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ORIGINAL PAPER

Identification and visualization of stimulus-specific transcriptional activity in cardiac hypertrophy in mice

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Abstract Identification of specific signaling pathways for cardiac hypertrophy in living animals is challenging because no methods have been established to directly observe sequential molecular signaling events at the transcriptional level during pathogenesis. Here, our aim was to develop a useful method for monitoring the specific signaling pathways involved in the development of cardiac hypertrophy in vivo. Expression profiling of the left ventricle by microarray was performed in 2 different mouse models of cardiac hypertrophy: mechanical pressure overload by transverse aortic constriction (TAC) and neurohumoral activation by angiotensin II (Ang II) infusion. To annotate the information on transcription factor-binding sites, we collected promoter sequences and identified significantly frequent transcription factor-binding sites in the promoter regions of coregulated genes from both models ($P < 0.05$, binomial probability). Finally, we injected a firefly luciferase vector plasmid containing each transcription factor-binding site into the left ventricle in both models. In the TAC and Ang II models, we selected 379 and 12 upregulated genes, respectively. Twenty binding sites for transcription factors, including activator protein 4, were identified in the TAC model, and 4 sites for transcription factors, including ecotropic viral integration 1, were identified in the Ang II model. GATA-binding sites were noted in both models of cardiac hypertrophy. Using the firefly luciferase reporter, we demonstrated the enhancement of transcriptional activity during the progression of cardiac hypertrophy using in vivo imaging in live mice. These results suggested that our approach was useful for the identification of unique transcription factors that characterize different models of cardiac hypertrophy in vivo.

Keywords In vivo imaging · Transcriptome · Hypertrophy

Introduction

Heart failure still remains one of the most frequent causes of death in industrialized countries [1]. Although early detection and intervention have been shown to improve prognosis, it is difficult to identify the molecular and genetic mechanisms of heart failure because of its complexity and similarity of clinical manifestations (e.g., cardiac dilatation and impaired cardiac contractility).

An initial cellular change in the pathogenesis of heart failure is cardiomyocyte hypertrophy, characterized by increased cell size, enhanced protein synthesis, and reactivation of fetal genes [2, 3]. In addition to mechanical stresses, several neurohumoral factors have been identified as potent hypertrophic agents, including angiotensin II (Ang II), endothelin, and catecholamines [4–6]. Because sustained activation of different signaling pathways commonly leads to cardiac hypertrophy, it is important to identify the specific signaling pathways that cause cardiac hypertrophy before the development of heart failure for

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individual risk stratification. However, no useful methods are currently available to monitor these sequential cardiac signaling pathways at the transcriptional level in vivo.

Microarray technology has enabled us to assay genes involved in cardiac hypertrophy and human heart failure on a broader and genomic scale [7, 8]; however, it may not be simple to identify key signaling molecules from a list of putative differentially expressed genes. Therefore, in the present study, we aimed to identify key transcription factors involved in cardiac hypertrophy by microarray analysis of promoter sequences of coregulated genes in mouse models of cardiac hypertrophy with different etiologies: mechanical pressure overload by transverse aortic constriction (TAC) and neurohumoral activation by Ang II infusion. In addition, since sequence-specific transcription factors are known to interact with co-activators to enhance their transcriptional activity [9], we focused on transcription factor-binding sites rather than transcription factors per se as the surrogate marker during the development of hypertrophy. We also aimed to follow the time course of activation of such transcription factors in live animals using a bioluminescence-based in vivo imaging system [10].

Experimental procedures

Animal preparation

Balb/c mice (6–8 weeks of age, both genders) were used in this study. They were housed individually in the research animal facility at Tohoku University. All experimental procedures were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals of Tohoku University.

