A wide range of antiviral drugs is currently available; however, drug-resistant viruses have begun to emerge and represent a potential public health risk. Here, we explored the use of compounds that inhibit or interfere with the action of essential host factors to prevent virus replication. In particular, we focused on the cyclin-dependent kinase 9 (CDK9) inhibitor, FIT-039, which suppressed replication of a broad spectrum of DNA viruses through inhibition of mRNA transcription. Specifically, FIT-039 inhibited replication of herpes simplex virus 1 (HSV-1), HSV-2, human adenovirus, and human cytomegalovirus in cultured cells, and topical application of FIT-039 ointment suppressed skin lesion formation in a murine HSV-1 infection model. FIT-039 did not affect cell cycle progression or cellular proliferation in host cells. Compared with the general CDK inhibitor flavopiridol, transcriptome analyses of FIT-039–treated cells revealed that FIT-039 specifically inhibited CDK9. Given at concentrations above the inhibitory concentration, FIT-039 did not have a cytotoxic effect on mammalian cells. Importantly, administration of FIT-039 ameliorated the severity of skin lesion formation in mice infected with an acyclovir-resistant HSV-1, without noticeable adverse effects. Together, these data indicate that FIT-039 has potential as an antiviral agent for clinical therapeutics.

**Introduction**

The number of available antiviral drugs has increased continually since the development of azidothymidine and acyclovir (ACV) in the 1970s (1, 2). However, this has resulted in the emergence of drug-resistant viruses, including HIV, hepatitis B, and the herpes simplex virus (HSV), which cause drug failures (3, 4). Although the drug resistance of HSV to ACV and related drugs, such as foscarnet or valacyclovir, rarely occurs in immunocompetent individuals, it has been more commonly reported in immunocompromised patients, including patients with HIV and transplant recipients (5). A focus on host cellular proteins (6–8) is necessary to overcome drug resistance to antiviral agents, because viruses often change their genomic sequences and acquire drug resistance (4). We have developed chemical inhibitors that target host kinases as antiviral agents, which prevent the replication of several viruses, including the hepatitis C virus, dengue virus, and Sindbis virus (9–12). To develop novel drugs, we have been recently focusing on cyclin-dependent protein kinases (CDKs), which are known to be used for virus replication (13–16).

CDKs comprise a family of serine/threonine kinases that play essential roles in the regulation of multiple cellular processes and can be divided into two major groups: CDK1, CDK2, CDK3, CDK4, and CDK6 are directly involved in cell cycle regulation, while CDK5, CDK7, CDK8, CDK9, CKD11, CDK12, and CDK13 have been implicated in the control of gene transcription (17, 18). The virus-specific regulation of CDK activity has been reported, as described below. CDK1 was shown to activate the expression of HSV-1 late genes (19), while CDK2 promoted the replication of adenovirus, papillomavirus, and herpes virus DNAs (7). CDK7 and CDK9 were shown to activate viral RNA transcription in HIV and HSV (7, 14, 15, 20–24). Feichtinger et al. recently described the interaction between CDK9/cyclin T1 and viral mRNA export factor pUL69 in human cytomegalovirus (HCMV), which belongs to the β subgroup of Herpesviridae (20). In mammalian cells, CDK9/cyclin T1 (or T2) is part of the subunit of the positive transcription elongation factor b (P-TEFb) complex and reportedly initiated the transcriptional elongation of genes by phosphorylating the carboxyterminal domain (CTD) of RNA polymerase II at Ser2 (25). These findings indicate that CDKs are also used in viral DNA replication and transcription.

Flavopiridol is a pan-specific CDK inhibitor that has been shown to inhibit CDK1, CDK2, CDK4, CDK5, CDK6, CDK7, and CDK9 and suppress the replication of HSV, HCMV, human adenovirus (HAdV), and HIV (15). Roscovitine, an oligo-specific CDK inhibitor that inhibits CDK1, CDK2, CDK5, CDK7, CDK9, and CDK12, suppresses the transcription of immediate-early genes (IEGs) of HCMV and prevents viral replication in vitro (14, 26–28). However, these broad-spectrum CDK inhibitors often negatively affect cell cycle progression and elicit adverse effects, which lead to difficulties in the development of antiviral drugs (29).

