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Identification of genes related to heart failure using global gene expression profiling of human failing myocardium

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ABSTRACT

Although various management methods have been developed for heart failure, it is necessary to investigate the diagnostic or therapeutic targets of heart failure. Accordingly, we have developed different approaches for managing heart failure by using conventional microarray analyses. We analyzed gene expression profiles of myocardial samples from 12 patients with heart failure and constructed datasets of heart failure-associated genes using clinical parameters such as pulmonary artery pressure (PAP) and ejection fraction (EF). From these 12 genes, we selected four genes with high expression levels in the heart, and examined their novelty by performing a literature-based search. In addition, we included four G-protein-coupled receptor (GPCR)-encoding genes, three enzyme-encoding genes, and one ion-channel protein-encoding gene to identify a drug target for heart failure using *in silico* microarray database. After the *in vitro* functional screening using adenovirus transfections of 12 genes into rat cardiomyocytes, we generated gene-targeting mice of five candidate genes, namely, *MYLK3, GPR37L1, GPR35, MMP23*, and *NBC1*. The results revealed that systolic blood pressure differed significantly between *GPR35*-KO and *GPR35*-WT mice as well as between *GPR37L1*-Tg and *GPR37L1*-KO mice. Further, the heart weight/body weight ratio between *MYLK3*-Tg and *MYLK3*-WT mice and between *GPR37L1*-Tg and *GPR37L1*-KO mice differed significantly. Hence, microarray analysis combined with clinical parameters can be an effective method to identify novel therapeutic targets for the prevention or management of heart failure.

Introduction

Heart failure is a multi-factorial condition with increasing prevalence worldwide; further, a significant increase has been observed in the mortality rate and economic impact associated with this condition. In the last 20 years, substantial development of treatment for heart failure, including angiotensin-converting-enzyme inhibitors [1] and beta-blockers [2,3], has greatly improved the prognosis of the patients with heart failure. However, despite these rapid advancements in the management of heart failure, effective treatment of end-stage heart failure without providing ventricular assistance or heart transplantation is still difficult. Investigation of new and unexplored targets for the prevention or treatment of heart failure is warranted. Global gene expression analysis using microarray technique has been used in the last decade to identify biomarkers or drug targets for heart failure [4–10]. Several gene expression signatures of heart failure have been identified by analyzing independent microarray datasets [11,12]. However, most of these analyses did not consider the severity of heart failure. Because the severity of heart failure may quantitatively reflect the expression levels of genes such as the natriuretic

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peptide-encoding gene, expression analysis combined with the severity of heart failure could be an appropriate method to identify heart failure-related genes. However, microarray analysis of genes expressed in failing myocardium while considering the severity of heart failure has not yet been reported.

Hence, we investigated the genes whose expression level correlated with clinical parameters such as pulmonary artery pressure (PAP), left ventricular ejection fraction (EF), and brain natriuretic peptide (BNP) mRNA level. Using this approach, we identified cardiac myosin light chain kinase as a novel heart failure-related gene [13]. Here, we describe newly identified several genes whose expression correlated with clinical parameters and additional genes encoding G-protein-coupled receptor genes (GPCRs), other enzymes and ion-channel proteins, and performed the functional analysis of these heart failure-related genes. This novel strategy involving the use of clinical parameters might find potential applications for the identification of disease-associated genes that could not be detected using conventional microarray techniques.

Materials and methods

Patient characteristics. We recruited 12 patients (11 males and 1 female) with heart failure and obtained written informed consent from them. The patients were diagnosed with severe chronic heart failure due to various cardiac diseases such as dilated cardiomyopathy and myocardial infarction [13]. The average age of patients was 55 ± 13 years. The plasma level of BNP, which is the best marker for the severity of heart failure, ranged from 80 to 2710 pg/ml. The mean PAP measured using a Swan-Ganz catheter 1–4 weeks before the operation varied between 16 and 59 mmHg. The average of EF determined by echocardiography on the day before the operation was 32.5% ± 12.4%.

