

# **Experimental infection of Japanese macaques with simian retrovirus 5**

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34 *Abbreviations:* KUPRI, Kyoto University Primate Research Institute; NIPS, National

35 Institute for Physiological Sciences; SRV, simian retrovirus; NBRP, national

36 bioresource project; HEK, human embryonic kidney; JM, Japanese macaques; BaEV,

37 baboon endogenous virus; MDTF, *Mus dunni* tail fibroblasts; MPMV, Mason-Pfizer

38 monkey virus.

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40 *Repositories:* Nucleotide sequences of pSRV547 has been deposited in GenBank with

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42

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## ABSTRACT

Recently, a large number of Japanese macaques (*Macaca fuscata*) died of an unknown hemorrhagic syndrome at Kyoto University Primate Research Institute (KUPRI) and an external breeding facility for National Institute for Physiological Sciences (NIPS). We previously reported that the hemorrhagic syndrome of Japanese macaques at KUPRI was caused by infection with simian retrovirus 4 (SRV-4); however, the cause of similar diseases that occurred at the external breeding facility for NIPS was still unknown. In this study, we isolated SRV-5 from Japanese macaques exhibiting thrombocytopenia and then constructed an infectious molecular clone of the SRV-5 isolate. When the SRV-5 isolate was inoculated into two Japanese macaques, severe thrombocytopenia was induced in one of two macaques within 22 days after inoculation. Similarly, the clone-derived virus was inoculated into the other two Japanese macaques, and one of two macaques developed severe thrombocytopenia within 22 days. On the other hand, the remaining two of four macaques survived as asymptomatic carriers even after administering an immunosuppressive agent, dexamethasone. As determined by real-time PCR, SRV-5 infected a variety of tissues in Japanese macaques, especially in digestive and lymph organs. We also identified the SRV-5 receptor as ASCT2, a neutral amino acid transporter in Japanese macaques. Taken together, we conclude that the causative agent of hemorrhagic syndrome occurred at the external breeding facility for NIPS was SRV-5.

## INTRODUCTION

Macaque monkeys have served as experimental models for biomedical research, mainly in the field of neuroscience. Japanese macaques (*Macaca fuscata*) are one of the macaque species endemic to Japan, and they have been preferentially used because of their gentle nature and high cognitive abilities [1]. The research use of Japanese macaques from wild populations has been restricted since 2001, and the national bioresource project (NBRP) has been started as a sustainable supply of Japanese macaques for laboratory use [1]. The National Institute for Physiological Sciences (NIPS) has therefore worked to establish breeding colonies of Japanese macaques, in collaboration with Kyoto University Primate Research Institute (KUPRI). Both institutes bred more than 350 Japanese macaques as a part of this project.

During 2001-2002 and 2008-2011, a large number of Japanese macaques kept at KUPRI died after exhibiting a hemorrhagic syndrome with symptoms such as anemia, facial pallor, bloody stool and nasal hemorrhage [2]. We have previously reported that the causative agent of this hemorrhagic disease occurred at KUPRI was simian retrovirus type 4 (SRV-4), which is known as D-type *Betaretrovirus* [3]. The hemorrhagic disease of Japanese macaques was originally thought to be a disease occurred only at KUPRI, but a similar disease was also found in an external breeding facility for NIPS. The first incidence was detected in June 2003, and consecutive deaths of Japanese macaques occurred between then and December 2014. Since both NIPS and KUPRI are the NBRP supply agencies, the disease had tremendous effects on the project.

The major symptoms of this disease were reduced appetite, lethargy, anemia, subcutaneous hemorrhaging and bleeding of the nasal mucosa and gums. After the

onset of symptoms, the disease followed a peracute course, with extremely high fatality rate. All the macaques that developed the disease had significant decreases in the platelet, leukocytes and erythrocytes counts. Since the platelet counts had dropped below 10,000 counts  $\mu\text{l}^{-1}$  in most cases, the clinical findings of subcutaneous and gingival hemorrhaging were considered to be consequences of increased bleeding tendencies accompanied with platelet reduction [4].

Since the first epidemic, we have attempted to determine the cause of this unknown hemorrhagic disease. Although extensive investigations suggested that this disease in Japanese macaques at the external breeding facility for NIPS is associated with simian retrovirus type 5 (SRV-5), the etiology still remained unclear.

In this study, we conducted experimental infection of SRV-5 on Japanese macaques and investigated the association between SRV-5 and the hemorrhagic syndrome. We isolated SRV-5 from Japanese macaques exhibiting thrombocytopenia and inoculated the SRV-5 isolate into two Japanese macaques. As a result, severe thrombocytopenia was induced in one of two virus-inoculated Japanese macaques within just 22 days. We then constructed an infectious molecular clone of the SRV-5 isolate and inoculated similarly into the other two Japanese macaques. The clone-derived virus also induced severe thrombocytopenia within 22 days. Although the remaining two of four macaques were administered with dexamethasone as an immunosuppressive agent, no clinical signs or decreased platelet counts were observed until the end of the experiment. The distribution of SRV-5 proviruses and viral RNAs in tissues revealed that SRV-5 infected a variety of tissues in Japanese macaques. We also demonstrated that SRV-5 utilizes a neutral amino acid transporter ASCT2 as a functional receptor in Japanese macaques. From these results, we

114 conclude that the hemorrhagic syndrome of Japanese macaques occurred at the  
115 external breeding facility for NIPS was caused by a single infection of SRV-5.

## RESULTS

**Isolation of SRV-5 from Japanese macaques exhibiting thrombocytopenia.** The hemorrhagic disease of Japanese macaques that occurred at KUPRI was caused by infection with SRV-4 [2, 3], thus we first suspected that SRV-4 is the causative agent of this disease occurred at the external breeding facility for NIPS. However, we could not detect SRV-4 proviruses and viral RNAs from any of the Japanese macaques exhibiting thrombocytopenia. Metagenomic sequencing analysis of RNAs isolated from plasma of a Japanese macaque with hemorrhagic disease revealed the presence of RNA sequences that are highly homologous to SRV-5 (Data not shown). PCR using SRV-5-specific primers revealed that the affected macaques were all positive for SRV-5 proviral DNAs. Thus, we attempted to isolate SRV-5 from stored plasma samples of SRV-5-positive Japanese macaques using human embryonic kidney (HEK) 293T cells, which are known to be susceptible to SRVs. Two weeks after inoculation, virus isolation was confirmed by PCR using SRV-5-specific primers. As a result, we succeeded in isolating SRV-5 from 2 of 6 plasma samples (Fig. 1a). We designated one of the SRV-5 isolates as strain A2.

**Construction of an infectious molecular clone of SRV-5.** Total genomic DNAs were isolated from HEK293T cells persistently infected with SRV-5 strain A2. We amplified the 5' and 3' halves of SRV-5 and reconstituted the clones as a complete provirus. We designated the reconstituted plasmid clone as pSRV547. To confirm the infectivity of the clone-derived virus, HEK293T cells were transfected with pSRV547. The culture supernatant was inoculated into naïve HEK293T cells two days post-transfection. Three weeks after inoculation, the presence of SRV-5 proviruses in the inoculated cells was confirmed by PCR using SRV-5-specific

primers (Fig. 1b). In parallel, the presence of infectious SRV-5 particles in the culture supernatant of HEK293T cells transfected with pSRV547 was confirmed by the LacZ marker rescue assay using TE671 cells (Fig. 1c). Using the genome sequence of pSRV547, we inferred the phylogenetic relationships among SRVs (Fig. 1d). As shown in the phylogenetic tree, the sequence of pSRV547 clustered with an SRV-5 strain isolated in Japan [5]. The result confirmed that the infectious molecular clone we constructed was SRV-5.

