Original Paper

Compared Analysis of LncRNA Expression Profiling in *pdk1* Gene Knockout Mice at Two Time Points

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Key Words

LncRNA • *pdk1* gene knockout • Heart failure • Microarray

Abstract

Background/Aims: Previous studies have indicated that long non-coding RNAs (lncRNA) are related to the occurrence and development of many human diseases, such as cancer and the HELLP and the brachydactyly syndromes. However, studies of lncRNA in heart failure have not yet been reported. Here, we investigated cardiac lncRNA expression profiles in the myocardial-specific knockout *pdk1* gene (KO) mouse model of heart failure. **Methods:** Cardiac samples were obtained from PDK1 KO and WT mice on postnatal (P) day 8 (P8) and day 40 (P40), and lncRNA expression profiles were analyzed by sequencing and screening using the Arraystar mouse lncRNA microarray. Quantitative real-time PCR analysis of these lncRNAs confirmed the identity of some genes. **Results:** Comparisons of the KO and control groups showed fold changes of >1.5 in the expression levels of 2,024 lncRNAs at P8, while fold changes of >2 in the expression levels of 4,095 lncRNAs were detected at P40. Nineteen lncRNAs were validated by RT-PCR. Bioinformatic and pathway analyses indicated that mkk7, a sense overlap lncRNA, may be involved in the pathological processes of heart failure through the MAPK signaling pathway. **Conclusion:** These data reveal differentially expressed lncRNA in mice with a myocardial-specific deletion of the *pdk1* gene, which may provide new insights into the mechanism of heart failure in PDK1 knockout mice.

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Introduction

Although technological advances have led to the improvement of heart failure (HF) treatments, it is still a major cause of morbidity and mortality as well as a significant burden on healthcare systems [1, 2].

HF, being a syndrome, not a disease, is made up of a series of pathological changes in the failing heart, in systems such as energy metabolism, ion channel functions, as well as growth and survival signaling pathways [3-5]. Problems in these complex systems will ultimately lead to fatal heart damage, although the processes by which this occurs remains somewhat elusive.

The 3-phosphoinositide-dependent protein kinase-1(PDK1) was first identified as a serine/threonine kinase. The mouse PDK1 (mPDK1) protein is composed of 559 amino acids and a COOH-terminal pleckstrin homology domain. Murine PDK1 mRNA is broadly expressed in all mouse tissues and in embryonic cells [6]. Previous studies have demonstrated that PDK1, through phosphorylation of the activated AGC protein kinase family and other protein kinases, has a key role in cell growth proliferation, migration and survival, as well as nutrient uptake and storage. These functions of PDK1 have been demonstrated in many types of cancer [7-9].

