Experimental infection of Japanese macaques with simian retrovirus 5

Rie Koide,[1] Rokusuke Yoshikawa,[2,3] Munehiro Okamoto,[4 ¶] Shoichi Sakaguchi,[5] Juri Suzuki,[4] Tadashi Isa,[6,7] So Nakagawa,[8] Hiromi Sakawaki,[9] Tomoyuki Miura,[10] Takayuki Miyazawa[1 ¶]

[1]Laboratory of Virus-Host Coevolution, Research Center for Infectious Diseases, Institute for Frontier Life and Medical Sciences, Kyoto University, Kyoto, Japan

[2]National Research Center for the Control and Prevention of Infectious Diseases (CCPID), Nagasaki University, Nagasaki, Japan

[3]Department of Emerging Infectious Diseases, Institute of Tropical Medicine (NEKKEN), Nagasaki University, Nagasaki, Japan

[4]Center for Human Evolution Modeling Research, Primate Research Institute, Kyoto University, Aichi, Japan

[5]Department of Microbiology, Osaka Medical College, Osaka, Japan

[6]Division of Neurobiology and Physiology, Department of Neuroscience, Kyoto University, Kyoto, Japan

[7]Section of NBR Promotion, and Department of Developmental Physiology, National Institute for Physiological Sciences, Aichi, Japan

[8]Department of Molecular Life Science, Tokai University School of Medicine, Kanagawa, Japan

[9]Non-human Primate Experimental Facility, Research Center for Infectious Diseases, Institute for Frontier Life and Medical Sciences, Kyoto University, Kyoto, Japan

[10]Laboratory of Primate Model, Research Center for Infectious Diseases, Institute for Frontier Life and Medical Sciences, Kyoto University, Kyoto, Japan

¶Correspondence to

Takayuki Miyazawa, takavet@infront.kyoto-u.ac.jp

Munehiro Okamoto, okamoto.munehiro.6w@kyoto-u.ac.jp

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Abbreviations: KUPRI, Kyoto University Primate Research Institute; NIPS, National Institute for Physiological Sciences; SRV, simian retrovirus; NBRP, national bioresource project; HEK, human embryonic kidney; JM, Japanese macaques; BaEV, baboon endogenous virus; MDTF, *Mus dunni* tail fibroblasts; MPMV, Mason-Pfizer monkey virus.

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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 hemorrahage [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

90 onset of symptoms, the disease followed a peracute course, with extremely high
91 fatality rate. All the macaques that developed the disease had significant decreases in
92 the platelet, leukocytes and erythrocytes counts. Since the platelet counts had
93 dropped below 10,000 counts μl^{-1} in most cases, the clinical findings of subcutaneous
94 and gingival hemorrhaging were considered to be consequences of increased bleeding
95 tendencies accompanied with platelet reduction [4].
96 Since the first epidemic, we have attempted to determine the cause of this
97 unknown hemorrhagic disease. Although extensive investigations suggested that this
98 disease in Japanese macaques at the external breeding facility for NIPS is associated
99 with simian retrovirus type 5 (SRV-5), the etiology still remained unclear.
100 In this study, we conducted experimental infection of SRV-5 on Japanese
101 macaques and investigated the association between SRV-5 and the hemorrhagic
102 syndrome. We isolated SRV-5 from Japanese macaques exhibiting thrombocytopenia
103 and inoculated the SRV-5 isolate into two Japanese macaques. As a result, severe
104 thrombocytopenia was induced in one of two virus-inoculated Japanese macaques
105 within just 22 days. We then constructed an infectious molecular clone of the SRV-5
106 isolate and inoculated similarly into the other two Japanese macaques. The clone-
107 derived virus also induced severe thrombocytopenia within 22 days. Although the
108 remaining two of four macaques were administered with dexamethasone as an
109 immunosuppressive agent, no clinical signs or decreased platelet counts were
110 observed until the end of the experiment. The distribution of SRV-5 proviruses and
111 viral RNAs in tissues revealed that SRV-5 infected a variety of tissues in Japanese
112 macaques. We also demonstrated that SRV-5 utilizes a neutral amino acid transporter
113 ASCT2 as a functional receptor in Japanese macaques. From these results, we

conclude that the hemorrhagic syndrome of Japanese macaques occurred at the 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 (1.8×10^5 $TCID_{50}$ ml^{-1}). 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^{-1}) (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