#### **Experimental infection of Japanese macaques with SRV-5 isolate and**

**molecularly cloned virus.** Two Japanese macaques (JM12 and JM14) were inoculated with SRV-5 isolate at a high dose ( $1.8 \times 10^5$  TCID<sub>50</sub> ml<sup>-1</sup>). Similarly, the other two Japanese macaques (JM13 and JM15) were inoculated with a molecularly cloned SRV-5 at the same dose. For each animal, either SRV-5 isolate or the clone-derived virus was inoculated intraperitoneally (15 ml) and intravenously (5 ml). By PCR, we detected proviruses in blood cells from 8 days post inoculation (dpi) in all virus-inoculated Japanese macaques (Fig. 2a to 2d). Real-time RT-PCR revealed that the viral copy numbers in plasma also stayed at a high level from 8 dpi ( $10^5$  to  $10^7$  copies ml<sup>-1</sup>) (Fig. 3a). The platelet counts dropped dramatically at 22 dpi in one macaque inoculated with the isolate and in another macaque inoculated with clone-derived virus (JM14 and JM15, respectively) (Fig. 3b), and these animals were euthanized at 24 dpi. At necropsy, we found gingival and subcutaneous bleeding in both of these animals (JM14 and JM15). The platelet counts of JM12 dropped severely at 36 dpi; however, the animal returned to a normal state from 50 dpi. The remaining animal (JM13) had no decreases in the platelet counts throughout the experiment. From 71 dpi, the surviving two macaques (JM12 and JM13) were

intramuscularly administered daily with dexamethasone (2mg kg<sup>-1</sup> of body weight) (Dexart®) as an immunosuppressive agent; however, no decreases in the platelet counts or any other clinical signs were observed until they were euthanized for necropsy at 100 dpi. We also observed that the leukocyte and erythrocyte counts were temporarily reduced in all animals (Fig. 3c and 3d).

**Antibody responses.** By immunoblot analysis, we examined antibody responses against SRV-5 in Japanese macaques. However, none of the Japanese macaques demonstrated antibody responses against SRV-5 (Fig. 4a to 4c).

**Tissue distribution of SRV-5.** At necropsy, body fluids were flushed out with phosphate-buffered saline to exclude blood from Japanese macaques. Tissue distribution of SRV-5 proviruses and viral RNAs revealed that SRV-5 infected a variety of tissues in Japanese macaques. By real-time PCR, we detected relatively high copy numbers of SRV-5 proviruses in small intestine, cecum, colon and bone marrow (Fig. 5a and 5b). Real-time RT-PCR was also carried out in various tissues of JM13 and JM15, and we detected relatively high copy numbers of SRV-5 mRNA in tonsil, thymus, spleen, cecum, colon and lymph nodes (Fig. 6a and 6b).

**Identification of the SRV-5 receptor as ASCT2.** SRVs share a common receptor on human cells with baboon and cat endogenous C-type viruses (BaEV and RD-114 virus), as determined by interference studies [6]. In humans, the receptor for these viruses has been identified as ASCT2, a neutral amino acid transporter [7, 8]. Here, we investigated the receptor for SRV-5 in Japanese macaques, rhesus macaques and cynomolgus macaques. We expressed ASCT1 or ASCT2 in *Mus dunni* tail

191 fibroblasts (MDTF) cells, which are not susceptible to SRVs (Fig. 7a). As a result,  
192 we demonstrated that SRV-5 utilizes ASCT2 of Japanese macaques, rhesus macaques  
193 and cynomolgus macaques as a functional receptor (Fig. 7b). In contrast, ASCT1  
194 from these macaques did not function as a receptor for SRV-5. It is known that  
195 carboxyl-terminal region (region C) in extracellular loop 2 of human ASCT2 is  
196 essential for cell entry of SRVs [9]. The homologies of the region C of human  
197 ASCT2 with those of Japanese macaque, rhesus macaque and cynomolgus macaque  
198 ASCT2 was 100% for the amino acid sequence (Data not shown).

## DISCUSSION

By experimental infection of Japanese macaques with SRV-5 isolate (wild-type) and molecularly cloned virus, we reproduced severe thrombocytopenia in two of four virus-inoculated macaques within 22 days. Since a replication-competent SRV-5 was also sufficient for inducing thrombocytopenia in Japanese macaques, we concluded that the hemorrhagic syndrome of Japanese macaques occurred at the external breeding facility for NIPS was caused by a single infection of SRV-5.

SRV belongs to the genus *Betaretrovirus*, and there are at least eight serotypes (SRV-1 to SRV-8) [10–14]. Mason-Pfizer monkey virus (MPMV), the prototype of SRV, was first isolated from a breast carcinoma of a rhesus monkey in 1970 [15, 16], and SRV infections have been reported in many Asian macaque species including rhesus macaques (*Macaca mulatta*), cynomolgus macaques (*Macaca fascicularis*) and pig-tailed macaques (*Macaca nemestrina*) [17, 18]. Although immunodeficiency-like diseases can occasionally occur, SRV infection is usually benign and asymptomatic in these macaques [19, 20].

SRV-5 infection was originally identified in rhesus macaques imported to the Oregon Regional Primate Research Center (Oregon, United States) from China [21]. There have been no reports of SRVs in wild Japanese macaques. Although it is not clear how SRV-5 was transmitted and induced diseases in Japanese macaques in this outbreak, SRV-5 may have been transmitted from other species of macaques to Japanese macaques, because Japanese macaques had been transiently housed close to breeding areas of rhesus macaques, cynomolgus macaques and marmosets at the external breeding facility for NIPS. As similar to the cases of SRV-4 infection at KUPRI [2, 3], we consider that the virus may have crossed the species barrier and infected Japanese macaques, leading to extremely severe symptoms. SRV-5 infected

a variety of tissues, especially digestive tissues, including colon and stomach. Thus, contact with feces or airborne droplets might be a route of SRV-5 infection from macaque species other than Japanese macaques.

The reason why SRV-5 infection leads to high mortality in Japanese macaques is still unknown at present. Interestingly, none of the Japanese macaques showing severe thrombocytopenia demonstrated antibody responses against SRV-5 (Fig. 4a to 4c), although we detected high copy numbers of SRV-5 viral RNAs in plasma (Fig. 3a). It has been shown in SRV-2 infection that viral loads tend to have an inverse relationship to neutralizing antibody levels so that lower neutralizing activities were found in monkeys with high viral load in rhesus macaques [22]. SRV-5 may impair the function of B cells, as observed in SRV-2 infection in rhesus macaques [23]. Since Japanese macaques are genetically close to the other Asian macaques belonging to the same genus *Macaca*, subtle genetic differences may explain the different outcomes after SRV-5 infection.

Notably, half of the infected macaques (2 out of 4) appeared to be asymptomatic carriers despite high copy numbers of SRV-5 viral RNAs. These macaques showed no clinical signs or decreased platelet counts even after administering dexamethasone as an immunosuppressant. Although dexamethasone is known to stimulate viral propagation by inhibiting host immune responses [22], the immunosuppression might not be sufficient to develop the disease in these macaques. The reasons for the asymptomatic infection of SRV-5 still remain unclear. By experimental infection of four Japanese macaques with SRV-4, all macaques exhibited severe thrombocytopenia, as shown in the previous study [3]. Although many factors may play relevant roles in the different clinical outcomes, SRV-5 may have lower pathogenicity compared with SRV-4 in Japanese macaques. The titer of SRV-5

inoculum used for the experimental infection was 2,000 times lower than that of SRV-4 infection in the previous experiment [3], and lower viral load of SRV-5 in infected animals might be related to the lower mortality.

In this experiment, we observed that two macaques that developed severe thrombocytopenia were both female, while male survived infection without any clinical symptoms. Based on the records, there has been no indication, suggesting that female animals are more susceptible to SRV-5. We consider that our observation about sex differences could likely be a coincidence, and individual differences of each macaque might have affected the susceptibility to SRV-5 infection.

