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Target Identification of MicroRNAs Expressed Highly and Regulated by Activin A in Human Embryonic Stem Cells

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1. Introduction

Since 1998, when the first human embryonic stem (hES) cell lines were reported (Thomson et al., 1998), a large number of hES cell lines have been derived from the inner cell mass of preimplantation embryos donated at *in vitro* fertilization (IVF) clinics (Guhr et al., 2006). These hES cell lines possess remarkable ability of both unlimited self-renewal and pluripotency to generate any cell type differentiated from three germ-layers ectoderm, mesoderm and endoderm. Thus, these hES cell lines have great potentials for cell therapies in regenerative medicine and experimental models for drug discovery and toxicity testing in addition to basic studies on stem cell biology and molecular embryogenesis (Wobus & Boheler, 2005).

The proliferation of undifferentiated hES cells can be maintained on either mitotically inactivated mouse embryonic fibroblasts (MEF) as feeder or Matrigel-coated plastic surfaces in MEF-conditioned medium (Xu et al., 2001). The continuous culture of undifferentiated hES cells either on MEF feeder or in the MEF-conditioned medium bears the risk of transmitting animal pathogens, and limits future medical applications of hES cells. A few human cell systems, including hES-derived fibroblast-like cells as feeder (Stojkovic et al., 2005), with capacity to support the growth of undifferentiated hES cells have been developed to replace the use of MEF. Activin A was previously reported to be necessary and sufficient for the maintenance of self-renewal and pluripotency of hES cells in long-term feeder- and serum-free culture (Xiao et al., 2006). It would be of interest to compare the gene expression profiles of these undifferentiated hES cells grown under these different conditions in order to better understand their common molecular mechanisms of unlimited self-renewal and pluripotency.

The genome-wide gene expression analyses using high-throughput microarray have been used to identify key “stemness” genes responsible for the unlimited self-renewal and pluripotency of hES cells (Ivanova et al., 2002; Ramalho-Santos et al., 2002; Sperger et al., 2003). A meta-analysis of 20 previously reported transcriptomes had identified 48 genes overexpressed in hES cells compared to differentiated cell types (Assou et al., 2007), and these 48 genes, including transcription factors such as OCT4 (also known as POU5F1), SOX2 and NANOG, may be responsible for the unlimited self-renewal and pluripotency of hES cells. However, molecular mechanisms involved in unlimited self-renewal and pluripotency of hES cells remain to be fully understood.

Recently, microRNAs (miRNAs) have been shown to play important roles in mammalian embryo development and cell differentiation. Mammalian genomes encode many hundreds of miRNAs, which are predicted to regulate expression of as many as 30% of protein-coding genes (Bartel, 2004; Griffiths-Jones et al., 2006; John et al., 2004; Landgraf et al., 2007). Although the biological functions of most miRNAs are unknown, some miRNAs appear to participate in determination of cell fate, in pattern formation in embryonic development, and in control of cell proliferation, cell differentiation and cell apoptosis in animals (Alvarez-Garcia & Miska, 2005; Kloosterman & Plasterk, 2006). The miRNAs negatively regulate the posttranscriptional expression by translational repression and/or destabilization of protein-coding mRNAs. The impact of miRNAs on protein output was recently shown that although some targets were repressed without detectable changes in mRNA levels, those translationally repressed by more than a third also displayed detectable mRNA destabilization, and, for the more highly repressed targets, mRNA destabilization usually comprised the major component of repression (Baek et al., 2008; Selbach et al., 2008). In human and mouse, several ES cell-specific miRNAs were identified (Houbaviy et al., 2003; Laurent et al., 2008; Suh et al., 2004), and these miRNAs were shown to play crucial roles in ES cell differentiation, lineage specification, and organogenesis, especially neurogenesis and cardiogenesis (Chen et al., 2007). It would be important to identify the target mRNAs of hES cell-specific miRNAs that will lead us to understand the complex and interesting networks of regulation in hES cells.

In my laboratory at Kaohsiung Medical University in Taiwan, five hES cell lines have been derived with institutional review board approval from preimplantation embryos donated at *in vitro* fertilization clinics in Taiwan (Li et al., 2006), and these lines have since been continuously cultured on mitotically inactivated MEF feeder in the hES medium for more than 44 passages and underwent freezing/thawing processes. All of five hES cell lines expressed characteristic undifferentiated hES markers such as SSEA-4, TRA-1-81, alkaline phosphatase, TERT, transcription factors POU5F1 (OCT4) and NANOG. The hES cell lines T1 and T3 possess normal female karyotypes, whereas lines T4 and T5 are normal male, but line T2 is male trisomy 12 (47XY,+12). The hES cell lines T1, T2, T3 and T5 were able to produce teratomas in SCID mice, and line T4 could only form embryoid bodies *in vitro*. Global gene expression profiling of these five hES cell lines indicated that the TGF β /activin branch components inhibin BC, ACVR2A, ACVR1 (ALK2), TGFBR1 (ALK5) and SMAD2 were found to be highly expressed in undifferentiated states of these five hES cell lines and decreased upon differentiation. These hES cell lines are useful for drug development and toxicity testing in addition to basic studies on human stem cell biology.

2. Target identification of hES cell-specific miRNAs

The undifferentiated state of hES-T3 cells grown on MEF feeder layer was indicated by positive staining of OCT4 and NANOG, and these undifferentiated hES-T3 cells were designated as T3MF. An autogenic feeder cells with capacity to support the growth of undifferentiated hES cells was established according to the previously published procedure (Stojkovic et al., 2005). hES-T3 (passage 19) cells were transferred into feeder-free and noncoated plate (10 cm) in DMEM supplemented with 10% FBS under 5% CO₂ at 37°C. After 10 days, cells appeared as fibroblast-like morphology, that is, flat cells with elongated nucleus and branching pseudopodia, and these differentiated fibroblast-like cells are designated as T3DF. These T3DF cells were passaged using trypsin (0.05%) every 4 days or

cryopreserved. After inactivation using mitomycin C (10 ug/ml), T3DF cells (passage 8) as feeder have been shown to support the undifferentiated growth of hES-T3 cells for more than 14 passages, and the hES-T3 colonies grown on T3DF feeder were stained positively for OCT4 and NANOG [Li et al. 2009].

The expression profiles of 250 human miRNAs in T3MF and T3DF cells were quantitated using TagMan MicroRNA Assays as described previously (Chen et al., 2005; Liang et al., 2007; Li et al., 2009), and the expression level of each miRNA was indicated as folds over U6 snRNA. The four hES cell-specific miRNAs miR-302d, miR-367, miR-372 and miR-200c were found to express abundantly (more than 20-fold U6 snRNA) in T3MF cells, but little (0.03-fold U6 snRNA) in T3DF cells (Table 1). These four miRNAs were also reported to be upregulated in hES cells (Laurent et al., 2008).

