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Induction of high STAT1 expression in transgenic mice with LQTS and heart failure

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Abstract

Cardiac-specific expression of the N1325S mutation of *SCN5A* in transgenic mouse hearts (TG-NS) resulted in long QT syndrome (LQTS), ventricular arrhythmias (VT), and heart failure. In this study we carried out oligonucleotide mircoarray analysis to identify genes that are differentially expressed in the TG-NS mouse hearts. We identified 33 genes in five different functional groups that showed differential expression. None of the 33 genes are ion channel genes. *STAT1*, which encodes a transcription factor involved in apoptosis and interferon response, showed the most significant difference of expression between TG-NS and control mice (a nearly 10-fold increase in expression, $P = 4 \times 10^{-6}$). The results were further confirmed by quantitative real-time PCR and Western blot analyses. Accordingly, many interferon response genes also showed differential expression in TG-NS hearts. This study represents the first microarray analysis for LQTS and implicates *STAT1* in the pathogenesis and progression of LQTS and heart failure.

Keywords: Cardiac sodium channel gene *SCN5A* mutation N1325S; Long QT syndrome (LQTS) and ventricular tachycardia; Microarray analysis; Dilated cardiomyopathy and heart failure; STAT1

The long QT syndrome (LQTS) is characterized by prolongation of the QT interval and T wave abnormalities on electrocardiograms (ECG) [1,2]. LQTS is associated with symptoms including syncope, seizures and sudden death caused by a specific ventricular arrhythmia, *torsade de pointes* [1,2]. One of the major genes identified for LQTS is the *SCN5A* gene on chromosome 3p21–23 (LQT3), which accounts for 10–20% LQTS cases [2,3]. *SCN5A* encodes a voltage-gated sodium channel $Na_v1.5$, which is mainly expressed in the heart and responsible for the generation and rapid propagation of electrical signals (action potentials) in cardiomyocytes [4,5]. Besides gain-of-function mutations associated with LQTS, loss of function mutations in *SCN5A* were demonstrated to be involved in the pathogenesis of both Brugada syndrome [6] and progressive cardiac conduction defects (PCCD) [7]. Mutations of *SCN5A* have also been reported to be involved in dilated cardiomyopathy/heart failure [8,9].

The N1325S mutation in *SCN5A* is a substitution of an asparagine residue by a serine at position 1325 in the intercellular region of domain III S4–S5 of $Na_v1.5$, and is one of the earliest mutations identified in LQT3 families [3]. It disrupts the Na^+ channel inactivation and generates the late persistent I_{Na} inward current. Overexpression of the N1325S mutation in *Xenopus* oocytes and HEK293 cells induced dispersed reopening in the late inactivation phase,

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which produced a late persistent inward sodium current [10,11]. We have expressed the *SCN5A* N1325S mutation in the mouse heart (TG-NS mice) [12]. The TG-NS transgenic mice showed prolongation of the QT interval on ECG and high incidences of spontaneous polymorphic VT followed by sudden cardiac death [12,13]. The electrophysiological studies of cardiomyocytes from the transgenic mice showed that the N1325S mutation produced a late sodium current and prolonged the cardiac action potential duration, which is expected to prolong the QT interval on ECG [12,14]. Recent studies also detected the phenotype of dilated cardiomyopathy and heart failure in TG-NS mice [15] as well as in a human patient with the N1325S mutation of *SCN5A* [14]. Age-dependent apoptosis and abnormal calcium handling were also demonstrated in the TG-NS cardiomyocytes, and are the likely causes of dilated cardiomyopathy and heart failure [15]. However, the molecular mechanism for cardiomyocyte apoptosis in TG-NS mice is not known. In this study we found that the expression of the *STAT1* gene was highly induced in TG-NS hearts, which may be a cause of apoptosis in these mice.

The *STAT1* gene encodes one of the signal transduction and activator of transcription factors (STATs) which are involved in transduction of signals from various ligands (cytokines, growth factors, stress-induced stimuli) to the nucleus through Janus tyrosine kinases (JAKs) or mitogen-activated protein (MAP) kinases [16]. Seven different STAT family members have been identified, which are activated by different cytokines [17]. *STAT1* mediates the response to interferon (IFN)-α and IFN-γ and has been shown to be pro-apoptotic [17]. STAT1-deficient mice are more susceptible to development of tumors, which implicates *STAT1* in oncogenesis [16]. No transgenic mice with over-expression of *STAT1* were developed, thus, the physiological effect for over-expression of *STAT1* is unknown.

