a dual-action compound that maintains the self-renewal and inhibits the differentiation of mouse ES cells by inhibiting RasGAP and ERK1[18]. Upon RasGAP inhibition, SC1 activates Ras signalling by promoting RasGTP accumulation, thereby activating PI3K signalling[19]. In addition, when SC1 is added to ES cell culture medium, it affects the expressions of Tet family genes, which are associated with DNA methylation; therefore, epigenetic modifications may occur in SC1-treated cells[20]. SC1 has been reported to substitute for LIF in conventional ES cell medium, and maintains the undifferentiated state of mouse ES cells at the fifth passages. The levels of the core pluripotent proteins Nanog, Oct4, Sox2 and SSEA1 are similar in cells grown in SC1-treated medium and LIF-treated medium[21]. The efficient isolation of ES cells derived from immunodeficient mice on refractory backgrounds is improved by the addition of SC1 to LIF-containing medium[22]. Importantly, SC1 rapidly induces a morphological change in pluripotent cells to compact the colony[23]. Although some reports have proposed a mechanism underlying the effects of SC1 on pluripotent cells, a genome-wide profile of SC1-treated EC cells is still lacking.

With the development of small RNA deep-sequencing technology, small chemical compounds and medicines have been shown to regulate the expression of mRNAs and microRNAs[24-27]. MicroRNAs are evolutionarily conserved, small noncoding RNAs, that have essential functions in maintaining the self-renewal or differentiation of mouse and human ES cells by repressing the translation of selected mRNAs[28-32]. An increasing number of highly enriched microRNA clusters have been analysed and identified in ES cells, such as miR15b/16, miR290, and miR302/267[33, 34]. Moreover, the efficiency of reprogramming has been improved by overexpressing specific microRNAs to significantly enhance the number of colonies of induced pluripotent stem cells (iPSCs)[35, 36]. Somatic cells have been reprogrammed into iPSCs using microRNAs alone, and the microRNA302/367 cluster is sufficient to induce pluripotent cells without exogenous transcription factors[35].

The mechanisms by which SC1 maintains the self-renewal of treated stem cells remain ambiguous. Therefore, mouse ES cells were treated with SC1 and microarray and deep-sequencing experiments were performed to explore the mechanism by which the undifferentiated state is maintained. Moreover, the functions of differentially expressed microRNAs were also screened and analysed in SC1-treated cells.

Materials and Methods

Reagents and antibodies

SC1, dimethyl sulfoxide (DMSO) and RA were purchased from Sigma-Aldrich (St. Louis, MO, USA). SC1 and RA were dissolved in DMSO at a stock concentration of 10mM. The primary antibodies used were

mouse anti-Erk1, anti-p-Erk1/2(Phospho-Thr202/Tyr204), mouse anti-Grb2, mouse anti-Sos2, mouse anti-Egr1(Santa Cruz Biotechnology, CA, USA), rabbit anti-Nanog, (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-Klf4 (Boster, Wuhan, China). Mouse anti-Gapdh, mouse anti- β -tubulin, and secondary antibodies were purchased from the Beyotime Institute of Biotechnology (Nantong, Jiangsu, China).

Cell culture and transfection

J1 mouse ES cells were purchased from ATTC (Manassas, VA, USA), and were cultured without feeder cells on 0.2% (w/v) gelatine-coated plates in ESC-SR medium [Knockout DMEM supplemented with 15% (v/v) Knockout Serum Replacement, 0.1 mM nonessential amino acids and 2-mercaptoethanol, 2 mM glutamine, which were purchased from Gibco (Invitrogen, Carlsbad, CA, USA), 50 U/mL penicillin, 50 μ g/mL streptomycin (Life Technologies Grand Island, NY, USA), and 103U/mL LIF (ESGRO, Millipore, USA)]. The 293T cells (cell bank of the Chinese Academy of Sciences, China) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% foetal bovine serum (PAN-Biotech, Adenbach, Germany) on 0.1% gelatine-coated plate. The two cell types were cultured at 37 °C in a 5% CO₂ atmosphere. Lipofectamine 2000(Life Technologies Inc., Carlsbad, CA) was used to transfect the cells according to the manufacturer's instructions.

RNA isolation, microarray-based gene expression profiling and small RNA deep-sequencing

J1 mouse ES cells were seeded in gelatin-coated six-well plates at 70%-80% confluence, and then SC1 was added to the ESC-SR media at a working concentration of 1 μ M. An equal volume of DMSO was added as a control. Three independent experiments were performed to prepare the samples. After a 6 hour treatment, the total RNA was extracted from cultured cells using Trizol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. RNA samples were checked and analysed by using an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA). Each qualified total RNA sample was divided into two aliquots, one for the whole-genome microarray expression profiling experiment and another for the small RNA deep-sequencing experiment. Qualified total RNA samples were used for the microarray experiment, and the specific methods and analytical procedures used for these two

Table 1. Primers used for genes qPCR analysis

Gene name		Primer sequences (5' to 3')
Nanog	Forward	CACCCACCCATGCTAGTCTT
	Reverse	ACCCTCAAACCTCCTGGTCCT
Oct3/4	Forward	AGAGGGAACCTCCTCTGAGC
	Reverse	TTCTAGCTCCTTCTGCAGGG
Sox2	Forward	GAGTGGAACTTTTGTCCGAGA
	Reverse	GAAGCGTGACTTATCCTTCTTCAT
Klf4	Forward	GGCGAGTCTGACATGGCTG
	Reverse	GCTGGACGCAGTGTCTTCTC
E-cadherin	Forward	CAGGTCTCCTCATGGCTTTTGC
	Reverse	CTTCCGAAAAGAAGGCTGTCC
β -catenin	Forward	ACCTACTCCCTCCTTTCTACTCC
	Reverse	CCGCAGGGCAGGATAAAAAAGT
Tcfcp2l1	Forward	CAGCCCGAACACTACAACCAG
	Reverse	CAGCCGGATTTCATACGACTG
Sox9	Forward	GAGCCGGATCTGAAGAGGGA
	Reverse	GCTTGACGTGTGGCTTGTTC
Wnt9b	Forward	CTGGTGCTCACCTGAAGCAG
	Reverse	CCGTCTCCTTAAAGCCTCTCTG
Klf8	Forward	TCAGAAAGTGGTTTCGATGCAG
	Reverse	AACAGAGCTGGGTTCTCCATT
Foxd1	Forward	CTTCTCCATCGAGAGCCTCAT
	Reverse	CTGTCCCTTGGTGCAGAGTC
Mapk4	Forward	GCCAGCGTCTACGGGTATG
	Reverse	GCGTCACTCAGAACGATCTTCTT
Lef1	Forward	AGAAATGAGAGCGAATGTCGTAG
	Reverse	CTTTGCACGTTGGGAAGGA
Myc	Forward	AGCCCTAGTGCTGCATGA
	Reverse	TCCACAGACACCACATCAATTTC
Egr1	Forward	CCACAACAACAGGGAGACCT
	Reverse	ACTGAGTGGCGAAGGCTTTA
Fgf5	Forward	AAGTAGCGCGACGTTTCTTC
	Reverse	CTGGAACTGCTATGTTCCGAG
Grb2	Forward	CCCTGTCCGTCAAGTTTGGAA
	Reverse	GGCATCTGTTCTATGTCCGTAA
Sos2	Forward	CAAGATGTTGAGGAACGAGTTCA
	Reverse	TGTCCACAGGTAGTAAGAGAGGA
Gapdh	Forward	TGTGAGGGAGATGCTCAGTG
	Reverse	TGTTCTACCCCAATGTGT

independent experiments were described previously [37, 38].