Because the clinical outcomes of viral infection depends on a complex interplay of viral and host factors, further study is needed to evaluate the clinical course of SRV-5 infection. In conclusion, our findings demonstrated that SRV-5 was the causative agent responsible for the hemorrhagic syndrome occurred at the external breeding facility for NIPS. This study also highlights the potential risk of cross-species transmission and emergence of new diseases in the closely related species. Preventive measures to avoid recurrence of SRV infection in Japanese macaques must be taken especially when Japanese macaques are housed with other species of macaques.

## METHODS

**Ethics statement.** Animal experiments were conducted in a biosafety level 3 animal facility after approved by the Committee on Experimental Use of Nonhuman Primates of the Institute for Frontier Life and Medical Sciences in Kyoto University (authorization numbers R15-13). All procedures were carried out following the guidelines for animal experiments of the Kyoto University Institutional Review Board. For blood collection and SRV-5 challenge, animals were anesthetized with ketamine (5 to 10 mg kg<sup>-1</sup> of body weight)/xylazine (0.25 to 2 mg kg<sup>-1</sup>). Euthanasia was performed by pentobarbital overdose via intravenous injection under ketamine (5 to 10 mg kg<sup>-1</sup>)/xylazine (0.25 to 2 mg kg<sup>-1</sup>) anesthesia. Two Japanese macaques (JM14 and JM15) were euthanized when the platelet counts became less than 40,000  $\mu\text{l}^{-1}$  at 24 dpi. At 100 dpi, the remaining macaques (JM12 and JM13) were also euthanized for necropsy, and the whole blood and tissues were collected from all animals.

**Cells.** HEK293T (human embryonic kidney cells) (ATCC, CRL-11268), TE671 (human rhabdomyosarcoma) and MDTF cells (*Mus dunni* tail fibroblasts) were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 units ml<sup>-1</sup>), and streptomycin (100  $\mu\text{g ml}^{-1}$ ) (Invitrogen, Carlsbad, CA). Peripheral blood mononuclear cells (PBMCs) were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FCS, 100 units ml<sup>-1</sup> of recombinant human interleukin-2, 50  $\mu\text{M}$  2-mercaptoethanol, L-glutamine (2 mM), nonessential amino acid solution (Invitrogen), sodium pyruvate (1 mM) (Invitrogen), and penicillin (100 units ml<sup>-1</sup>) and streptomycin (100  $\mu\text{g ml}^{-1}$ ) (Invitrogen).

289 **Virus isolation from infected Japanese macaques.** Plasma samples were inoculated  
 290 into HEK293T cells. Two weeks after inoculation, genomic DNAs were isolated from  
 291 the inoculated cells using a QIAamp DNA blood minikit (Qiagen, Valencia, CA) and  
 292 then subjected to PCR analysis as described below.

293 **PCR to detect SRV-5 proviruses.** To detect SRV-5, partial SRV-5 proviral DNAs were  
 294 amplified using SRV-5 *env*-specific primers (forward primer, 5' -  
 295 AGGGAGATCGAAAAGAGCAGTTCAG - 3'; reverse primer, 5' -  
 296 TAGGGTTCACCGCGATCTTCC - 3'). The PCR was carried out using ExTaq  
 297 polymerase (TaKaRa, Ohtsu, Shiga, Japan) according to the manufacturer's instructions.  
 298 The PCR conditions were 98°C for 1 min, followed by 45 cycles of amplification,  
 299 consisting of denaturation at 98°C for 10 s, annealing at 60°C for 30 s and extension at  
 300 72°C for 30 s, and then a final extension at 72°C for 5 min.

301 **Construction of an infectious molecular clone of SRV-5.** Total genomic DNAs were  
 302 isolated from HEK293T cells persistently infected with SRV-5 using a QIAamp DNA  
 303 blood minikit (Qiagen). To construct an infectious molecular clone of SRV-5 strain A2,  
 304 we amplified the 5' and 3' halves of SRV-5 (designated SRV-5a and SRV-5b,  
 305 respectively) and cloned into a pcDNA3.1 vector (Invitrogen). The clone fragments  
 306 SRV-5a and SRV-5b were amplified using primers corresponding to the fragment SRV-  
 307 5a (forward primer, 5'-CAAGCTGGCTAGTTAGTGGCGCAGTCCCT-3'; reverse  
 308 primer, 5'-TCAGCGGGTTTAAACAGGACAAACCCCTG-3') and the fragment SRV-  
 309 5b (forward primer, 5'-CCAGGGGTTTGTCTCCTAGGTG-3'; reverse primer, 5'-  
 310 TCAGCGGGTTTAAACTGTCCCGTCCTGCG -3') from total genomic DNA. The PCR  
 311 was carried out using PrimeSTAR GXL DNA polymerase (TaKaRa) according to the

312 manufacturer's instructions. The amplification settings for SRV-5a were 98°C for 3 min  
313 followed by 30 cycles of amplification, consisting of denaturation at 98°C for 10 s,  
314 annealing at 60°C for 15 s, and extension at 68°C for 40 s, and then final extension at  
315 68°C for 5 min. The amplification settings for SRV-5b were 98°C for 3 min followed by  
316 30 cycles of amplification, consisting of denaturation at 98°C for 10 s, annealing at 60°C  
317 for 15 s, and extension at 68°C for 1 min, and then final extension at 68°C for 5 min.  
318 PCR was carried out in 200- $\mu$ l thin-walled tubes using a thermal cycler (C1000 Thermal  
319 Cycler; Bio-Rad). Then, the fragments were reconstructed to be a proviral form in the  
320 pcDNA3.1 using Infusion enzyme (TaKaRa). To confirm the infectivity of the clone-  
321 derived virus, HEK293T cells were transfected with 1  $\mu$ g of pSRV547 using  
322 Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The  
323 culture supernatant was inoculated into uninfected HEK293T cells two days post-  
324 transfection. Three weeks after inoculation, the presence of SRV-5 proviruses in the  
325 inoculated cells was confirmed by PCR using SRV-5 *env* specific primers. In parallel,  
326 the presence of infectious SRV-5 particles was confirmed by the LacZ marker rescue  
327 assay using TE671 cells as described below.

328 **LacZ marker rescue assay.** pSRV4 $\psi$ LacZ (a LacZ-expressing reporter plasmid with a  
329 SRV-4 packaging signal) was previously constructed by replacing the nucleotides from  
330 positions 801 to 7594 of an infectious molecular clone of SRV4 (the transcription  
331 initiation site was defined as position 1) with SV40 promoter-*nls*LacZ using In-Fusion  
332 HD Cloning kit (Clontech, Mountain View, CA) [3]. To confirm the presence of  
333 infectious SRV-5 particles, HEK293T cells infected with SRV-5 were transfected with  
334 pSRV4 $\psi$ LacZ using Lipofectamine 2000 (Invitrogen) according to the manufacturer's

instructions. Two days after transfection, each culture supernatant with 8  $\mu\text{g ml}^{-1}$  of Polybrene (Sigma-Aldrich) was filtered through a 0.45 $\mu\text{m}$  filter unit (Acrodisc; Pall Co., Ann Arbor, MI) and then inoculated into TE671 cells. Two days after inoculation, the cells were fixed with 1% glutaraldehyde and stained with 1mg  $\text{ml}^{-1}$  X-Gal.

