Stem Cell Research (2014) 12, 338–353

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/scr

miR-200c and GATA binding protein 4 regulate human embryonic stem cell renewal and differentiation☆

Hsiao-Ning Huang[a,b], Shao-Yin Chen[a], Shiaw-Min Hwang[c], Ching-Chia Yu[a], Ming-Wei Su[d,e], Wei Mai[d,e], Hsei-Wei Wang[f,g,h,i,j], Wei-Chung Cheng[f,k], Scott C. Schuyler[l], Nianhan Ma[m], Frank Leigh Lu[n], Jean Lu[a,b,d,h,o,*]

[a] *Genomics Research Center, Academia Sinica, Taipei, Taiwan*
[b] *Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei, Taiwan*
[c] *Bioresource Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan*
[d] *National RNAi Platform/National Core Facility Program for Biotechnology, Taipei, Taiwan*
[e] *Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan*
[f] *VGH-YM Genomic Research Center, National Yang-Ming University, Taipei, Taiwan*
[g] *Institute of Biomedical Informatics, National Yang-Ming University, Taipei, Taiwan*
[h] *Institute of Microbiology and Immunology, National Yang-Ming University, Taipei, Taiwan*
[i] *Cancer Research Center, National Yang-Ming University, Taipei, Taiwan*
[j] *Department of Education and Research, Taipei City Hospital, Taipei, Taiwan*
[k] *Division of Pediatric Neurosurgery, Neurological Institute, Taipei Veterans General Hospital, Taipei, Taiwan*
[l] *Department of Biomedical Science, College of Medicine, Chang Gung University, Taoyuan County, Taiwan*
[m] *Institute of Systems Biology and Bioinformatics, National Central University, Taoyuan, Taiwan*
[n] *Department of Pediatrics, National Taiwan University Hospital, National Taiwan University Medical College, Taipei, Taiwan*
[o] *Genomics and System Biology Program, College of Life Science, National Taiwan University, Taipei, Taiwan*

Received 27 April 2013; received in revised form 11 November 2013; accepted 20 November 2013
Available online 3 November 2013

Abstract Human embryonic stem cells (hESCs) are functionally unique for their self-renewal ability and pluripotency, but the molecular mechanisms giving rise to these properties are not fully understood. hESCs can differentiate into embryoid bodies (EBs) containing ectoderm, mesoderm, and endoderm. In the miR-200 family, miR-200c was especially enriched in undifferentiated hESCs and significantly downregulated in EBs. The knockdown of the miR-200c in hESCs downregulated Nanog expression, upregulated GATA binding protein 4 (GATA4) expression, and induced hESC apoptosis. The knockdown of GATA4 rescued hESC apoptosis induced by downregulation of miR-200c. miR-200c directly targeted the 3′-untranslated

Abbreviations: hESCs, human embryonic stem cells; EB, embryoid body; TGF-β, transforming growth factor-β; FGF, fibroblast growth factor; miRNAs, microRNAs; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

* Corresponding author at: Genomics Research Center, Academia Sinica, 128 Academia Road, Sec2, IBMS, N401F, Nankang, Taipei 115, Taiwan. Fax: +886 2 2789 9587.
E-mail address: jeanlu@gate.sinica.edu.tw (J. Lu).

1873-5061/$ - see front matter
http://dx.doi.org/10.1016/j.scr.2013.11.009

region of GATA4. Interestingly, the downregulation of GATA4 significantly inhibited EB formation in hESCs. Overexpression of miR-200c inhibited EB formation and repressed the expression of ectoderm, endoderm, and mesoderm markers, which could partially be rescued by ectopic expression of GATA4. Fibroblast growth factor (FGF) and activin A/nodal can sustain hESC renewal in the absence of feeder layer. Inhibition of transforming growth factor-β (TGF-β)/activin A/nodal signaling by SB431542 treatment downregulated the expression of miR-200c. Overexpression of miR-200c partially rescued the expression of Nanog/phospho-Smad2 that was downregulated by SB431542 treatment. Our observations have uncovered novel functions of miR-200c and GATA4 in regulating hESC renewal and differentiation.

Introduction

Isolated from the inner cell mass of blastocysts, embryonic stem cells (ESCs) are characterized by their ability for unlimited self-renewal and pluripotency. ESCs are able to develop into almost all cell types in the body. Thus, ESCs are important for the study of both developmental biology and regenerative medicine. Human embryonic stem cells (hESCs) presumably preserve the molecular signatures of early development. Cultured in suspension, hESCs spontaneously form three dimensional spheroid aggregates called embryoid bodies (EBs) that differentiate into ectoderm, mesoderm, and endoderm, which mimic the developmental stages of the embryo from the blastocyst to gastrulation and the egg-cylinder formation (Desbaillets et al., 2000; Itskovitz-Eldor et al., 2000; Leahy et al., 1999). The observation of parallel tissue-specific gene expression patterns and signals during EB formation and embryo differentiation supports the hypothesis that ESC differentiation into EBs can serve as a tool to investigate the differentiation processes of different lineages (Keller et al., 1993; Risau et al., 1988; Sanchez et al., 1991; Wang et al., 1992). However, our knowledge about the molecular basis of the hESC–EB transition is still in its infancy. Only limited molecular details have been revealed. For example, the suppression of PI3-Kinase delays the formation of compact mouse EBs by 12 h (Gurney et al., 2011), and knockout of GATA6 induces apoptotic gene expression in mouse EBs without abrogating EB formation (Rong et al., 2012).

Transforming growth factor-β (TGF-β)/activin A/nodal signaling is essential for the self-renewal and pluripotency of hESCs (James et al., 2005). TGF-β, activin A, and nodal all belong to the TGF-β superfamily, and regulate Smad2/Smad3 signaling pathways (Heldin et al., 1997; Vallier et al., 2005). TGF-β/activin A signaling directly regulates the Nanog promoter through Smads (Xu et al., 2008). Inhibition of TGF-β/activin A/nodal signaling by SB431542, an inhibitor of TGF-β type I activin receptor-like kinase (ALK) receptors, downregulates phosphorylation of Smad2 and/or Smad3, and the expression of Oct4 and Nanog (Besser, 2004; Inman et al., 2002; James et al., 2005; Valdimarsdottir and Mummery, 2005). Activin A/nodal and the basic fibroblast growth factor (bFGF) pathways cooperate to maintain pluripotency of hESCs in the absence of feeder cells (Vallier et al., 2005). Furthermore, phosphorylation of Smad2/Smad3 induced by TGF-β signaling is decreased during early differentiation (James et al., 2005).

MicroRNAs (miRNAs) are another class of critical regulators in development. miRNAs are small (18–25 nucleotides in length), endogenous non-coding RNA molecules that regulate target genes either by degradation of mRNA transcripts or by inhibition of mRNA translation (Lee and Shin, 2012; Nelson et al., 2003). miRNAs have been proposed to play important roles in cell fate decisions and embryonic development (Gill et al., 2011; Wang et al., 2012b). Knockout of dicer, an enzyme required for miRNA biogenesis, leads to embryonic lethality in mice on day 7.5 (Bernstein et al., 2003). DGCR8 is an RNA-binding protein that functions together with the RNase III enzyme Drosha in processing of miRNAs. Mouse ESCs without DGCR8 or dicer display defects in differentiation and proliferation (Kanellopoulou et al., 2005; Murchison et al., 2005; Suh and Blelloch, 2011; Wang et al., 2007). However, the functions of the ESC miRNAs are not fully characterized (Wang et al., 2009).

The miR-200 family of miRNAs includes miR-200a, miR-200b, miR-200c, miR-141, and miR-429. Among them, miR-200a and miR-141, but not miR-200c, were observed to be regulated by c-Myc in mouse ESCs (Lin et al., 2009). The overexpression of miR-200a and miR-141 attenuated mouse ESC differentiation upon the removal of leukemia inhibitory factor (LIF) (Lin et al., 2009). In hESCs, the miR-302–367 cluster was shown to regulate cell growth, metabolism, and transcription (Barroso-del Jesus et al., 2009). The combination of miR-200c, miR-302s, and miR-369s reprogram both mouse and human somatic cells into a pluripotent ESC-like state (induced pluripotent stem cells, iPSCs) (Miyoshi et al., 2011; Samavarchi-Tehrani et al., 2010). Oct4 and Sox2 can regulate miR-200 family expression and mesenchymal–epithelial transition during iPSC generation (Wang et al., 2013). However, the functional roles of the miR-200 family in hESCs have not yet been determined.

In this paper, we have discovered a critical role for miR-200c in hESC renewal and the differentiation of all three developmental lineages that is partially mediated by directly targeting GATA4, and observed that miR-200c was reciprocally regulated by the TGF-β/activin A/nodal-Smad pathways.

Materials and methods

Materials

All cell culture reagents and qRT-PCR (quantitative real-time polymerase chain reaction) reagents were purchased from Invitrogen (Carlsbad, CA, USA), and all chemicals were obtained from Sigma (St. Louis, MO, USA), unless otherwise specified.

Cell lines and culture conditions

The hESC line H9 was purchased from WiCells (Madison, WI, USA) (Thomson et al., 1998), while HUES6 cells were kindly provided by Dr. Douglas A. Melton (Harvard University, Boston, MA, USA) (Cowan et al., 2004). hESC lines were maintained in

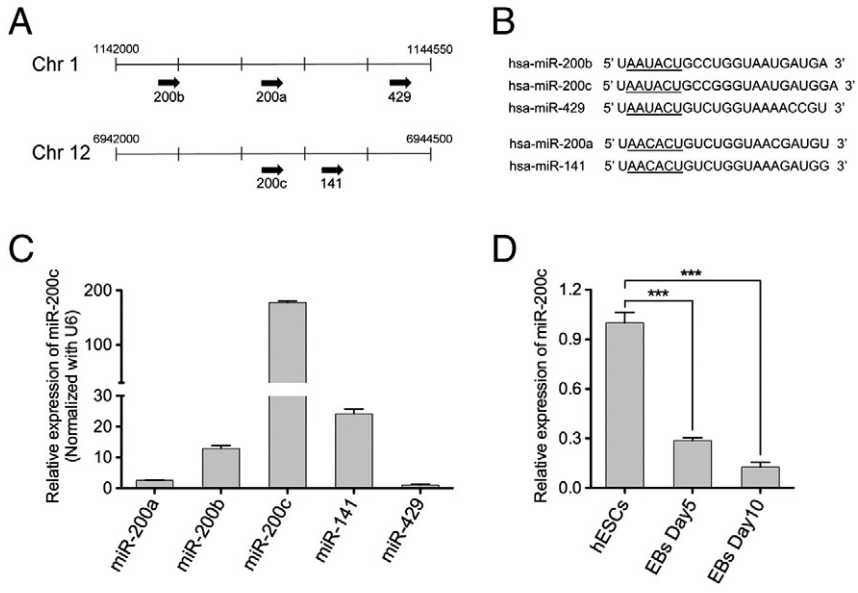


Figure 1 The location, sequences, and expression levels of the miR-200 family in H9 cells were investigated. **(A)** Schematic diagram of the chromosomal locations of the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429). **(B)** Mature miRNA sequences of the miR-200 family. The seed sequence is underlined. **(C)** Abundant expression of miR-200c in undifferentiated H9 cells. miR-200 family expression levels were detected by qRT-PCR. **(D)** Downregulation of miR-200c upon hESC differentiation. The detection of miR-200c expression levels in undifferentiated hESCs and EBs day 5 and day 10 by qRT-PCR. The samples were normalized against undifferentiated hESCs. In all figures, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 20% knockout serum replacement, 2 mM L-glutamine, 1% nonessential amino acids, 4 ng/mL human bFGF, and 0.1 mM 2-mercaptoethanol. For the feeder-free culture, hESCs were seeded on the culture plates coated with Matrigel Matrix (BD Biosciences, San Jose, CA, USA), and the cells were cultured with conditioned medium of MEF (C57BL/6). HEK293T cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Thermo, Wilmington, DE, USA). All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