The genome-wide mRNA expression of T3MF and T3DF cells was determined using Affymetrix human genome U133 plus 2.0 GeneChip (Li et al., 2009). The original data had been deposited to NCBI database, and the GEO series number is GSE9440. The most expressed genes of T3MF cells include the undifferentiated hES markers such as OCT4, SOX2 and NANOG, and many of the most expressed mRNAs of T3MF cells were found to be in common with the 48 over-expressed genes in undifferentiated hES cells reported previously (Assou et al., 2007). The most expressed genes of differentiated T3DF cells include genes TRPS1, KLF13, MBNL2, MTMR3, NF1B, RAB6A, MARCKS, DDEF1, MBNL1 and QKI, which were later identified to be targets of four ES cell-specific miRNAs miR-302d, miR-372, miR-200c and/or miR-367 (Table 2).

miRNAs	T3MF/			Specificity	Chromosome
	T3MF	T3DF	T3DF		
hsa-miR-302d	204.60	0.01	20460	hES	4q25
hsa-miR-367	136.40	0.01	13640	hES	4q25
hsa-miR-372	27.80	0.01	2780	hES	19q13.42
hsa-miR-200c	27.73	0.03	937	hES	12p13.31
hsa-miR-20b	44.68	0.06	791		Xq26.2
hsa-miR-26b	24.02	0.07	349		2q35
hsa-miR-302c*	13.82	0.01	1382	hES	4q25
hsa-miR-302a*	1.29	0.01	129	hES	4q25
hsa-miR-302b*	0.11	0.01	11	hES	4q25
hsa-miR-371	3.25	0.01	325	hES	19q13.42
hsa-miR-373*	0.21	0.01	21	hES	19q13.42
hsa-miR-368	0.01	0.01	1	hES	14q32.31

Table 1. Expression levels of hES cell-specific miRNAs in T3ES and T3DF cells. This table is modified from Li et al. (2009).

The targets of the four hES cell-specific miRNAs miR-302d, miR-367, miR-372 and miR-200c miRNAs in hES cells are not known previously (Griffiths-Jones et al., 2006; John et al., 2004; Landgraf et al., 2007), and their potential targets were predicted using the methods of PicTar and TargetScanS (Sethupathy et al., 2006). The expression levels of these predicted target mRNAs in T3MF and T3DF cells were analyzed by the Volcano plot, and the differentially expressed genes were defined by more than 3-folds of changes (T3DF/T3MF) and a p-value cutoff of 0.05. The 58 differentially expressed genes were found to express more than 3-folds

miR-	miR-	miR-	miR-	Gene Symbol	T3MF	T3DF	T3DF/ T3MF	Description	UniGene
302d	372	200c		TRPS1	4.21	81.92	19.5	trichorhinophalangeal syndrome I	Hs.657018
302d	372	200c		KLF13	6.13	113.7	18.5	Kruppel-like factor 13	Hs.525752
302d	372	200c		MBNL2	1.47	19.69	13.4	muscleblind-like 2 (Drosophila)	Hs.657347
302d	372	200c		MTMR3	0.99	11.28	11.4	myotubularin related protein 3	Hs.474536
302d	372		367	NFIB	0.67	12.88	19.3	nuclear factor I/B	Hs.644095
302d	372			RAB6A	2.37	16.23	6.9	RAB6A, member RAS oncogene family	Hs.503222
302d	372			ZNF238	1.76	9.84	5.6	zinc finger protein 238	Hs.69997
302d	372			MEF2C	0.95	9.62	10.1	myocyte enhancer factor 2C	Hs.654474
302d	372			PURB	1.03	8.49	8.3	purine-rich element binding protein B	Hs.349150
302d	372			NR4A2	1.52	7.61	5	nuclear receptor subfamily 4, group A, member 2	Hs.563344
302d	372			TAL1	1.67	7.46	4.5	T-cell acute lymphocytic leukemia 1	Hs.658150
302d	372			PPP3R1	1.21	5.62	4.7	isoform	Hs.280604
302d	372			FNDC3A	1.34	5.02	3.8	fibronectin type III domain containing 3A	Hs.508010
302d	372			ZFP91	0.65	4.79	7.3	zinc finger protein 91 homolog (mouse)	Hs.524920
302d	372			C11orf9	0.81	4.54	5.6	chromosome 11 open reading frame 9	Hs.473109
302d	372			IQSEC2	0.99	4.38	4.4	IQ motif and Sec7 domain 2	Hs.496138
302d	372			RBL1	1.24	4.31	3.5	retinoblastoma-like 1 (p107)	Hs.207745
302d	372			ZMYND11	0.82	3.76	4.6	zinc finger, MYND domain containing 11	Hs.292265
302d	372			ESR1	1.14	3.47	3	estrogen receptor 1	Hs.208124
302d	372			LMO7	0.99	3.13	3.2	LIM domain 7	Hs.207631
302d		200c		ZFHx4	0.27	8.46	31.8	zinc finger homeodomain 4	Hs.458973
302d			367	RUNX1	1.16	4.02	3.5	leukemia 1)	Hs.149261
302d				UBE3A	1.24	4.16	3.3	small nuclear ribonucleoprotein polypeptide N	Hs.654383
		200c	367	KLF4	0.52	5.15	10	Kruppel-like factor 4 (gut)	Hs.376206
		200c	367	C6orf62	0.87	3.97	4.6	chromosome 6 open reading frame 62	Hs.519930
		200c	367	SYNJ1	1.14	3.83	3.3	synaptojanin 1	Hs.473632
		200c		MARCKS	1.58	26.97	17.1	myristoylated alanine-rich protein kinase C substrate	Hs.519909
		200c		DDEF1	1.51	12.34	8.2	development and differentiation enhancing factor 1	Hs.655552
		200c		MBNL1	0.67	10.55	15.7	muscleblind-like (Drosophila)	Hs.478000
		200c		ARHGDI A	0.89	10.17	11.4	Rho GDP dissociation inhibitor (GDI) alpha	Hs.159161
		200c		QKI	0.94	9.55	10.2	quaking homolog, KH domain RNA binding (mouse)	Hs.510324
		200c		BAT3	1.07	7.32	6.9	HLA-B associated transcript 3	Hs.440900
		200c		TSC22D2	0.99	6.51	6.6	TSC22 domain family, member 2	Hs.644065
		200c		MAP2	0.57	5.58	9.7	microtubule-associated protein 2	Hs.368281
		200c		CNOT4	0.94	5.54	5.9	CCR4-NOT transcription complex, subunit 4	Hs.490224
		200c		SOX1	1.44	4.33	3	SRY (sex determining region Y)-box 1	Hs.202526
		200c		ETS1	0.81	4.09	5	(avian)	Hs.369438
		200c		TMEFF2	0.58	3.8	6.5	domains 2	Hs.144513
		200c		ANKRD15	0.92	3.55	3.9	DKFZp451J1819)	Hs.306764
		200c		RAP2C	0.84	3.39	4.1	RAP2C, member of RAS oncogene family	Hs.119889
		200c		SNAP25	0.89	3.36	3.8	synaptosomal-associated protein, 25kDa	Hs.167317
		200c		DMD	1.05	3.35	3.2	types)	Hs.495912
		200c		FBXO33	0.68	3.33	4.9	F-box protein 33	Hs.324342
		200c		VASH1	0.6	3.32	5.6	vasohibin 1	Hs.525479
		200c		SEMA6D	0.61	3.27	5.3	(semaphorin) 6D	Hs.511265
		200c		GIT2	0.95	3.15	3.3	CDNA clone IMAGE:5272062	Hs.434996
		200c		GATA2	0.76	3.1	4.1	GATA binding protein 2	Hs.367725