In this study, we developed the CDK9 inhibitor, FIT-039, which suppressed the replication of HSV and other DNA viruses, including...
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HCMV and HAdV. It also suppressed the replication of ACV-resistant HSV-1, not only in vitro, but also in vivo. Our ongoing preclinical studies have shown no significant adverse effects. These findings suggest that FIT-039 is a promising compound for the therapeutic treatment of drug-resistant HSVs and other DNA viruses.

Results

Requirement of CDK9 for HSV-1 replication but not for host cell cycle progression. To establish whether CDK9 was required for HSV-1 replication, we investigated the replication level of HSV-1 in HeLa cells transfected with CDK9 siRNA or scramble dsRNA as a negative control (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI73805DS1). The cells were infected with HSV-1 at a MOI of 0.1 PFU per cell, and the copy number of HSV-1 genomic DNA was measured using realtime PCR. Knockdown of CDK9 significantly suppressed the replication of HSV-1 genomic DNA (Supplemental Figure 1B), which demonstrated the necessity for CDK9 in the replication of HSV-1. In addition, knockdown of CDK9 had a similar effect on cell cycle progression to that of the negative control, whereas CDK4 reduced the number of S-phase cells (Supplemental Figure 1, C and D). These results support our hypothesis that CDK9 is a potential target for anti-HSV-1 drug discovery.

Identification of a CDK9-selective inhibitor. To obtain a selective inhibitor for CDK9, we performed in vitro screening assays on our original synthetic chemical library to identify small molecules that suppressed the kinase activity of CDK9 but not that of CDK4. As a result, we found a good candidate, N-[5-fluoro-2-(1-piperidinyl)phenyl]sonicotiniothioamide (Figure 1A), which we refer to herein as FIT-039 (Figure 1A), which we refer to herein as FIT-039. This compound suppressed the in vitro kinase activity of CKD9/cyclin T1 with an IC50 value of 5.8 μM but did not have a marked inhibitory effect on CDK4/cyclin D3-up to 30 μM (Figure 1B). FIT-039 also did not inhibit other CDKs, including CDK2/cyclinB1, CDK2/cyclinE1, CDK5/p25, CDK6/cyclinD3, or CDK7/cyclinH/MAT (Supplemental Figure 2). We checked the effect of FIT-039 on other kinases and found that FIT-039 potently suppresses 8 kinases (GSK3ß, PKN1, haspin, p70s6K, DYRK1B, GSK3α, IRR, and DYRK3) other than CDK9 on the 332-kinase panel (Figure 1C and Supplemental Table 1).

To examine whether FIT-039 bound to the ATP-binding pocket of CDK9, we analyzed the inhibitory action of FIT-039 on CKD9/cyclin H/MAT (Supplemental Figure 2). We checked the effect of FIT-039 on host cellular transcriptome. HeLa cells were infected with HSV-1 at a MOI of 0 or 0.1 PFUs per cell and then were treated with FIT-039 or flavopiridol for 3 hours. FIT-039 decreased phosphorylated CTD in the infected or noninfected cells to a level lower than that shown by flavopiridol (Figure 2A). Accordingly, FIT-039 reduced the expression levels of HSV-1 IEGs and early and late genes (Figure 2B and Supplemental Figure 4). IEGs are transcribed immediately after the viral infection, and their encoded proteins regulate the transcription of early and late viral genes (30). These results indicated that the antiviral mechanism may be attributed to the inhibition of the viral transcription through suppression of the CTD phosphorylation of RNA polymerase II.

Effect of FIT-039 on transcriptome of host cells. We next evaluated the effect of FIT-039 on host cellular transcriptome. HeLa cells were incubated with FIT-039 or flavopiridol at the concentration of IC50 for 24 hours, and cellular RNAs were collected and subjected to microarray analysis. The solvent DMSO was used as a negative control. We compared divergences in gene expression between FIT-039 and flavopiridol with scatter plots (Figure 2, C and D) and F tests. We found that fold changes in FIT-039/DMSO were smaller than those in flavopiridol/DMSO. The F test showed that the difference between SDs of these 2 data sets was statistically significant (P < 10(-10), Figure 2E), which implied that FIT-039 produced a less
which demonstrated that flavopiridol caused a severe adverse effect. On the other hand, a preclinical study showed that FIT-039 did not affect body weight gain in mice administrated with an overdose of this compound (1,000 mg/kg/d) for 14 days (Figure 2G), and no changes were observed in biological markers in their blood (Supplemental Table 2).

