Generation of Hypertension-Associated STK39 Polymorphism Knockin Cell Lines With the Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 System

Shintaro Mandai, Takayasu Mori, Eisei Sohara, Tatemitsu Rai, Shinichi Uchida

Abstract—Previous genome-wide association studies identified serine threonine kinase 39 (STK39), encoding STE20/SPS1-related proline/alanine-rich kinase, as one of a limited number of hypertension susceptibility genes. A recent meta-analysis confirmed the association of STK39 intronic polymorphism rs3754777 with essential hypertension, among previously reported hypertension-associated STK39 polymorphisms. However, the biochemical function of this polymorphism in the mechanism responsible for hypertension is yet to be clarified. We generated rs3754777G>A knockin human cell lines with clustered regularly interspaced short palindromic repeats-mediated genome engineering. Homozygous (A/A) and heterozygous (G/A) knockin human embryonic kidney cell lines were generated using a double nickase, single-guide RNAs targeting STK39 intron 5 around single-nucleotide polymorphism, and a 100-bp donor single-stranded DNA oligonucleotide. Reverse transcription polymerase chain reaction with sequencing analyses revealed the identical STK39 transcripts among the wild-type and both knockin cell lines. Quantitative reverse transcription polymerase chain reaction showed increased STK39 mRNA expression, and immunoblot analysis revealed increases in total and phosphorylated STE20/SPS1-related proline/alanine-rich kinase with increased phosphorylated Na–K–Cl cotransporter isoform 1 in both knockin cell lines. The largest increases in these molecules were observed in the homozygous cell line. These findings indicated that this intronic polymorphism increases STK39 transcription, leading to activation of the STE20/SPS1-related proline/alanine-rich kinase–solute carrier family 12A signaling cascade. Increased interactions between STE20/SPS1-related proline/alanine-rich kinase and the target cation–chloride cotransporters may be responsible for hypertension susceptibility in individuals with this polymorphism. 

Key Words: clustered regularly interspaced short palindromic repeats ■ hypertension ■ single nucleotide polymorphism ■ SLC12A transporters ■ SPAK protein

Hypertension is by nature a complex disorder determined by multiple genetic and environmental factors; the pathophysiology is not fully understood, although the contribution of genetic factors to blood pressure (BP) variation is estimated as 30% to 50%.1 Recent genome-wide association studies (GWASs) have identified genes susceptible to hypertension2–6 that would potentially contribute to understanding of the pathogenesis as well as discovery of novel therapeutic targets. However, despite the increasingly accumulating genetic data, there have been only a few replicated findings identifying risk variants.2–7 Racial or population heterogeneity or low statistical power of the studies is a potential explanation, and linkage disequilibrium can influence the results of GWAS analyses. Moreover, the variants detected with GWAS analyses generally have weak effects on disease development.8 Therefore, experimental verification of the biochemical function of each gene identified in genetic studies is essential for confirmation of candidate genes.

Serine threonine kinase 39 (STK39), encoding STE20/SPS1-related proline/alanine-rich kinase (SPAK), was implicated as the first hypertension susceptibility gene in a GWAS in whites by Wang et al.4 This study revealed the association of 2 single-nucleotide polymorphisms (SNPs; rs3754777A>G in intron 5 and rs6749447 in intron 1) with BP increase. Subsequently, a third polymorphism, rs35929607 in STK39 intron 2, was identified as a hypertension-associated variant,9 and this SNP was in complete linkage disequilibrium with rs3754777 4 After several replicating10–13 and conflicting14,15 findings reporting the link between these SNPs and hypertension in various races or populations, a meta-analysis confirmed that rs3754777 is significantly associated with essential hypertension after adjustments for multiple confounders.16
We have previously shown that the with-no-lysine kinase-oxidative stress responsive 1/SPAK–solute carrier family 12A (SLC12A) signaling cascade plays a pivotal role in the regulation of BP under normal and pathogenic conditions.\textsuperscript{15–21} In particular, phosphorylation and activation of the SLC12A cotransporters (Na–Cl cotransporter/Na–K–Cl cotransporter 1/2 [NKCC1/NKCC2]) have been shown to depend completely on SPAK and oxidative stress responsive 1 activation.\textsuperscript{4} However, the biochemical functions of these polymorphisms have been speculated to modulate the function of these cotransporters, resulting in BP elevation.\textsuperscript{4} However, the biochemical functions of these polymorphisms and the mechanisms of their contribution to hypertension remain to be clarified.

