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Human embryonic stem cells express a unique set of microRNAs

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Abstract

Human embryonic stem (hES) cells are pluripotent cell lines established from the explanted inner cell mass of human blastocysts. Despite their importance for human embryology and regenerative medicine, studies on hES cells, unlike those on mouse ES (mES) cells, have been hampered by difficulties in culture and by scant knowledge concerning the regulatory mechanism. Recent evidence from plants and animals indicates small RNAs of approximately 22 nucleotides (nt), collectively named microRNAs, play important roles in developmental regulation. Here we describe 36 miRNAs (from 32 stem-loops) identified by cDNA cloning in hES cells. Importantly, most of the newly cloned miRNAs are specifically expressed in hES cells and downregulated during development into embryoid bodies (EBs), while miRNAs previously reported from other human cell types are poorly expressed in hES cells. We further show that some of the ES-specific miRNA genes are highly related to each other, organized as clusters, and transcribed as polycistronic primary transcripts. These miRNA gene families have murine homologues that have similar genomic organizations and expression patterns, suggesting that they may operate key regulatory networks conserved in mammalian pluripotent stem cells. The newly identified hES-specific miRNAs may also serve as molecular markers for the early embryonic stage and for undifferentiated hES cells.

Keywords: Human embryonic stem cell; microRNA

Introduction

Embryonic stem (ES) cell lines were first derived from mice and are now available from a variety of mammalian systems, including human. They are characterized by nearly unlimited self-renewal in an undifferentiated state under defined culture conditions while retaining differentiation capacity (Evans and Kaufman, 1981; Martin, 1981; Smith, 2001). During differentiation in vitro, ES cells are able to develop into various kinds of specialized somatic cell types and recapitulate processes of early embryonic development. Thus, ES cells hold promise as an unlimited source for various clinical and biotechnological applications (Brustle, 1999; Martin, 1981; Li et al., 1998; Pera et al., 2000).

ES cell lines were first established in mice in 1981 (Evans and Kaufman, 1981; Martin, 1981) and have been used as a model system to study mammalian ES cells. Currently, a few molecular regulators are known to participate in the self-renewal and pluripotency of mouse ES (mES) cells. A POU family transcription factor Oct4, the classical marker of all pluripotent cells, is specifically expressed in pre-implantation embryos, epiblast, germ cells, and pluripotent stem cell lines including ES cells, embryonic germ (EG) cells, and embryonic carcinoma (EC) cells (Palmieri et al., 1994; Yeom et al., 1996). Oct4 plays a critical role in the establishment and maintenance of pluripotent cells in a pluripotent state (Nichols et al., 1998; Niwa et al., 2000; Pesce et al., 1998). Leukemia inhibitory factor (LIF) can maintain self-renewal

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of mES cells through activation of Stat3 (Niwa et al., 1998). Oct4 and Stat3 each interact with various cofactors and regulate the expression of multiple target genes (Niwa, 2001). Two other transcription factors, Sox2 and FoxD3, have been shown to be essential for pluripotency in mice embryos (Avilion et al., 2003; Hanna et al., 2002). More recently, it was found that the homeoprotein Nanog is capable of maintaining mES cell self-renewal independently of LIF/Stat3 (Chambers et al., 2003; Mitsui et al., 2003).

The first human ES (hES) cell line was established only recently (Thomson et al., 1998), and 12 lines are publicly available worldwide (NIH Human Embryonic Stem Cell Registry). Despite their great potential, hES cells have not been a prolific source of information. This is mainly due to the technical difficulties in cell culture. Maintaining and expanding hES cells require laborious and skill-intensive procedures. Moreover, the population-doubling time of hES cells is almost three times longer than that of mES cells (Amit et al., 2000). There exist apparent differences in the characteristics of hES cells compared to mES cells in many aspects, including the regulation of self-renewal. Of the regulators found in mice, only a few including Oct4 play similar regulatory roles in hES cells. Others such as LIF do not affect hES cells in maintaining their self-renewal (Reubinoff et al., 2000). Dissecting the regulatory mechanism in hES cells will greatly enhance the understanding of stem cells as well as their application.

Recent advances in small RNA research have implicated microRNAs (miRNAs) as important regulators of development and differentiation. miRNAs constitute a large family of noncoding small RNAs of approximately 22 nucleotides (nt) in length. Our understanding of miRNA function originates from studies of the developmentally regulated miRNAs *lin-4* (Olsen and Ambros, 1999; Lee et al., 1993; Wightman et al., 1993) and *let-7* (Reinhart et al., 2000) in *Caenorhabditis elegans*. By binding and inhibiting the translation of the target mRNA, the *lin-4* and *let-7* RNAs play an important role in regulating the timing of larval development. Another example is *bantam* RNA from *Drosophila melanogaster*, which is expressed in a temporal and tissue-specific manner during development, suppressing apoptosis and stimulating cell proliferation by inhibiting translation of hid mRNA (Brennecke et al., 2003). Several mouse miRNAs including miR-181 were recently shown to modulate hematopoiesis (Chen et al., 2003). In plants, miRNAs show a high degree of complementarity to transcription factors that are significant in development (Aukerman and Sakai, 2003; Chen, 2003; Llave et al., 2002b; Palatnik et al., 2003; Rhoades et al., 2002). These miRNAs induce target mRNA cleavage or translational repression, thereby facilitating plant development and organogenesis.

The expression of miRNAs is often regulated in tissue-specific and developmental stage-specific manners (Aravin et al., 2003; Krichevsky et al., 2003; Lagos-Quintana et al., 2002; Pasquinelli et al., 2000; Sempere et al., 2003), although the regulatory mechanism is still largely unknown. We have previously shown that miRNAs are transcribed as long primary transcripts (termed pri-miRNAs) (Lee et al., 2002). These primary transcripts are first trimmed into approximately 70 nt stem-loop forms (called pre-miRNAs) by the RNase III type protein, Drosha, in the nucleus (Lee et al., 2003). Following this initial processing, pre-miRNAs get exported to the cytoplasm by Exportin-5 (Lund et al., 2003; Yi et al., 2003) and are subject to a second processing to generate the final product of approximately 22 nt mature miRNAs by another RNase III type protein Dicer (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). This stepwise processing and compartmentalization may allow for the fine regulation of miRNA biogenesis at multiple steps.

