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Research Paper

Differential Expression Profile of Long Non-coding RNAs during Differentiation of Cardiomyocytes

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Abstract

Many long non-coding RNAs (lncRNAs) are species specific and seem to be less conserved than protein-coding genes. Some of them are involved in the development of the lateral mesoderm in the heart and in the differentiation of cardiomyocytes. The purpose of the study was to investigate the expression profiles of lncRNAs during the differentiation of P19 cells into cardiomyocytes, with a view to studying the biological function of lncRNAs and their involvement in the mechanism of heart development. First, we observed the morphology of P19 cells during differentiation using an inverted microscope. Then, cardiac troponin T (cTnT) expression was detected to validate that the cells had successfully differentiated into cardiac myocytes by real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) and western blotting. Lastly, the expression profile of lncRNA genes was obtained using an lncRNA microarray and real-time RT-PCR analyses. The microarray results showed that 40 lncRNAs were differentially expressed, of which 28 were upregulated and 12 were downregulated in differentiated cardiomyocytes. The differentially expressed lncRNAs were further validated. Our results illustrated a critical role of lncRNAs during the differentiation of P19 cells into cardiac myocytes, which will provide the foundation for further study of the biological functions of lncRNAs and the mechanism of heart development.

Key words: lncRNAs; differentiation; caridiomyocytes; microarrays.

Introduction

The heart is the first functional organ that is developed in the process of embryonic development. It is very important for development that the heart is healthy. Numerous studies have revealed the accurate regulation of key molecular pathways during embryonic development, particularly in the cardiovascular system. Haploinsufficiency of essential genes often leads to cardiac malformations [1], which are the most common major congenital defects, with a prevalence of approximately eight in every 1,000 newborn infants [2].

The human transcriptome is composed of not only a large set of protein-coding messenger RNAs (mRNA), but also many non-protein coding transcripts that have structural, regulatory or unknown functions. Over the last decade, much attention focused on the microRNAs (miRNAs), a class of small non-coding RNAs that are involved in various biological and pathological processes [3, 4]. More recently, long non-coding RNAs (lncRNAs), generally defined as non-coding RNAs of more than 200nt in length without known protein-coding function [5],

have risen to prominence, with central roles in a diverse range of functions in cell biology [6,7]. In contrast to miRNAs, lncRNAs have not been fully investigated. A handful studies have indicated that dysregulation of lncRNAs result in aberrant gene expression associated with cancers [8-10]. Although an increasing number of lncRNAs have been characterized, the role of lncRNAs in the differential of cardiomyocytes has not been investigated.

The P19 mouse embryonal carcinoma cell line is multipotent and can differentiate into cardiac myocytes with embryoid body formation in the presence of dimethylsulfoxide (DMSO) [11]. Thus, P19 cells have been used to study cardiac-specific transcription factors and upstream signaling pathways in cardiac differentiation [12-14]. Therefore, P19 cells are a suitable model for studying cardiac differentiation at the molecular and functional levels [15].

In this study, we initially identified differentially expressed lncRNAs during the differentiation of P19 cells using an lncRNA microarray. We subsequently validated the microarray results by real-time quantitative reverse transcription PCR (real-time qRT-PCR) for specific differentially expressed lncRNAs.

Results and Discussion

P19 cells differentiation

P19 cells differentiate into cardiac myocytes in the presence of DMSO. The efficiency of differentiation depends on the prior formation of non-adhering aggregates [17]. We observed and photographed the morphological changes in P19 cells using an inverted microscope to investigate the process of P19 cell differentiation. We discovered that P19 cells aggregated during the first 4 days and there were beating cell colonies on day 10 (Figure 1). The myocyte differentiation marker cTnT was detected on day 0 and day10, respectively, to validate that the cells had differentiated into cardiac myocytes. As shown in Figure 2 and Figure 3, mRNA expression and protein expression of cTnT were much higher on day 10 compared to day 0. Thus, the beating cell colonies generated on day 10 of P19 cell differentiation, and the high expression of cTnT, demonstrated that the cells had differentiated into cardiac myocytes.

lncRNA microarray.