Microarray analysis is an unbiased approach to study expression of thousands of genes simultaneously in a system. To date, no microarray analysis or other large-scale gene expression studies have been performed for LQTS, either in the humans or mice. Here, we took advantage of our mouse model for LQTS, the TG-NS mice, to explore global gene expression re-programming in these mice. We used mouse oligonucleotide microarrays with 22,690 unique genes to determine gene expression differences between TG-NS and non-transgenic control mice. A surprisingly large number of genes showed differential expression between the two types of mice, which may be partly caused by the marked up-regulation of transcription factor STAT1 as validated by RT-PCR and Western blot analyses. These results implicate *STAT1* in the pathogenesis and progression of LQTS and heart failure and offer insights into the observation of cardiomyocyte apoptosis in TG-NS mice.

Materials and methods

Transgenic mice. Human mutant *SCN5A* gene with the LQTS-causing mutation N1325S was expressed in the mouse heart using a cardiac specific promoter, the mouse α-myosin heavy chain (α-mMHC) promoter, and we named this line of transgenic mice as TG-NS. Transgenic mice with cardiac-specific expression of wild type *SCN5A*, TG-WT, were also lately created. The creation of TG-NS and TG-WT mice was reported by us previously [12,18], and they carry the comparable number of the transgenes and have a comparable level of *SCN5A* expression. Genotyping of the positive TG-NS mice was performed by polymerase chain reactions (PCR) using genomic DNA isolated from mouse tails/toes using the tail lysis buffer (50 mM Tris–HCl, 100 mM EDTA, 100 mM NaCl, 1% SDS). We used PCR primers 5′-TGT CCG GCG CTG TCC CTG CTG-3′ and 5′-CTC ATG CCC TCA AAT CGT GAC AGA-3′ for specific amplification of the *SCN5A* transgene and primers 5′-GGC ACC TGC TGC AAC GCT CTT T-3′ and 5′-GGT GGG CAC TGG AGT GGC AAC TT-3′ for amplification of *AGGF1* that serves as an internal control for quality of mouse genomic DNA. PCR was performed using standard procedures.

Microarray analysis. Total RNA was prepared from heart tissues of the non-transgenic control and TG-NS mice. First, heart tissues were homogenized by a polytron homogenizer (PT3100, Dispersing and Mixing Technology by Kinematica). Total RNA was then isolated using the TRIzol reagent (Invitrogen). The integrity and purity of the RNA was confirmed visually on a 1% denaturing agarose gel, and by measuring the optical density ratio (A260/A280). Double-stranded complementary DNA (ds-cDNA) was synthesized from 15 μg of total RNA using the Superscript Choice System (*Invitrogen*) with an HPLC-purified oligo-dT primer containing a T7 RNA polymerase promoter (GENSET, La Jolla, CA) as instructed by the manufacturer. The cDNA was extracted by the Phase Lock Gel (PLG) kit (Eppendorf) and purified by ethanol precipitation. *In vitro* transcription was performed with 1 μg of ds-cDNA using the ENZO BioArray RNA Transcript Labeling kit (ENZO Diagnostics). Fragmentation of biotinylated cRNA (20 μg), hybridization, washing, and staining were performed following the instructions by Affymetrix by the CWRU Gene Expression Core Facility. The Mouse Genome MOE430A arrays (Affymetrix) were used. Each array contains ~22,690 genes.

Statistical analysis. Microarray data was extracted from scanned images. GeneSpring 7.0 (Silicogenetics) was used to compare the data from three transgenic mice with those from three non-transgenic control littermates. All samples were considered as one group of replicates. The algorithm to generate a list of genes that showed a statistically significant difference between the two groups was described previously [19,20]. The median value for group comparisons was used. All raw data with a score less than zero were set to zero. Genes were further filtered by an absolute call: present (P) or marginally present (M) in the two groups for the up-regulated genes and down-regulated genes.

Quantitative real-time PCR (RT-PCR). Quantitative RT-PCR was performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Total RNA was extracted from hearts using TRIzol (Invitrogen). Reverse transcription was performed with 5 μg of RNA using the Superscript Choice System (Invitrogen). Primers spanning exon-intron junctions were designed to avoid amplification of genomic DNA. PCR conditions were 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Fluorescence changes were monitored with SYBR Green PCR Supermix (VWR) after every cycle, and melting curve analysis was performed at the end of 40 cycles to verify PCR product (0.5 °C/s increase from 55–99 °C with continuous fluorescence reading). The 18S gene was used to normalize samples for comparison. To quantify changes in gene expression, the $\Delta\Delta C_t$ method was used to calculate the relative fold changes as previously described [21].