More than 300 miRNAs have been reported in diverse eukaryotic organisms so far (Aravin et al., 2003; Dostie et al., 2003; Grad et al., 2003; Lagos-Quintana et al., 2001, 2002, 2003; Lai et al., 2003; Lau et al., 2001; Lee and Ambros, 2001; Lee et al., 1993; Lim et al., 2003b; Llave et al., 2002a; Mourelatos et al., 2002; Park et al., 2002; Reinhart et al., 2000, 2002). The majority of miRNA genes were discovered through cDNA cloning from size-fractionated RNA samples. Recently, additional miRNA genes have been identified using computational procedures from vertebrates, *C. elegans* and *Drosophila*. A bioinformatic study suggested that there exist 200–255 miRNAs in humans, accounting for almost 1% of the predicted genes (Lim et al., 2003a). If the prediction is correct, about 100 miRNA genes remain to be identified in humans because 152 miRNAs have been reported, of which 109 miRNAs have been experimentally validated (Brennecke and Cohen, 2003). miRNAs that are expressed only in specific developmental stages or conditions would be difficult to be cloned or validated.

To identify miRNAs that may be involved in the regulation of hES cells, we cloned small RNAs in the range of 17–26 nt from hES cells, resulting in 36 RNAs with characteristics of miRNAs. Of the 36 cloned RNAs, 16 were identical to previously reported miRNAs from various mammalian adult tissues and cell lines. Recently, a comparable study was carried out using mouse ES cells, reporting 15 novel miRNAs (Houbaviy et al., 2003). Comparison to this study shows that three miRNAs are common between the two data sets from human and mouse ES cells. Of the 17 novel miRNAs identified in our study, 11 miRNAs are homologous to those cloned from mES cells. Most of the conserved miRNAs are specifically expressed in ES cells, implicating that these miRNAs are important regulators in mammalian embryonic development.

Materials and methods

Human ES cell culture

Human ES cells (SNU-hES3 and MIZ-hES1) were maintained in DMEM/F12 (Gibco BRL) supplemented with

20% (v/v) serum replacements (Gibco BRL), penicillin (100 IU/ml, Gibco BRL) and streptomycin (100 μg/ml, Gibco BRL), 0.1 mM nonessential amino acids (Gibco BRL), 0.1 mM Mercaptoethanol (Sigma), and 4 ng/ml basic FGF (R&D). Media were changed daily. Human ES cell colonies were cultured on a feeder layer of mouse STO (ATCC CRL-1503) cells pretreated with mitomycin C (Sigma) and were manually detached and transferred onto new STO feeders every 5–6 days. HS-3 mouse ES cells were grown under standard condition.

Differentiation of human ES cells

To prepare embryoid bodies (EBs), whole colonies of hES cells were detached by glass pipette, transferred onto petri dishes coated with pluronic F-127 (Sigma), and

Table 1
MicroRNAs cloned from human embryonic stem cells

ID[a]	Sequence[b]	Observation[c]		Size	Chromosome	Conservation[d]	Expression[e]
		1st	2nd				
miR-302b*	ACUUUAACAUGGAAGUGCUUUCU	1	1	23	4	Mm	00011
miR-302b	UAAGUGCUUCCAUGUUUUAGUAG	9	36	23	4	Mm	00011, 000221
miR-302c*	UUUAACAUGGGGGUACCUGCUG	1		22	4	Mm	00011
miR-302c	UAAGUGCUUCCAUGUUUCAGUGG	7	3	23	4	Mm	00012
miR-302a*	UAAACGUGGAUGUACUUGCUUU	3	3	22	4	Mm	00012
miR-302a	UAAGUGCUUCCAUGUUUUGGUGA	6	17	23	4	Mm, Rn	00023, 001223
miR-302d	UAAGUGCUUCCAUGUUUGAGUGU	1	12	23	4	Mm	00023, 000112
miR-367	AAUUGCACUUUAGCAAUGGUGA	2	3	22	4	Mm, Rn	00011
miR-200c	UAAUACUGCCGGGUAAUGAUGGA		4	23	12	Mm	00010, 000110
miR-368	ACAUAGAGGAAAUUCCACGUUU	1		22	14	Mm	00010, 000110
miR-154*	AAUCAUACACGGUUGACCUAUU		1	22	14	Mm	000110
miR-369	AAUAAUACAUGGUUGAUCUUU	1		21	14	Mm	no signal
miR-370	GCCUGCUGGGGUGGAACCUGG		1	21	14	Mm	00S000
miR-301	CAGUGCAAUAGUAUUGUCAAAGC	1		23	17	Mm , Rn, Fr	21111
miR-371	GUGCCGCCAUCUUUUGAGUGU		2	21	19	Mm	002120
miR-372	AAAGUGCUGCGACAUUUGAGCGU		1	23	19	Mm	000230
miR-373*	ACUCAAAAUGGGGGCGCUUUCC		1	22	19	Mm	00SSS0
miR-373	GAAGUGCUUCGAUUUUGGGGUGU		1	23	19	Mm	000120
miR-296	AGGGCCCCCCCUCAAUCCUGU		1	21	20	Mm	00SSS
miR-374	UUAUAAUACAACCUGAUAAGUG	2		22	X	Mm	11000
sum		35	87				
let-7a	UGAGGUAGUAGGUUGUAUAGUU		1	22	9, 11, 17, 22		33000
miR-16	UAGCAGCACGUAAAUAUUGGCG		2	22	13		22112
miR-17-5p	CAAAGUGCUUACAGUGCAGGUAGU		1	24	13		11112
miR-19b	UGUGCAAAUCCAUGCAAAACUGA	7	1	23	13, X		111111
miR-21	UAGCUUAUCAGACUGAUGUUGAC		4	23	17		32111
miR-26a	UUCAAGUAAUCCAGGAUAGGCU		3	22	3		221112
miR-29	CUAGCACCAUCUGAAAUCGGUU		1	22	7		12SSSS
miR- 29b	UAGCACCAUUUGAAAUCAGUG		1	21	7		11000
miR-92	UAUUGCACUUGUCCCGGCCUG	1	1	21	13, X		21112
miR-103	AGCAGCAUUGUACAGGGCUAUG	1	2	22	5		21112
miR-124a	UUAAGGCACGCGGUGAAUGCCA	3	1	22	8		00SSS
miR-130a	CAGUGCAAUGUUAAAAGGGCAU		1	22	11		12223
miR-134	UGUGACUGGUUGACCAGAGGGG	1		22	14		00SSS0
miR-135-2	UAUGGCUUUUUAUUCCUAUGUGA		1	23	12		no signal
miR-136	ACUCCAUUUGUUUUGAUGAUGGA	1		23	14		no signal
miR-222	AGCUACAUCUGGCUACUGGGUCUC	1	1	24	X		11SSS1
sum		15	21				