Bioinformatics analysis (GO and KEGG pathway analysis)

Data regarding the differentially expressed gene were selected based on a fold change ($FC \geq 2$ or $FC \leq 0.5$) and a statistically significant difference ($p \leq 0.05$). Database for Annotation, Visualization, and Integrated Discovery (DAVID, <https://david.ncifcrf.gov/>) was used to perform gene ontology (GO) and KEGG pathway analyses.

Table 2. Primers used for microRNAs qPCR analysis

microRNAs name		Primer sequences (5' to 3')
Universal (miRNA)	Forward	TGAATCGAGCACCAGTTACGCATGCCGAGGTCGACTTCCTAGA
U6	Reverse	CTCGCTTCGGCAGCAC
miR122-5p-F	Reverse	TGGAGTGTGACAATGGTGTGTTG
miR331-3p-F	Reverse	GGCCCTGGGCTATCCT
miR339-5p-F	Reverse	TCCCTGTCTCCAGGAGCTCAC
miR124-5p-F	Reverse	GGTAAGGCACGCGTGAAT
miR296-3p-F	Reverse	GAGGGTTGGGTGGAGGCTCTCC
miR92a-2-5p	Reverse	AGGTGGGGATTGGTGGCATT
miR423-3p	Reverse	AGCTCGGTCTGAGGCCCTCAGT
miR485-5p	Reverse	GGGAGAGGCTGGCCGTGA
miR320-3p	Reverse	GGGAAAAGCTGGGTTGAGA
miR382-5p	Reverse	GAAGTTGTTCTGGTGGATTCTG
miR744-5p	Reverse	TGCGGGCTAGGGCTAACAG

Reverse transcription PCR (RT-PCR) and quantitative real-time PCR (qPCR) analysis

Total RNAs were extracted using Trizol reagent. For the gene expression analysis, total mRNAs (1 μ g) was reverse-transcribed to cDNAs using the First-strand cDNA Synthesis SuperMix (Trans, Beijing, China), according to the manufacturer's instructions. For the microRNA expression level analysis, the expression was validated by poly(A)-tailed qPCR. Qualified total RNAs (2 μ g) were reverse-transcribed using the miScript II RT Kit (Qiagen GmbH, Hilden, Germany). Expression levels of genes and microRNAs were assessed by qPCR using SYBR Premix Ex Taq II Kit (TaKaRa, Dalian, China) on an ABI StepOnePlus PCR System (Applied Biosystems, Foster City, CA, USA). All qPCR experiments were performed at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. Gapdh and U6(a small nuclear RNA) were used as the controls to normalize the expression of test genes and microRNAs. The $2^{-\Delta\Delta CT}$ method was used to analyse relative changes in the expression of genes and microRNAs. All primer sequences used for qPCR are provided in Table 1 and Table 2.

Western Blot analysis

The differently treated cells were lysed in RIPA lysis buffer (Beyotime, Jiangsu, China) supplemented with protease inhibitors (Roche, Mannheim, Germany). The total proteins in the cell lysates were quantified using a BCA protein analysis kit (Beyotime, Jiangsu, China), and then the protein concentration was adjusted to the sample with the lowest protein concentration, to ensure that the total protein concentrations were the same in each sample. The total protein samples were hyperthermally denatured, separated on 10% or 12% polyacrylamide gels and transferred to PVDF membranes (Millipore, MA, USA) for 1.5 or 2 h at 100 V. Membranes were blocked in TBST (10 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween-20, pH 7.0) containing 10% non-fat milk at room temperature for 3-4 h. Then, the membranes were incubated with primary antibodies overnight at 4 °C, and then incubated with secondary antibody for 2 h at room temperature after washing three times with TBST for 10 min each. Following three additional washes for 10 min each, the immunoblots were revealed by autoradiography using SuperSignal West Pico substrate (Pierce/Thermo Scientific, Rockford, IL, USA) or the Gel Doc™ XR+ Gel Documentation System (Bio-Rad, CA, USA). The relative amount of each protein was quantified with ImageJ software. And housekeeping proteins were used for normalization.

Alkaline phosphatase (AP) staining

J1 mouse ES cells were cultured on 24-well plates before staining. The media were removed from cells that had been treated with SC1 or DMSO for 6 h. Cells were washed three times with PBS, and then fixed with immunol staining fix solution for 20 min. After washing three times with immunostaining washing solution, the fixed cells were incubated with a BCIP/NBT working solution for 20 min in the dark, and then washed three times with distilled water to interrupt staining. The BCIP/NBT alkaline phosphatase colour development kit and other reagents were purchased from the Beyotime Institute of Biotechnology (Nantong, Jiangsu, China).

MicroRNA analyses and candidate gene predictions

Two online software packages, miRBase (<http://www.mirbase.org/>) and TargetScan (http://www.targetscan.org/vert_71/), were used to predict candidate target genes for microRNAs. The identification of genes by both software packages predicted which genes were analysed and screened. Mature microRNA sequences were identified using the miRBase website, and the predicted complementary pairing of the target region and microRNAs was identified using the TargetScan website.

Plasmid constructs

The 3'-UTR sequences of the predicted target genes containing the wild type (WT) or mutated (Mut) binding sites were cloned into the psiCHECK-2 vector (Promega, Madison, Wisconsin, USA). The miR-124-3p mimics and inhibitors were purchased from RiboBio Co (Guangzhou, Guangdong, China). For microRNA experiments, cells were transfected with the indicated mimics (50 nM final concentrations) and inhibitors (100 nM final concentrations) for 24 h. The primer sequences used for plasmid constructs are shown in Table 3.