Table 2. Expression levels of 58 target mRNAs predicted by four miRNAs. This table is modified from Li et al. (2009).

miR-	miR-	miR-	miR-	Gene Symbol	T3DF/			Description	UniGene
					T3MF	T3DF	T3MF		
367				SMAD6	1.5	6.23	4.1	SMAD family member 6	Hs.153863
367				CPEB4	0.56	4.76	8.6	cytoplasmic polyadenylation element binding protein 4	Hs.127126
367				WWP2	1.08	4.28	4	WW domain containing E3 ubiquitin protein ligase 2	Hs.408458
367				CACNA1C	0.97	4.2	4.3	subunit	Hs.118262
367				TEF	0.73	4.16	5.7	thyrotrophic embryonic factor	Hs.181159
367				FMR1	0.98	4.05	4.1	fragile X mental retardation 1	Hs.103183
367				DNAJB12	0.87	4.02	4.6	DnaJ (Hsp40) homolog, subfamily B, member 12	Hs.696014
367				ZNF287	1.05	3.81	3.6	zinc finger protein 287	Hs.99724
367				HAS3	1.15	3.79	3.3	hyaluronan synthase 3 isoform b	Hs.592069
367				ATXN3	1.12	3.48	3.1	ataxin 3	Hs.532632

Table 2. continued.

of overall mean in T3DF cells (Table 2). Therefore, these 58 abundantly differentially expressed mRNAs are very likely to be the targets of four abundantly expressed miRNAs miR-302d, miR-372, miR-200c and/or miR-367 in hES cells (Li et al. 2009). Recently, 253 target mRNAs of mouse miR-290 cluster (corresponding to human miR-302 cluster) were found by the transcriptome analysis of ES cells lacking Dicer of miRNA processing enzyme (Sinkkonen et al., 2008). A comparison of these mouse 253 targets with the 58 targets of human miR-302d, miR-367, miR-372 and/or miR-200c revealed four common targets TRPS1, MBNL1, MBNL2 and NF1B.

The three genes TRPS1, KLF13 and MBNL2 were found to express extremely abundantly (more than 20-fold overall mean) in T3DF cells in which miR-302d, miR-372 and miR-200c had almost no expression (Tables 1 and 2), whereas these three genes TRPS1, KLF13 and MBNL2 exhibited relatively low mRNA expression in T3MF cells in which miR-302d, miR-372 and miR-200c were expressed abundantly (more than 20-fold U6 snRNA). Therefore, the TRPS1, KLF13 and MBNL2 genes are very likely to be the common targets of three abundantly expressed miRNAs miR-302d, miR-372 and miR-200c in hES cells. To demonstrate directly whether TRPS1, KLF13 and MBNL2 were indeed the targets of miR-302d and miR-372, the luciferase reporter vectors harboring the amplified 3'UTR fragments of target genes were cotransfected with and without miRNA precursor of either miR-302d or miR-372 in HEK293T cells (Li et al., 2009). The potential binding structures of the 3'UTR of TRPS1, KLF13 and MBNL2 genes by miRNAs miR-302d and miR-372 were predicted using PicTar program. The base-pairings between miRNAs and their targets, as well as the constructions of luciferase reporter vectors, were shown in Fig. 1A and B. The miR-302d specifically suppressed the luciferase activity to 57, 62 and 80% of reporter vector harboring TRPS1, KLF13 and MBNL2, respectively. The miR-372 inhibited the luciferase activity to 86, 37 and 47% for TRPS1, KLF13 and MBNL2, respectively (Fig. 1C). These results implied that miR-302d and miR-372 were able to regulate negatively the same targets due to their same seed sequence. Furthermore, in order to confirm the predicted miRNA binding sites, the 4-bp mutations of miR-302d and miR-372 binding sites on KLF13 and MBNL2 genes were generated by site-directed mutagenesis. The results of these reporter assays showed that miR-302d and miR-372 were indeed able to suppress specifically the luciferase activities of the reporter vectors harboring the predicted binding sites of wild-type 3'UTR of KLF13 and MBNL2, but not mutant-types (Fig. 1D).

The GeneOntology of the 58 abundantly differentially expressed targets by hES cell-specific miRNAs miR-302d, miR-372, miR-200c and/or miR-367 was analyzed (Li et al., 2009). 48