Microarray analysis and subsequent in silico functional analysis. RNA was extracted from myocardium samples of 12 heart failure patients who had undergone either Batista or Dor surgeries. RNA samples of non-failing hearts were purchased from Biochain, Inc. Complementary RNA (cRNA) was prepared from RNA samples and hybridized to HG-U95 Affymetrix GeneChip (Affymetrix, US). The expression data were analyzed using Microarray Analysis Suite version 5.0 software. Among all the genes detected on the microarray, we selected the genes whose expression was significantly different in the failing and non-failing myocardial samples ($p < 0.005$). From these genes, we selected genes whose expression was correlated with PAP, EF, and BNP mRNA level, with 0.7 being the cutoff value of the correlation coefficient. The values of PAP, EF, and BNP mRNA level used for the correlation analysis were normalized to their median during the measurements. Subsequently, the functional analysis of datasets was performed using Ingenuity Pathway Analysis (Ingenuity® Systems; www.ingenuity.com), and the biological functions most significant to the dataset were identified.

Cell culture. Cardiomyocytes were harvested before the experiments from 2- to 3-day-old neonatal rats and cultured as described in previous studies [14]. Briefly, primary cardiomyocytes isolated from neonatal rats were grown in Dulbecco's modified Eagle medium/F12 (Gibco) supplemented with 10% fetal calf serum for 72 h, and then cultured in a serum-free condition for 24 h.

Adenovirus generation and transfection. Adenovirus constructs encoding the genes of interest were generated using the ViraPower Adenoviral Expression System (Invitrogen, US) according to the manufacturer's method. Adenovirus vectors were transfected to cultured cardiomyocytes for 12 h according to the published protocol.

In vitro functional analysis of genes. Cultured rat cardiomyocytes were infected by adenovirus vectors. After 24 h, hypertrophic reaction, cell viability, and cellular morphology were assessed. Hypertrophic reaction was determined by estimating the incorporation of [^{3}H]phenylalanine. In brief, [^{3}H]phenylalanine was added to the culture medium at the final concentration of 0.1 μCi/ml, and the cells were incubated for an additional 24 h. Then, the incorporation of [^{3}H]phenylalanine was determined by counting the radioactivity of each sample with a liquid scintillation counter. The viability of cardiomyocytes was evaluated by the Alamar blue assay according to the manufacturer's method. The morphology of cardiomyocytes was evaluated 24 h after adenovirus transfections.

Generation of transgenic and knockout mice. To generate transgenic mice, open reading frame of each gene, namely, *Mylk3*, *Gpr37l1*, or *Nbc1* was amplified from mouse cDNA by PCR, with Sal I site linker on each end, and cloned into Sal I site of alpha-MHC clone 26 vector. Then the DNAs used in the microinjections were released from the vector by digestion with NotI and were microinjected into fertilized eggs of mouse. Founder mice were identified by PCR analysis with appropriate primers. To develop *Gpr37l1* knockout mice, the targeting vector was assembled to replace the exon 1 and 2 by neomycin selection cassete resulting in the absence of *Gpr37l1* protein. W9.5 ES cells were electroporated with linearized targeting vector. ES cell clones with successful homologous recombination was determined by the PCR and subsequent direct sequence. From these targeted ES cells, the chimera mice were bred to C57 BL/6 females to generated F1 and F2 offsprings were obtained. The *Gpr37l1* null mice were determined by PCR genotyping of F2 offsprings. The knockout mice of *Gpr35* and *Mmp23* (*the* mouse ortholog of MMP23B) were purchased from Deltagen, Inc. (California, US).

Invasive blood pressure measurement. The phenotype of the gene-targeted mice was examined. Before sacrificing the mice, their hemodynamic parameters were evaluated. The mice were anesthetized and ventilated, and a Millar catheter was inserted via right carotid artery. The left-ventricular systolic and end-diastolic pressures were measured. Then, the mice were sacrificed and the weight of the whole body and heart was determined.