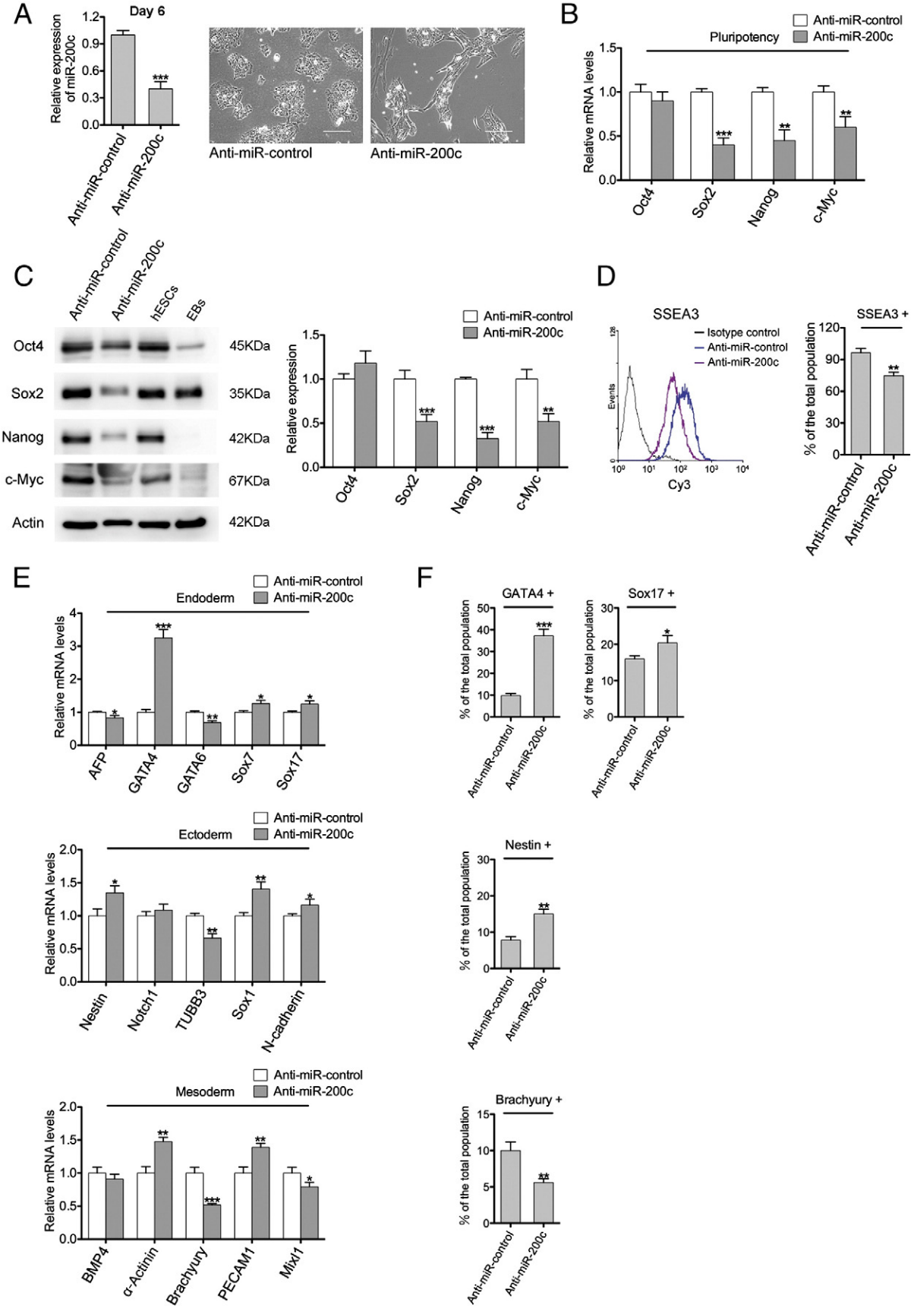
Embryoid body formation

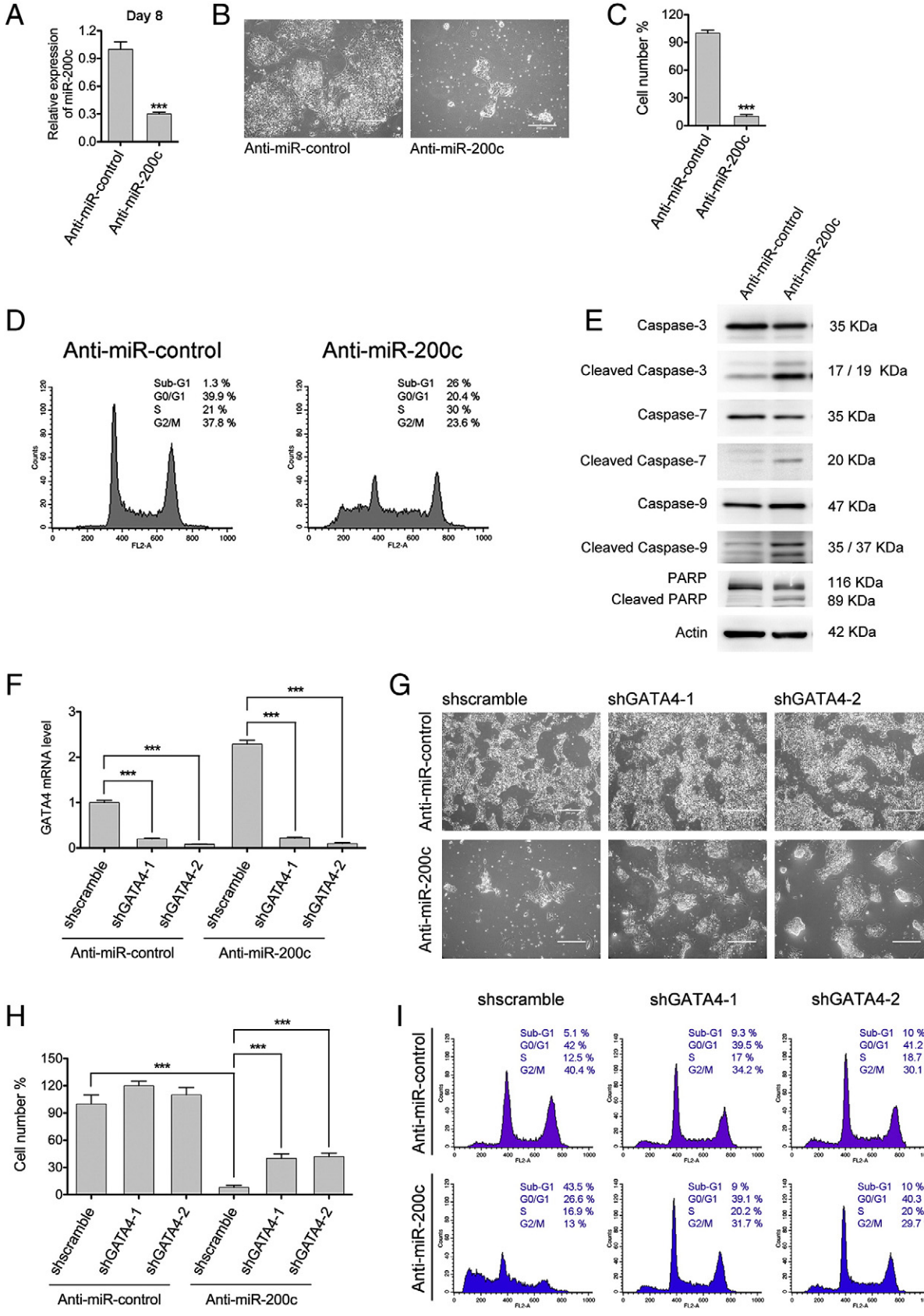
To form EBs, hESCs were detached with 1 mg/mL collagenase IV and the cell clumps were cultured in DMEM supplemented with 10% FBS by the "hanging drop" method for 4 days. The volume of a drop was 20 μL. Drops were placed on the lids of petri-dishes (Corning, Lowell, MA, USA). The medium was changed every two days. For immunofluorescence assay and flow cytometry, these EBs (4 days) were transferred to a 24-well plate coated with 0.2% gelatin (Sigma) and cultured for another 2 days.

Lentivirus production and hESC infection

Lentivirus production was performed in HEK293T cells using TurboFect (Fermentas, Glen Burnie, MD, USA). One day before transfection, 4×10^5 cells per well were plated in 6-well plates. Then cells were respectively transfected with 1 μg of the following plasmids: GATA4 overexpression plasmids (cDNA of GATA4 was amplified from the pCR4-TOPO-GATA4 plasmid (GeneCopoeia, Rockville, MD, USA), and then placed into the pLKO_AS3w.bsd vector (National RNAi Core Facility, Taipei, Taiwan)), miR-200c overexpression plasmid (National RNAi Core Facility), shGATA4 (small hairpin targeted GATA4, shGATA4-1:TRCN0000020424, 5′-CCAGAGA TTCTGCAACACGAA-3′; shGATA4-2:TRCN0000329713, 5′-GGA CATAATCACTGCGTAATC-3) (National RNAi Core Facility), anti-miR-200c plasmid (miRZip-Anti-miR-200c miRZip™ Lentivector-based Anti-MicroRNAs plasmids, System Biosciences, Mountain View, CA, USA), and the vector controls along with 1 μg helper plasmids (pCMVR8.91 and pMD.G: 10:1)

Figure 2 The expression of miR-200c was crucial for maintaining hESC pluripotency. H9 cells were transfected with anti-miR-control or anti-miR-200c oligonucleotides (day 6 post-transfection) in undifferentiated medium. **(A)** The expression of anti-miR-200c downregulated the expression of miR-200c transcripts measured by qRT-PCR (left panel) and induced morphological change (right panel). The scale-bar equals 200 μm. **(B)** The expression of anti-miR-200c downregulated the expression of Nanog, Sox2, and c-Myc transcripts as measured by qRT-PCR. **(C)** The expression of anti-miR-200c downregulated the expression of Nanog, Sox2, and c-Myc proteins as measured by Western blot analyses (left panel). Quantified results from Western blot analyses are shown (right panel). **(D)** The expression of anti-miR-200c downregulated the expression of SSEA3. Flow cytometry was performed (left panel), where quantified results of flow cytometry are shown (right panel). **(E)** Downregulation of miR-200c altered the expression amounts of the transcripts of ectoderm, endoderm, and mesoderm markers. qRT-RCR was performed. **(F)** Downregulation of miR-200c altered the protein expression levels of differentiation markers which was detected by flow cytometry.