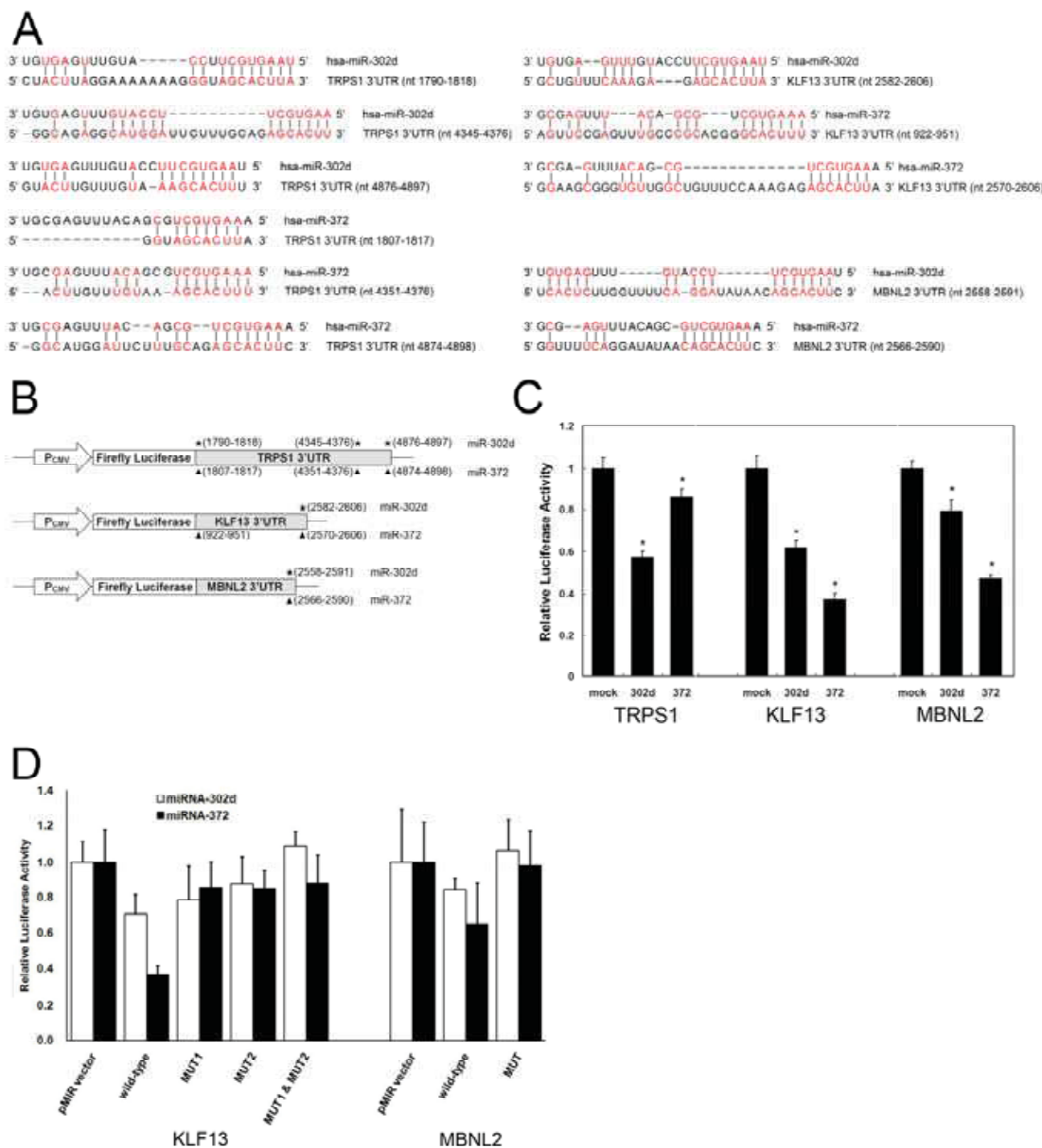


Fig. 1. TRPS1, KLF13 and MBNL2 targets of miR-302d and miR-372.

(A) Predicted binding sites of miR-302d and miR-372 within the 3'UTRs of TRPS1, KLF13 and MBNL2. (B) The construction of luciferase reporter vectors. (C) The effects of miR-302d and miR-372 on the luciferase activity of TRPS1, KLF13 and MBNL2 reporter vectors. (D) The effects of miR-302d and miR-372 on the luciferase activity of reporter vectors containing wild-type and mutant 3'UTRs of KLF13 and MBNL2. This figure is adapted from Li et al. (2009).

(p-value of 6.09E-10) of these 58 targets are localized in nucleus, and 39 (p-value of 4.38E-10) of them are involved in regulation of gene expression processes. The molecular functions of the 58 targets of four miRNAs expressed abundantly in hES cells were analyzed using GeneSpring GeneOntology Browser and MetaCore Analytical Suite. The top four molecular functions of eight categories obtained by GeneSpring were found to be the same to the top four molecular functions of eight categories obtained by MetaCore (Table 3). All of the top four molecular

functions are involved in gene transcription. Among the other different categories between GeneSpring and MetaCore, zinc ion binding and sequence-specific DNA binding are also involved in gene transcription. In other words, the molecular functions of approximately half of these 58 targets are involved in gene transcription in hES cells.

Molecular functions	GeneSpring		MetaCore	
	Genes	p-Value	Genes	p-Value
Transcription factor activity	18	1.03E-08	28	8.87E-16
Transcription regulator activity	20	2.81E-07	30	1.40E-13
Nucleic acid binding	29	2.78E-06	38	5.03E-10
DNA binding	22	1.80E-05	31	2.01E-09
Zinc ion binding	19	0.000595		
Calmodulin binding	4	0.0039		
Ciliary neurotrophic factor receptor binding	1	0.00495		
Calmodulin inhibitor activity	1	0.00495		
Protein N-terminus binding			6	2.65E-07
Estrogen receptor activity			3	2.67E-07
Sequence-specific DNA binding			12	1.91E-06
Nitric-oxide synthase regulator activity			3	5.53E-06

Table 3. Molecular functions of 58 abundantly differentially expressed targets. This table is adapted from Li et al. (2009).

3. Target identification of miRNAs up- and down-regulated by activin A

The hES-T3 cells (passage 36) were cultured on the inactivated MEF feeder (designated as T3MF) in hES medium (containing 4 ng/ml bFGF) and feeder-free Matrigel in MEF-conditioned medium with additional 4 ng/ml bFGF (designated as T3CM) for 14 and 12 more passages, respectively. The T3MF and T3CM cells were stained positively for OCT4 and NANOG, indicating that both T3MF and T3CM cell populations contained very high proportions of undifferentiated hES cells. When hES-T3 cells were grown on feeder-free Matrigel in hES medium (without bFGF) supplemented with 5, 10 and 25 ng/ml activin A, many cells around the edges, as well as the center, of colonies differentiated into fibroblast-like cells with much less staining of OCT4 and NANOG, and these cells were able to proliferate only two more passages, indicating that bFGF is indispensable for maintenance of self-renewal of hES cells. However, the hES cells cultured on feeder-free Matrigel in hES medium containing both 4 ng/ml bFGF and 5 ng/ml activin A (designated as T3BA) were able to proliferate for seven more passages, and most of these T3BA cells were stained positively for both OCT4 and NANOG (Tsai et al., 2010).

The genome-wide mRNA expression of T3MF, T3CM and T3BA cells was determined using Affymetrix human genome U133 plus 2.0 GeneChip (Tsai et al., 2010). The original data have been deposited to NCBI database, and the GEO series number is GSE16910. The average values of expressed mRNAs from T3MF, T3CM and T3BA cells were compared by scatter plots (Fig. 2A, B). The Pearson correlation coefficient of $R^2 = 0.9934$ between T3MF and T3CM cells indicates their very similar expression profiles of mRNAs (Fig. 2A), and only 49 and 17 genes were found to be abundantly (more than 3-folds of overall mean)