LncRNA microarrays are powerful tools for studying the biological function of lncRNAs. We conducted lncRNA microarray analysis on P19 cells at day 0 and day 10 of DMSO exposure. According to the microarray data, we selected lncRNAs that were upregulated by more than five-fold and downregulated by more than three-fold. In addition, poorly conserved lncRNAs were excluded. The conservation of lncRNAs was determined using the online Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Ultimately, 40 differentially expressed lncRNAs that were highly conserved in cardiac myocytes (day 10) compared to normal P19 cells (day 0) were identified (Table 1). Among them, 28 were upregulated and 12 were downregulated. We randomly chose five upregulated lncRNAs (ENSMUST00000159006, uc009byc.1, AK089560, ENSMUST00000101005, ENSMUST00000124503) and three downregulated lncRNAs (uc007keu.1, AK028257, BC030682) for qRT-PCR validation (Table 2).

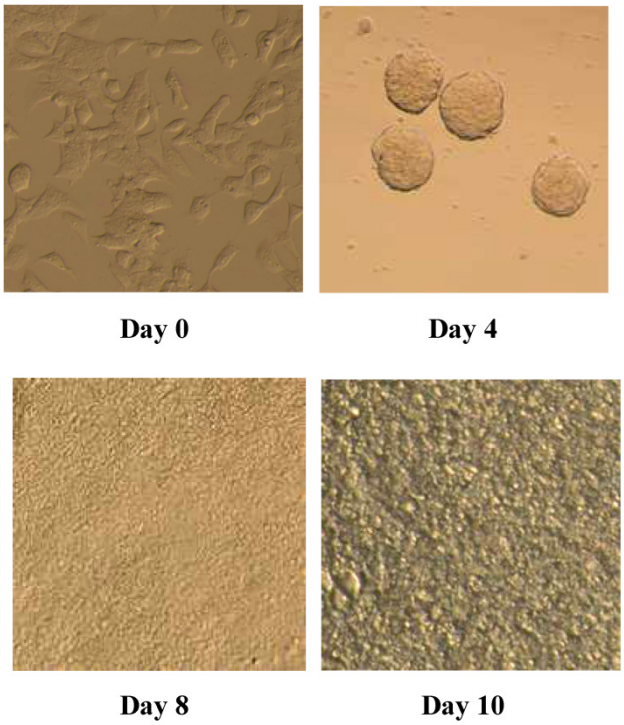

Figure 1: Morphology of P19 cells during differentiation into cardiac myocytes (day 0, day 4, day 8, day 10). P19 cells were aggregated for 4 days and colonies of beating cells were observed on day 10 under an inverted microscope, as described in Materials and methods.

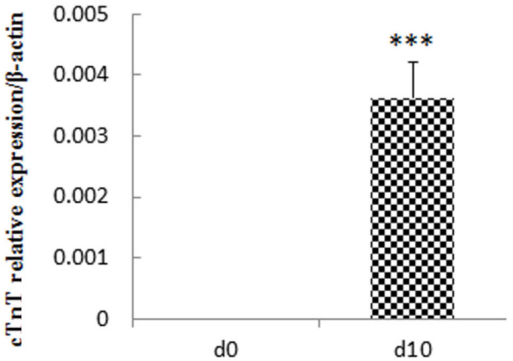

Figure 2: Relative expression of cTnT at day 10 compared with day 0. The experiment was repeated three times with consistent results. ***p<0.001

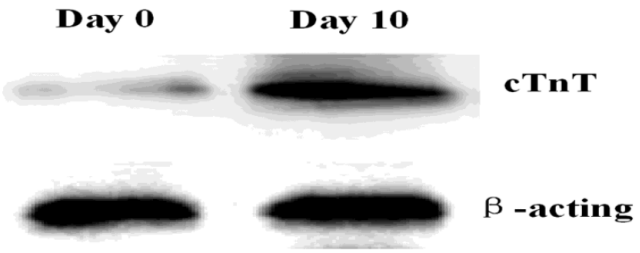

Figure 3: Expression of the cTnI protein in P19 cells. Total proteins were isolated from P19 cells and analyzed by western blotting. Lane 1, day 0; Lane 2, day 10. The experiment was repeated three times with consistent results.

Table 1. 40 differentially expressed lncRNAs.