Dual-luciferase reporter assay

The sources of the pathway reporter vectors pAP1-TA-luc, pISRE-TA-luc, pP53-TA-luc, pCRE-TA-luc, pMyc-TA-luc, pNFAT-TA-luc, pSRE-TA-luc, pSuperTOPFlash-TA-luc Luc and the negative control pTA-luc were described previously [39, 40], and pRL-SV40 (Promega) was used as an internal control. After 30 h, transfected cells were treated with 1 μ M SC1 or an equal volume of DMSO for an additional 6 h. The 293T cells were cotransfected with mmu-miR124-3p mimics or the negative control along with the reporter plasmids for 24 hours to identify the target genes. The firefly and Renilla luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Statistical analysis

The experimental data are presented as means $\pm$ standard deviations (SD). The statistical analysis of differences between two groups was performed by using an independent t-test, an analysis of variance (ANOVA) was used to analyse three or more groups, and $P < 0.05$ was considered statistically significant.

Results

SC1 maintains the compact morphology of J1 mouse ES cell colonies and elevates expression levels of core pluripotent factors

Alkaline phosphatase activity is higher in undifferentiated pluripotent cells. Cells were cultured with 1 μ M SC1 or an equal volume of DMSO for 6 or 24 h to clarify the effects of SC1 on mouse ES cells. The AP activity was higher in SC1-treated mouse EC cells than in DMSO-treated cells at 6 and 24 h (Fig. 1A). SC1 significantly changed the morphology of the ES cell colonies. At 6 h, SC1-treated colonies were smoother and more compact, and the results were more obvious at 24 h. High expression of pluripotent genes is a critical property of ESCs [41]. RA induces the differentiation of embryonic stem cells by altering mRNA and microRNA expression [24]. Mouse ES cells were treated with RA (1 μ M) with or without LIF in ESC-SR medium for 18 h, and 1 μ M SC1 was added to the medium for another 6 h to investigate whether SC1 inhibited RA-induced differentiation. Mouse ES cells that had been cultured in the presence of SC1 for 6 h showed significantly higher expression levels of the core pluripotency factors Nanog and Klf4 compared with treatment with RA alone for 24 h,

Table 3. Primers used for Plasmid Constructs

primers name		Primer sequences (5' to 3')
mus-Grb2-xho1-F	Forward	CCGCTCGAGAAGAGCCGGATTCTCACACAC
mus-Grb2-Not1-R	Reverse	ATAAGAATGCGGCCGCCACTGAGTCTGAGCTGAAGCG
mus-Sos2-xho1-F	Forward	CCGCTCGAGAACCCTCTGACCGTCTTCTAGC
mus-Sos2-Not1-R	Reverse	ATAAGAATGCGGCCGCAGGTTTGACAATGGCAGTTGG
mus-Egr1-xho1-F	Forward	CCGCTCGAGACAGCCCTTCACTTACCATCC
mus-Egr1-Not1-R	Reverse	ATAAGAATGCGGCCGCGGTAACCACAACACTCCAACCTCC
Mut-Grb2-3'utr-R	Forward	CCCTTGAAATAAGACACCTTACATATTTAAA
Mut-Grb2-3'utr-F	Reverse	TTTAAATATGTAAGGTGTCTTATTTCGAAGGG
Mut-Sos2-3'utr-R	Forward	CACTGCAAAACAACCTTACAATCTT
Mut-Sos2-3'utr-F	Reverse	GTTTCATTAAGATTGTAAGGTTGTTTTGC
Mut-egr1-3'utr-R	Forward	CGTGTCACACAACCTTACACAAGA
Mut-egr1-3'utr-F	Reverse	TTTCACGTCTTGTTGAAGGTTGTGT

Fig. 1. SC1 treatment promotes self-renewal and alters the morphology of mouse ES cells colonies. A: Alkaline phosphatase staining of J1 mouse ES cells cultured in ESC-SR medium with DMSO or 1 μ M SC1 for 6 h and 24 h. B and C: SC1 inhibits the RA-induced differentiation of J1 mouse ES cells. Cells were treated with 1 μ M SC1 for 6 h and/or 1 μ M RA for 24 h in the presence or absence of LIF in ESC-SR medium; an equal volume of DMSO was added to control samples. Expression levels of the Nanog and Klf4 proteins were analysed by western blotting.

Fig. 2. SC1 promotes the expression pluripotency factors. J1 mouse ES cells were treated with 1 μ M SC1 or an equal volume of DMSO for 6 h and the expression levels of Nanog, Oct4, Sox2, Klf4, E-cad and Ctnnb1 were analysed by qPCR. Data are presented as the means $\pm$ SD of three independent experiments (* p <0.05). Gapdh was used for normalization.

regardless of whether LIF was added (Fig. 1B and 1C).

Thus, qPCR was performed to examine Nanog, Oct4, Sox2 and Klf4 expression level in mouse ES cells when SC1 treating for 6 h. It was found that SC1 can elevate the expression of all four ES cell markers (Fig. 2). In addition, SC1 can also significantly promote the expression of factor E-cadherin associated with cell-to-cell adhesion, which does not affect the expression of Ctnnb1(β -catenin) (Fig.2).

Based on these results, SC1 maintains the self-renewal of J1 mouse ES cells, improves colony morphology, and increases the expression levels of core pluripotent and adhesion genes. Moreover, SC1 also inhibits the RA-induced differentiation of mouse ES cells.

Fig. 3. SC1 regulates the expression of genes associated with self-renewal and differentiation according to the whole-genome gene expression profile. A: Heat map of representative differentially expressed genes related to pluripotency and differentiation in J1 mouse EC cells treated with or without 1 μ M SC1. High expression levels are shown in red and low expression is shown in green. Six selected genes were analysed by qPCR to confirm the reliability of the microarray data. J1 mouse ES cells were cultured with 1 μ M SC1 in ESC-SR medium; an equal volume of DMSO was added as a control. Data are presented as the means $\pm$ SD of three independent experiments; Gapdh was used to normalized the qPCR data. B: The column chart shows upregulated genes. C: The chart shows downregulated genes.

Transcription factors required for self-renewal and differentiation are regulated by SC1

A gene expression microarray analysis was performed using J1 mouse ES cells cultured with or without SC1 and differentially expressed genes were compared to better understand the effects of SC1 on transcription in mouse ES cells. Four hundred sixty differentially expressed genes were identified in SC1-treated J1 mouse ES cells compared with DMSO-treated cells at 6 h, of which 248 genes were upregulated ($FC \geq 2$, $p \leq 0.01$) and 212 were downregulated ($FC \leq 0.5$, $p \leq 0.01$). The stem cell maintenance genes Nanog, Tcfcp2l1, Wnt10a, Sox9 and Klf8 showed increased expression in SC1-treated cells compared with control groups, and the expression of the differentiation genes Egr1, Mapk4, Lef1, Fgf5 and Dusp4 was decreased. Remarkably, SC1 altered the expression of a number of genes associated with cell adhesion, such as Itgb3, Camk2n1, Pcdh21, Calcoco2 and Cdh10 (Fig. 3A). Consistent with the microarray data, the qPCR analysis confirmed the SC1-induced expression of these upregulated and downregulated genes (Fig. 3B and 3C).