PDK1 knockout (KO) mice exhibit embryonic fatality at day E9.5 due to multiple abnormalities including lack of somites, forebrain and neural crest-derived tissues. However, PDK1 hypomorphic mice, which exhibit a partial loss of gene function, were 40–50% smaller than the control animals, and their organ volumes were reduced proportionately [10, 11]. In the heart tissues specifically, PDK1 KO mice developed and suddenly died of heart failure between 5 and 11 weeks of age. In addition, heart muscle mass was markedly reduced in KO mice due to a reduction in cardiomyocyte volume rather than cardiomyocyte cell number [12-14]. In this study, we investigated the role of long non-coding RNA (lncRNAs) in the *pdk1* gene knockout mouse model of heart failure. These mice start expressing the α -myosin heavy chain recombinase in cardiomyocytes at postnatal day 7, develop heart failure at postnatal day 40 and die at approximately 5 to 8 weeks of age [14, 15]. However, both the heart function and histomorphology are normal at P8 [14]. Long non-coding RNAs (lncRNAs) have recently emerged as key players in many rapidly growing areas of research, including epigenetics, hormone signaling, development, stem cell biology, cancer, brain function and plant biology [16-26]. lncRNAs are typically 1,000–10,000 residues in length and have little or no ability for protein coding [27, 28]. An lncRNA can be classified into five major categories, based on their relative positions to neighboring protein coding genes: (1) sense, when overlapping one or more exons of another transcript on the same strand; (2) antisense, when overlapping one or more exons of another transcript on the opposite strand; (3) bidirectional, when the expression of it and a neighboring coding transcript on the opposite strand is initiated in close genomic proximity; (4) intronic, when it is derived wholly from within an intron of a second transcript; and (5) intergenic, when it lies within the genomic interval between two genes [21]. Previous studies have indicated that lncRNAs have a wide range of biological functions mediated via many poorly understood molecular mechanisms, which include cis-tethering or cis-targeting, trans-targeting, allosteric modification and functions as enhancers, decoys, scaffold, and co-activators or co-repressors of gene regulation [28-33]. Existing research on the role of lncRNA in cardiac development and disease has focused on the expression of lncRNA-SRA transcripts in dilated cardiomyopathy [34, 35], as well as the regulation and induction of the lncRNA-ALC-1 antisense by ALC-1 in hypertrophic ventricles [36]. Other studies have found lncRNA expression in the myocardium from infants with Tetralogy of Fallot [37]. More recent studies have investigated the role of lncRNA, Braveheart, in cardiovascular lineage commitment [38]. Another tissue-specific lncRNA, Fendrr, has been found to be an essential regulator of heart and body wall development in the mice [39]. However, investigations of the role of lncRNA in cardiac development and pathophysiology are rare, with no current reports on its impact in heart failure.

In this study, we compared the lncRNA expression profiles between *pdk1* gene KO mice and matched control mice (intact *PDK1*) at postnatal day 8 (P8) and day 40 (P40). A small number of mice were evaluated by RT-PCR in a total of six pairs of heart tissue samples from KO and control mice. Our results suggest that lncRNAs may participate in the progression of HF and provide key new molecular insights into the mechanisms may underlying HF.

Materials and Methods

Experimental animals and tissue collection

The generation of *pdk1* gene knockout mice has been previously described [14]. Our experimental animal facility has been accredited by the AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) and the IACUC (Institutional Animal Care and Use Committee) of the Model Animal Research Center of Nanjing University approved all animal protocols (AP) used in this study (AP#: ZY10). Specimens were snap-frozen in liquid nitrogen and subsequently stored at -80°C. Total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA) according to the instructions provided by the manufacturer.

Microarray and data analysis

DNA microarray. The Arraystar mouse lncRNA Array v2.0 was designed for profiling both lncRNAs and protein coding RNAs of the mouse genome. A total of 31,423 lncRNAs were collected from authoritative data sources including RefSeq, UCSC Knowngenes, Ensembl and other related literature. The microarray analysis was performed by KangChen Bio-tech, Shanghai, PR China. For each microarray study, RNA of 3 mouse heart tissue samples from control and KO groups were pooled, and used in hybridization. The array experiment was repeated twice on two different sets of mouse-derived tissues.

RNA labeling and array hybridization. Three samples were hybridized, two biological replicates for each condition (cardiac tissues of control and *pdk1* KO mouse, respectively). Sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology, CA, USA) with minor modifications. Briefly, mRNA was purified from total RNA after removal of rRNA (mRNA-ONLY™ Eukaryotic mRNA Isolation Kit, Epicentre). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias utilizing a random priming method. The labeled cRNAs were purified by RNeasy Mini Kit (Qiagen). The concentration and specific activity of the labeled cRNAs (pmol Cy3/μg cRNA) were measured by NanoDrop ND-1000. One μg of each labeled cRNA was fragmented by adding 5 μl 10× Blocking Agent and 1 μl of 25× Fragmentation Buffer. The mixture was then heated at 60°C for 30 min before the addition of 25 μl 2× GE Hybridization buffer to dilute the labeled cRNA. Hybridization solution (50 μl) was dispensed into the gasket slide and assembled with the lncRNA expression microarray slide. The slides were incubated for 17 hours at 65°C in an Agilent Hybridization Oven. The hybridized arrays were washed, fixed and scanned using the Agilent DNA Microarray Scanner (part number G2505C). The microarray analysis was performed by KangChen Bio-tech, Shanghai, PR China.