Therapeutic activity of FIT-039 in mice. The therapeutic effect of FIT-039 was evaluated in the murine zosteriform spread model, which mimics recurrent cutaneous herpetic disease. Figure 2F,
Figure 2. FIT-039 inhibited the transcription of HSV-1 IEGs. (A) HEK293 cells were infected with HSV-1 at a MOI of 0.1 PFUs per cell, and then the cells were treated with FIT-039 (30 μM) or flavopiridol (1 μM) for 3 hours. RNA polymerase II was detected by antibody against RNA polymerase II with Western blotting analysis. P-CTD, phosphorylated RNA polymerase II CTD. (B) HSV-1–attached cells were incubated for 3 hours with the indicated compounds. HSV-1 IEGs were analyzed by RT-PCR. (C and D) Overlaid scatter plots comparing mRNA expression. Two comparisons of the expression signals (C) between the FIT-039 and DMSO and (D) between the flavopiridol and DMSO treatments were analyzed. Two-fold variations in expression signals were plotted in red. (E) F tests comparing the divergences of gene expression between FIT-039 (red line) and flavopiridol (blue line). (F) HeLa cells were treated with FIT-039 and flavopiridol at a concentration 10-fold higher than that of the IC_{50} value of anti-HSV-1 for 24 hours. The cells were stained with propidium iodide and analyzed by flow cytometry. (G) Two-week repeated-dose oral toxicity study in rats. FIT-039 (1,000 mg/kg) or the solvent (carboxymethylcellulose) were administrated orally to male rats once a day for 14 days. Body weights and general conditions were determined for 28 days from the first administration. Flavo, flavopiridol.
HSV-2 and HCMV. FIT-039 suppressed the replication of HSV-2 in a dose-dependent manner, and its potency was similar to that of ACV (Figure 4, A and B). FIT-039 also inhibited HCMV genomic DNA replication, similar to ganciclovir (GCV) (Figure 4C). Furthermore, compared with cidofovir (CDV), FIT-039 potently inhibited the genomic DNA replication of HAdV type 5, which was obtained from a patient’s throat (Figure 4D), as well as type 53 (Figure 4E) and type 19 and type 37 (data not shown), which indicated that FIT-039 inhibited the replication of DNA viruses other than Herpesviridae.

We also observed that FIT-039 reduced the expression of E1A, an early gene of HAdV, whereas CDV could not (Figure 4F). These results demonstrated that FIT-039 suppressed the replication of a broad spectrum of DNA viruses.

Therapeutic effect of FIT-039 on mice infected with ACV-resistant HSV-1. We next examined the antiviral effect of FIT-039 on ACV-resistant HSV-1. In the plaque reduction assay, FIT-039 significantly reduced the plaque number in Vero cells infected with ACV-resistant HSV-1, whereas GCV did not, due to cross resistance (Figure 5A). Furthermore, the effect of FIT-039 on ACV-resistant HSV-1 infection was also evaluated in the murine zosteriform spread model, in which treatment with ACV ointment did not prevent the development of lesions (Figure 5B). In contrast, treatment with the FIT-039 ointment prevented zosteriform spread (Figure 5B) and rescued mice from lethality (Figure 5C), which indicated that FIT-039 was also therapeutically effective against ACV-resistant HSV-1.

Discussion
In this study, we successfully identified the novel CDK9 inhibitor FIT-039 and showed that CDK9 is a promising drug target for a broad spectrum of DNA viruses. CDK9 paired with cyclin T1 forms the human P-TEFb complex and stimulates productive transcrip-
tion through phosphorylation of the CTD of RNA polymerase II and viral proteins, such as the ICP22 of HSV. The ICP22-CDK9 complex was reported previously to phosphorylate the CTD of RNA polymerase II and regulate the transcription of HSV (20, 22). A previous study showed that CDK9 was localized to nuclear viral transcriptosomes at the immediate-early stage of the HCMV infection, which allowed the active transcription of HCMV IEGs (14). The suppression of HAdV replication by FIT-039 demonstrated that HAdVs also depend on the CDK9-regulated transcription elongation machinery for their synthesis of viral RNAs. Vijayalingam et al. reported recently that the HAdV large-E1A protein activated viral transcription by recruiting the RNA polymerase II mediator complex (33); however, the effect of CDK9 on large-E1A–mediated transcription remains to be clarified. The above data suggest that these viruses take advantage of the viral protein CDK9 complexes to hijack the host transcription and perform the viral-specific transcription.