166 intramuscularly administered daily with dexamethasone (2mg kg^{-1} of body weight)
167 (Dexart®) as an immunosuppressive agent; however, no decreases in the platelet
168 counts or any other clinical signs were observed until they were euthanized for
169 necropsy at 100 dpi. We also observed that the leukocyte and erythrocyte counts
170 were temporarily reduced in all animals (Fig. 3c and 3d).
171
172 **Antibody responses.** By immunoblot analysis, we examined antibody responses
173 against SRV-5 in Japanese macaques. However, none of the Japanese macaques
174 demonstrated antibody responses against SRV-5 (Fig. 4a to 4c).
175
176 **Tissue distribution of SRV-5.** At necropsy, body fluids were flushed out with
177 phosphate-buffered saline to exclude blood from Japanese macaques. Tissue
178 distribution of SRV-5 proviruses and viral RNAs revealed that SRV-5 infected a
179 variety of tissues in Japanese macaques. By real-time PCR, we detected relatively
180 high copy numbers of SRV-5 proviruses in small intestine, cecum, colon and bone
181 marrow (Fig. 5a and 5b). Real-time RT-PCR was also carried out in various tissues
182 of JM13 and JM15, and we detected relatively high copy numbers of SRV-5 mRNA
183 in tonsil, thymus, spleen, cecum, colon and lymph nodes (Fig. 6a and 6b).
184
185 **Identification of the SRV-5 receptor as ASCT2.** SRVs share a common receptor on
186 human cells with baboon and cat endogenous C-type viruses (BaEV and RD-114
187 virus), as determined by interference studies [6]. In humans, the receptor for these
188 viruses has been identified as ASCT2, a neutral amino acid transporter [7, 8]. Here,
189 we investigated the receptor for SRV-5 in Japanese macaques, rhesus macaques and
190 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

224 a variety of tissues, especially digestive tissues, including colon and stomach. Thus,
225 contact with feces or airborne droplets might be a route of SRV-5 infection from
226 macaque species other than Japanese macaques.
227 The reason why SRV-5 infection leads to high mortality in Japanese macaques is
228 still unknown at present. Interestingly, none of the Japanese macaques showing
229 severe thrombocytopenia demonstrated antibody responses against SRV-5 (Fig. 4a to
230 4c), although we detected high copy numbers of SRV-5 viral RNAs in plasma (Fig.
231 3a). It has been shown in SRV-2 infection that viral loads tend to have an inverse
232 relationship to neutralizing antibody levels so that lower neutralizing activities were
233 found in monkeys with high viral load in rhesus macaques [22]. SRV-5 may impair
234 the function of B cells, as observed in SRV-2 infection in rhesus macaques [23].
235 Since Japanese macaques are genetically close to the other Asian macaques belonging
236 to the same genus *Macaca*, subtle genetic differences may explain the different
237 outcomes after SRV-5 infection.
238 Notably, half of the infected macaques (2 out of 4) appeared to be asymptomatic
239 carriers despite high copy numbers of SRV-5 viral RNAs. These macaques showed
240 no clinical signs or decreased platelet counts even after administering dexamethasone
241 as an immunosuppressant. Although dexamethasone is known to stimulate viral
242 propagation by inhibiting host immune responses [22], the immunosuppression might
243 not be sufficient to develop the disease in these macaques. The reasons for the
244 asymptomatic infection of SRV-5 still remain unclear. By experimental infection of
245 four Japanese macaques with SRV-4, all macaques exhibited severe
246 thrombocytopenia, as shown in the previous study [3]. Although many factors may
247 play relevant roles in the different clinical outcomes, SRV-5 may have lower
248 pathogenicity compared with SRV-4 in Japanese macaques. The titer of SRV-5

249 inoculum used for the experimental infection was 2,000 times lower than that of SRV-
250 4 infection in the previous experiment [3], and lower viral load of SRV-5 in infected
251 animals might be related to the lower mortality.
252 In this experiment, we observed that two macaques that developed severe
253 thrombocytopenia were both female, while male survived infection without any
254 clinical symptoms. Based on the records, there has been no indication, suggesting
255 that female animals are more susceptible to SRV-5. We consider that our observation
256 about sex differences could likely be a coincidence, and individual differences of each
257 macaque might have affected the susceptibility to SRV-5 infection.
258 Because the clinical outcomes of viral infection depends on a complex interplay of
259 viral and host factors, further study is needed to evaluate the clinical course of SRV-5
260 infection. In conclusion, our findings demonstrated that SRV-5 was the causative
261 agent responsible for the hemorrhagic syndrome occurred at the external breeding
262 facility for NIPS. This study also highlights the potential risk of cross-species
263 transmission and emergence of new diseases in the closely related species. Preventive
264 measures to avoid recurrence of SRV infection in Japanese macaques must be taken
265 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^{-1} of body weight)/xylazine (0.25 to 2 mg kg^{-1}). Euthanasia was performed by pentobarbital overdose via intravenous injection under ketamine (5 to 10 mg kg^{-1})/xylazine (0.25 to 2 mg kg^{-1}) anesthesia. Two Japanese macaques (JM14 and JM15) were euthanized when the platelet counts became less than 40,000 μl^{-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^{-1}), and streptomycin (100 μg 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^{-1} of recombinant human interleukin-2, 50 μM 2-mercaptoethanol, L-glutamine (2 mM), nonessential amino acid solution (Invitrogen), sodium pyruvate (1 mM) (Invitrogen), and penicillin (100 units ml^{-1}) and streptomycin (100 μg 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'-CCAGGGGTTTGTCCTAGGTG-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