[a] miRNAs that were newly identified in this study are listed in the upper panel. The exceptions are the miRNAs recently cloned from mES cells and indicated in brackets. Lines shadowed in yellow indicate the miRNAs expressed in both ES cells and EC cells. Lines shadowed in blue indicate the miRNAs expressed in ES cells but not in other cells including EC cells.

[b] The longest clone is presented.

[c] Number of the clones found in each library.

[d] The presence of homologous stem loops in the mouse (Mm), rat (Rn), and pufferfish (Fr) is indicated. Note that these homologues are the only predicted ones based on the genomic sequences.

[e] Expression patterns determined by Northern blot analysis. Single digit numbers indicate the relative band intensities of given miRNA in different cell lines as shown in Fig. 2 and do not give information about the relative levels of different miRNAs. Five digits represent band intensities from HeLa, STO, mES, SNU-hES1, and hEC, consecutively. Six digits represent band intensities from HeLa, STO, mES, SNU-hES1, Miz-hES3, and hEC, consecutively. S indicates a smear around approximately 22 nt that makes it difficult to judge the expression level of the given miRNA.

incubated for 10 days. The media for EB were identical to the hES media except that it lacked bFGF. Every 2 days, media were changed using a pipette. To further differentiate EBs made from SNU-hES3, they were plated onto tissue culture plates coated with poly-L-ornithin (0.01% (v/v))/fibronectin (5 g/ml (w/v)). Cells were further incubated for 5 days in N2 supplement medium containing 20 ng/ml bFGF and the medium was changed daily. Confluent cells were manually detached, and then pipetted using yellow tips and transferred onto new plates coated with poly-L-ornithin/fibronectin. Cells were cultured for 5 days in N2 medium containing 20 ng/ml bFGF. When the cells reached confluency, they were trypsinized and split 2:1 or 3:1 at new poly-L-ornithin/fibronectin-coated plates.

miRNA cloning and bioinformatic analysis

Total RNA was prepared from each cell line with TRIzol regent (Gibco BRL). Cloning of miRNA was performed as described (Lagos-Quintana et al., 2001). Database searches were performed at the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1990) and ENSEMBL server (http://www.ensembl.org) (Hubbard et al., 2002). Genomic sequences covering the region identical to the cloned miRNAs were examined by using MFOLD (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/) to predict the secondary structure (Zuker et al., 1999). Sequence alignment between miRNA sequences was performed by using CLUSTALW (http://www.ebi.ac.uk/clustalw/) (Higgins and Sharp, 1988).

Northern blot analysis

Total RNA (100 μg) from each cell line was loaded on a 12.5% denaturing polyacrylamide gel. The resolved RNA was transferred to a Zeta-Probe GT blotting membrane (Bio-Rad) overnight. Oligodeoxinucleotides labeled at the 5′ end with ^{32}P-γ-ATP were used as probes. Prehybridization and hybridization were carried out using ExpressHyb Hybridization Solution (Clontech) according to the manufacturer's instruction. The sequences of the probes are: 5′-AGAAAGCACTTCCATGTTAAAGT-3′ (miR-302b*), 5′-CTACTAAAACATGGAAGCACTTA-3′ (miR-302b), 5′-CAGCAGGTACCC-CCATGTTAAA-3′ (miR-302c*), 5′-