The IC_{50} value of FIT-039 for CDK9 inhibition is higher than the EC_{50} value of FIT-039 for anti-HSV-1 activity. The IC_{50} values of kinases show the comparative specificity of the compound but cannot be compared directly with the effect in vivo, because the kinase assay condition may not recapitulate the intracellular environments. However, we cannot deny the possibility that the antiviral activity of FIT-039 is due to a synergistic effect by inhibiting kinase(s) other than CDK9. The CTD phosphorylation is catalyzed by CDK7, CDK8, CDK11, CDK12, and CDK13, in addition to CDK9. Our preliminary experiment using siRNA against these CDKs showed that knockdown of CDK11 or CDK13 suppressed the HSV-1 replication, though the suppression was not as potent as that of CDK9 (data not shown). Furthermore, FIT-039 potently inhibited kinases, as shown in the large-panel kinase screening (see Supplemental Methods and Supplemental Table 1). GSK3 (34), PAK3 (35), DYRK3 (36), Pim1 (37), PKG1 (38), p70s6k (39), Flt3 (40), and MSK1 (41) were reported to be involved in the replication of various viruses. In addition, 6BIO, an inhibitor of GSK3, and its derivatives were reported to suppress the replication of HSV-1 and HIV (42, 43).

Figure 4. FIT-039 suppressed the replication of HSV-2, HCMV, and HAdVs. (A) FIT-039 suppressed the plaque formation caused by the HSV-2 infection in a dose-dependent manner. HSV-2 plaque reduction assay was performed in Vero cells treated with the indicated concentrations of FIT-039 and ACV. (B) FIT-039 suppressed the replication of HSV-2 genomic DNA in a dose-dependent manner. HSV-2 replication was analyzed by real-time PCR at the indicated concentrations of FIT-039. ACV was used as a positive control. (C) FIT-039 suppressed the replication of HCMV genomic DNA in a dose-dependent manner. HCMV replication was analyzed by real-time PCR at the indicated concentrations of FIT-039 and CDV. (D) HAdV type 5 and (E) HAdV type 53 were examined. CDV was used as a positive control. (F) FIT-039 inhibited transcription of the HAdV gene E1A. A549 cells were infected with HAdV-5 and treated with FIT-039 at 10 μM for 6 hours. The cells were subjected to RT-PCR. In B–E, each point represents the average ± SD of the results from 3 experiments performed in duplicate. *P < 0.001, versus DMSO treatment, Student’s t test.
FIT-039 selectively inhibited viral transcription with minimal effect on cellular transcription, indicating the possibility that cellular transcription is regulated not only by CDK9 but also other CDKs, such as CDK7, CDK8, CDK11, CDK12, and CDK13, and that viral gene expression may more depend on the ICP22-CDK9 complex than cellular genes. Thus, it is conceivable that FIT-039 produced less effect on cellular transcription than on viral transcription. This study suggests that FIT-039 is a promising drug against a broad spectrum of DNA viruses.

In addition to DNA viruses, cyclin T1/CDK9 has been shown to interact with RNA viral proteins, such as the influenza virus polymerase of influenza A virus and Tat proteins of HIV, and facilitate its association with cellular RNA polymerase II (44, 45). Flavopiridol inhibited the transcription elongation activity and suppressed replication of the influenza virus (46, 47). The anti-influenza virus activity of FIT-039 was also examined. FIT-039 suppressed the replication of influenza virus H1N1 (Supplemental Figure 6). FIT-039 inhibited the HIV-Tat–dependent transcription (Supplemental Figure 7), which is regulated by CDK9 (48, 49). Furthermore, FIT-039 also suppresses the replication of HIV (M. Okamoto and M. Baba, et al., unpublished observations). These studies demonstrate the possibility that FIT-039 suppresses the replication of RNA viruses as well as DNA viruses. A drug such as FIT-039, which selectively targets the key molecules in host cells and exhibits an antiviral effect on a broad spectrum of viruses, may be able to cope with new types of viruses emerging in the future.

