博士論文

Characterization of pluripotent cells by profiling microRNA expression pattern in human and mouse ES and iPS cells

(ES、iPS細胞におけるmicroRNA発現パターンの解析による幹細胞のキャラクタリゼーション)

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Abstract

Using quantitative PCR-based miRNA arrays, I have comprehensively analyzed the expression profiles of miRNAs in human and mouse embryonic stem (ES), induced pluripotent stem (iPS), and somatic cells. Immature pluripotent cells used for miRNA profiling were purified using the SSEA-1 or SSEA-4 antibody. Hierarchical clustering and consensus clustering by nonnegative matrix factorization showed separation of two major clusters, human ES/iPS cells and other cell groups, as previously reported. Principal components analysis (PCA) that segregates miRNAs into these two groups identified miR-187, 299-3p, 499-5p, 628-5p, and 888 as new miRNAs that specifically characterize human ES/iPS cells. Detailed direct comparisons of miRNA expression levels in human ES and iPS cells showed that several miRNAs included in the chromosome 19 miRNA cluster were more strongly expressed in iPS cells than in ES cells. Similar analysis was conducted with mouse ES/iPS cells and somatic cells, and several miRNAs were suggested to be ES/iPS cell-specific, which have not been reported to be expressed in mouse ES/iPS cells. ES/iPS cells in humans and mice showed quite similar average expression levels of miRNAs. Expression levels of miRNA showed drastic and different patterns of changes during embryoid body formation. In summary, my miRNA expression profiling, encompassing human and mouse ES and iPS cells, would give various perspectives in understanding the miRNA core regulatory networks which regulate the characteristics of pluripotent cells.

Abbreviations

miRNA	microRNA
ES	embryonic stem
iPS	induced pluripotent stem
SSEA-1	stage-specific embryonic antigen-1/ CD15
SSEA-4	stage-specific embryonic antigen-4
b-FGF	basic fibroblast growth factor
EGF	epidermal growth factor
TGF-β	transforming growth factor-β
LIF	leukemia inhibitory factor
OSKM	Oct3/4, Sox2, Klf4, c-myc
C19MC	chromosome 19 miRNA cluster
HC	Hierarchical clustering
NMF	Non-matrix factorization
PCA	Principal component analysis
PC	Principal Component

Introduction

Pluripotent stem cells were first discovered in the inner cell mass of blastocyst-stage embryos [1]. Embryonic stem (ES) cells can be expanded in vitro while maintaining its undifferentiated properties and possessing the capacity to differentiate into various types of cells. The unique pluripotent characteristics offered by ES cells bring hope to the goal of regenerative medicine; generation of tissues or organs in vitro and usage in the treatment of various diseases. However, utilization of ES cells for clinical purposed is challenging due to several ethical issues pertaining to the used of embryos. To tackle this issue, a group from Kyoto University has identified a set of transcription factors that's responsible for the maintenance of ES cells identity. The human-made pluripotent cells which were first reprogrammed from mouse somatic cells with the introduction of Oct3/4, Sox2, Klf-4, and c-Myc (OSKM) were then termed as induced pluripotent stem (iPS) cells [2]. The OSKM are pluripotent associated transcription factors that are conserved between mouse and human, and were shown to successfully reprogram fibroblast cells to iPS cells [2, 3]. The use of OSKM has been shown to cause global reversion of the somatic epigenome to ES cell state, resulted in the generation of iPS cells that are epigenetically similar to ES cells in terms of the expression of cell surface markers, differentiation capacity, teratoma formation, germline transmission and chimera development [4]. These criteria are phenotypically used to define or distinguish ES and iPS cells from somatic cells.

Thanks to the great potential offer by iPS cells, rapid progresses have been done in investigating the capacity of other somatic cells to be reprogrammed into pluripotent cells. Several studies have explored the use of neural stem cells [5], blood cells [6], renal

tubular cells from urine [7], and others as a promising source to generate iPS cells in human. On the other hand, several groups are also focusing on the improvement of the reprogramming efficiency by using small molecule or chemical compounds such as valproic acid [8] and vitamin C [9] that alter DNA methylation or histone modification. Furthermore, studies on improving the methods of delivering the reprogramming factors have received a lot of attention. The reprogramming delivery systems are now not limited to only retrovirus [2], but expanded to the use of lentivirus [10], adenovirus [11], sendai virus [12], small RNA [13] and recombinant protein [14].

However, differences in the choice of somatic cells sources and reprogramming methods could cause the variations among iPS cells and affect the application of iPS cells, particularly for the clinical use. On the other hand, many studies have focused on the examination of the regulatory networks linking chromatin structure and gene expression programs [15], as well as mRNA and microRNA (miRNA) expression profiles [15,16] of iPS cells and ES cells, and improved the understanding of genomic and epigenomic networks underlying reprogramming, self-renewal, and cell fate decisions. One regulatory component that has received increasing attention is miRNAs. A miRNA is transcribed from a miRNA gene into a primary miRNA with stem loop structure, followed by generation of a 60-70 nt long precursor miRNA (pre-miR) before forming a mature miRNA with 18-22 nt. Mature miRNAs have the ability to regulate many target genes and control gene expression through the translational repression and degradation [17]. miRNAs are expressed at different levels in a wide range of cells, including ES [18-20], iPS [21], and somatic cells [21]. Differential expression of miRNAs can be used as markers to classify cell types such as iPS and ES cells. Previous studies have identified several stem cell-specific miRNAs that are expressed abundantly in human and mouse

pluripotent cells. This includes members of the miR-302 cluster [20-23], which are transcribed as a single transcript from the same miRNA cluster and highly related to each other. Other known stem cell-specific miRNA clusters are the chromosome 19 microRNA cluster (C19MC) including miR-517a, miR-519b, miR-520b, miR-520b, and miR-521 which are highly expressed only in human stem cells [18], and the miR-290 cluster with high expression levels in mouse stem cells [19].

Considering the importance of miRNAs to serve as a cell signature, I further focused on the identification of new miRNAs that can be used to define the pluripotency in ES and iPS cells. The identification of new miRNAs is important to improve the current understanding of miRNA regulation in regards to the reprogramming, maintenance of the self-renewal and determination of cell lineage. The selection of method is very crucial to obtain a reliable miRNA profiling and miRNAs can be detected by various methods such as sequencing [18], Northern blot [19], microarray [20,21], and others. Each technique has its advantages and disadvantages; sensitivity, cost, sequence dependence, and possible artifact from contamination. Therefore, different approaches and/or experimental settings may result in different miRNA expression profiling.

The miRNA profiling of pluripotent and differentiated cell lines of human and mouse cells has not been analyzed yet at the same time. In this study, I took the advantage of the miRNA array system with optimized experimental settings to improve the current understanding of miRNA profiles in both pluripotent and non-pluripotent cells. I identified new miRNAs, which are specific to pluripotent cells from human, and mouse. I also focused on the miRNAs that can be used to distinguish between human and mouse, which will provide an insight into the similarity and differences between the human and mouse pluripotent cells in term of miRNA expression profiles.

Materials and Methods

Culture of pluripotent stem cells

Human ES and iPS cell lines were used in conformity with the guidelines for derivation and utilization of human ES cells outlined by The Ministry of Education, Culture, Sports, Science and Technology, Japan. The human ES (HUES3) was kindly provided by Dr. Michiue (the Uni. of Tokyo). Other human pluripotent cell lines were provided by Stem Cell Bank of Institute of Medical Science, The University of Tokyo. The human pluripotent cells used in this work were previously published, and are listed in Table 1 with references. Human iPS cells were maintained as described previously with some modification [2]. Briefly, human iPS and two human ES (khES3, H1) cells were maintained in DMEM/F12 (Sigma) supplemented with 20% knockout serum replacement (Invitrogen), 1% MEM non-essential amino acid (Invitrogen), 1% L-Glutamine (Invitrogen), 0.2% 2-mercaptoethanol (Invitrogen) and 5ng/ml basic-FGF (Upstate). For splitting cells, 0.05% Trypsin-EDTA (Sigma) was used with or without 10μM Rock inhibitor (TOCRIS). Other human ES cells (HUES3, HUES8, and HUES10) were maintained under feeder free condition as previously described [25] with modification. Cells were dissociated using CTK solution (ReproCells). All human ES and iPS cells were maintained, and sorted for RNA analysis by members of the Stem Cell Bank.

The B6 iPS#1 and #3 (unpublished) were kindly provided by Prof. Nakauchi and Dr. Otsu (Institute of Medical Science, The University of Tokyo). The SP_miPS and Nanog-iPS38 cells were proved by Prof. Yamanaka (Kyoto University). The mouse ES cells (CCE) was kindly provided by Dr. Nichikawa (CDB) while K3 B6 and ES_RED were provided by Stem Cell Bank (Institute of Medical Science, The University of Tokyo). The mouse iPS

cells were maintained in iPS medium consisting of DMEM high glucose (GIBCO), 15% fetal calf serum (GIBCO), 2% HEPES buffer solution (Nacalai Tesque), 1% L-glutamine (Nacalai Tesque), 1% non-essential amino acid (GIBCO), penicillin/streptomycin (P/S), 1000 U/ml of Leukemia Inhibitory Factor (ESGRO, Chemicon), and 0.2% β-mercaptoethanol (SIGMA). The cells were cultured on 0.1% gelatin-coated plates with monolayer of mitomycin-C treated mouse embryonic fibroblast (MEF). Mouse ES cells (ES_K3_B6 and ES_RED) were cultured in the same culture conditions as iPS cells while ES_CCE was cultured in the same culture medium but without the feeder cells.