TAC model

Transverse aortic constriction was performed as previously reported [11]. In brief, mice were anesthetized with pentobarbital sodium [15 mg/kg, intraperitoneal (IP) injection] and ventilated with a mouse ventilator. The transverse aorta was constricted at the upper left sternal border by ligation with a 7-silk surgical thread and 27-gauge needle, which was removed thereafter. Sham-operated controls underwent an identical procedure without TAC. At 2 weeks after the procedure, the pressure gradient between the right and left carotid arteries was measured with a polyethylene tube connected to a pressure transducer to validate the model [11].

Ang II model

Subcutaneous Ang II infusion was performed in mice as previously reported [12]. In brief, under general anesthesia with pentobarbital sodium (15 mg/kg, IP), an osmotic minipump (model 2002; Alzet, Colorado City, CO, USA) was implanted subcutaneously, slightly posterior to the scapula. Ang II was dissolved in 0.9 % NaCl at concentrations sufficient to allow an infusion rate of 2.0 mg/kg/day, known to produce hypertension and cardiac hypertrophy [12]. Control mice received a vehicle (saline) via an osmotic minipump.

Hemodynamic measurements

Systolic and diastolic blood pressures and heart rate were measured every other day after TAC or Ang II minipump implantation for 15 days by a noninvasive computerized tail-cuff system (MK-2000, Muromachi Kikai, Tokyo, Japan). After 2 weeks, the weights of the whole heart and the left ventricle (LV) were measured, and the LV was harvested. For gene-chip analysis, RNA was extracted from the LV at 1 week after TAC or Ang II minipump implantation.

Plasmid construction

Three DNA fragments containing the target transcription factor-binding sites were ligated to firefly luciferase reporter vector pGL3-Basic (Promega, Madison, WI, USA) at BglII or SmaI restriction sites. The sequences of the 3 fragments were as follows: GATA, 5′-CAGATAGAGAAA TCAGATAGAGAAATCAGATAGAGAAATCAGATAG AGAAATCAGATAGAGAAATCAGATAGAG-3′; activator protein 4 (AP4), 5′-AGATCTCAGCTGCAGCTGC AGCTGCAGCTGAGATCT-3′; ecotropic viral integration 1 (Evi1), 5′-GACAAGATAAGTAAGACAAGACAAGTA AGATAAGATAAGTAAGATAAGACAAGTAA-3′.

Gene-chip preparation and analysis

One week after TAC or Ang II minipump implantation, total RNA was extracted from the LV using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA integrity and purity were electrophoretically verified by ethidium bromide staining and measurement of the optical density (OD) 260/OD280 absorption ratio. Next, 5 μg RNA was reverse-transcribed to double-stranded cDNA, and labeled cRNA was then synthesized by incubation of cDNA with biotin-labeled ribonucleotides. Fifteen micrograms cRNA was hybridized to the mouse genome M430 2.0 Array (Affymetrix, Santa Clara, CA, USA) for 16 h, and the array was then scanned for signal intensity. The arrays were washed and stained with streptavidin–phycoerythrin and scanned with an Affymetrix GeneChip Scanner 3000. Array background, Q values, and mean intensities were within the acceptable ranges for all the

arrays. Raw data was first analyzed using GeneChip Operating Software (GCOS). Further analysis was carried out using GeneSpring version 7.2 (Silicon Genetics, Redwood City, CA, USA). For normalization, data measurements <0.01 were set to 0.01, and each measurement was divided by the 50th percentile of all measurements in that sample. We filtered data using a signal confidence ('present' flag) and extracted genes that were upregulated in hypertrophic models by more than twofold compared with controls. In addition, upregulated genes with raw values <100 (low expression) and within a standard deviation of $+5$ from the mean expression level of each gene in 4 controls were excluded. Microarray data are available at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE26671.