A
Day 6
Relative expression of miR-200c
Anti-miR-control
Anti-miR-200c
B
Pluripotency
Anti-miR-control
Anti-miR-200c
Relative mRNA levels
Oct4
Sox2
Nanog
c-Myc
C
hESCs
EBs
Oct4
Sox2
Nanog
c-Myc
Actin
45KDa
35KDa
42KDa
67KDa
42KDa
Relative expression
D
SSEA3
Isotype control
Anti-miR-control
Anti-miR-200c
Events
Cy3
SSEA3 +
% of the total population
E
Endoderm
AFP
GATA4
GATA6
Sox7
Sox17
Ectoderm
Nestin
Notch1
TUBB3
Sox1
N-cadherin
Mesoderm
BMP4
α-Actinin
Brachyury
PECAM1
Mixl1
F
GATA4 +
Sox17 +
Nestin +
Brachyury +


A
Day 8
Relative expression of miR-200c
1.2
0.9
0.6
0.3
0.0
***
Anti-miR-control
Anti-miR-200c
B
Anti-miR-control
Anti-miR-200c
C
Cell number %
120
90
60
30
0
***
Anti-miR-control
Anti-miR-200c
D
Anti-miR-control
Sub-G1 1.3 %
G0/G1 39.9 %
S 21 %
G2/M 37.8 %
Counts
FL2-A
Anti-miR-200c
Sub-G1 26 %
G0/G1 20.4 %
S 30 %
G2/M 23.6 %
Counts
FL2-A
E
Anti-miR-control
Anti-miR-200c
Caspase-3
35 KDa
Cleaved Caspase-3
17 / 19 KDa
Caspase-7
35 KDa
Cleaved Caspase-7
20 KDa
Caspase-9
47 KDa
Cleaved Caspase-9
35 / 37 KDa
PARP
116 KDa
Cleaved PARP
89 KDa
Actin
42 KDa
F
GATA4 mRNA level
3
2
1
0
***
***
***
***
***
shscramble
shGATA4-1
shGATA4-2
shscramble
shGATA4-1
shGATA4-2
Anti-miR-control
Anti-miR-200c
G
shscramble
shGATA4-1
shGATA4-2
Anti-miR-control
Anti-miR-200c
H
Cell number %
150
120
90
60
30
0
***
***
***
shscramble
shGATA4-1
shGATA4-2
shscramble
shGATA4-1
shGATA4-2
Anti-miR-control
Anti-miR-200c
I
shscramble
shGATA4-1
shGATA4-2
Anti-miR-control
Sub-G1 5.1 %
G0/G1 42 %
S 12.5 %
G2/M 40.4 %
Sub-G1 9.3 %
G0/G1 39.5 %
S 17 %
G2/M 34.2 %
Sub-G1 10 %
G0/G1 41.2 %
S 18.7 %
G2/M 30.1 %
Anti-miR-200c
Sub-G1 43.5 %
G0/G1 26.6 %
S 16.9 %
G2/M 13 %
Sub-G1 9 %
G0/G1 39.1 %
S 20.2 %
G2/M 31.7 %
Sub-G1 10 %
G0/G1 40.3 %
S 20 %
G2/M 29.7 %
Counts
FL2-A

(National RNAi Core Facility). 16–24 h later, the medium was replaced, and 72 h after transfection the supernatant was harvested. For infections, 4×10^4 cells were transduced with a multiplicity of infection (MOI) of 10. Cells were seeded on Matrigel-coated dishes, and later incubated in the supernatants containing lentivirus. hESCs were cultured with MEF conditioned medium containing the lentivirus for 16 h in the presence of polybrene (8 μg/mL). Then the hESCs were placed in a fresh conditioned medium containing 2 μg/mL puromycin.

Transfection assay

hESCs were trypsinized and seeded at 4×10^4 cells per well in 12-well plates. After 2 h of incubation at 37 °C, cells were transfected with 80 nM of miR-200c inhibitor (locked nucleic acid (LNA) antisense oligonucleotides against miR-200c, 5′-CCATCATTACCCGGCAGTATT-3′) (Exiqon, Woburn, MA, USA), or a negative control inhibitor (scrambled sequence LNA oligonucleotide, 5′-GTGTAACACGTCTATACGCCCA-3′) (Exiqon) using Lipofectamine RNAiMax transfection reagent (Invitrogen). The medium was changed every day. To assay knockdown efficiency of miRNAs, total RNA was collected 6 days after transfection. For overexpression of miR-200c, 80 nM of either miR-200c mimics (Pre-miR™ miRNA Precursor, 5′-UAAUACUGCCGGGUAAUGAUGGA-3′) or a control mimic (Pre-miR™ miRNA Precursor, Negative Control #1) were used (Ambion, Carlsbad, CA, USA). For SB431542 inhibitor (Sigma) treatment, H9 cells were transfected with control mimics or miR-200c mimics and were cultured in a conditioned medium with or without SB431542 for 6 days. The medium was changed every day.

Luciferase reporter assay

The luciferase reporter plasmid containing the 3′-untranslated region (3′-UTR) of GATA4 (HmiT007183-MT01; GeneCopoeia, Rockville, MD, USA) and the vector control (CmiT000001-MT01; GeneCopoeia) were purchased. Mutations in the GATA4 3′-UTR were generated using PCR-based site-directed mutagenesis. In brief, the GATA4 3′-UTR mutant reporter plasmids were constructed by mixing PCR reagents and the primers, which created a one base pair change in the predicted miR-200c seed sequence-targeted regions. The sequence of the GATA4 3′-UTR was changed from 5′-CAGTATT-3′ to 5′-CGGTATT-3′, or from 5′-CAGTATT-3′ to 5′-CAGTATG-3′. HEK293T cells were transfected with the GATA4 3′-UTR wild type and mutant plasmids (1 μg) in the presence of control miRNA mimics (80 nM) or miR-200c mimics (80 nM) using Lipofectamine™ RNAiMAX (Invitrogen). Cells were selected by 2 μg/mL puromycin (Sigma). Cells were harvested at 48 h post-selection and luciferase activity assays were performed using the Luciferase Reporter Assay System and *Renilla* luciferase assays following the manufacturer's instructions (Promega, Madison, WI, USA). The activities were measured using a VICTOR3 luminometer (PerkinElmer Technologies, Waltham, MA, USA). The Firefly luciferase activity was normalized against the *Renilla* luciferase activity.

RNA extraction and quantitative real-time PCR

For miRNA analyses, total RNA was isolated using the mirVana miRNA isolation kit according to the manufacturer's instructions (Ambion). A TaqMan MicroRNA Assay was used to quantify the levels of miR-200 family expression (Applied Biosystems, Carlsbad, CA, USA). All miRNA data were normalized against a small nuclear RNA control (U6 snRNA). For quantification of mRNA, total RNA and real-time PCR analyses were performed as described (Wang et al., 2012a). Primer sequences are listed in Supplemental Table S1.

Western blot analysis

The Western blot analyses were performed as previous described (Wang et al., 2012a). Primary antibodies including anti-β-actin (A5441; Sigma), anti-Oct4 (sc-9081; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Nanog (3580; Cell Signaling Technology, Danvers, MA, USA), anti-Sox2 (2748; Cell Signaling Technology), anti-c-Myc (sc-40; Santa Cruz Biotechnology), anti-GATA4 (TA500253; Origene, Rockville, MD, USA), anti-p-Smad2 (3108; Cell Signaling Technology), anti-Smad2 (5339; Cell Signaling Technology), and apoptosis antibody kit (9915; Cell Signaling Technology) were used.

Immunofluorescence assay

The immunofluorescence assays were performed as previous described (Wang et al., 2012a). Cells were stained with anti-GATA4 (sc-9053; Santa Cruz Biotechnology), anti-GATA6 (5851; Cell Signaling Technology), anti-Nestin (AB5922; Chemicon, Billerica, MA, USA), anti-TUBB3 (T5076; Sigma), anti-BMP4 (4680; Cell Signaling Technology), or anti-Brachyury (AF2085; R&D Systems) antibodies in the presence

Figure 3 Downregulation of GATA4 rescued the apoptosis triggered by anti-miR-200c- in H9 cells in undifferentiated medium. hESCs were infected with anti-miR-control, anti-miR-200c, shscramble, or shGATA4 lentivirus, and the cells were harvested on day 8 or day 9 post-infection. **(A)** The expression levels of miR-200c transcripts were downregulated by anti-miR-200c transduction. **(B)** The morphology of anti-miR transduced hESCs was observed. The scale-bar equals 200 μm. **(C)** The expression of anti-miR-200c reduced hESC cell numbers measured by trypan blue exclusion assay. **(D)** The expression of anti-miR-200c induced hESC apoptosis. Cell cycle distribution and sub-G1 populations were assayed by propidium iodide (PI) staining. hESCs were infected with anti-miR-control or anti-miR-200c lentivirus. **(E)** Downregulation of miR-200c increased the expression of cleaved form of caspase-3, -7, -9, and PARP. Western blot analysis was performed. **(F)** The infection of two different shGATA4 lentivirus (shGATA4-1 and shGATA4-2) downregulated the expression of GATA4 mRNA. The expression levels of GATA4 were examined by qRT-PCR. **(G)** The downregulation of GATA4 rescued the apoptosis of hESCs induced by the decrease of miR-200c. The morphology lentivirus-infected hESCs are shown. The scale-bar equals 200 μm. **(H)** The knockdown of miR-200c reduced H9 cell renewal could be partially rescued by downregulation of GATA4 with shRNAs (trypan blue exclusion assays). **(I)** The knockdown of miR-200c triggered apoptosis in H9 cells could be rescued by the downregulation of GATA4 with shRNAs. PI staining was performed to analyze the percentage of sub-G1 cells and the cell cycle distribution.

of 2% BSA in PBS overnight at 4 °C. After washing with PBS, cells were incubated with Alexa Fluor® 555 anti-rabbit IgG, anti-mouse IgG, or anti-goat IgG (Invitrogen) for 1 h at room temperature.

Flow cytometry

Cells were disassociated with trypsin and incubated with primary antibodies which included anti-GATA4 (sc-9053; Santa Cruz Biotechnology), anti-GATA6 (5851; Cell Signaling Technology), anti-Nestin (AB5922; Chemicon), anti-TUBB3 (T5076; Sigma), anti-BMP4 (4680; Cell Signaling Technology), anti-Brachyury (AF2085; R&D Systems), anti-Sox17 (09-038; Chemicon), or anti-SSEA3 (MC-631; DSHB) for 1 h at room temperature. The cells were then incubated with Alexa Fluor® 555 anti-rabbit IgG (Abs/Em maxima: 555/565 nm), anti-mouse IgG, anti-goat IgG, or anti-rat IgG (Invitrogen) for 1 h at room temperature. For cell cycle and subG1 phase analysis, 10^5 cells from each sample were trypsinized, washed with PBS, and fixed in cold 90% methanol (Merck). The cells were then treated with RNaseA (Sigma) and stained with propidium iodide (PI) (Sigma). The intensity of fluorescence was measured with a Flow Cytometer (Caliber, Becton Dickinson, Franklin Lakes, NJ, USA), and analyzed using BD CellQuest™ Pro Software.

Statistical analyses

All the statistical results are calculated from at least three biological replicates that were performed independently at different days, and data are reported as the mean value ± standard deviation. Significance in the differences was assessed by the unpaired Student's *t*-test.

Results

Examination of miR-200 family expression levels in hESCs

The miR-200 family contains miR-200a, miR-200b, miR-200c, miR-141, and miR-429. miR-200a, miR-200b, and miR-429 are located on chromosome 1, whereas miR-200c and miR-141 are located on chromosome 12 (Fig. 1A). The miR-200 family contains common sequences at the 5′-end of the miRNA (Fig. 1B, underlined). To investigate the potential roles of the miR-200 family in hESCs, the expression levels of each miRNA in undifferentiated hESCs were compared. By quantitative reverse transcription polymerase chain reaction (qRT-PCR), miR-200c was the most abundant miRNA among the miR-200 family expressed in undifferentiated H9 and HUES6 hESC lines (Fig. 1C and Supplementary Fig. S1A). Thus, we chose to focus our investigation on miR-200c. We confirmed that miR-200c was downregulated upon hESC differentiation into EBs by qRT-PCR in H9 and HUES6 cells (Fig. 1D and Supplementary Fig. S1B). These results suggest a potential role for miR-200c in the maintenance of the undifferentiated state of hESCs.