manufacturer's instructions. The amplification settings for SRV-5a were 98°C for 3 min followed by 30 cycles of amplification, consisting of denaturation at 98°C for 10 s, annealing at 60°C for 15 s, and extension at 68°C for 40 s, and then final extension at 68°C for 5 min. The amplification settings for SRV-5b were 98°C for 3 min followed by 30 cycles of amplification, consisting of denaturation at 98°C for 10 s, annealing at 60°C for 15 s, and extension at 68°C for 1 min, and then final extension at 68°C for 5 min. PCR was carried out in 200-μl thin-walled tubes using a thermal cycler (C1000 Thermal Cycler; Bio-Rad). Then, the fragments were reconstructed to be a proviral form in the pcDNA3.1 using Infusion enzyme (TaKaRa). To confirm the infectivity of the clone-derived virus, HEK293T cells were transfected with 1 μg of pSRV547 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The culture supernatant was inoculated into uninfected 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 *env* specific primers. In parallel, the presence of infectious SRV-5 particles was confirmed by the LacZ marker rescue assay using TE671 cells as described below.

LacZ marker rescue assay. pSRV4ψLacZ (a LacZ-expressing reporter plasmid with a SRV-4 packaging signal) was previously constructed by replacing the nucleotides from positions 801 to 7594 of an infectious molecular clone of SRV4 (the transcription initiation site was defined as position 1) with SV40 promoter-*nls*LacZ using In-Fusion HD Cloning kit (Clontech, Mountain View, CA) [3]. To confirm the presence of infectious SRV-5 particles, HEK293T cells infected with SRV-5 were transfected with pSRV4ψLacZ using Lipofectamine 2000 (Invitrogen) according to the manufacturer's

335 instructions. Two days after transfection, each culture supernatant with 8 μg ml^{-1} of
336 Polybrene (Sigma-Aldrich) was filtered through a 0.45μm filter unit (Acrodisc; Pall Co.,
337 Ann Arbor, MI) and then inoculated into TE671 cells. Two days after inoculation, the
338 cells were fixed with 1% glutaraldehyde and stained with 1mg ml^{-1} X-Gal.
339 **Phylogenetic analyses.** The genome sequences of SRV-5 infectious molecular clone
340 was determined by a commercial DNA sequencing service (FASMAC, Kanagawa,
341 Japan). The nucleotide sequences of SRVs were also obtained from the NCBI database
342 (accession numbers: M12349 for mason-Pfizer monkey virus (MPMV); U85505 for
343 simian endogenous retrovirus (SERV); M11841 for SRV1; AF126467 and M16605 for
344 SRV2; AB920339 and FJ971077 for SRV4; AB611707 for SRV5). Alignment of
345 nucleotide sequences were performed using L-INS-i program in the MAFFT suite [24].
346 The gapped regions were removed by using trimAl [25]. A maximum-likelihood
347 phylogenetic tree was constructed using RAxML version 8.0 based on the general time
348 reversible model with gamma-distributed rate variation across sites [26].
349 **Real-time PCR and real-time reverse transcription PCR (RT-PCR).** Total genomic
350 DNAs and RNAs were extracted from various tissues of Japanese macaques using a
351 QIAamp DNA minikit (Qiagen) and RNeasy minikit (Qiagen), respectively, according to
352 the manufacture's instructions. All RNA samples were treated with DNase I (Roche
353 Diagnostics GmbH, Mannheim, Germany) to exclude contamination of cellular DNA.
354 Real-time PCR and real-time reverse transcription PCR (RT-PCR) were performed by
355 using Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA) and
356 Power SYBR green RNA-to-CT 1-step kit (Applied Biosystems), respectively, according
357 to the manufacturer's instructions. The primer pairs used were as follows: forward