Preparation of mouse embryonic fibroblasts (MEF) and tail-tip fibroblasts (TTF)

MEF and TTF were isolated and described in detail in previous paper [2]. Briefly, MEF were obtained by mincing the E12 embryo without internal organs followed by digestion in 0.05% trypsin-EDTA, and strained through 70 μm cell strainer (Falcon, BD Bioscience). Single cells obtained were cultured until confluence. TTF were obtained from adult tail tip by culturing tailbone that was cleaned by removing surrounding muscle and skin. The cleaned bones were then placed on the culture dish and medium was carefully added to the dish. The tail-tip was left undisturbed for two days before fresh medium was added. MEF and TTF were maintained in DMEM (Nacalai Tesque) supplemented with 10% FCS and 0.5% penicillin/streptomyci

Table 1 Cell lines used in this study

Sample name	*Abbreviation*	*Reprogramming conditions*	*Cell source*	*Cell condition*	*Ref*
Undifferentiated human embryonic stem cells (hES cells)					
HUES3	HUES3	-	female	P26-3-3 sen	58
HUES8	HUES8	-	female	P17-4-7, TELA	58
HUES10	HUES10	-	female	P12-13, TELA	58
KhES-3	khES3, khES3_RI	-	female	+/- RI	-
HI	H1	-	female	P57	59
Undifferentiated human induced pluripotent stem cells (hiPS cells), all cells were sorted by SSEA-4					
tkDA 3-4	DA, DA_RI	OSKM, retroV	adult dermal fibroblasts	+/- RI	65
TkCB 7-4	CB, CB_RI	OSKM, retroV	cord blood	+/- RI	65
tkCB Sev9	CB_sev9	OSKM, Sendai V	cord blood		65
tkPB Sev2	PB	OSKM, Sendai V	peripheral blood, CD34-rich		65
tkDN 4-M	DN	OSK, retroV	neonatal dermal fibroblasts		65
TkT 3 V1-7	TKT	OSK, retroV	peripheral blood T cells		60
H25 4 Sev	H254	OSKM, Sendai V	CD8+ T cell clone		60
Embryoid bodies (EBs) and somatic cells					
tkCB7-1 EBd7	EB_d7	-	Differentiated from tkCB7-1 iPS	-	-
tkCB7-1 EBd14	EB_d14	-		-	-
tkCB7-1 EBd21	EB_d21	-		-	-
HEK293	HEK293	-	human embryonic kidney 293	-	61
HeLa	HeLa	-	Adenocarcinoma cervix cell line	-	62
Y79	Y79	-	Retinoblastoma cell line	-	26
HDF Adult	HDF_AD	-	Dermal fibroblast cell	-	-
HDF Neonatal	HDF_N	-		-	-
PBMC_M	PBMC_M	-	Peripheral blood mono nuclear cells		
PBMC_T	PBMC_T	-			-
tkDA3-4 NSC	DA_NSC	-	Neural stem cells from tkDA3-4 iPS	-	-
tkCB Sev9 NSC	CB_NSC	-	Neural stem cells from tkCB Sev9 iPS	-	-
Undifferentiated mouse embryonic stem cells (miPS cells), all cells were sorted by SSEA-1					
CCE	ES_CCE	-	129S6/SyEyTac		1
K3 B6	ES_K3_B6	-	129Sv x B6		-
(2)RED2i	ES_RED	-	129S6/B6-F1		63
Undifferentiated mouse induced pluripotent stem cells (miPS cells), all cells were sorted by SSEA-1					
SP miPS	SP_iPS	OSKM, retroV	MEF (B6)		65
Nanog-ips 38	Nanog_iPS	OSKM, retroV	MEF (B6)	Sorted GFP+	-
B6 iPS#1	B6_iPS_1	OSKM, retroV	MEF (B6)		-
B6 iPS#3	B6_iPS_3	OSKM, retroV	MEF (B6)		-
Embryoid bodies and others					
EB_ SP	EB_SP_iPS	-	SP miPS	Day 15 EBs	-

EB_ Nanog 38	EB_Nanog_iPS	-	Nanog-38 ips	Day 15 EBs	-
mouse embryonic fibroblast	MEF		ICR		64
tail tip fibroblast	TTF		ICR		-

Abbreviation: OSKM; Oct3/4, Sox2, Klf4, c-Myc, retroV; retrovirus, sendai V; sendai virus, RI; Rock Inhibitor

Culture of cell lines, primary cells, and preparation of PBMC

HeLa and HEK293 cells were maintained in DMEM (Nacalai Tesque) supplemented with 10% FCS and 0.5% penicillin/streptomycin. Y79 [26] was obtained from the Riken Cell Bank (identification number RCB1645) and maintained in RPMI1640 (Nacalai Tesque) supplemented with 10% FCS and penicillin/streptomycin. Peripheral blood mononuclear cells (PBMC) were prepared by members of Stem Cell Bank (Institute of Medical Science, University of Tokyo) using a standard density gradient-separation technique. Documented informed consent was obtained. This study has been performed according to the Declaration of Helsinki, and the process involved has also been approved by the institutional review board (Institute of Medical Science, University of Tokyo Ethics Committee reference No. 20-8-0826). Human adult and fetal dermal cells (HDF_AD and HDF_N) were purchased from Cell Applications Inc. through Japanese trader TOYOBO and maintained in DMEM supplemented with 10% FBS, L-glutamine and 0.5% penicillin/streptomycin.

Neural induction of human iPS cells

We prepared neural induction according to protocol that was described previously [27] Briefly, human iPS cell cultures were dissociated using 0.25% trypsin, and plated on gelatin for 1 h at 37°C in the presence of Rock inhibitor (Y-27632, Wako) to remove

MEF. The nonadherent iPS cells were plated on Matrigel (Becton, Dickinson and Company) coated dishes at a density of 10,000 cells/cm^2 in MEF-conditioned iPS-medium supplemented with 10 ng/ml of b-FGF (Peprotech) and Rock inhibitor. The iPS cells were allowed to expand for 3 days, and the initial differentiation was induced by replacing media with knockout serum replacement media supplemented with 10 μM TGF-β inhibitor (SB431542, Tocris) and 200 ng/ml of Noggin (R&D). From day 4, increasing amounts of N2/B27 medium (Neurobasal, 1% N2 supplement, 2% B27 supplement, 1% L-Glutamine, P/S) was added to the culture every 2 days (25%, 50%, 75%). Upon day 10 of differentiation, cells were passaged en bloc onto Matrigel-coated dishes in N2/B27 media supplemented with 10 ng/ml b-FGF and 10 ng/ml EGF. The growing cells were dissociated and passaged every 7-10 days in the N2/B27 media supplemented with b-FGF and EGF. To examine neurogenic potential of these cells, differentiation was induced by the removal of growth factors and the addition of FCS. Multi-lineage differentiation was confirmed by immunostaining of neuronal (βIII tubulin) and astroglial (S-100β) markers, thereby iPS-derived cells were defined as neural stem/progenitor cells (NSCs).

Preparation of immature pluripotent cells

We cultured human and mouse iPS cells at appropriate density and were grown exponentially on 6-cm dishes containing pre-irradiated MEF feeder cells. On day 4 or 5, the cells were harvested by trypsinization and stained with anti-SSEA-4 (for human cells) or -SSEA-1 (for mouse cells) antibodies. Subsequently, the SSEA-4 or SSEA-1 positive cells ($5x10^5$ cells/tube) were sorted by FACS (Moflo, Dako Cytomation) into collection

tubes containing 200 μl of 2.5% FCS in PBS. Cells were immediately collected as pellets by centrifugation, snap frozen in liquid nitrogen, and stored at -80°C until used. Mouse ES, CCE cells are cultured in feeder free system. Feeder free culture of human ES cells were done by Dr. Michiue (Univ. Tokyo).

Preparation of embryoid bodies (EBs)

EB formation of human iPS cells was carried out following previously reported procedures [28]. The EBs were harvested at day 7, 14 and 21, stained and sorted for SSEA-4 negative cell by members of the Stem Cell Bank. Mouse EBs were obtained by culturing iPS cells on a petri dish in the absence of LIF. Briefly, iPS cells were detached and collected cells were cultured for 30 minutes in a gelatin coated tissue culture dish to separate iPS cells from MEF feeder cells. Then, suspension cells were collected and further cultured as suspension in non-coated petri dishes. At day 15 of differentiation, cells were harvested, stained and sorted for SSEA-1 negative cells. Cells were all prepared under RNase-free condition.

RNA extraction and miRNA examination

Total RNA was extracted according to the manufacturer protocol using miRVana miRNA isolation kit (Ambion). In some cases as noted in the manuscript, cells were underwent RNA purification using RNeasy Plus Micro Kit (Qiagen). RNA (500 ng) was reverse transcribed using Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems) and Megaplex RT primers Human Pool A or Rodent Pool A (Applied Biosystems). Then, cDNA was mixed with EagleTaq Master Mix with Rox (ROCHE) and was dispensed into

each port of the TaqMan human or rodent MicroRNA Array A card v2.0 (Applied Biosystems). Human Array A card contains primers for 381 miRNAs including 3 positive control miRNAs, and 1 negative control primer. Rodent Array A card contains 341 primers for mouse miRNAs including 5 positive control miRNAs, and 1 negative control primer. Each miRNA card was run for real-time PCR using ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). The results were analyzed with SDS 2.4 and RQmanager 1.2.1 software (Applied Biosystems).

Data Analysis

Hierarchical clustering (HC), non-negative matrix factorization (NMF) and principal component analysis (PCA) were done using MeV 4.8.1 (Multi Experiment Viewer) software (http://www.tm4.org/mev/) according to the manufacturer's instruction. The miRNAs expression data was clustered using average hierarchical clustering using Euculidean distance. Average value of distance of each miRNA was used to measure the cluster-to-cluster distance. NMF [29] was computed on miRNA expression profiles. We determined the minimum number of metaprofiles necessary to separate pluripotent cells from differentiated cells. PCA [30] was carried out on all genes under investigation to determine expression trends within the samples. A sample trend is shown in a scatter plot of the principal components PC1, PC2 and PC3.

Results

miRNA Expression Profiling of Pluripotent Stem Cells Using Quantitative PCR Arrays

To perform a comprehensive profile of miRNA expression patterns in human pluripotent stem cells, I used six ES cell lines, nine iPS cell lines, EBs at three different time points from one iPS cell line, two iPS cell-derived NSCs, and four primary tissues. Three cancer cell lines were also used as control somatic cells. For mouse samples, I profiled three ES cell lines, four iPS cell lines, two iPS-derived EBs, MEF, and TTF. The human iPS cells were generated by three or four reprogramming factors, and were transduced using different delivery methods and source of cells (Table 1). I used human and mouse arrays covering 377 and 335 miRNAs, respectively.

To begin with, I optimized a protocol to prepare cells for the accurate examination of miRNA expression patterns. Since some of the iPS and ES cells were cultured on MEF feeder cells, I examined whether the purification of the iPS cells is necessary or not. I compared the miRNA expression profiles of the unsorted and the sorted iPS cells using The SSEA-4 or SSEA-1 antibody. The miRNA expression profiles using the purified samples gave lower Ct values in the most of the miRNAs analyzed. This indicated that the contamination of the RNA from feeder cells reduced the sensitivity of detection. Hence, I purified human and mouse pluripotent cells with the SSEA-4 or SSEA-1 antibody before subsequent analysis in this study.

