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MicroRNA profiling during germline differentiation of mouse embryonic stem cells

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Abstract

MicroRNAs are new classes of small non-coding regulatory RNAs which control degradation or suppress translation of its target mRNAs by sequence complementarity. Mature microRNAs are enriched in embryonic stem cells and play important roles in controlling stem cell self-renewal as well as control of differentiation. There is significant evidence that microRNAs are involved in the regulation of stem cell differentiation. The male mouse Embryonic Stem Cell line C57BL6/J with normal karyotype 46, XY was used for profiling microRNA expression in undifferentiated mouse embryonic stem cells (mESCs) and mESCs which were differentiated to germ line cells to determine and compare differences in microRNA expression before and after differentiation. Also, testis tissue samples of a 5-day-old mouse and a mature mouse was used as *in vivo* control. Profiling was performed by quantitative real-time PCR using locked nucleic acid microRNA-specific LNA™-enhanced primers. After data analysis and comparison of results profiled microRNAs expression, three microRNAs, mmu-miR-21, mmu-miR-21* and mmu-miR-16 showed 50.31, 43.76 and 46.77-fold change increase of expression, respectively, in differentiated mESCs in comparison with undifferentiated state with significant p-value (Average p-value $p<0.001$ for each members of microRNAs). Expression of Let-7 microRNA family increased in differentiated state when compared with undifferentiated mESCs (Average p-value<0.0001 for each members of family). The levels of expression all other profiled microRNAs were significantly higher in undifferentiated in comparison with differentiated mESCs and their expression was down regulated after differentiation. (Average p-value <0.003 for each members of microRNAs).

Key words: MicroRNA, Embryonic Stem Cell, Self-renewal, Germ line Differentiation.

Introduction

MicroRNAs are a novel class of short endogenous (on average 22 nucleotides long) non-coding RNAs that are transcribed by RNA polymerase II and can play important regulatory and developmental roles in evolution and stability of many mRNAs in transcriptional and post-transcriptional levels of gene expression. The microRNA pathway has emerged as one of the most extensively investigated pathways of the past decade. They act by direct binding to their target sites on mRNAs (1-7). Mature microRNAs are processed products from larger microRNA precursor transcripts (8). Biogenesis and function of microRNAs are highly regulated and this regulation may be cell type specific. Expression of microRNAs has been identified in human cancers which suggested function of them as a tumor repressor gene, regulate response to chemotherapy and control of signalling pathways (9-10). Some microRNAs are tissue specific and preferentially expressed in embryonic stem cells and embryonic tissues (7, 11). Mature microRNAs which are abundant in stem cells can control stem cell self-renewal as well as their differentiation towards different lineages (12). Aside from transcription factors, signaling pathways and other different signaling and regulatory molecules, up- and down-regulation of microRNAs can be important in maintenance of pluripotency and differentiation of embryonic stem cells (13). Despite identification of huge numbers of microRNAs, the functions of most of these microRNAs still remain to be discovered. MicroRNAs are well conserved in eukaryotic organisms and they are considered as one of the major regulators of gene expression. As a novel group of endogenous small RNAs, the evolution of microRNAs in vertebrates and their role in developmental biology is being well documented. Expression and profiling of microRNAs can be assayed by quantitative real-time PCR, but their small size and wide range of melting temperatures pose a special challenge in designing experimental primers and probes for their detection and quantification with acceptable sensitivity and specificity. Detection of mature microRNAs with PCR has been technically challenging because of their small size and homology in sequence. Hybridization properties of LNA-modified oligonucleotide probes have proven to be highly useful for determining the expression of microRNAs in cells and tissues. By applying proprietary LNA™ technology in PCR primers, profiling and expression analysis is performed with high sensitivity and specificity. LNAs, locked nucleic acids , are a class of nucleic acid analogs in which the furanose ring of the ribose is 'locked' with a methylene bridge

Table 1. Name, sequence and accession number profiled mature mouse microRNAs.