Data analysis. Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed with using the GeneSpring GX v11.5.1 software package (Agilent Technologies). After quantile normalization of the raw data, lncRNAs and mRNAs with at least two out of two samples with flags in Present or Marginal ("All Targets Value") were chosen for further data analysis. Differentially expressed lncRNAs and mRNAs between two samples were identified through fold change filtering. Hierarchical Clustering was performed using the Agilent GeneSpring GX software (version 11.5.1). GO analysis and pathway analysis were performed using in the standard enrichment computation method.

Functional group analysis. Pathway analysis is a functional analysis that maps genes to KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (<http://www.genome.jp/kegg/>). The *P*-value (EASE-score, Fisher *P*-value or Hypergeometric *P*-value) denotes the significance of the pathway correlated to the conditions. Lower *P*-values indicate greater significance of the correlation (recommended *P*-value cut-off is 0.05.)

Quantitative real-time PCR

Total RNA was isolated from P8 and P40 mouse heart tissue using TRIzol (Invitrogen, Carlsbad, CA). The reverse transcription mixture contained 2 μg total RNA, 1 μl oligo(dT), 1 μl 10 mM dNTP mixture, 4 μl 5× buffer, 2 μl 0.1 M DTT, 1 μl M-MLV and an appropriate amount of RNase-free H₂O to a total volume of 20 μl. The reaction was performed at 37°C for 2 min and 37°C for 50 min, followed by heat inactivation at 70°C for 15 min. Quantitative real-time PCR (qPCR) was performed on an ABI StepOne Plus real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) with 1 μl reverse transcription product, 0.25 μl forward

Fig. 1. The expression profiles of lncRNAs and mRNAs were compared between the PDK1 KO group and the paired normal Control group at P40. The scatter plot is a visualization method used for assessing the lncRNA (A) and mRNA (B) expression variations between KO and Control samples. The values of the X and Y axes in the scatter plot are the averaged normalized signal values of the group (log2 scale). The green lines are fold change lines (the default fold change given is 2.0).

Table 1. Relative differential lncRNA and mRNA expression from microarray analysis of P40 cardiac tissue samples

Sample	Long non-coding RNA					
ID		Fold change >2	Fold change>6-10	Fold change >10	Total	Change lncRNA
P40	Upregulation	1916	116	62	2094	4059
	Downregulation	1707	110	148	1965	
Sample	Message RNA					
ID		Fold change >2	Fold change>6-10	Fold change >10	Total	Change lncRNA
P40	Upregulation	973	55	30	1158	3377
	Downregulation	959	140	120	2219	

and reverse primer (Table 4), and 5 μ l SYBR-Green real-time PCR master mix. The PCR cycling parameters were as follows: 95°C for 10 s; 55 cycles of 95°C for 5 s, 60°C for 31 s, 72°C for 20 s; 78°C for 20 s. The level of lncRNA was calculated relative to GAPDH (internal control) using the $2^{-\Delta\Delta C_t}$ method. Six mice samples were used for biological replicates and every sample for three technical replicates. The primers are listed in Table 5.

Statistical analysis

All data were expressed as mean $\pm$ standard deviation. Statistical analysis was performed with the Student's *t*-test for comparison of two groups and analysis of variance (ANOVA) for multiple comparisons. In both cases, differences with $P < 0.05$ were considered statistically significant. The statistical significance of microarray results was analyzed in terms of fold change using the Student's *t*-test. False discovery rate (FDR) was calculated to correct the *P*-value. Fold changes ≥ 2 or >1.5 , or ≤ 0.25 ($P < 0.01$) were used as threshold values used to screen differentially expressed lncRNAs and mRNAs.