We are now preparing a clinical trial for FIT-039 in patients infected with ACV-resistant HSV-1 and thus investigating toxicities of FIT-039. Although mice with knockout of the target kinases, which FIT-039 potently inhibited in this study, are known to show the phenotypes raised in Supplemental Table 3, our ongoing preclinical studies have indicated that FIT-039 does not induce significant adverse effects under a high-dose oral administration protocol in rats and dogs (Supplemental Figure 8), mutagenic toxicity (Supplemental Tables 4 and 5), or irritation activity on the skin. These results are consistent with the effects of FIT-039 on cells, which does not affect cell cycle progression or cell viability.

Considering the results of toxicity tests, FIT-039 could be available for systemic treatment. However, the topical medicines described in this manuscript have following advantages: (a) the drug is easily delivered to the infectious site, and (b) the risk of adverse effects can be further reduced. Therefore, FIT-039 ointment is also unlikely to cause any side effect in the preclinical study. Furthermore, FIT-039 has an agreeable pharmacokinetic profile (Supplemental Figure 9). These results indicate that FIT-039 could be developed as oral preparations against viral infectious diseases, such as herpetic encephalitis, HCMV infectious mononucleosis,
and HAdV pharyngitis. These outcomes indicate that FIT-039 is a promising novel antiviral agent for clinical therapeutics.

Methods

Viruses and cells. The HSV strains used were HSV-1 (F strain) and HSV-2 (G strain), which were provided by Y. Kawaguchi, University of Tokyo, Tokyo, Japan. The HCMV strain used was the Towne strain, as well as HAdV-19, HAdV-36, and HAdV-53, provided by K. Aoki and S. Ohno, Hokkaido University, Sapporo, Japan. The same procedure was applied to human CDK4 (PDB IDs: 2W9Z, 2W96) but failed to produce a reasonable complex model. Thus, the CDK4 structure was superimposed on that of CDK9 in the CDK9/FIT-039 complex.

Plaque reduction assay. Wild-type and ACV-resistant HSV-infected Vero cells were incubated with 150 PFUs per 1 ml for 1 hour at 37°C, and the culture media were then changed to fresh media containing various concentrations of FIT-039 and ACV. After 2 days of incubation, the infected cells were fixed with 100% methanol and stained with crystal violet solution (2.5% crystal violet, 30% ethanol, and 1% ammonium oxalate). The number of plaques was counted under a microscope.

Quantitative PCR analysis. HeLa cells, HFL-1 cells, and A549 cells were infected with HSVs, HCMV, and HAdVs, respectively, at a MOI of 0.1, with chemical compounds. HSV-, HCMV-, and HAdV-infected cells were incubated for 24, 72, and 24 hours, respectively. After incubation, these cells were harvested and their DNA was isolated. HSVs, HCMV, and HAdVs, genomic DNA, and cellular GAPDH were quantified by real-time PCR. Analyses of HSV-1, HSV-2, HAdV-5, and GAPDH were performed using FastStart Universal Probe Master (ROX) (Roche Applied Science). Analysis of HAdV-19 was performed using FastStart Universal SYBR Green Master (Roche Applied Science). PCR was performed with an initial denaturation reaction at 95°C for 1 minute and then amplified with 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The amplification was monitored on Step One Plus (Applied Biosystems Inc.). The primers used are shown in Supplemental Table 5.

MTT assay. Cytotoxicity was evaluated by the MTT reduction assay. HeLa cells were seeded at a concentration of 1 x 10^4 cells per well in 96-well tissue culture plates and grown at 37°C for 1 day. The culture medium was replaced by fresh medium containing FIT-039 at various concentrations (1–50,000 nM), and cells were grown for a further 24 hours. After incubation, the water-soluble tetrazolium salt, WST-8 (Nacalai Tesque Inc.), solution was added to media. Cells were incubated at 37°C for 2 hours, followed by plate reading on an ARVO X5 microplate reader (PerkinElmer Inc.), using a test wavelength of 450 nm and reference wavelength of 600 nm.

