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miRNA-222 Modulates Differentiation of Mouse Embryonic Stem Cells

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ABSTRACT : MicroRNAs (miRNAs) function as a key regulator of diverse cellular functions. To find out novel miRNAs that promote the differentiation of mouse embryonic stem cells (mESCs), we compared the miRNAs expression profiles of mESCs under self-renewal vs. differentiation states. We noticed that miR-222 was highly expressed during the differentiation of mESCs. Quantitative RT-PCR analysis revealed that expression of miR-222 was up-regulated during the embryonic bodies formation and retinoic acid -dependent differentiation. When miR-222 was suppressed by antogomiR-222, the differentiation of mESCs was delayed compared to control. Self-renewal marker expression or cell proliferation was not affected but the expression of lineage specific marker was suppressed by the treatment of miR-222 inhibitor during the differentiation of mESCs. Taken together, these results suggest that miR-222 functions to promote the differentiation of mESCs by regulating expression of differentiation related genes.

Key words : Differentiation, Embryonic stem cells, MicroRNAs, miR-222

INTRODUCTION

MicroRNAs (miRNAs) is consisted with small 19- to 25-ribonucleic acid (RNA) molecule and found in eukaryotic cells. miRNAs regulates post-transcriptional process of gene expression by binding to complementary sequences on target messenger RNA transcripts (mRNAs). Binding of miRNAs to target sequences usually results in the inhibition of translation process or induces mRNA degradation. Hundreds of miRNAs have been found in various tissues, and in many case, miRNAs are key players on cellular function, including the pathogenesis of several human diseases (Kato & Slack, 2008).

Embryonic stem cells (ESCs) are pluripotent cells derived from inner cell mass or epiblast of preimplantation embryos. ESCs are able to differentiate into all cell types except placenta (Evans & Kaufman, 1981; Thomson et al., 1998). Because of their plasticity and potentially unlimited capacity for self-renewal, ESCs are excellent materials for regenerative medicine and developmental research. Recently, miRNAs are described a main component for the transition of ESCs from self-renewal into differentiation state. Using ESCs null for the Dicer gene, a key molecule for miRNAs biogenesis, cell cycle progression was slowed down by the absence of miRNAs, which leads to the abnormal differentiation (Kanellopoulou et al., 2005; Murchison et al., 2005). On the contrary, miR-195 down regulates the G2-M checkpoint inhibitory kinase WEE1, an inhibitor of the G2 cyclin B-Cdk complex. In this case, miRNAs plays the counteract inhibitor of the cell cycle (Qi et al., 2009). In addition, some miRNAs (miR-134, 141, 200, 296 and 470) target master genes of ESC pluripotency and self-renewal, such as *Oct4*, *Sox2*, *Nanog* and *Myc*. The consorted activity of these miRNAs regulates maintenance of self-renewal or differentiation of ESCs (Tay et al., 2008; Lin et al., 2009). Recently, a number of reports have shown that miRNAs also function in reprogramming. miR-290 and miR-302 cluster control the expression of self-renewal related genes and then inducing reprogramming (Barroso-delJesus et al., 2008; Card et al., 2008; Marson et al., 2008).

In this study, we analyzed the expression profiles of miRNAs during the differentiation of mouse embryonic stem cells (mESCs). We found that miR-222 was prominently upregulated during the differentiation of mESCs. MicroRNA

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inhibitor-mediated suppression of miR-222 transcripts delayed the differentiation of mESCs. We found that the expression of self-renewal genes or cell proliferation was not affected by the miR-222. Instead, the reduced miR-222 level resulted in the suppression of differentiation related markers such as *Nestin*. Taken together, we suggest that the function of miR-222 in mESCs is to facilitate the differentiation of the cells by regulating the expression of differentiation related genes.

MATERIALS AND METHODS

1. Reagents and Cell Culture

The mESCs line J1 (cat # SCRC-1010) was purchased from ATCC (Manassas, VA, http://www.atcc.org). These cells were maintained on feeder- free and 0.1% gelatin coated petri-dishes in Dulbecco's modified Eagle's medium (DMEM, Gibco Invitrogen, Carlsbad, CA, http://www.invitrogen.com) supplemented with 10% horse serum (Gibco Invitrogen), 1x non-essential amino acids (Life Technologies), 2 mM glutamine, 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, St Louis, MI, http://www.sigmaaldrich.com), 100 U/㎖ penicillin, 100 ㎍/㎖ streptomycin (Gibco Invitrogen), and 1,000 U/㎖ LIF (Chemicon, Temecula, CA, http://www._chemicon.com). To induced differentiation, we formed Embryonic bodies (EBs) in LIF-deficient ESCs medium (as described above) by suspension culture and media changed every two days. To induce spontaneous differentiation, mESCs were cultured in LIF-deficient ESC medium with 500 nM all-trans retinoic acid (RA) (Lee et al., 2009).