**Phylogenetic analyses.** The genome sequences of SRV-5 infectious molecular clone was determined by a commercial DNA sequencing service (FASMAC, Kanagawa, Japan). The nucleotide sequences of SRVs were also obtained from the NCBI database (accession numbers: M12349 for mason-Pfizer monkey virus (MPMV); U85505 for simian endogenous retrovirus (SERV); M11841 for SRV1; AF126467 and M16605 for SRV2; AB920339 and FJ971077 for SRV4; AB611707 for SRV5). Alignment of nucleotide sequences were performed using L-INS-i program in the MAFFT suite [24]. The gapped regions were removed by using trimAl [25]. A maximum-likelihood phylogenetic tree was constructed using RAxML version 8.0 based on the general time reversible model with gamma-distributed rate variation across sites [26].

**Real-time PCR and real-time reverse transcription PCR (RT-PCR).** Total genomic DNAs and RNAs were extracted from various tissues of Japanese macaques using a QIAamp DNA minikit (Qiagen) and RNeasy minikit (Qiagen), respectively, according to the manufacture's instructions. All RNA samples were treated with DNase I (Roche Diagnostics GmbH, Mannheim, Germany) to exclude contamination of cellular DNA. Real-time PCR and real-time reverse transcription PCR (RT-PCR) were performed by using Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA) and Power SYBR green RNA-to-CT 1-step kit (Applied Biosystems), respectively, according to the manufacturer's instructions. The primer pairs used were as follows: forward

primer, 5'-GACAGCACGTTCCAAAAGGAAAATC-3' and reverse primer, 5'-CTGGAAAGATGTCTTTAGGA-3', for amplification of SRV-5 *gag* gene. For quantification, standard curves were generated for each gene by serial dilution of the plasmids. The copy ratio of each gene was normalized relative to the amount of a validated endogenous control Japanese macaque's GAPDH DNA or mouse Actb DNA. Quantitation was performed in triplicate for each sample, and all values are presented as means  $\pm$  standard errors of the means (SEM).

**Experimental infection of Japanese macaques with SRV-5.** Male (JM12 and JM13) and female (JM14 and JM15) Japanese macaques were used for experimental infection. Virus inoculation was carried out under anesthesia by intramuscular injection of a mixture solution of ketamine chloride (Ketalar; Daiichi Sankyo, Tokyo, Japan) at 5 to 10 mg kg<sup>-1</sup> and xylazine chloride (Celactal; Bayer Healthcare, Leverkusen, Germany) at 1.5 to 2.0 mg kg<sup>-1</sup>. Blood was routinely collected from animals by venipuncture at 0, 8, 15, 22, 24, 36, 43, 50, 61, 71, 78, 85, 92 and 100 dpi. Various tissues (cerebrum, epencephalon, lung, heart, thymus, stomach, liver, kidney, pancreas, spleen, small intestine, large intestine, testis, ovary, prostate gland, uterus, skin, muscle, and lymph nodes [mesentery, axillary, mandibular, and inguinal]) were collected at necropsy.

**(i) Experiment 1.** To prepare SRV-5 inoculum, SRV-5 strain A2 isolated from a Japanese macaque was inoculated into uninfected HEK293T cells. Two weeks after inoculation, the culture supernatants were harvested and filtered through a 0.45- $\mu$ m filter unit (Pall). To exclude contamination of cellular DNA from the culture supernatant, the samples were treated with DNase I (Roche). Two Japanese macaques (JM12 and JM14)

were inoculated with the SRV-5 isolate ( $1.8 \times 10^5$  TCID<sub>50</sub> ml<sup>-1</sup>) intraperitoneally (15ml) and intravenously (5ml).

**(ii) Experiment 2.** To prepare clone-derived virus of SRV-5, HEK293T cells were transfected with an infectious molecular clone termed pSRV547. Two weeks after inoculation, the culture supernatant was harvested, filtered through a 0.45-μm filter unit (Pall) and then treated with DNase I (Roche). Two Japanese macaques (JM13 and JM15) were inoculated with the virus derived from pSRV547 ( $1.8 \times 10^5$  TCID<sub>50</sub> ml<sup>-1</sup>) intraperitoneally (15 ml) and intravenously (5 ml).

**Immunoblot analysis.** To detect antibodies against SRV-5, plasma samples from four Japanese macaques we investigated were subjected to immunoblot analysis using the concentrated culture supernatant of HEK293T cells infected with SRV-5 as antigens. In the previous study, we generated rabbit anti-SRV CA and anti-Env antibodies using synthetic peptides, and they were used as positive control antibodies [3]. Immunoblot analysis was performed as described previously [3] using 1:100 dilutions of plasma samples and a 1:10,000 dilution of horseradish peroxidase-conjugated anti-human IgG antibody (GE Healthcare, Buckinghamshire, United Kingdom).

**Cloning of ASCT1 and ASCT2 cDNA from three macaque species.** cDNAs encoding ASCT1 and ASCT2 molecules of rhesus macaques were cloned as previously described [3]. Briefly, RNAs were extracted from PBMCs of a rhesus macaque, and cDNA was synthesized with random primers according to the manufacturer's instructions. The entire ASCT1 and ASCT2 genes were amplified by PCR and inserted into pACGFP-N1 vector (Clontech, Mountain View, CA) to produce pMmASCT1/GFP and pMmASCT2/GFP. The expression plasmids (termed pJmASCT1/GFP, pJmASCT2/GFP,

403 pCyASCT1/GFP and pCyASCT2/GFP) for ASCT1 and ASCT2 from Japanese macaques  
404 and cynomolgus macaques, respectively, were reported previously [3].

405 **Functional assay of ASCT1 and ASCT2 as SRV-5 receptors.** To confirm whether  
406 SRV-5 utilizes ASCT1 and ASCT2 of Japanese macaque, rhesus macaque and  
407 cynomolgus macaque as functional receptors, pJmASCT1/GFP, pJmASCT2/GFP,  
408 pMmASCT1/GFP, pMmASCT2/GFP, pCyASCT1/GFP, pCyASCT2/GFP, or pACGFP-  
409 N1 was transfected into MDTF cells using Lipofectamine 2000 (Invitrogen) according to  
410 the manufacturer's instructions. MDTFs expressing ASCT1-GFP, ASCT2-GFP or GFP  
411 only were seeded in 12-well plates at  $10^5$  cells per well 1 day before inoculation.  
412 Infectivity of SRV-5 to these cells was confirmed by real-time PCR using SRV-5 *gag*-  
413 specific primers.

414 **Nucleotide sequence numbers.** Nucleotide sequences of pSRV547 has been deposited  
415 in GenBank with accession number, LC426347.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

Animal experiments were approved by the Committee on Experimental Use of Nonhuman Primates of the Institute for Frontier Life and Medical Sciences in Kyoto University (authorization numbers R15-13). All procedures were performed according to the Kyoto University Institutional Guidelines in addition to the guidelines of Japanese law.

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## Figure legends

**Fig. 1.** Virus isolation from SRV-5-positive Japanese macaques. (a) Detection of SRV-5 proviral DNAs from HEK293T cells inoculated with plasma samples of Japanese macaques JM-a [ID: J07036] (lane 1), JM-b [J08031] (lane 2), JM-c [J08042] (lane 3), JM-d [J08036] (lane 4), JM-e [J08011] (lane 5), JM-f [J08013] (lane 6). N.C., negative control (uninfected HEK293T cells). (b and c) Confirmation of the infectivity of the virus derived from an infectious molecular clone of SRV-5 isolate termed pSRV547 by PCR using SRV-5 *env*-specific primers (b) and LacZ marker rescue assay (c). N.C., negative control (uninfected HEK293T cells). (d) Phylogenetic tree of complete nucleotide sequences of SRV-5 isolates using the maximum-likelihood method. Genome sequence derived from the SRV-5 infectious clone (pSRV547) is marked with black dot. Numbers on the nodes represent the bootstrap values (1,000 resamplings). MPMV, Mason-Pfizer monkey virus (SRV-3); SERV, simian endogenous retrovirus.