MicroRNA name	Sequence	Accession number mature sequence
mmu-let-7a	UGAGGUAGUAGGUUGUAUAGUU	MIMAT0000521
mmu-let-7b	UGAGGUAGUAGGUUGUGUGGUU	MIMAT0000522
mmu-let-7c	UGAGGUAGUAGGUUGUAUGGUU	MIMAT0000523
mmu-let-7d	AGAGGUAGUAGGUUGCAUAGUU	MIMAT0000383
mmu-let-7f	UGAGGUAGUAGAUUGUAUAGUU	MIMAT0000525
mmu-let-7e	UGAGGUAGGAGGUUGUAUAGUU	MIMAT0000524
mmu-let-7g	UGAGGUAGUAGUUUGUACAGUU	MIMAT0000121
mmu-let-7i	UGAGGUAGUAGUUUGUGCUGUU	MIMAT0000122
mmu-miR-17	CAAAGUGCUUACAGUGCAGGUAG	MIMAT0000649
mmu-miR-18a	UAAGGUGCAUCUAGUGCAGAUAG	MIMAT0000528
mmu-miR-18b	UAAGGUGCAUCUAGUGCUGUUAG	MIMAT0004858
mmu-miR-19a	UGUGCAAAUCUAUGCAAAACUGA	MIMAT0000651
mmu-miR-19b	UGUGCAAAUCCAUGCAAAACUGA	MIMAT0000513
mmu-miR-19b-1*	AGUUUUGCAGGUUUGCAUCCAGC	MIMAT0017065
mmu-miR-92a-2*	AGGUGGGGAUUGGUGGCAUUAC	MIMAT0004635
mmu-miR-92b	UAUUGCACUCGUCCCGGCCUCC	MIMAT0004899
mmu-miR-302a	UAAGUGCUUCCAUGUUUUGGUGA	MIMAT0000380
mmu-miR-302b	UAAGUGCUUCCAUGUUUUAGUAG	MIMAT0003374
mmu-miR-302c	AAGUGCUUCCAUGUUUCAGUGG	MIMAT0003376
mmu-miR-302d	UAAGUGCUUCCAUGUUUGAGUGU	MIMAT0003377
mmu-miR-302a*	ACUUAAACGUGGUUGUACUUGC	MIMAT0004579
mmu-miR-302b*	ACUUUAACAUGGGAAUGCUUUCU	MIMAT0003373
mmu-miR-290-3p	AAAGUGCCGCCUAGUUUUAAGCCC	MIMAT0004572
mmu-miR-290-5p	ACUCAAACUAUGGGGGCACUUU	MIMAT0000366
mmu-miR-291a-3p	AAAGUGCUUCCACUUUGUGUGC	MIMAT0000368
mmu-miR-291a-5p	CAUCAAAGUGGAGGCCCUCUCU	MIMAT0000367
mmu-miR-291b-5p	GAUCAAAGUGGAGGCCCUCUCC	MIMAT0003189
mmu-miR-292-3p	AAAGUGCCGCCAGGUUUUGAGUGU	MIMAT0000370
mmu-miR-292-5p	ACUCAAACUGGGGGCUCUUUUG	MIMAT0000369
mmu-miR-294	AAAGUGCUUCCCUUUUGUGUGU	MIMAT0004574
mmu-miR-293	AGUGCCGCAGAGUUUGUAGUGU	MIMAT0000371
mmu-miR-294*	ACUCAAAAUGGAGGCCCUAUCU	MIMAT0004574
mmu-miR-295	AAAGUGCUACUACUUUUGAGUCU	MIMAT0000373
mmu-miR-295*	ACUCAAAUGUGGGGCACACUUC	MIMAT0004575
mmu-miR-20a	UAAAGUGCUUAUAGUGCAGGUAG	MIMAT0000529
mmu-miR-20a*	ACUGCAUUACGAGCACUUAAAG	MIMAT0004627
mmu-miR-20b	CAAAGUGCUCAUAGUGCAGGUAG	MIMAT0003187
mmu-miR-20b*	ACUGCAGUGUGAGCACUUCUAG	MIMAT0004788
mmu-miR-21	UAGCUUAUCAGACUGAUGUUGA	MIMAT0000530
mmu-miR-21*	CAACAGCAGUCGAUGGGCUGUC	MIMAT0004628
mmu-miR-124	UAAGGCACGCGGUGAAUGCC	MIMAT0000134
mmu-miR-16	UAGCAGCACGUAAAUAUUGGCG	MIMAT0000527
mmu-miR-182	UUUGGCAAUGGUAGAACUCACACCG	MIMAT0000211
mmu-miR-130a	CAGUGCAAUGUUAAAAGGGCAU	MIMAT0000141
mmu-miR-467e	AUAAGUGUGAGCAUGUAUAUGU	MIMAT0005293

connecting the 2'-O atom with the 4'-C atom. LNAs are constrained in the ideal conformation for Watson-Crick binding (14-16). This modification cause significant thermal stability and an increase in melting temperature with excellent properties in discrimination of mismatch sequences with an enhanced binding affinity to its targets. We propose that microRNAs are likely important regulators for stem cell self-renewal. The purpose of this study was differential analysis of microRNA expression in undifferentiated mESCs and comparison of results with differentiated state toward germ line cells in the presence of retinoic acid (RA) to elucidate differences microRNA gene expression and specify possible role of related microRNAs in control of self-renewal and differentiation.