Downregulation of miR-200c decreases pluripotency markers and promotes the expression of GATA4 in hESCs

To explore potential functional roles of miR-200c, anti-miR-200c or an irrelevant anti-miR control was expressed in hESCs. Sixty percent of miR-200c was knocked down in hESCs as measured by qRT-PCR (Fig. 2A, left panel). After downregulation of miR-200c at day 6, hESCs differentiated and the cellular morphology changed into a spindle-like shape (Fig. 2A, right panel). Furthermore, the mRNA and protein expression levels of hESC pluripotency markers Sox2, Nanog, and c-Myc were decreased in H9 cells (Figs. 2B, C). The regulation of Nanog protein levels by miR-200c was also observed in HUES6 cells (Supplementary Fig. S1C). In addition, Oct4 was downregulated at day 7 in H9 cells (Supplementary Fig. S1D). The expression of pluripotency marker SSEA3 was also decreased in anti-miR-200c-treated cells which was revealed by FACS analysis (Fig. 2D and Supplementary Fig. S1E). Interestingly, the expression level of Nanog was decreased first at day 2, after miR-200c downregulation (Supplementary Fig. S1F). These data suggested that miR-200c is crucial for hESC renewal.

We then investigated if miR-200c knockdown could influence the differentiation of hESCs cultured in the presence of a conditioned medium and the anti-differentiation factor bFGF. By qRT-PCR analysis, the expression levels of three different lineage markers including AFP, GATA4, GATA6, Sox7, Sox17 (endoderm), Nestin, Notch, TUBB3, Sox1, N-cadherin (ectoderm), and BMP-4, α-Actinin, Brachyury, PECAM1, Mixl1 (mesoderm) were examined (Fig. 2E). Among all the differentiation markers examined by qRT-PCR and flow cytometry, the expression of GATA4 was enhanced the most (Figs. 2E, F, top panel). Notably, GATA4 was upregulated immediately at day 2 after miR-200c downregulation (Supplementary Fig. S1F). The expression levels of Nestin, Sox1, α-Actinin, PECAM1, and Sox17 were slightly increased in the anti-miR-200c-expressing cells (Figs. 2E, F). These observations revealed that miR-200c may function in hESC renewal by maintaining the expression of pluripotency genes and by preventing the expression of differentiation genes.

The miR-200c-GATA4 pathway regulates hESC apoptosis

To investigate if miR-200c was required for hESC renewal over the long-term, cells were infected with anti-miR-200c lentivirus and the knockdown efficiency of anti-miR-200c as measured by qRT-PCR. A 70% reduction in miR-200c was observed in cells infected with anti-miR-200c lentivirus (Fig. 3A). In anti-miR-200c-expressing cells, the cell numbers were significantly decreased compared to the control cells after 8 days of infection (Figs. 3B, C). This phenomenon was not observed at day 6 post-infection (data not shown). In order to investigate whether knockdown miR-200c increased cell death or regulated the cell cycle, we performed PI staining and Western blot analyses on apoptotic markers. The downregulation of miR-200c induced apoptosis evidenced by an increase in the sub-G1 population from 1.3% to 26% (anti-miR-control vs. anti-miR-200c) (Fig. 3D), and the upregulation of the cleaved form of caspase-3, -7, and -9, and

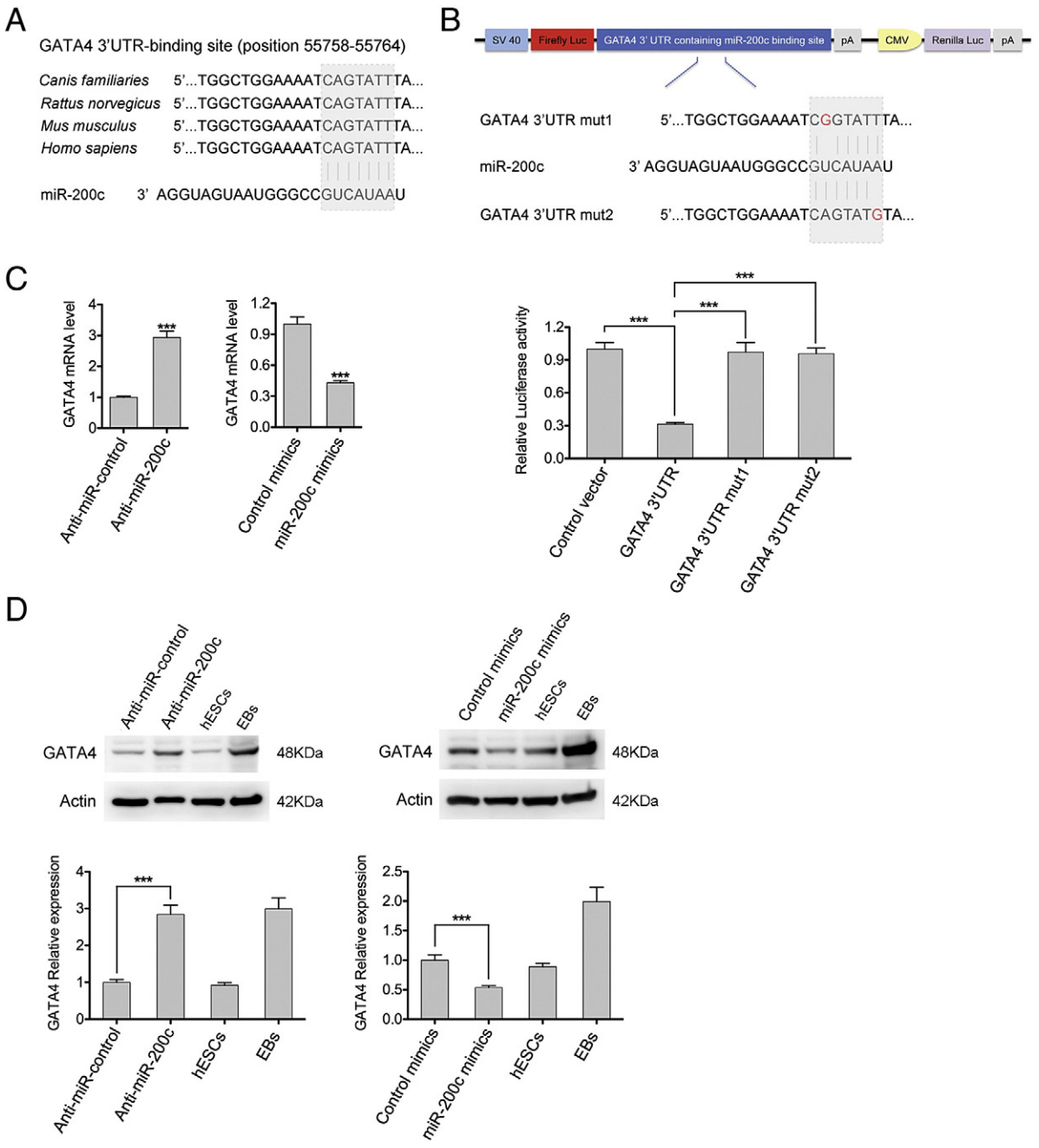
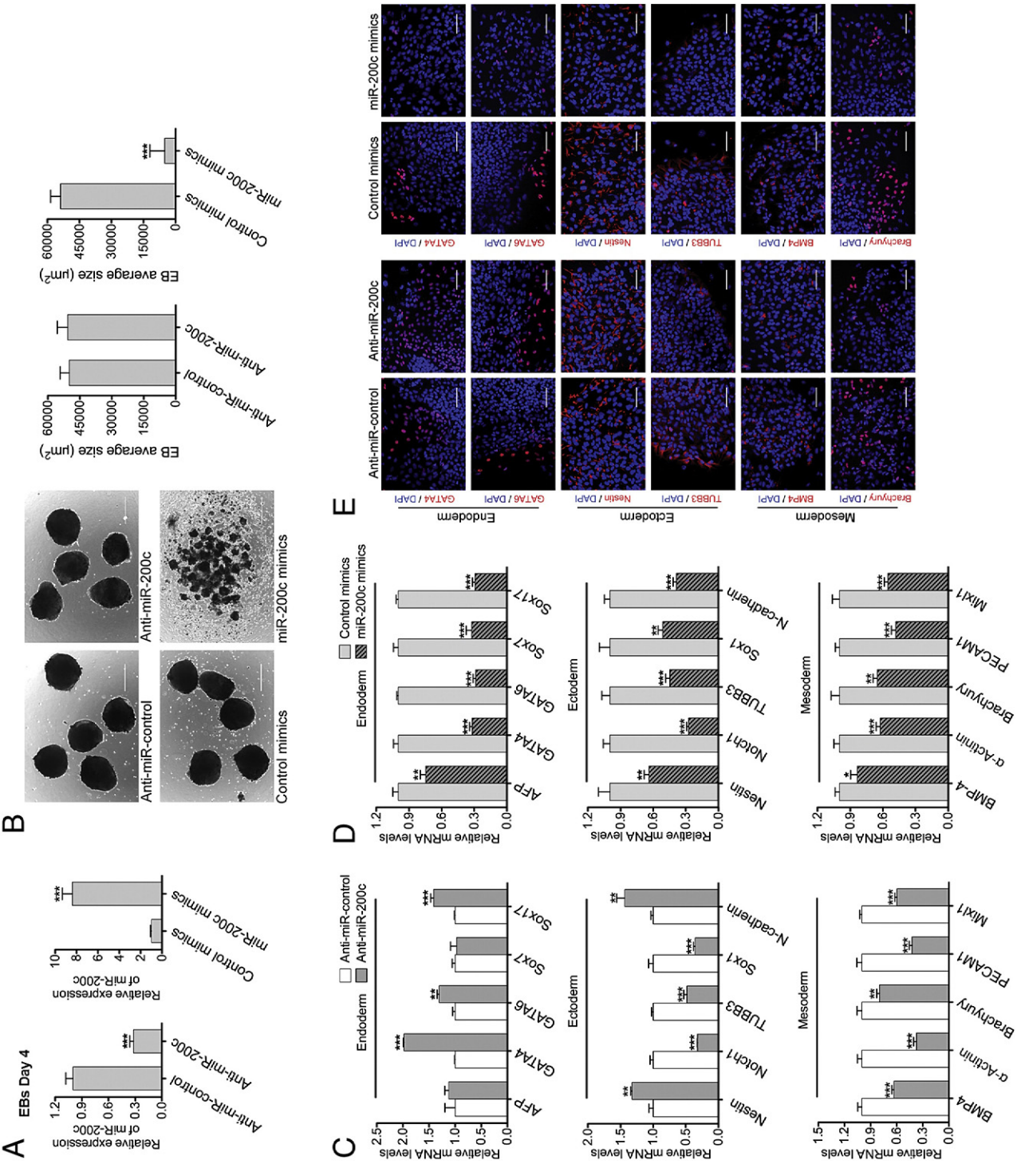


Figure 4 miR-200c directly targeted GATA4. **(A)** The miR-200c putative target site in the 3′-UTR element of GATA4 (genome sequence 55758–55764) of different species appears to be conserved. **(B)** miR-200c inhibited the luciferase activity of the cells transfected with GATA4 3′-UTR reporter plasmid, but not with reporter plasmids mutated at the putative binding site of miR-200c. Schematic representation of the reporter constructs and the two mutation sites are shown (red) (top panel). The GATA4 reporter assays were performed HEK293T cells transfected with miR-200c mimics and reporter plasmids (bottom panel). **(C)** miR-200c regulated the expression levels of GATA4 transcripts (qRT-PCR). **(D)** miR-200c regulated the expression levels of GATA4 proteins (Western blot). The relative expression levels of GATA4 was normalized against the expression levels of actin, and the GATA4 expression levels of the anti-miR-control transfectants for the left panel, and control mimics transfectants for the right panel.