Statistical analysis. Unpaired Student's *t*-test was used for comparing the two groups. Results are expressed as means ± SEM, and *p* value less than 0.05 was considered statistically significant.

Results

Identification of heart failure-related genes by expression analysis using clinical parameters

We performed microarray analysis of the genes expressed in failing myocardium obtained from 12 patients with heart failure and the genes expressed in non-failing myocardium from two normal objects whose characteristics were reported in the previous study [13]. Although all patients were diagnosed with chronic heart failure, the plasma BNP level, which is an index of the severity of heart failure, ranged from 80 to 2710 pg/ml, suggesting that the severity of heart failure varied extensively among the patients. This marked difference in the severity of heart failure reflects the fact that the gene expression patterns in the 12 patients were not uniform, as shown in Fig. 1A. Thus, we analyzed gene expression profiles of failing myocardium using clinical parameters representing the severity of heart failure. We identified 166 and 194 genes whose expressions were correlated with PAP and BNP mRNA level, respectively (Fig. 1B and Supplementary Tables S1, S2). Among these, 49 genes correlated with both PAP and BNP mRNA level (Fig. 1C). The expression of only two genes, namely, *FMO2* and *LMAN1L*, correlated with the EF. We investigated the functional categories of these genes by performing Ingenuity Pathway Analysis. The number of genes in each group, functional categories, and

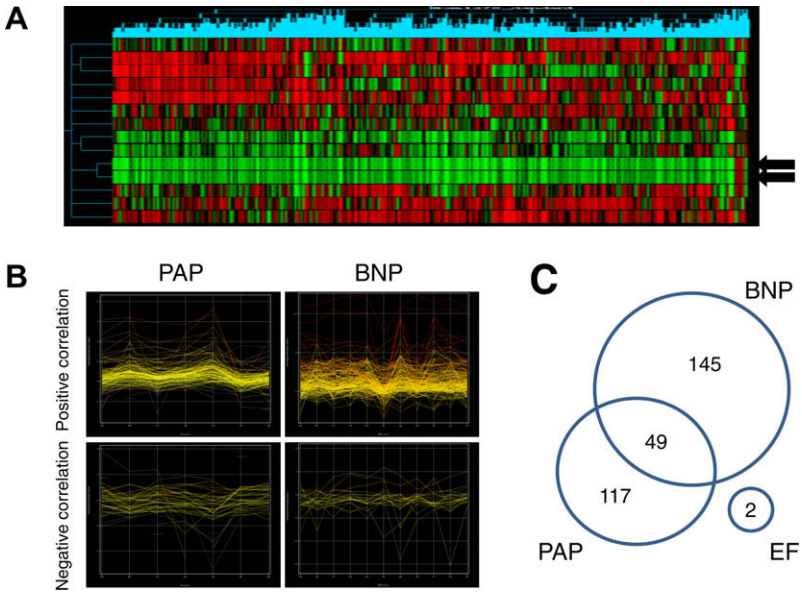

Fig. 1. The gene expression profile of human failing or non-failing myocardium. Gene expression levels of myocardial samples from 12 patients with severe heart failure and from two normals were analyzed using microarray. (A) Heat maps showing the genes with differential expression between the 12 failing myocardial samples and the two non-failing myocardial samples. Red color indicates upregulated gene expression. Green color indicates downregulated gene expression. Arrows indicate non-failing samples. (B) Expression profile of positively or negatively correlated genes to pulmonary artery pressure (PAP) or brain natriuretic peptide (BNP) mRNA level ($r > 0.7$). (C) Venn diagram of genes correlated with PAP, BNP, and ejection fraction.

Table 1
Datasets of genes whose expressions were correlated to clinical parameters.