358 primer, 5'- GACAGCACGTTCCAAAAGGAAAATC -3' and reverse primer, 5'-
359 CTGGAAAGATGTCTTTAGGA -3', for amplification of SRV-5 *gag* gene. For
360 quantification, standard curves were generated for each gene by serial dilution of the
361 plasmids. The copy ratio of each gene was normalized relative to the amount of a
362 validated endogenous control Japanese macaque's GAPDH DNA or mouse Actb DNA.
363 Quantitation was performed in triplicate for each sample, and all values are presented as
364 means ± standard errors of the means (SEM).
365 **Experimental infection of Japanese macaques with SRV-5.** Male (JM12 and JM13)
366 and female (JM14 and JM15) Japanese macaques were used for experimental infection.
367 Virus inoculation was carried out under anesthesia by intramuscular injection of a
368 mixture solution of ketamine chloride (Ketalar; Daiichi Sankyo, Tokyo, Japan) at 5 to 10
369 mg kg^{-1} and xylazine chloride (Celactal; Bayer Healthcare, Leverkusen, Germany) at 1.5
370 to 2.0 mg kg^{-1}. Blood was routinely collected from animals by venipuncture at 0, 8, 15,
371 22, 24, 36, 43, 50, 61, 71, 78, 85, 92 and 100 dpi. Various tissues (cerebrum,
372 epencephalon, lung, heart, thymus, stomach, liver, kidney, pancreas, spleen, small
373 intestine, large intestine, testis, ovary, prostate gland, uterus, skin, muscle, and lymph
374 nodes [mesentery, axillary, mandibular, and inguinal]) were collected at necropsy.
375 **(i) Experiment 1.** To prepare SRV-5 inoculum, SRV-5 strain A2 isolated from a
376 Japanese macaque was inoculated into uninfected HEK293T cells. Two weeks after
377 inoculation, the culture supernatants were harvested and filtered through a 0.45-μm filter
378 unit (Pall). To exclude contamination of cellular DNA from the culture supernatant, the
379 samples were treated with DNase I (Roche). Two Japanese macaques (JM12 and JM14)

were inoculated with the SRV-5 isolate (1.8×10^5 $TCID_{50}$ ml^{-1}) 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 (1.8×10^5 $TCID_{50}$ ml^{-1}) 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.

436 **References**

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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 JM 15 (△) 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 ± 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.

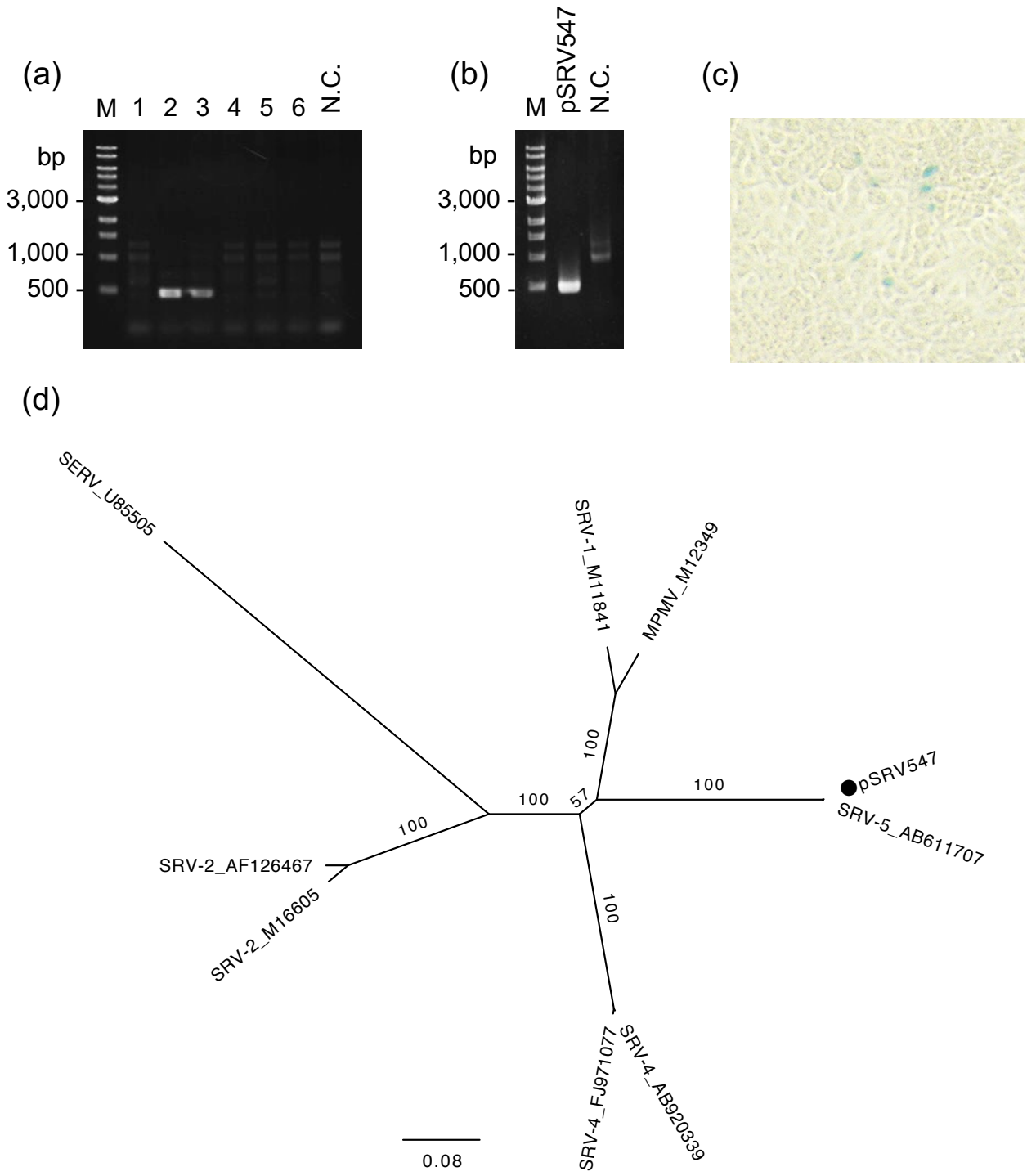
Fig. 4. Antibody responses against SRV-5 in Japanese macaques. (a to c) Immunoblot analyses were carried out using concentrated SRV-5 as the antigen. Numbers shown above the lanes indicate dpi. α-CA, rabbit anti-SRV CA antibody; α-Env, rabbit anti-SRV Env antibody. Anti-SRV-5 antibodies were not observed 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 ± 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 ± 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 ± SEM of data from three independent experiments.