PARP (Fig. 3E). In addition, cell cycle analysis via PI staining, revealed that the downregulation of miR-200c also decreased the G0/G1 phases from 39.9% to 20.4%, and the G2/M phases from 37.8% to 23.6% (Fig. 3D).

To investigate the roles of GATA4 in anti-miR-200c mediated hESC apoptosis, GATA4 was knocked down by two independent short hairpin RNAs (shRNAs) in H9 and HUES6 cells expressing anti-miR-200c or an anti-miR-control (Fig. 3F and Supplementary Fig. S2A). GATA4 downregulation partially restored the cell numbers (Figs. 3G, H, and Supplementary Figs. S2B, C), and almost fully rescued the sub-G1 population induced by the miR-200c knockdown from 43.5% to 9–10% (Fig. 3I). In addition, the downregulation of GATA4 also restored the G0/G1 population from


A
EBs Day 4
Relative expression of miR-200c
Anti-miR-control
Anti-miR-200c
Control mimics
miR-200c mimics
B
Anti-miR-control
Anti-miR-200c
Control mimics
miR-200c mimics
EB average size (μm²)
Anti-miR-control
Anti-miR-200c
Control mimics
miR-200c mimics
C
Anti-miR-control
Anti-miR-200c
Endoderm
Ectoderm
Mesoderm
Relative mRNA levels
AFP
GATA4
GATA6
Sox7
Sox17
Nestin
Notch1
TUBB3
Sox1
N-cadherin
BMP-4
α-Actinin
Brachyury
PECAM1
Mixl1
D
Control mimics
miR-200c mimics
E
Anti-miR-control
Anti-miR-200c
Control mimics
miR-200c mimics
Endoderm
Ectoderm
Mesoderm
GATA4 / DAPI
GATA6 / DAPI
Nestin / DAPI
TUBB3 / DAPI
BMP4 / DAPI
Brachyury / DAPI

26.6% to 39.9–40.3%, and the G2/M population from 13% to 29.7–31.7% (Fig. 3I). Thus the downregulation of GATA4 almost completely blocked the apoptosis triggered by anti-miR-200c and restored the cell cycle (Fig. 3I). These data indicated that the apoptosis triggered by anti-miR-200c functions partially through GATA4 expression. To further assess whether the overexpression of GATA4 affected pluripotency markers, qRT-PCR measurements were performed after GATA4 overexpression. The overexpression of GATA4 decreased Sox2 and Nanog expression levels (Supplementary Figs. S2D-F).

GATA4 is a direct target of miR-200c

A prediction of miR-200c direct target genes was performed employing the TargetScan 4.2 algorithm (http://www.targetscan.org). One of the predicted candidate genes was GATA4. The seed sequence of miR-200c matched with a 3′-untranslational region (3′-UTR) sequence of GATA4, and displayed conservation among different mammalian species, including human, mouse, dog, and rat (Fig. 4A). The ability of miR-200c to regulate the 3′-UTR of GATA4 was evaluated by Firefly luciferase/*Renilla* luciferase reporter assays in HEK293 cells. The 3′-UTR of GATA4 was cloned into the 3′-end of a Firefly luciferase reporter gene driven by SV40, which was followed by a *Renilla* luciferase reporter gene driven by the CMV promoter (Fig. 4B, top panel). *Renilla* luciferase activity was used to normalize the transfection efficiency. Compared to the miR-control, the expression of miR-200c-mimics downregulated GATA4 3′-UTR luciferase activity by 70% (Fig. 4B). The function of miR-200c was abolished with two different single point mutations that were introduced separately into the putative target site of miR-200c in the GATA4 3′-UTR region (Fig. 4B). These results are consistent with the observations that expression of GATA4 was increased upon miR-200c knockdown in hESCs as detected by qRT-PCR and flow cytometry (Figs. 2E, F). To confirm the inverse correlation between miR-200c and GATA4, western blot analyses were performed. The downregulation of miR-200c led to an increase in GATA4 protein levels, and the overexpression of miR-200c decreased GATA4 protein levels (Figs. 4C, D). These observations provide the first evidence linking miR-200c to the direct regulation of a developmental gene GATA4.

miR-200c overexpression inhibits hESC differentiation and EB formation

To further assess the biological significance of miR-200c in hESC differentiation, EB formation was monitored while miR-200c was either knocked down or overexpressed in H9 and HUES6 cells. By qRT-PCR, the expression level of miR-200c was successfully downregulated in the presence of anti-miR-200c anti-sense oligonucleotides, while the expression level of miR-200c was increased employing miR-200c mimics (Fig. 5A and Supplementary Fig. S3A). Interestingly, in both H9 and HUES6 cells, the overexpression of miR-200c significantly decreased the sizes of EBs (Fig. 5B and Supplementary Fig. S3B). Overexpression of miR-200c led to the downregulation of most of the differentiation markers for the three germ layers examined in our system (Figs. 5D, E, Supplementary Figs. S3D, S4B), indicating that miR-200c has an inhibitory role in EB formation and hESC differentiation. However, stem cell marker expression levels were the same in the control mimic and miR-200c mimic groups (Supplementary Fig. S4D). In contrast, knocked down of miR-200c did not cause obvious changes in EB size (Fig. 5B and Supplementary Fig. S3B). miR-200c knockdown led to elevated expression levels of endoderm markers (GATA4, Sox17), and the downregulation of several ectoderm markers (Notch, TUBB3, and Sox1), and mesoderm markers (BMP-4, α-Actinin, and PECAM1) (Figs. 5C, E, Supplementary Figs. S3C, S4A, and S4C). The endoderm markers do enhance the most compared to the ectoderm and mesoderm markers (Fig. 5C and Fig. S3C).

Ectopic overexpression of GATA4 partially restores EB formation inhibited by the overexpression of miR-200c

To explore whether the inhibition of GATA4 affected EB formation, two independent anti-GATA4 shRNAs were used (Figs. 6A, B). GATA4 downregulation reduced the sizes of EBs by 70% (Fig. 6C), which was similar to the effects of miR-200c overexpression (Fig. 5B and Supplementary Fig. S3B). To further investigate whether the downregulation of GATA4 by miR-200c hampers EB formation, a rescue experiment was performed. hESCs were infected with GATA4-expressing viruses or vector control viruses, in the presence of miR-200c mimics or control mimics. Moderate overexpression of GATA4 was confirmed by qRT-PCR and Western blot analyses (Figs. 6D, E). Ectopic overexpression of GATA4 partially rescued the efficiency of EB formation blocked by the overexpression of miR-200c (Fig. 6F). Interestingly, GATA4 overexpression not only upregulated the expression levels of several endoderm markers (AFP, GATA4, and Sox7), but also increased the expression of some ectoderm markers (Nestin, Notch, and Sox1), and mesoderm markers (BMP-4, α-Actinin, and PECAM1) (Supplementary Fig. S5). By contrast, the expression levels of other endoderm markers (GATA6 and

Figure 5 miR-200c overexpression hampered the EB formation efficiency in H9 cells in differentiated medium. **(A)** The transfection of anti-miR-200c downregulated the expression levels miR-200c transcripts while the transfection of miR-200c mimics increased the miR-200c amounts in EB. **(B)** The overexpression of miR-200c decreased the average size of EBs. The morphology of the EBs transfected with anti-miR control, anti-miR-200c, control mimics, or miR-200c mimics were examined (left panel). The scale-bar equals 200 μm. The sizes of the EBs were measured by ImageJ and are presented as the averages of the surface area (right panel). 30–50 EBs were measured for each condition. **(C)** The downregulation of miR-200c altered the expression levels of several differentiation markers in EB (qRT-PCR). **(D)** The overexpression of miR-200c downregulated the expression levels of differentiation markers for all three germ layers in EB (qRT-PCR). **(E)** Changes in miR-200c levels affected the expression levels of differentiation markers. Immunofluorescence staining was performed in anti-miR-200c transfectants (left panel) or miR-200c mimics transfectants (right panel) in EBs. Lineage specific markers were stained with Alexa 555. The nuclei were stained with DAPI. The scale-bar equals 200 μm.

Sox17) and mesoderm makers (Brachyury and Mixl1) were downregulated when GATA4 was overexpressed in miR-200c mimic expressing EBs (Supplementary Fig. S5).

The overexpression of miR-200c partially restores the expression levels of Nanog and phospho-Smad2 downregulated by SB431542

TGF-β/activin A/nodal signaling is essential for the maintenance of pluripotency in hESCs (James et al., 2005), where inhibition of TGF-β/activin A/nodal signaling by SB431542 downregulates the expression of Oct4 and Nanog (James et al., 2005; Vallier et al., 2005). To investigate if TGF-β/activin A/nodal signals regulate miR-200c expression, hESCs were treated with SB431542. SB431542 treatment changed the cellular morphology of hESCs into a spindle shape that is very similar to the cell morphology observed upon miR-200c-knockdown in hESCs (compare Figs. 7A and 2A). Expression levels of miR-200c, Nanog, and Oct4 were also decreased after SB431542 treatment (Figs. 7B, C). The effectiveness of SB431542 in inhibiting TGF-β/activin A/nodal signals in these experiments was confirmed by the downregulation of phospho-Smad2 (Fig. 7C). Overexpression of miR-200c in the presence of SB431542 restored hESC cell morphology (Fig. 7D), and partially rescued the expression levels of phospho-Smad2 and Nanog in both H9 and HUES6 cells (Fig. 7E and Supplementary Fig. S6). This indicates that TGF-β/activin A/nodal can regulate miR-200c expression, and miR-200c can modulate the TGF-β/activin A/nodal signals.

Discussion

miR-200c was the most abundant miRNA of the miR-200 family in HUES6 and H9 hESC cell lines (Fig. 1 and Supplementary Fig. 1), which is consistent with the reported observations of miR-200c expression by microRNA profiling (Li et al., 2009). The downregulation of miR-200c during hESC differentiation, but not other members of miR-200 family, has been consistently observed during microarray profiling of multiple hESC lines (Lakshmipathy et al., 2007; Li et al., 2009; Ren et al., 2009; Suh et al., 2004), and was confirmed by our data in HUES6 and H9 cells (Fig. 1 and Supplementary Fig. S1). The knockdown of miR-200c downregulated Nanog and SSEA3 expression and increased GATA4 expression (Fig. 2 and Supplementary Fig. S1). Furthermore, the downregulation of Sox2 and c-Myc by the knockdown of miR-200c was observed in H9 cells but not in HUES6 cells, which may reflect the variations in the renewal and differentiation signals in different hESC lines (Osafune et al., 2008; Wang et al., 2012c) (Fig. 2, and Supplementary Fig. S1). The functions of miR-200c in cell expansion are varied in different cell types. miR-200c overexpression inhibits the clonal expansion of breast cancer stem cells, suppresses the growth of embryonal carcinoma cells (Shimono et al., 2009), and reduces cell proliferation of developing submandibular gland and melanoma cells (Liu et al., 2012; Rebustini et al., 2012). By contrast, miR-200c promotes the proliferation of transformed pancreatic cells (Korpal et al., 2011; Yu et al., 2010).