**Fig. 2.** Detection of SRV-5 proviral DNAs in blood from Japanese macaques experimentally infected with SRV-5. (a to d) Genomic DNAs were isolated from blood of four Japanese macaques (JM12 [a], JM13 [b], JM14 [c] and JM15 [d]) and subjected to PCR using SRV-5 *env*-specific primers. Numbers above lanes indicate dpi.

**Fig. 3.** Dynamics of SRV-5 infection of Japanese macaques. JM12 (●) and JM14 (●) were inoculated with SRV-5 strain A2. JM13 (△) and JM15 (△) were inoculated with a cloned virus derived from pSRV547. (a) The copy numbers of SRV-5 RNAs in plasma samples. SRV-5 viral RNAs were quantified by real-time RT-PCR using SRV-5 *gag*-

specific primers. Values are the means  $\pm$  SEM from three independent experiments. (b to d) Hematological analyses of experimentally infected Japanese macaques. Blood samples were routinely collected, and the counts of platelets (b), leukocytes (c) and erythrocytes (d) were measured with a hematocytometer.

**Fig. 4.** Antibody responses against SRV-5 in Japanese macaques. (a to c) Immunoblot analyses were carried out using concentrated SRV-5 as the antigen. Numbers shown above the lanes indicate dpi.  $\alpha$ -CA, rabbit anti-SRV CA antibody;  $\alpha$ -Env, rabbit anti-SRV Env antibody. Anti-SRV-5 antibodies were not observed in any Japanese macaques (JM12 [a], JM13 [b], JM14 and JM15 [c]) experimentally infected with SRV-5. Because none of the macaques showed detectable antibodies against SRV-5, the samples after 61 dpi has been omitted from the analysis.

**Fig. 5.** Detection and quantification of SRV-5 proviral DNAs in various tissues by real-time PCR. (a and b) Quantification of SRV-5 proviral DNAs in various tissues of four Japanese macaques (JM12 and JM13 [a] and JM14 and JM15 [b]) by real-time PCR using SRV-5 *gag*-specific primers. Each value was normalized to the amount of Japanese macaque's GAPDH DNAs and expressed as the means  $\pm$  SEM of data from three independent experiments. Ly, lymph node.

**Fig. 6.** Detection and quantification of SRV-5 viral RNAs in various tissues by real-time RT-PCR. (a and b) Total RNAs were isolated from various tissues of two Japanese macaques (JM13 [a] and JM15 [b]), and SRV-5 viral RNAs were quantified by real-time

RT-PCR using SRV-5 *gag*-specific primers. Quantification was performed in triplicate for each sample, and all values were normalized to the amount of Japanese macaque's GAPDH DNAs and presented as the means  $\pm$  SEM of data from three independent experiments. Ly, lymph node.

**Fig. 7.** Functional assay of ASCT1 and ASCT2 of Japanese macaques, cynomolgus macaques and rhesus macaques as SRV-5 receptors. (a) Expression of ASCT1-GFP, ASCT2-GFP and GFP in MDTF cells detected by fluorescence microscopy. (b) Virus infection assay was performed in MDTF cells transiently expressing ASCT1 and ASCT2 from Japanese macaques (JmASCT1 and JmASCT2), cynomolgus macaques (CyASCT1 and CyASCT2) and rhesus macaques (MmASCT1 and MmASCT2). Infectivity of SRV-5 was quantified by real-time PCR using SRV-5 *gag*-specific primers. Each value was normalized to the amount of mouse Actb DNAs and expressed as the means  $\pm$  SEM of data from three independent experiments.

