

**Pathophysiological significance of *Stim1* mutation in sympathetic response to stress and cardiovascular phenotypes in SHRSP/Izm: in vivo evaluation by creation of a novel gene knock-in rat using CRISPR/Cas9**

Batbayar Odongoo<sup>a\*</sup>, Hiroki Ohara<sup>a\*</sup>, Davis Ngarashi<sup>a,d</sup>, Takehito Kaneko<sup>b,e</sup>, Yayoi Kunihiro<sup>b,c</sup>, Tomoji Mashimo<sup>b,c,f</sup>, Toru Nabika<sup>a</sup>

<sup>a</sup>Department of Functional Pathology, Faculty of Medicine, Shimane University, Izumo, Japan

<sup>b</sup>Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Kyoto, Japan

<sup>c</sup>Institute of Experimental Animal Sciences, Graduate School of Medicine, Osaka University, Osaka, Japan

<sup>d</sup>Present address: Department of Physiology, School of Medicine, Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania

<sup>e</sup>Present address: Laboratory of Animal Reproduction and Development, Faculty of Science and Engineering, Iwate University, Morioka, Japan

<sup>f</sup>Present address: Laboratory Animal Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

\* Contributed equally to this work.

Correspondence: Hiroki Ohara, Department of Functional Pathology, Faculty of Medicine, Shimane University, 89-1 Enya-cho, Izumo, Japan

E-mail: [oharah@med.shimane-u.ac.jp](mailto:oharah@med.shimane-u.ac.jp)

## Abstract

Genetic approach using rat congenic lines between SHRSP/Izm and WKY/Izm identified stromal interaction molecule 1 (*Stim1*), an essential component of store-operated  $\text{Ca}^{2+}$  entry (SOCE), as a promising candidate gene responsible for the exaggerated sympathetic response to stress in SHRSP. Since SHRSP has a nonsense mutation in *Stim1* resulting in the expression of a truncated form of STIM1 that caused reduction of SOCE activity in primary cultured cerebral astrocytes, we created SHRSP/Izm knocked-in with the wild-type *Stim1* (KI SHRSP) by the CRISPR/Cas9 method to investigate whether the functional recovery of STIM1 would mitigate sympatho-excitation to stress *in vivo* in SHRSP. No potential off-target nucleotide substitutions/deletions/insertions were found in KI SHRSP. Western blotting and fluorescent  $\text{Ca}^{2+}$  imaging of astrocytes confirmed wild-type STIM1 expression and restored SOCE activity in astrocytes from KI SHRSP, respectively. Blood pressure (BP) measured by the tail cuff method at 12, 16, and 20 weeks of age did not significantly differ between SHRSP and KI SHRSP, while the heart rate of KI SHRSP at 16 and 20 weeks of age was significantly lower than that of age-matched SHRSP. Unexpectedly, the sympathetic response to stress (evaluated with urinary excretion of norepinephrine under cold stress and BP elevation under cold/restraint stress) did not significantly differ between SHRSP and KI SHRSP. The present results indicated that the functional deficit of STIM1 was not a genetic determinant of the exaggerated sympathetic response to stress in SHRSP and that it would be necessary to explore other candidates within the congenic fragment on chromosome 1.

Key words: SHRSP, CRISPR/Cas9, *Stim1*, store-operated  $\text{Ca}^{2+}$  entry (SOCE), sympathetic response, stress

## Introduction

Hypertension is one of the most critical risk factors for cerebro-cardiovascular diseases. A large number of genetic/environmental factors are involved in the pathogenesis of this complex disease in humans, and stress in daily life is one of important environmental factors; masked/white-coat hypertension are a clinically well-known example of stress-related hypertension, which are associated with an increased risk of cardiovascular events (1-3).