	PAP	EF	BNP mRNA level
Positive correlation			
Number	124	1	175
Function	Cardiovascular system development and function Cell death	–	Cardiovascular system development and function Cell cycle
Representative genes	*ARNT*, *MYOCD*, *SMARCA4* *BGN*, *CFLAR*, *EEF2*, *MTPN*	*LMAN1L*	*BTG1*, *NPPA*, *NPPB*, *SERPNF1* *CKS1B*, *DDR2*, *FCGR2B*, *FN1*
Negative correlation			
Number	42	1	19
Function	Skeletal and muscular system development and function Cellular assembly and organization	–	Skeletal and muscular system development and function Cellular assembly and organization
Representative genes	*PIK3R1*, *PRKAR1A*, *SLMAP* *C19ORF20*, *RAB9A*, *SYNGAP1*, *TTN*	*FMO2*	*ACTC1*, *RBBP4*, *TTN*

The function of gene sets was analyzed by Ingenuity Pathway Analysis.
PAP, pulmonary artery pressure; EF, ejection fraction; BNP, brain natriuretic peptide.

representative genes are shown in Table 1. Interestingly, both gene sets correlated positively with PAP and BNP mRNA level were most associated with the same functional category of "cardiovascular system development and function", although the included genes were different. Similarly, the gene sets correlated negatively with both PAP and BNP mRNA level had most association with common functional categories of "skeletal and muscular system development and function" and "cellular assembly and organization".

Selection of 12 genes for in vitro screening

Among the genes selected using clinical parameters, we selected those genes that showed high expression levels in the heart by performing microarray analysis. On the basis of their novelty determined by a literature-based search, we selected four genes for further investigation (Table 2). Concurrently, to identify possible drug targets, we included four orphan GPCRs and four additional genes (three enzyme-encoding genes and one ion-channel protein-encoding gene) in the further analysis. The *RHOQ* and *STK38* genes were selected based on their correlation with BNP mRNA level and PAP, respectively. *GPR161* and *NBC1* were selected owing to their high expression level in the heart. *GPR37L1*, *GPR35*, *F2RL2*, and *MMP23B* were selected because of their high expression level in the heart, and their association with the cardiac diseases-related genes listed in the database was determined by *in silico* analysis.

Functional analysis of genes on the basis of adenovirus-mediated overexpression of proteins in neonatal rat cardiomyocytes

To determine which of the selected genes were associated with the physiological functions of the heart, we first generated adenovirus vectors for each gene listed in Table 2 and transfected these vectors into neonatal rat cardiomyocytes. Next, we evaluated the hypertrophic reaction, viability, and morphology of the transfected cardiomyocytes. Among the 12 selected genes, three adenovirus-mediated genes decreased the incorporation of [^{3}H]phenylalanine in neonatal rat cardiomyocytes (Table 2); the expression of one

Table 2
In vitro functional screening of the 12 candidate genes.

Probe set ID	Gene symbol	Gene name	Criteria for selection	*p* value	[^{3}H]PA intake	Fluorescence of Alamar blue	Cellular morphology
Genes relevant to clinical parameters							
75678_at	*MYLK3*	Myosin light chain kinase 3	Correlation with PAP (r = 0.792)	0.00262	No change	No change	Spiking
49333_at	*XPR1*	Xenotropic and polytropic retrovirus receptor	Correlation with PAP (r = 0.765), GPCR, change in CHF	0.00045	No change	No change	No change
38435_at	*PRDX4*	Peroxiredoxin 4	Correlation with BNP (r = 0.863)	0.00024	Increased	Decreased	No change
45314_at	*SMOC2*	SPARC related modular calcium binding 2	Correlation with both PAP and BNP (r = 0.715 and 0.758, respectively)	0.00444	No change	No change	No change
Genes encoding orphan GPCRs							
35544_at	*GPR37L1*	G-protein-coupled receptor 37 like 1	Orphan GPCR, downregulated in CVD	>0.005	Decreased	Decreased	Apoptosis
31700_at	*GPR35*	G-protein-coupled receptor 35	Orphan GPCR, upregulated in MI	0.00216	Decreased	Decreased	Hypertrophy
45204_at	*F2RL2*	Coagulation factor II (thrombin) receptor-like 2	GPCR, change in CVD	>0.005	Increased	No change	No change
40299_at	*GPR161*	G-protein-coupled receptor 161	GPCR, expression in heart	>0.005	Decreased	Decreased	No change
Genes encoding interesting enzymes or ion-channels							
38950_at	*MMP23B*	Matrix metallopeptidase 23B	Family of MMP, change in CHF	>0.005	No change	Decreased	No change
35285_at	*NBC1*	Na^+–HCO^{3-} cotransporter 1	Expression in heart	>0.005	No change	Decreased	No change
87788_at	*RHOQ*	Ras homolog gene family, member Q	Expression in DCM, correlation with BNP (r = 0.711)	>0.005	No change	No change	No change
78801_at	*STK38*	Serine/threonine kinase 38	Kinase activity, correlation with PAP (r = 0.736)	>0.005	No change	No change	No change