Thus, SC1 functions to maintain ES cell self-renewal and inhibit differentiation by upregulating the expression of pluripotency-related genes and downregulating the expression of differentiation-related genes.

Comparison of GO Terms and KEGG Pathways in which SC1-regulated transcripts are involved

Functional annotation of these upregulated and downregulated genes was performed using the DAVID online program to survey the functions of the differentially expressed genes in SC1-treated cells identified in the microarray profile. SC1-induced

Fig. 4. Multiple GO term and signal transduction pathways were regulated by SC1. GO annotation of SC1-regulated genes. A and B: Comparison of the GO terms for upregulated genes (A) or downregulated genes (B) identified by the gene expression microarray. The identified biological processes (BP), cellular components (CC) and molecular functions (MF) are shown as $-\log P$ values and are presented in a bar chart. C: KEGG pathway analysis of differentially expressed genes in SC1-treated J1 mouse ES cells. Results were ranked according to the number of SC1-regulated genes. D: a dual-luciferase reporter assay was used to identify signal transduction pathways that were regulated by SC1. Cells were cotransfected with pathway reporter vectors, negative (pTA-luc) and internal control (pRL-SV40) vectors using Lipofectamine 2000. Thirty hours after transfection, 1 μ M SC1 or an equal volume of DMSO was added to medium and incubated for an additional 6 h. Data are presented as the means $\pm$ SD of three independent experiments. (*: $p < 0.05$; **: $p < 0.01$).

upregulated genes were significantly enriched for terms linked to cell proliferation, cell fate commitment, glycosaminoglycan binding and biosynthetic processes (Fig. 4A). SC1-induced downregulated genes were highly enriched for terms associated with glycoprotein metabolism, morphogenesis, embryonic development and transcription factor activity (Fig. 4B). SC1-regulated genes were involved in the PI3K-Akt signalling pathway, Ras signalling pathway, focal adhesions and signalling pathways regulating the pluripotency of stem cells (Fig. 4C). We performed a dual-luciferase reporter assay using reporter vectors for signal transduction intermediates to identify key signalling pathways that may be influenced by SC1. SC1 negatively regulated the MAPK/ERK and Ca^{2+} /NFAT signalling pathways, and positively regulated cell cycle signalling pathways. No significant changes were observed in other pathways following a 6 h SC treatment (Fig. 4D).

Taken together, GO terms and KEGG pathway analysis of differentially expressed genes indicated that SC1 either directly alters the expression of pluripotency or differentiation factors or regulates different signalling pathways to maintain the self-renewal of mouse ES cells, particularly by inhibiting the MEK/ERK pathway.

Moreover, the changes in the expression of genes related to cell morphology and adhesion suggest that these pathways may be a biological basis for the rapid changes in cell morphology.

Fig. 5. Overview of small RNA deep-seencing data and the microarray analysis. A: Comparison of the known microRNA expression profiles between DMSO- and SC1-treated samples. The scatter plots show the distribution of the detected microRNAs in J1 mouse ES cells treated with 1 μ M SC1 or equal volume of DMSO for 6 h. Significantly regulated microRNAs with a 1.5-fold change are marked red (upregulated) and green (downregulated). B: Representative microRNAs that were altered by the 6 h treatment 1 μ M SC1; the results were validated by real-time PCR. The qPCR data for differentially expressed microRNAs were normalized to U6. Error bars indicate the means $\pm$ SD of three independent experiments, *: $p < 0.05$ compared with controls. C: Venn diagram showing the differentially expressed microRNAs in SC1-treated (red) and PD-treated (green) J1 mouse ES cells. Venn diagram showing the same changes in microRNAs, including global differentially expressed, upregulated and downregulated microRNAs in SC1- and PD-treated cells.

Small RNA deep-seencing of SC1-treated mouse ES cells

Alterations in both MEK/ERK signalling and mRNAs after the SC1 treatment have been shown to play pivotal roles in regulating the self-renewal of mouse ES cells. MEK/ERK-related microRNAs have not yet been investigated. Small RNA sequencing was performed on J1 mouse ES cells treated with 1 μ M SC1 or an equal volume of DMSO for 6 h using small RNA deep-seencing technology to identify MEK/ERK signaling-related microRNAs in mouse ES cells. The detailed sequencing methods are described in a previous study [25]. According to the scatter plots, the overall trend of microRNA expression changed after SC1 stimulation (Fig. 5A). The levels of several differentially expressed microRNAs were confirmed by qPCR (Fig. 5B). PD0325901 (PD) is an inhibitor of the MEK/ERK pathway. Our previous study reported small RNA deep-seencing results from PD-treated mouse ES cells [37]. Therefore, the general differences in differentially expressed microRNAs in SC1- and PD-treated mouse ES cells were analysed. After comparing the expression of cells treated with these two small molecules, Venn diagram was constructed to show the global differentially, upregulated and downregulated microRNAs between SC1- and PD-treated mouse ES cells. Notably, miR302a-5p expression was inhibited by SC1, but increased by PD (Fig. 5C).

SC1-mediated upregulation of mmu-miR124-3p in mouse ES cells inhibits the MEK/ERK pathway

SC1 effectively inhibited MEK/ERK signalling pathway, but we have not clearly determined whether microRNAs whose expression was altered by SC1 also inhibit this pathway.

Fig. 6. mmu-miR124-3p is a representative upregulated microRNA that affects the Erk1 signalling pathway. The biological function of miR124-3p was screened a representative significantly upregulated microRNAs after SC1-treated cells. A: KEGG pathway analysis of predicted target genes using the DAVID online system; results were ranked according to the number of genes. B: A dual-luciferase reporter assay was used to identify signalling transduction pathways regulated by miR124-3p. Cells were cotransfected with pathway reporter vectors or the negative control (pTA-luc), and mimics NC or miR124-3p mimics using Lipofectamine 2000 for 36 h; cells were cotransfected with pRL-SV40 as an internal control. Data are presented as the means $\pm$ SD of three independent experiments. (*: $p < 0.05$; **: $p < 0.01$). J1 mouse ES cells were transfected with mimics NC or miR124-3p mimics for 24 h. C: miR124-3p represses the Erk1 signalling pathway. Western blots showing the expression levels of the Erk1 and phosphorylated Erk1/2 proteins. D: miR124-3p regulates Nanog expression. Western blot analysis of the Nanog protein.