Regulation	lncRNA	chromosomal localization	RNA length	Start locus	Stop locus
28 up-regulated lncRNAs	AK158639	chr2	417	169458512	169458928
	uc007vie.1	chr15	4168	12959401	12963569
	ENSMUST00000159006	chr6	253	52108522	52112019
	AK166199	chr13	1351	16122089	16123440
	AK052877	chr19	1301	30638978	30640279
	uc009biz.1	chr6	606	36664928	36665534
	uc009pal.1	chr9	3270	41388823	41400910
	uc009eqi.1	chr6	2534	143777066	143779600
	ENSMUST00000101005	chr6	1302	119912384	119913686
	ENSMUST00000124503	chr11	454	35163570	35164287
	AK020106	chr11	802	69615637	69616437
	AK142834	chrX	2388	118021841	118024227
	uc007cpz.1	chr1	2470	135197537	135200007
	uc007prv.1	chr13	2198	21925626	21929399
	uc009byc.1	chr6	545	52122879	52124051
	AK078053	chr18	1378	36459754	36461132
	NR_024257	chr2	4066	9802872	9808394
	AK089560	chr5	2683	13525726	13528408
	AK142308	chr18	1262	37965377	37966636
	AK046177	chr13	606	117639650	117640255
	AK135062	chr7	2464	104057136	104059598
	AK138321	chr11	2303	47744849	47747149
	uc008sdp.1	chr4	3039	22409926	22412965
	uc008fug.1	chr18	948	83172461	83173409
	AK028129	chr3	2428	96043315	96045742
	uc008xbx.1	chr5	802	34516538	34517340
	uc008euf.1	chr18	1629	43480650	43482279
	ENSMUST00000127359	chr14	344	47007193	47008957
	uc007keu.1	chr11	1635	75565382	75579340
	AK033485	chr1	2241	54532201	54534442
	uc007pyj.1	chr13	1179	28700386	28977221
	uc008sac.1	chr4	1060	11893711	11921427
12 down-regulated lncRNAs	AK137254	chr7	5124	127773275	127778400
	AK028257	chr14	272	55735163	55735434
	BC030048	chr17	1092	35087185	35088238
	BC030682	chr7	1343	71031236	71032537
	ENSMUST00000117553	chr2	1125	111840336	111841461
	ENSMUST00000172121	chr6	291	64941211	64941502
	AK010244	chr2	1771	125082798	125084785
	uc008mcn.1	chr2	1771	125082798	125084785

Table 2. lncRNAs differentially expressed between cardiomyocytes that differentiated from P19 cells (day 10) compared with normal cells (day 0).

up-regulated lncRNA	fold change	GeneSymbol	down-regulated lncRNA	fold change	GeneSymbol
ENSMUST00000159006	46.21	Gm15051	uc007keu.1	8.07	Ywhae
uc009byc.1	21.50	AK142386	AK028257	4.71	
AK089560	15.47		BC030682	3.4	
ENSMUST00000101005	6.29	Wnk1			
ENSMUST00000124503	5.11	Gm12122			

Validation of differentially expressed lncRNAs

We performed real-time qRT-PCR expression analysis on P19 cells at day 0 and day 10 to confirm the microarray results. Using GAPDH as a normalization control, the statistics demonstrated that four out of the five upregulated lncRNAs (P=0.038, 0.000016, 0,022 and 0.017 for ENSMUST00000159006, uc009byc.1, AK089560, ENSMUST00000124503, respectively) and two of the three downregulated lncRNAs (P=0.00012 and 0.001 for AK028257 and uc007keu.1, respectively) showed significantly different expressions (Figure 4). In addition, we validated the eight differentiated expressed lncRNAs at different time points during the differentiation. D0, d4, d6, d8, d10 were chosen as the time points. As shown in the figure 5, the expressed trends during the differentiation are consistent with the comparison between d0 and d10. The major difference might be the beginning time of the change was different.

Discussion

Congenital heart defects (CHD) are the most common major congenital malformation, accounting for approximately 40% of perinatal deaths and more than one fifth of deaths in the first month of life [18]. Although many studies have focused on heart development in recent decades, details of the mechanism remain unclear [19, 20]. P19 cells are isolated from an experimental embryo-derived mouse teratocarcinoma and can differentiate into cardiac myocytes with embryoid body formation in the presence of DMSO [21]. Thus, we simulated heart development in vitro by differentiating P19 cells into cardiomyocytes.