In this study, to investigate whether SPAK itself and the downstream targets are modulated by this polymorphism, we generated \textit{STK39} rs3754777 knockin human embryonic kidney (HEK293T) cell lines using the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system, a recently developed genome-engineering technology with high efficiency.\textsuperscript{22–24} We report that the rs3754777 knockin cell lines exhibited increases in \textit{STK39} mRNA and protein expression and increased phosphorylation of SPAK, leading to increased phosphorylation of NKCC1. These findings suggest that the common SNP rs3754777 in \textit{STK39} intron 5, with allele frequency as high as 10% to 30% globally,\textsuperscript{4,5,16} increases BP via activation of SPAK and target cation–chloride cotransporters. To the best of our knowledge, this study is the first to clarify the biochemical function of the essential hypertension-associated polymorphism rs3754777 by the generation of knockin human cell lines using the CRISPR/Cas9 system. Here, we also showed the efficacy of this methodology for functional analyses of causative variants, including intrinsic SNPs.

**Materials and Methods**

**Plasmid Construction**

A plasmid encoding hCas9-D10A and a guide RNA cloning vector were purchased from Addgene (no. 41816 and no. 41824, respectively). Using the CRISPR Design Tool (http://crisp.mit.edu), 23-bp sequences of 2 pairs of single-guide RNAs (sgRNAs A and B) for the downstream targets were designed and incorporated into gRNA cloning vectors using Gibson Assembly (New England Biolabs Inc) according to the sgRNA synthesis protocol provided by the Church laboratory at Harvard Medical School. A 100-nt donor ssODN (Table 1) containing the target SNP was purchased from Eurofins Genomics (Japan).

**Cell Culture and Transfection**

Cell culture and transfection were performed according to a previous protocol.\textsuperscript{23} HEK293T cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37°C and 5% CO\textsubscript{2} in a humidified incubator. HEK293T cells were seeded in 6-well plates 24 hours before transfection and were ≈40% confluent at transfection. The cells were transfected with a 0.1-μg Cas9-D10A plasmid, a 0.1-μg donor ssODN using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The cells were split into 3 identical 100-mm dishes at 90% confluency 3 days post transfection. After a 3-day additional incubation, genomic DNA was extracted with DNeasy (Qiagen).

**Isolation of Monoclonal Knockin Cell Lines**

Before cell isolation, we assessed the targeted knockin efficacy using next-generation sequencing to determine whether it was feasible to isolate knockin cells from heterogeneous transfected cells. The genomic region around the targeted SNP of DNA extracted from the heterogeneous transfected cells was amplified by polymerase chain reaction (PCR), and ultradep deep sequencing of the amplicons was performed with a MiSeq Personal Sequencer (Illumina, San Diego, CA). Knockin efficiency was ≈1%. A 2-step cell isolation was performed using PCR amplification with a specific primer set (Tables 1 and 2, 666-bp amplicons) followed by 37°C overnight digestion with a 4-base cutter, XspI (Takara Bio Inc, Otsu, Japan; Figure 1A and 1B). The 666-bp PCR amplicon of the targeted knockin cell line was digested into 2 bands of 232 and 434 bp with XspI. First, heterogeneous cells that were transfected with Cas9-D10A plasmid, sgRNA cloning vectors A and B, and a donor ssODN were individually split into 96-well plates at a concentration of 10 cells/well and heterogeneous colonies containing targeted cells in high proportions were isolated using PCR amplification and XspI digestion. Subsequently, monoclonal knockin cell lines were further isolated by picking single colonies using a similar isolation method.