The TGF-β/activin A/nodal pathway cooperates with bFGF to maintain hESC renewal, where TGF-β/activin A/nodal can be secreted in mouse embryonic fibroblast (MEF)-conditioned medium (James et al., 2005; Vallier et al., 2005). This signal triggers the phosphorylation of Smad2 and Smad3, and then activates Nanog promoter activity (Vallier et al., 2009; Xu et al., 2008). It was reported that miR-200c is upregulated significantly in hESCs cultured with activin A, while hESCs cultured with MEF-conditioned medium only expressed little amount of miR-200c (Tsai et al., 2010). However, in our system, hESCs cultured with conditioned medium of MEF abundantly express miR-200c (Fig. 1 and Supplementary Fig. S1). This discrepancy might be due to a difference in hESC lines or because of the compositions of MEF-conditioned media. The treatment of cells with SB431542, an inhibitor of TGF-β/activin A/nodal signaling pathway, led to the downregulation of miR-200c transcripts (Fig. 7C). This is consistent with the finding that the overexpression of Smad3 in a human gastric cancer cell line induces the expression of miR-200c, miR-200a, and miR-200b (Ahn et al., 2012). However, even the expression of phospho-Smad2 was almost completely abolished by treatment with SB431542, where only half of the miR-200c was downregulated (Fig. 7). It is possible that additional pathway(s) are also involved in the regulation of miR-200c expression.

Interestingly, it has been demonstrated that the overexpression of the miR-200b–200a–429 cluster in the differentiation condition significantly repressed neuron induction and upregulated the meso-endoderm genes (Du et al., 2013). By contrast, we have observed that the overexpression of miR-200c is sufficient to block hESC differentiation into all three lineages during EB formation (Fig. 5, Supplementary Figs. S3 and S4). This discrepancy in the observations might be due to differences in the overexpressed gene(s) (miR-200b–200a–429 vs. miR-200c), and/or differences in assay conditions (such as specific lineage induction vs. EB formation, harvest time day 6 and day 10 vs. day 4). In addition, we observed that miR-200c is essential for blocking the expression of differentiated genes in undifferentiated hESCs (Fig. 2). Downregulation of miR-200c could upregulate GATA4 (endoderm) significantly, and Nestin (ectoderm), Sox1 (ectoderm), α-Actinin (mesoderm), and PECAM1 (mesoderm) moderately (Fig. 2). Our observations demonstrate that miR-200c is required for undifferentiated hESC renewal and endogenous Nanog expression, and the overexpression of miR-200c blocks the differentiation of all three germ layers (Figs. 2, 5, and Supplementary Figs. S1, S3, S4), which is different from mouse ESCs, where miR-200c upregulate ectoderm markers (Xu et al., 2008).

The reduction of miR-200c in hESCs led to an increase in GATA4 expression and apoptosis. These phenomena can be rescued by the downregulation of GATA4 by two different shRNAs against GATA4 (Figs. 2, 3, and Supplementary Fig. S2). GATA4 belongs to a member of the GATA family of zinc-finger transcription factors. Members of this family recognize the GATA motif (5′-A/TGATAA/G-3′), which is present in the promoters of many genes involved in embryogenesis and in myocardial differentiation (Bresnick et al., 2010; Molkentin, 2000). In mouse GATA4 knockout ESCs, visceral endoderm differentiation is abolished during EB and teratoma formation, but other lineages are not affected (Capo-Chichi et al., 2005; Soudais et al., 1995). GATA4 knockout EBs lose features including phagocytic vacuoles, lipid droplets, and apical tight junctions (Soudais et al., 1995). The ectopic expression of GATA4 induces mouse ESCs to differentiate into extra-embryonic endoderm (Fujikura et al., 2002). The aggregation

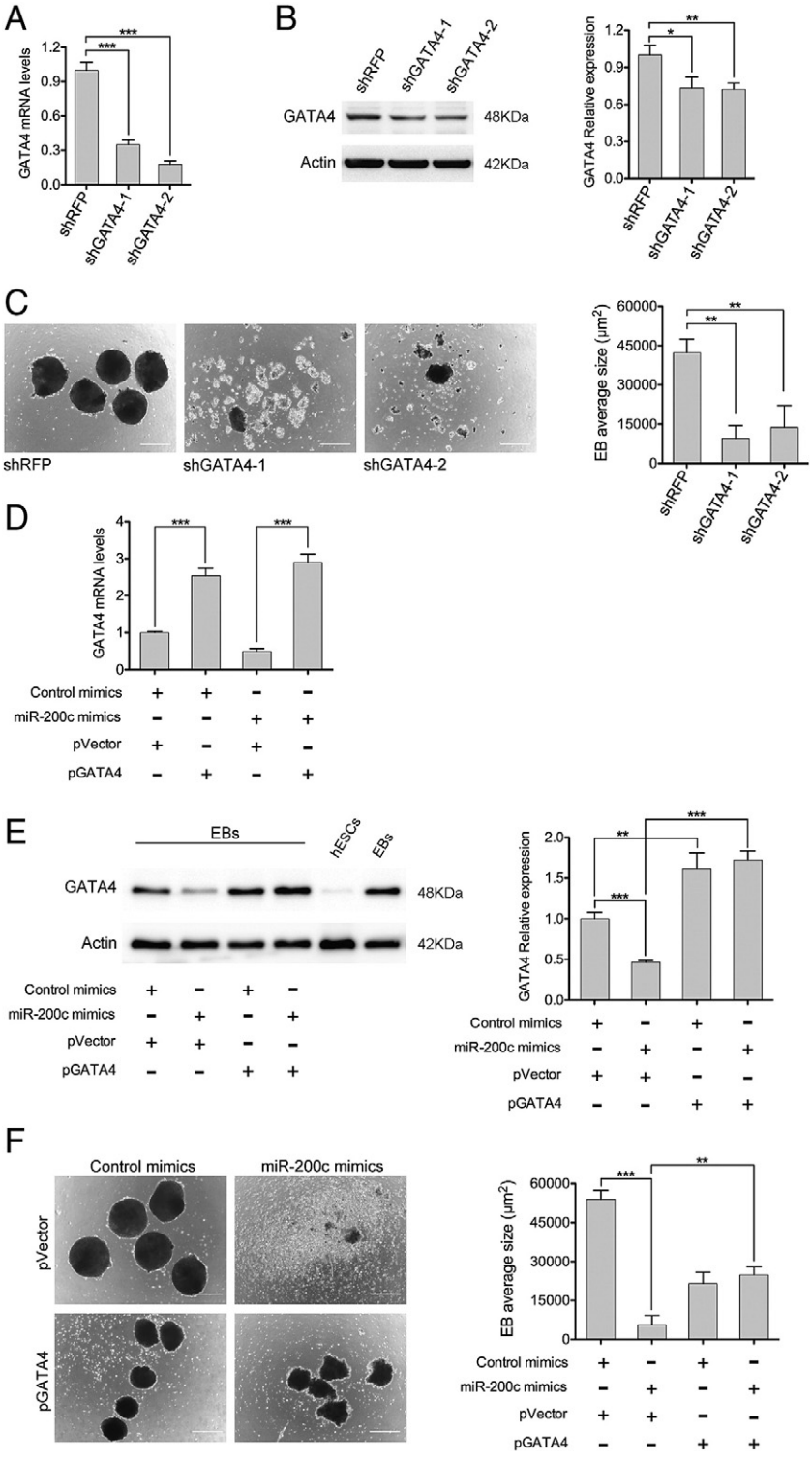


Figure 6 The expression of GATA4 was required for EB formation and overexpression of GATA4 can rescue EB formation inhibited by the overexpression of miR-200c. **(A)** The downregulation of GATA4 mRNA by the infection with the two different shGATA4 lentivirus (shGATA4-1 and shGATA4-2) was measured by qRT-PCR. shRFP is a negative control that did not target a cellular protein. **(B)** The downregulation of GATA4 protein by shGATA4 lentivirus. The results of Western blot analyses are shown in left panel, while the quantified results are shown in the right panel. **(C)** The downregulation of GATA4 repressed EB formation. Morphology of the harvested EBs is shown (left panel). The scale-bar equals 200 μm. Sizes of the EBs were quantified with the ImageJ program (right panel). **(D)** The transduction of GATA4 lentivirus increased the expression of GATA4 transcripts (qRT-PCR). **(E)** The transduction of GATA4 lentivirus increased the expression levels of GATA4 protein. Western blot analyses were performed with EBs (left panel), and the quantification results of three replicates are shown in the right panel. **(F)** The overexpression of GATA4 partially rescued EB formation in miR-200c mimics transfectants. The morphology of EBs is shown. The scale-bar equals 200 μm. The sizes of EBs were measured using the ImageJ program (right panel).

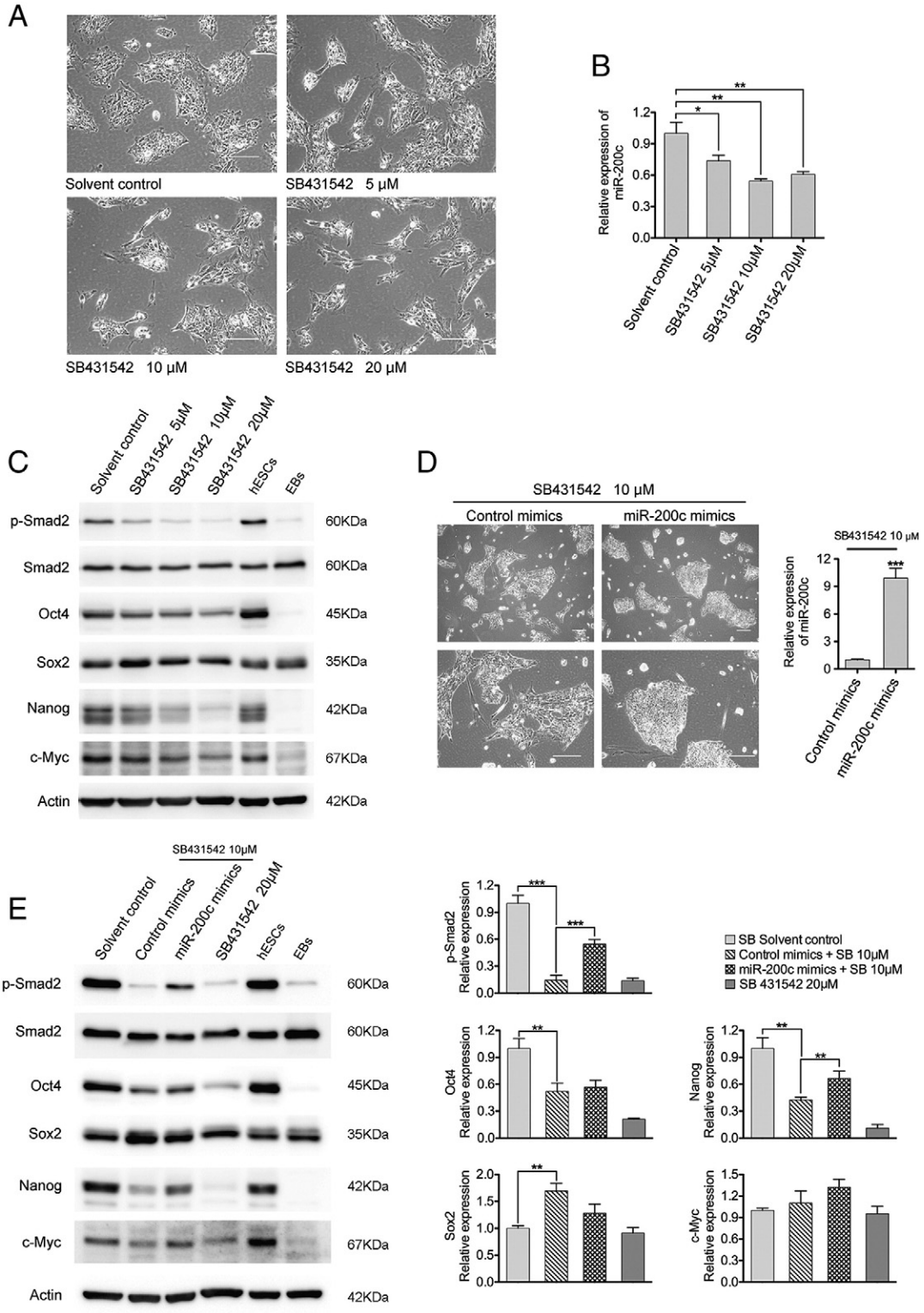


Figure 7 Overexpression of miR-200c partially rescued the undifferentiated morphology and the expression of Nanog and phosphor-Smad2 altered by the treatment of the TGF-β pathway inhibitor SB431542 in H9 cells. **(A)** H9 hESCs treated with SB431542 for six days induced a morphological change. The scale-bar equals 200 μm. **(B)** The downregulation of miR-200c in H9 cells treated with SB431542 (qRT-PCR). **(C)** The downregulation of the expression levels of phopho-Smad2, Nanog, and c-Myc proteins in H9 cells treated with SB431542 were analyzed by Western blot. **(D)** The overexpression of miR-200c restored the morphological change induced by SB431542 (left panel). The scale-bar equals 200 μm (upper panel) or 100 μm (lower panel). qRT-PCR quantification of miR-200c expression levels in H9 cells (right panel). **(E)** The overexpression of miR-200c partially restored the expression levels of Nanog and phopho-Smad2 downregulated by SB431542 (Western blot). The western blot results of miR-200c mimics and control mimics transfectants are shown in the left panel, and the quantified results are shown in the right panel.