We predicted the genes targeted by microRNAs using the TargetScan program, and analysed cell signalling transduction pathways using the KEGG pathway analysis to investigate the functions of the upregulated microRNAs. Many target genes of miR124-3p were involved in the MEK/ERK pathway (Fig. 6A). We performed a dual-luciferase reporter assay using signal transduction reporter vectors to confirm which signalling pathway were regulated by miR124-3p in mouse ES cells. The signal transduction reporter plasmids and miR124-3p mimics or mimics NC were cotransfected in J1 mouse ES cells for 36 h. As shown in Fig. 6B, miR-124-3p significantly inhibited the MEK/ERK signalling pathway in mouse ES cells. J1 mouse ES cells were transfected with miR124-3p mimics and mimics NC for 24 h. The core MEK/ERK pathway protein Erk1 was significantly downregulated compared to the control, according to the western blot results. The trend for phosphorylated Erk1/2 protein was consistent with the Erk1 protein (Fig. 6C). Furthermore, western blots showed that transfection of miR124-3p mimics increased Nanog levels in J1 mouse ES cells (Fig. 6D).

Based on these results, SC1 inhibits the MEK/ERK signalling pathway by increasing miR124-3p expression. In addition, miR124-3p increases the levels of the Nanog protein to improve pluripotency in mouse ES cells by inhibiting the MEK/ERK pathway.

Fig. 7. Grb2, Sos2 and Egr1 mRNAs are direct targets of miR124-3p. **A:** A bioinformatic analysis was used to predict the mouse miR124-3p seed sequences and the 3'UTR sequences of the WT and mutated Grb2, Sos2 and Egr1 genes. **B:** 293T cells were cotransfected with a psiCheck2 vector carrying Grb2/Sos2/ Egr1 WT or mutant constructs (Mut) and miR124-3p mimics, and mimics NC were used as a negative control. A dual-luciferase reporter assay was performed and data are presented as the means $\pm$ SD of three independent experiments, **: $p < 0.01$. mimics NC or miR124-3p mimics was transfected, and inhibitor NC or miR124-3p inhibitor and miR124-3p mimics was cotransfected in J1 mouse ES cells for 24 h. **C:** qPCR analysis of the expression levels of the Grb2, Sos2 and Egr1 genes, which were normalized to Gapdh expression. **D:** Western blot analysis of the levels of the Grb2, Sos2 and Egr1 proteins. **E:** Schematic of miR124-3p biosynthesis and functions in maintaining the undifferentiated state of mouse ES cells. SC1 inhibits the MEK/ERK signalling pathway to maintain the self-renewal of mouse ES cells by promoting miR124 expression. → means active and ⊥ means inactive.

miR124-3p inhibits the MEK/ERK pathway by indirectly targeting key intermediates: Grb2, Sos2 and Egr1

MicroRNAs are small noncoding RNAs (containing approximately 21–25 nucleotides) that are highly conserved throughout evolution, and function in RNA silencing and post-transcriptional modifications [42]. A bioinformatics analysis using miRBase and TargetScan was performed to identify specific target genes of the MEK/ERK pathway that contain a miR124-3p-binding site. Then, the scores of the predicted target genes were evaluated, and the miR124-3p-binding site was monitored using a bioinformatics analysis. Finally, three potential target genes involved in the MEK/ERK pathway were screened from the high-scoring group. The Grb2, Sos2 and Egr1 mRNAs were predicted to be potential targets of mmu-miR124-3p (MIMAT0000134) through interactions with complementary sequences at the 3'UTR of these three genes (the binding site sequences in the 3'UTR of the genes were: Grb2: from 646 to 653, Sos2: from 793 to 811, and Egr1: from 749 to 767) (Fig. 7A). The partial 3'UTR sequence (the regions upstream and downstream of the binding site are no less than 200 nucleotides) of these three genes were cloned into a luciferase reporter vector (psiCheck2), and mutant miR124-3p-binding sites in the 3'UTR sequences were also cloned into the psiCheck2 vector. Each of these three genes was cotransfected in 293T cells with miR124-3p mimics for 24 h and assessed using 3'UTR luciferase reporter assays. We observed miR124-3p-dependent suppression of luciferase activity when cells expressed the Grb2/Sos2/Egr1 3'UTR vectors. However, mutation of the miR124-3p-binding site abrogated the

Fig. 8. The quantitative analysis of western blot bands was performed by ImageJ. A and B: The quantitative of the Nanog and Klf4 proteins bands were analysed in the Fig. 1B and 1C. C: The quantitative of the Erk1 and p-Erk1/2 proteins bands were analysed in the Fig. 5C. D: The quantitative of the Nanog proteins bands were analysed in the Fig. 5D. E: The quantitative of the Grb2, Sos2 and Egr1 proteins bands were analysed in the Fig. 6D.

inhibition of luciferase activity (Fig. 7B). We further examined whether the overexpression of miR124-3p mimics and inhibitors changed the expression of these genes in mouse ES cells to confirm that these three genes are putative target of miR124-3p. Therefore, we transfected cells with the miR124-3p mimics alone or cotransfected them with the miR124-3p mimics and miR124-3p inhibitor for 24 h. Transfection with the miR124-3p mimics significantly decreased the expression of these three genes, as shown by qPCR. In contrast, the qPCR analysis showed that the expression of each of these three genes was significantly increased after cells were transfected with miR124-3p mimics and inhibitor compared to cells transfected with miR124-3p mimics and the inhibitor NC (Fig. 7C). A western blot analysis of the protein levels 24 h after transfection showed consistent results (Fig. 7D). The suppression of the MEK/ERK signalling pathway has been shown to promote the self-renewal of mouse ES cells [43, 44]. According to the experimental results, SC1 promotes the expression of miR124-3p and miR124-3p inhibits MEK/ERK pathway by suppressing the expression of key factors in this pathway: Grb2, Sos2 and Egr1. Therefore, SC1 inhibits the MEK/ERK pathway in various ways, which not only directly inhibits the transcription factor Erk1, but also increases miR124-3p expression (Fig. 7E).

As shown in Fig. 8, the quantitative analysis of all western blot bands was performed by ImageJ.

Discussion

Natural and synthetic small molecules have been used to control and manipulate the fates of stem cells by targeting signal transduction pathways and affecting epigenetic modifications. According to several studies, many small molecules can be used as drugs to directly activate stem cell differentiation and self-renewal [45-47]. A heterocyclic compound SC1 has attracted our attention because it inhibits differentiation and promotes the self-renewal of mouse stem cells in the absence of serum, feeder cells and LIF [18, 21]. AP staining confirmed that SC1 alone effectively promotes mouse ES cell self-renewal (Fig. 1A), and increases the expression of core pluripotent genes after 6 h. Thus, SC1 is a fast and efficient small molecule that promotes the self-renewal of stem cells.