Results

lncRNAs are aberrantly expressed in the heart failure group compared with the matched normal group

To study the potential role of lncRNA using the HF mice model, we used microarray analysis to determine the lncRNA and mRNA expression profiles of P40 *pd1* gene KO mice with HF (Fig. 1 A, B). Using the sample pool consisting of more than 31,423 lncRNAs, we first assessed the lncRNA expression profile in heart tissue of the HF group and the matched normal mouse group (Table 1). A total of 4,059 lncRNAs were differentially (fold change >2 ,

Fig. 2. The expression profiles of lncRNAs and mRNAs were compared between KO and Control groups at P8. The scatter plot is a visualization method used for assessing the lncRNA (A) and mRNA (B) expression variations between KO and Control samples. The values of the X and Y axes in the scatter plot are the averaged normalized signal values of the group (log2 scale). The green lines are fold change lines (the default fold change given is 1.5).

Fig. 3. Differential expression of lncRNAs in cardiac tissue samples obtained at postnatal days 8 and 40 was validated by quantitative real-time PCR; (A) P8 and (B) P40. The quantitative real-time RT-PCR reactions were repeated three times for every lncRNA. The level of lncRNA was calculated relative to GAPDH (internal control) using the $2^{-\Delta\Delta C_t}$ method. All reactions were run at least in triplicate. Error bars indicate standard error. * $P < 0.05$, ** $P < 0.01$.

$P < 0.05$) expressed in the KO group compared to the control group. Among these, 2,094 lncRNAs were upregulated (>2-fold) in experimental group compared to the control group, while 1,965 lncRNA were downregulated.

LncRNAs are also differentially expressed in pdk1 gene knockout mouse without heart failure

To investigate the implications of differential lncRNAs expression in HF mice, we analyzed the lncRNA and mRNA expression profiles in those P8 *pdk1* KO mice that did not develop heart failure (Fig. 2A, B). Similar to the data from P40, 2,024 lncRNAs were differentially (fold change >1.5, $P < 0.05$) expressed between the *pdk1* gene knockout and matched normal groups. Among these, 1,224 lncRNA were upregulated (>1.5-fold in experimental group compared to the controls), while 800 lncRNAs were downregulated.

Fig. 4. Comparison of microarray and qPCR data for differentially expressed lncRNAs in cardiac tissue samples obtained at postnatal days 8 and 40; (A) P8 and (B) P40. The heights of the columns in the chart represent the log-transformed median fold changes (KO/Control) in expression. The qPCR results are generally consistent with microarray data.

Fig. 5. Comparison of qPCR data for differentially expressed lncRNAs in cardiac tissue samples between P(8) and P(40). The real-time RT-PCR reactions were repeated three times for every lncRNA. The level of lncRNA was calculated relative to GAPDH (internal control) using the $2^{-\Delta\Delta C_t}$ method. All reactions were run at least in triplicate. Error bars indicate standard error. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Real-time quantitative PCR validation

From the P8 differentially expressed lncRNAs, we randomly selected one downregulated lncRNA (Gm15791) and four upregulated lncRNAs (R74862, AK204442, AK087371 and Lsm3). From the P40 differentially expressed lncRNAs, we also randomly selected two downregulated lncRNA (Gm12660 and AK04367) and four upregulated lncRNA (Uc.92, AK141141, AK089315 and Gm15265) for qPCR validation (Fig. 3A, B). The qPCR and microarray data were in accordance (Fig. 4 A, B).