of mouse ESCs downregulates Nanog expression and induces primitive endoderm differentiation (Hamazaki et al., 2004). In addition to the presence in the early embryo, GATA4 also has been associated with the development of the heart, ovary, and liver (Haworth et al., 2008; Huang et al., 2011; Kuo et al., 1997; Kyronlahti et al., 2008; Molkentin et al., 1997).

To our knowledge, no prior research has evaluated the functional role of GATA4 in hESC apoptosis and EB differentiation. The most relevant clues have come from a study in which EBs were formed under hypoxia and show enhanced differentiation into primitive endoderm with an increased expression of GATA4 on the EB periphery (Lim et al., 2011). In our study, the overexpression of miR-200c or the knockdown of GATA4 significantly reduces the size of EBs (Figs. 5, 6, and Supplementary Fig. S3), while the overexpression of GATA4 can partially rescue the EB formation inhibited by the overexpression of miR-200c (Fig. 6). The ectopic expression of miR-200c inhibits hESC differentiation, and downregulates markers for all three germ layers (Fig. 5, Supplementary Figs. S3 and S4), while the overexpression of GATA4 is able to rescue the expression of endoderm markers (AFP, Sox7), ectoderm markers (Nestin, Sox1, Notch), and mesoderm markers (BMP4, α-Actinin, PECAM1) (Supplementary Fig. S5). Interestingly, unlike the roles of GATA4 in mouse ESCs, which functions are more limited to the primitive endoderm differentiation (Fujikura et al., 2002), overexpression of GATA4 also activated ectoderm and mesoderm markers in hESCs (Supplementary Fig. S5). Our observations imply that GATA4 expression levels might be critical in determining the size of EBs, since both the GATA4 overexpression and downregulation decreased the size of EBs (Figs. 6C, F). The molecular mechanisms underlying how the miR-200c-GATA4 balance regulates hESC aggregation and EB formation remain to be determined.

GATA4 functions differently in different cell types and can serve as a transcriptional activator or repressor (Hua et al., 2009; Wang et al., 2005; Zhang et al., 2007). GATA4 is a negative regulator of astrocyte proliferation (Agnihotri et al., 2009). The overexpression of GATA4 induced the expression of cyclin-dependent kinase inhibitor p15INK4B, down-regulated cyclin D1 expression, and arrested the cell cycle at G0/G1 phases (Agnihotri et al., 2009). In our study, the inhibition of GATA4 rescued the apoptosis triggered by knocking down miR-200c (Fig. 3). This result suggests that, similar to the observation in astrocytes, GATA4 is a negative regulator of hESC renewal. In contrast, GATA4 is associated with the formation of the heart and enhances the proliferation of cardiomyocytes through the regulation of cyclin D2 and Cdk4 (Rojas et al., 2008), which suggests that the functions of GATA4 are cell-context dependent.

Recently, it was shown that GATA4 binds to a distal enhancer located 9 kb upstream of the Nanog gene, and inhibits Nanog transcription and mouse iPSC formation (Serrano et al., 2013). The same binding site is also present in human Nanog promoter (Serrano et al., 2013). Using computer analysis, several potential GATA4-binding sites were identified in the c-Myc promoter (data not shown). We have observed that the knockdown of miR-200c down-regulated Nanog/c-Myc expression levels and inhibited hESC renewal (Figs. 2, 3, and Supplementary Figs. S1, S2). Since miR-200c directly inhibits GATA4 expression (Fig. 4), these observations suggest the possibility that miR-200c maintains pluripotency by inhibiting GATA4 functions that block Nanog and c-Myc expression. In summary, we found novel functions for miR-200c and GATA4 in regulating human embryonic stem cell renewal and differentiation.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2013.11.009.

Acknowledgements

This work was supported by the National Health Research Institutes (NHRI-EX100-10025SI), National Science Council (NSC 100-2314-B-001-002, NSC 100-2321-B-001-039, NSC 100-2353-B-080-001, NSC 101-2311-B-182-001, NSC 102-2311-B-182-004, NSC 101-2321-B-001-020), the Chang Gung Memorial Hospital (CMRP-D190213, CMRP-D1B0311), the Taiwan Ministry of Education (EMRP-D1B0221), National Central University (102G907-2), and Academia Sinica. We thank Dr. Ming Ji Fann (Yang Ming University, Taipei, Taiwan), Dr. Jin-Yuh Shew (Academia Sinica Taipei, Taiwan), Dr. Ching Hwa Tsai (National Taiwan University, Taipei, Taiwan), and Dr. Jeou-Yuan Chen (Academia Sinica, Taipei, Taiwan) for their suggestions and input in the manuscript.

References

Agnihotri, S., Wolf, A., Picard, D., Hawkins, C., Guha, A., 2009. GATA4 is a regulator of astrocyte cell proliferation and apoptosis in the human and murine central nervous system. Oncogene 28, 3033–3046.

Ahn, S.M., Cha, J.Y., Kim, J., Kim, D., Trang, H.T., Kim, Y.M., Cho, Y.H., Park, D., Hong, S., 2012. Smad3 regulates E-cadherin via miRNA-200 pathway. Oncogene 31, 3051–3059.

Barroso-del Jesus, A., Lucena-Aguilar, G., Menendez, P., 2009. The miR-302–367 cluster as a potential stemness regulator in ESCs. Cell Cycle 8, 394–398.

Bernstein, E., Kim, S.Y., Carmell, M.A., Murchison, E.P., Alcorn, H., Li, M.Z., Mills, A.A., Elledge, S.J., Anderson, K.V., Hannon, G.J., 2003. Dicer is essential for mouse development. Nat. Genet. 35, 215–217.

Besser, D., 2004. Expression of nodal, lefty-a, and lefty-B in undifferentiated human embryonic stem cells requires activation of Smad2/3. J. Biol. Chem. 279, 45076–45084.

Bresnick, E.H., Lee, H.Y., Fujiwara, T., Johnson, K.D., Keles, S., 2010. GATA switches as developmental drivers. J. Biol. Chem. 285, 31087–31093.

Capo-Chichi, C.D., Rula, M.E., Smedberg, J.L., Vanderveer, L., Parmacek, M.S., Morrisey, E.E., Godwin, A.K., Xu, X.X., 2005. Perception of differentiation cues by GATA factors in primitive endoderm lineage determination of mouse embryonic stem cells. Dev. Biol. 286, 574–586.

Cowan, C.A., Klimanskaya, I., McMahon, J., Atienza, J., Witmyer, J., Zucker, J.P., Wang, S., Morton, C.C., McMahon, A.P., Powers, D., Melton, D.A., 2004. Derivation of embryonic stem-cell lines from human blastocysts. N. Engl. J. Med. 350, 1353–1356.

Desbaillets, I., Ziegler, U., Groscurth, P., Gassmann, M., 2000. Embryoid bodies: an in vitro model of mouse embryogenesis. Exp. Physiol. 85, 645–651.

Du, Z.W., Ma, L.X., Phillips, C., Zhang, S.C., 2013. miR-200 and miR-96 families repress neural induction from human embryonic stem cells. Development 140, 2611–2618.

Fujikura, J., Yamato, E., Yonemura, S., Hosoda, K., Masui, S., Nakao, K., Miyazaki Ji, J., Niwa, H., 2002. Differentiation of embryonic stem cells is induced by GATA factors. Genes Dev. 16, 784–789.

Gill, J.G., Langer, E.M., Lindsley, R.C., Cai, M., Murphy, T.L., Kyba, M., Murphy, K.M., 2011. Snail and the microRNA-200 family act in opposition to regulate epithelial-to-mesenchymal transition and germ layer fate restriction in differentiating ESCs. Stem Cells 29, 764–776.

Gurney, S.M., Forster, P., Just, U., Schwanbeck, R., 2011. Suppression of the PI3K subunit p85alpha delays embryoid body development and inhibits cell adhesion. J. Cell. Biochem. 112, 3573–3581.

Hamazaki, T., Oka, M., Yamanaka, S., Terada, N., 2004. Aggregation of embryonic stem cells induces Nanog repression and primitive endoderm differentiation. J. Cell Sci. 117, 5681–5686.

Haworth, K.E., Kotecha, S., Mohun, T.J., Latinkic, B.V., 2008. GATA4 and GATA5 are essential for heart and liver development in *Xenopus* embryos. BMC Dev. Biol. 8, 74.

Heldin, C.H., Miyazono, K., ten Dijke, P., 1997. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature 390, 465–471.

Hua, G., Zhu, B., Rosa, F., Deblon, N., Adelaide, J., Kahn-Perles, B., Birnbaum, D., Imbert, J., 2009. A negative feedback regulatory loop associates the tyrosine kinase receptor ERBB2 and the transcription factor GATA4 in breast cancer cells. Mol. Cancer Res. 7, 402–414.

Huang, P., He, Z., Ji, S., Sun, H., Xiang, D., Liu, C., Hu, Y., Wang, X., Hui, L., 2011. Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. Nature 475, 386–389.

Inman, G.J., Nicolas, F.J., Callahan, J.F., Harling, J.D., Gaster, L.M., Reith, A.D., Laping, N.J., Hill, C.S., 2002. SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol. Pharmacol. 62, 65–74.

Itskovitz-Eldor, J., Schuldiner, M., Karsenti, D., Eden, A., Yanuka, O., Amit, M., Soreq, H., Benvenisty, N., 2000. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol. Med. 6, 88–95.

James, D., Levine, A.J., Besser, D., Hemmati-Brivanlou, A., 2005. TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. Development 132, 1273–1282.

Kanellopoulou, C., Muljo, S.A., Kung, A.L., Ganesan, S., Drapkin, R., Jenuwein, T., Livingston, D.M., Rajewsky, K., 2005. Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. Genes Dev. 19, 489–501.

Keller, G., Kennedy, M., Papayannopoulou, T., Wiles, M.V., 1993. Hematopoietic commitment during embryonic stem cell differentiation in culture. Mol. Cell. Biol. 13, 473–486.