2. Micro-array for miRNA Expression

To comparing miRNA expression levels between undifferentiation and differentiation conditions, we used the PANArray™ miRNA expression profiling kit (PANAGENE, PM-2001, Korea, http://www.panagene.com). For Array, total RNA was extracted from mESCs using TRIzol (Invitrogen), and 400 ng of total RNA was denaturated at 95℃ for 5 min. The denatured RNA sample was hybridized using specific buffer at 55℃, 4 hours and then, washed two times. The next labeling process was performed by using bound miRNA as enzymatic ligation with pCP-Cy3. After washing steps, the sample was scanning by GenePix4000B Axon Instruments.

3. Quantitative Analysis of miRNA Expression and Suppression of miRNA Expression by AntagomiR Transfection

For analysis of miRNA expression, total RNA was isolated from undifferentiated and RA-induced differentiated ESCs with PureLink RNA Mini Kit (Ambion, http://www.Ambion.com). From each sample, 10 ng of total RNA was used to synthesize single-stranded cDNA with TaqMan MicroRNA reverse transcription kit and miR-222 primer (ABS-4393387, GeneID:002276, Applied Biosystems, Foster City, CA, USA, http://www.appliedbiosystem.com) according to the manufacturer's instructions. Real-time PCR was carried out in triplicate with Rotor-Gene (Corbett Research, http://www.corbettlifescience.com). AntagomiR for miR-222 was purchased from Ambion (ABS-4427975, Gene ID: 000397) and transfection was performed according to the manufacturer's instructions using Lipofectamin 2000 (Invitrogen).

4. RNA Extraction and Real-time PCR

Total RNA was extracted from mESCs using TRIzol (Invitrogen), and 2 ㎍ of total RNA was reverse-transcribed into cDNA using the SuperScriptII™ First-Strand Synthesis System (Invitrogen), according to the manufacturer's instructions. Real-time PCR was carried out in triplicate with the Quantitect SYBR Green PCR kit (Qiagen, Valencia, CA, http://www.qiagen.com) and CFX96 Real-time System (Bio-Rad Laboratories, Richmond, CA http://www.bio-rad.com). For quantification, target genes were normalized with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. The PCR primers used in this study are listed in Table 1.

5. Alkaline Phosphatase Activity Assay

To analysis alkaline phosphatase activity, we used the

Table 1. Primers used for realtime RT-PCR in this study

	Primer sequences (5' to 3')	
	Forward	Reverse
GAPDH	gggtgtgaaccatgagaa	gtcttctgggtggcagtgat
Oct3/4	ctgagggccaggcaggagcacgag	ctgtagggagggcttcgggcactt
Sox2	ggttacctcttcctcccactccag	tcacatgtgcgacaggggcag
Nanog	gatgctctgcacagaggctg	caccgcttgcacttcaccct
Nestin	ctgcaggccactgaaaagt	ttccaggatctgagcgatct
Gata4	gatgggacgggacactacctg	acctgctggcgtcttagattt
VE-cadherin	tcctctgcatcctcactatcaca	gtaagtgaccaactgctcgtgaat

alkaline phosphatase (AP) assay kit (86-R, Sigma Aldrich). In briefly, cells were fixed by citrate-acetone-formaldehyde solution for 1min, and treated stain solution for 15 min. The stain solution was composed by sodium nitrate solution, FRV-alkaline solution and AS-BI alkaline solution. After staining, cells were washed twice using dPBS (Dulbecco's phosphate-buffered saline, 14190-250, Invitrogen) and observed.

6. Statistical Analysis

Graphical data are presented as means ± SD. Each experiment was performed at least three times and subjected to statistical analysis. Statistical significance between two groups was determined using Student's *t*-test. A p value < 0.05 was considered significant. Statistical analysis was performed using the SAS statistical package v.9.13 (SAS Inc., Cary, NC, http://www.sas.com/).