Compared analysis of lncRNAs microarray and validated of several lncRNAs

To identify the specific lncRNAs that participate in the development of heart failure, we analyzed the microarray data of the two experimental time-points (P8 and P40). A total of 93 lncRNAs were consistently upregulated, while 52 were consistently downregulated. Furthermore, 136 lncRNAs were downregulated at P40 after the upregulation at P8 and 51 lncRNAs were downregulated at P8 before upregulation at P40. Moreover, qPCR revealed five consistently upregulated lncRNAs (UC.378, BC060302, Gm12960, AK037803 and Gm12475), as well as three lncRNAs (Gm15791, AK040557 and MKK7) that were downregulated at P8

Table 2. The RT-PCR results were consistent with the microarray data

		mean fold change	
gene name	regulation	qpcr	chip
P8 Gm15971	down	-19.186	-8.795761
P8 AK040557	down	-1.09886	-0.8860555
P8 MKK7	down	-2.99873	-1.6066321
P8 Gm12960	up	1.234282	1.5142047
P8 AK037803	up	1.686044	1.5943521
P8 Gm12475	up	1.768452	3.0566463
P8 Uc.378	up	1.685338	1.5790685
P8 BC060302	up	2.706037	1.5210909
P40 Gm15971	up	2.759753	3.870869
P40 AK040557	up	3.825017	7.016618
P40 MKK7	down	-1.05613	-1.475715
P40 Gm12960	up	3.543601	4.1962757
P40 AK037803	up	1.515246	1.817615
P40 Gm12475	up	3.485707	2.1022792
P40 Uc.378	up	2.556295	2.35287
P40 BC060302	up	2.739006	2.02075

before being upregulated in P40 (Fig. 5). The results were consistent with the microarray data (Table 2).

Bioinformatics analysis indicates that MAPK and cell adhesion molecule signaling pathways are involved in the pathology of heart failure pathological process

Pathway analysis of the differentially expressed lncRNAs at P40 in the HF group compared to the controls (which have more associated genes and a lower *P*-value, <0.05) revealed that 10 different pathways corresponding to the target genes, the top three being MAPK, cell adhesion molecules and the tight junction signaling pathway (Table 3). Pathway analysis of P8 data showed roughly similar results (Table 4). In both cases, the MAPK and cell adhesion molecule signaling pathways showed the most significant changes.

Discussion

Previous studies have indicated that lncRNAs are involved in many human diseases, such as cancer, HELLP syndrome, brachydactyly syndrome, as well as hypertrophic and dilated cardiomyopathies [25, 34–36, 40, 41]. One study found that ALC-1 antisense mRNA is expressed in human hypertrophic, but not in normal, ventricles. Furthermore, it was shown that higher antisense to sense ALC-1 mRNA ratios are associated with lower ALC-1 protein expression [36]. Subsequently, the Steroid Receptor RNA Activator (SRA) transcript, which is a long non-coding RNA, functions as a coactivator of nuclear receptor signaling, as well as being an important component of gene insulator complexes found in human dilated cardiomyopathy [34, 35]. Recent studies have also suggested that lncRNAs participate in

Table 3. Pathways represented among differentially expressed lncRNAs at P40 (microarray data)