(a)
M 1 2 3 4 5 6 N.C.
bp
3,000
1,000
500
(b)
M pSRV547 N.C.
bp
3,000
1,000
500
(c)
(d)
SERV_U85505
SRV-1_M11841
MPMV_M12349
pSRV547
SRV-5_AB611707
SRV-2_AF126467
SRV-2_M16605
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SRV-4_AB920339
100
57
100
100
100
100
0.08

Fig. 1

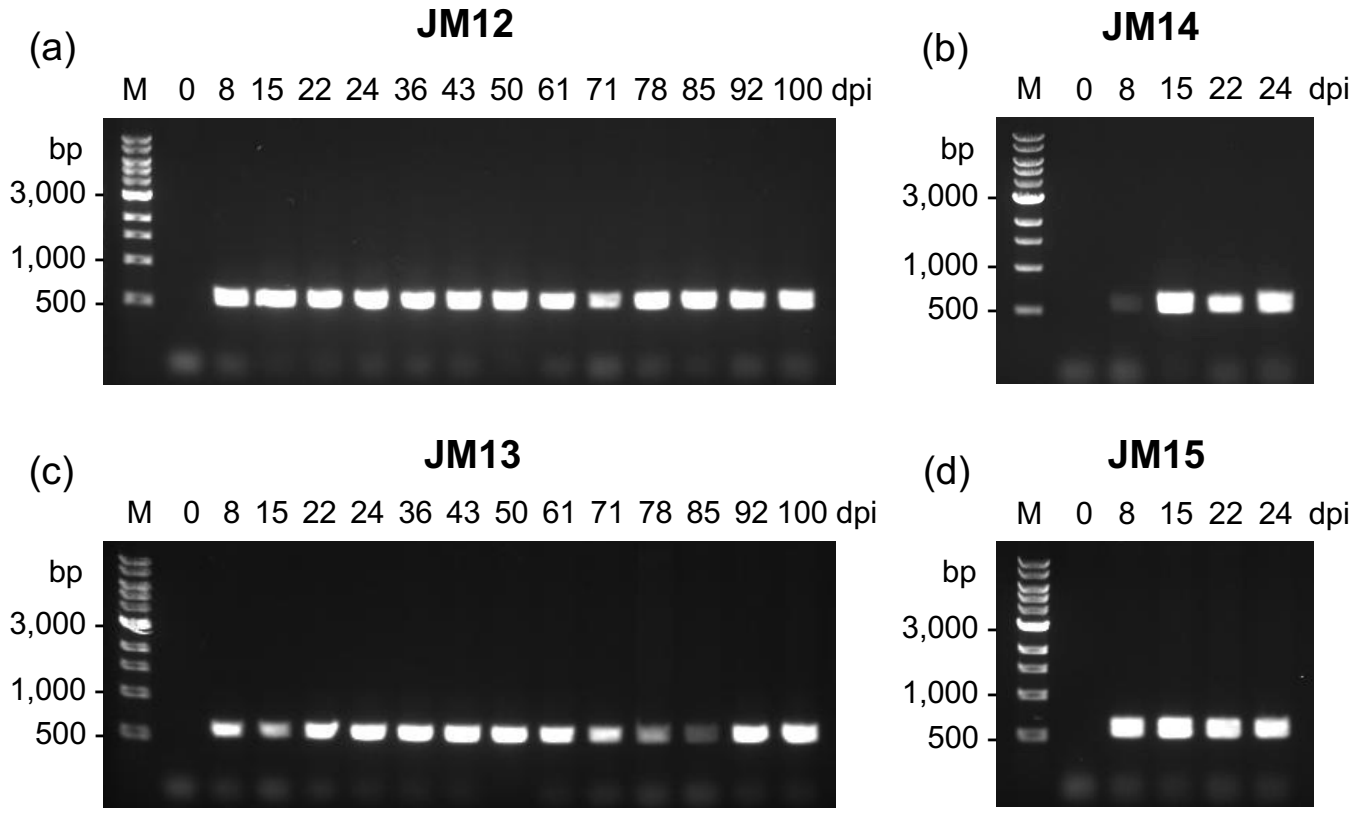

(a)
JM12
M 0 8 15 22 24 36 43 50 61 71 78 85 92 100 dpi
bp
3,000
1,000
500
(b)
JM14
M 0 8 15 22 24 dpi