Materials and methods

We have performed mouse microRNAs profiling using LNA™-enhanced specific real-time PCR primers to analyze expression mature microRNAs. Profiled microRNAs are listed in Table 1. Necessary microRNA information was obtained from the following websites; miRanda (http://www.microrna.org/), miRBase (http://www.mirbase.org/), DIANA LAB (diana.cslab.ece.ntua.gr) and miRecords (mirecords.biolead.org).

We used the mouse embryonic stem cell line C57BL/6J with normal male (46, XY) karyotype (obtained from Invitrogen, USA), which was harboring a fusion construct (Stra8-EGFP), consisting of the coding region of the enhanced green fluorescent protein under the control of the 1.4 kb promoter region germ line specific gene Stra8 (a gift from Dr. Karim Nayernia), to sort differentiated embryonic stem cell populations which were successfully imported to meiosis by fluorescence-activated cell sorter FACS Aria (BD Biosciences). Previous data reported that the 1.4 kb flanking promoter region of the Stra8 gene is able to direct reporter gene expression specifically in premeiotic stage of germ cells (17).

Embryonic stem cell culture

Mouse Embryonic Stem Cell C57BL6/J was cultured in an undifferentiated state on mitomycin C-inactivated mouse embryonic fibroblast (MEF) as feeder layer cells to expand and increase cell colony number with knock-out Dulbecco's modified Eagle's medium (KO-DMEM,GIBCO-BRL) supplemented with 15% ES-FBS, 2 mM L-glutamine (GIBCO-BRL), 100 u/ml penicillin/streptomycin (GIBCO-BRL), 50 mM b-mercaptoethanol (Promega), 1% nonessential amino acid (NEAA; GIBCO-BRL), and 10^3 U/ml leukemia inhibitory factor (LIF) (18).

Differentiation of mESC toward germ line lineage and cell sorting

To differentiate mES cells, cells were trypsinized and cultured on 0.1% gelatin-coated culture dish (gelatin, Sigma) in the absence of LIF. Differentiation was induced by all-trans retinoic acid (RA, Sigma-Aldrich).

Table 2. RT-PCR primer sequences used for each gene marker.

Stage	Target gene	Primer	Sequence	Product Size
Pre-meiotic	Nanog	Forward	CTGTGGGAAGGCTGCGGCTCAC	330 bp
		Reverse	GAAGACGCAGCCTCTGTGCAGA	
	oct-04	Forward	CTGAAGCAGAAGGAGGATCACC	180 bp
		Reverse	TCGAACCACATCCTTCTCTAGCC	
	Dazl	Forward	CAGGCATATCCTCCTTATCCAAG	263 bp
		Reverse	TGTATGCTTCGGTCCACAGAC	
	Vasa	Forward	CGTTGAATACAGCGGGGATTTC	257bp
		Reverse	GCAGTGTTGTAACGTCAGCATT	
Meiotic	Sycp3	Reverse	CCGGAGCCGCTGAGCAAACA	436 bp
		Reverse	CCAGTTCCCACTGCTGCAACAC	
Post-meiotic	Prm1	Forward	CTCACAGGTTGGCTGGCTCGAC	195 bp
		Reverse	CGGCGACGGCAGCATCTTCG	
Housekeeping gene control	Pgm1	Forward	GCTTCGATGCGAGAGCTCAC	190 bp
		Reverse	TGCGACACGGTGTACGGCAC	

Stock lyophilized retinoic acid was diluted in ES-DMSO to give a stock solution of 10^{-1} M. Retinoic acid was added to the medium at a final concentration of 10^{-5} M to induce differentiation. After 72 hours of RA induction, GFP-positive cells were selected using fluorescence-activated cell sorter (FACS). Briefly, cells were trypsinized and after pipetting up and down several times they were filtered through a 40 µm falcon cell strainer to create single-cell suspension. Cell sorting was performed with about 6×10^6 mouse embryonic stem cells using FACS Aria flow cytometric cell sorter (FACS Aria, BD Biosciences). Simultaneously, one culture dish with about 3×10^6 mESC C57BL6/J cells harboring construct Stra8-EGFP were cultured on 0.1% gelatin-coated culture dish in presence of 10^3 U/ml LIF and absence of RA and considered as the negative control cell population for cell sorting. Sorted pure GFP-positive cells were cultured on 0.1% gelatin-coated culture dish and designated as day-1 and were kept in presence of RA in final concentration of 0^{-5} M for 30-days to continue their differentiation.