The stroke-prone spontaneously hypertensive rat (SHRSP) is one of the best disease models for hereditary hypertension and cerebral stroke (4-5). It has long been known that SHRSP shows hyper-responsiveness to various forms of stress with enhanced sympathetic nerve activity (6-10). Importantly, accumulating evidence in the last two decades has shown that oxidative stress-induced sympatho-excitation in the rostral ventrolateral medulla (RVLM), a brainstem region controlling sympathetic activity and blood pressure (BP), is a plausible molecular mechanism for severe hypertension in SHRSP (11-14). Identification of genes related to the sympathetic excitation in SHRSP would provide an important clue to uncover the pathophysiological basis of essential hypertension in humans as well as rodents, however, the genetic determinants remain unclear.

Quantitative trait locus (QTL) analysis and construction of congenic strains have been widely used as a set of genetic approaches for identifying genes associated with cardiovascular traits in hypertensive rats (15). We previously showed that a major BP QTL existed in rat chromosome (chr) 1 through a genome-wide linkage analysis using F2 generation cross derived from SHRSP and normotensive Wistar-Kyoto (WKY) rats (16). Then, we created reciprocal congenic lines between SHRSP and WKY for the BP QTL on chr1 and revealed that the chr1 QTL was implicated in the pathophysiology of exaggerated sympathetic response to stress in SHRSP (17-19) with

possible involvement of hyperactivity of the RVLM (20). Subcongenic analysis successfully narrowed down the candidate region to a 1.2 Mbp fragment on the chr1 QTL, finally, stromal interaction molecule 1 (*Stim1*) was identified as the most promising candidate gene responsible for the sympathoexcitation to stress in SHRSP according to the existence of a nonsense mutation (c.1918C>T, p.Arg640X) in this gene resulting in the truncated STIM1 expression in SHRSP (21).

STIM1 is an endoplasmic reticulum (ER)-resident transmembrane protein and an essential component in store-operated  $\text{Ca}^{2+}$  entry (SOCE). When  $\text{Ca}^{2+}$  store in the ER lumen is depleted, STIM1 translocates to the plasma membrane in order to induce SOCE by opening  $\text{Ca}^{2+}$  channels (ORAI1 and TRPCs), and this results in both  $\text{Ca}^{2+}$ -dependent intracellular signaling and the refilling of  $\text{Ca}^{2+}$  in the ER particularly in non-excitabile (i.e., not neuronal nor muscle) cells (22, 23). STIM1 is expressed ubiquitously in the body and plays important roles in the pathogenesis of various diseases, such as cardiac hypertrophy, type 2 diabetes, immunodeficiency, and autoimmunity, as well as in physiological  $\text{Ca}^{2+}$  homeostasis (24-29).

In an *in vitro* study, we showed that the mutant STIM1 caused a decrease in SOCE activity in primary culture of cerebral astrocytes (30). Astrocytes are the most abundant glial cell type in the brain and control neuronal functions via metabolic regulation of the neurotransmitter pool of glutamate and GABA (31). Moreover, previous studies have suggested that astrocytes in RVLM play a pivotal role in controlling sympathetic activities via angiotensin II type 1 receptor-mediated mechanisms (32, 33).

We therefore hypothesized that decreased SOCE activity in astrocytes was associated with the sympatho-excitation mechanisms in SHRSP, and expected that the functional recovery of STIM1 would improve the exaggerated sympathetic response to stress in SHRSP. To examine this hypothesis *in vivo*, *Stim1* knock-in SHRSP/Izm (KI SHRSP), in which the mutated *Stim1* was substituted for the wild-type *Stim1* by the gene-editing technology using CRISPR/Cas9, was

created in the present study. Effects of the 'knock-in' on stress response as well as on other cardiovascular phenotypes were evaluated in this strain.

## Materials and Methods

### Animals

SHRSP/Izm was provided by the Disease Model Cooperative Research Association (Kyoto, Japan) and used as the control strain throughout this study. *Stim1* knock-in SHRSP/Izm was created as described below. Male rats were used in all experiments. Rats were kept under controlled living conditions at a room temperature of  $23\pm 2^{\circ}\text{C}$ , with  $55\pm 10\%$  humidity and a 12-h light-dark cycle (lights on at 07:00 and off at 19:00). Rats used in experiments were fed the Stroke-Permissive (SP) diet (Funabashi Farm Co., Ltd., Funabashi, Japan) after weaning at 4 weeks of age.