bp
3,000
1,000
500
(c)
JM13
M 0 8 15 22 24 36 43 50 61 71 78 85 92 100 dpi
bp
3,000
1,000
500
(d)
JM15
M 0 8 15 22 24 dpi
bp
3,000
1,000
500

Fig. 2

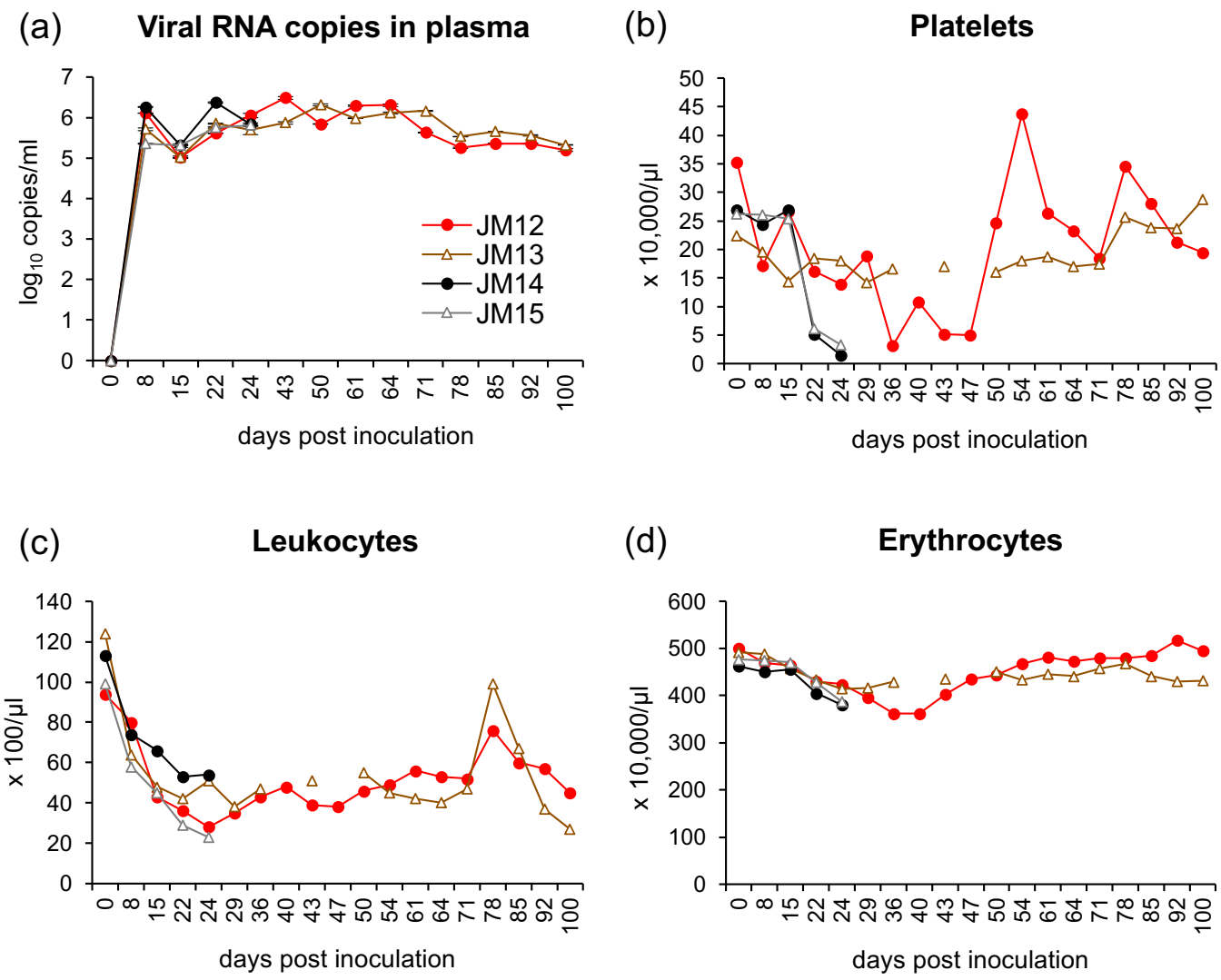

(a)
Viral RNA copies in plasma
log10 copies/ml
JM12
JM13
JM14
JM15
days post inoculation
(b)
Platelets
x 10,000/µl
days post inoculation
(c)
Leukocytes
x 100/µl
days post inoculation
(d)
Erythrocytes
x 10,000/µl
days post inoculation