Detection of gene marker specific transcripts

Total cellular RNA was extracted from cells and testicular tissue according to the manual (TriPure RNA isolation kit, Roche Applied Sciences, Germany). Total RNA extracted from samples of undifferentiated mESC, and mESC after 5-days, 12-days, 21-days and 30-days after RA induction to check expression of different gene markers. Total of 1 µg RNA was reverse transcribed into cDNA by MMLV reverse transcriptase (Fermentas) and random hexamers according to the manufacturer's instructions of the cDNA synthesis kit (RevertAid ™ First Strand cDNA Synthesis Kit, Fermentas). Housekeeping gene, mouse Phosphoglucomutase-1 (pgm1), was used to check quality and the amplification reaction of cDNAs. In order to check the different stages consisting of pre-meiotic, meiotic and post-meiotic of embryonic stem cells differentiation, we used specific forward and reverse primers to amplify the genes nanog, oct-4 (Octamer-binding transcription factor 4), Dazl (Deleted in azoospermia-like), Vasa, Sycp3 (Synaptonemal complex protein 3) and Prm1 (protamine 1). The PCR amplification condition for all mentioned genes was performed in 5 minutes at 95° C followed by 30 cycles of 95 ° C for 30 seconds, 60 °C for 30 seconds, 72 °C for 30 seconds, and final extension at 72°C for 10 minutes. Each PCR round was performed in a final reaction volume of 25 µl. The sequences of primers are listed in Table 2. PCR products were separated on a 2% agarose gel containing ethidium bromide and then visualized under ultraviolet light. The results were considered as positive or negative.

MicroRNA extraction, cDNA Synthesis and Real-time PCR

Expression microRNA profiling was analysed in undifferentiated mouse Embryonic Stem Cells C57BL6/J and differentiated mESC toward germ line lineage. Also, to compare obtained results, two samples of 5-day-old mouse testis and mature mouse testis were profiled as *in vivo* control samples. Total RNA was extracted with miRCURY ™ RNA isolation kit-Cell & Plant (version 2.0) for purification of total RNA that includes the microRNAs (Exiqon, Vedbaek, Denmark) according to the manufacturer's protocol. Before cDNA synthesis, concentrated RNA samples were measured by NanoDrop 1000 (Thermo Scientific, Wilmington) and diluted to 5 ng/µl with nuclease free water as recommended in the protocol manual kit. For each sample, all microRNAs are polyadenylated and reverse transcribed into cDNA in a single reaction step. Procedure was performed by miRCURY LNA™ Universal first strand cDNA synthesis kit (version 4.1) in 60 minutes at 42˚C followed for 5 minutes at 95˚C (Exiqon, Vedbaek, Denmark). Then, Real-time PCR for mature microRNAs was performed with SYBR Green master mix (Exiqon, Vedbaek, Denmark) by initial denaturation at 95°C for 10 minutes, 40 cycles at 95°C for 10 seconds, 60°C for 60 seconds with final melting curve analysis starting from 60°C proceeding to 95°C to evaluate the specificity of the amplification products. The experiments were done in triplicate for each microRNA in designated panels. Also, three inter-plate calibrators (UniSp3-IPC) and one RNA Spike-in RNA (UniSp6) were considered to minimize the analytical variations and to quality control experiment. To normalize the real-time data, U6 snRNA housekeeping gene was selected as endogenous reference gene. Signals were detected with Real-Time PCR instrument ABI StepOnePlus (Applied Biosystems).

Statistical real-Time PCR data analysis

Expression results were analysed with GenEX software version 2.5 (MultiD Analyses AB, Sweden). Data for each sample was normalized for U6snRNA

gene. Statistical Analysis of data was conducted by T-test. The data of undifferentiated mESCs were compared with data of differentiated mESCs and also the results of 5-day-old mice were compared with mature mouse testis and for each cases standard deviation (STDEV), standard error of the mean (SEM), p-value and linear fold change in gene expression were calculated. The data was analyzed by the $\Delta\Delta C_T$ method. Fold change of each microRNA was calculated by the equation $2^{-\Delta\Delta CT}$ method. Data was considered significant when p-value less than 0.05.

Results

GFP-positive embryonic stem cells were checked under a fluorescent microscope (Olympus IX53 Inverted Motorized Microscope, USA) after 72h of RA induction (Fig. 1).

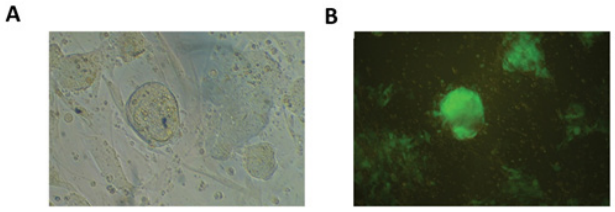

Figure 1. Colonies of mESCs C57BL6/J which harboring fusion construct (Stra8-EGFP) were cultured on 0.1% gelatin-coated culture dish in absence of LIF and presence of retinoic acid to induce differentiation in final concentration of 10^{-5} M for 72 hours and GFP-Positive mESCs were observed under a fluorescent microscope. (A) bright field image, (B) fluorescence image. 400 × Magnification.