Identification of specific frequent transcription factor-binding sites

We collected the promoter sequences of 18,218 mouse transcripts from DBTSS (http://dbtss.hgc.jp/) [13] and identified transcriptional sites on the promoter sequences with MOTIF (http://motif.genome.jp/) by using TRANSFAC (http://www.biobase-international. com/) [14]. We calculated the frequency of each transcription factor-binding site in the promoter sequences as the number of promoter regions containing the transcription factor-binding site among a set of genes divided by the total number of genes in that set. The frequency of each transcription factor-binding site in the promoter regions was statistically compared between a set of upregulated genes in each model and all sets of genes in that model by calculating binomial probability with R version 2.7 (http://www.r-project.org/).

In vivo imaging

Under general anesthesia with pentobarbital sodium (15 mg/kg, IP), we directly injected 5 μg of plasmid DNA in 30 μL PBS into the LV of beating hearts without thoracotomy; the expression of the reporter gene in cardiac myocytes was confirmed as previously described [15]. Two days after the DNA injection, the animals were anesthetized with 2 % inhaled isoflurane, and luciferin was intraperitoneally administered at a dose of 150 mg/kg. Cardiac luciferase activity was assessed by the intensity of light emitted from the heart with an in vivo imaging system (IVIS100, Xenogen, Alameda, CA, USA) for 30 min after the injection of luciferin [16]. The peak signal from the region of interest in the left thorax was subtracted from that in the right lower abdomen. Imaging parameters were as follows: field, 10 cm; exposure time, 2 min; number of binnings, 8; and f1/stop, 1. For display, the luminescent image (pseudocolor) was overlaid on a photographic image that showed the anatomic landmarks. Two days after injection of the DNA plasmid (day 0), the animals, whose hearts emitted bioluminescence, underwent TAC or implantation of an Ang II minipump (2.0 mg/kg/day). Some animals also underwent implantation of a vehicle (saline) infusion minipump as a sham operation. The imaging and measurement of hemodynamic variables were performed every other day for 15 days (day 1–15). The relative cardiac transcriptional activity was expressed as the ratio of the activity on each day to that on day 0 (Fig. 1).

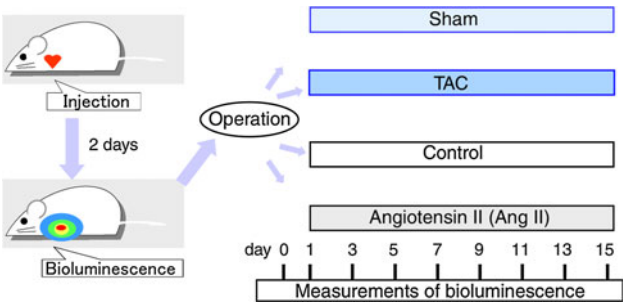

Fig. 1 Protocol of the bioluminescence measurements for each model

Statistical analysis

The results are presented as the mean ± SEM. Hemodynamic variables and relative cardiac transcriptional activity were analyzed with 2-way repeated-measures ANOVA followed by Tukey's honestly significant difference (HSD) post-test for comparisons of serial blood pressure measurements among the groups. Binomial probability was calculated to identify significantly frequent transcription factor-binding sites in the promoter regions of the coregulated genes of both models. Differences with P values of <0.05 were considered to be statistically significant.

Results

LV hypertrophy

At 2 weeks after TAC or Ang II minipump implantation, the ratio of the LV weight to body weight significantly increased in both models compared with the control and sham-operated groups (Table 1). This was similar to the results of previous studies [11]. Mean blood pressure was significantly higher only in the Ang II-infused group and was slightly decreased in the TAC group throughout the experimental period (Fig. 2). In contrast, heart rate remained unchanged in all groups (see Supplementary Figure).