Next, I validated the reproducibility of the qPCR array method, and analysed the miRNA expression patterns of three independent samples from tkCB7-4 cells using the human array. They showed good reproducibility, especially for the miRNAs known to be expressed at relatively high levels in pluripotent cells. In summary, there were 124

miRNAs with average ΔCt values of less than 10, 115 miRNAs of which showed standard deviations (SD) of less than 1. Although nine miRNAs showed SD more than 10, they were caused by false negative or false positive samples. Due to the high reproducibility of this methods, all data were taken from single experiment

Average ΔCt values for miRNAs were compared between human ES and iPS cell group (hES/hiPS-g) and the somatic cell, cancer cell, and NSC group (hSomatic-g). I found statistically significant differences between the two groups and listed the top 50 miRNAs with by low average ΔCt values (high expression levels) in hES/hiPS-g (Fig. 1A, blue bar), with the corresponding values in hSomatic-g (Fig. 1A, red bar). The list included the miRNAs that were previously reported to be highly expressed in ES or iPS cells, such as miR-302 cluster [18,20,21,23,24], miR-17-92 cluster miRNAs [18,21], and C19MC members [18], as expected. More than half of the top 50 miRNAs in hES/hiPS-g showed higher expression levels than in hSomatic-g. Previous studies have shown that p53 and its downstream effector p21 are induced during reprogramming, and minimizing the expression of p53 and p21 enhances iPS cell formation [32-36]. The miRNAs that were reported to suppress p21 [37] showed high expression levels both in hES/hiPS-g and in hSomatic-g (Fig. 1A, purple asterisks). Next, I listed the top 50 miRNAs based on the differences in ΔCt values between hES/hiPS-g and hSomatic-g (Fig. 1B). Notably, the list contained 28 human-specific miRNAs including 23 members of the C19MC. Since 94 of 381 miRNAs are human-specific in the array, human-specific miRNAs were enriched in the highly expressed group of miRNAs in hES/hiPS-g (56% vs 25%). On the contrary, only two human-specific miRNAs were included in the top 50 miRNAs with higher expression levels in hSomatic-g than in hES/hiPS-g (Fig. 1C). Members of the let-7 group,

which are involved in developmental timing and expressed at higher levels in fibroblasts than in ES cells, were on the list (Fig. 1C), as reported previouly [21].

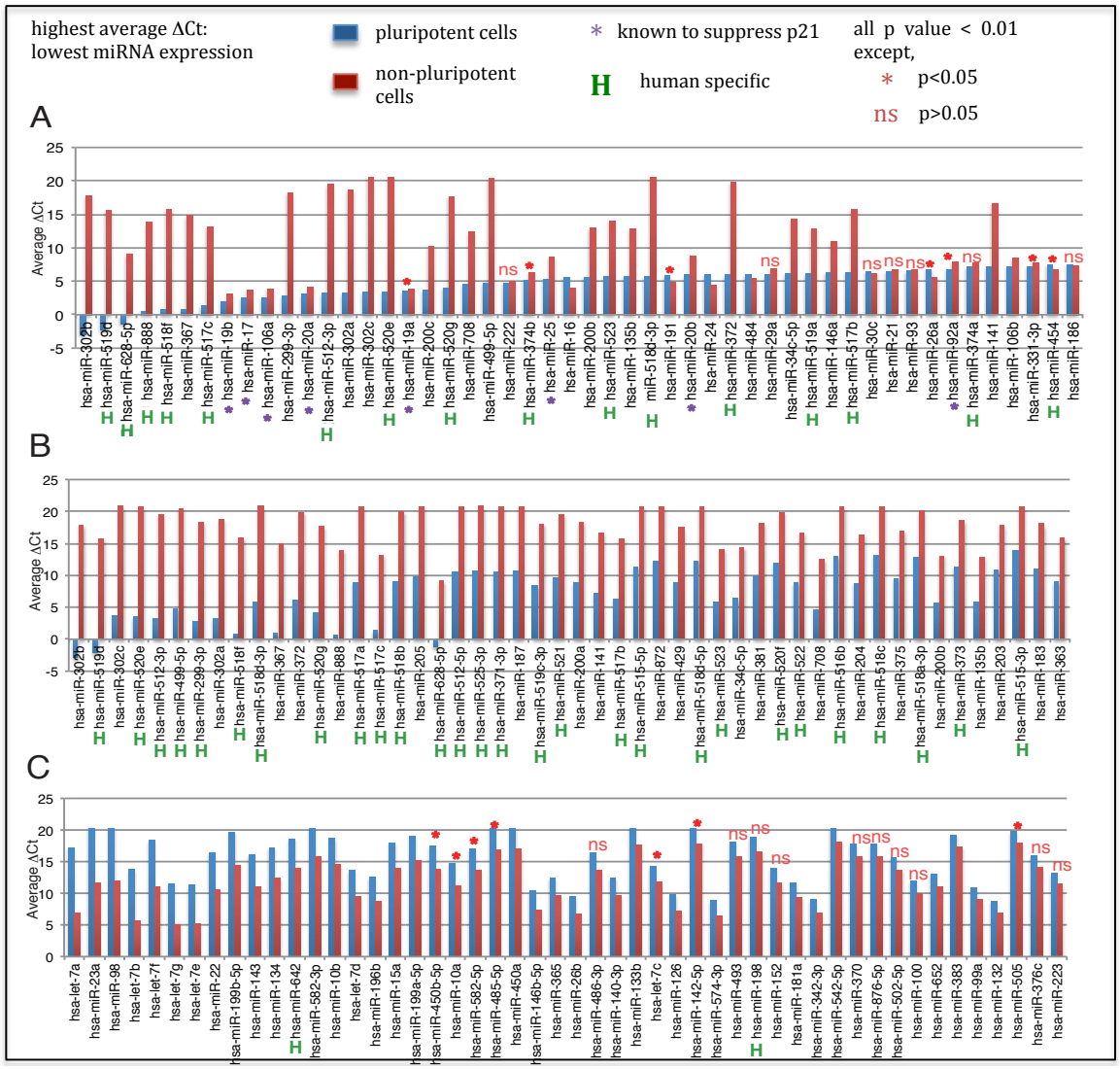


Fig. 1 Comparison of the expression level of miRNAs in human pluripotent stem cells and human somatic cells. The expression level of miRNAs in various cells was examined by qPCR based array, and the ΔCt value was calculated as described. Average of ΔCt of immature ES/iPS (hES/hiPS-g, blue bar) and somatic cells and iPS derived NSCs (hSomatic-g, red bar) was calculated with standard deviation. A. Top 50 miRNAs from the highest expression level in ES/iPS are shown. miRNAs labeled with "H" are human specific miRNAs. B, C. Top 50 miRNAs from biggest difference of ΔCt between hES/hiPS-g and hSomatic-g are shown, as well as values in hES/hiPS-g is bigger (B) and in hSomatic-g is bigger (C). In A-C. p value: non marked bars < 0.01. * <0.05, ns not significant. Purple asterisk indicates miRNAs which have suppressive to p21 expression [37].

Analysis of Human miRNA Profiles

For clustering analysis, I have selected miRNAs that have Ct values greater than 30 in all samples. Following that, 118 miRNAs were excluded, and the remaining 263 miRNAs were subjected to HC (Fig. 2A) and NMF (Fig. 2B) based on ΔCt values [29]. Result showed that both analyses clearly segregate ES/iPS cells from other cells (Fig. 2A, B). The somatic cells, which consist of peripheral blood mononuclear cells (PBMC) and dermal fibroblast cells, were closely clustered together, but clearly separated from the ES/iPS cells. In the ES and iPS cell lines, three of the human ES cell lines were closely clustered. However, the clustering did not group the iPS cells based on cell of origin, methods, or use of ROCK inhibitor (Fig. 2A). This result suggests causing the iPS cells to acquire another set of miRNAs that exclusively belong to the iPS cells.

Next, I further analyzed the miRNA expression profile using PCA [30,38]. Result showed that the first component (PC1) showed clear segregation of ES/iPS and other cells (Fig. 2C). The second and third components segregated dermal fibroblasts and PBMCs (Fig. 2C, D). The PCA listed eigenvector values of each miRNAs, and the miRNAs with eigenvector values greater than 2 are shown in Table 2. Most of the listed miRNAs are members of the miR-302 cluster and two human-specific C19MC clusters (the miR-371/372/373 and miR-512~ clusters), and these miRNAs were previously reported to be expressed in ES and iPS cells [18, 20-24, 39,40]. Among the list, miR-187, 299-3p, 499-5p, 628-5p, and 888, have not been reported reported to characterize ES or iPS cells. The miR-299-3p was previously described in ES [40], but not in iPS cells. Other studies also fail to detect miR-299-3p in ES or iPS cells, perhaps because of the insensitivity of the microarray technique used [21]. However, in this study, I have clearly showed that miR-

187, 299-3p, 499-59, 638-5p, and 888 are strongly expressed in all ES/iPS cell lines, but not in other cells, including both primary cells and cancer cell lines (Fig. 2E).

Table 2: Human miRNAs with eigenvectors of the first component of more than 2 in absolute value.

miRNA	eigenvector
hsa-miR-302b	4.18252
hsa-miR-519d	3.6681
hsa-miR-520e	3.60713
hsa-miR-499-5p	3.46634
hsa-miR-302c	3.45615
hsa-miR-299-3p	3.35883
hsa-miR-512-3p	3.13687
hsa-miR-302a	3.04599
hsa-miR-518f	3.03613
hsa-miR-367	2.74841
hsa-miR-888	2.64427
hsa-miR-520g	2.54294
hsa-miR-372	2.4097
hsa-miR-517a	2.24895
hsa-miR-517c	2.19304
hsa-miR-628-5p	2.18733
hsa-miR-518b	2.03204
hsa-miR-187	2.01046
hsa-miR-525-3p	2.00243