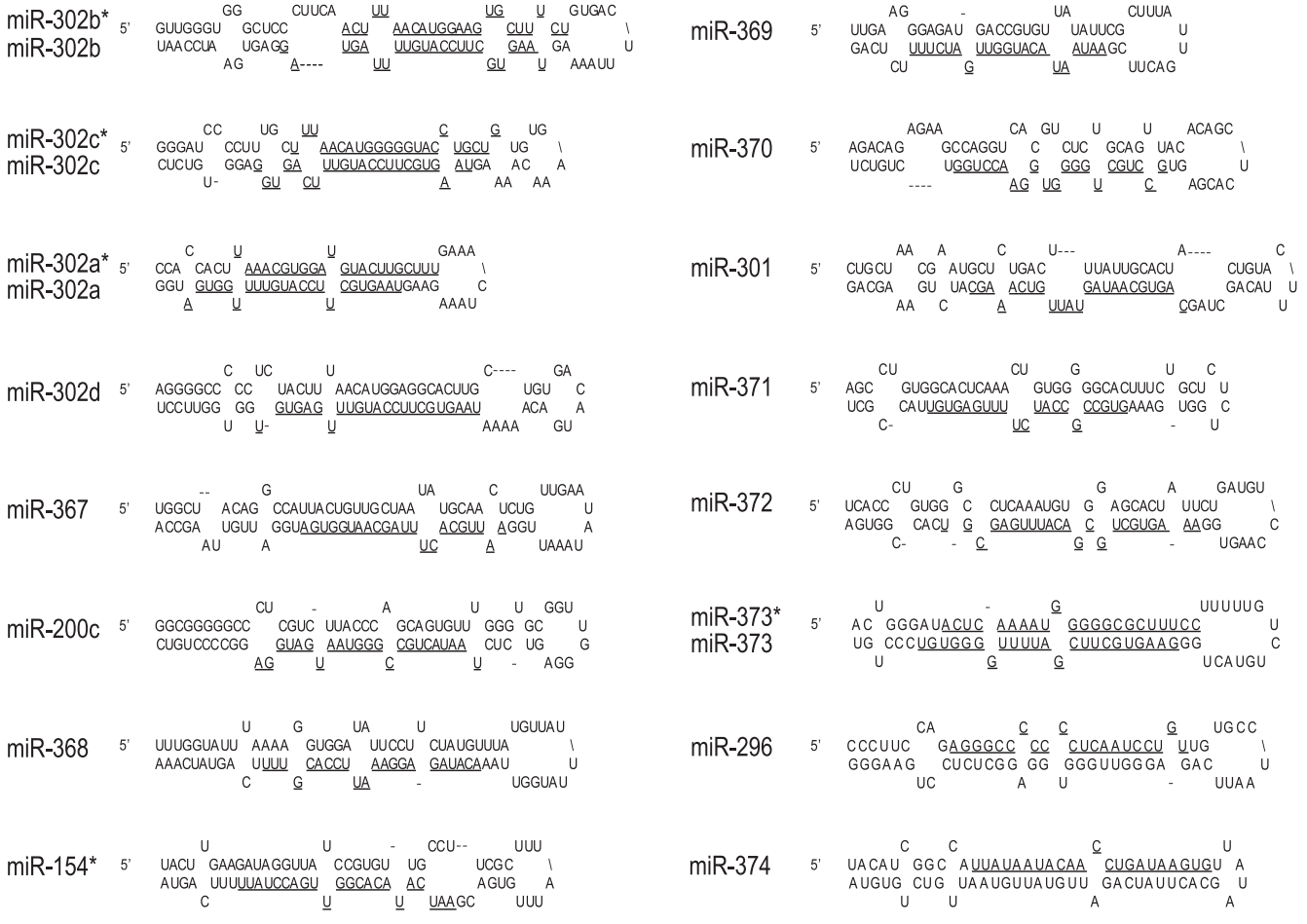

Fig. 1. Predicted structures of miRNA precursors. RNA secondary structure prediction was performed using MFOLD (version 3.1) and manually refined to accommodate G/U wobble base pairs in helical segments. The miRNA sequences are underlined. The actual termini of the pre-miRNAs have not been verified experimentally and may be different from the presented.

CCACTGAAACATGGAAGCACTTA-3′ (miR-302c), 5′-AAAGCAAGTACTACCACGTTTA-3′ (miR-302a*), 5′-TCACCAAAACATGGAAG-CACTTA-3′ (miR-302a), 5′-ACACTCAAACATGGAAGCACTTA-3′ (miR-302d), 5′-TCACCATTGCTAAAGTGCAATT-3′ (miR-367), 5′-TCCATCATTACCCGGCAGTATTA-3′ (miR-200c), 5′-AAACGTGGAATTTCCTCTATGT-3′ (miR-368), 5′-AATAGGTCAACCGTGTATGATT-3′ (miR-154*), 5′-AAAGATCAACCATGTATTATT-3′ (miR-369), 5′-CCAGGTTCCACCCCAGCAGGC-3′ (miR-370), 5′-GCTTTGA-CAATACTATTGCACTG-3′ (miR-301), 5′-ACACTCAAAAGATGGCGGCAC-3′ (miR-371), 5′-ACGCTCAAATGTCGCAGCACTTT-3′ (miR-372), 5′-ACACCCCAAA-ATCGAAGCACTTC-3′ (miR-373), 5′-GGAAAGCGCCCCCATTTTGAGT-3′ (miR-373*), 5′-ACAGGATTGAG-GGGGGGCCCT-3′ (miR-296), 5′-CACTTATCAGGTTGTATTATAA-3′ (miR-374), 5′-AAC-TATACAACCTACTACCTCA-3′ (let-7a), 5′-CGCC-AATATTTACG-TGCTGCTA-3′ (miR-16), 5′-ACTACCTGCACTGTAAGCACTTTG-3′ (miR-17-5p), 5′-TCAGTTTTGCATGGAT-TTGCACA-3′ (miR-19b), 5′-GTCAACATCAGTCTGATAAGCTA-3′ (miR-21), 5′-AGCCTATCCTGGATTACTTGAA-3′ (miR-26a), 5′-AACCGATTTCAGATGGA-GCTAG-3′ (miR-29), 5′-CACTGATTTCAAATGG-TGCTA-3′ (miR-29b), 5′-CAGGCCGGGACAAGTGCA-ATA-3′ (miR-92), 5′-CAT-AGCCCTGTACAATGCTGCT-3′ (miR-103), 5′-TGGCATTCACCGCGTGCCTTAA-3′ (miR-124a), 5′-ATGCCCTTTTAACATTGCACTG-3′ (miR-130a), 5′-CCCCTCTGGTCAACCAGTCACA-3′ (miR-134), 5′-TCACATAGGAATAAAAAGCCATA-3′ (miR-135-2), 5′-TCCATCATCAAAACAAATGGAGT-3′ (miR-136), 5′-GAGACCCAGTAGCCAGATGTAGCT-3′ (miR-222).