PAP, pulmonary artery pressure; GPCR, G-protein-coupled receptor; CHF, congestive heart failure; BNP, brain natriuretic peptide; CVD, cardiovascular disease; MI, myocardial infarction; DCM, dilated cardiomyopathy; PA, phenylalanine. *p* value indicates the significance of the difference between the gene expression level of failing and non-failing myocardium.

gene promoted [^{3}H]phenylalanine incorporation; and the overexpression of six genes lowered the viability of cardiomyocytes, which was evaluated by Alamar blue assay. We also evaluated the phenotype of transfected cardiomyocytes. Unlike control cells, *MYLK3*-adenovirus-transfected cardiomyocytes were spike shaped. The overexpression of *GPR37L1* induced apoptosis of cardiomyocytes. The transfection of *NBC1*-adenoviral vectors modified the beating rate of cardiomyocytes (data not shown). Then, we analyzed each gene that encoded a distinct cardiomyocyte phenotype by developing gene-targeted mouse models.

In vivo analysis using transgenic and knockout mice

To study the *in vivo* role of the selected genes, we developed genetically modified mice: three transgenic (Tg) mice for *Mylk3*, *Gpr37l1*, or *Nbc1* and three knockout (KO) mice for *Gpr37l1*, *Gpr35*, or *Mmp23*. We estimated hemodynamic parameters using Miller catheter and the heart weight (HW)/body weight (BW). As shown in Fig. 2A, we found that the blood pressure of *Gpr37l1*-KO mice was significantly higher than that of *Gpr37l1*-Tg mice by 61.7 mmHg ($p < 0.01$). Further, the blood pressure of *Gpr35*-KO mice was higher than that of wild type (WT) littermate by 37.5 mmHg ($p < 0.01$). Overexpression with or knockout of *Mylk3*, *Mmp23*, or *Nbc1* did not result in a significant change in the systolic blood pressure. The HW/BW of *Mylk3*-Tg mice was lower than that of *Mylk3*-WT mice (Fig. 2B). The HW/BW was higher in *Gpr37l1*-KO mice than in *Gpr37l1*-Tg mice. The HW/BW in mice with *Nbc1*, *Gpr35*, or *Mmp23* manipulations did not show any difference. These data showed that modification of *Gpr37l1*, *Gpr35*, or *Mylk3* can produce a distinct cardiovascular phenotype *in vivo*.

Discussion

The present study identified heart failure-related genes using a novel strategy that was different from the conventional microarray analysis approach. Firstly, we constructed global gene expression profiles to analyze the gene expression in 12 human samples of failing myocardium and two samples of non-failing myocardium. Secondly, we prepared datasets of heart failure-related genes associated with the severity of heart failure; this approach is unique to our study and has not been published before. Thirdly, we selected four genes from these datasets by microarray analysis and a literature-based search. We also included four orphan GPCR genes and four other genes with high expression in the heart as possible drug targets for heart failure treatment. Fourthly, we screened the *in vitro* functions of these 12 genes by achieving adenovirus-mediated overexpression of these genes in rat cardiomyocytes. Finally, we generated gene-targeted mouse models of the five selected genes and screened the *in vivo* functions of these genes. Our novel strategy using a microarray analysis revealed three potential targets, namely, *MYLK3*, *GPR37L1*, and *GPR35* for diagnosing and managing heart failure.