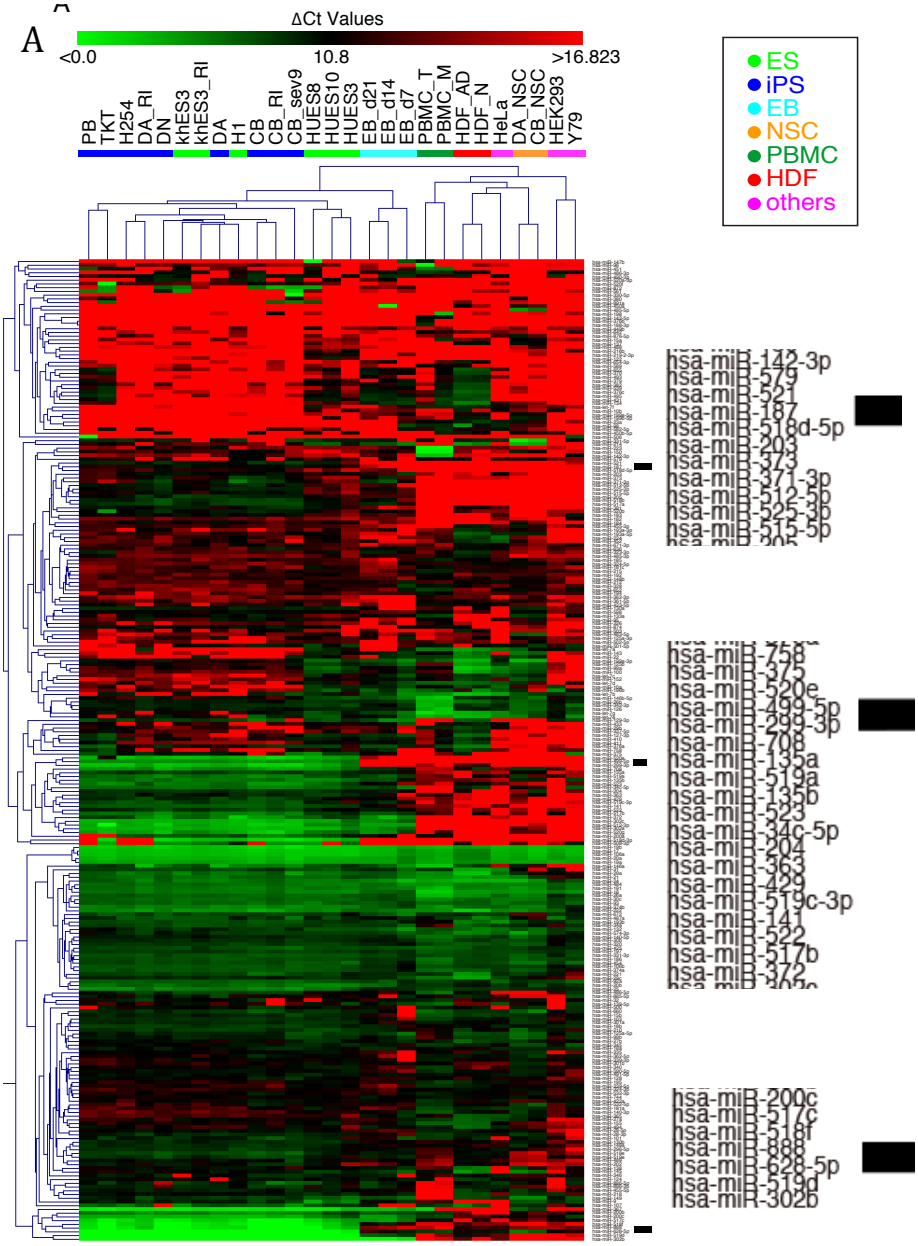


Fig. 2 Clustering analyses of the expression pattern of miRNA in human pluripotent cells, differentiated cells, and somatic cells. (A) Comparison of relative expression levels of 263 miRNAs in human pluripotent cells, differentiated cells, and somatic cells. Red indicates low expression and green indicates high expression.

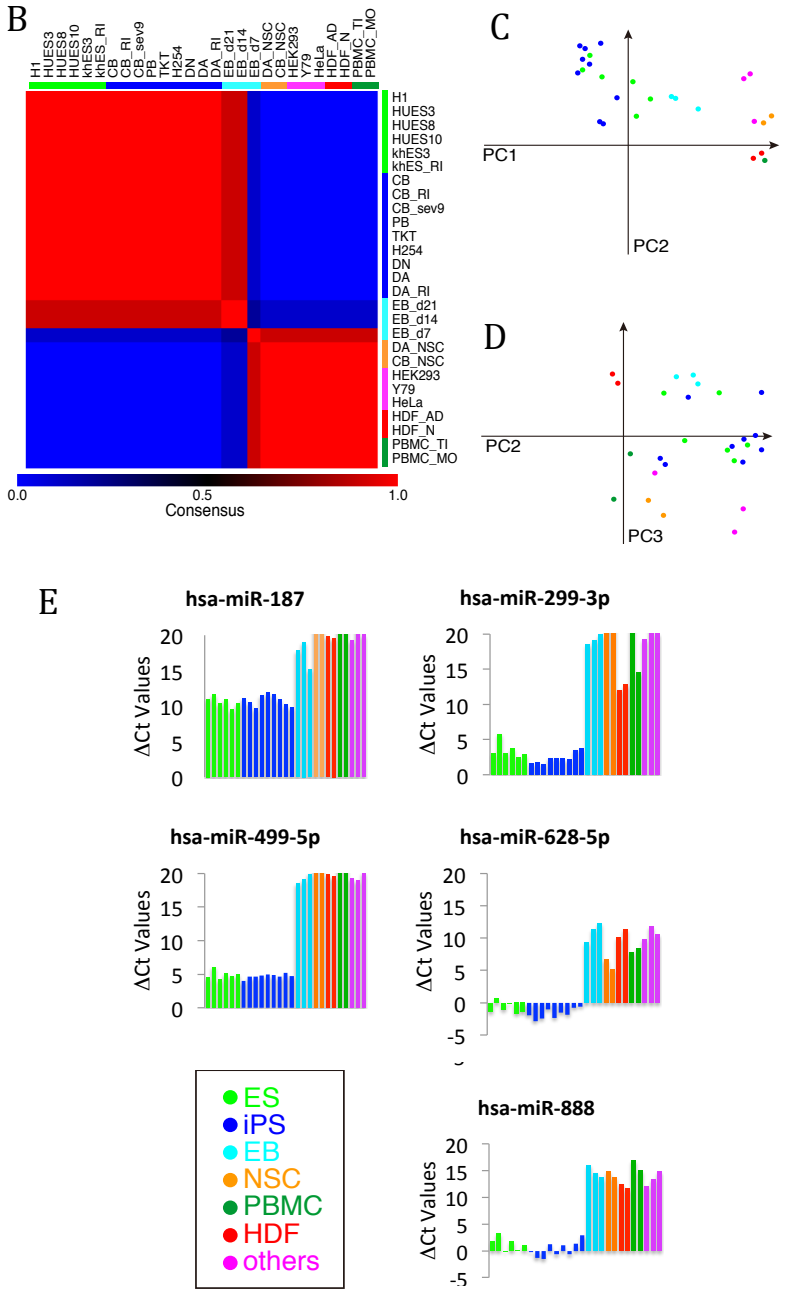


Fig. 2 Clustering analyses of the expression pattern of miRNA in human pluripotent cells, differentiated cells, and somatic cells. (B) Consensus clustering of cells by NMF with two metaprofiles. The NMF-transformed miRNA profiles distinguished two clusters. (C, D) PCA was done using the same set of miRNA above. The first three components clustered cells (the first and second in C, the second and third in D) are shown. The first component accounts for 44.90%, the second for 8.83%, and the third for 8.13% of the system variance. (E) Expression levels of newly identified miRNAs are shown.

Analysis of Mouse miRNA Profiles

As in the human analysis, the Ct values of each miRNA were normalized to mammalian U6. A simple comparison of the average ΔCt values of ES/iPS cells with the average ΔCt values of MEF and TTF showed that members of the 290 and 302 clusters are more highly expressed in ES/iPS cells than in MEF/TTF (Fig. 3A, B). On the other hand, miRNAs that are highly expressed in MEF/TTF are listed in Fig. 3C. Members of let-7 (a, b, c, d, e), and 11 other miRNAs (miR-100, -10a, -10b, -132, -143, -181a, -196b, -199a-5p, -23a, -383, -505) are also listed as miRNA that are expressed at high level in somatic cells in human analysis (Fig. 1C). The result suggests that these miRNAs are expressed at low levels in pluripotent cells, and conserved in both human and mouse.

Similar to human clustering analysis, miRNAs with Ct values greater than 30 in all samples were excluded. The remaining 201 miRNAs (out of 335 miRNAs) were further processed for clustering analysis. Clustering by HC and NMF showed clear separation of ES/iPS cells from MEF/TTF, except for ES_CCE cells (Fig. 4A, B). The PCA also showed good separation, and the first component (PC1) contributed significantly to distinguished these two groups of cells (Fig. 4C, D). The eigenvectors of all miRNAs were obtained from PC1, and miRNAs with absolute values greater than 2 are listed in Table 3. Among the listed miRNAs, the members of miR-290 and 302 clusters are well documented as ES and/or iPS cell-specific miRNAs [41]. However, the miR-133b, 200a, 23a, and 743b-5p have not been previously mentioned to characterize ES or iPS cells. The ΔCt values of these miRNAs showed that the expression of miR-133b and 23a was lower in ES/iPS cells than in MEF/TTF, while miR-200a showed much higher expression in ES/iPS cells than in MEF/TTF (Fig. 4E). In contrast, miR-743-5p showed no difference,

probably because this miRNA had an extremely low value in TTF. However, similar to human cell analysis, the PCA failed to segregate iPS and ES to different categories.

Table 3: Mouse miRNAs which have eigenvectors of the first component more than 2 in absolute value.

miRNA	eigenvector
mmu-miR-292-3p	3.42786
mmu-miR-295	3.33589
mmu-miR-293	3.05248
mmu-miR-291a-3p	3.02925
mmu-miR-302b	2.81198
mmu-miR-302a	2.572
mmu-miR-294	2.52447
mmu-miR-200a	2.51061
mmu-miR-290-3p	2.36788
mmu-miR-302d	2.29456
mmu-miR-367	2.22163
mmu-miR-302c	2.21755
mmu-miR-291b-5p	2.01874
mmu-miR-133b	-2.04838
mmu-miR-23a	-2.27597
mmu-miR-743b-5p	-2.2998