RT-PCR

Two to 5 μg of total RNA from the indicated cells was used for the first-strand cDNA synthesis with SUPERSCRIPT (Gibco BRL). Primers used for first-strand synthesis are: for miR-302b*−1−2*−2−3*−3−4−5, 5′-ATTCTGTC-ATTGGCTTAACAATCCATCACC-3′ was used for first-strand synthesis, and this primer (reverse) and 5′-GGGC-TCCCTTCAACTTTAAC-3′ (forward) were used for PCR amplification. For miR-371−13−14*−14, 5′-TGGTTCGT-GATGCCCTACTCAAACAGGGAC-3′ was used for first-strand synthesis, and this primer (reverse) and 5′-CGATCG-CCGCCTTGCCGCAT-3′ (forward) were used for PCR amplification. For miR-30a, 5′-TTCAGCT-TTGTAAAAA-TGTATC AAAGAGAT-3′ was used for first-strand synthesis, and this primer (reverse) and 5′-ATTGCTGTTTGAAT-GAGGCTTCAGTACTTT-3′ (forward) were used for PCR amplification. For let-7a-1, 5′-TTTCTATCAGACCGCCT-GGATGCAGACTTT-3′ was used for first-strand synthesis, and this primer (reverse) and 5′-GATTCCTTTTCACCA-TTCACCCTGGATGTT-3′ (forward) were used for PCR amplification. For Oct4, 5′-CTGAAGCAGAAGAGGATC-AC-3′ (forward) and 5′-GACCACATCCTTCTCGAGCC-3′ (reverse) were used for PCR amplification. For GAPDH, 5′-TGTCATCAATGGAAATCCCATCACC-3′ (forward) and 5′-CATGAGTCCTTCCACGATACCAAA G-3′ (reverse) were used for PCR.

Results

MicroRNAs cloned from human embryonic stem cells

To identify miRNAs expressed in hES cells, two independent cDNA libraries were generated by directional cloning method using size fractionated RNA (17−26 nt) from undif-

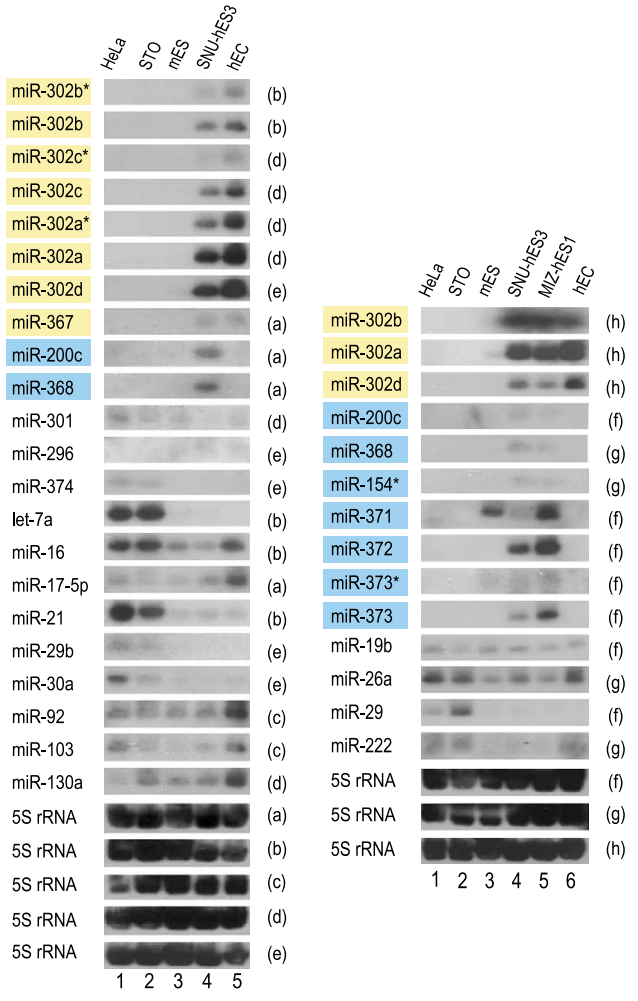

Fig. 2. Northern blot analysis of miRNAs cloned from human embryonic stem cells. Total RNA from HeLa, mouse feeder cell line (STO), mouse embryonic stem cell line (mES), two human embryonic stem cell lines (SNU-hES3 and Miz-hES1), and human embryonic carcinoma cell line (hEC) was blotted and probed with 5′-radiolabled oligodeoxynucleotide complementary to the indicated miRNA. 5S rRNA serves as a loading control. The alphabets (a−h) in brackets on the right indicate the membranes used for probing. miRNAs expressed in ES cells as well as in EC cells are shadowed in yellow, while miRNAs expressed specifically in ES cells are shadowed in blue.

ferentiated hES cells, SNU-hES3 (registered at the Korea Stem Cell Research Center). hES cells were grown on a feeder layer of the STO cell line pretreated with mitomycin C. To assess the undifferentiating status, the steady-state level of Oct4 mRNA was determined by RT-PCR, and the cells were stained for alkaline phosphatase. In addition, the expression of cell surface markers including stage-specific embryonic antigen SSEA-1, SSEA-3, and SSEA-4 was determined. The undifferentiated SNU-hES3 cells expressed a high level of Oct4 (Fig. 4), alkaline phosphatase, SSEA-3, and SSEA-4 throughout this study (data not shown).

Sequences were obtained for 1475 small cDNAs that resulted in 733 non-redundant sequences. Approximately 70% of these sequences corresponded to the expressed strand of loci that encode longer, previously identified coding or noncoding RNAs such as tRNAs and rRNAs. To distinguish miRNAs from degradation products or small interfering RNAs (siRNAs), we evaluated the ability of RNA containing the clones to fold into stem-loop by using the MFOLD program (Zuker et al., 1999). Thirty-six RNAs were found in the stems of strong hairpin structures (Table 1 and Fig. 1). Although some abundant RNAs appeared as many as 45 times, half of the potential miRNAs were represented by only one clone, suggesting that these RNAs are relatively less abundant in hES cells or that our miRNA data set may not represent the complete pool of miRNAs present in hES cells.

Of the 36 cloned miRNAs, 16 were identical to previously reported miRNAs from various mammalian adult tissues and cell lines (Table 1, lower panel). Interestingly, the remaining 20 RNAs were represented by the majority (77%) of the clones (122 of 158 clones) (Table 1, upper panel). This presents a striking contrast to a recent study using a similar cloning method, where 91% of approximately 600 clones matched previously identified miRNAs (Lagos-Quintana et al., 2003). The primary difference was in the source of RNA; various tissues of 18.5-week-old adult mice and the human osteoblast sarcoma cell line Saos-2 (Lagos-Quintana et al., 2003). In accordance with the prediction that some cells such as the cells in early developmental stage may express specific miRNAs (Lagos-Quintana et al., 2003), our data show that hES cells are highly enriched with a distinct set of miRNAs unlike the cells in adult tissues or other cell lines.