Whole-genome expression profiling of SC1-treated mouse ES cells showed that many pluripotency-related genes were upregulated and differentiation-related genes were downregulated (Fig. 3A). For example, *Tcfcp2l1* promotes the self-renewal of ES cells by upregulating *Nanog* expression[48]. *Foxd1* controls *Dax1* expression to maintain pluripotency, and *Foxd1* knockdown reduces the number of iPSCs[49]. *Egr1* is a target of the FGF-ERK signalling pathway and is associated with stem cell differentiation[5, 50, 51]. *Fgf5* is a specific ectodermal marker whose expression level is upregulated during stem cell differentiation *in vitro*[52].

The MEK/ERK signalling pathway plays an indispensable role in enhancing the differentiation of mouse ES cells[53]. This pathway also suppresses the expression of pluripotency genes to enable endodermal differentiation[54]. As shown in our previous study, the MEK inhibitor PD enhances the self-renewal capacity of mouse ES cells by modulating the expression of the key pluripotent genes and ESC-specific microRNAs[37]. SC1 is an effective small molecule inhibitor of the MEK/ERK pathway that inhibits *Erk1/2* activation. Moreover, SC1 also inhibits *RasGAP* to activate that PI3K pathway and maintain the self-renewal of ES cells[18, 19]. The KEGG pathway analysis confirmed that SC1-regulated genes are involved in the PI3K-Akt signalling pathway, Ras signalling pathway and signalling pathways regulating stem cells pluripotency (Fig. 4C). Luciferase reporter assays also confirmed that SC1 inhibits MEK/ERK signalling pathways (Fig.4D).

In addition, the morphology of SC1-treated mouse ES cell colonies became compact and smoother. Thus, SC1 may enhance cell adhesion. SC1 has been reported to promote the expression of the cell adhesion molecule E-cadherin, but overexpression of E-cadherin alone is insufficient to initiate the formation of EC cells colonies[19]. According to the expression profile, the expression of a large number of adhesion-related genes was changed, suggesting that the enhanced adhesion of SC1-treated ES cells may be mediated by multiple factors. Furthermore, many terms were associated with glycoprotein by the GO term analysis (Fig. 4B). Cell adhesion occurs due to the action of transmembrane glycoproteins; thus, we have a hint that the future study of SC1-induced cell adhesion may focus on cell membrane glycoproteins.

MicroRNAs are widely expressed in both plants and animals[55]. They regulate the expression of multiple target genes by repressing translation and inducing mRNA degradation[56]. MicroRNAs also play a pivotal role in determining stem cell fate. Many pluripotency-maintaining microRNAs share a conserved seed sequence and are crucial components involved in maintaining the self-renewal of stem cells[33]. The expression levels of some microRNAs were changed in SC1-treated samples (Fig. 5A). Some differentially expressed microRNAs were related to the maintenance of pluripotency. For example, *mmu-miR122-5p* affects the differentiation of stem cells by targeting *Tbr1*[57]; *miR382-5p* controls stem cell differentiation by repressing *Mxd1*[58]. The expression of *miR296-3p* was decreased in SC1-treated mouse ES cells, and was increased during ES cell differentiation[59]. Previous experiments also confirmed that transfection of *miR-296* mimics suppressed *Nanog* levels in J1 mouse ES cells[37].

Notably, *miR124-3p* is one of the significantly upregulated microRNAs in SC1-treated mouse ES cells. *PAX6* promotes cell migration by downregulating *miR124* during mouse ES cell differentiation[60]. *miR124* also represses the osteogenic differentiation of mesenchymal stem cells by targeting *Dlx5*, *Dlx3* and *Dlx2*. These findings may provide important information about the expression of *miR124* during stem cell differentiation. J1 mouse ES cells were transfected with *miR124-3p* mimics, and the results showed that *miR124-3p* increased the levels of the *Nanog* protein by WB experiments (Fig. 6D). Thus, *miR124-3p* promotes mouse ES cells pluripotency by controlling the expression of pluripotency factor.

Moreover, one of the most important reasons for our further study of *miR124-3p* is that it may affect the MEK/ERK pathway, based on the KEGG analysis of predicted target genes. The result was confirmed using a luciferase reporter assays (Fig. 6B). Three high-scoring predicted genes in MEK/ERK pathway were screened to further investigate the mechanism by which *miR124-3p* inhibited the MEK/ERK pathway. Based on our results, *miR124-3p*

inhibits MEK/ERK pathway by targeting Grb2, Sos2 and Egr1. Grb2 was initially discovered as the adapter protein between the epidermal growth factor receptor (EGFR) and the Ras-mitogenactivated protein kinase (MAPK) pathway[61]. The Grb2 downregulation represses Nanog expression, and further represses the differentiation of mouse ES cells into endoderm and mesoderm[12, 62]. Grb2 forms a Grb2-Sos complex with the mammalian SOS homolog. This complex in turn regulates the EGFR-ERK signalling pathway[63]. As we have mentioned above, Egr1 is an important target of the MEK/ERK signalling pathway. In conclusion, miR124-3p inhibits the MEK/ERK pathway by inhibiting the expression of important genes: Grb2, Sos2 and Egr1.

Based on our finding, SC1 not only regulates the expression of transcripts related to pluripotency and differentiation but also affects the expression of genes related to cell adhesion. Meanwhile, SC1 also alters the expression of some microRNAs, some of which are related to the self-renewal and differentiation of stem cells. In particular, miR124-3p significantly inhibits the MEK/ERK pathway by targeting Grb2, Sos2 and Egr1 to maintain the pluripotency of ES cells.

Disclosure Statement

The authors have declared that no conflicts of interest exist.