Western blot analysis. To determine the expression level of the STAT1 protein, total proteins were extracted from mouse hearts. Hearts were homogenized with Polytron, and lysed on ice with the lysis buffer (0.5% NP-40, 20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA). The protein concentration was measured using the Bradford method (Bio-Rad). Equal amounts of protein extracts were separated on 10% SDS–polyacrylamide gels by electrophoresis. Western blot analysis was performed as described previously [5]. The blots were incubated with agitation at room temperature in the presence of a rabbit polyclonal anti–STAT1 antibody (Santa Cruz Biotechnology) (diluted in 1:500 in

0.3% BSA in PBST). The signal was detected using enhanced chemiluminescence (ECL kit, Amersham Biosciences). An anti-β-actin antibody and an anti-GAPDH antibody (Sigma–Aldrich) were used as loading controls at 1:1000 dilution in PBST.

Results

Identification of genes differentially expressed in the hearts from TG-NS mice

We investigated the gene expression profiles from three TG-NS mice and three age- and sex-matched control mice (6–8 months of age, male) using the Affymetrix Mouse Genome MOE430A Arrays. 2492 of 22,690 genes showed significant expression differences between the two groups if $P \leqslant 0.05$ (Welch *t*-test). Because of the large number of genes identified, filters exceeding 2- and 5-fold changes and several different *P* values were applied. The results are summarized in Table 1. With $P \leqslant 10^{-5}$, only two genes, *STAT1* encoding signal transduction and activator of transcription factor 1 and *DLM-1* encoding Z-DNA binding protein 1, showed an expression difference between two groups of mice. At *P* value of 10^{-4}, 11 genes showed differential expression of $\geqslant$5-fold, 9 up-regulated and 2 down-regulated (9↑, 2↓). At *P* value of 10^{-3}, 33 genes (31↑, 2↓) showed expression differences of $\geqslant$5-fold. The number of genes increased to 14 at $P \leqslant 10^{-4}$ and 65 at $P \leqslant 10^{-3}$ if the cut off expression difference was set to 2-fold (Table 1).

Table 1
Summary data for the number of genes showing differential expression in TG-NS hearts

Fold difference of expression	*P* value			
	0.01	0.001	0.0001	0.00001
Up-regulated genes				
2-fold	470	54	12	2
5-fold	101	31	9	2
Down-regulated genes				
2-fold	306	11	2	0
5-fold	53	2	2	0

Our further analysis was focused on genes showing a large differential expression difference, i.e. those showing 5-fold expression differences and *P* value of $\leqslant$0.001. As a result, 31 genes were found to be up-regulated and 2 genes down-regulated in TG-NS mice (Table 1). These genes can be divided into five functional groups: 11 genes are involved in interferon-responses; 4 genes are related to apoptosis and inflammation; 4 genes may mediate other immune responses; 4 genes encode enzymes; 11 unknown genes (Table 2 and Supplementary Table 1).

Validation of microarray results by real-time PCR (RT-PCR)

To verify the results from the microarray analysis, quantitative RT-PCR was performed with 9 additional TG-NS and 7 control mice (6–8 months of age). Results from RT-PCR analysis of 6 highly significant genes, including *STAT1*, *Usp18*, *Oas1 1G*, *Helicard*, *Trim34 delta*, and *Phgdh*, are shown in Table 3. The real-time PCR results generally confirmed the results from microarray analysis. Linage regression analysis demonstrated a strong positive correlation between the two technological platforms with $R = 0.93$.

STAT1 showed the most significant differential expression in TG-NS mice

STAT1 is the gene showing the most significant expression difference between TG-NS and control mice with a *P* value of 4.0×10^{-6} and a fold difference of 9.8 (Table 2). Thus, in addition to the RT-PCR analysis described above

Table 2
Genes showing differential expression in TG-NS hearts by microarray analysis