End-stage heart failure caused by a variety of cardiovascular diseases including hypertension, cardiomyopathy, and ischemic heart disease features a common phenotype of reduced cardiac function and dilated cardiac chamber. This result strongly suggested the existence of common genes during the development of heart failure, including the genes encoding natriuretic peptides. To identify novel diagnostic or therapeutic targets for heart failure, such as natriuretic peptides, several microarray analyses of genes expressed in failing myocardium have been performed in the last decade by comparing the gene expression levels between different pairs of samples, such as non-failing versus failing hearts [4–6], failing hearts before versus after placement of left-ventricular assisting device [7,8], hypertrophic versus failing hearts [9], ischemic versus non-ischemic hearts [10]. However, the severity of heart failure is not fixed, but varies from mild to severe heart failure in these studies. To identify the therapeutic targets for heart failure effectively, we believe that it is important to consider the severity of heart failure with microarray data analysis. In this study, we prepared new datasets of heart failure-associated genes that were selected from gene expression profiles of 12 human failing myocardial samples using clinical parameters. A number of genes were associated with PAP, which is an index for the severity of heart failure, whereas only two genes correlated with EF, which is an index for cardiac contractility. This result implies that the stress caused to the heart, and not the ability of cardiac contraction, regulates gene expression in heart failure. We also selected heart failure-related genes whose expression correlated to

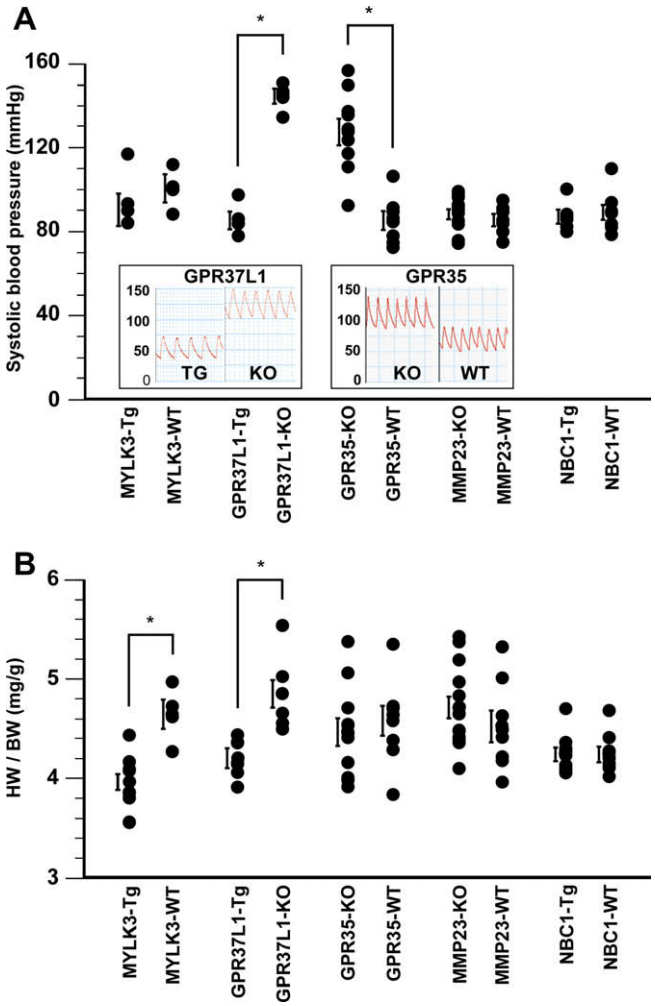

Fig. 2. *In vivo* functional analysis using gene-targeting mice of the *Mylk3*, *Gpr37l1*, *Gpr35*, *Mmp23*, and *Nbc1* genes. Blood pressure and heart weight (HW)/body weight (BW) of transgenic (Tg), knockout (KO) and their wild type (WT) littermate mice of each gene were investigated. Values are means ± SEM. $^{*}p < 0.01$. (A) Systolic blood pressure measured using Millar catheter inserted via right carotid artery. The monitoring chart shows representative data of *Gpr37l1*- and *Gpr35*-manipulated mice. (B) HW/BW ratio of each gene-targeting mouse.