RESULTS AND DISCUSSION

1. miRNAs Expression Profile during the Differentiation of mESCs

miRNAs are emerging as master regulators of stemness properties of ESCs (Gustavo & Juan, 2010; Junlin et al., 2009). To elucidate the function of miRNAs during the differentiation of mESCs, we compared the miRNA expression profiles between mESCs in self-renewal state and RA-induced differentiating cells by PANArray^{TM}. We noticed that only three miRNAs were significantly activated at 2 days after RA induced differentiation of mESCs, and the expression of miR-222 was the most activated miRNAs (Fig. 1). miR-222 is reported as a member of onco-miRs, which are related to the malignancy of the tumors (Tsunoda et al., 2011; Quintavalle et al., 2011). In several human cancer cells, miR-221 and miR-222 activate signaling pathway involved in cancer cell survival and anti-cancer drug resistance (Shah & Calin, 2011; Silvia et al., 2011). In addition, miR-221 and 222 inhibit expression of epithelial-related genes and activate expression of mesenchymal-specific genes, which leads to cell migration and invasion (Acunzo et al., 2011; Stinson et al., 2011). Cardinali et al. (2009) and Staszel (2011) et al. showed that miR-221 and 222 control skeletal muscle differentiation and endothelial cell pathophysiology. Despite several studies reported the function of miR-222 in cancer, the role of miR-222 in ESCs has not been reported. To confirm the result of miRNAs array, we quantitatively analyzed the expression level of miR-222 in 2 days old EBs and spontaneously differentiated EBs. As shown in Figure 2A, the miR-222 expression was not activated in EBs but it was significantly increased during the differentiation of EBs. Next, we examined the expression of miR-222 during RA induced differentiation of mESCs. miR-222 expression was highly activated from 2 days after the differentiation and the increased expression was maintained until 6 days after RA-induced differentiation. These results suggest that the expression of miR-222 is related to the

	Normalized Ratio of expression miRNA
	RA-D2/RA-D0
miR-122	0.76
miR-370	0.87
miR-22	0.88
miR-103	0.89
miR-202	0.90
miR-206-3	0.91
miR-208b	0.91
miR-23b	0.93
miR-23a	0.94
miR-137	0.94
miR-342-3p	0.96
miR-133b	0.97
miR-198	0.97
miR-452	0.98
miR-30d	1.00
miR-197	1.00
miR-200c	1.01
miR-143	1.01
miR-142-5p	1.02
miR-214	1.02
let-7b	1.03
miR-183	1.04
miR-127	1.04
miR-215	1.05
miR-127-5p	1.06
let-7c	1.07
miR-107	1.08
miR-150	1.09
miR-181c	1.09

	Normalized Ratio of expression miRNA
	RA-D2/RA-D0
miR-296-3p	1.09
miR-153	1.10
miR-188-3p	1.10
miR-138	1.10
miR-124a	1.10
miR-135a	1.12
miR-145	1.12
miR-1	1.12
let-7i	1.12
miR-194	1.13
miR-216a	1.13
miR-196b	1.13
miR-125a	1.13
miR-133a	1.13
miR-185	1.14
miR-136	1.15
miR-141	1.15
miR-371-5p	1.15
miR-186	1.16
miR-188-5p	1.17
miR-30c	1.17
miR-26a	1.18
miR-190b	1.18
miR-149	1.19
miR-181b	1.19
miR-95	1.19
miR-130b	1.20
miR-210	1.20
miR-17-3p	1.20

	Normalized Ratio of expression miRNA
	RA-D2/RA-D0
miR-25	1.09
miR-106b	1.21
miR-134	1.21
miR-9*	1.22
miR-30a	1.24
miR-372	1.25
miR-151	1.25
miR-181d	1.26
miR-34a	1.27
miR-205	1.28
miR-7	1.28
miR-140-3p	1.28
miR-203	1.28
miR-132	1.28
miR-488	1.30
miR-199a	1.30
miR-302a	1.32
miR-27a	1.32
miR-148a	1.35
miR-182	1.35
miR-193a-5p	1.37
miR-130a	1.39
miR-296-5p	1.40
miR-30b	1.41
miR-219-5p	1.45
miR-155	1.48
miR-96	**1.54**
miR-368	**1.87**
miR-222	**1.91**

Fig. 1. Fold increases of miRNAs expression during the RA-induced differentiation of mESCs. mESCs were differentiated with 500 nM RA for 3 days and miRNA expression profile in the differentiated cells was compared with that of mESCs under self-renewal culture condition.