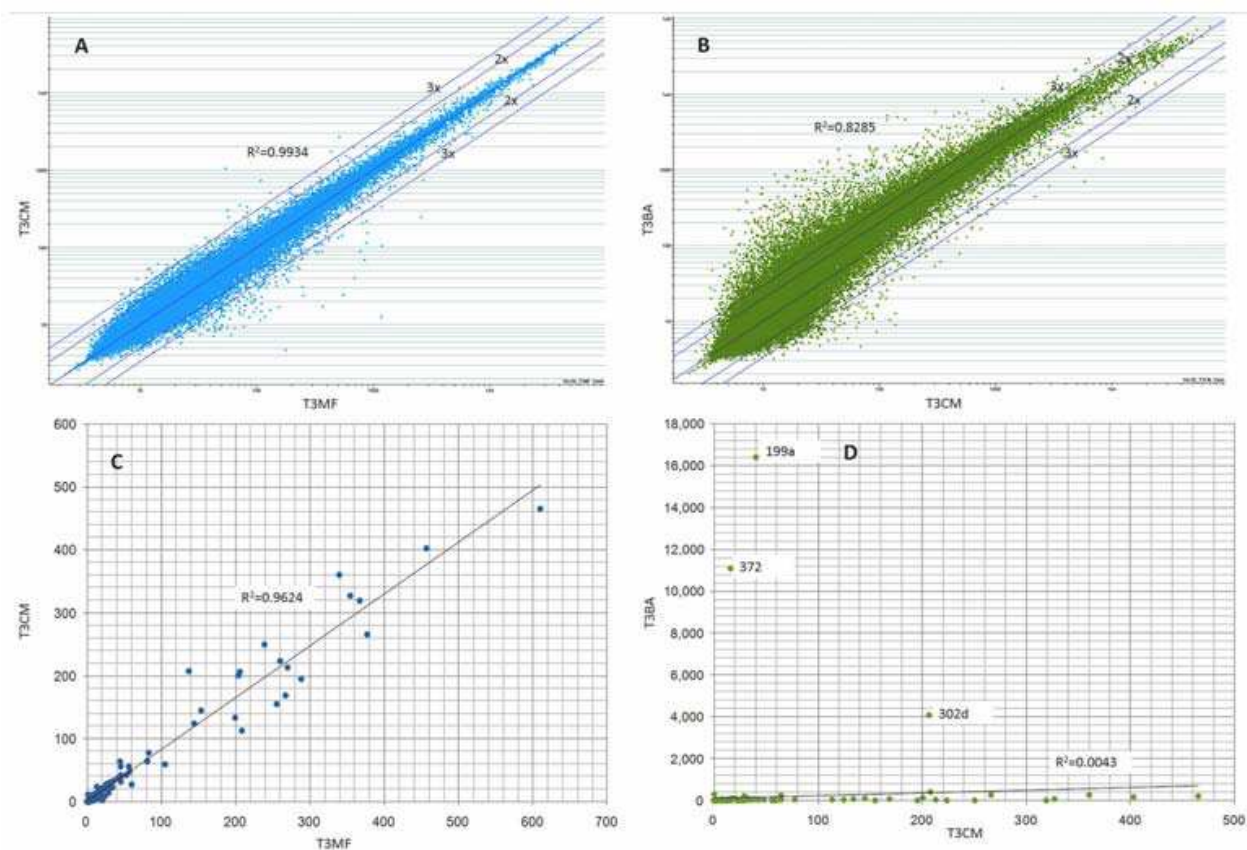


Fig. 2. Scatter plot and correlation analyses of mRNAs and miRNAs among T3MF, T3CM and T3BA cells.

(A) mRNAs of T3MF and T3CM; (B) mRNAs of T3BA and T3CM; (C) miRNAs of T3MF and T3CM; (D) miRNAs of T3BA and T3CM. This figure is adapted from Tsai et al. (2010).

differentially (more than 3-folds of changes) expressed in T3MF and T3CM cells, respectively. These results indicated that the unlimited self-renewal and pluripotency of hES-T3 cells can be maintained by continuous culture on either inactivated MEF feeder in hES medium (containing 4 ng/ml bFGF) or feeder-free Matrigel in MEF-conditioned medium (containing additional 4 ng/ml bFGF). The correlation value of $R^2 = 0.8285$ between T3BA and T3CM cells suggests less similarity between their mRNA profiles (Fig. 2B), and 589 and 58 genes were abundantly differentially expressed in T3BA and T3CM cells, respectively.

The expression profiles of 250 human miRNAs in T3MF, T3CM and T3BA cells were quantitated using TaqMan microRNA Assays as described previously (Chen et al., 2005; Liang et al., 2007; Tsai et al., 2010), and the expression level of each miRNA was indicated as folds over U6 snRNA. The Pearson correlation coefficient of $R^2 = 0.9624$ between T3MF and T3CM cells indicates their very similar miRNA expression profiles (Fig. 2C), while no correlation ($R^2 = 0.0043$) was found between T3BA and T3CM cells (Fig. 2D). When the three highly expressed miRNAs miR-199a, miR-372 and miR-302d were excluded from analysis, low correlation ($R^2 = 0.3541$) was still observed for the remaining 247 miRNAs of T3BA and T3CM cells. These results indicate that four hES cell-specific miRNAs miR-372, miR-302d,

miR-367 and miR-200c, as well as three other miRNAs miR-199a, miR-217 and miR-19a, were over-expressed in T3BA cells, whereas five miRNAs miR-19b, miR-221, miR-222, let-7b and let-7c were under-expressed in T3BA cells compared with T3CM cells (Table 4).

miRNAs	T3MF	T3CM	T3BA	T3BA/ T3CM	T3CM/ T3BA	Specificity
A group (up-regulated)						
hsa-miR-372	27.97	15.65	11079.38	707.9	0	hES
hsa-miR-302d	205.43	206.53	4071.21	19.7	0.1	hES
hsa-miR-367	136.63	207.63	397.69	1.9	0.5	hES
hsa-miR-200c	27.76	28.94	201.26	7	0.1	hES
hsa-miR-199a	44.84	39.81	16380.51	411.5	0	
hsa-miR-217	0.01	0.01	296.13	29613	0	
hsa-miR-19a	43.74	63.94	233.47	3.7	0.3	
B group (down-regulated)						
hsa-miR-19b	366.63	319.07	2.56	0	124.6	
hsa-miR-221	254.57	154.9	2.93	0	52.9	
hsa-miR-222	287.47	195.53	6.73	0	29.1	
hsa-let-7b	259.6	223.2	12.12	0.1	18.4	
hsa-let-7c	237.9	250.27	15.17	0.1	16.5	

Table 4. Levels of miRNAs expressed highly in T3MF, T3CM and T3BA cells
This table is modified from Tsai et al. (2010).

Gene Symbol	miR-372	miR-302d	miR-367	miR-200c	miR-19a	miR-199a	miR-217	Gene Description
NR4A2	372	302d			19a		217	nuclear receptor subfamily 4, group A, member 2
ERBB4	372	302d			19a			v-erb-a erythroblastic leukemia viral oncogene homolog 4
CXCR4	372	302d						chemokine (C-X-C motif) receptor 4
PCDH9			367					protocadherin 9
TMEFF2				200c				transmembrane protein with EGF- & 2 follistatin-like dom 2
CD24					19a			CD24 molecule
COX6A1						199a		cytochrome c oxidase subunit VIa polypeptide 1
TAL1	372	302d						T-cell acute lymphocytic leukemia 1
GRIA3			367					glutamate receptor, ionotropic, AMPA 3
PRDM1				200c	19a			PR domain containing 1, with ZNF domain
MYT1				200c	19a			myelin transcription factor 1
EIF4G2					19a			eukaryotic translation initiation factor 4 gamma, 2
CHMP4B					19a			chromatin modifying protein 4B

Table 5. The 13 genes targeted by the activin A up-regulated miRNAs in T3BA cells.
This table is modified from Tsai et al. (2010).