### Creation of *Stim1* knock-in SHRSP/Izm

The CRISPR/Cas9-mediated creation of *Stim1* knock-in SHRSP/Izm (KI SHRSP, formally SHRSP- *Stim1*<sup>em1Izm</sup>) was performed in Kyoto University as described previously (34). The local committee for Animal Research in Kyoto University approved the procedure of genome editing using CRISPR/Cas9. Briefly, guide RNA targeting the *Stim1* gene was designed using the CRISPR design tool (<http://crispr.mit.edu/>). The target sequence selected was 5'-GCAGGGTAGCTGA(stop)AACACAC-3' and the underlined sequence shows the premature stop codon found in SHRSP/Izm resulting in the expression of a truncated form of STIM1.

## Measurement of BP and heart rate (HR)

Male rats at 12 weeks of age were used (KI SHRSP; n=15, SHRSP; n=10). BP and HR were measured by the tail-cuff method (BP-98A, Softron, Tokyo, Japan) under a 37°C condition with a warmer (THC-3, Softron). The average of 5 readings was taken for each measurement. Periodical measurements at 4-week intervals (12, 16, and 20 weeks of age) were also performed (KI SHRSP; n=6, SHRSP; n=10, also see Table 2). Decreased number of examined KI SHRSP at 16 weeks of age is due to that 9 KI SHRSP were sacrificed for other purpose such as tissue sample collection for western blotting after BP measurement at 12 weeks of age. In addition, rats showing representative stroke signs (i.e., seizures, paralytic gait, and akinesia) were not examined at 20 weeks of age.

## Isolation of cerebral astrocytes and Ca<sup>2+</sup> imaging

Neonatal rats (1-3 days old) were sacrificed by the inhalation of CO<sub>2</sub> and the cerebral cortex was dissected out. Cerebral astrocytes were obtained with the shaking method as previously described (27). Dulbecco's modified Eagle's medium (DMEM, Cat. No. 048-30275, Wako, Osaka, Japan) containing 10% fetal bovine serum (Biowest, Nuaille, France) and 1% Penicillin-Streptomycin-Amphotericin B suspension (Wako) were used to culture cells.

Ca<sup>2+</sup> imaging of astrocytes was performed as previously described (30). Cells were observed using a confocal fluorescent microscope (FV1000-D, Olympus, Tokyo, Japan) followed by a serial medium exchange, as shown in Figure 2. Data analyses were performed using FV10-ASW software (Olympus). SOCE activity was defined as changes in fluorescence intensity before and

after the  $\text{Ca}^{2+}$  re-addition and calculated as follows:  $F_{7.5\text{min}} - F_{6.5\text{min}}$ ,  $F$  = fluorescence intensity at the indicated time.

#### Physiological evaluation of the stress response

Responses to restraint and cold stress were examined as previously described (21). Briefly, restraint stress was imposed by placing rats for 3 h in a stainless-steel holder adjusted to the rat's body size. Regarding cold stress, a rat was placed in a cage kept at 4 °C for 3 h (in telemetry experiments) or for 6 h (in the collection of urine samples). In the collection of urine samples, one milliliter of 1.0 N HCl was added to collection tubes to avoid the degradation of norepinephrine (NE). All experiments to evaluate the stress response were performed in the afternoon with the light on. Urinary NE was measured by SRL (Tokyo, Japan).

BP and HR changes under restraint and cold stress were monitored with the telemetry system, HD-S10 (Data Science Inc., St. Paul, MN). Surgical implantation of the telemetry probes was performed at 11-12 weeks of age and at least 7 days of recovery period was given to the rats after operation. BP and HR were monitored for 10 s every 10 min during the experiment. The averages of baseline BP (no stress) and BP under stress were calculated from the data obtained between 10:00-11:00 and between 11:00-14:00, respectively. Changes in BP ( $\Delta\text{BP}$ ) and HR ( $\Delta\text{HR}$ ) were calculated as the difference between the averaged BP during the periods with and without stress.