**Immunoblotting**

The wild-type (WT) and knockin HEK293T cell lines were grown in a 6-well plate for 24 hours, and 16 hours after serum starvation they were lysed with lysis buffer (50 mmol/L Tris–HCl [pH 7.5], 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, 1% Triton X-100, 0.27 mol/L sucrose, 1 mmol/L dithiothreitol, and protease inhibitor cocktail [Roche Diagnostics]) for 30 minutes at 4°C. Lysates were centrifuged at 12000g for 5 minutes, and supernatants were diluted with 2× SDS sample buffer (Cosmo Bio USA) and denatured at 60°C for 20

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**Table 1. Primer Sequences Used for CRISPR/Cas9-Genome Engineering**

<table>
<thead>
<tr>
<th>CRISPR/Cas9 System</th>
<th>A pair of 60-nt</th>
<th>ssODNs containing</th>
<th>sgRNA-A</th>
<th>A pair of 60-nt</th>
<th>ssODNs containing</th>
<th>sgRNA-B</th>
<th>100-nt Donor</th>
<th>ssODN</th>
</tr>
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<tr>
<td></td>
<td>5′-TTTCTTGCGTTATATATCT</td>
<td>TGGGAGAAAGCAGAAACCC</td>
<td>GTAAAAAGCCACGAGACTCC-3′</td>
<td>5′-TTTCTTGCTATATATCT</td>
<td>TGGGAGAAAGCAGAAACCC</td>
<td>GGAGTCTGTTGCTTTTAC-3′</td>
<td>5′-CAACATTCCACTTCTCCCCCGATCTGCTGCTTTCTACCCAGAGCACGAG</td>
<td>TGGTGAAGAGGACAGAGACTCCG-3′</td>
</tr>
</tbody>
</table>

CRISPR indicates clustered regularly interspaced short palindromic repeats; sgRNA, single-guide RNA; and ssODN, single-stranded DNA oligonucleotide.
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minutes. The protein extracts were separated by SDS–PAGE, electrically transferred to a nitrocellulose membrane, and probed with the following primary antibodies: rabbit antiphosphorylated SPAK,26 rabbit anti-SPAK (Cell Signaling, Danvers, MA), rabbit antiphosphorylated NKCC1 (T206),20 mouse anti-NKCC1 (T4; Hybridoma Bank, University of Iowa, Iowa City, IA), and rabbit antiactin antibody (Cytoskeleton, Denver, CO). Alkaline phosphatase–conjugated anti-IgG antibodies (Promega, Madison, WI) were used as secondary antibodies. Band densities of the proteins were quantified using ImageJ software (National Institutes of Health).

Quantitative Real-Time PCR

Total RNA was extracted with the RNeasy Mini Kit (Qiagen) from cultured cells according to the manufacturer’s instructions and treated with RNase-free DNase Set (Qiagen). cDNA was produced with ReverTra Ace (TOYOBO, Japan). Quantitative real-time PCR was performed on a Thermal Cycler Dice Real Time System Lite TP700 (Takara Bio Inc, Otsu, Japan) using SYBR Premix Ex Taq II (Takara Bio Inc). The primers used in this study are described in Table 2. PCR amplification consisted of 40 cycles at 95°C for 5 s and at 60°C for 30 s after an initial denaturation step at 95°C for 30 s. All reactions were performed in duplicate, and the relative mRNA expression levels of each target gene were normalized with β-actin as an internal control.

Data Analysis

Statistical analyses were performed using 1-way ANOVA followed by Bonferroni test among >2 groups or unpaired t test between 2 groups. All data are presented as mean±SEM, and P<0.05 was considered to be statistically significant.

Table 2. Primer Sequences Used for Quantitative Real-Time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<tr>
<td>ACTB (β-actin)</td>
<td>5'-TGGGACGCGACAGGAGATGC-3'</td>
<td>5'-TCCACACGGGATCTTGCACGC-3'</td>
</tr>
<tr>
<td>STK39 exons 5–6</td>
<td>5'-GGGTGAGGATGGTCTGACGATC-3'</td>
<td>5'-CATGACCTGGAGGACGATCC-3'</td>
</tr>
<tr>
<td>STK39 exons 1–3</td>
<td>5'-ACGGGTACGACGAGCTCAGG-3'</td>
<td>5'-TGCTGACGACTCATGAGGC-3'</td>
</tr>
<tr>
<td>STK39 exons 13–15</td>
<td>5'-TCTTGGTGCCGGAACTGCG-3'</td>
<td>5'-AGCAAGAGAGCTCTTCAGG-3'</td>
</tr>
<tr>
<td>SLC12A2 (NKCC1)</td>
<td>5'-GATCACTACGGCCACAGAC-3'</td>
<td>5'-CCCCATTGCAAAGAGCATCC-3'</td>
</tr>
</tbody>
</table>

NKCC1 indicates Na–K–Cl cotransporter isoform 1; PCR, polymerase chain reaction; SLC12A2, solute carrier family 12 (sodium/potassium/chloride transporter), member 2; and STK39, serine threonine kinase 39.