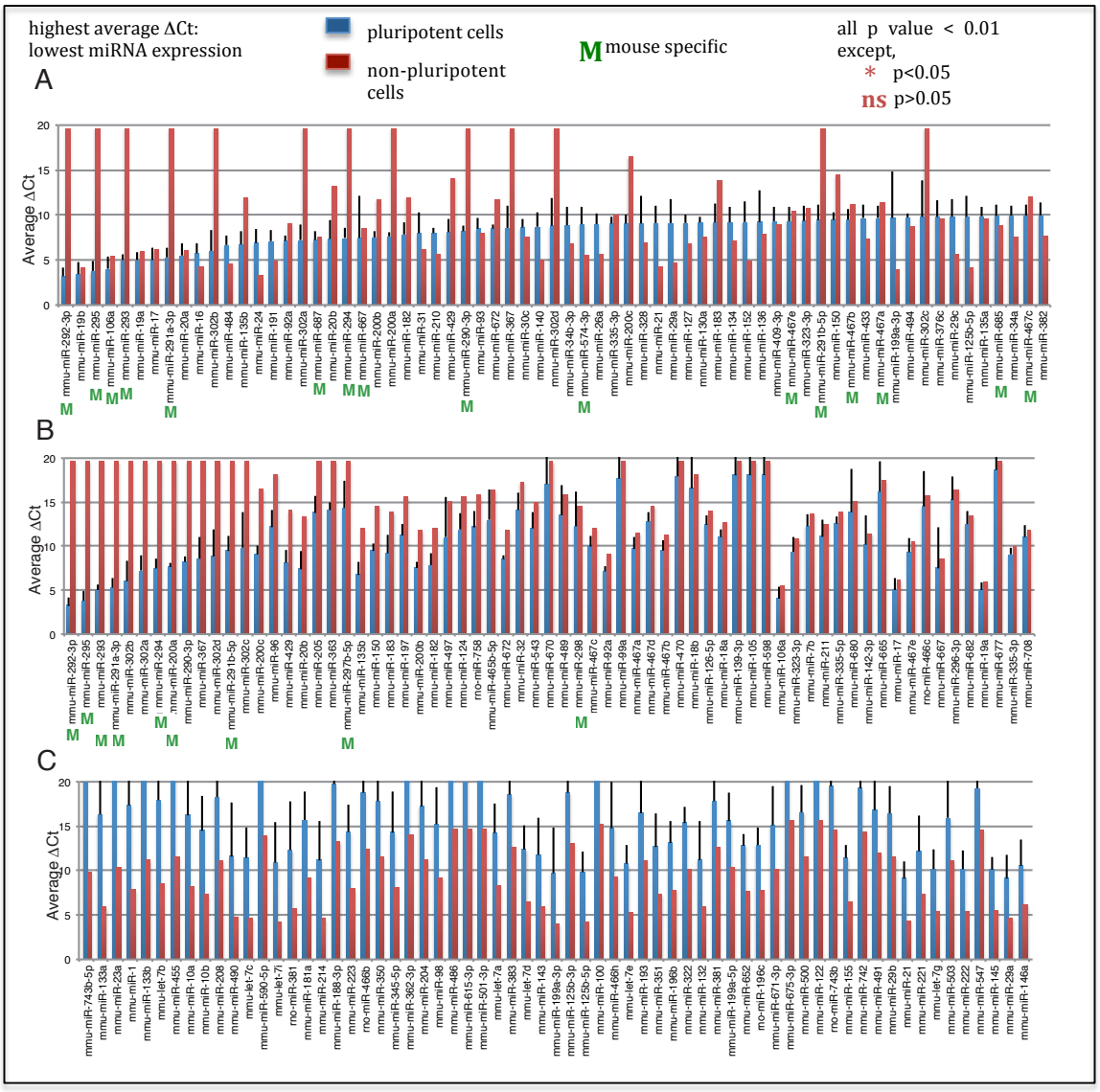


Fig. 3 Comparison of the expression level of miRNAs in mouse pluripotent stem cells and mouse somatic cells. Expression level of miRNAs in various cells was examined by qPCR based array, and the ΔCt value was calculated as described. Average values with standard deviation of ΔCt of immature mouse ES/iPS are shown in blue bars. Average values of ΔCt of MEF and TTF are shown in red bars. A. Top 50 miRNAs from the highest expression level in ES/iPS are shown. miRNAs labeled with "M" are mouse specific miRNAs. B, C. Top 50 miRNAs from the biggest difference of ΔCt between mES/miPS-g and mSomatic-g are shown, as well as values in mES/miPS-g is bigger (B) and in mSomatic-g is bigger (C).

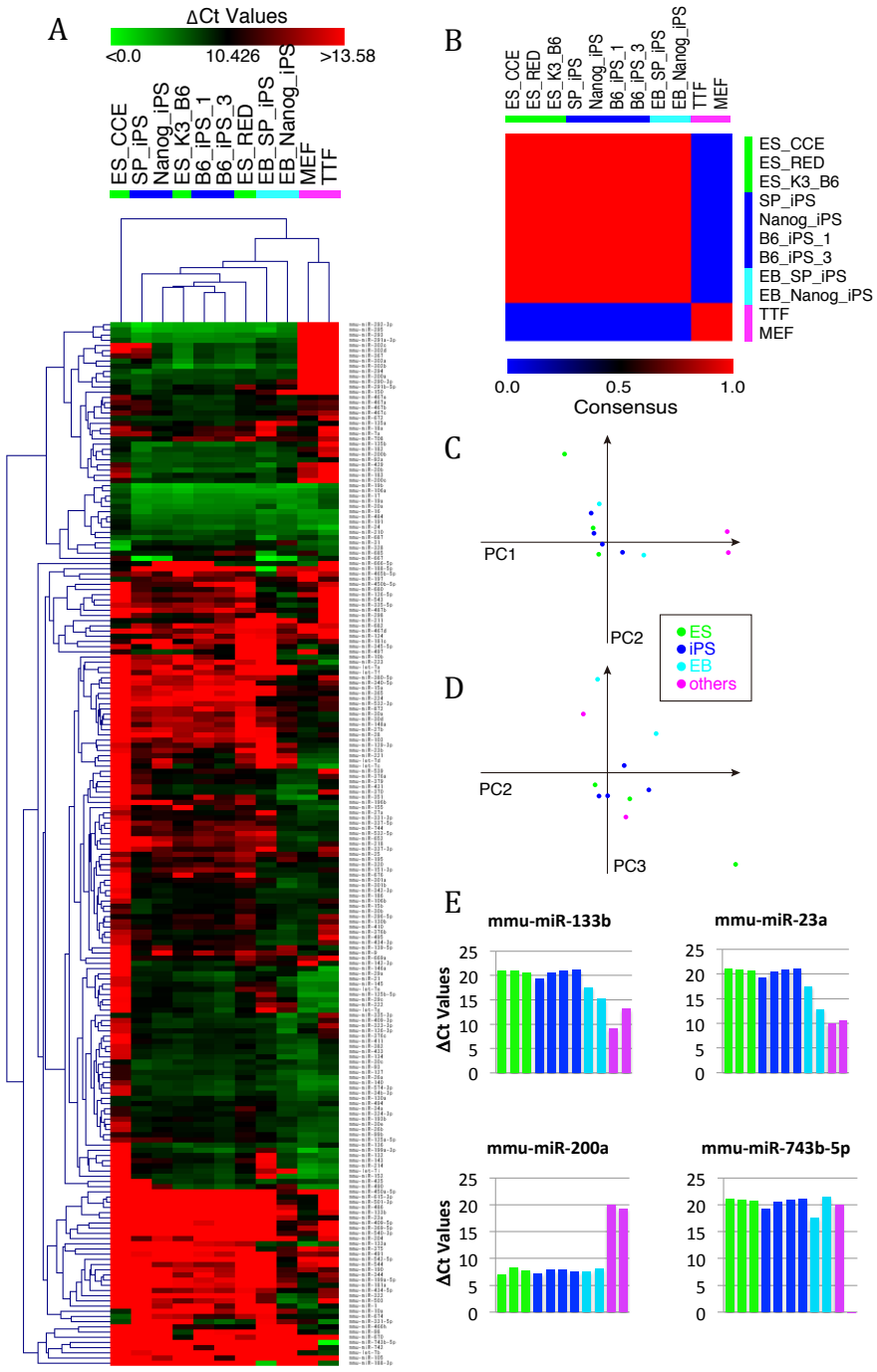


Fig. 4 Clustering analyses of the expression pattern of miRNA in mouse pluripotent cells, differentiated cells, and somatic cells. (A) Comparison of relative expression levels of 201 miRNAs in mouse pluripotent cells, and somatic cells. Red indicates low expression and green indicates high expression. (B) Consensus clustering of cells by NMF with two metaprofiles. The NMF-transformed miRNA profiles distinguished two clusters. (C, D) PCA was done using the same set of miRNA as above. The first three components clustered cells (the first and second in C, the second and third in D) are shown. The first component accounts for 41.99%, the second for 16.62%, and the third for 11.08% of the system variance. (E) Expression levels of newly identified miRNAs are shown.

Identification of miRNAs Distinguishing ES and iPS Cells

A previous study has shown that ES and iPS cells can be distinguished by gene expression signatures [16]. To further analyzed and understand these cells, I further focused on identification of miRNAs that can distinguish human ES and iPS cells. For that, I conducted HC and NMF analyses using ΔCt values for ES and iPS cells under several different sets of conditions. Result showed that both HC or NMF failed to separate ES and iPS cells. Further analysis by PCA also produced a similar result, with no identification of miRNAs to clearly distinguish human ES and iPS cells. Therefore, I decided to perform a simple comparison based on average ΔCt values for ES and iPS cells. The miRNAs that showed statistically significant differences between the two groups are shown in Figure 5A. Strikingly, most C19MC members showed higher expression in iPS than in ES cells (Fig. 5A). A previous study also reported the differential expression of miRNAs C19MC, but with higher expression in ES than in iPS cells [21]. However, in that study, single types of each ES and iPS cell lines were compared, indicating that their result may only be true for that particular type of cell, and does not reflect the ES and iPS cells in general. A previous study also identified other miRNAs that are differentially expressed in ES and iPS cells. The study by Wilson et al. reported that the miR-886-5p was expressed at higher in iPS than in ES cells. However, in this study, the average ΔCt values of miR-886-5p was shown to express at similar levels in iPS (9.2, SD. 2.8) and ES cells (9.2, SD. 1.7). A study by Chin et al. listed 16 miRNAs that are differentially expressed in human ES and iPS cells [16]. However, only three of them showed similar trends in our study. The group used oligo DNA-based arrays (Ohio State University Comprehensive Cancer Center) to profile their miRNAs, and I cannot deny that different miRNA analysis protocols contribute to the different results observed.

In mouse cells, comparison of mouse ES and iPS cells identified several miRNAs that are expressed at significantly different levels in ES and iPS cells, with members of the let-7 and miR-30 families more highly expressed in iPS than in ES cells (Fig. 5B).