Gene Symbol	miR-			let-		Gene Description
	19b	221	222	7b	7c	
ACTN1	19b					actinin, alpha 1
ADRB2				7b	7c	adrenergic, beta-2-, receptor, surface
AMMECR1					7c	Alport syndrome, mental retardation, gene 1
AMT				7b		aminomethyltransferase
ARFGEF1	19b					ADP-ribosylation factor guanine nucleotide-exchange factor 1
ARIH2	19b					ariadne homolog 2 (Drosophila)
ATXN7L1	19b					ataxin 7-like 1
CALB1				7b	7c	calbindin 1, 28kDa
CCND2	19b			7b	7c	cyclin D2
CDC25A				7b	7c	cell division cycle 25 homolog A (S. pombe)
CHD7		221	222	7b	7c	chromodomain helicase DNA binding protein 7
CLASP2	19b			7b	7c	cytoplasmic linker associated protein 2
CLASP2	19b			7b	7c	cytoplasmic linker associated protein 2
COIL				7b	7c	coilin
CPNE8		221	222			copine VIII
DACH1		221	222			dachshund homolog 1 (Drosophila)
DTNA	19b					dystrobrevin, alpha
EFNB2	19b					ephrin-B2
EIF2C1	19b			7b	7c	eukaryotic translation initiation factor 2C, 1
FAM46B	19b					family with sequence similarity 46, member B
FIGN	19b				7c	fidgetin
FRS2	19b	221				fibroblast growth factor receptor substrate 2
GRSF1	19b					G-rich RNA sequence binding factor 1
GULP1	19b					GULP, engulfment adaptor PTB domain containing 1
HIC2				7b	7c	hypermethylated in cancer 2
ILF3	19b					interleukin enhancer binding factor 3, 90kDa
KCMF1	19b	221	222			potassium channel modulatory factor 1
KLF13	19b					Kruppel-like factor 13
KRAS	19b					v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
MPPED2	19b					metallophosphoesterase domain containing 2
MYCL1	19b					v-myc myelocytomatosis viral oncogene homolog 1,
NAV3	19b					neuron navigator 3
NRG1	19b		222			neuregulin 1
NRK	19b	221	222	7b	7c	Nik related kinase
PCDHA9	19b	221	222			protocadherin alpha 9
PGM2L1				7b	7c	phosphoglucomutase 2-like 1
POGZ		221	222	7b	7c	pogo transposable element with ZNF domain
PRPF38B				7b	7c	PRP38 pre-mRNA processing factor 38 domain containing B
PTBP2		221				polypyrimidine tract binding protein 2
PTEN		221				phosphatase and tensin homolog
RGS16				7b	7c	regulator of G-protein signalling 16
SLC1A2	19b		222			solute carrier family 1, member 2
SMARCD1				7b	7c	SWI/SNF related, matrix associated, member 1
SOCS1	19b					suppressor of cytokine signaling 1
SOX4	19b		222			SRY (sex determining region Y)-box 4
STRBP				7b	7c	spermatid perinuclear RNA binding protein
VANGL2			222	7b		vang-like 2 (van gogh, Drosophila)
VAV3	19b			7b	7c	vav 3 oncogene
ZBTB10	19b			7b	7c	zinc finger and BTB domain containing 10
ZNF518						zinc finger protein 518

Table 6. The 50 genes targeted by the activin A down-regulated miRNAs
This table is modified from Tsai et al. (2010).

The potential targets of four abundantly expressed hES cell-specific miRNAs miR-372, miR-302d, miR-367 and miR-200c, as well as three other over-expressed miRNAs miR-19a, miR-199a and miR-217, were predicted by the PicTar (4-way) and TargetScanS with a cutoff *p* value less than 0.05. The expression levels of 13 target mRNAs were found to be inversely to their miRNAs (Tsai et al., 2010). Seven abundantly (more than 3-folds of overall mean) differentially (more than 3-folds of changes) expressed genes NR4A2, ERBB4, CXCR4, PCDH9, TMEFF2, CD24 and COX6A1, as well as six other genes TAL1, GRIA3, PRDM1, MYT1, EIF4G2 and CHMP4B, were found to be targets of miR-372, miR-302d, miR-367, miR-200c, miR-19a, miR-199a and/or miR-217 (Table 5). It may be noted that the NR4A2, TMEFF2 and TAL1 were also included in the 58 target genes of hES cell-specific miR-372, miR-302d, miR-367 and/or miR-200c (Li et al., 2009). The five miRNAs miR-19b, miR-221, miR-222, let-7b and let-7c were under-expressed in T3BA cells compared with T3CM cells, and their 50 target mRNAs (Table 6) were also found to exhibit inverse expression levels (Tsai et al. 2010).

The two target genes NR4A2 and ERBB4 were chosen to be validated by luciferase assay, since they expressed abundantly (more than four folds of overall mean in T3CM cells) and highly differentially (more than 7-fold changes of T3CM/T3BA) (Table 5). The potential miRNA binding sites of the 3' UTRs of NR4A2 and ERBB4 genes were predicted using PicTar and TargetScanS programs. The 3' UTR of NR4A2 was found by both methods to contain two potential miR-372 binding sites, four miR-302d binding sites and one miR-19a binding site, but miR-217 binding site was predicted by TargetScanS only. The 3' UTR of ERBB4 was found by both methods to have one site for miR-302d and two sites for miR-19a, but miR-372 binding site was predicted by TargetScanS only. The base-pairing between miRNAs and their target mRNAs, as well as the construction of luciferase reporter vectors, are shown in Fig. 3A, B. To demonstrate directly whether NR4A2 and ERBB4 genes were indeed the targets of miR-372, miR-302d, miR-19a and/or miR-217, the luciferase reporter vectors harboring the 3'UTRs of target genes were cotransfected with pSilencer vector containing miRNAs precursor or not in HEK293T cells (Tsai et al., 2010). The luciferase activity of reporter vector harboring NR4A2 3'UTR was inhibited to 37%, 38%, 53% or 33% by miR-372, miR-302d, miR-19a or miR-217, respectively. In the case of ERBB4, the luciferase activity was suppressed to 58%, 74% or 81% by miR-372, miR-302d or miR-19a, respectively (Fig. 3C). These results implied that NR4A2 gene was inhibited much stronger than ERBB4 gene by miR-372, miR-302d and miR-19a.

The mRNAs expressed more than three-folds of overall mean from T3BA and T3CM cells were also analyzed for the network and signaling pathways by using MetaCore Analytical Suite (Tsai et al., 2010). Besides 969 common genes, 1,396 and 153 genes were found to be unique for T3BA and T3CM cells, respectively (Fig. 4A), and the top 3 scored pathways (with lowest *p*-value) involved in regulation of cell cycle were highly regulated by activin A (Fig. 4B). Activin A has been reported to inhibit cell proliferation and activate cell differentiation, although it was also shown to participate in maintenance of pluripotency (Beattie et al., 2005; James et al., 2005). Activin A through its receptor ActR2B increased the expression of OCT4 and NANOG, as well as LEFTY-1 and LEFTY-2, to regulate stem cell maintenance. The abundantly differentially expressed target genes NR4A2, ERBB4 and CXCR4 of miRNAs miR-372, miR-302d, miR-19a and/or miR-217 highly induced by activin A in T3BA cells were further analyzed using IPA for their involvements in network and signaling pathways. The NR4A2 in nucleus and CXCR4 associated with plasma membrane