A recent study identified 53 miRNAs from mouse ES cells, of which 15 miRNAs are novel (Houbaviy et al., 2003). Three miRNAs are common between the novel miRNA data sets from mouse and human ES cells; miR-296 (miR-296), miR-301 (miR-301), and miR-302 (miR-302a). Thus, we here report 17 novel miRNAs exclusive of the three common miRNAs.

Most of the novel miRNAs from hES are expressed specifically in hES cells

To validate the expression of the cloned miRNAs, Northern blot analysis was performed using total RNAs from undifferentiated hES cells, mES cells, and the hEC cell line, NTERA2. To confirm the expression of miRNAs from hES cells, we employed another hES cell line, MIZ-hES1, that is registered in the NIH Human Embryonic Stem Cell Registry. As negative controls, total RNAs from HeLa cells and STO feeder cells were used (Fig. 2). Of the 16 newly identified miRNAs, 14 miRNAs are expressed in a hES cell-specific manner; miR-302b*, miR-302b, miR-302c*, miR-302c, miR-302a*, miR-302d, miR-367, miR-200c, miR-368, miR-154*, miR-371, miR-372, miR-373*, and miR-373. miR-302a that was previously reported in mES cells as miR-302 (Houbaviy et al., 2003) is also expressed specifically in mES, hES, and hEC cells. miR-296 (miR-296), previously cloned from mES cells, appeared as smear that

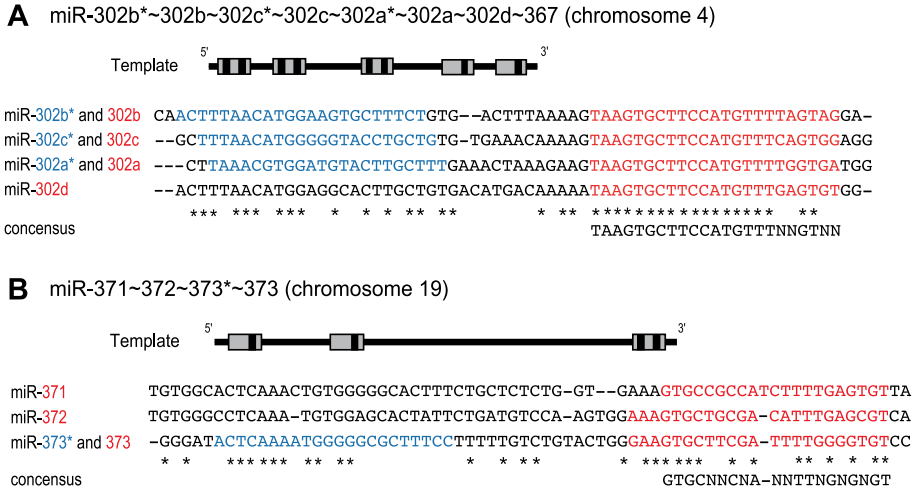

Fig. 3. Multiple sequence alignment of the genomic DNA segments corresponding to the miRNA clusters. (A) miR-302b*–1–2*–2–3*–3–4–5 (chromosome 4). (B) miR-371–13–14*–14 (chromosome 19). The positions of dominantly expressed mature miRNAs are highlighted in red and the positions of weakly expressed mature miRNAs are shown in blue. Conserved residues are indicated with asterisks. Sequences were aligned with CLUSTALW and manually refined.

made it difficult to judge the specificity of expression. Two miRNAs showed no ES cell-specific expression; miR-301 (miR-301) was detectable in all samples tested and miR-374 was mainly expressed in HeLa and STO cell lines. The remaining two clones (miR-369 and miR-370) could not be detected. They might be expressed only at very low levels.

Conserved miRNA gene families are organized as gene clusters

Genomic loci for 12 miRNAs are found in two gene clusters. Eight miRNA loci (miR-302b, miR-302b*, miR-302c, miR-302c*, miR-302a, miR-302a*, miR-302d, and miR-367) are located within an about 700 bp region on chromosome 4. Another four loci (miR-371, miR-372, miR-373, and miR-373*) are found within a 1050 bp region on chromosome 19. Sequence comparison of these miRNAs shows that the miRNAs from a given cluster are highly related (Fig. 3).

Four miRNAs from the chromosome 4 cluster (miR-302b, miR-302c, miR-302a (miR-302), and miR-302d) are highly homologous to each other. Their sequence similarity is greatest in the 5′ portions of the miRNA sequences as is the case with the *lin-4* and *let-7* families. This finding is consistent with the hypothesis that target recognition occurs primarily via 5′ sequences (Lai, 2002). These related miR-