Table 1 Basic characteristics of the cardiac hypertrophy model mice

	Sham	TAC	Control	Ang II
N	23	37	23	27
Body weight (g)	18.6 ± 0.4	18.2 ± 0.5	18.6 ± 0.4	18.1 ± 0.6
LV weight (mg)	65.1 ± 0.4	89.2 ± 0.5*	63.2 ± 0.5	85.7 ± 0.7*
LV/BW weight ratio (10^{-3})	3.5 ± 0.4	4.9 ± 0.5*	3.4 ± 0.5	4.7 ± 0.6*

Results are expressed as the mean ± SEM

TAC transverse aortic constriction, *Sham* sham operation, *Ang II* angiotensin II, *LV* left ventricle, *Control* control without angiotensin II infusion

* $P < 0.01$, TAC versus sham, Ang II versus control

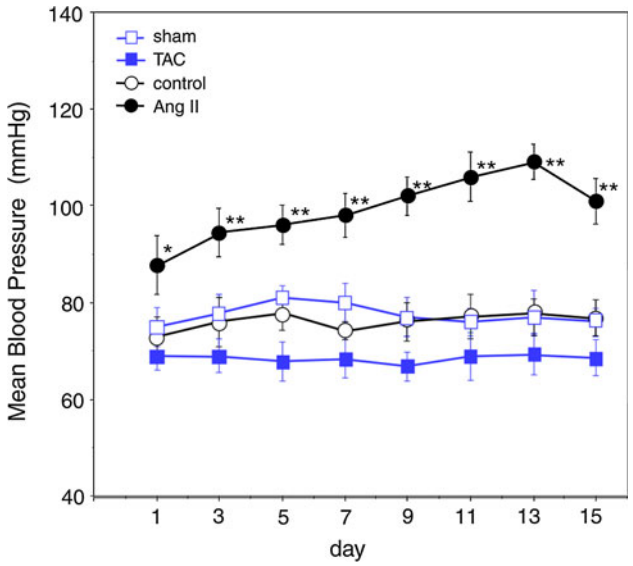

Fig. 2 Time-course of mean systemic arterial pressure: Ang II-treated mice had a significantly higher mean blood pressure than controls. In contrast, TAC mice showed a slightly reduced mean blood pressure. *$P < 0.05$ Ang II versus control. **$P < 0.01$ Ang II versus control

Transcriptional analysis

Next, we aimed to identify transcriptional signaling pathways involved in the development of cardiac hypertrophy. Therefore, since cardiac hypertrophy was fully apparent at 2 weeks after TAC or Ang II minipump implantation, we extracted RNA from the LV in both models at 1 week after these procedures in order to detect differences in gene expression between the models. Expression profiling by gene-chip microarrays was subsequently performed. Of the 45,101 probe sets on the chips, 24,428 and 23,510 had flag calls of "present" in the TAC and Ang II models, respectively. Among them, 1,005 genes in the TAC model and 706 in the Ang II model were upregulated by more than twofold. To exclude false-positive results, we filtered these genes with a 5-standard deviation excess from the mean expression level in the controls, resulting in the selection of 379 genes in the TAC model and 12 genes in the Ang II model (see Supplementary Table).

Table 2 Frequent transcriptional binding sites in the promoter sequence of the gene sets in both models

TRANSFAC PWM	Transcription factor	*P* value
TAC model		
M00189	Activator protein 2	<0.0001
M00008	Stimulating protein 1	<0.0001
M00196	Stimulating protein 1	0.0003
M00050	E2F	0.0004
M00255	GC box elements	0.0016
M00024	E2F	0.0055
M00180	E2F	0.0061
M00005	Activator protein 4	0.0077
M00072	CP2	0.0126
M00084	MZF-1	0.0170
M00227	v-Myb	0.0227
M00004	c-Myb	0.0234
M00053	c-Rel	0.0266
M00083	MZF1	0.0286
M00076	GATA	0.0348
M00051	NF-κB (p50)	0.0350
M00175	Activator protein 4	0.0357
M00287	Nuclear factor Y	0.0362
M00252	Cellular TATA box elements	0.0380
M00183	c-Myb	0.0452
Ang II model		
M00158	HNF4	0.0148
M00126	GATA	0.0311
M00080	EVI1	0.0414
M00128	GATA	0.0437