#### Statistical analysis

The values shown are means  $\pm$  S.D. Statistical analyses were performed using the unpaired Student's *t*-test. A value of  $p < 0.05$  was considered to be significant.

## Results

### Establishment of SHRSP-*Stim1*<sup>emIzm</sup>

Two heterozygous female rats for the *Stim1* allele were created by CRISPR/Cas9, and were then backcrossed with a male SHRSP/Izm. A heterozygous male and female of the F1 generation were then crossed with each other, and we finally obtained 3 homozygous rats (♂1 and ♀2) for subsequent procedures. As shown in Figure 1A, the nonsense mutation (c.1918C>T, p.Arg640X) in the *Stim1* sequence of SHRSP/Izm was substituted into the wild-type nucleotide sequence.

Expression of the wild-type STIM1 (hereafter referred as STIM1<sup>WT</sup>) was confirmed in brainstem lysates from KI SHRSP (Fig. 1B). The protein level of STIM1 was significantly greater in KI SHRSP than in SHRSP, which was consistent with previous findings (Fig. 1B, also see ref. 21). This result implied that the truncated form of STIM1 (hereafter referred as STIM1<sup>SHRSP</sup>) was unstable and degraded more rapidly.

### SOCE activity was improved in astrocytes from KI SHRSP

Cerebral astrocytes from KI SHRSP expressed STIM1<sup>WT</sup> at a significantly greater level than those from SHRSP (Fig. 2A). This result was consistent with the results obtained from brainstem lysates (Fig. 1B). SOCE activity was evaluated in cultured astrocytes using the fluorescent intracellular Ca<sup>2+</sup> indicator and the results showed that SOCE activity was significantly greater in KI SHRSP astrocytes than in SHRSP astrocytes (Fig. 2B). This observation was also supported by a greater expression of cyclooxygenase-2 (*Cox-2*), a downstream target of SOCE (30), in astrocytes from

KI SHRSP when compared with those from SHRSP (Fig.S1). Collectively, the results indicated functional recovery of STIM1 in KI SHRSP.

#### Physiological parameters of KI SHRSP

Table 1 summarizes age-dependent changes in body weight (BW), systolic BP (SBP), diastolic BP (DBP) and HR in the two strains. BW of KI SHRSP at 12 and 16 weeks of age was significantly lower than that of age-matched SHRSP. HR of KI SHRSP at 16 and 20 weeks of age was significantly lower than that of SHRSP as well. SBP and DBP showed no significant differences between the two strains at all examined ages.

#### Urinary excretion of NE under cold stress

Urinary NE is commonly used to evaluate sympathetic activity in rodents (6, 33). Urinary NE excretion was measured to evaluate sympathetic activity to cold stress as described previously (17-19, 21). Urinary NE in KI SHRSP at RT was significantly lower than that in SHRSP (Fig. 3). However, no significant inter-strain difference was found in urinary NE under cold stress and increase in NE by cold stress ( $\Delta$ NE) did not differ significantly between the two strains either (Fig. 3).

#### Changes in BP and HR under the cold/restraint stress

Changes in BP (SBP and DBP) and HR under cold or restraint stress were monitored using the telemetry system. Baseline BP, BP under cold/restraint stress, and changes in BP ( $\Delta$ BP) between

the baseline and under the stresses did not significantly differ between the two strains (Fig. 4A and B). The absence of inter-strain difference in baseline BP by the telemetry analysis was consistent with the results obtained using the tail-cuff method (see Table 1). Changes in HR ( $\Delta$ HR) under restraint stress were significantly greater in SHRSP than in KI SHRSP, while those under cold stress were not (Fig. 4A and B).