Figure 1. Generation of human embryonic kidney (293T) cell lines carrying the hypertension-associated serine threonine kinase 39 (STK39) polymorphism, rs3754777, using the clustered regularly interspaced short palindromic repeats/Cas9 system. A, Outline of the targeting strategy for generating knockin cell lines using a Cas9-D10A expression vector, a 100-bp donor single-stranded DNA oligonucleotide (ssODN), and a pair of constructed single-guide RNA (sgRNA) encoding vectors targeting single-nucleotide polymorphism located in intron 5. The 666-bp polymerase chain reaction amplification region for XspI digestion is shown. B, Amplification of genomic DNA with specific primers yielded a 666-bp DNA fragment. In this fragment, an XspI site (rs3754777) is located 232 bp from the 5' end of the forward primer. Agarose electrophoresis shows the establishment of heterozygous and homozygous knockin cell lines. C, Sequencing analysis of the 666-bp amplicon reveals heterozygous and homozygous single-base substitutions of G to A. *Indicates a point mutation.
Results
Generation of the 2 HEK293T Cell Lines Carrying the Homozygous and Heterozygous Variant in STK39 Intron 5
To precisely validate the effect of the hypertension-associated SNP (rs3754777) in the STK39 gene on biochemical function in cultured cells, we generated knockin cell lines carrying SNP (A>G) in the STK39 gene using the CRISPR/Cas9-mediated genome-editing system (24–26). In this system, we prepared a double-nickase (Cas9-D10A) plasmid and sgRNA plasmids for the double nicking–targeted sites flanking SNP and ssODN that provide a mutated allele sequence. An outline of the targeting strategies is presented in Figure 1A.

Targeted cells were isolated by PCR amplification followed by Xspl digestion at a specific site surrounding the targeted SNP (Figure 1A and 1B). We finally succeeded in establishing 2 knockin cell lines carrying the targeted mutation (G>A) with heterozygous and homozygous alleles. The single-base substitution of G to A in each cell line was confirmed by conventional Sanger sequencing (Figure 1C).

STK39 mRNA Expression Was Increased in rs37354777 Knockin Cell Lines
When an intronic SNP affects the expression levels of the gene containing the intron, SNP often causes alternative transcription.27 We first investigated whether alternative splicing of STK39 occurred in knockin cells. Amplicon sizes of reverse transcription PCR spanning exons 5 to 7 were similar between WT and knockin cell lines (Figure 2A). Furthermore, as shown in Figure 2B, we conducted Sanger sequencing for...

![Image](http://hyper.ahajournals.org/)

Figure 2. Serine threonine kinase 39 (STK39) mRNA expression is increased in rs3754777 knockin cell lines. A, Reverse transcription polymerase chain reaction (RT-PCR) fragment size of STK39 exons 5 to 7 shows no difference among the groups. B, Sequences of the RT-PCR fragment of each knockin cell lines were identical to that of the wild-type (WT). C, Quantification of the STK39 exon 5 to 6, 1 to 3, and 13 to 15 transcripts and Na–K–Cl cotransporter isoform 1 (NKCC1) by real-time RT-PCR (n=6 per experimental group). The homozygous knockin cell line shows the largest increase in STK39 transcription, followed by the heterozygous cell line. mRNA levels are normalized by β-actin. Values are presented as mean±SEM. *P<0.05; **P<0.005 vs the WT cell line. §P<0.05 versus the heterozygous cell line.)
those amplicons and confirmed that no insertions or deletions were present in the boundary regions between exons 5 and 6 or 7 and 8. We also confirmed that the sequence of exon 6 was identical between groups. This finding indicated that alternative splicing, including emergence of cryptic exons, had not occurred in heterozygous or homozygous knockin cell lines. Furthermore, we evaluated the STK39 mRNA expression level in each cell line by quantitative reverse transcription PCR. We found that the STK39 mRNA expression level was significantly increased in knockin cells. As shown in Figure 2C, we prepared 3 pairs of primers for quantification (one for exons 5–6 and the others for exons 1–3 and 13–15), and a similar increase in STK39 mRNA expression was observed in all assays. The homozygous cell line showed the largest increase followed by the heterozygous cell line. No differences in NKCC1 transcript abundance were observed among the groups. To examine whether β-actin mRNA expression influenced the results of STK39 expression, we measured the absolute β-actin expressions as well as the relative STK39 expression (exons 5–6) normalized to GAPDH expression. As shown in Figure S1 in the online-only Data Supplement, absolute mRNA expression of Actb, encoding β-actin, was not significantly different among the groups. When normalized to GAPDH, the largest increase in STK39 mRNA level remained significant (Figure S1), and the increase appeared larger than when normalized to β-actin expression (Figure 2).