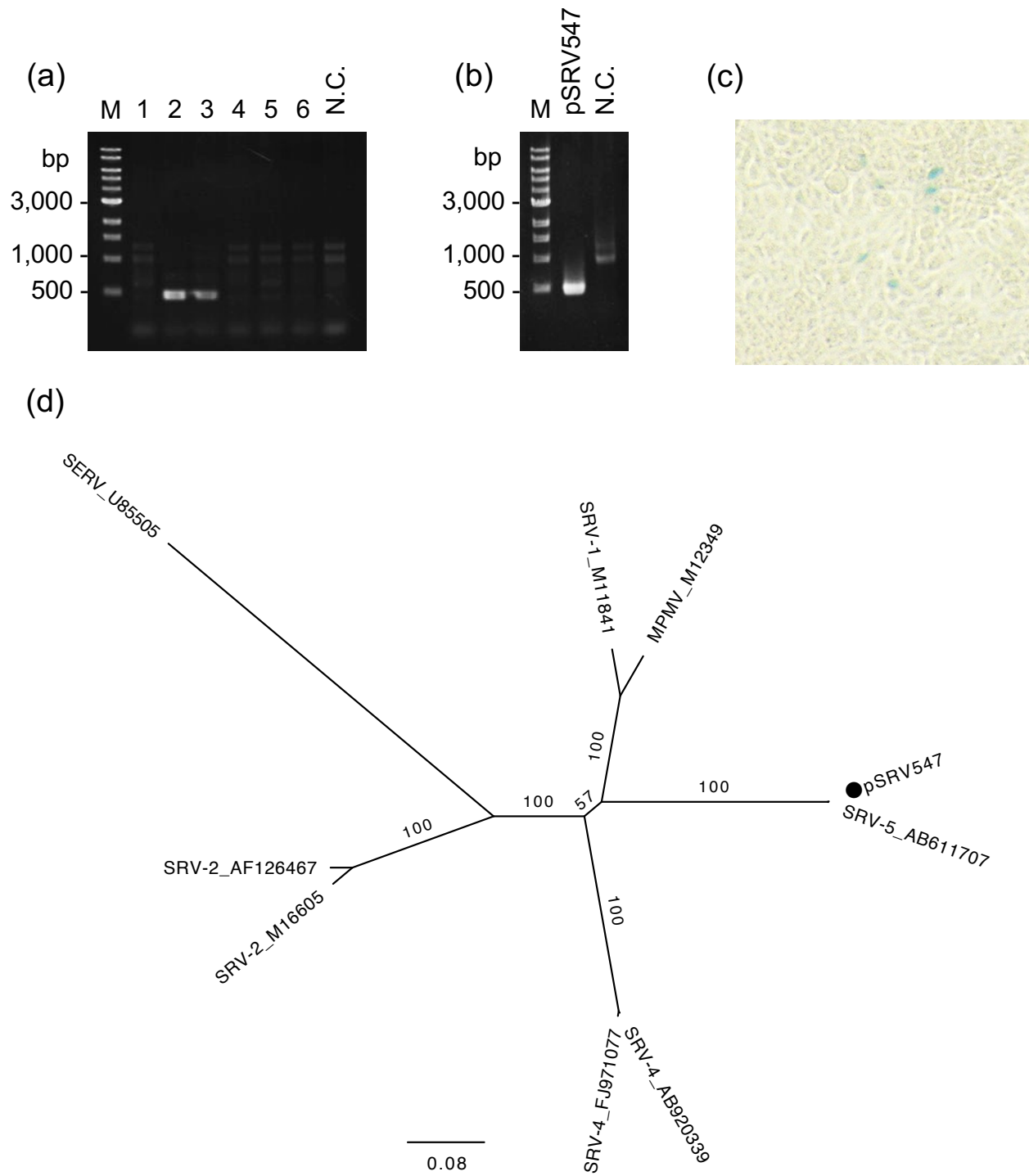


Fig. 1

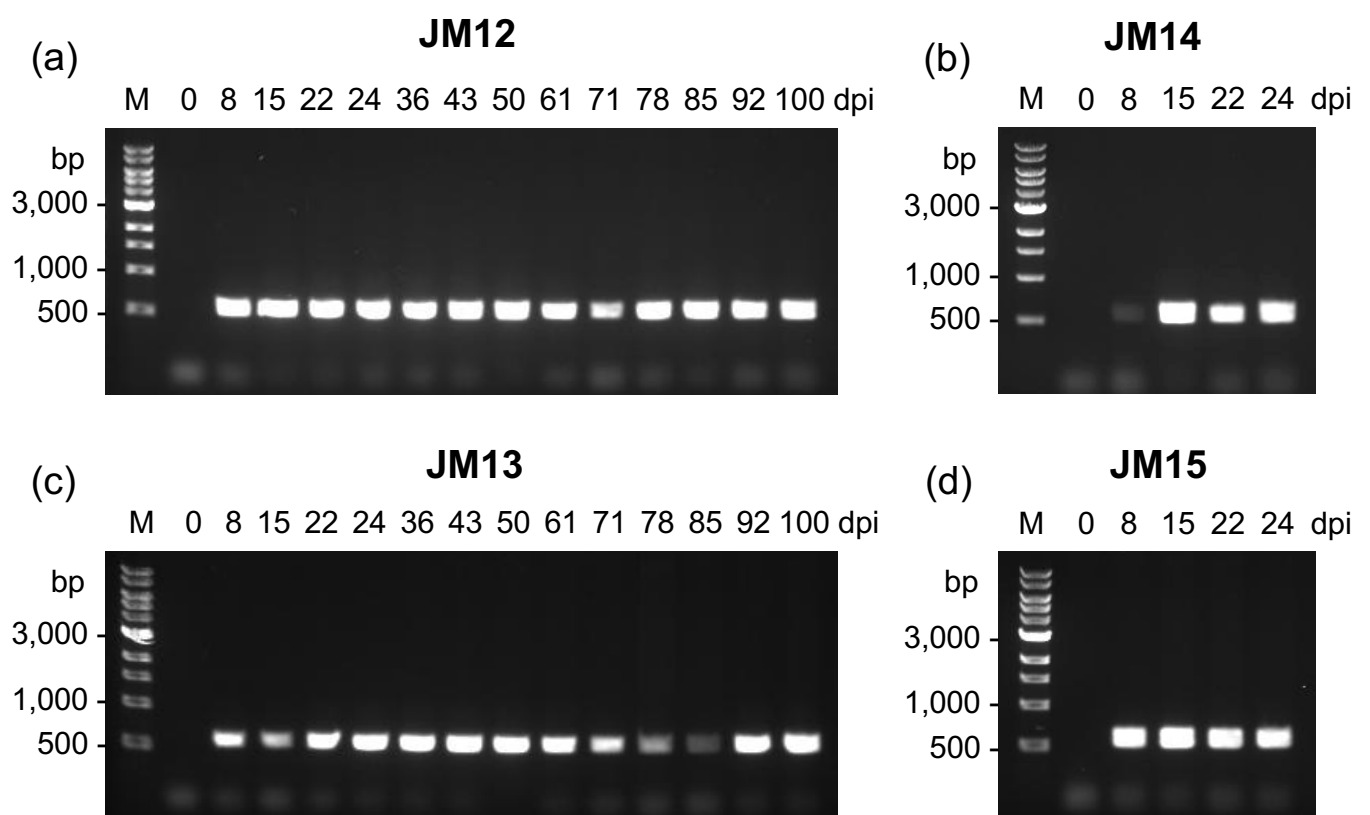


Fig. 2

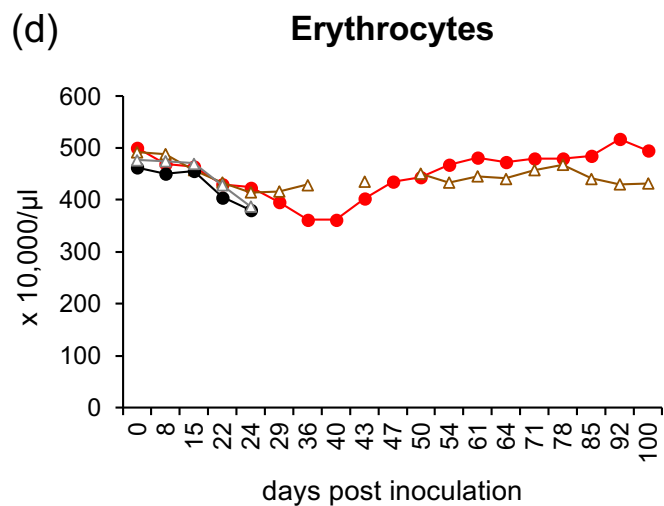
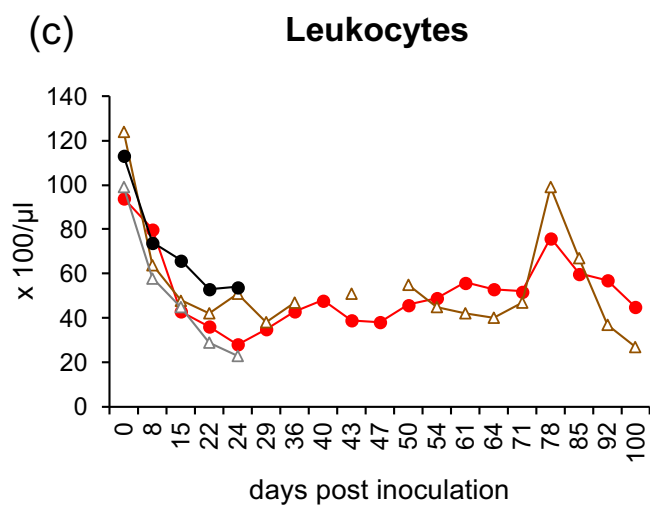
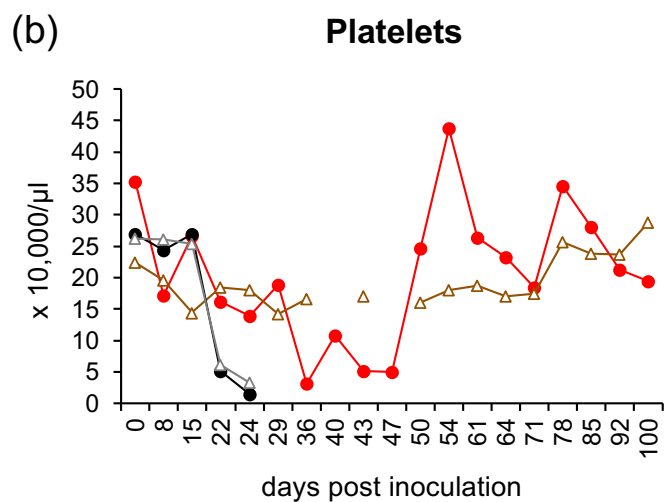
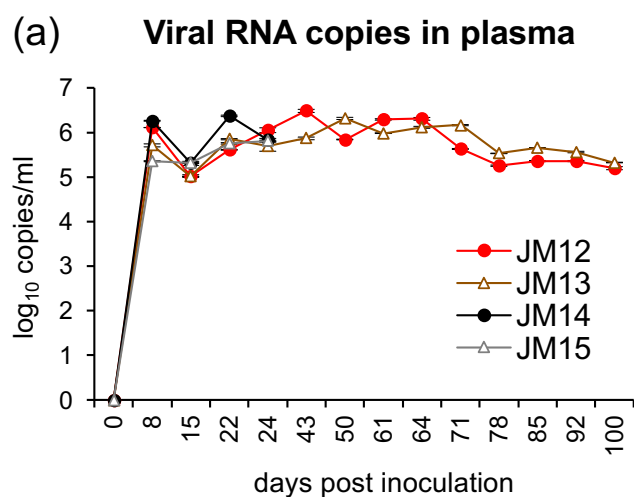