To identify putative transcription factor-binding sites specific to each cardiac hypertrophy model, we collected 1,500 bp of the promoter sequences of 18,218 murine genes from DBTSS. The binding sites were searched by MOTIF on the basis of the information derived from TRANSFAC. By comparison of the selected coregulated genes in each model with all genes whose promoter sequences were available in DBTSS, we identified significantly frequent transcription factor-binding sites overrepresented in the promoter regions of the coregulated genes for cardiac hypertrophy (Table 2). GATA-binding sites were observed in both models of cardiac hypertrophy. Twenty overlapping binding sites, including those for AP4 and specificity protein-1 (SP-1), were extracted in the TAC

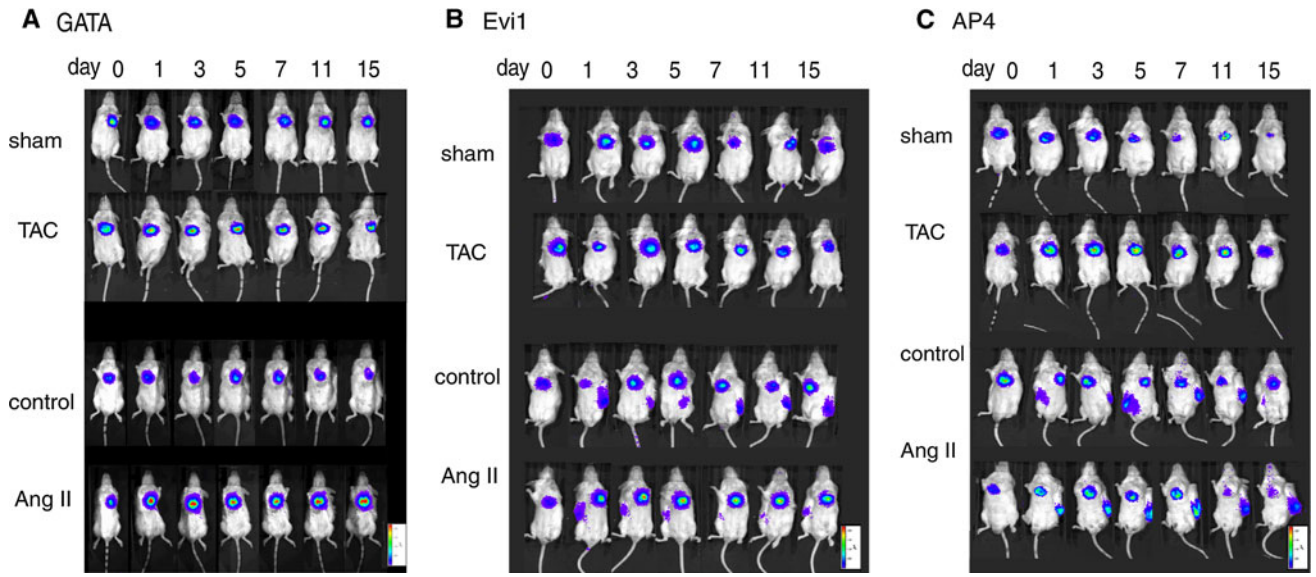

Fig. 3 Serial in vivo imaging in cardiac hypertrophy models: *Each panel* shows the in vivo imaging of the cardiac reporter plasmid containing GATA (**a**), Evi1 (**b**), or AP4 (**c**) binding sites in control (*Control*), Ang II-infused (*Ang II*), sham (*Sham*), and TAC-operated (*TAC*) mice. The *color bar* on the *right side* represents the total photons per animal (photons/se; *red* is the strongest and *purple* is the weakest). The signal intensity for the cardiac GATA reporter gene was enhanced in both Ang II and TAC mice (**a**). In contrast, the signal intensity of the Evi1 reporter gene was enhanced only in Ang II mice (**b**), while that of the AP4 reporter gene was enhanced only in TAC mice (**c**)

model, while Evi1 and hepatocyte nuclear factor 4 (HNF4) were identified in the Ang II model (Table 2).