Moreover, to determine whether the SNP-induced increase in STK39 transcripts is based on upregulation of transcription or post-transcriptional modulations, mRNA stability was assessed through actinomycin D treatment. As shown in Figure S2, the percentages of remaining STK39 mRNA were not significantly different between the WT and homozygous knockin cell line at every time point after exposure to actinomycin D. This finding suggests that this intronic SNP increases STK39 mRNA expression level via increase in transcription.

STK39 rs3754777 Knockin Cell Lines Showed Increases in Phosphorylated and Total SPAK and in Phosphorylated NKCC1

To determine whether SNP affects phosphorylation level or total protein expression of SPAK, we performed immunoblot analysis. We further investigated whether phosphorylation levels or total expression of NKCC1, widely recognized as the substrate of SPAK, was modulated in the knockin cell lines.

As shown in Figure 3A and 3B, total (t) and phosphorylated (p) SPAK were increased in both knockin cell lines compared with the WT. Increase in phosphorylated SPAK seemed to depend on its increased expression, given that phosphorylation levels adjusted by total expression were not significantly different among the groups (Figure 3B). Furthermore, phosphorylated NKCC1 was significantly increased in both knockin cell lines compared with the WT. The most marked increases in pSPAK, tSPAK, and pNKCC1 were observed in the homozygous cell line followed by the heterozygous cell line. These findings indicate that SNP increases SPAK protein expression, resulting in increased phosphorylation of SPAK.

Figure 3.

Activation of STE20/SPS1-related proline/alanine-rich kinase (SPAK)–solute carrier family 12A cascade in rs3754777 knockin cell lines. A, Representative immunoblots of total (t) and phosphorylated (p) SPAK, and Na–K–Cl cotransporter isoform 1 (NKCC1). B, Densitometric analysis of immunoblots (n=6 per experimental group) showed the increased pSPAK, tSPAK, and pNKCC1 in both the knockin cell lines. The largest increases of these molecules are observed in the homozygous cell line. Values are presented as mean±SEM. *P<0.05; **P<0.005 vs the wild-type cell line.
and NKCC1, and that the activated SPAK–SLC12A signaling is primarily accounted for by increased STK39 transcripts.

To determine whether the activation of the SPAK–SLC12A cascade because of this SNP is observed in other multiple independent clones and to confirm this biochemical function of the SNP, we additionally generated 5 homozygous knockin cell lines using the CRISPR/Cas9 system. Multiple WT cell lines that were subjected to these procedures were also isolated, and used as control cell clones for comparison with the knockin clones. As shown in Figure S3, tSPAK, pSPAK, and pNKCC1 expression level was also significantly increased in these clones. Consistent findings with those in a single homozygous and heterozygous clone (Figure 3) represent more reliable evidence, suggesting STK39 rs3754777-induced activation of SPAK–SLC12A cascade. We also compared the protein expression of these control cell lines with that of the WT cell line that was used as the control in the initial experiments and was not subjected to transfection and isolation procedures. Figure S4 shows that pSPAK and pNKCC1 expression was highly similar between the WT cell line used as the control in the initial experiments (WT) and the multiple control clones, which were subjected to transfection and isolation procedures (control). This finding indicates that transfection procedures did not alter the cell functions.

### Discussion

Over the past several years, GWASs have revealed a host of new loci associated with susceptibility to a wide variety of complex diseases. They represent a highly efficient approach to identify novel disease susceptibility genes. However, contrary to expectation, few hypertension susceptibility genes have been identified. Several explanations include the presence of fewer risk alleles with large effects on hypertension than those found for other diseases, or complex multiple underlying diseases in hypertensive patients. These underlying diseases can be strong confounders that influence BP and affect results of GWAS analyses. STK39, encoding SPAK, was implicated as one of the limited number of hypertension susceptibility genes; however, contrary to expectation, few hypertension susceptibility genes have been identified. Several explanations include the presence of fewer risk alleles with large effects on hypertension than those found for other diseases, or complex multiple underlying diseases in hypertensive patients. These underlying diseases can be strong confounders that influence BP and affect results of GWAS analyses. STK39, encoding SPAK, was implicated as one of the limited number of hypertension susceptibility genes; however, contrary to expectation, few hypertension susceptibility genes have been identified.