This study focused on determining the lncRNAs expression profile during cardiomyocyte differentiation and explaining the differences between cardiomyocytes and undifferentiated P19 cells. We identified 40 differentially expressed lncRNAs (28 upregulated and 12 downregulated). Real-time qRT-PCR validated four of five upregulated and two of three downregulated lncRNAs.

Some researchers have demonstrated that the expression of many lncRNAs is different during development and that their functions range from the control of pluripotency to lineage specification [22, 23]. In theory, lncRNAs have intrinsic cis-regulatory capacity, which has been confirmed and whose mechanism has been described. Increasing numbers of reports show that lncRNAs can play a role in both cis and trans [24, 25], and more direct experimental studies are required to determine the precise proportion of cis regulators. Regulating the expression of some lncRNAs may influence the expression of their neighboring protein-coding genes, including several master regulators of cellular differentiation [26-28]. It is in the early stage that the role of lncRNAs in heart development has attracted much attention from researchers. Indeed, two lncRNAs, *Fendrr* and Braveheart (Bvht), were recently uncovered to be involved in the development of the lateral mesoderm in the heart and the differentiation of cardiac myocytes, respectively [29, 30]. Deficiency of *Fendrr*, particularly in the nascent lateral plate mesoderm, can result in a thin ventricular wall of the heart. *Fendrr* regulates the expression of certain core transcription factors in heart development by modulating the epigenetic profile of cells to generate cardiac hypoplasia [29]. In a similar way to *Fendrr*, *Bvht* interacts with SUZ12, a component of PRC2, to alter cardiomyocytes differentiation and retain the cardiac phenotype in neonatal cardiomyocytes [30].

Most of the four up-regulated and two down-regulated lncRNAs have no official Human Genome Nomenclature Committee symbol and their function is still unclear. However, some studies have been shown that Ywhae play a critical role in many diseases, such as HIV neurocognitive impairment [31], neuronal migrational defects [32], bipolar disorder [33] and endometrial stromal sarcoma [34].

Although we have identified some differentially expressed lncRNAs during the cardiac differentiation, it is too early for us to confirm their relationship with cardiac malformation. Therefore, subgroup analysis of lncRNAs should be performed to explore this relationship in the future. In addition, most lncRNAs have a distinct spatial and temporal specificity in the process of organismal differentiation and development. It has been shown that lncRNAs have different expression patterns in different parts of the brain [35].

We should sample more cells from different times in the process of differentiation, such as d4, d6 and d8, to examine alterations in lncRNA expression in the early stage of differentiation.