Fig. 3

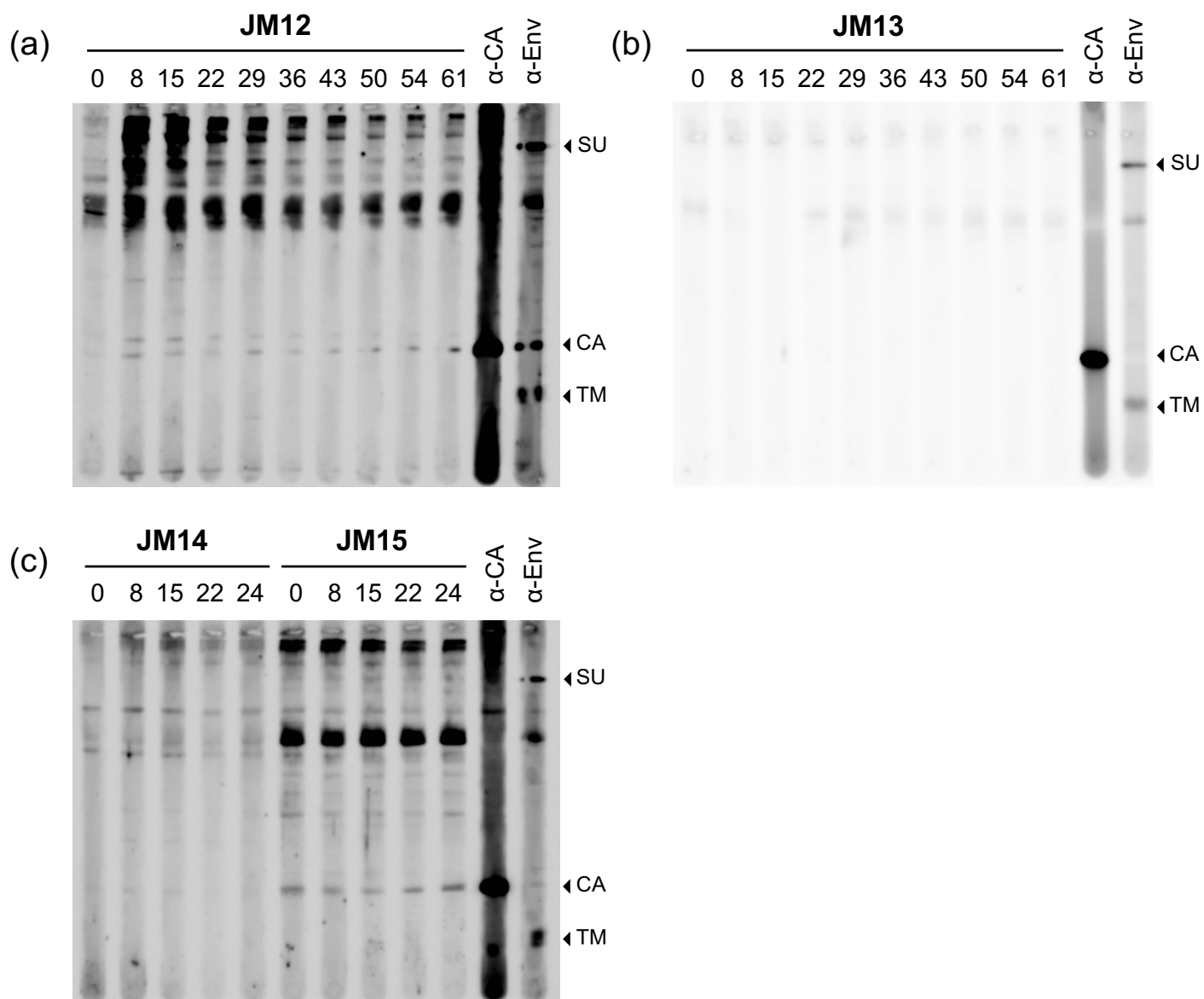


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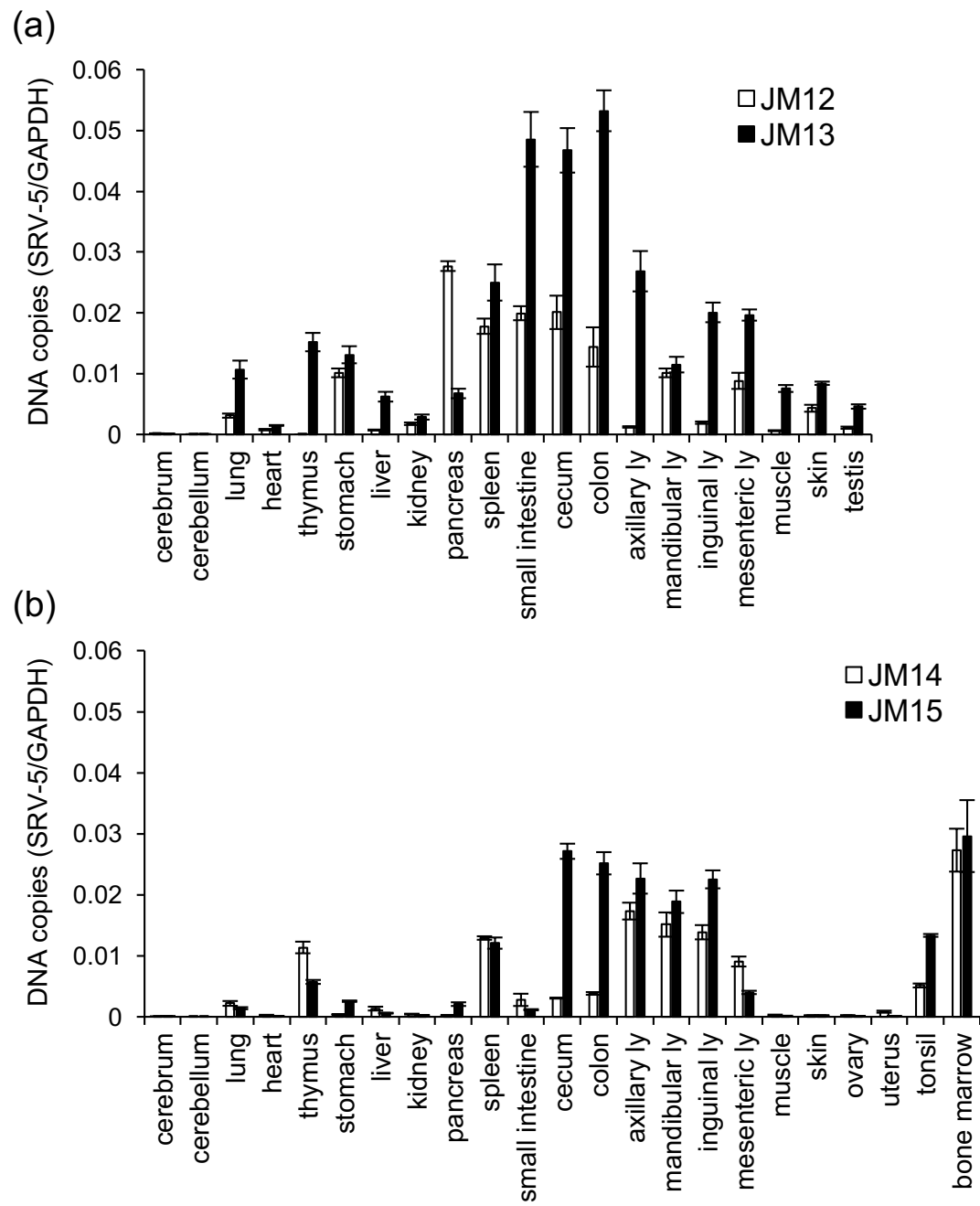