Term	Count	P-value	Genes
MAPK signaling pathway	35	0.04749578	BDNF,BRAF,CACNA2D1,CACNB1,CACNG3,DUSP4,ELK1,FGF13,FGF18,GNA12,GNG12,MAP3K2,MAP3K7,MAPK8IP3,MAPT,NFKB2,NR4A1,NTRK2,PAK2,PLA2G12A,PLA2G4B,PPM1B,PPP3CA,PPP3CB,PPP3R1,PRKCC,PTPN5,PTPRR,RAP1B,RASA1,RASA2,RASGRF1,RASGRP,RRAS2,TAOK2
Tight junction	31	0.000005429382	ACTN3,ASH1L,CDK4,CLDN14,CLDN22,CLDN3,CLDN4,CLDN6,CSDA,CTNNA1,CTTN,EPB4,EPB4.1L1,EXOC4,F11R,GNAI1,GNAI2,INADL,MYH1,MYH9,MYL2,PARD3,PPP2R2B,PRKCC,PTEN,RAB3B,RHOA,RRAS2,SPNB2,TJP1,VAPA
Regulation of actin cytoskeleton	29	0.04497438	ABI2,ACTN3,APC,ARPC5,BRAF,CFL2,CHRM4,DOCK1,FGF13,FGF18,GNA12,GNG12,GRLF1,GSN,IQGAP2,ITGA9,ITGB1,MYH9,MYL2,NCKAP1,PAK2,PFN2,PIK3CB,PIK3CG,PPP1CB,PTK2,RHOA,RRAS2,WASF1
Cell adhesion molecules (CAMs)	28	0.0005315351	CADM3,CD8B1,CLDN14,CLDN22,CLDN3,CLDN4,CLDN6,CNTN1,F11R,GLG1,H2-AA,H2-D1,H2-EB1,H2-M10.3,H2-M10.6,ITGA9,ITGB1,MPZL1,NCAM1,NEGR1,NLGN1,NRXN1,NRXN2,PDCD1,PTPRC,SDC4,SELPLG,VCAN
Calcium signaling pathway	25	0.0440985	CADM3,CD8B1,CLDN14,CLDN2,CLDN3,CLDN4,CLDN6,CNTN1,F11R,GLG1,H2-AA,H2-D1,H2-EB1,H2-M10.3,H2-M10.6,ITGA9,ITGB1,MPZL1,NCAM1,NEGR1,NLGN1,NRXN1,NRXN2,PDCD1,PTPRC,SDC4,SELPLG,VCAN
Wnt signaling pathway	24	0.0148108	CADM3,CD8B1,CLDN14,CLDN22,CLDN3,CLDN4,CLDN6,CNTN1,F11R,GLG1,H2-AA,H2-D1,H2-EB1,H2-M10.3,H2-M10.6,ITGA9,ITGB1,MPZL1,NCAM1,NEGR1,NLGN1,NRXN1,NRXN2,PDCD1,PTPRC,SDC4,SELPLG,VCAN
Spliceosome	23	0.005362725	ACIN1,CDC40,GM5803,HNRNPA1,HNRNPA3,HNRNPu,PRPF3,PRPF38B,PRPF4,PRPF40B,RBM17,RBM22,RBMX,RBMXRT,SF3B1,SMNDC1,SNRNP70,SNW7,SRSF1,SRSF4,SRSF9,THOC4,WBP11
mRNA surveillance pathway	20	0.001131179	ACIN1,CPSF3,CSTF1,DAZAP1,ETF1,FIP1L1,GSPT1,NXT2,PABPC1,PABPC1L,PABPC4L,PNN,PPP1CB,PPP2R2B,PPP2R5C,RNPS1,SMG7,THOC4,UFPF2,WIBG
Toll-like receptor signaling pathway	10	0.04471018	AKT2,CD14,IFNA4,IL1B,IRAK4,MAPK,PIK3CD,PIK3CG,TLR5,TLR7
Inositol phosphate metabolism	7	0.04117666	IMPAD1,INPP5B,IPPK,PIK3C2B,PIK3C2G,PIK3CD,PIK3CG

heart development and disease. For example, *Fenrr*, a tissue-specific lncRNA, is an essential regulator of heart and body wall development in the mouse. *Fenrr* binds to both PRC2 and TrxG/MLL complexes, and acts as a modulator of chromatin signatures that define gene activity [39]. However, the pathogenesis of HF remains unclear, and there are no reports describing lncRNA expression profiles in the *pdk1* gene KO mouse model of heart failure.