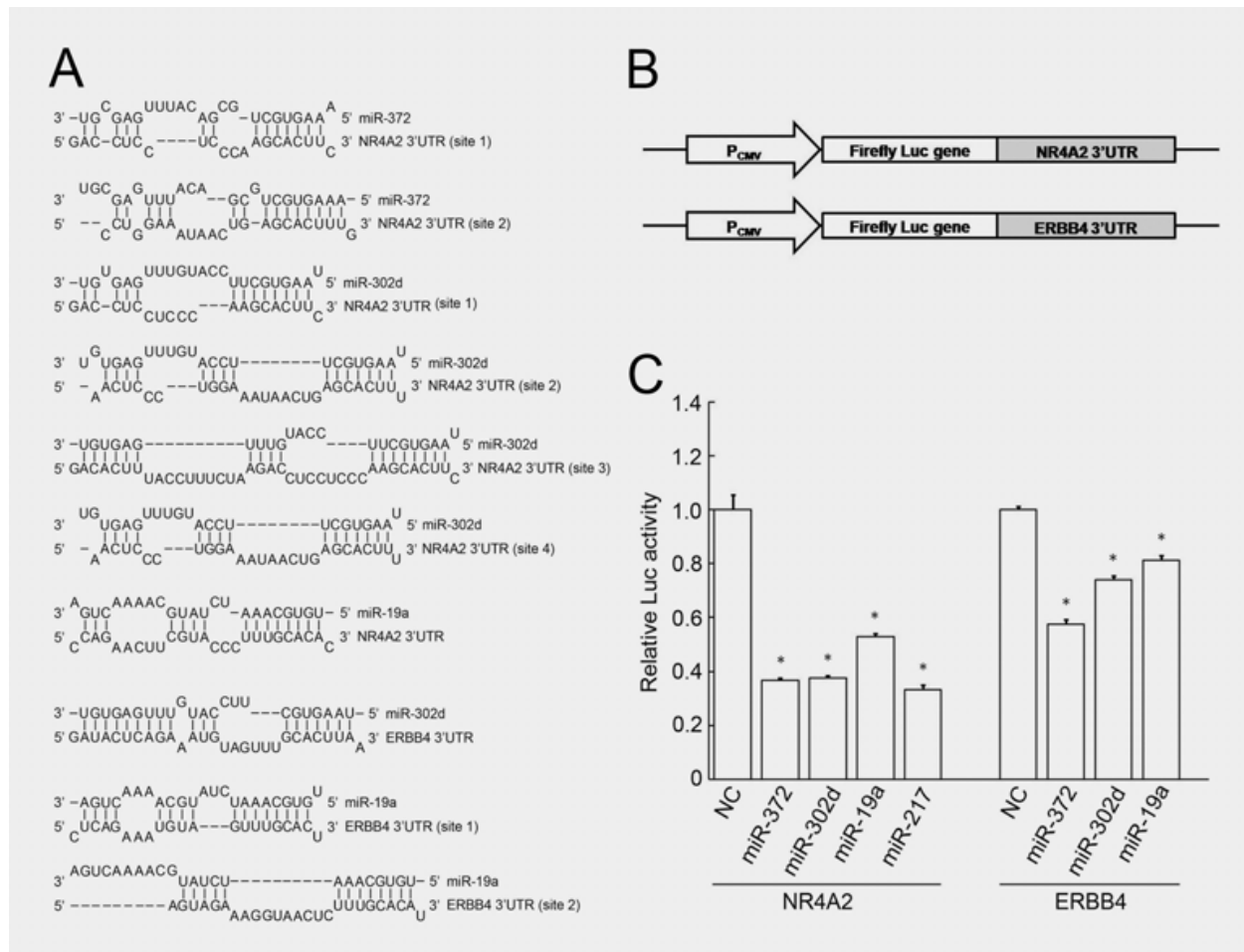


Fig. 3. Luciferase experiments to validate NR4A2 and ERBB4 targets (A) Predicted binding sites of miR-372, miR-302d and miR-19a within the 3'UTRs of NR4A2 and ERBB4. (B) The construction of luciferase reporter vectors. (C) The effects of miR-372, miR-302d, miR-19a and/or miR-217 on the luciferase activity of NR4A2 and ERBB4 reporter vectors.

This figure is adapted from Tsai et al. (2010).

were found to be commonly regulated by both EGF and TNF, while the membrane associated ERBB4 is regulated by EGF only. The NR4A2, also known as NURR1, is essential for the differentiation of the midbrain dopaminergic neurons, and it was reported to cooperate with PITX3 in promoting the terminal maturation of human and murine embryonic stem cell cultures to a dopamine neuron phenotype, and neither factor alone induced differentiation (Martinat et al., 2006). The ERBB4 is a transmembrane receptor tyrosine kinase that regulates cell proliferation and differentiation. The ERBB4 and its ligand heregulin are essential for neuronal development. The ERBB4 was reported to express at high levels in rat subventricular zone and rostral migratory system and to play a role in neuroblast tangential migration and olfactory interneuronal placement (Anton et al., 2004). The CXCR4, a chemokine receptor, is a crucial effector of the transcriptional pathway specifying mouse ventral motor neurons, and it controls the precision of initial motor axon trajectories (Lieberman et al., 2005). The CXCR4 has also been used as a biomarker of definite endoderm which is induced by activin A.