## Discussion

We previously showed that a BP QTL on chr 1 affected sympathetic response to stress in SHRSP, and identified STIM1, an essential  $\text{Ca}^{2+}$  storage sensor in the ER, as a promising candidate for this pathophysiological phenotype (16, 21, 30). Given the ubiquitous expression of STIM1 and its multifaced roles in various cells including neurons and astrocytes (36), it was very difficult to explore roles of STIM1 on responsiveness to stress and the underlying molecular mechanisms *in vitro*. Therefore, we created *Stim1* knock-in SHRSP using the CRISPR/Cas9 technology to rescue STIM1 function *in vivo*, and tried to clarify whether the functional deficit in STIM1<sup>SHRSP</sup> was the genetic determinant of the exaggerated sympathetic response to stress in SHRSP. In both brainstem lysates and cultured astrocytes from KI SHRSP, we confirmed the STIM1<sup>WT</sup> expression at a greater protein level when compared with SHRSP (Fig. 1 and 2). In addition, an increase in SOCE activity in KI SHRSP was confirmed by a  $\text{Ca}^{2+}$  imaging analysis (Fig. 2). These changes were considered to be due to the introduced wild-type *Stim1* since no off-target changes were observed in KI SHRSP on most probable target sites (Table 1). In spite of the recovery of SOCE activity in STIM1<sup>WT</sup>-introduced SHRSP cells, STIM1<sup>WT</sup> had no effects on the hyper-responsiveness to stress as well as on the basal BP in SHRSP *in vivo* (Table 2, Fig. 3 and 4). This suggested that *Stim1* was not the causal gene, and the responsible gene(s) would be somewhere

else in the chr1 congenic region (21). On the other hand, we found that basal urinary NE at room temperature and HR under tail-cuff measurement or restraint stress in KI SHRSP were significantly lower than those in SHRSP. Although these observations imply reduced sympathetic activity in KI SHRSP under particular conditions, more evidence is needed to obtain a robust answer.

Nevertheless, it is of interest that difference in HR between SHRSP and KI SHRSP were observed not under the cold stress but under the restraint stress in the telemetry experiments and in the tail-cuff measurement of BP; as restriction of active movement of rats using holders is necessary for measurement by the tail-cuff method (see Materials and Methods), the tail-cuff measurement and the telemetry measurement under the restraint stress are partly in common in terms of 'restriction'. The results on HR might therefore suggest that KI SHRSP was resistant not to physical stress (cold stress) but to mental stress (restraint stress). STIM1 plays an important role in cardiac pacemaking by regulation of the Ca<sup>2+</sup> dynamics in the sinoatrial cells (37), however, the underlying mechanisms for the HR differences under the restraint stress are currently unclear. Elevated resting HR is associated with risk of cardiovascular events in humans (38). Thus, chronic repeated exposure to the restraint stress may result in inter-strain differences in cardiovascular events between SHRSP and KI SHRSP. This hypothesis, including the molecular basis, should be verified by further in-depth investigation.

BW was significantly lower in KI SHRSP (Table 2). According to a previous study, *Stim1*<sup>+/-</sup> mice (with lower STIM1 activity) showed BW reduction when compared with wild-type mice due to disordered development of skeletal muscle (27). In an opposite way, KI SHRSP (with the wild-type STIM1) was significantly smaller than SHRSP (with the truncated STIM1) at 12 and 16 weeks of age. It is currently unclear that whether *Stim1* genotype contributes to this phenotypic characteristic because BW would be largely influenced by environmental factors (number of

littermates, daily amounts of mother's milk/food intake, and social status among rats living together in the cage) in the postnatal growth phase (39-42).

Pathophysiological functions of STIM1 in vasculature have been investigated in several studies; two independent studies by Kassan et al. (43) and Nishimoto et al. (44) revealed that endothelial cells (ECs)-specific *Stim1* knockout (KO) mice exhibited 1) decreases in eNOS activation and NO production in ECs, 2) impaired endothelium-dependent vasodilation, and 3) elevated BP. In contrast, vascular smooth muscle cells (VSMCs)-specific *Stim1* KO mice showed decrease in Ang II-induced cardiovascular events (hypertension, cardiac hypertrophy, perivascular fibrosis, and endothelial dysfunction) (26). These observations indicated that pathophysiological roles of STIM1 were opposite in different cell types, and thus, it is not surprising that systemic (not cell-specific) substitution of the wild-type for the truncated *Stim1* did not affect basal BP in SHRSP (Table 2 and Fig. 4). In this context, 'natural mutant' models (e.g. SHRSP) and 'artificial mutant' models (e.g. conditional KO mice) should be interpreted in a separate way.