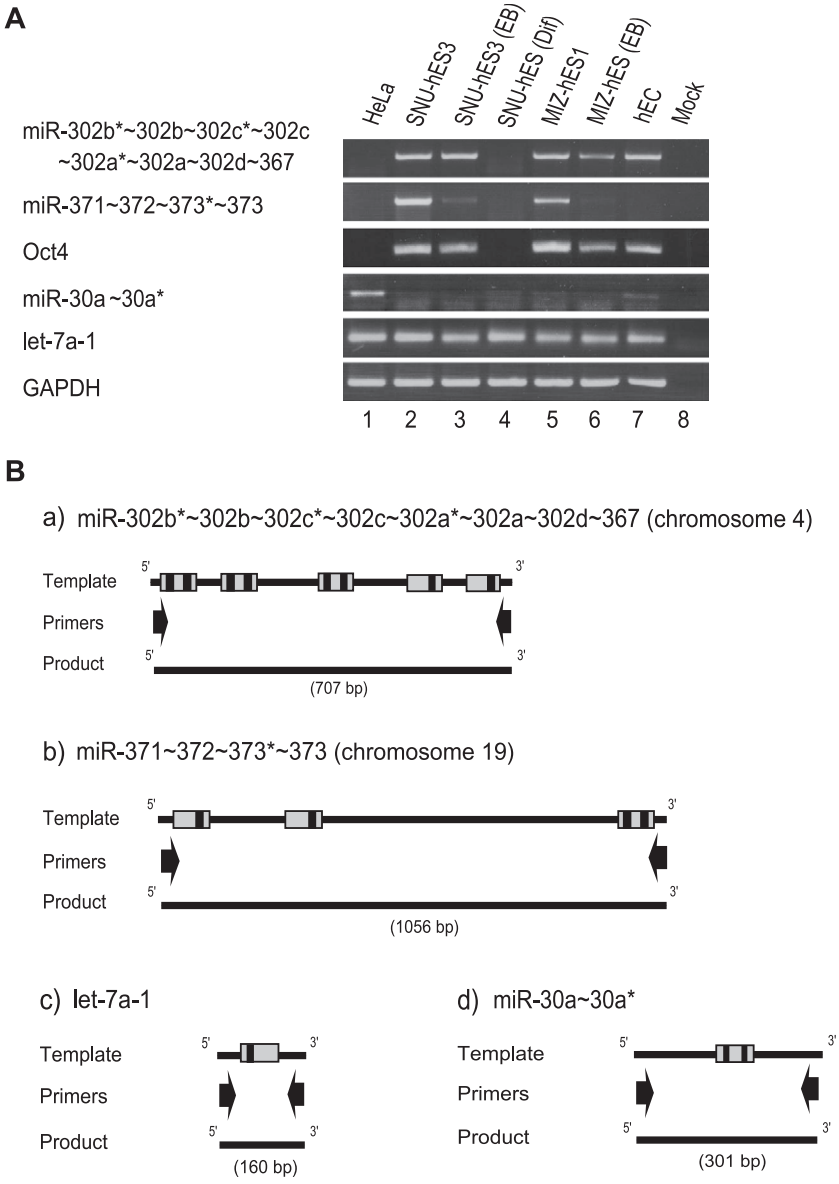

Fig. 4. Specific expression of miRNA gene clusters in undifferentiated human embryonic stem cells. RT-PCR analysis of total RNA from HeLa, two human embryonic stem cell lines (SNU-hES3 and Miz-hES1), embryoid body derived from SNU-hES3 (SNU-hES3 (EB)) or Miz-hES1 (Miz-hES1 (EB)), differentiated cells derived from EBs (SNU-hES3 (Dif)), and human embryonic carcinoma cell line (hEC). Total RNA was reverse transcribed into first-strand cDNA, which was then subjected to PCR using primers specific to the indicated miRNAs, human Oct4, and GAPDH. Genomic organization of miRNA gene clusters is schematically depicted.

NAs may recognize a consensus target sequence and hence act on the same mRNAs or different mRNAs with conserved binding sites. Therefore, recognition of these miRNA gene families should help in the identification of putative mRNA targets. The consensus sequence for these clustered miRNAs is 5′-UAAGUGCUUCCAUGUUUNNGUNN-3′ (Fig. 3A). While these miRNAs are the most abundant ones in hES cells, their murine homologue miR-302 appears to be less abundant in mES cells (Houbaviy et al., 2003). Interestingly, additional sequences related to this family are found in mouse chromosome 3 (Table 1). These putative mouse homologues form stem-loop structures and are in a gene cluster. Three other related miRNAs (miR-371, miR-372, and miR-373) are also found in a cluster on chromosome 19 (Fig. 3B). These miRNAs have been predicted by Houbaviy et al. (2003) based on the sequence similarity to mouse miR-291–295, although the expression has not been verified. Mouse miR-291–295 are located as one cluster spanning 2.2 kb (Houbaviy et al., 2003). It is noted that the miRNAs from the two gene clusters (for instance, miR-302a/miR-302 on chromosome 4 and miR-372 on chromosome 19) are similar to some extent, implicating that they may have originated from a common ancestral miRNA gene.

To examine the expression patterns of the miRNA clusters during hES cell differentiation, RT-PCR was carried out. Because clustered miRNA genes are generally transcribed into polycistronic primary transcripts (pri-miRNAs) (Lee et al., 2002), the primers were chosen to bind outside the boundary of the predicted approximately 70 nt stem-loop clusters so that we could detect pri-miRNAs covering the entire cluster. PCR products of the expected size (707 and 1056 bp, respectively) were detected from two hES cell lines (Fig. 4A, lanes 2 and 5), indicating that these clusters are indeed single transcriptional units.

Clustered miRNAs are rapidly downregulated during differentiation

The steady-state levels of primary transcripts from the two gene clusters decreased when hES cells developed into embryoid bodies (EBs) (Fig. 4A, lanes 3 and 6). EBs were prepared by culturing hES cells for 10 days in the presence of hES-cultured media without bFGF. The levels were reduced further in differentiated cells derived from EBs (Fig. 4A, lane 4). These results demonstrate that the clustered miRNAs are expressed specifically in hES cells and are rapidly downregulated during differentiation. To assess the degree of differentiation, we determined the steady-state levels of Oct4 mRNAs by RT-PCR (Fig. 4A). Interestingly, downregulation of chromosome 19 cluster (miR-12–13–14*–14) precedes that of Oct4.

The polycistronic transcripts from the chromosome 4 cluster, but not those from the chromosome 19, were detected in hEC cells (Fig. 4A, lane 7), which is consistent with the results from Northern blot analysis (Fig. 2). The control miRNA (miR-30a), which had not been cloned from hES cells, was detected in HeLa cells but only barely shown in other cells in accordance with Northern results (Figs. 2 and 4). Pri-let-7a-1 transcript was expressed in all of the tested cell lines (Fig. 4), although mature let-7a-1 appeared only in HeLa and STO cell lines (Fig. 2), which suggests that the processing of let-7a-1 may be regulated posttranscriptionally. It would be of great interest to understand how the expression of miRNAs is regulated during development.