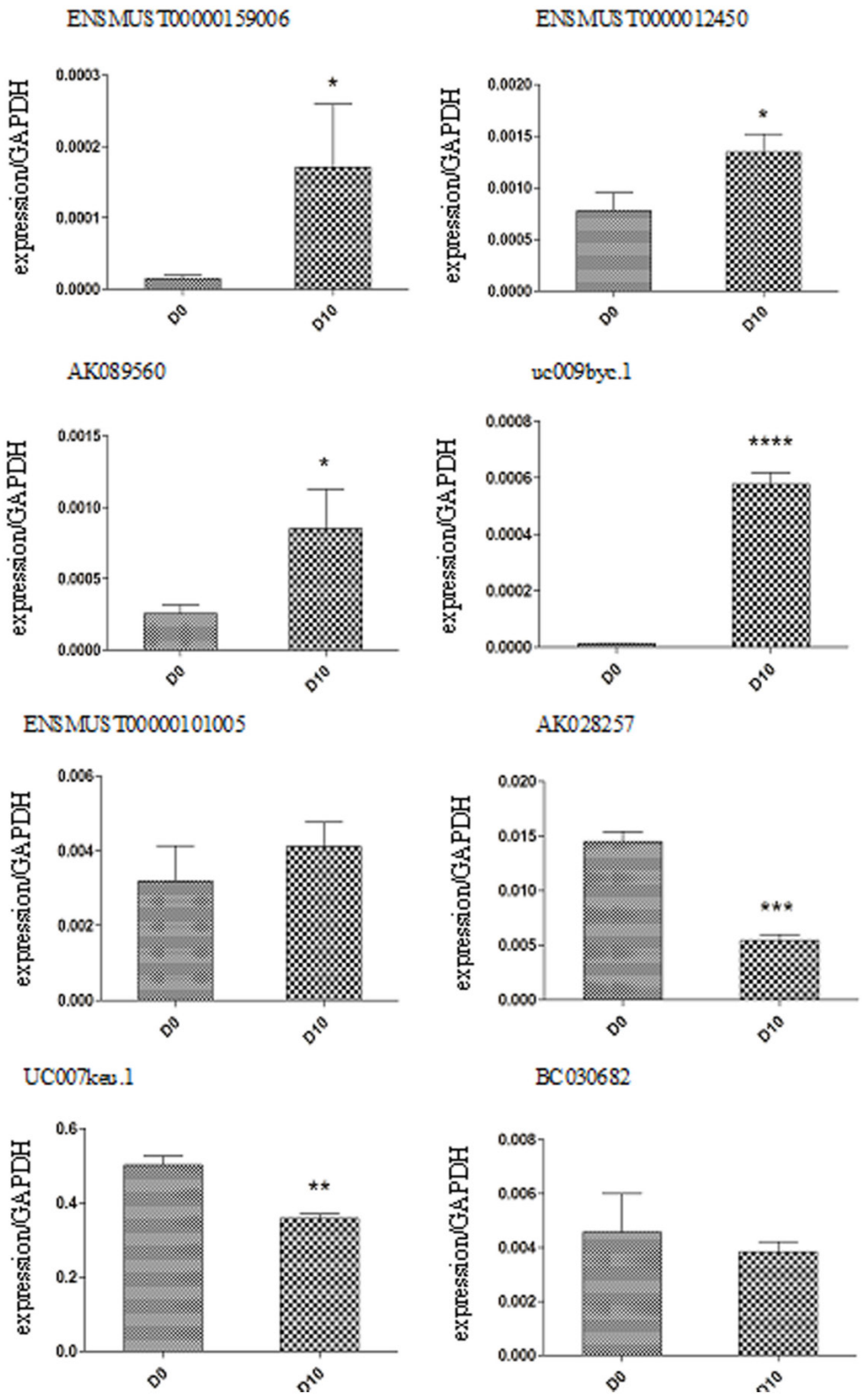

Figure 4: Validation of lncRNA microarray data using real-time RT-PCR. The real-time RT-PCR reactions were repeated three times for every lncRNA. * $p < 0.05$, **$p<0.01$, ***$p<0.001$.