Accession No.	Symbol	Gene	*P* value	Fold of change
(A) Genes involved in interferon (IFN)-signaling pathways				
NM_008332.1	*Ifit 2*	Interferon-induced protein with tetratricopeptide repeats 2	1.3×10^{-4}	43.9
NM_011909.1	*Usp18*	Ubiquitin specific protease 18	6.5×10^{-5}	31.8
NM_010501.1	*Ifit 3*	Interferon-induced protein with tetratricopeptide repeats 3	8.3×10^{-4}	31.5
NM_008331.1	*Ifit 1*	Interferon-induced protein with tetratricopeptide repeats 1	2.2×10^{-4}	26.0
NM_016850.1	*Irf 7*	Interferon regulatory factor 7	1.3×10^{-4}	18.6
NM_018734.1	*Gbp3*	Guanylate nucleotide binding protein 3	1.8×10^{-4}	13.5
AF136520	*Dlm-1*	Tumor stroma and activated macrophage protein DLM-1	8.6×10^{-6}	12.9
NM_009283	*STAT1*[a]	Signal transducer and activator of transcription 1	4.0×10^{-6}	9.8
AB067533.1	*Oasl 9*	2,5-Oligoadenylate synthetase-like 9	9.8×10^{-4}	6.6
BC018470	Oasl 1G	2′–5′ Oligoadenylate synthetase 1G	2.0×10^{-4}	6.5
AY090098.1	*Isg 12*	Interferon stimulated gene 12	2.7×10^{-4}	6.2
(B) Genes with a potential role in apoptosis and inflammation				
NM_009283	*STAT1*[a]	Signal transducer and activator of transcription 1	4.0×10^{-6}	9.8
AY075132	*Helicard*	Helicard	2.1×10^{-5}	7.9
AF220142	*Trim34 delta*	Tripartite motif protein 34 delta	8.1×10^{-5}	5.5
NM_126166	*Tlr3*	Toll-like receptor 3	2.5×10^{-4}	5.4

[a] *STAT1* is involved in both IFN signaling and apoptosis.

Table 3
Conformation of results from microarray analysis by RT-PCR

Gene	Microarray[a]	RT-PCR[b]
STAT1: Signal transducer and activator of transcription 1	9.8	8.4
Usp18: Ubiquitin specific protease 18	31.8	65.9
Oas1 1G: 2′–5′ Oligoadenylate synthetase 1G	6.5	14.9
Helicard: Helicard	7.9	3.4
Trim34 delta: Tripartite motif protein Trim34 delta	5.5	7.9
Phgdh: 3-phosphoglycerate dehydrogenase	53.8	66.3

[a] Data of fold difference of expression between 3 TG-NS and 3 control mice.
[b] Data of fold difference of expression between 9 TG-NS and 7 control mice; no overlapping of samples between the microarray and RT-PCR studies.

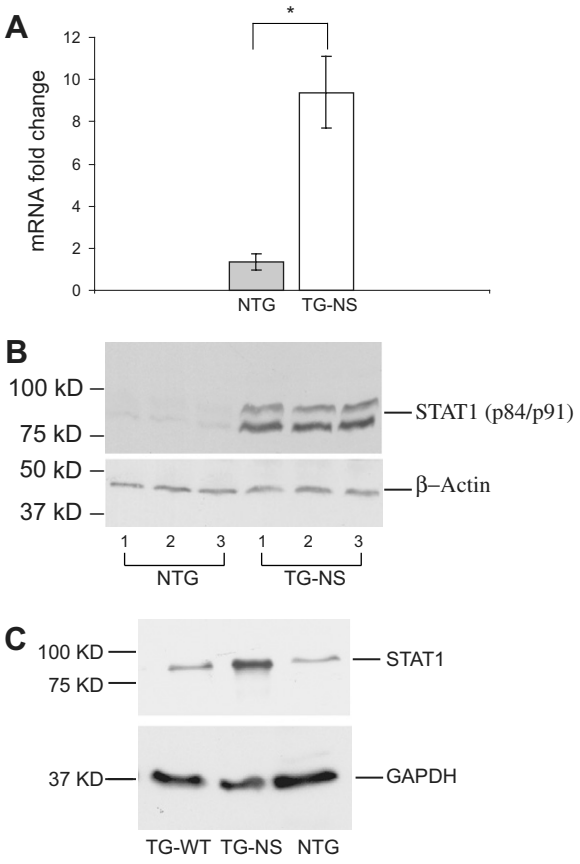

Fig. 1. Markedly increased expression of *STAT1* in TG-NS hearts. (A) Quantitative RT-PCR analysis with RNA isolated from 9 TG-NS and 7 NTG (non transgenic control) hearts (age = 6–8 months). Data were normalized to 18S RNA expression in the same samples and reported as fold changes (mean ± SE) from levels in control mice (*$P = 0.0016$). (B) Western blot analysis of STAT1 from three NTG and three TG-NS hearts (age = 6–8 months). β-Actin was used as loading control. The experiment was repeated three times and similar results were obtained. (C) Western blot analysis of STAT1 from non-transgenic control (NTG), TG-NS, and TG-WT hearts (age = 6–8 months). The experiment was repeated twice and similar results were obtained. Note that TG-NS and TG-WT have the comparable copy number of the transgene and a similar level of expression of the cardiac sodium channel (see Reference by Zhang et al. [18]). The only difference between these two types of mice is that TG-NS mice carry the mutant N1325S *SCN5A* and TG-WT mice carry the wild type *SCN5A*.