Discussion

We have cloned small RNAs from hES cells, resulting in 36 RNAs with known characteristics of miRNAs. Of the 36 cloned RNAs, 16 were identical to previously reported miRNAs from various mammalian adult tissues and cell lines. Of the remaining 20 miRNAs, 3 miRNAs are identical to those cloned from mouse ES cells (Houbaviy et al., 2003).

Comparison to the study on mouse ES-specific miRNAs revealed interesting similarities between the two miRNA pools from hES and mES cells. Many of the ES-specific miRNA genes are highly related to each other and organized as clusters. The miR-302b, miR-302c, and miR-302d on chromosome 4 appear to be the close homologues of miR-302 that was cloned from mES cells (Houbaviy et al., 2003). We found that there are additional miRNA-like sequences related to miR-302 (Table 1). Mouse miR-302 and its related sequences also form a gene cluster on mouse chromosome 3. Another set of miRNAs, miR-371, miR-372, miR-373*, and miR-373 on chromosome 19, is the human homologues of mouse miR-290, miR-291-s, miR-291-as, miR-292-s, miR-292-as, miR-293, miR-294, and miR-295 expressed in mouse ES cells (Houbaviy et al., 2003). It is intriguing that two hES-specific miRNA clusters are conserved in the mouse genome. Although the numbers of the homologous genes are different and the sequences are variable in human and mouse clusters, which may implicate divergence of the conserved regulatory pathways, these conserved miRNAs are likely to play central roles in the regulation of mammalian ES cells.

It should be noted, however, that a considerable proportion of the cloned miRNAs from human and mouse ES cells are different from each other; 7 out of 20 miRNAs identified in our study do not have apparent homologues among those cloned from mES cells. Conversely, 5 out of 15 novel miRNAs from mES cells (Houbaviy et al., 2003) do not have related sequences in our data set. This may be because the cloning was not intensive enough to identify the complete set of miRNAs in these cell lines. These results may also implicate fundamental differences between the regulatory networks in hES cells and mES cells.

The expression patterns of miRNAs cloned from hES cells can be classified into four groups. (1) miRNAs that are expressed in ES cells as well as in EC cells; miR-302b*, miR-302b, miR-302c*, miR-302c, miR-302a*, miR-302a, miR-302d, and miR-367 (shadowed in yellow in Table 1

and Fig. 2). These miRNAs may have conserved roles in mammalian pluripotent stem cells. (2) miRNAs that are expressed specifically in ES cells but not in other cells including EC cells; miR-200c, miR-368, miR-154*, miR-371, miR-372, miR-373*, and miR-373 (shadowed in blue in Table 1 and Fig. 2). These miRNAs may have functions specific to ES cells. It would be interesting to dissect the molecular basis for the differences between the two pluripotent stem cells; ES and EC cells. (3) miRNAs that are rare in ES cells but abundant in HeLa and STO cells; let-7a, miR-301 (miR-301), miR-374, miR-21, miR-29b, and miR-29. These stage-specific miRNAs may play roles in the regulation of development and differentiation, like let-7 in *C. elegans* (Reinhart et al., 2000). (4) The last class consists of miR-16, miR-17-5p, miR-19b, miR-26a, miR-92, miR-103, miR-130a, and miR-222. These are expressed in most tested cell lines so they may contribute to basic cellular functions (Fig. 2).

The expression pattern of the cluster of miR-12–13–14*–14 (chromosome 19) is particularly interesting because downregulation of this cluster becomes evident more rapidly than that of Oct4, which is the earliest marker for ES cells known so far (Fig. 4A). It is tempting to speculate that these miRNAs may be the primary regulators of embryonic stem cell maintenance or differentiation, which act before other known factors including Oct4. These miRNAs may define the very early stage of embryonic development that has not been recognized before.

For most miRNAs, RNA from only one side of the miRNA precursor is typically cloned or detected on Northern blot. In this study, four miRNA genes (miR-302b, miR-302c, miR-302a, and miR-373 hairpins) yielded small RNAs corresponding to both strands of the stems (Table 1 and Fig. 1). However, the frequencies of the clones of the two opposite strands are not equal, indicating that there is a certain degree of asymmetry in the abundance of these miRNAs. In the case of miR-302b and miR-302c, the ratios between the cloning frequencies of the two sides were 22.5:1 and 10:1, respectively. According to the standard nomenclature (Ambros et al., 2003), the less abundant miRNA of each pair was designated with an asterisk mark. Recent studies using siRNA duplexes demonstrated that this asymmetry comes from asymmetric degradation of the opposing strand following Dicer processing. According to this, the strand with the less stable 5′ end has a better chance to survive (Khvorova et al., 2003; Schwarz et al., 2003). Sixty-nine percent of our novel miRNA precursors are clearly in agreement with this, indicating that this rule is generally valid although the mechanism of strand selection for miRNA may be more complex than that for siRNA.

It is intriguing that ES cells are such rich sources of novel miRNAs. Previous cloning efforts intensively searched for miRNA genes using cultured mammalian cells and adult tissues. It would be an interesting quest to look for additional miRNAs in other rare cell types. For instance, small RNA expression profiling of various stem cells or mouse embryos from different developmental stages would be highly informative. ES cell-specific miRNAs appear to be less conserved than other previously reported miRNAs from adult tissues and cell lines. miRNAs found in our study have their homologues only in other mammals, but not in invertebrates such as *C. elegans* and *Drosophila*. The low degree of conservation may partially explain the reason why these miRNAs have not been identified until now. They may have escaped bioinformatics database searches, which partially rely on phylogenetic conservation.

None of the miRNAs cloned in our study complements perfectly to known mRNAs or ESTs, suggesting that these miRNAs may act as translational inhibitors through imperfect pairing to their target mRNAs. It would be important to identify the target mRNAs of ES-specific miRNAs that will lead us to understand the complex and interesting networks of regulation in ES cells.

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