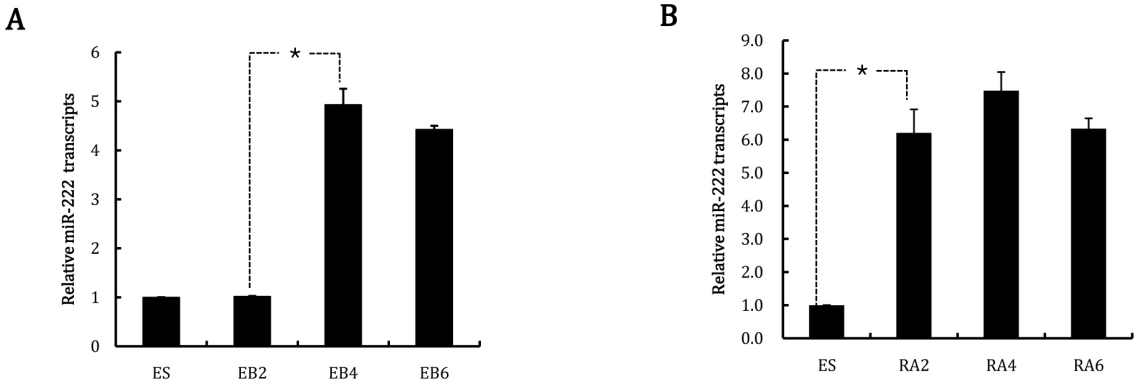

Fig. 2. miR-222 expression pattern during the differentiation of mESCs. (A) Quantitative PCR analysis of miR-222 in mESCs, EBs and differentiated EBs (4 days or 6 days) (B) Quantitative PCR analysis of miR-222 in mESCs and cells spontaneously differentiated with RA for 2, 4 or 6 days (RA2, RA4, RA6). Quantitative miRNAs levels were normalized against U6. All values are means ± S.D. from at least triplicate experiments. * Indicates significant (P<0.05) results based on Student's T-test analyses.

differentiation of mESCs.

2. Suppression of miR-222 Expression Delays Differentiation of mESCs

To study the role of miR-222 for initiation of mESCs' differentiation, we suppressed miR-222 using antagomiR-222 and then examined the differentiation pattern of mESCs. We first confirmed that transfection of antagomiR-222 in mESCs successfully suppressed the expression of miR-222 and the inhibition effect was maintained until 24 hrs after RA-induced differentiation (Fig. 3A). Interestingly, we noticed that the differentiation of mESCs transfected with antagomiR-222 was delayed compared to control when cells were examined 24 hrs after the induction of differentiation (Fig. 3B, top pannel). miR-222 knock-downed cells maintained ESC like morphology whereas control cells showed typical differentiated cell morphology. Alkaline phosphatase (AP) staining clearly showed that antagomiR-222 transfected cells were less differentiated compared to control (Fig. 3B, bottom pannel). Taken together, we concluded that suppression of miR-222 in mESCs delays the differentiation of mESCs, which implies that miR-222 functions to promote the differentiation of mESCs.

3. The Effect of miR-222 Suppression on the Expression of Self-renewal or Differentiation Related Genes

To analyze underlying mechanism of differentiation retardation induced by suppression of miR-222 expression, we first examined the expression level of self-renewal related genes such as *Oct4*, *Sox2* and *Nanog*, which are prominent genes that are essential to maintain the self-renewality of ESCs. Unexpectedly, the expression level of *Oct4*, *Sox2* and *Nanog* in antogomir-222 transfected mESCs was similar to those of control cells (Fig. 4A). Therefore, we next examined the expression of marker genes of lineage differentiation. As shown in Figure 4B, the expression of *Nestin*, a marker of neural differentiation, was significantly low in antagomir-222 transfected cells compared to control whereas expression of *Gata4* and *VE-cadherin*, markers of mesodermal differentiation, were comparable to control. These results suggest the possible role of miR-222 to regulate the differentiation associated genes. Since we induced differentiation of mESCs by treating RA, we suspect that most of mESCs were differentiated into neural lineage (Fraichard et al., 1995). This would explain why *Nestin* expression is modulated by miR-222 whereas the expression of *Gata4* or *VE-cadherin* was not affected. In gastric