Korpal, M., Ell, B.J., Buffa, F.M., Ibrahim, T., Blanco, M.A., Celia-Terrassa, T., Mercatali, L., Khan, Z., Goodarzi, H., Hua, Y., Wei, Y., Hu, G., Garcia, B.A., Ragoussis, J., Amadori, D., Harris, A.L., Kang, Y., 2011. Direct targeting of Sec23a by miR-200s influences cancer cell secretome and promotes metastatic colonization. Nat. Med. 17, 1101–1108.

Kuo, C.T., Morrisey, E.E., Anandappa, R., Sigrist, K., Lu, M.M., Parmacek, M.S., Soudais, C., Leiden, J.M., 1997. GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. Genes Dev. 11, 1048–1060.

Kyronlahti, A., Ramo, M., Tamminen, M., Unkila-Kallio, L., Butzow, R., Leminen, A., Nemer, M., Rahman, N., Huhtaniemi, I., Heikinheimo, M., Anttonen, M., 2008. GATA-4 regulates Bcl-2 expression in ovarian granulosa cell tumors. Endocrinology 149, 5635–5642.

Lakshmipathy, U., Love, B., Goff, L.A., Jornsten, R., Graichen, R., Hart, R.P., Chesnut, J.D., 2007. MicroRNA expression pattern of undifferentiated and differentiated human embryonic stem cells. Stem Cells Dev. 16, 1003–1016.

Leahy, A., Xiong, J.W., Kuhnert, F., Stuhlmann, H., 1999. Use of developmental marker genes to define temporal and spatial patterns of differentiation during embryoid body formation. J. Exp. Zool. 284, 67–81.

Lee, D., Shin, C., 2012. MicroRNA-target interactions: new insights from genome-wide approaches. Ann. N. Y. Acad. Sci. 1271, 118–128.

Li, S.S., Yu, S.L., Kao, L.P., Tsai, Z.Y., Singh, S., Chen, B.Z., Ho, B.C., Liu, Y.H., Yang, P.C., 2009. Target identification of microRNAs expressed highly in human embryonic stem cells. J. Cell. Biochem. 106, 1020–1030.

Lim, H.J., Han, J., Woo, D.H., Kim, S.E., Kim, S.K., Kang, H.G., Kim, J.H., 2011. Biochemical and morphological effects of hypoxic environment on human embryonic stem cells in long-term culture and differentiating embryoid bodies. Mol. Cells 31, 123–132.

Lin, C.H., Jackson, A.L., Guo, J., Linsley, P.S., Eisenman, R.N., 2009. Myc-regulated microRNAs attenuate embryonic stem cell differentiation. EMBO J. 28, 3157–3170.

Liu, S., Tetzlaff, M.T., Cui, R., Xu, X., 2012. miR-200c inhibits melanoma progression and drug resistance through down-regulation of BMI-1. Am. J. Pathol. 181, 1823–1835.

Miyoshi, N., Ishii, H., Nagano, H., Haraguchi, N., Dewi, D.L., Kano, Y., Nishikawa, S., Tanemura, M., Mimori, K., Tanaka, F., Saito, T., Nishimura, J., Takemasa, I., Mizushima, T., Ikeda, M., Yamamoto, H., Sekimoto, M., Doki, Y., Mori, M., 2011. Reprogramming of mouse and human cells to pluripotency using mature microRNAs. Cell Stem Cell 8, 633–638.

Molkentin, J.D., 2000. The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. J. Biol. Chem. 275, 38949–38952.

Molkentin, J.D., Lin, Q., Duncan, S.A., Olson, E.N., 1997. Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. Genes Dev. 11, 1061–1072.

Murchison, E.P., Partridge, J.F., Tam, O.H., Cheloufi, S., Hannon, G.J., 2005. Characterization of Dicer-deficient murine embryonic stem cells. Proc. Natl. Acad. Sci. U. S. A. 102, 12135–12140.

Nelson, P., Kiriakidou, M., Sharma, A., Maniataki, E., Mourelatos, Z., 2003. The microRNA world: small is mighty. Trends Biochem. Sci. 28, 534–540.

Osafune, K., Caron, L., Borowiak, M., Martinez, R.J., Fitz-Gerald, C.S., Sato, Y., Cowan, C.A., Chien, K.R., Melton, D.A., 2008. Marked differences in differentiation propensity among human embryonic stem cell lines. Nat. Biotechnol. 26, 313–315.

Rebustini, I.T., Hayashi, T., Reynolds, A.D., Dillard, M.L., Carpenter, E.M., Hoffman, M.P., 2012. miR-200c regulates FGFR-dependent epithelial proliferation via Vldlr during submandibular gland branching morphogenesis. Development 139, 191–202.

Ren, J., Jin, P., Wang, E., Marincola, F.M., Stroncek, D.F., 2009. MicroRNA and gene expression patterns in the differentiation of human embryonic stem cells. J. Transl. Med. 7, 20.

Risau, W., Sariola, H., Zerwes, H.G., Sasse, J., Ekblom, P., Kemler, R., Doetschman, T., 1988. Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies. Development 102, 471–478.

Rojas, A., Kong, S.W., Agarwal, P., Gilliss, B., Pu, W.T., Black, B.L., 2008. GATA4 is a direct transcriptional activator of cyclin D2 and Cdk4 and is required for cardiomyocyte proliferation in anterior heart field-derived myocardium. Mol. Cell. Biol. 28, 5420–5431.

Rong, L., Liu, J., Qi, Y., Graham, A.M., Parmacek, M.S., Li, S., 2012. GATA-6 promotes cell survival by up-regulating BMP-2 expression during embryonic stem cell differentiation. Mol. Biol. Cell 23, 3754–3763.

Samavarchi-Tehrani, P., Golipour, A., David, L., Sung, H.K., Beyer, T.A., Datti, A., Woltjen, K., Nagy, A., Wrana, J.L., 2010. Functional genomics reveals a BMP-driven mesenchymal-to-epithelial transition in the initiation of somatic cell reprogramming. Cell Stem Cell 7, 64–77.

Sanchez, A., Jones, W.K., Gulick, J., Doetschman, T., Robbins, J., 1991. Myosin heavy chain gene expression in mouse embryoid bodies. An in vitro developmental study. J. Biol. Chem. 266, 22419–22426.

Serrano, F., Calatayud, C.F., Blazquez, M., Torres, J., Castell, J.V., Bort, R., 2013. Gata4 blocks somatic cell reprogramming by directly repressing Nanog. Stem Cells 31, 71–82.

Shimono, Y., Zabala, M., Cho, R.W., Lobo, N., Dalerba, P., Qian, D., Diehn, M., Liu, H., Panula, S.P., Chiao, E., Dirbas, F.M., Somlo, G., Pera, R.A., Lao, K., Clarke, M.F., 2009. Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. Cell 138, 592–603.

Soudais, C., Bielinska, M., Heikinheimo, M., MacArthur, C.A., Narita, N., Saffitz, J.E., Simon, M.C., Leiden, J.M., Wilson, D.B., 1995. Targeted mutagenesis of the transcription factor GATA-4 gene in mouse embryonic stem cells disrupts visceral endoderm differentiation in vitro. Development 121, 3877–3888.

Suh, N., Blelloch, R., 2011. Small RNAs in early mammalian development: from gametes to gastrulation. Development 138, 1653–1661.

Suh, M.R., Lee, Y., Kim, J.Y., Kim, S.K., Moon, S.H., Lee, J.Y., Cha, K.Y., Chung, H.M., Yoon, H.S., Moon, S.Y., Kim, V.N., Kim, K.S., 2004. Human embryonic stem cells express a unique set of microRNAs. Dev. Biol. 270, 488–498.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., Jones, J.M., 1998. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145–1147.

Tsai, Z.Y., Singh, S., Yu, S.L., Kao, L.P., Chen, B.Z., Ho, B.C., Yang, P.C., Li, S.S., 2010. Identification of microRNAs regulated by activin A in human embryonic stem cells. J. Cell. Biochem. 109, 93–102.

Valdimarsdottir, G., Mummery, C., 2005. Functions of the TGFbeta superfamily in human embryonic stem cells. Apmis 113, 773–789.

Vallier, L., Alexander, M., Pedersen, R.A., 2005. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. J. Cell Sci. 118, 4495–4509.

Vallier, L., Mendjan, S., Brown, S., Chng, Z., Teo, A., Smithers, L.E., Trotter, M.W., Cho, C.H., Martinez, A., Rugg-Gunn, P., Brons, G., Pedersen, R.A., 2009. Activin/Nodal signalling maintains pluripotency by controlling Nanog expression. Development 136, 1339–1349.

Wang, R., Clark, R., Bautch, V.L., 1992. Embryonic stem cell-derived cystic embryoid bodies form vascular channels: an in vitro model of blood vessel development. Development 114, 303–316.

Wang, L., Tanaka, S., Ramirez, F., 2005. GATA-4 binds to an upstream element of the human alpha2(I) collagen gene (COL1A2) and inhibits transcription in fibroblasts. Matrix Biol. 24, 333–340.

Wang, Y., Medvid, R., Melton, C., Jaenisch, R., Blelloch, R., 2007. DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. Nat. Genet. 39, 380–385.

Wang, Y., Keys, D.N., Au-Young, J.K., Chen, C., 2009. MicroRNAs in embryonic stem cells. J. Cell. Physiol. 218, 251–255.

Wang, C.H., Ma, N., Lin, Y.T., Wu, C.C., Hsiao, M., Lu, F.L., Yu, C.C., Chen, S.Y., Lu, J., 2012a. A shRNA functional screen reveals Nme6 and Nme7 are crucial for embryonic stem cell renewal. Stem Cells 30, 2199–2211.

Wang, J., Cao, N., Yuan, M., Cui, H., Tang, Y., Qin, L., Huang, X., Shen, N., Yang, H.T., 2012b. MicroRNA-125b/Lin28 pathway contributes to the mesendodermal fate decision of embryonic stem cells. Stem Cells Dev. 21, 1524–1537.

Wang, Z., Oron, E., Nelson, B., Razis, S., Ivanova, N., 2012c. Distinct lineage specification roles for NANOG, OCT4, and SOX2 in human embryonic stem cells. Cell Stem Cell 10, 440–454.

Wang, G., Guo, X., Hong, W., Liu, Q., Wei, T., Lu, C., Gao, L., Ye, D., Zhou, Y., Chen, J., Wang, J., Wu, M., Liu, H., Kang, J., 2013. Critical regulation of miR-200/ZEB2 pathway in Oct4/Sox2-induced mesenchymal-to-epithelial transition and induced pluripotent stem cell generation. Proc. Natl. Acad. Sci. U. S. A. 110, 2858–2863.

Xu, R.H., Sampsell-Barron, T.L., Gu, F., Root, S., Peck, R.M., Pan, G., Yu, J., Antosiewicz-Bourget, J., Tian, S., Stewart, R., Thomson, J.A., 2008. NANOG is a direct target of TGFbeta/activin-mediated SMAD signaling in human ESCs. Cell Stem Cell 3, 196–206.

Yu, J., Ohuchida, K., Mizumoto, K., Sato, N., Kayashima, T., Fujita, H., Nakata, K., Tanaka, M., 2010. MicroRNA, hsa-miR-200c, is an independent prognostic factor in pancreatic cancer and its upregulation inhibits pancreatic cancer invasion but increases cell proliferation. Mol. Cancer 9, 169.

Zhang, C., Ye, X., Zhang, H., Ding, M., Deng, H., 2007. GATA factors induce mouse embryonic stem cell differentiation toward extraembryonic endoderm. Stem Cells Dev. 16, 605–613.