In this study, we conducted microarray analysis of the lncRNA expression profile in mice with myocardial-specific *pdk1* gene KO at day P8 and P40. Differential lncRNA expression profiles were detected at the two time-points between the PDK1 KO and matched control groups. Of the 4,059 lncRNAs that were differentially expressed in the PDK1 KO group, only 2,024 lncRNAs were differentially expressed in those without HF, indicating that these lncRNAs may be involved in the initiation and progression of HF. Furthermore, comparison analysis of differential lncRNA expression in the PDK1 KO group at P8 and P40 was conducted to determine which lncRNAs are involved in HF development and progression. We randomly selected a small proportion of the total lncRNAs detected in the six *pdk1* gene knockout heart samples and their paired tissue samples for validation by qPCR. In total, 19 lncRNAs were evaluated by qPCR to validate the consistency of the test. Finally, bioinformatics analysis was conducted to explore the biological function of the identified lncRNA targets in HF progression. Pathway analysis indicated that the MAPK signaling pathway is involved in heart failure progression. Members of the MAPK family are involved in the regulation of a large variety of cellular processes, such as cell growth, differentiation, development, cell cycle progression, as well as cell death and survival. The major groups of MAPKs found in cardiac tissue include the extracellular signal-regulated kinases (ERKs), the stress-activated/c-Jun

Table 4. Pathways represented among differentially expressed lncRNAs at P8 (microarray data)

Term	Count	P-value	Genes
MAPK signaling pathway	13	0.034314	CACNG6,CRKL,DUSP16,DUSP8,EGF,FGF23,NGG12,IKBKG,MYC,NGF,NTF3,PRKACB,RPS6KA2
ErbB signaling pathway	8	0.002668727	CACNG6,CRKL,DUSP16,DUSP8,EGF,FGF23,NGG12,IKBKG,MYC,NGF,NTF3,PRKACB,RPS6KA2
Gap junction	7	0.01065806	ADCY9,EGF,GJD2,PRKACB,SRC,TUBB2A,TUBB2C
PPAR signaling pathway	6	0.02416941	ACADM,ACSL1,CYP4A31,FABP2,HMGCS2,UCP1
Cell adhesion molecules (CAMs)	20	0.000003991549	CADM1,CADM3,CDH5,CLDN10,H2-AAH2-AB1,H2-BL,H2-D1,H2-K1,H2-M3,H2-Q6,H2-Q7,H2-Q8,H2-T10,H2-T22,H2-T23,NCAM1,NLGN2,NRXN2,SELE
Calcium signaling pathway	18	0.0005380389	ATP2B1,ATP2B3,BST1,CACNA1E,CAMK2B,CAMK4,CD38,DRD5,EDNRB,ITPKB,ITPR1,NOS2,P2RX7,PDE1C,PPP3CA,PRKCB,PTAFR,SPHK1
Regulation of actin cytoskeleton	15	0.03746045	BAIAP2,CHRM4,FGD3,FGF20,FGF3,IQGAP2,ITGA10,ITGB6,MAPK1,MAPK3,NCKAP1L,PAK6,PFN3,TIAM2,WASF1
Toll-like receptor signaling pathway	14	0.00008043762	CCL3,CCL4,CCL5,CXCL10,CXCL9,IFNA13,IL12B,IRF3,IRF7,MAPK1,MAPK3,TAB1,TIRAP,TLR5
Endocrine and other factor-Regulative calcium reabsorption	6	0.03007296	AP2S1,ATP2B1,CALB1,CLTC,DNM1,PRKCB