(Fig. 1A and Table 3), Western blot analysis was also used to validate the finding of increased STAT1 expression in TG-NS mice compared to control littermate mice (Fig. 1B). Western blot analysis demonstrated that the basal expression level of the STAT1 protein in non-transgenic control mice was low, however, it dramatically increased in TG-NS hearts (Fig. 1B).

Recently, we created and studied transgenic mice with cardiac-specific expression of wild type *SCN5A* (TG-WT) (in contrast to TG-NS with mutant *SCN5A* containing the N1325S mutation) [18]. TG-WT mice did not develop LQTS, VT, or heart failure [18], thus they could serve as an excellent control for TG-NS mice (note that the TG-WT mice were not available when the microarray project started). Western blot analysis was used to compare the expression level of the STAT1 protein between TG-NS to TG-WT mice. As shown in Fig. 1C, the expression level of STAT1 in TG-WT heart was comparable to that in non-transgenic control hearts, but much lower than that in TG-NS hearts. These results suggest that induction of STAT1 expression is specific to TG-NS mice with the *SCN5A* mutation N1325S.

Discussion

Microarray analysis is a large scale study that can provide unbiased assessment of expression of thousands of genes in a cell or tissue simultaneously. To date, no microarray analysis has been reported for LQTS either in the humans or mice. We performed a microarray study using a mouse model for LQTS, the TG-NS mice with cardiac expression of the *SCN5A* mutation N1325S associated with LQTS. Microarray analysis revealed 33 genes showing marked differential expression between TG-NS and wild type mice (>5-fold difference, $P \leqslant 0.001$). The results from microarray analysis were almost identical to that from the follow-up RT-PCR analysis using independent samples for all selected genes. Our results suggest the involvement of expression remodeling in the progression of LQTS. Furthermore, the results from this study suggest that in addition to its effects on basic biophysical properties of the cardiac sodium channel, the N1325S mutation has a more profound effect on cardiomyocytes *in vivo*.

The gene that showed the most significant difference between TG-NS and wild type mice is *STAT1*. Follow-up RT-PCR and Western blot analysis confirmed that expression of *STAT1* was markedly increased in TG-NS myocytes compared to non-transgenic control cells. Further analysis showed that STAT1 protein expression was also much higher in TG-NS hearts than in TG-WT hearts with cardiac expression of wild type *SCN5A* (Fig. 1C). STAT1 is a key signaling protein that functions as a transducer of cytokine signaling and as a sensor responding to cellular stresses, in particular, IFN response [17]. Thus, it was interesting to note that many genes involved in the IFN response also showed

increased expression in TG-NS myocytes, for example, *Usp18*, *Ifit1*, *Ifit2*, *Ifit3*, Irf7, *Gbp3*, *Dlm-1*, *Oasl 9, Oasl 1G*, and *Isg 12* that all showed many fold increases of expression (Table 2). The molecular mechanism for induction of high *STAT1* expression in TG-NS hearts is not clear, however, abnormal handling of intracellular calcium transients detected in TG-NS cardiomyocytes [14] may be a likely cause.

STAT-1 has been shown to induce apoptosis. Human fibroblast cells deficient in *STAT1* were resistant to TNF-α-induced apoptosis [22]. Neonatal rat cardiomyocytes subjected to ischemia for 4 h showed increased expression of *STAT1*, and cardiomyocytes transfected with *STAT1* showed increased apoptosis with exposure to ischemia [23]. A trend of increased apoptosis in the absence of exposure to ischemia was also detected in cardiomyocytes transfected with a *STAT1* construct compared to control cells transfected with the vector (Fig. 3A in Stephanou et al. [23]). Because age-dependent apoptosis has been detected in TG-NS mouse hearts [15], we speculate that increased expression of *STAT1* may be a cause for apoptosis in these mice.

In summary, our microarray analysis of a transgenic mouse model for LQTS, TG-NS, demonstrated that gene expression remodeling existed in these mice. Of a particular interest was the finding of highly increased *STAT1* expression, which may provide insights into the findings of cardiomyocyte apoptosis in TG-NS mice and high risk of heart failure in these mice.

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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.2007.04.119.

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