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References

- 1 Evans MJ, Kaufman MH: Establishment in culture of pluripotential cells from mouse embryos. *Nature* 1981;292:154-156.
- 2 Martin GR: Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* 1981;78:7634-7638.
- 3 Bradley A: Embryonic stem cells: proliferation and differentiation. *Curr Opin Cell Biol* 1990;2:1013-1017.
- 4 Hirai H, Karian P, Kikyo N: Regulation of embryonic stem cell self-renewal and pluripotency by leukaemia inhibitory factor. *Biochem J* 2011;438:11-23.
- 5 Weinberger L, Ayyash M, Novershtern N, Hanna JH: Dynamic stem cell states: naive to primed pluripotency in rodents and humans. *Nat Rev Mol Cell Biol* 2016;17:155-169.
- 6 Katoh M: Network of WNT and other regulatory signaling cascades in pluripotent stem cells and cancer stem cells. *Curr Pharm Biotechnol* 2011;12:160-170.
- 7 Ougland R, Jonson I, Moen MN, Nesse G, Asker G, Klungland A, Larsen E: Role of ALKBH1 in the Core Transcriptional Network of Embryonic Stem Cells. *Cell Physiol Biochem* 2016;38:173-184.
- 8 Ying QL, Wray J, Nichols J, Batlle-Morera L, Doble B, Woodgett J, Cohen P, Smith A: The ground state of embryonic stem cell self-renewal. *Nature* 2008;453:519-523.
- 9 Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH: Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med* 2004;10:55-63.
- 10 Kunath T, Saba-El-Leil MK, Almousaileakh M, Wray J, Meloche S, Smith A: FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. *Development* 2007;134:2895-2902.
- 11 Stavridis MP, Lunn JS, Collins BJ, Storey KG: A discrete period of FGF-induced Erk1/2 signalling is required for vertebrate neural specification. *Development* 2007;134:2889-2894.
- 12 Hamazaki T, Kehoe SM, Nakano T, Terada N: The Grb2/Mek pathway represses Nanog in murine embryonic stem cells. *Mol Cell Biol* 2006;26:7539-7549.
- 13 Shi X, Wu Y, Ai Z, Liu X, Yang L, Du J, Shao J, Guo Z, Zhang Y: AICAR sustains J1 mouse embryonic stem cell

self-renewal and pluripotency by regulating transcription factor and epigenetic modulator expression. *Cell Physiol Biochem* 2013;32:459-475.

- 14 Du J, Wu Y, Ai Z, Shi X, Chen L, Guo Z: Mechanism of SB431542 in inhibiting mouse embryonic stem cell differentiation. *Cell Signal* 2014;26:2107-2116.
- 15 Jiang BH, Zheng JZ, Vogt PK: An essential role of phosphatidylinositol 3-kinase in myogenic differentiation. *Proc Natl Acad Sci U S A* 1998;95:14179-14183.
- 16 Park HS, Hwang I, Choi KA, Jeong H, Lee JY, Hong S: Generation of induced pluripotent stem cells without genetic defects by small molecules. *Biomaterials* 2015;39:47-58.
- 17 Hou P, Li Y, Zhang X, Liu C, Guan J, Li H, Zhao T, Ye J, Yang W, Liu K, Ge J, Xu J, Zhang Q, Zhao Y, Deng H: Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds. *Science* 2013;341:651-654.
- 18 Chen S, Do JT, Zhang Q, Yao S, Yan F, Peters EC, Scholer HR, Schultz PG, Ding S: Self-renewal of embryonic stem cells by a small molecule. *Proc Natl Acad Sci U S A* 2006;103:17266-17271.
- 19 Letso RR, Stockwell BR: Chemical biology: renewing embryonic stem cells. *Nature* 2006;444:692-693.
- 20 Wu Y, Guo Z, Liu Y, Tang B, Wang Y, Yang L, Du J, Zhang Y: Oct4 and the small molecule inhibitor, SC1, regulates Tet2 expression in mouse embryonic stem cells. *Mol Biol Rep* 2013;40:2897-2906.
- 21 Xiong W, Gao Y, Cheng X, Martin C, Wu D, Yao S, Kim MJ, Liu Y: The use of SC1 (Pluripotin) to support mESC self-renewal in the absence of LIF. *J Vis Exp* 2009. DOI:10.3791/1550
- 22 Yang W, Wei W, Shi C, Zhu J, Ying W, Shen Y, Ye X, Fang L, Duo S, Che J, Shen H, Ding S, Deng H: Pluripotin combined with leukemia inhibitory factor greatly promotes the derivation of embryonic stem cell lines from refractory strains. *Stem Cells* 2009;27:383-389.
- 23 Du J, Wu Y, Liu Y, Ai Z, Wu H, Shi X, Guo Z, Zhang Y: E-cadherin is critical for SC1-induced colony growth of F9 embryonic carcinoma cells. *Cell Physiol Biochem* 2014;33:501-512.
- 24 Zhang J, Gao Y, Yu M, Wu H, Ai Z, Wu Y, Liu H, Du J, Guo Z, Zhang Y: Retinoic Acid Induces Embryonic Stem Cell Differentiation by Altering Both Encoding RNA and microRNA Expression. *PLoS One* 2015;10:e0132566.
- 25 Shi X, YongyanWu, Ai Z, Du J, Cao L, Guo Z, Zhang Y: MicroRNA modulation induced by AICA ribonucleotide in J1 mouse ES cells. *PLoS One* 2014;9:e103724.
- 26 Wang J, Masika J, Zhou J, Wang J, Zhu M, Luo H, Hu X, Zhang L, Tang M, Gao L, Hescheler J, Liang H: Traditional Chinese medicine baicalin suppresses mESCs proliferation through inhibition of miR-294 expression. *Cell Physiol Biochem* 2015;35:1868-1876.
- 27 Yin M, Yuan Y, Cui Y, Hong X, Luo H, Hu X, Tang M, Hescheler J, Xi J: Puerarin Suppresses the Self-Renewal of Murine Embryonic Stem Cells by Inhibition of REST-MiR-21 Regulatory Pathway. *Cell Physiol Biochem* 2015;37:527-536.
- 28 Luo W, Sehgal A: Regulation of circadian behavioral output via a MicroRNA-JAK/STAT circuit. *Cell* 2012;148:765-779.
- 29 Gao Y, Han Z, Li Q, Wu Y, Shi X, Ai Z, Du J, Li W, Guo Z, Zhang Y: Vitamin C induces a pluripotent state in mouse embryonic stem cells by modulating microRNA expression. *FEBS J* 2015;282:685-699.
- 30 Yamaguchi S, Yamahara K, Homma K, Suzuki S, Fujii S, Morizane R, Monkawa T, Matsuzaki Y, Kangawa K, Itoh H: The role of microRNA-145 in human embryonic stem cell differentiation into vascular cells. *Atherosclerosis* 2011;219:468-474.
- 31 Jain AK, Allton K, Iacovino M, Mahen E, Milczarek RJ, Zwaka TP, Kyba M, Barton MC: p53 regulates cell cycle and microRNAs to promote differentiation of human embryonic stem cells. *PLoS Biol* 2012;10:e1001268.
- 32 Zhang Z, Xiang D, Wu WS: Sodium butyrate facilitates reprogramming by derepressing OCT4 transactivity at the promoter of embryonic stem cell-specific miR-302/367 cluster. *Cell Reprogram* 2014;16:130-139.
- 33 Gangaraju VK, Lin H: MicroRNAs: key regulators of stem cells. *Nat Rev Mol Cell Biol* 2009;10:116-125.
- 34 Wang Y, Keys DN, Au-Young JK, Chen C: MicroRNAs in embryonic stem cells. *J Cell Physiol* 2009;218:251-255.
- 35 Anokye-Danso F, Trivedi CM, Juhr D, Gupta M, Cui Z, Tian Y, Zhang Y, Yang W, Gruber PJ, Epstein JA, Morrissey EE: Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. *Cell Stem Cell* 2011;8:376-388.
- 36 Judson RL, Babiarz JE, Venere M, Blueloch R: Embryonic stem cell-specific microRNAs promote induced pluripotency. *Nat Biotechnol* 2009;27:459-461.
- 37 Ai Z, Shao J, Shi X, Yu M, Wu Y, Du J, Zhang Y, Guo Z: Maintenance of Self-Renewal and Pluripotency in J1 Mouse Embryonic Stem Cells through Regulating Transcription Factor and MicroRNA Expression Induced by PD0325901. *Stem Cells Int* 2016;2016:1792573.
- 38 Wu Y, Liu F, Liu Y, Liu X, Ai Z, Guo Z, Zhang Y: GSK3 inhibitors CHIR99021 and 6-bromindirubin-3'-oxime inhibit microRNA maturation in mouse embryonic stem cells. *Sci Rep* 2015;5:8666.