In vivo imaging of myocardial transcriptional activities

We injected a firefly luciferase vector plasmid containing GATA-, AP4-, or Evi1-binding sequences into the LVs of living mice to determine whether their transcriptional activity was specifically enhanced in vivo. Injection of these plasmids into the LV induced sustained bioluminescence following the IP administration of luciferin. We sequentially evaluated the relative transcriptional activity of the targeted transcription factors by monitoring the intensity of bioluminescence emitted from the in vivo heart for 15 days. GATA activity was enhanced in both models (Fig. 3a). In contrast, Evi1 activity was increased only in the Ang II model (Fig. 3b) and that of AP4 was increased only in the TAC model (Fig. 3c). Figure 4 shows the different patterns of transcriptional activity in each model. In the TAC model, GATA and AP4 activities, but not Evi1 activity, were significantly enhanced from day 1 after TAC (Fig. 4a). In contrast, in the Ang II model, GATA and Evi1 activities, but not AP4 activity, were significantly enhanced from day 1 (Fig. 4b).

Discussion

In the present study, we were able to obtain serial in vivo measurements of stimulus-specific transcriptional activity during the development of cardiac hypertrophy in mice. The present approach may be useful to evaluate individual stimuli in the pathogenesis of cardiac hypertrophy, an early phase of heart failure caused by multifactorial disorders.

Identification of transcription factor-binding sites specific to diverse stimuli of cardiac hypertrophy

It is an important but difficult challenge to demonstrate how the expression of thousands of genes in cardiomyocytes is regulated in response to diverse stimuli. Clustering genes from microarray data is not sufficient to clarify the pathological relevance of gene regulation, and further analysis is required to identify key signaling pathways. The major problem for accurate analysis of large array data is the lack of reliable methods to identify bona fide changes in gene expression [17]. The fold-change method is biologically equivocal because the variability of gene expression is intensity specific [18]. Balancing the type I error rate and the power is another challenging problem [19]. To reduce false-positive results, we calculated each gene-specific variance in the control using repeated measurements and selected genes that were upregulated by more than twofold, with an excess of 5 standard deviations of the control ($P < 0.000001$), which is more stringent than Bonferroni correction [20]. This criterion drastically reduced the number of upregulated genes in each model (from 1,005 to 379 in the TAC model and from 706 to 12 in the Ang II model).