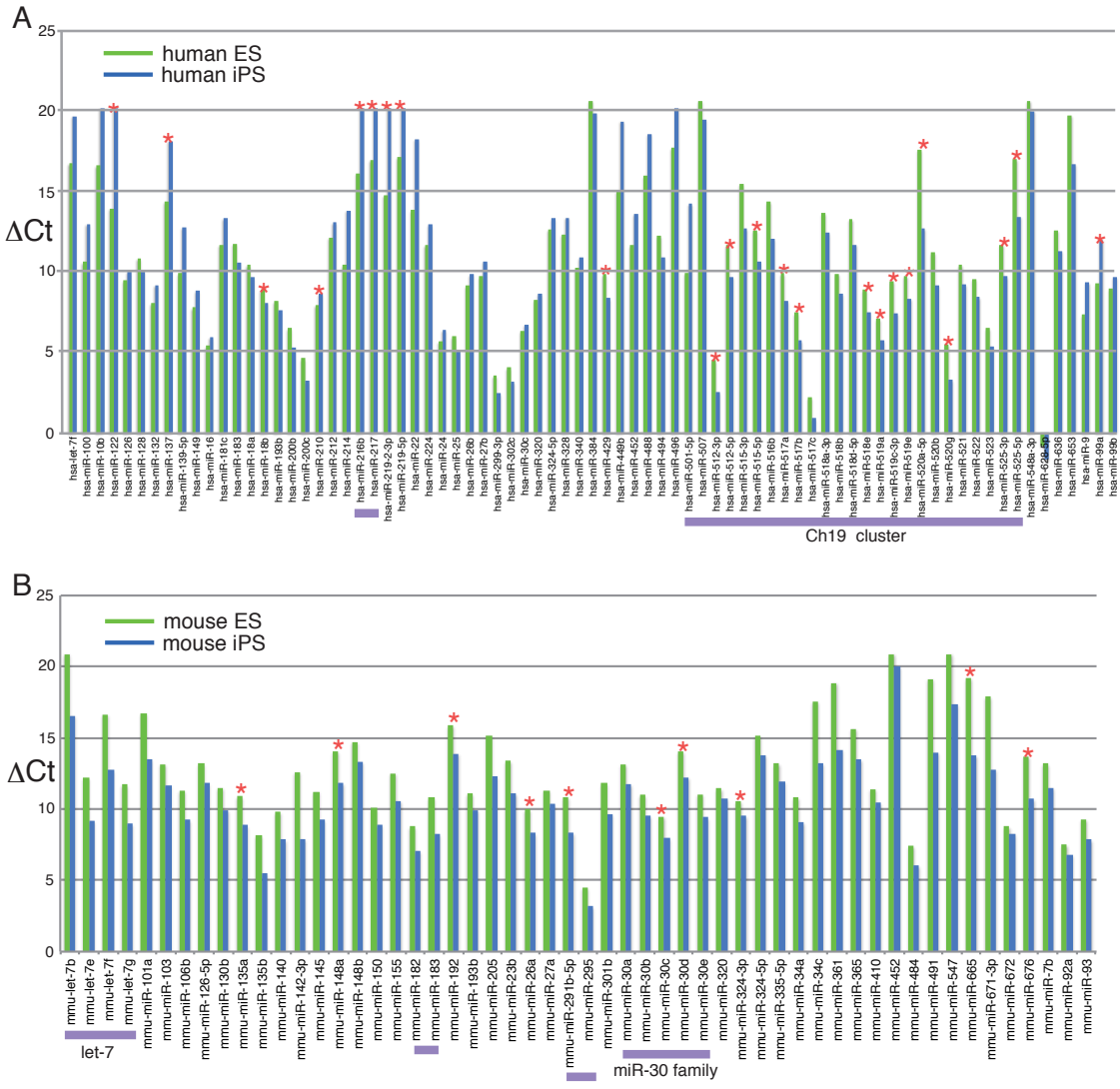


Fig. 5 Comparison of miRNA expression levels of ES and iPS cells. A, B. Average ΔCt of human (A) or mouse (B) ES and human (A) or mouse (B) iPS are shown. miRNAs that have less than 0.05 p value between ES and iPS cells are listed by their miRNA number. Clustered miRNAs are indicated by a violet mark under the bar. p value: non marked bars < 0.01. *(red asterisk) <0.05

Comparison of Human and Mouse ES/iPS Cell-Specific miRNAs

Human and mouse ES/iPS cells are in different states of pluripotency. Specifically, mouse ES/iPS cells are in naïve pluripotency while human ES/iPS cells are in the primed state [42-44]. Mice have primed pluripotent stem cells called epiblast stem cells [4,44], and differences in gene expression patterns have been reported. To further compared these two types of cells at the miRNA level, I identified miRNAs with the highest expression levels (lowest ΔCt values) in human and mouse ES/iPS cells. Figure 6A shows the top 60 miRNAs in mouse ES/iPS and human ES/iPS cells. Green asterisks indicate housekeeping miRNAs, the miRNAs that showed a difference of less than 2 in the ΔCt values between ES/iPS-g and somatic-g (Fig. 1). The miRNAs with differences in rank of greater than 50 are highlighted in pink. Expression levels (ΔCt) of miRNAs that have a difference of more than 50 difference in mouse and human ranking and more than 2 ΔCt value difference with somatic cells are shown in Figure 6B. Most of the top-ranked miRNAs specific to ES/iPS cells are either mouse-specific or human-specific. In the mouse list (Fig. 6A), six of the top eight mouse-specific miRNAs are members of the miR-290 cluster. The human miR-371 cluster was predicted to exist based on sequence similarity to members of the mouse miR-290 cluster [45,46]. One of the members of the miR-371 cluster, the miR-372, is ranked 33rd in the human miRNA list (Fig. 6A). In humans, the C19MC were originated evolutionarily from the miR-371-373 cluster [47], and some C19MC members were shown to share the same seed sequence with miR-372, 373, and other ES/iPS enriched miRNAs such as miR-302 cluster [48,49]. Members of the miR-302 cluster were ranked 11th, 17th, 34th, and 59th in the mouse list, and 1st, 30th, and 33rd in the human list. On the other hand, the miR-367, which is a distantly related member of the miR-302 cluster [45], was 37th in the mouse list and 17th in the

human list. This indicates that large numbers of miRNAs that are orthologs or have an identical seed sequence are among the most abundant miRNAs in both mice and humans. A previous study has shown that miR-302, miR-372, and miR-292 share redundant activity by targeting to TGF-β, and promotes cells reprogramming [46]. In addition, members of the miR-17 cluster and the miR-302 cluster, which share the same sequence, were also shown to mediate reprogramming by targeting TGFβr2 and p21 during the mesenchymal-to-epithelial transition during the initiation stage of reprogramming [13]. The miR-182, 708, and 499-5p, which showed a large difference of expression between human iPS/ES and somatic cells, showed very little difference in mouse case (Fig. 6B). However, previous literature did not explain the specific expression of these miRNAs in human and mouse pluripotent cells.

Fig. 6 Comparison of miRNAs between human and mouse pluripotent stem cells
(A) The top 60 miRNAs from the lowest average ΔCt value of human and mouse pluripotent stem cells are listed. Ranking number in human and mouse are compared, and additional information is indicated in the panel below of the figure. (B) Expression levels (ΔCt) of miRNAs which are differentially expressed in human and mouse ES/iPS. Listed miRNAs are labeled with pink color but not with green asterisk in the panel A, which means there are miRNAs with more than 50 differences in mouse and human ranking, and at the same time, have more than 2 ΔCt value difference with somatic cells.

A

Ranking from lowest ΔCt		mouse miRNA	Ranking in human		human miRNA	Ranking in mouse
1		mmu-miR-292-3p			hsa-miR-302b	11
2	*	mmu-miR-19b	8		hsa-miR-519d	
3		mmu-miR-295			hsa-miR-628-5p	
4	*	mmu-miR-106a	10		hsa-miR-888	
5		mmu-miR-293			hsa-miR-518f	
6	*	mmu-miR-19a	17		hsa-miR-367	31
7	*	mmu-miR-17	9		hsa-miR-517c	
8		mmu-miR-291a-3p		*	hsa-miR-19b	2
9	* *	mmu-miR-20a	12	*	hsa-miR-17	7
10	*	mmu-miR-16	25	*	hsa-miR-106a	4
11		mmu-miR-302b	1		hsa-miR-299-3p	
12	*	mmu-miR-484	34	*	hsa-miR-20a	9
13		mmu-miR-135b	28		hsa-miR-512-3p	
14	*	mmu-miR-24	33	*	hsa-miR-302a	17
15	*	mmu-miR-191	30	*	hsa-miR-302c	59
16	* *	mmu-miR-92a	44		hsa-miR-520e	
17		mmu-miR-302a	14	*	hsa-miR-19a	2
18	*	mmu-miR-687			hsa-miR-200c	39
19		mmu-miR-20b	31		hsa-miR-520g	
20		mmu-miR-294			hsa-miR-708	98
21	*	mmu-miR-667			hsa-miR-499-5p	241
22		mmu-miR-200b	26	*	hsa-miR-222	71
23		mmu-miR-200a	72		hsa-miR-374b	
24		mmu-miR-182	203		hsa-miR-25	
25	*	mmu-miR-31	55	*	hsa-miR-16	10
26	*	mmu-miR-210	63		hsa-miR-200b	
27		mmu-miR-429	77		hsa-miR-523	
28		mmu-miR-290-3p			hsa-miR-135b	13
29	*	mmu-miR-93	42		hsa-miR-518d-3p	
30	*	mmu-miR-672	59	*	hsa-miR-191	15
31		mmu-miR-367	6		hsa-miR-20b	19
32	*	mmu-miR-30c	40	*	hsa-miR-24	14
33	*	mmu-miR-140	98		hsa-miR-372	
34		mmu-miR-302d	*	*	hsa-miR-484	12
35	*	mmu-miR-34b-3p	*	*	hsa-miR-29a	
36	*	mmu-miR-574-3p		*	hsa-miR-34c-5p	210
37	*	mmu-miR-26a	43		hsa-miR-519a	
38	*	mmu-miR-335-3p	*		hsa-miR-146a	
39		mmu-miR-200c	18		hsa-miR-517b	
40	*	mmu-miR-328	189	*	hsa-miR-30c	32
41	*	mmu-miR-21	41	*	hsa-miR-21	41
42	*	mmu-miR-29a	35	*	hsa-miR-93	85
43	*	mmu-miR-127	169	*	hsa-miR-26a	37
44	*	mmu-miR-130a	96	*	hsa-miR-92a	16
45		mmu-miR-183	138	*	hsa-miR-374a	
46		mmu-miR-134	260		hsa-miR-141	205
47		mmu-miR-152	215	*	hsa-miR-106b	77
48	*	mmu-miR-136	258	*	hsa-miR-331-3p	99
49	*	mmu-miR-409-3p	*	*	hsa-miR-454	
50	*	mmu-miR-467e		*	hsa-miR-186	86
51	*	mmu-miR-323-3p	168	*	hsa-miR-425	126
52		mmu-miR-291b-5p		*	hsa-miR-107	303
53		mmu-miR-150	186	*	hsa-miR-193b	82
54	*	mmu-miR-467b		*	hsa-miR-221	136
55	*	mmu-miR-433	211	*	hsa-miR-31	25
56	*	mmu-miR-467a		*	hsa-miR-34a	65
57	*	mmu-miR-199a-3p	110		hsa-miR-487a	
58	*	mmu-miR-494	154		hsa-miR-518e	
59		mmu-miR-302c	15		hsa-miR-672	30
60		mmu-miR-367c	243		hsa-miR-519c-3p	

Remarks

mouse/human specific
more than 50 difference in ranking

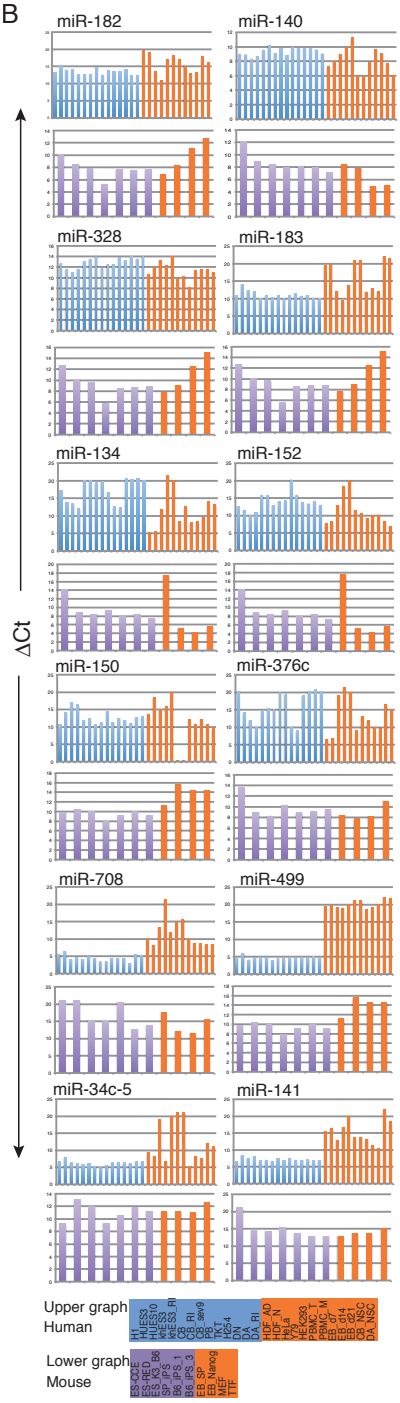
all miRNAs are highly expressed in ES/iPS than in somatic except:

* ES/iPS < somatic
* equally expressed
* a primer is not available in the array

miRNAs that are highly regulated in previous report (Jouneau et al, 2012, RNA)

* higher in ES than EpiSCs
* higher in EpiSCs than ES

B



Changing Levels of miRNAs when iPS cells Differentiated

Next, I examined the expression levels of miRNAs during iPS cells differentiation (Fig. 7). The iPS cells were differentiated through the formation of EBs, and the differentiated cells were harvested at three different time points. The expression levels of most of the miRNA were drastically changed after EB formation, and the miRNAs were divided into two groups: downregulated (Fig. 7A–D) and upregulated (Fig. 7E–G) after EB formation. Figure 7A shows miRNAs that were expressed at high levels in human ES/iPS cells (denoted by immature EBs, d 0) and at low levels in EBs at day 7, 14, and 21). One of the miRNAs in the list, the miR-369-3p, was previously reported to reprogram human and mouse cells to pluripotency by direct transfection of mature double stranded miRNAs [31]. The newly identified ES/iPS cell-specific miRNAs, miR-299, -499-5p, -628-5p, and -888, were also included in the list, further showing that these miRNAs are more highly expressed in immature cells than in differentiating or differentiated cells, and may be useful for iPS cells reprogramming. Another group of miRNAs were expressed at high levels in immature EBs, but showed decreased expression at day 7; these later increased when the cells underwent further differentiation at days 14 and 21 (Fig. 7B). Similarly, miRNAs shown in Figures 7C and 7D were expressed at a high level in iPS cells but showed a significant decrease in expression on day 7. Some miRNAs were upregulated as the cells differentiated on day 14 and 21 (Fig. 7C), while some miRNAs constantly expressed at low levels on both day 7 and day 14 and only increased at al later stage of differentiation (Fig. 7D). By contrast, the miRNAs that are important in the regulation of cell differentiation were expressed at a low level in immature iPS cells but showed significantly increased expression on day 7 and at a later stage of differentiation (Fig. 7E). The other groups of miRNAs were lowly expressed in both immature cells and cells at an

early stage of differentiation but significantly increased on day 14 and remained expressed at a high level on day 21 of differentiation (Fig. 7F, G). The members of the let-7 cluster, which were previously reported on [40,50,51], showed sharp increases in their miRNA expression on day 7 of differentiation and remained expressed at a later stage of differentiation (Fig. 7H). The differential expression of miRNAs observed during iPS cell differentiation shows that miRNAs are highly regulated in pluripotent cells and play an important role in regulating cell differentiation.

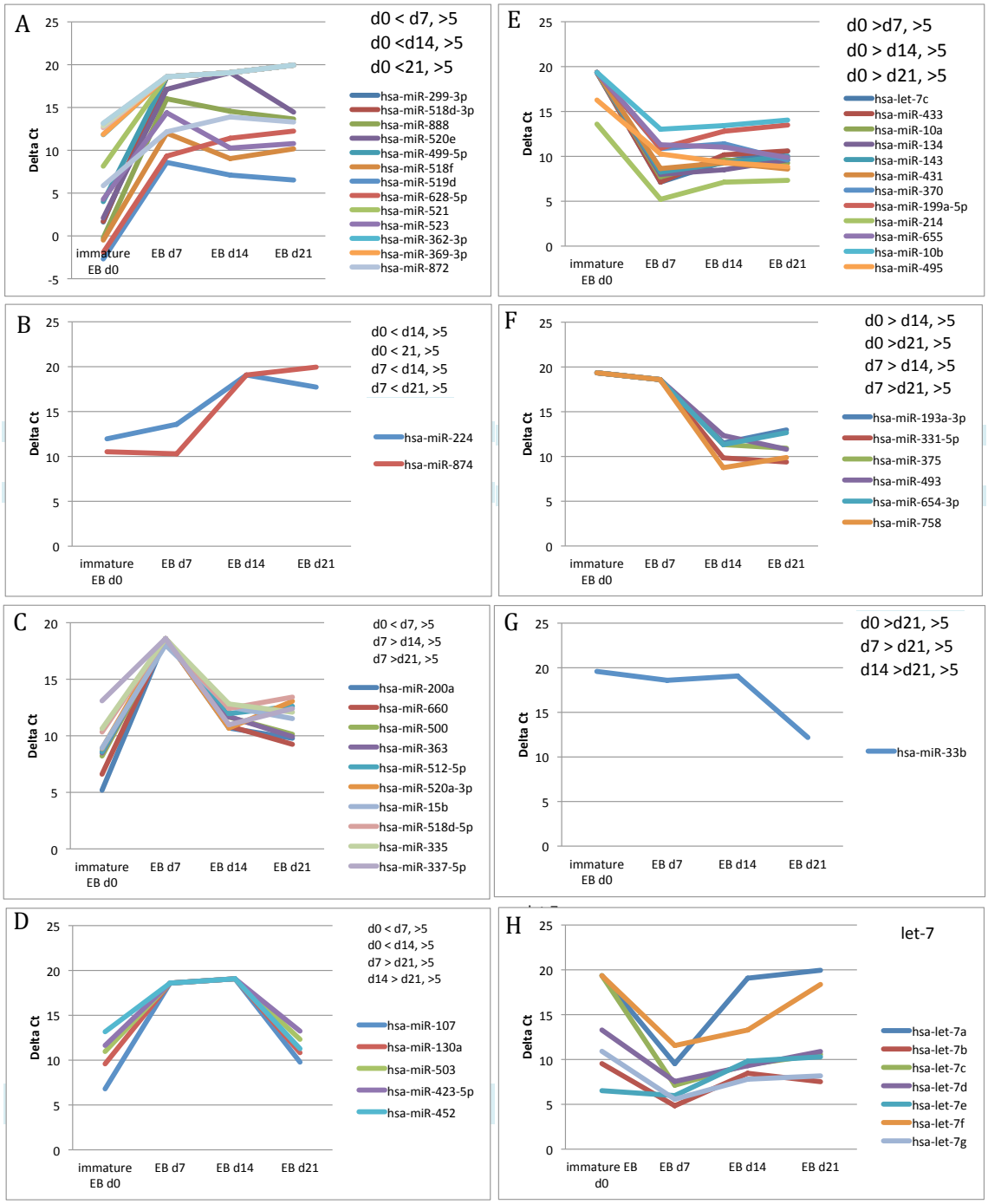


Fig. 7 Alteration of level of miRNAs during formation of human EB. Human EB was formed, and the expression profile of miRNAs was examined at days 7, 14, and 21 after starting the formation of EB. Levels of miRNAs were categorized by patterns of changing of expression (ΔCt values).

Discussion

miRNA has received much attention in recent years in basic and applied science. For pluripotent cells, miRNA is expected to be a powerful tool from a technical perspectives as well as in terms of biological interests. Profiling miRNA expression patterns in ES and iPS cells is critical. Several technologies are available for miRNA profiling, and each of them may be better than others in terms of sensitivity, cost efficiency, sequence dependence, or avoidance of potential contamination by artifacts. The selection of a technique with a different approach and experimental setting may explain the fundamental differences observed, especially when a variety of pluripotent and differentiated cells from different species is used. Thus, for this study, I took advantage of a miRNA array system that offers a consistent setting when applied to different types of cells in both humans and mice. Thus far, no study had profiled miRNA expression in human and mouse ES and iPS cells at the same time. Hence, I comprehensively analyzed miRNA expression patterns in purified ES and iPS cells and somatic cells, in both species. As I am attempted to establish a reliable database, I carefully examined the reproducibility of the results obtained by the qPCR-based method and the effects of sample preparation on the quality of the results. I found that RNA prepared from a mixture of iPS cells and feeder cells gave weaker signals than RNA obtained from purified iPS cells. Therefore, I prepared all pluripotent cells by purification, using a cell sorter and SSEA-4 (human) or SSEA-1 (mouse) antibody. The resulting comprehensive data allowed me to compare different subsets of pluripotent cells and identify several miRNAs that had not previously been reported to characterize ES/iPS cells.

In this study, I have identified miR-187, 299-3p, 499-5p, 628-5p, and 888 as new miRNAs to distinguish between pluripotent and non-pluripotent cells. These miRNAs

showed nearly negligible values or were not examined in previous studies [21,22]. An interesting question to ask is: Why was this study able to identify these miRNAs, and why were others unable to do so? The answer may lie in the technique used for analysis in this study: a very stable, high-sensitivity analysis was applied. The iPS cells cultured on MEF feeder cells were sorted for SSEA-4 or SSEA-1 to ensure that there was no contamination of cells from the feeder cells. The purification of cells is necessary as cDNA isolated from feeder cells can dilute the absolute number of miRNAs present in the iPS cells. So far, no other study has employed this purification method for miRNA expression profiling. This may further answer why I can identify the miR-187, 299-3p, 499-5p, 628-5p, and 888.

Another important issue to clarify is whether or not the identification of these miRNAs was influenced by the selection of cells used as a control. In this study, I am using various types of ES and iPS cells. I am comparing them with non-pluripotent cells consisting of peripheral blood, human-dermal fibroblast cells, iPS-derived EBs, iPS-derived NSCs and several epithelial-associated cancer cells. As such, does this analysis, which includes samples from cancer cells, interfere with the overall miRNA expression profile and contribute to the identification of miR-187, 299-3p, 499-5p, 628-5p, and 888? To answer the question, I used a similar experimental setting with PCA and analyzed the samples without the cancer cell line, HEK293, Y79, and Hela. As a result, I observed a similar trend in cell distribution where PCA clearly showed a clear segregation of ES/iPS cells with other non-pluripotent cells. These two groups of cells were distinguished by PC1 with the same list of miRNAs as recorded in Table 2, except for miR-187. The miR-187 showed an eigenvectors value of 1.86, less than 2, which is the minimum number I had selected to distinguish pluripotent and non-pluripotent cells. The eigenvector values from this PCA showed that the selection of cells used as a control has little impact on the final

outcome that contributes to the identification of miR-187, 299-3p, 499-59, 628-5p, and 888. In a different view, I also applied a similar PCA to the ES/iPS cells and compared it with control cells consisting of only HDF and PB cells. The HDF and PB cells were selected as controls since all iPS cells used in this work were reprogrammed from fibroblast and blood-related cells. Interestingly, I obtained similar results as observed in the first data analysis indicating that the newly identified miRNAs are indeed highly expressed in ES/iPS rather than in non-pluripotent cells. Moreover, the selection of cells as controls do not significantly contribute to the identification of miR-187, 299-3p, 499-5p, 628-5p, and 888.