We generated homozygous and heterozygous knockin human cell lines carrying the essential hypertension-associated STK39 polymorphism rs3754777 in intron 5 by CRISPR/Cas9-mediated genome engineering and found that this polymorphism increases SPAK mRNA expression and the protein levels of total and phosphorylated SPAK, leading to increased phosphorylation of NKCC1. These findings suggest that this SNP causes activation of the SPAK–SLC12A signaling, resulting in increased sodium reabsorption and BP increase. To the best of our knowledge, this is the first study to investigate the biochemical function of a hypertension-associated STK39 polymorphism and may explain hypertension susceptibility in subjects carrying this polymorphism.

Of interest is that the homozygous cell line showed the most remarkable increases among SPAK transcripts, phosphorylated SPAK, and NKCC1 followed by the heterozygous cell line. This finding is compatible with the observation of human essential hypertension, in which subjects with the homozygous allele were more frequently hypertensive than those with the heterozygous allele. Moreover, we could exclude the possibility of nonspecific effects of genome editing using CRISPR by investigating 2 independent knockin cell lines. In general, causative SNPs detected with GWAS are tag SNPs and it is possible that a nearby SNP in linkage disequilibrium is the true causal variant. However, considering that the tag SNP knocked-in in our study actually increased SPAK transcription, we infer that this SNP is the causal SNP. We and other groups have previously shown that the with-no-lysine kinase–SPAK–Na–Cl cotransporter/NKCC signaling cascade plays a pivotal role in BP regulation in the kidney and aorta. This study suggests that subjects with rs3754777 develop an activation of this cascade primarily based on increased SPAK transcription, leading to hypertension. A previous study showed a link between genetic variants and salt-sensitive BP elevation in Korean population and identified this SNP as one of the variants associated with salt sensitivity. However, the physiological mechanism whereby alterations in these pathways promote salt-sensitive hypertension have not been resolved and it is not clear whether the mutations cause salt sensitivity through effects of the mutation on renal tubular sodium reabsorption or on vascular responses to salt or both.

Another advantage of this study was the use of genome editing with the CRISPR/Cas9 system. When the targeted SNP is a deep intronic mutation, similar to that in our case, it is usually not easy to experimentally investigate the biochemical effects of the mutation. CRISPR/Cas9 system, a recently established technology, enabled such an investigation. It represents a highly efficient tool with respect to ease of design and high specificity for targeted sites via DNA–RNA instead of DNA–protein recognition, compared with zinc-finger nucleases or transcription activator–like effector nucleases. In this study, we selected a double-nicking strategy, in which the D10A mutant Cas9 nickase (Cas9-D10A) is specifically guided to the target site by a pair of appropriately spaced guide RNAs and introduces single-strand nicks on both strands of the genomic DNA; this is in contrast to an sgRNA-guided double-stranded break at the target site by the WT Cas9 nuclease. This difference results in high genome-editing specificity, and the double-nicking strategy minimizes off-target activity. Furthermore, it was not necessary to insert a selection cassette, such as a green fluorescent protein cassette, because a restriction enzyme digestion-based selection method could be adopted. This strategy made it possible to analyze the effects of SNP alone. We have shown that the CRISPR/Cas9 system offers an efficient strategy for generation of knockin cell lines and functional analyses of disease causal variants acquired from GWAS data even if they are located in introns. The sequence around this polymorphism is not conserved in other animal models such as rodents, and thus it may be difficult to study in vivo function of this SNP in rodent models, such as mice.

Although we have suggested the activation of SPAK–SLC12A signaling primarily based on increased STK39 transcription, the biological mechanism of the regulation of STK39...
transcription by this intronic SNP is unknown. Reverse transcription PCR and sequencing analysis showed no insertions or deletions in cDNA sequence and no emergence of cryptic exons in either homozygous and heterozygous knockin cell lines (Figure 2A and 2B), suggesting that splicing abnormalities because of this polymorphism are unlikely. Furthermore, transcriptional regulatory elements such as enhancers are unlikely to be located near this SNP, given the absence of binding sites of transcription factors or microRNAs in in silico analyses.