RT-PCR and quantitative RT-PCR analysis. HeLa cells and A549 cells were infected with HSV-1 and HAdV-5, respectively, at a MOI of 0.1, with chemical compounds. HSV-1- and HAdV-5-infected cells were incubated for 3 hours or 6 hours, respectively, following total RNA extraction using Sepasol RNA-I Super (Nacalai Tesque Inc.). Reverse transcription was performed with PrimeScript Reverse Transcriptase (Takara Bio Inc.), using oligo (dT) primer. The IEGs of HSV-1, E1A of HAdV, and cellular GAPDH were then amplified by PCR. PCR was performed using PrimeStar HS DNA Polymerase with GC Buffer (Takara Bio Inc.). PCR was performed with an initial denaturation...
reaction at 95°C for 1 minute and then amplified with 27 cycles of 98°C for 10 seconds, 60°C for 5 seconds, 72°C for 1 minute. This amplification was monitored on a T100 Thermal Cycler (Bio-Rad). The primers used are shown in Supplemental Tables 6 and 7.

**Microarray analysis.** HeLa cells were plated in a 6-cm culture dish at a density of 1 x 10⁶ cells per dish. Cells were incubated with FIT-039 or flavopiridol at 4 μM or 800 nM, respectively, following total RNA extraction using the RNeasy Mini Kit (QIAGEN), per the manufacturer’s instructions. RNA was prepared for microarray expression analysis by the GeneChip 3′ IVT Expression Kit (Affymetrix Inc.) according to the manufacturer’s instructions. The prepared RNA was hybridized and stained onto the Human Genome U133 Plus 2.0 array (Affymetrix Inc.) using the GeneChip Hybridization Wash and Stain Kit (Affymetrix Inc.). Signals were detected using the GeneChip Scanner 3000G (Affymetrix Inc.). These data were analyzed with Perl scripts and a statistical package, R (http://www.r-project.org/).

**Two-week repeat-dose oral toxicity study.** Male 5-week-old Wistar rats were purchased from Charles River Laboratories Inc. FIT-039 (1,000 mg/kg) or the solvent (carboxymethylcellulose) was administered orally to male rats once a day for 14 days, and these animals were followed for 14 days from the final administration. Three rats were assigned to each experimental group. Body weights and general conditions were determined for 28 days from the first administration. Hematology tests were performed and the organ weights were noted on day 28.

**Ointment supplement with chemical compounds.** The 5% ACV ointment, which contained polyethylene glycol, glycerol, and ACV, was provided by Rakool Pharmaceutical Marketing Co. Ltd. Japan. We used 10% lactic acid, in addition to polyethylene glycol and glycerol, to dissolve FIT-039 in ointment.

**Mouse skin HSV-1 or ACV-resistant HSV-1 infection model.** Male 5-week-old ICR mice were purchased from Charles River Laboratories Inc. The left mid-flank of each mouse was depilated with a chemical depilatory hair remover. The naked skin was scratched 1 or 2 days later with a 27-gauge needle, and 20 μl of the HSV-1 suspension of 2 x 10⁴ PFUs was applied to the scratched area. Each ointment was administered to mice (5 mice per group) as follows. For experiment 1 (topical treatment, the effect of different concentrations), FIT-039 prepared in the ointment formulation at concentrations of 1.25%, 2.5%, 5%, and 10% (w/w) was topically applied to the scratched area (30 mg/area/dose) 3 hours after the HSV-1 infection and twice daily for 12 days. For experiment 2 (topical treatment, therapeutic efficacy of FIT-039 in skin ACV-resistant HSV-1-infected mice), 10% FIT-039 ointment was applied topically to the scratched area (30 mg/area/dose) 3 hours after the ACV-resistant HSV-1 infection and twice daily for 12 days. The placebo (ointment base) and 5% ACV ointment applied twice daily were used as control. In both experiments, the development of skin lesions and mortality were continuously observed twice daily for 12 days after the HSV-1 or ACV-resistant HSV-1 infection and scored as follows: 0, no lesion; 2, vesicles in a local region; 4, erosion and/or ulceration in a local region; 6, mild zosteriform lesion; 8, moderate zosteriform lesion; 10, severe zosteriform lesion; 14, death.

**Database repository.** Gene expression profiling data were deposited in GEO (accession no. GSE57429).

**Statistics.** Numerical values are reported as mean ± SD unless stated otherwise. Statistical significance was determined with a 2-tailed Student’s t test. A P value of less than 0.05 was considered significant.

**Study approval.** All animal protocols were reviewed and approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University.

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