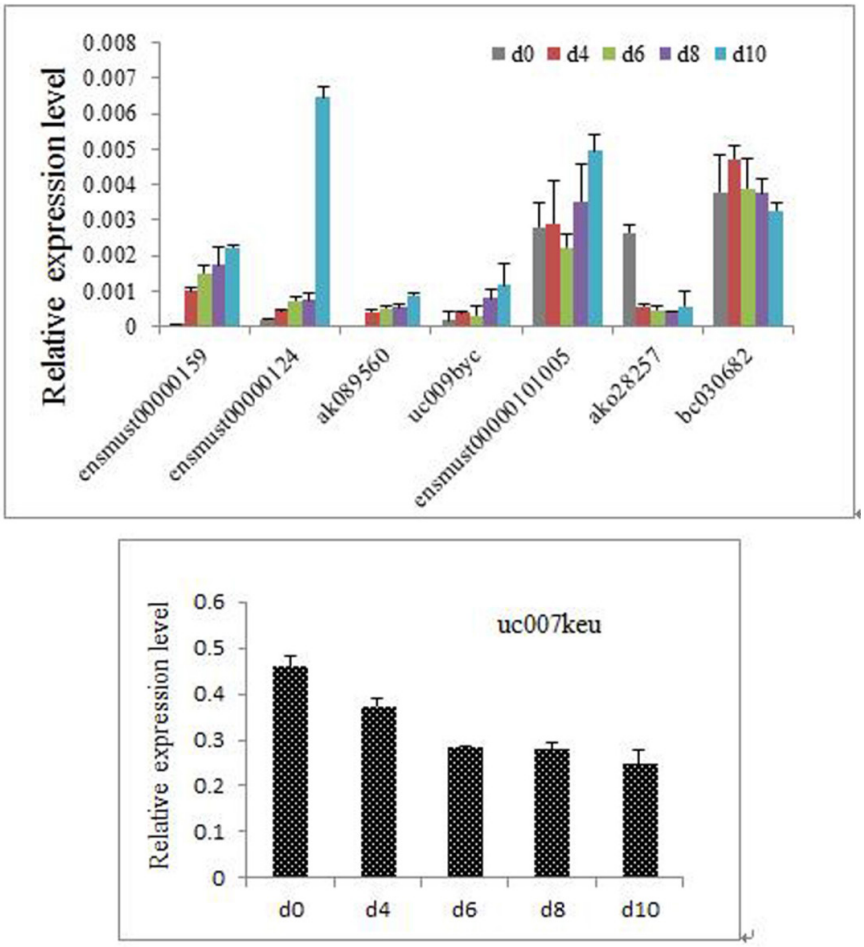

Figure 5. The 8 differentiated expressed lncRNAs at different time points of the differentiation. (Because the relative expression of uc007keu was much higher than the other lncRNAs, we performed two histograms for clarity and aesthetic feeling.)

Materials and methods

P19 cell culture and induction of differentiation

P19 cells were bought from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in complete medium (α-MEM + 10% fetal bovine serum, FBS + 100 U/ml penicillin + 100ug/ml streptomycin, pH 7.2-7.4) (α-MEM, FBS, penicillin and streptomycin, Gibco-BRL, Grand Island, NY, USA) in a 5% CO_2 atmosphere at 37°C. During differentiation, P19 cells were maintained in suspension as aggregates for 4 days in complete medium containing 1% dimethylsulfoxide (DMSO, Sigma, St. Louis, MO, USA) in bacteriological dishes. On day 4, the cell aggregates were transferred to cell culture flasks and then adherently cultivated from the 5^{th} to the 10^{th} day without DMSO. The culture medium was replaced every 2 days. We harvested cells on differentiation day 0 and day 10. The morphological changes in P19 cells were observed under an inverted microscope (Nikon Eclipse TE300, Tokyo, Japan) equipped with phase-contrast objectives and a digital camera (Nikon E4500).

Quantitative real time-PCR (qPCR) and western blotting

Total RNA was extracted from the harvested cells using a mirVana extraction kit (Ambion, Austin, TX, USA), following the manufacturer's protocol. RNA was measured using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE, USA) to assess its quantity and quality, and stored at -80°C. In general, we simultaneously performed RNA extraction and cDNA transcription for all subjects. The total RNA was reverse-transcribed to cDNA with a High-Capacity cDNA reverse transcription kit (Applied Biosystems, Austin, USA.). According to the manufacturer's protocol, we used 1μg of mRNA to activate 20μl of the reverse transcription reaction. The reaction comprised 25°C for 10min, 37°C for 120min, 85°C for 5 min and a hold at 4°C. Subsequently, real-time PCR was performed in triplicate for each sample and included no-template negative controls. For the final volume of 20 μl reaction, 1 μl of synthe-

sized cDNA was mixed with 8 μl of diethylpyrocarbonate (DEPC)-treated water, 10 μl of TaqMan Gene Expression Master Mix and 1 μl of cardiac troponin T (cTnT) / β-actin TaqMan Gene Expression Assay (Applied Biosystems, cTnT ID: Mm01290256_m, β-actin ID: Mm00607939_s1). The reaction conditions comprised 50°C for 2min, 95°C for 10min; followed by 40 cycles of 95°C for 15s and 60°C for 1min on the ABI 7500 Real-Time PCR system (Applied Biosystems). β-actin was used as a reference to obtain the relative expression of cTnT, which was determined with the comparative cycle threshold (CT) ($2^{-\Delta CT}$) method, in which $\Delta CT = C_{T\ cTnT} - C_{T\ \beta\text{-actin}}$.

A monoclonal rabbit anti-cTnT antibody and a monoclonal rabbit anti-β-actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cells were broken using the lysis buffer provided in the total protein extraction kits (KeyGen, Inc., China). The lysate supernatant was obtained after centrifugation at 14000×g for 30min at 4°C. We then measured the protein concentration with a BCA protein detection kit (KeyGen, Inc., China). Western blotting was conducted as previously described [16].