the BNP mRNA level, which is the best known indicator of heart failure. The approach used in our study can help in efficient identification of the diagnostic or therapeutic targets for heart failure rather than only comparing two types of samples such as failing versus non-failing myocardium. Among the genes from these new datasets, we focused on the genes exhibiting high expression in heart tissues and finally selected four genes for performing the screening of functional analysis *in vitro*. The expression level of *MYLK3* gene was highly correlated to PAP, and this gene was detected only in the heart tissue. Recently, we reported that *MYLK3* plays a crucial role in sarcomere assembly via phosphorylation of myosin regulatory light chain 2V (MLC2v) [13]. We also showed that the knockdown of *MYLK3* by using a morpholino oligo caused immature sarcomere formation leading to ventricular dilation in zebrafish. These results indicate that *MYLK3* is strongly associated with the pathophysiology of heart failure. Chan et al. also reported that MYLK3 phosphorylates MLC2v and regulates sarcomere organization [15]. These reports affirm the reliability of our original strategy that involves the microarray analysis of failing myocardium. Among these genes, most genes including *XPR1*, *PRDX4*, and *SMOC2* have not been reported to link with cardiovascular phenotypes and were not included in many gene expression profiles published previously.

Next, we performed *in vivo* functional analysis of five selected genes, and we found that gene-targeted mouse models of *Mylk3*, *Gpr37l1*, or *Gpr35* showed the cardiovascular phenotype. As described above, *Mylk3* plays a crucial role in failing heart. In this study, we identified two GPCRs, namely, *Gpr37l1* and *Gpr35*, whose modification affects systolic blood pressure or HW/BW. To our knowledge, this is the first report about the role of these genes in cardiovascular system.

GPCRs constitute one of the largest protein families, but many GPCRs remain to be orphaned. GPR35 is now known to have some ligands such as kynurenic acid (KYNA) [16], zaprinast [17], and 5-nitro-2-(3-phenylpropylamino) benzoic acid [18]. These agonists mobilize intracellular calcium concentration. Therefore, lowering systolic blood pressure in *Gpr35*-KO mice can be induced by modulating calcium release from calcium-storing organelles. Among the three agonists, only KYNA is produced endogenously as a metabolite of tryptophan. Although GPR35 gene expression is supposed to be specific to immune cells and gastrointestinal tract, we found that GPR35 gene expression increased in failing myocardium. In an inflammatory state, interferon γ induces idolemine 2,3-dioxygenase, a rate-limiting enzyme involved in tryptophan degradation, resulting in a substantial increase in KYNA. Inflammation is thought to be involved in the pathogenesis of dilated cardiomyopathy as well as myocardial infarction. Hence there is a possibility that a KYNA-GPR35 signaling plays a role in the pathogenesis of cardiovascular diseases.

Unlike GPR35, GPR37L1 is still orphaned. However, we found that *Gpr37l1*-KO mice showed significant high blood pressure and high HW/BW as compared to Tg mice, which implies the existence of cardiovascular-related function of *Gpr37l1*. Identification of the ligand and the function of this orphan receptor are awaited.

Although no significant phenotype was observed in *Mmp23* and *Nbc1*-Tg mice, we have been investigating their cardiac function in pathological condition such as myocardial infarction or hypertension and determined their detrimental effect on heart failure (data not shown).

In the present study, we determined 12 novel heart failure-related genes by integrating an original method with parameters that indicated disease severity. Further, we assessed these possible targets of drug discovery. *MYLK3*, *GPR37L1*, and *GPR35* were the newly identified targets that play an interesting role in the cardiovascular system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.01.076.

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