Table 5. LncRNA primers for quantitative real-time PCR analysis

Gene name	Forward (5' - 3')	Reverse (5' - 3')
PDK1	AAAACAAAGTTCCGTATG	TCGTCGTCCTGAAATGTA
GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA
Gm15971	ATACTACCCACCTGACTTTG	CATCAGCCATACCACATAC
R74862	TCTGTCTCCAAAGAACCCTA	AACACCATCAAGCCCACT
AK204442	AACACCATCAAGCCCACT	TAGTTCTTTCCCTTGTGG
AK087371	CTCCGCTTGCTCCTT	CTTCTGCTCCCGTGGT
Lsm3	CGGCCTAAGGTTTCTGAGTT	GGTATTGGTGGTCTGTTGCT
Uc.92	CTTGGTGCGGAGGTTATG	TGATGGACACGGCAGAAG
Gm12660	GGACAAGTGGGTCTAGGTTCA	GGTTGGTCTGGCATCTTTG
AK141141	GACGCAGCTAGATTTCCG	CCATTGCCTGGTTGTTTG
AK043267	CCTGCCTTCTGTTTGGAC	ATTGGTAGCCTTGCTCTG
AK089315	AGGGTTGTGGTTGTAGGG	CTGCGATGATGACGATGA
Gm15265	GGGGTGAGCTGGAAAGGA	CAGGCACAACGGGAACAT
Uc.378	AGACCACCAAAGATTCCC	CCTGAGCCATACCAGAT
BC060302	GCCTCCATTGCAGGAACA	GCTTCACTGGGTCTGGTTGT
Gm12960	GGCAGCAGAGGTAGTTAT	TATCCACCACCCTTCCAT
AK037803	GCGAGCCTTCCAGACAAA	TCTTCGGAGCGATTACAGC
Gm12475	TCGCCATCTCCAGTTCCT	CAGCCTTCTTCATCCACAAA
AK040557	CTGCGATGGTACGAGGAC	CAATCAGTGGCTGCGAAA
MKK7	GGCACCTTCATCACCAC	CAATCGCCACAGTCATCTT

NH2-terminal kinases (SAPK/JNKs), p38-MAPK, and ERK5/big MAPK 1 (BMK1). Activation of the MAPK families in the heart plays a key role in the pathogenesis of various processes, such as myocardial hypertrophy and its transition to heart failure, ischemic and reperfusion injury, as well as cardio-protection conferred by ischemia- or pharmacologically-induced preconditioning [42]. Some previous studies have elucidated the biological role of MAPK signaling cascades during the development of hypertrophy and its transition to heart failure

[43-45]. In our study, biological analysis suggested that the sequence of *mkk7*, a sense overlap lncRNA, stretches from 4238828 to 4251420 on the chromosome 8 sense strand. Furthermore, *map2k7*, a gene in the proximity of *mkk7*, is also on the chromosome 8 sense strand start, and stretches from 4238739 to 4247897, while dual-specific mitogen-activated protein kinase kinase 7 (MKK7), encoded by the *map2k7* gene, is a member of the MAPK family and activates JNK isoforms [46]. Using transgenic mouse models with cardiac-specific and temporally regulated expression of activated mutants MKK7, Scherise and Ota demonstrated that MKK7, along with other specific pathways, contribute specifically to different aspects of HF pathology. [47]. Similarly, Wang et al. showed that expression of both wild-type and constitutive mutants of MKK7, as well as specific activation of the JNK pathway, led to the induction of the hypertrophic responses in the heart [48]. Here, we found that *mkk7* was downregulated ($p < 0.05$) in cardiomyocytes at P8 in the PDK1 KO group compared to the control group; however, this effect did not reach the level of statistical significance at P40. Furthermore, lncRNAs have been shown to regulate target gene expression by a trans-mechanism [30, 31]. Therefore, our data indicate that *mkk7*, a sense overlap lncRNA, may play an important role in the development and progression of heart failure through the MAPK signaling pathway.

We determined that lncRNAs were abnormally expressed in *pd1* gene knockout mice and identified several lncRNAs that potentially participate in the initiation and progression of heart failure. Furthermore, we showed that the regulatory roles of these lncRNAs in the pathogenesis of heart failure may be associated with the MAPK signaling pathways. However, further investigations are required to clarify the exact signaling pathways that utilize lncRNAs to regulate the development of heart failure, and to elucidate the molecular mechanisms and biological functions of lncRNAs in heart failure pathology.

Conflict of Interest

The authors declare no conflict of interest.

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