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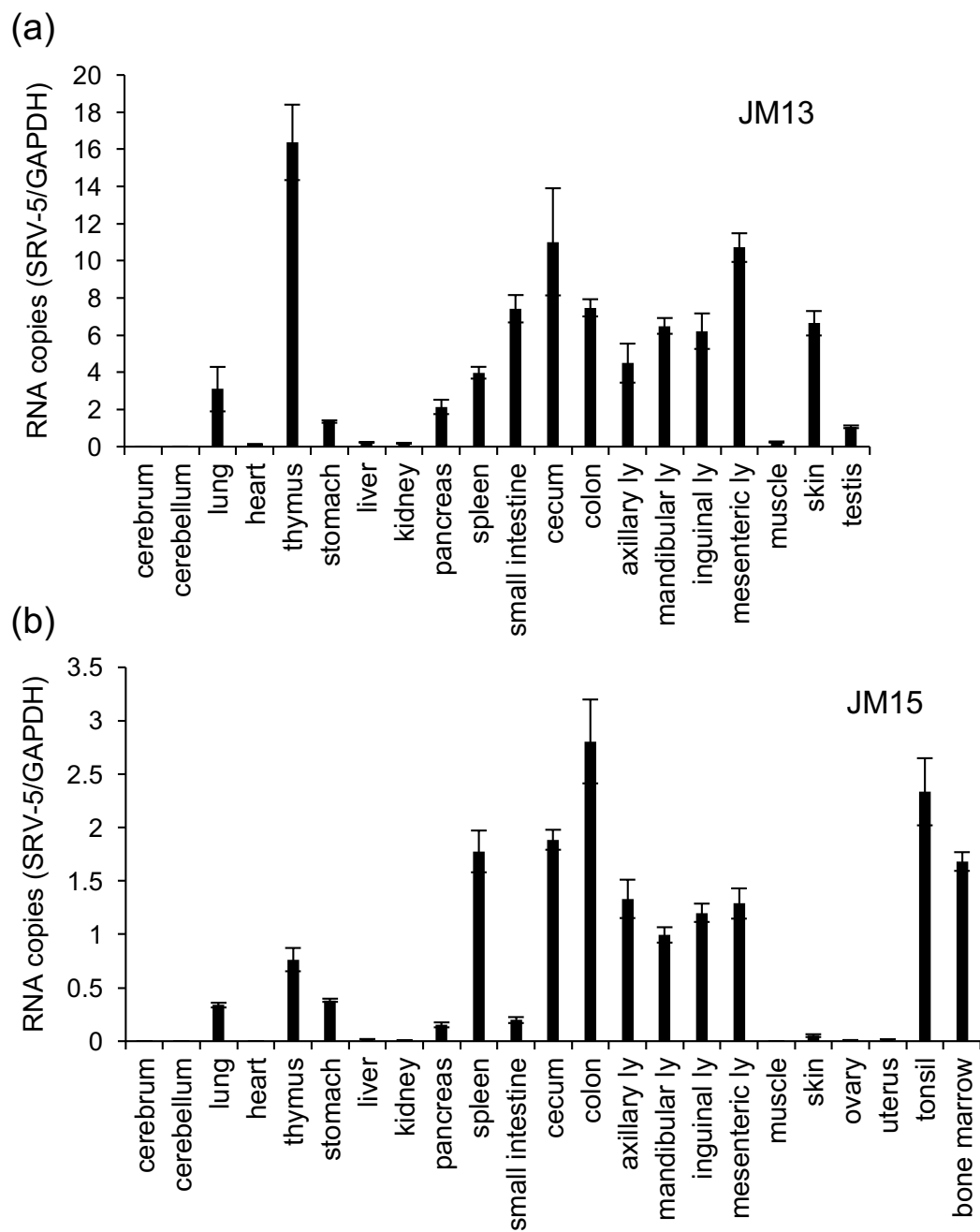


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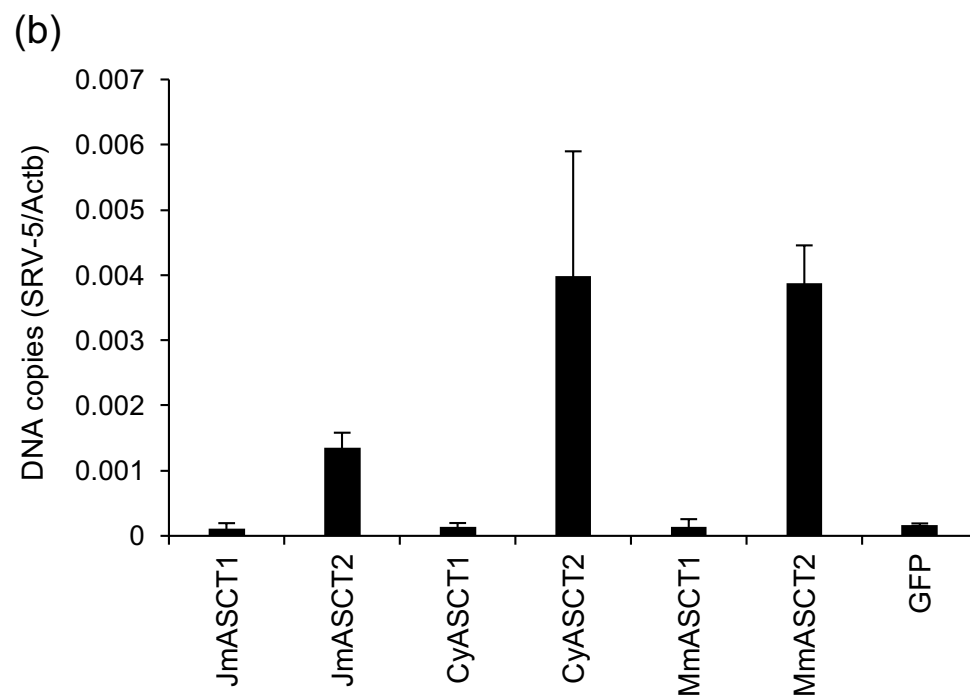
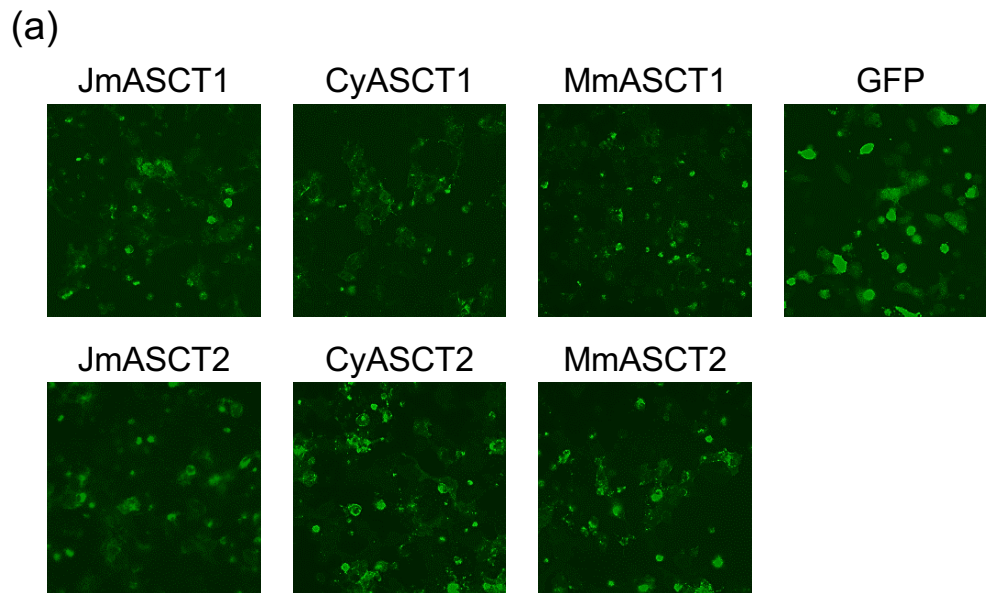


Fig. 7