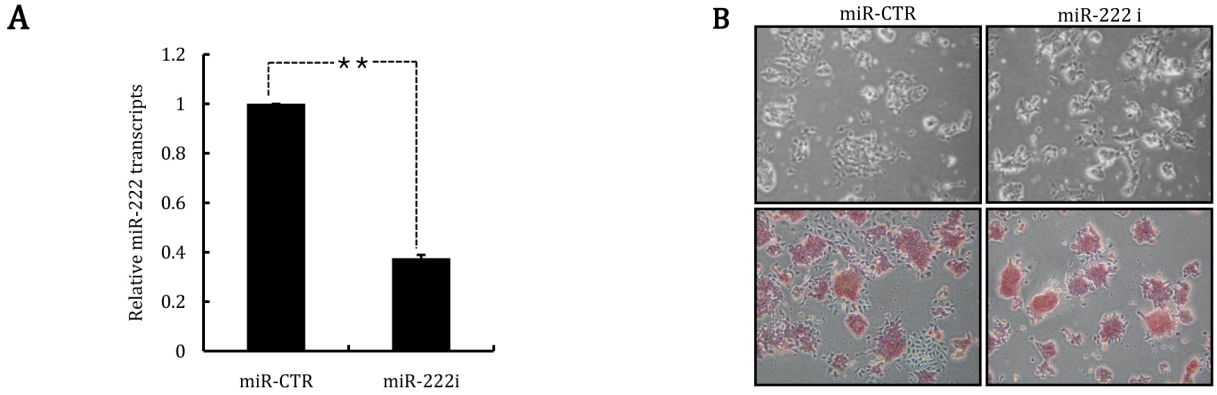

Fig. 3. The effect of miR-222 suppression on the differentiation of mESCs. Suppression of miR-222 by lipofectamine mediated transfection of antagomiR for miR-222 (miR-222i). AntagomiR was transfected into mESCs and differentiation was induced 24 hrs after transfection by removing LIF and adding RA. The expression of miR-222 (A) or cell morphology (B) was analyzed 24 hrs after RA mediated differentiation. All values are means ± S.D. from at least triplicate experiments. ** Indicates significant (P<0.01) results based on Student's T-test analyses. Abbreviations: miR-CTR, mESCs transfected with miRNA non-specific control; miR-222i, mESCs transfected with antagomir-222.

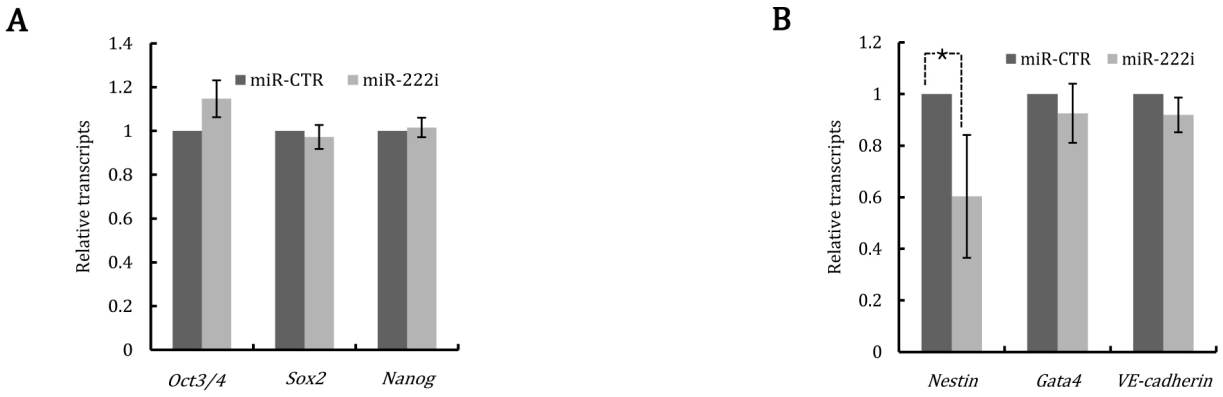

Fig. 4. The effect of miR-222 suppression on the expression of self-renewal or differentiation related genes. As in Figure 3, antagomir was transfected into mESCs and cells were differentiated for 24 hrs to analyze the expression of self-renewal related genes (A) or differentiation related genes (B). Quantitative gene expression levels were normalized against GAPDH. All values are means ± S.D. from at least triplicate experiments. * Indicates significant (P<0.05) results based on Student's T-test analyses. Abbreviations: miR-CTR, mESCs transfected with miRNA non-specific control; miR-222i, mESCs transfected with antagomir-222.

cancer, miR-222 is reported as a key regulator of cell cycle related genes (Kim et al., 2009). However, cell cycle of antagomir-222 transfected cells was similar with that of control suggesting that the growth retardation induced by the inhibition of miR-222 was not caused by cell cycle perturbation (data not shown).

Taken together, we suggest that miR-222 is a novel miRNA that regulates the expression of differentiation related genes, which probably leads to the promotion of differentiation of mESCs. Since miRNAs are negative regulator of gene expression, miR-222 is expected to regulate differentiation related genes via indirect mechanism. Therefore, it will be important to elucidate the direct downstream target of miR-222, which connects the miR-222 activity to the activation of differentiation related genes. Previously, several miRNAs were identified as regulators to maintain stemness and to activate differentiation of ESCs (Gustavo et al., 2010; Kathryn et al., 2010; Tarantino et al., 2010). It would be interesting how the function of miR-222 is connected with other miRNAs known to induce differentiation of ESCs.

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