Percentage of GFP expressing stem cells were analysed by Fluorescence activating cell sorting after 72h RA induction. From population of mESCs which were subjected to FACS, about 42.74% of cells were GFP-positive (B in fig 2). Fluorescence activated cell sorting was used to isolate GFP-positive from GFP-negative embryonic stem cells.

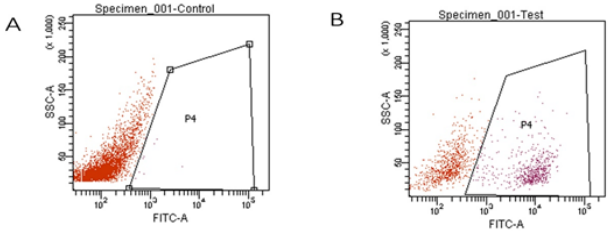

Figure 2. GFP-positive mESC57BL6/J cells were sorted by using Fluorescence-Activated Cell Sorting (FACS) Aria II Flow Cytometer. (A) Negative control samples included the mESCs C57BL6/J population harboring construct Stra8-EGFP which were cultured on 0.1% gelatin-coated culture dish in presence of 10^3 U/ml LIF and absence of RA, (B) Population mES C57BL6/J cells before cell sorting with 42.74% percentage GFP-positive mES cells after 72h RA induction (Gate p4) .

Specific gene markers for different stages of premeiotic, meiotic and post-meiotic were checked by RT-PCR. Expression of Dazl, Oct-4 and Vasa as premeiotic gene markers were seen in samples of RNA extracted at days 5, 12, 21 and 30. Also, the expression of Oct-4, a pluripotency marker in mESCs, was positive in undifferentiated mESCs. Expression of meiotic marker Sycp3 was positive in samples of RNA extracted at days 12, 21 and 30. Post-meiotic marker protamine (Prm1) expressed in RNA samples extracted at days 21 and 30. Expression of nanog gene, a transcription factor involved in self-renewal of undifferentiated embryonic stem cells (19), was positive only in RNA extracted from undifferentiated mESCs and differentiated mESCs at day 5, but was negative during differentiation in RNA samples extracted on other days (Fig. 3).

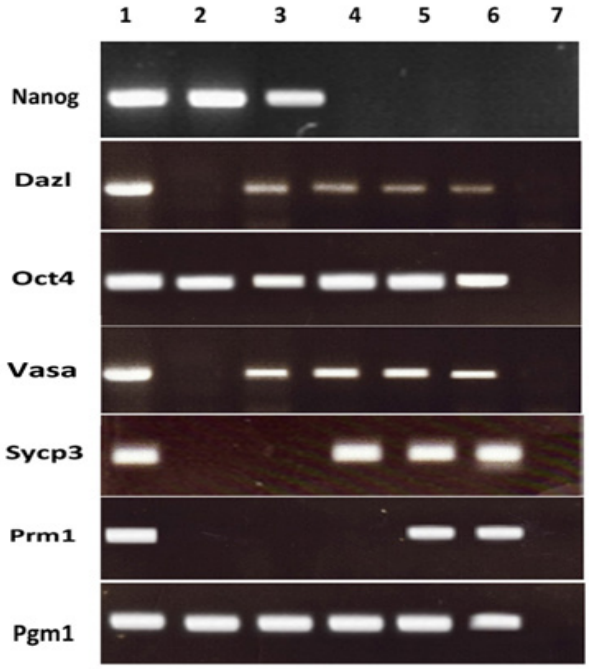

Figure 3. Expression marker genes in different stages of differentiation of mouse embryonic stem cell C57BL6/J toward germ line lineage checked by RT-PCR. Mature mouse testis tissue was used as positive control sample (lane1), Undifferentiated mESC C57BL6/J in absence of retinoic acid induction (lane2), differentiated mESC after 5-days of retinoic acid induction (lane 3), differentiated mESC after 12-days retinoic acid induction (lane 4), differentiated ESC after 21- days retinoic acid induction (lane 5), differentiated ESC after 30-days retinoic acid induction (lane 6), and negative control sample without template (lane7). Retinoic acid used in final concentration 10^{-5} molar. Sorted GFP-Positive cells cultured on 0.1% gelatin-coated culture dish and designated as day-1. Different times for RNA extraction and RT-PCR started from this time.
Dazl; deleted in azoospermia-like, Oct4; octamer-binding protein 4, Sycp3; synaptonemal complex protein 3, prm1; protamine 1.