Fig. 3

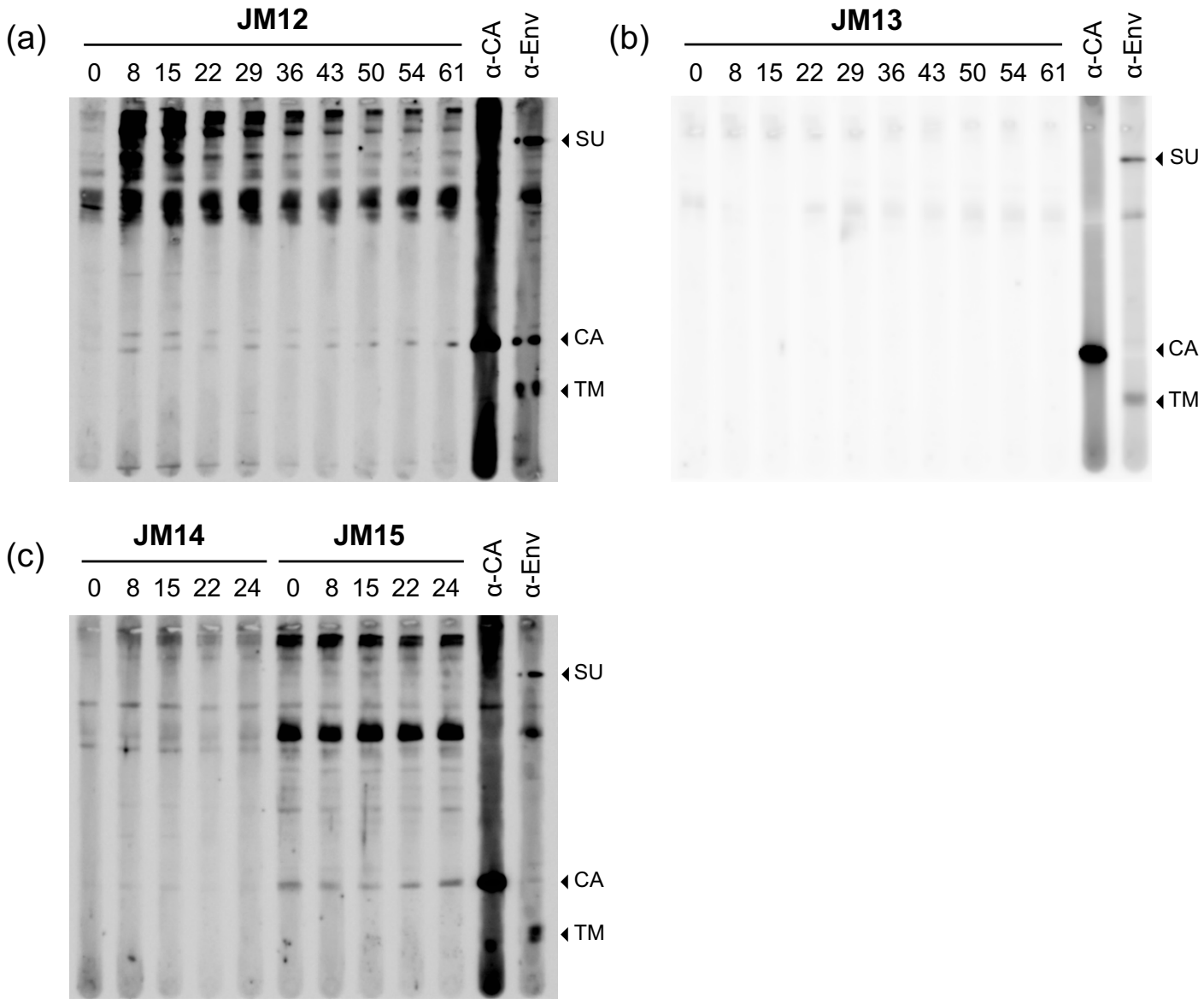

(a)
JM12
0 8 15 22 29 36 43 50 54 61
α-CA
α-Env
SU
CA
TM
(b)
JM13
0 8 15 22 29 36 43 50 54 61
α-CA
α-Env
SU
CA
TM
(c)
JM14
JM15
0 8 15 22 24 0 8 15 22 24
α-CA
α-Env
SU
CA
TM