- 39 Ai Z, Shao J, Wu Y, Yu M, Du J, Shi X, Shi X, Zhang Y, Guo Z: CHIR99021 enhances Klf4 Expression through beta-Catenin Signaling and miR-7a Regulation in J1 Mouse Embryonic Stem Cells. *PLoS One* 2016;11:e0150936.
- 40 Wu H, Wu Y, Ai Z, Yang L, Gao Y, Du J, Guo Z, Zhang Y: Vitamin C enhances Nanog expression via activation of the JAK/STAT signaling pathway. *Stem Cells* 2014;32:166-176.
- 41 Chen CY, Cheng YY, Yen CY, Hsieh PC: Mechanisms of pluripotency maintenance in mouse embryonic stem cells. *Cell Mol Life Sci* 2017;74:1805-1817.
- 42 Ambros V: The functions of animal microRNAs. *Nature* 2004;431:350-355.
- 43 Burdon T, Stracey C, Chambers I, Nichols J, Smith A: Suppression of SHP-2 and ERK signalling promotes self-renewal of mouse embryonic stem cells. *Dev Biol* 1999;210:30-43.
- 44 Nichols J, Silva J, Roode M, Smith A: Suppression of Erk signalling promotes ground state pluripotency in the mouse embryo. *Development* 2009;136:3215-3222.
- 45 Schugar RC, Robbins PD, Deasy BM: Small molecules in stem cell self-renewal and differentiation. *Gene Therapy* 2008;15:126-135.
- 46 Schmoele A-C, Huebner R, Beller M, Rolfs A, Frech MJ: Small Molecules in Stem Cell Research. *Curr Pharm Biotechnol* 2013;14:36-45.
- 47 Lu J, Kong X, Luo C, Li KK: Application of Epigenome-Modifying Small Molecules in Induced Pluripotent Stem Cells. *Med Res Rev* 2013;33:790-822.
- 48 Ye S, Li P, Tong C, Ying QL: Embryonic stem cell self-renewal pathways converge on the transcription factor Tfc2l1. *EMBO J* 2013;32:2548-2560.
- 49 Koga M, Matsuda M, Kawamura T, Sogo T, Shigeno A, Nishida E, Ebisuya M: Foxd1 is a mediator and indicator of the cell reprogramming process. *Nat Commun* 2014;5:3197.
- 50 Maegawa M, Arao T, Yokote H, Matsumoto K, Kudo K, Tanaka K, Kaneda H, Fujita Y, Ito F, Nishio K: EGFR mutation up-regulates EGR1 expression through the ERK pathway. *Anticancer Res* 2009;29:1111-1117.
- 51 Press T, Viale-Bouroncle S, Felthaus O, Gosau M, Morsczeck C: EGR1 supports the osteogenic differentiation of dental stem cells. *Int Endod J* 2015;48:185-192.
- 52 Chen J, Lu Z, Cheng D, Peng S, Wang H: Isolation and characterization of porcine amniotic fluid-derived multipotent stem cells. *PLoS One* 2011;6:e19964.
- 53 Shaul YD, Seger R: The MEK/ERK cascade: from signaling specificity to diverse functions. *Biochim Biophys Acta* 2007;1773:1213-1226.
- 54 Hamilton WB, Brickman JM: Erk signaling suppresses embryonic stem cell self-renewal to specify endoderm. *Cell Rep* 2014;9:2056-2070.
- 55 Chen K, Rajewsky N: The evolution of gene regulation by transcription factors and microRNAs. *Nat Rev Genet* 2007;8:93-103.
- 56 Shukla GC, Singh J, Barik S: MicroRNAs: Processing, Maturation, Target Recognition and Regulatory Functions. *Mol Cell Pharmacol* 2011;3:83-92.
- 57 Yang Y, Shen Z, Sun W, Gao S, Li Y, Guo Y: The role of miR-122-5p in negatively regulating T-box brain 1 expression on the differentiation of mouse bone mesenchymal stem cells. *Neuroreport* 2017;28:367-374.
- 58 Zini R, Rossi C, Norfo R, Pennucci V, Barbieri G, Ruberti S, Rontauroli S, Salati S, Bianchi E, Manfredini R: miR-382-5p Controls Hematopoietic Stem Cell Differentiation Through the Downregulation of MXD1. *Stem Cells Dev* 2016;25:1433-1443.
- 59 Sun M, Yan X, Bian Y, Caggiano AO, Morgan JP: Improving murine embryonic stem cell differentiation into cardiomyocytes with neuregulin-1: differential expression of microRNA. *Am J Physiol Cell Physiol* 2011;301:C21-30.
- 60 Fang J, Zhang T, Liu Y, Li Y, Zhou S, Song D, Zhao Y, Feng R, Zhang X, Li L, Wen J: PAX6 downregulates miR-124 expression to promote cell migration during embryonic stem cell differentiation. *Stem Cells Dev* 2014;23:2297-2310.
- 61 Lowenstein EJ, Daly RJ, Batzer AG, Li W, Margolis B, Lammers R, Ullrich A, Skolnik EY, Bar-Sagi D, Schlessinger J: The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. *Cell* 1992;70:431-442.
- 62 Liu Y, Liu Q, Jia W, Chen J, Wang J, Ye D, Guo X, Chen W, Li G, Wang G, Deng A, Kang J: MicroRNA-200a regulates Grb2 and suppresses differentiation of mouse embryonic stem cells into endoderm and mesoderm. *PLoS One* 2013;8:e68990.
- 63 Myers MG, Jr., Wang LM, Sun XJ, Zhang Y, Yenush L, Schlessinger J, Pierce JH, White MF: Role of IRS-1-GRB-2 complexes in insulin signaling. *Mol Cell Biol* 1994;14:3577-3587.