In order to analyze our microRNAs results, we categorized our samples into 2 groups of *in vivo* and *in vitro* with each group consisting of 2 members. The *in vitro* group included samples of undifferentiated and differentiated and the *in vivo* group included samples of 5-day-old mouse testis and mature mouse testis. microRNA. Heat map of 4 samples for all profiled microRNAs is depicted (Fig. 4). Real-time data were analyzed with T-test calculation between members of the *in vitro* (undifferentiated vs differentiated) and *in vivo* (5-day-old mouse testis vs mature mouse testis) group. The microRNAs clusters miR-17-92 miR-290-295 miR-302 family and also mmu-miR-130a, mmu-miR-182, mmu-miR-124, mmu-miR-20b, mmu-miR-467e, mmu-miR-20a, mmu-miR-20a*, mmu-miR-20b*, shown high level of expression in undifferentiated state and 5-day-old mouse testis, but down-regulated after differentiation(average p-

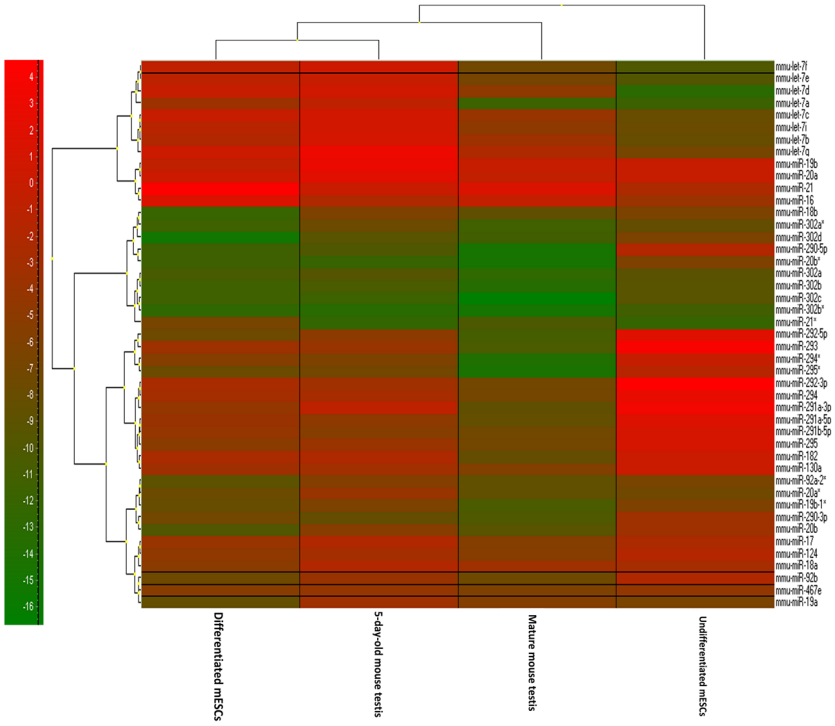

Figure 4. The heat map diagram which displays hierarchical clustering of microRNAs in 4 groups of samples undifferentiated mESC differentiated mESC after 30 days RA induction, 5-day-old mouse testis and mature mouse testis. Each row represents one microRNA gene and each column represents one sample. The microRNA clustering tree is shown on the left. Up-regulated microRNAs were shown as red and down-regulated microRNAs were shown as green.