**Perspectives**

This study showed that the hypertension-associated STK39 polymorphism rs3754777 causes increased STK39 mRNA expression, leading to the activation of an SPAK–SLC12A cascade. Activation of the target cation–chloride cotransporters in the kidneys and arteries may be responsible for hypertension susceptibility in subjects with this polymorphism. To the best of our knowledge, this is the first report to clarify the biochemical function of a hypertension-associated STK39 polymorphism and may explain hypertension susceptibility in subjects carrying this polymorphism.

**Acknowledgments**

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**References**


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**Novelty and Significance**

**What Is New?**

- This is the first study to identify the biochemical function of the essential hypertension-associated intronic single-nucleotide polymorphism rs3754777 by generation of knockin human cell lines using the clustered regularly interspaced short palindromic repeats/Cas9 system.

- The essential hypertension-associated serine threonine kinase 39 (*STK39*) polymorphism rs3754777 activates an STE20/SPS1-related proline/alanine-rich kinase–solute carrier family 12A cascade via increased *STK39* transcription. This finding suggests that increased interactions between STE20/SPS1-related proline/alanine-rich kinase and target cation–chloride cotransporters contribute to hypertension in subjects with this polymorphism.

**What Is Relevant?**

- The *STK39* polymorphism rs3754777 is a global common single-nucleotide polymorphism with allele frequency as high as 10% to 30%. This polymorphism may partially account for human essential hypertension.

- This study indicates that the clustered regularly interspaced short palindromic repeats/Cas9 system provides an efficient strategy for functional analyses of disease causal variants, including deep intronic single-nucleotide polymorphisms.

**Summary**

The essential hypertension-associated *STK39* polymorphism rs3754777 causes increased *STK39* mRNA expression, leading to the activation of an STE20/SPS1-related proline/alanine-rich kinase–solute carrier family 12A cascade. Increased interactions between STE20/SPS1-related proline/alanine-rich kinase and the target cation–chloride cotransporters may contribute to hypertension in subjects with this polymorphism.
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Generation of Hypertension-Associated STK39 Polymorphism Knock-in Cell Lines with the CRISPR/Cas9 System

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Short title: SNP in STK39 activates a SPAK-SLC12A cascade

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Materials and methods

Generation of additional homozygous knock-in cell lines carrying STK39 SNP rs3754777
To generate the additional homozygous knock-in clones, cell culture, transfection, and subsequent isolation of knock-in cell lines were performed as described in the Materials and methods. Multiple wild-type cell lines that were subjected to these procedures were also isolated, and used as the control cell clones for comparison to the knock-in clones. To examine the protein expressions of total (t) and phosphorylated (p) STE20/SPS1-related proline/alanine-rich kinase (SPAK) and Na–K–Cl cotransporter isoform 1 (NKCC1) in the control and knock-in clones, immunoblotting analysis was performed as described in the Materials and methods. Moreover, to determine whether unexpected effects of the transfection procedures or transfected vectors influence the results in the initial experiments, we also compared the protein expression of these control cell lines with that of the wild-type cell line, which was used as the control in the initial experiments and was not subjected to transfection and isolation procedures.

STK39 mRNA stability assay
To examine STK39 mRNA stability, cells were treated with DMEM containing actinomycin D (5 μg/ml), an inhibitor of transcription, dissolved in dimethylsulfoxid (DMSO). At several time points (0hr, 6hr, 12hr, 24hr) after exposure to actinomycin D, the decay of STK39 mRNA was measured by quantitative real time PCR with specific primers for STK39 transcripts exons 5-6 (Table 1).

Data analysis
Statistical analyses were conducted using one-way ANOVA with Bonferroni’s test among more than two groups or unpaired t test between two groups. All data are presented as mean ± SEM, and P < 0.05 was considered to be statistically significant.
Results

*STK39* SNP rs3754777-induced increases in phosphorylated and total SPAK and phosphorylated NKCC1 were shown in multiple independent knock-in clones

To confirm the biochemical function of this intronic SNP on activation of the SPAK-SLC12A cascade, we additionally generated homozygous knock-in cell lines using the CRISPR/Cas9 system. As shown in Figure S3A, five additional clones were isolated by PCR amplification followed by XspI digestion at a specific site surrounding the targeted SNP. The homozygous clone 1 is the one in the initial experiment. The 5 control wild-type clones were also purified using these procedures.