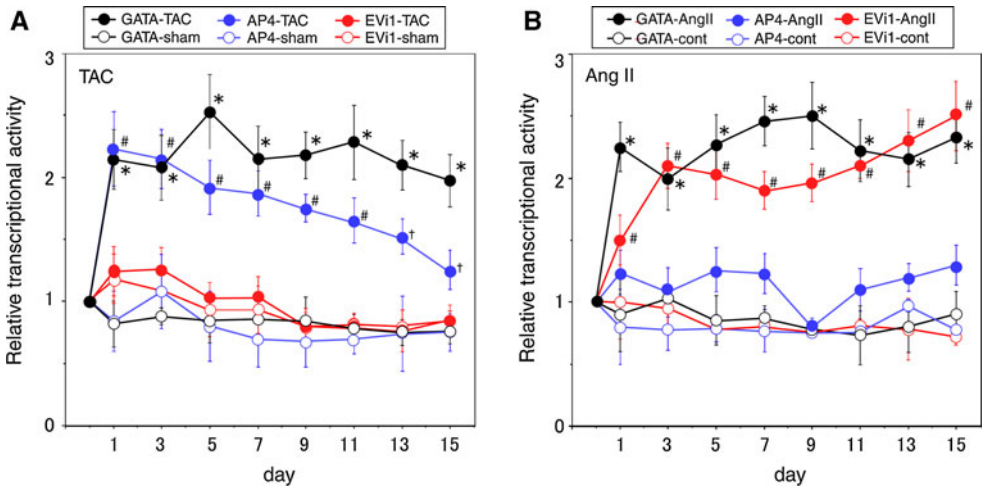

Fig. 4 In vivo transcriptional activity of identified transcription factor binding sites in different cardiac hypertrophy models: *Each figure* shows the transcriptional activity of GATA, Evi1, and AP4 cardiac reporter genes in the TAC model with sham operation (**a**) and the Ang II model with control treatment (**b**). The activity of GATA was significantly enhanced in both models. In contrast, the activity of Evi1 was enhanced only in the Ang II model, while that of AP4 was enhanced only in the TAC model. $*P < 0.01$ GATA Ang II versus control (*cont*) and TAC versus sham. $\#P < 0.01$ AP4 TAC versus sham and Evi1 Ang II versus control (*cont*). $\dagger P < 0.05$ AP4 Ang II versus control (*cont*)

Comparison of the promoter regions for transcription factor-binding sites between upregulated and unchanged genes led to the identification of several putative transcription factor-binding sites in the present animal models of cardiac hypertrophy. Indeed, GATA binding sites were similarly detected in both models. The GATA family is well known to play a crucial role in the progression of heart failure. Hautala et al. [21, 22] reported that both pressure overload and Ang II enhanced GATA activity in the development of cardiac hypertrophy. These results favor our present method to identify transcription factors closely associated with the development of cardiac hypertrophy.

Serial in vivo imaging to evaluate stimulus-specific transcriptional activity in the cardiac hypertrophy models

In the present study, we were able to identify several putative binding sites of transcription factors, including AP4 and SP1 in the TAC model and Evi1 and HNF4 in the Ang II model. Because the presence of transcription factor-binding sites in the promoter region of a gene does not prove that the corresponding transcription factor is actually activated, we verified the transcriptional activity in living animals during the progression of cardiac hypertrophy using an in vivo imaging system. Our strategy involved noninvasive, sensitive, and real-time measurement of transcriptional activity in the development of cardiac hypertrophy. Moreover, the present method focused on early heart failure, in which transcriptional changes more accurately reflect a specific myocardial response to primary injury preceding the pathological change of hypertrophy. Transcriptional activation through GATA-, AP4-, and Evi1-binding sites was observed from day 1 after TAC and/or Ang II infusion, suggesting that these transcription factors were directly related to pressure overload by TAC or a systemic increase in Ang II levels.

GATA has been reported to be involved in the progression of cardiac hypertrophy and heart failure in previous studies. Additionally, consistent with our current data, inhibition of Ang II type 1 receptor by olmesartan has been shown to completely abolish the transcriptional activity of GATA in an Ang II model [23]. Thus, since we observed the involvement of GATA during the development of cardiac hypertrophy using our described method, these data confirmed the technical plausibility and accuracy of this method. In contrast to GATA, the function of AP4 and Evi1 in the molecular mechanisms of heart remains to be elucidated. Evi1 is a nuclear protein containing a zinc finger motif, originally identified as a common locus of retroviral integration in myeloid tumors [24]. Recently, Evi1 was reported to work with GATA6 as a putative regulator of the calreticulin gene in neonatal rat ventricular cardiomyocytes [25]. On the other hand, the AP4 protein is a ubiquitous and repressive transcription factor [26] that is reportedly regulated by c-myc [27], which plays a crucial role in cellular proliferation, differentiation, apoptosis, and cell cycle progression [28]. In cardiomyocytes, biomechanical stretch induces c-myc [29]. Therefore, c-myc may be involved in AP4 transcriptional activity in response to

pressure overload in the heart. Nonetheless, the signaling pathways of these models may overlap [3]. Kee et al. [30] reported that inhibition of histone deacetylase (HDAC) blocks rat cardiac hypertrophy induced by either Ang II infusion or aortic banding. HDAC is profoundly involved in gene expression by direct interaction with DNA-binding transcription factors, such as MyoD and myocyte enhancing factor 2 (MEF2), and represses their transcriptional activity via deacetylation [31, 32]. Considering that inhibition of deacetylation has a similar effect in both models, HDAC may work downstream of the signaling pathways in response to diverse stimuli. Future studies should elucidate the interactions of HDAC with Evi1 and/or AP4 in each model.