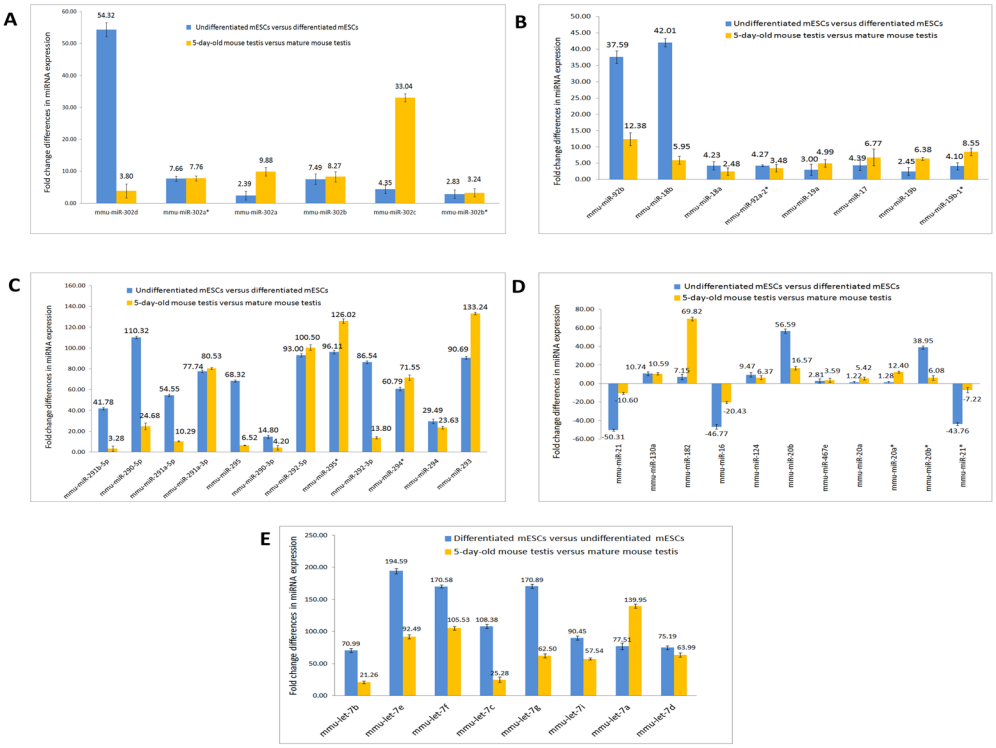

Figure 5. Variation of gene expression for each microRNA in samples undifferentiated mESCs, differentiated mESCs, 5-days-old mouse testis and mature mouse testis. Numbers upon each bar graph show differences in fold change expression. Profiled microRNAs were shown in samples undifferentiated mESC vs differentiated (Blue bars) and 5-day-old mouse testis vs mature mouse testis (yellow bars). (A) miR-302family, (B) cluster 17-92, (C) cluster miR-290-295. (D) microRNAs mmu-miR-21, mmu-miR-130a, mmu-miR-182, mmu-miR-16, mmu-miR-124, mmu-miR-20b, mmu-miR-467e, mmu-miR-20a, mmu-miR-20a*, mmu-miR-20b*, mmu-miR-21*. Expression mmu-miR-21, mmu-miR-16 and mmu-miR-21* is up-regulated after differentiation, (E) The comparison of profiled microRNAs Let-7 family in samples differentiated mESCs vs undifferentiated (Blue bars) and 5-day-old mouse testis vs mature mouse testis (Yellow bars) , expression this family is up-regulated after differentiation. Errors bars represent standard deviation (SD).

value p<0.003 for each members of cluster) and mature mouse testis (average p-value p<0.001 for each members of cluster). Our results demonstrate that expression level microRNAs mmu-miR-21, mmu-miR-21* and mmu-miR-16 were increased 50.31, 43.76 and 46.77 fold change, respectively, in differentiated mESCs with significant p-value in comparation to undifferentiated state (average p-value p<0.001). Also in our *in vivo* control samples, expression microRNAs mmu-miR-21, mmu-miR-21* and mmu-miR-16 in sample 5-day-old mouse testis increased 10.6, 7.22 and 20.43 fold change respectively in comparison to mature mouse testis (average p-value P<0.003 for each microRNA). Expression profiled microRNAs Let-7 family increased significantly after differentiation in comparison to undifferentiated mESC (Average p-value<0.0001 for each members of family) and sample 5-day-old mouse testis in comparison to mature mouse testis shown higher level of expression (average p-value<0.01 for each members of family) (Fig. 5).

Discussion

MicroRNA molecules are one of the important regulators of gene expression due to downregulation of gene expression by inhibiting translation (2, 20). MicroRNAs have been shown to play important roles in establishment and maintenance of pluripotency, proliferation and differentiation of embryonic stem cells (ESCs) (21-22). Our results show differentiation level of microRNA expression in undifferentiated mESC and differentiated mESC. Identification of differentially expressed microRNAs can elucidate their function and importance in self-renewal and differentiation of ESCs. Overall roles and significances of microRNAs in embryonic stem cells were demonstrated by knock-out ESCs model of essential components of microRNA processing and maturation, like Dicer, Drosha or DGCR8 which result in longer cell cycle time and show delay in cell cycle progression with defects in G1 to S transition and defects in differentiation and self-renewal of mESCs (23-24).

Mouse microRNA cluster 290–295 is a 2.2-kb region on chromosome 7 of the mouse genome and mostly characterized by the hexamer seed sequence "AAGUGC" which suggests members of this cluster are redundantly directed against the same targets (7, 8, 25, 26). Expression of this cluster is regulated by c-Myc (25). They cause rapid proliferation of ES cells with acceleration from G1 to S transition by targeting cyclin kinase inhibitors (CKIs) (2). The cluster of miR-290-295 is the most abundant microRNA cluster in mESC which is responsible for about 60-70% of all microRNAs in mESC (13). This cluster has high level of expression in embryonic stem cells which can suggest specific expression and important role of cluster miR-290-295 in maintaining pluripotency and self-renewal of embryonic stem cells (11, 27, 28). In current study, cluster miR-290-295 members are expressed dominantly in undifferentiated embryonic stem cells which can be an emphasis on importance this cluster in maintaining pluripotency. The inhibitors of the cyclinE/cdk2 pathway which regulate cell cycle progression such as p21 (Waf1/Cip1), Lats2 and retinoblastomalike 2 protein (Rbl2) are validated targets of miR-290 cluster (29-31). Dkk-1 gene, one of the inhibitors of the Wnt pathway, is another validated target of this cluster which can explain role of cluster miR-290-295 in pluripotency state in stem cell rather than the differentiated state (32). In mouse embryonic stem cells, activation of the Wnt pathway was shown to be adequate in maintaining self-renewal (33).