Figure S3B shows the representative immunoblots in multiple independent clones with the wild-type allele or polymorphism. As shown in Figure S3C, the mean values of tSPAK, pSPAK, and pNKCC1 expression level in multiple clones were significantly higher in the homozygous knock-in clones than the control. These consistent findings with the initial experiments in a single homozygous and heterozygous clone (Fig. 2 and 3) represents more reliable evidence of *STK39* rs3754777-induced activation of SPAK-SLC12A cascade.

Furthermore, Figure S4 shows that pSPAK and pNKCC1 expression was almost similar between the wild-type cell line which was used as the control in the initial experiments (WT) and the multiple wild-type clones which were subjected to transfection and isolation procedures (Control). This finding indicates that transcription procedures or transfected vectors did not particularly alter the cell functions.

Not modulation of mRNA stability, but upregulation of transcription is likely to explain the increased *STK39* transcripts due to the SNP rs3754777

To determine whether the SNP-induced increase in *STK39* transcripts is based on upregulation of transcription or post-transcriptional modifications, mRNA stability was examined through actinomycin D treatment at several time points.

As shown in Figure S2, the percentages of remaining *STK39* mRNA were not significantly different between the wild-type and homozygous knock-in cell line at every time point after exposure to actinomycin D. This finding suggests that this intronic SNP is likely to increase *STK39* mRNA expression level via increase in transcription.

*STK39* mRNA expression was increased in the rs3754777 knock-in cell lines independently of housekeeping gene expression
To examine whether β-actin mRNA expression influenced the results of STK39 expression (Fig. 2), we measured the absolute β-actin expressions among the wild-type, heterozygous and homozygous knock-in cell lines as well as the relative STK39 expression normalized to GAPDH expression.

As shown in Figure S1, absolute mRNA expression of Actb, encoding β-actin, was not significantly different among the groups. Furthermore, if normalized to GAPDH, the largest increase in STK39 mRNA level remained significant (Fig S1), and the increase is much larger than when normalized to β-actin expression (Fig. 2).
Figure S1. STK39 mRNA expression is increased in the rs3754777 knock-in cell lines independently of housekeeping gene expression.

Absolute β-actin expression was not significantly different among the wild-type, heterozygous, and homozygous knock-in cell lines (n = 6 per experimental group). The homozygous knock-in cell line exhibited the largest increase in STK39 mRNA level moralized to GAPDH (n = 6 per experimental group). Values are presented as mean ± SEM. *P < 0.005 versus the wild-type cell line. §P < 0.005 versus the heterozygous cell line.
Figure S2. *STK39* mRNA stability assay.

The percentages of remaining *STK39* mRNA were not significantly different between the wild-type and homozygous knock-in cell line at every time point after exposure to actinomycin D (5 μg/ml). Open circles represent the mRNA expression levels in the wild-type cell line, and solid circles represent the values in the homozygous knock-in cell line. Values are presented as mean ± SEM from duplicates of 4 independent experiments.
**Figure S3.** Activation of SPAK-SLC12A cascade in multiple independent rs3754777 knock-in clones.

A, Amplification of genomic DNA with specific primers yielded a 666-bp DNA fragment. In this fragment, an XspI site (rs3754777) is located 232 bp from the 5′ end of the forward primer. Agarose electrophoresis shows 5 control clones and 6 homozygous knock-in clones. B, Representative immunoblots of total (t) and phosphorylated (p) SPAK, and NKCC1. C, Densitometric analysis of immunoblots (n = 5 in control clones, and n = 6 in homozygous knock-in clones) showed the increased pSPAK, tSPAK, and pNKCC1 in knock-in clones. Values are presented as mean ± SEM. *P < 0.05 versus the control clones.
**Figure S4.** Similarity of protein expressions between the wild-type cell line and the isolated control clones.

Representative immunoblots of phosphorylated SPAK, and NKCC1. These protein expressions are highly similar between the wild-type cell line which was used as the control in the initial experiments (WT) and the multiple independent wild-type clones which were subjected to transfection and isolation procedures (Control).