Construction of the lncRNA microarray

We pooled three replicate samples of cells on day 0 and day 10 of DMSO exposure, respectively, to perform lncRNA microarray analysis. Total RNA was isolated from the two samples as above and was quantified using a NanoDrop spectrophotometer (NanoDrop). RNA integrity was assessed by standard denaturing agarose gel electrophoresis. Each sample was then amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias, using a random priming method. The labeled cRNAs were hybridized onto the Mouse LncRNA Array v2.0 (8 x 60K, Arraystar). The hybridized arrays were washed, fixed and scanned with using the Agilent DNA Microarray Scanner (part number G2505C). Data were extracted using Agilent Feature Extraction software (version 11.0.1.1). Quantile normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies). Differentially expressed LncRNAs between the two samples were identified by Fold Change filtering. The threshold set for upregulated lncRNAs was more than five-fold and for downregulated lncRNAs it was more than three-fold. The lncRNAs discussed in this article were carefully collected from the most authoritative databases, such as RefSeq, UCSC Knowngenes, Ensembl and many related literature.

Validation of differentially expressed lncRNAs

Total RNA extraction and cDNA transcription were conducted as above. For real-time PCR, we added 1μl of cDNA to 12.5μl of SYBR-Green Gene Expression Master Mix (Applied Biosystems, Inc), 10.5μl f DEPC-treated water and 0.5μl of reverse and forward primers. cDNA was amplified for 50 cycles on the ABI 7500 Real-Time PCR system (Applied Biosystems). The primers sequences used are listed in Table 3. GAPDH was used as a reference to obtain the relative expression of target lncRNAs which was determined with the comparative cycle threshold (CT) ($2^{-\Delta CT}$) method, in which $\Delta CT = C_{T\ target\ lncRNA} - C_{T\ GAPDH}$.

Table 3. Primers for real-time RT-PCR.

Gene name	Primers	Tm (℃)
ENSMUST00000159006	P5:GGAGCTGACTTGGAGCACTG	60
	P3:AACAGACCTCTTGCCAGTTCA	
uc009byc.1	P5:AACTTGCGTCTGGAGTTGGG	60
	P3:CCCAGAATAGCAGCACCTCA	
AK089560	P5:ATGCTTTCCCAGGGTGTGTT	60
	P3:GGCTAGGATTTCCCGACGAG	
ENSMUST00000101005	P5:TGTTGATACAGCCTCAGTCCAT	60
	P3:GTTGGAAGTGGCGAGTTTGG	
ENSMUST00000124503	P5:GACACGAAGAAGAACCACATCA	60
	P3:GCCTGCGAGGATTCTATTTATT	
uc007keu.1	P5:AAAATGTGATTGGAGCCAGAAG	60
	P3:GTCCTCTCCTCCCTTGTTTTCT	
AK028257	P5:CTCTCCTCTCCGCTTCTCTCT	60
	P3:CATCCCAGCACAAATCAATGT	
BC030682	P5:GACCTGGCTCTTCCTCAT	60
	P3:TTCCATCTGTCCGTTCTG	
GAPDH	P5:ATTCAACGGCACAGTCAA	60
	P3:CTCGCTCCTGGAAGATGG	

Statistical analysis

All statistical analyses were performed using the Student's t-test with SPSS software version 13.0 (SPSS, Inc, Chicago, IL, USA). P-values less than 0.05 were considered statistically significant, and all the statistical tests were two-sided.

Conclusion

In conclusion, we identified a set of lncRNAs that were aberrantly expressed in cardiomyocytes compared to undifferentiated P19 cells, which will provide the foundation for the further study of the biological function of lncRNAs and the mechanism of heart development.

Abbreviations

lncRNAs: long non-coding RNAs; cTnT: cardiac troponin T; RT-PCR: reverse transcriptase polymerase chain reaction; mRNA: messenger RNAs; miRNA: microRNA; DMSO: dimethylsulfoxide; CHD: con-

genital heart defects; DEPC: diethylpyrocarbonate.

Acknowledgments

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Competing Interests

The authors have declared that no competing interest exists.

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