Cluster miR-290-295 also has function in promoting DNA methylation and can prevent Rbl2 function as a repressor of de novo DNA methyltransferase by inhibition of its expression (34) which can suggest role of this cluster in epigenetic regulations in embryonic stem cells.

Cluster miR-17-92 and miR-302 family members are expressed at high levels in mouse embryonic stem cells (7, 8). Their over-expression can induced the pluripotent stem (iPS) cell state and accelerate mesenchymal-to-epithelial (MET) transition by directly targeting TGF-β receptor II and p21 (Waf1/Cip1) (35-36). Members of cluster miR-17-92 can control differentiation embryonic stem cells via targeting STAT3 mRNA, a known transcription factor in stem cells, (37). In this study, cluster miR-17-92 and miR-302 family shown high levels of expression in undifferentiated mESC and their expression is down-regulated in differentiated mESCs. This can emphasize function of cluster miR-17-92 and miR-302 family in keeping pluripotency and self-renewal capacity of stem cells rather than differentiation.

In our experiment, microRNAs mmu-miR-21, mmu-miR-21* and mmu-miR-16 showed increased expression in differentiated mESCs. Higher level of expression following induction of differentiation can indicate potential roles of these microRNAs in stem cell differentiation and somatic cells. In addition, for finding validates targets of these three microRNAs the website http://mirtarbase.mbc.nctu.edu.tw/ was used. According to this web site, for miR-21 six validate target gene (Fasl, Peli, Pdcd4, Spry2, Pten and Reck) and for miR-16 thirteen validate target gene (Wnt3a, Ccnd1, Bcl2, Ccnt2, Arl2, Cadm1, App, Mdm4, Vegfa, Jun, Jag1, Slc6a4, Ccne1) have been identified. These validate target genes can somehow elucidate importance miR-21 and miR-16 in regulation differentiation ESCs and somatic cell maturation.

The slightly higher level of expression in other profiled microRNAs, including mmu-miR-130a, mmu-miR-182, mmu-miR-124, mmu-miR-20b, mmu-miR-467e, mmu-miR-20a, mmu-miR-20a*, mmu-miR-20b*, were determined in undifferentiated mESCs vs differentiated. The expression analysis of these miRNAmicroRNAs in differentiated mESCs toward germ line lineage have been done only in this experiment and even though these microRNAs are not ESCs specific,their function in ESCs remain to be understood and needs further functional studies.

Let-7 family consists of twelve members which are expressed from eight distinct genomic loci (38). Let-7 can regulate the expression of RAS and high mobility group A2 (HMGA2) oncogenes as the major oncogenic targets of Let-7 and also suppress expression of cell cycle regulators such as CDC25A, CDK6 and cyclin D1 (39-40). Previous microRNA profiling data shown microRNAs of the Let-7 family have target genes such as N-myc, C-myc, Sal4 in embryonic stem cells (41).

In our findings, members of microRNAs let-7 family were almost absent in undifferentiated mouse embryonic stem cells and there is increased expression upon differentiation which can suggest their importance in controlling self-renewal and promoting differentiation. Expressed members of the Let-7 family can induce differentiation and it is worth testing that inhibition of let-7 family by an antisense inhibitor may be able to promote pluripotency in embryonic stem cells. Lin28 is an RNA binding protein that has been well characterized for regulation of let-7 processing in undifferentiated embryonic stem cells with inhibitory effect on let-7 processing. The expression of Lin28 is regulated with miR-290 cluster (21, 42). Decreased expression of miR-290 during differentiation led to decreased levels of Lin-28 and increased expression and maturation of the Let-7 microRNA family (21). This can suggest that functions cluster miR-290 and Let-7 family in control self-renewal and differentiation Es cells is in contrast to each other. Finally, we conclude microRNAs are crucial for self-renewal and behavior of embryonic stem cells. Our hypothesis is based on the observation that distinct sets of microRNAs are specifically expressed in pluripotent ES cells but not in differentiated. Identification of differentially expressed microRNAs and their relation to mRNA targets in embryonic stem cells before and during differentiation can lead to better understand of molecular mechanisms involved in controlling stem cell fate, development, proliferation and differentiation.

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