Although previous studies have reported cardiac in vivo imaging using the luciferase reporter gene, the present study has several new findings. Wu et al. [16] directly injected an adenovirus vector containing the luciferase gene into the rat heart for in vivo imaging. They showed continuous expression of the luciferase gene in normal rats, but did not study transcriptional activity in a cardiac disease model. In contrast, in the present study, we demonstrated serial measurements of the stimulus-specific transcriptional activity that precedes the development of hypertrophy, which would be useful to provide insights into the disease mechanism. Tillmanns et al. [33] observed the cardiac transcriptional activity of NF-κB after myocardial infarction by using transgenic mice systemically overexpressing NF-κB. However, since NF-κB was ubiquitously expressed in their transgenic mouse model, luminescence emitted from organs other than the heart was unavoidable. On the other hand, our present method utilized a simple direct injection of a naked plasmid, which yielded substantial expression of the luciferase reporter gene only in the heart with a resulting high signal/noise ratio. Thus, our method is useful for measuring transcriptional activity in the heart and will have applications in a variety of other studies.

Limitations of the study

Several limitations of the present study should be mentioned. First, because of the combinatorial approach using expression data from microarrays and promoter analysis, there was a chance of incorrect multiplication. However, in the present study, we addressed this issue by applying strict cut-off levels to the microarray data and validated the results carefully using the in vivo imaging approach. In fact, we performed stimulus-specific monitoring to distinguish between the different cardiac hypertrophy models. Second, we did not measure the nuclear protein expression of these transcription factors, which could make it difficult to understand the mechanisms of induction of cardiac hypertrophy by transcription factors. However, in the present study, we placed more emphasis on transcriptional activity related to transcription factor-binding sites rather than transcription factors per se as the surrogate marker during the development of hypertrophy because sequence-specific transcription factors are known to interact with co-activators that enhance their transcriptional activity [14]. Further studies are needed to elucidate the detailed mechanisms of the development of cardiac hypertrophy. Third, the number of extracted genes in the TAC and Ang II models was not balanced. However, blood pressure in Ang II mice rose beginning on the day after implantation of the Ang II infusion pump, and consequently, LV weight in Ang II model mice was significantly higher than that in controls. Thus, this imbalance may result from our strict selection criteria, which sought to exclude mild changes in gene expression rather than the incomplete development of cardiac hypertrophy in the AngII model. Fourth, our analysis did not allow the identification of any novel promoter sequences that have not yet been registered in DBTSS. However, our primary goal was to show a new method to identify specific signaling pathways involved in different triggers of cardiac hypertrophy and to monitor transcriptional activity in living animals. Once established, this methodology will be useful to demonstrate the involvement of novel transcription factors.

Future studies

Identification and monitoring of specific signaling pathways involved in the development of a disease would allow for the investigation of novel therapeutic targets in personalized medicine. Visualization of specific signaling pathways in living humans is a major hurdle in this objective. However, identification of candidate therapeutic targets in animal models is the first step to achieve this goal.

Conclusions

In the present study, we were able to identify stimulus-specific transcriptional activity in response to TAC or Ang II infusion by comparing the promoter regions of transcription factor-binding sites in coregulated genes with those of unchanged genes in silico. Furthermore, we quantitatively evaluated the stimulus-specific transcriptional activity occurring during the development of cardiac hypertrophy, i.e., AP4 for the TAC model and Evi1 for the Ang II model. The present approach may be useful to accurately target the diverse stimuli of cardiac hypertrophy.

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Conflict of interest The authors declare no conflicts of interest regarding this work.

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