To predict the regulatory activities of newly identified miRNAs, I focused on identifying the seed sequence of the miRNAs, which is responsible for binding to the 3'UTRs of target genes. The seed sequence of the newly identified miRNAs was compared with known ES/iPS-specific miRNAs. Most of the known pluripotent-specific miRNAs shared the same seed sequence, which demonstrated a redundant function of these pluripotent-specific miRNAs. The seed sequence of AAGUGC is shared among miR-302b-3p, miR-373, miR-520e, miR-519c-3p, miR-520a-3p, and miR-520b; AGUGCC in miR-371-3p, miR-515-3p and miR-519e; and AAGUG in miR-17, 201a, 93, 106a, 106b, 20b and 519d. However, none of the newly identified miRNAs shared the same seed sequence with these known pluripotency-specific miRNAs. Among all listed miRNAs highly expressed in pluripotent cells (Fig. 1A), miR-200c does not share any seed sequence with the ES/iPS-specific miRNAs but has been shown to induce cell reprogramming. Meanwhile, the miR-200c, with a seed sequence of AAUACUG, was reported to work together with miR-302 to induce iPS cell reprogramming using mature, double-stranded miRNAs [32]. The regulatory activity shown by miR-200c with miR-302 proved that miRNAs are

highly expressed in pluripotent cells but do not possess the same seed sequence as ES/iPS-specific miRNAs still playing an important roles in regulation of reprogramming and perhaps in the maintenance of pluripotency. These miRNAs may work in combination with ES-specific miRNAs to regulate certain genes during reprogramming, maintenance of self-renewal, or regulation of cell differentiation.

Pluripotent cells share characteristics with tumor cells in several different ways. For example, both cells have been shown to form teratomas when injected into nude mice, and these criteria have been commonly used to characterize iPS cell pluripotency [2,3]. In regards to reprogramming, suppression of p53 and UTF1, which are disrupted in tumor cells, have been shown to accelerate and induce reprogramming [34,66]. In addition, the reprogramming factors such as c-myc, Klf-4, and Nanog are oncogenes that were dysregulated in various types of cancer [67-69]. Hence, it is not surprising to see miRNAs specifically expressed in ES cells playing important roles in the regulation of cancer cells [70-72]. Several studies have reported miR-302 cluster to be overexpressed in germ-cell tumors and may serve as a potential serum bio-marker [73]. In addition, miR-302 cluster was also reported to regulate self-renewal and proliferation of glioma-initiating cells, cervical carcinoma cells, and endometrial cancer cells [74-76]. The regulatory activities of the miR-302 cluster showed that miRNAs highly expressed in pluripotent cells have redundant activities in cancer cells. Interestingly, the newly identified miRNAs, miR-187, 299-3p, 499-5p, 628-5p, and 888 were also reported in association with diseases and cancers in various papers and databases such as miR2diseaseBase and HMDD [52-55, 70-72]. However, whether or not these newly identified miRNAs are important with regard to iPS or ES cells still remains unanswered.

In this study, I found that the newly identified miRNAs were expressed at high levels in pluripotent cells but not in non-pluripotent cells. With the exemption of miR-187, all newly identified miRNAs were in the top 21 miRNAs that were highly expressed in pluripotent cells. The miR-628-5p and miR-888 were at rank 3 and 4, respectively. This indicates that these miRNAs may be expressed at high levels in order to control various genes in association with pluripotency. To clarify further the roles of miR187, 299-3p, 499-59, 628-5p and 888, I identified their potential target genes using several public databases. These included miRanda, miRDB, miRWalk, RNA22 and TargetScan (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html). The databases predicted various physiological functions for the miRNAs associated with cell-cycle regulation, cell growth, apoptosis, cell differentiation, and other factors, which were previously reported to be regulated during cell reprogramming [46]. The miR-499-5p was predicted by the microRNA.org database to regulate CDK19, which involves cell-cycle regulation, RAB5C in vesicular transport, and ARHGAP26 in the cell-signaling process [77]. These predicted target genes of miR-499-5p were shown to down-regulate during cell reprogramming suggesting that upregulation of miR-499-5p during cell transition from somatic to pluripotent is necessary to regulate reprogramming process.

The functional activities of miRNAs can be verified via several means based on three main subjects: reprogramming, maintenance of pluripotency, and determination of cell lineage. The analysis can be performed by manipulating endogenous levels of miRNAs using the overexpression or knockdown approach, through a plasmid-based method, or via a commercially available short-stranded miRNA mimic or antagonist. In the reprogramming process, somatic cells such as fibroblast cells can be used as a reprogramming source. The cells can be reprogrammed with miRNAs in the presence or

absence of commonly used OSKM transcription factors. The reprogramming factors can be delivered to the cells using several methods [10-14]. Meanwhile, the effect of miRNAs on the efficiency of reprogramming can be monitored by observing the number of iPS cell colonies. Moreover, the quality of the generated iPS cells can be evaluated through several means as previously discussed [2,3,5]. In regards to self-renewal and pluripotency, the same evaluation methods in combination with an analysis at the molecular level can be applied to the established iPS cells that were overexpressed or suppressed with the miRNAs [15]. In order to study the effect of miRNAs during the regulation of cell differentiation, the manipulated iPS can be cultured in the absence of feeder cells or growth factors such as LIF or b-FGF. The regularly used method involves inducing the cells to differentiate into embryoid bodies, which allows the cells to differentiate into all three main germ layers that develop during embryogenesis: the ectoderm, endoderm, and mesoderm [78]. The effect of miRNAs during iPS cell differentiation can be accessed using qRT-PCR or immunohistochemistry by examining the expression of pluripotency marker such as Rex1 or Nanog, and cell specific marker such as Pdx1, Sox9 or Ngn3 [25]. Specifically, the process is carried out by measuring the level of pluripotency markers or cell-specific markers as mentioned earlier [79,80]. Exploring the regulatory activities of these newly identified miRNAs might unravel new molecular mechanisms that underly iPS cell reprogramming, the maintenance of self-renewal, and differentiation.

Differences in miRNA expression patterns between ES and iPS cells were one of the focuses of the current study. At first, I used the clustering and PCA methods to segregate ES and iPS. However, the analysis failed to segregate ES and iPS cells. Hence, I further analyzed the cells by comparing the two groups of cells based on ΔCt. Simple comparison of average values for human ES and iPS cells identified several miRNAs with statistically

significant differences in expression between those group of cells. Among them, C19MC members showed higher expression levels in iPS than in ES cells. C19MC harbors the largest cluster of miRNA genes that developed in a recent mammalian evolution [47,56]. It spans a genomic region of about 100 kb, which contains 39 miRNAs. A common enhancer for C19MC miRNAs may contribute to differences in the expression levels between ES and iPS cells; however, mechanisms regulating C19MC miRNA transcription have not been well characterized. C19MC originated evolutionally from the miR-371-373 cluster, the human ortholog of the mouse 290 cluster [47]. However, the miR-371-373 cluster (humans and mice) and miR-290 cluster (mice) did not show significant differences in expression between iPS and ES. The presence of abundant miRNA with similar seed sequences in C19MC indicates the generation of novel miRNAs during primate evolution, which may have led to functional diversification [47]. Therefore, higher expression of C19MC members, but not human miR-371-373 or mouse miR-290 members, in iPS cells indicates that the acquired functions of C19MC members may contribute to the biological significance of different expression levels in ES and iPS cells. Through examination of the SD values, I found that most miRNAs that show statistically significant differences between ES and iPS cells have relatively high SD values. In this study, I had analyzed large numbers of cells and detected statistically significant differences in several miRNAs. However, whether these differences reflect the difference between ES and iPS cells should be examined carefully. Previous works at the gene expression profiles suggested that ES and iPS cells are distinctly different [17, 81]. However, more recent work focusing on the global chromatin structure and gene expression data of ES and iPS cells showed that the transcriptional programs of ES and iPS cells exhibit little variation and did not serve to distinguish ES from iPS cells [15].

The variation or similarities observed may be due to the cells or methods used for the analysis, which may answer why current findings contradict each other.

By analyzing miRNAs in both human and mouse pluripotent cells, I sought to identify differences in miRNA expression patterns between humans and mice. I am concerned as to whether a comparison of Ct values obtained using different primers (human and mouse) would give meaningful results, but the ranks of miRNAs were quite similar in humans and mice (Figs. 1, 3). This is probably because the qPCR conditions for each primer set were well adjusted to give proper Ct values, as the supplier claimed. Among common human and mouse miRNAs, not many miRNAs showed significant differences in expression between humans and mice. Whether these miRNAs correlate with the differences in pluripotency levels between humans and mice is an interesting issue. Previous work examining miRNA expression in naïve (ES cells) and primed (epiblast stem cell, EpiSC) mouse pluripotent stem cells revealed that several distinct miRNAs are differentially expressed in ES cells and EpiSCs [57]. Hierarchical clustering of miRNA expression profiles in two ES cells lines and three EpiSC lines showed that the three EpiSC samples clustered closely together and could be discriminated from the ES cells [57]. Among 987 miRNAs whose expression differed between ES cells and EpiSCs, 226 miRNAs were more highly expressed in ES cells, while 76 miRNAs were more highly expressed in EpiSCs [57]. However, among the most abundant differentially expressed miRNAs (19 miRNAs with higher expression in ES cells and 22 miRNAs with higher expression in EpiSCs), three and four miRNAs that are more highly expressed in ES and iPS cells, respectively, showed similar trends in our comparison of human and mouse miRNAs (Fig. 6). However, further characterization of these miRNAs are necessary to improve understanding of the molecular mechanisms underlie stem cell pluripotency.

Conclusion

MiRNA expression pattern of various types of pluripotent and non-pluripotent cells analyzed by qRT-PCR based miRNA profiling showed clear separation of two major clusters, ES/iPS cells and other cell group, demonstrating that miRNA have significant roles in defining cells identity. Further analyses lead to identification of new miRNAs that characterized human ES/iPS cells, miR-187, 299-3p, 499-5p, 628-5p, and 888. Several miRNAs including chromosome 19 miRNA cluster found to be more strongly expressed in human iPS cells than in ES cells. Similar analyses conducted in mouse ES/iPS cells and somatic cells also suggest differentially expressed miRNAs in ES and iPS cells. Time course tracing of miRNA levels during embryoid body formation further highlight the regulatory activities of miRNAs during development. Expression pattern of the miRNA may serve as a valuable tool evaluate the level of pluripotency among ES and iPS cells, and it may suggest that miRNAs are a functionally important molecule to regulate iPS or ES cells pluripotency and differentiation. Further understanding of miRNA regulation in combination of gene expression profiling may provide a better understanding to the mechanisms involved in regards to cells reprogramming, maintenance of pluripotency and regulation of cells differentiation.

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