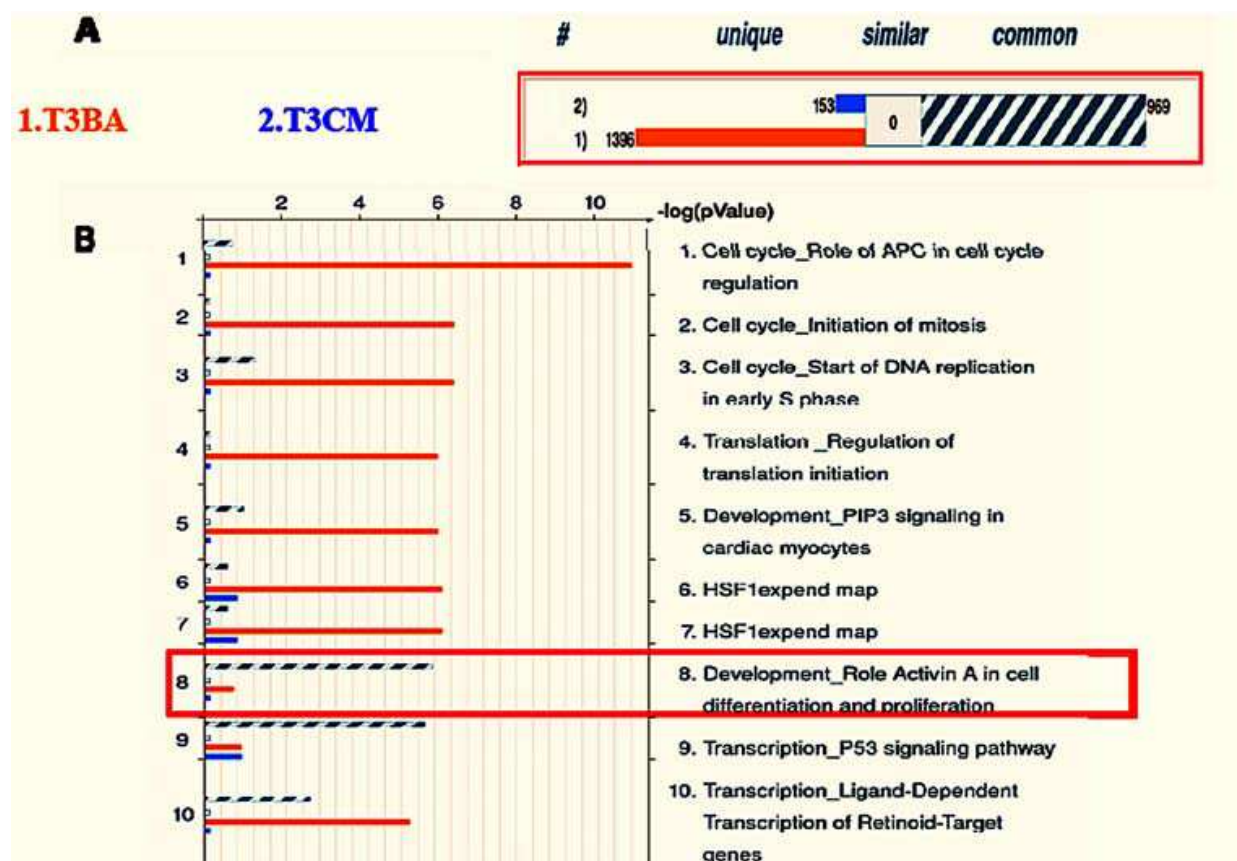


Fig. 4. Comparison of gene expression and GeneGo canonical pathway maps among T3CM and T3BA cells.

(A) The parameters for comparison are set at threshold of 3 with p-value of 0.05. The common genes are indicated by blue/white strips. The unique genes are marked as color band: (1) T3BA, orange; (2) T3CM, blue. (B) The top 10 GeneGo canonical pathway maps among T3BA and T3CM cells. The degree of “relevance” to different GeneGo ontology categories is defined by p-value, so that the lower random p-value gets higher priority. This figure is adapted from Tsai et al. (2010).

The seven miRNAs miR-372, miR-302d, miR-367, miR-200c, miR-19a, miR-199a and miR-217 were found to be highly up-regulated by activin A in T3BA cells (Table 4 and Fig. 2D). The expression of hES cell-specific miR-302 cluster was previously shown to be regulated by OCT4 and SOX2 (Card et al., 2008). Thus, activin A through its receptor ActR2B increased indirectly the expression of OCT4 to induce the expression of hES cell-specific miR-372, miR-302d, miR-367 and miR-200c. The miR-199a was found to be most abundant in T3BA cells, and its target COX6A1 expressed extremely abundantly in T3MF and T3CM cells was down-regulated in T3BA cells. The miR-199a is encoded by duplicated genes located within the intron of dynamin genes on chromosomes 1 and 19. Since the miR-199a genes are

positioned in opposite direction to the dynamin genes, they may be transcribed from their own promoters. The expression of miR-199a was also reported to be controlled by transcription factor TWIST-1 via an E-box promoter element (Lee et al., 2009), and how activin A indirectly regulate TWIST-1 remains to be elucidated. It may be noted that miR-199a and miR-199a* (processed from the same miRNA precursor) were recently reported to down-regulate the MET proto-oncogene and its downstream effector extracellular signal-regulated kinase 2 (ERK2) gene resulting in inhibiting cell proliferation of tumor cells (Kim et al., 2008). The miR-199a and miR-199a* were also shown to inhibit the mRNA translation of I κ B kinase β required for NF- κ B activation in ovarian cancer cells (Chen et al., 2008). The miR-217 was also found to be highly expressed in T3BA, but not at all in T3MF and T3CM cells (Table 4). It is of interest that the miR-217 was reported to be linked to tumorigenesis in pancreatic ductal adenocarcinoma (Szafranska et al., 2007) and used as one of biomarkers to discriminate benign and malignant pancreatic tissues (Szafranska et al., 2008).

4. Conclusions

The hES-T3 cells with normal female karyotype, one of five hES cell lines derived in my laboratory in Taiwan, were maintained their undifferentiated growth on the inactivated MEF feeder (T3MF), in MEF-conditioned medium (T3CM) and hES medium supplemented with both bFGF and activin A (T3BA). Autogenic feeder fibroblast (T3DF) cells with capacity to support the undifferentiated growth of hES cells were spontaneously differentiated from hES-T3 cells. The expression profiles of miRNAs and mRNAs from these four cell types (T3MF, T3CM, T3BA & T3DF) were quantitatively determined, and many target mRNAs of miRNAs expressed highly and regulated by activin A in hES cells were identified by the inverse expression levels of miRNAs to their target mRNAs. Approximately half of the 58 targets by hES cell-specific miRNAs miR-372, miR-302d, miR-367 and/or miR-200c were involved in gene transcription. Four hES cell-specific microRNAs miR-372, miR-302d, miR-367 and miR-200c, as well as three other microRNAs miR-199a, miR-19a and miR-217, were found to be up-regulated, whereas five miRNAs miR-19b, miR-221, miR-222, let-7b and let-7c were down-regulated by activin A. Thirteen abundantly differentially expressed mRNAs, including NR4A2, ERBB4, CXCR4, PCDH9, TMEFF2, CD24 and COX6A1 genes, targeted by seven over-expressed miRNAs were identified. The NR4A2, ERBB4 and CXCR4 target genes were further found to be regulated by EGF and/or TNF. The 50 abundantly differentially expressed genes targeted by five under-expressed miRNAs were also identified. The abundantly expressed mRNAs were also analyzed for the network and signaling pathways, and roles of activin A in cell proliferation and differentiation were found. These findings will help elucidate the complex signaling networks which maintain the unlimited self-renewal and pluripotency of hES cells. The autogenic fibroblasts derived from hES cells will be useful to reduce the risk of animal pathogens in the future medical applications of hES cells.

5. Acknowledgements

I thank Drs. P.-C. Yang, S.-L. Yu, S. Singh and Y.-H. Liu for their productive collaborations; Z.-Y. Tsai, L.-P. Kao, B.-Z. Chen and B.-C. Ho for their hard work; and M.P. Li for copy

editing. The tables and figures are modified/adapted with permission from Li et al. (2009) and Tsai et al. (2010). My research has been supported by grants from National Science Council of Taiwan.

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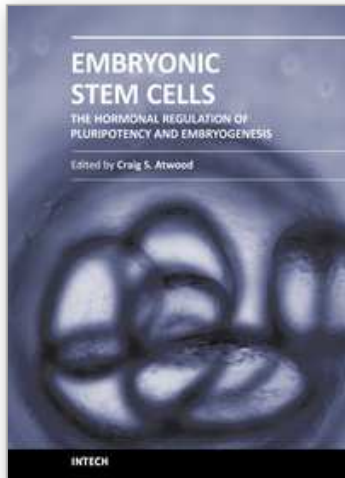
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ISBN 978-953-307-196-1

Hard cover, 672 pages

Publisher InTech

Published online 26, April, 2011

Published in print edition April, 2011

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