In conclusion, the rescue of the truncated STIM1 in SHRSP did not reduce the exaggerated sympathetic response to stress although it ameliorated SOCE activity at a cellular level. To the best of our knowledge, this is the first study to create a knock-in SHRSP in which a natural mutation was genetically rescued. As we still have other candidate genes in the chr1 congenic region besides *Stim1* (21), further investigation is necessary, which would contribute to our understanding of the pathological basis of essential hypertension in humans.

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#### Disclosure of interest

The authors report no conflict of interest.

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#### Figure legends

Fig. 1 Establishment of *Stim1* knock-in SHRSP/Izm and confirmation of STIM1 protein expression in brainstem lysates.

(A) Genomic sequence of *Stim1* around the nonsense mutation site. SHRSP/Izm has a nonsense mutation (c.1918C>T, p.Arg640X) causing the expression of the truncated form of STIM1 (STIM<sup>SHRSP</sup>) with a 46-amino acid deletion, while the mutation was substituted to the normal sequence (CGA, coding Arg) in *Stim1* knock-in SHRSP/Izm (KI SHRSP). Arrows indicate the c.1918C>T site. (B) Western blotting showed the expression of wild-type STIM1 (STIM1<sup>WT</sup>) in KI SHRSP and the protein expression in KI SHRSP was significantly greater than that in SHRSP. Note that STIM1<sup>SHRSP</sup> has a lower molecular weight than STIM1<sup>WT</sup> due to the deletion of 46 C-

terminal amino acids. N=5 for each strain. \* $p < 0.05$ .

Fig. 2  $\text{Ca}^{2+}$  imaging in cerebral astrocytes for the evaluation of SOCE activity.

(A) Cerebral astrocytes from KI SHRSP expressed  $\text{STIM1}^{\text{WT}}$  at a significantly greater expression level when compared with those from SHRSP, which was consistent with the results in Fig. 1B. N=5 for each strain. (B) Evaluation of SOCE activity by  $\text{Ca}^{2+}$  imaging. Left panels show representative fluorescent images at the indicated time points in KI SHRSP and SHRSP astrocytes. Experiments were performed in triplicate and the averaged time course of Fluo-8 intensity is shown in the middle panel. In the statistical analysis, 15-19 cells were analyzed in each image obtained (in total, KI SHRSP; 49 cells, SHRSP; 45 cells). Data shown are representative of three independent experiments. 0 Ca; no  $\text{Ca}^{2+}$ , 2 Ca; 2 mM of  $\text{Ca}^{2+}$ , TG; thapsigargin. \* $p < 0.05$ . Scale bar = 50  $\mu\text{m}$ .

Fig. 3 Urinary norepinephrine excretion under room temperature or cold stress.

Rats were kept at room temperature or 4 °C for 3 h to collect urine samples and the urinary excretion of norepinephrine (NE) was examined as a marker for sympathetic activity. RT; room temperature.  $\Delta\text{NE}$ ; changes in urinary NE before and after cold stress. Numbers in parentheses indicate the number of rats tested. \* $p < 0.05$ . n.s.; not significant.

Fig. 4 Telemetric recording of blood pressure and heart rate under restraint or cold stress.

Rats were abdominally implanted with telemetry probes to record periodical changes in systolic blood pressure (SBP), diastolic BP (DBP) and heart rate (HR) under (A) cold or (B) restraint stress. Red lines with open circles; KI SHRSP, blue lines with open triangles; SHRSP. Graphs show averaged baseline SBP/DBP/HR (with no stress), averaged SBP/DBP/HR under the cold or

restraint stress, and changes in SBP/DBP/HR ( $\Delta$ SBP,  $\Delta$ DBP,  $\Delta$ HR) before and after the stress.

Numbers in parentheses indicate the number of rats tested. \* $p$ <0.05. n.s.; not significant.