Fig. 4

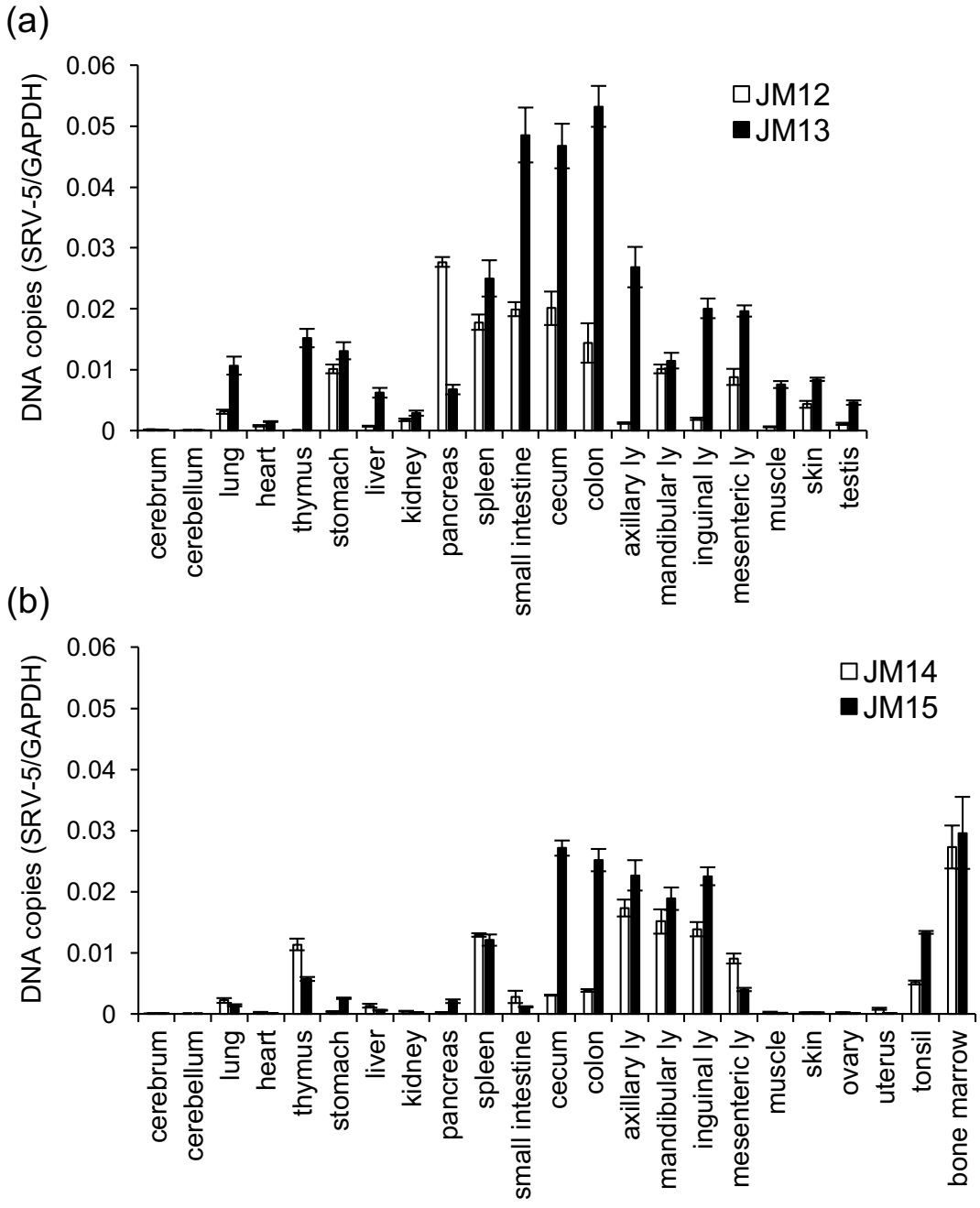

(a)
DNA copies (SRV-5/GAPDH)
0.06
0.05
0.04
0.03
0.02
0.01
0
JM12
JM13
cerebrum
cerebellum
lung
heart
thymus
stomach
liver
kidney
pancreas
spleen
small intestine
cecum
colon
axillary ly
mandibular ly
inguinal ly
mesenteric ly
muscle
skin
testis
(b)
DNA copies (SRV-5/GAPDH)
0.06
0.05
0.04
0.03
0.02
0.01
0
JM14
JM15
cerebrum
cerebellum
lung
heart
thymus
stomach
liver
kidney
pancreas
spleen
small intestine
cecum
colon
axillary ly
mandibular ly
inguinal ly
mesenteric ly
muscle
skin
ovary
uterus
tonsil
bone marrow

Fig. 5

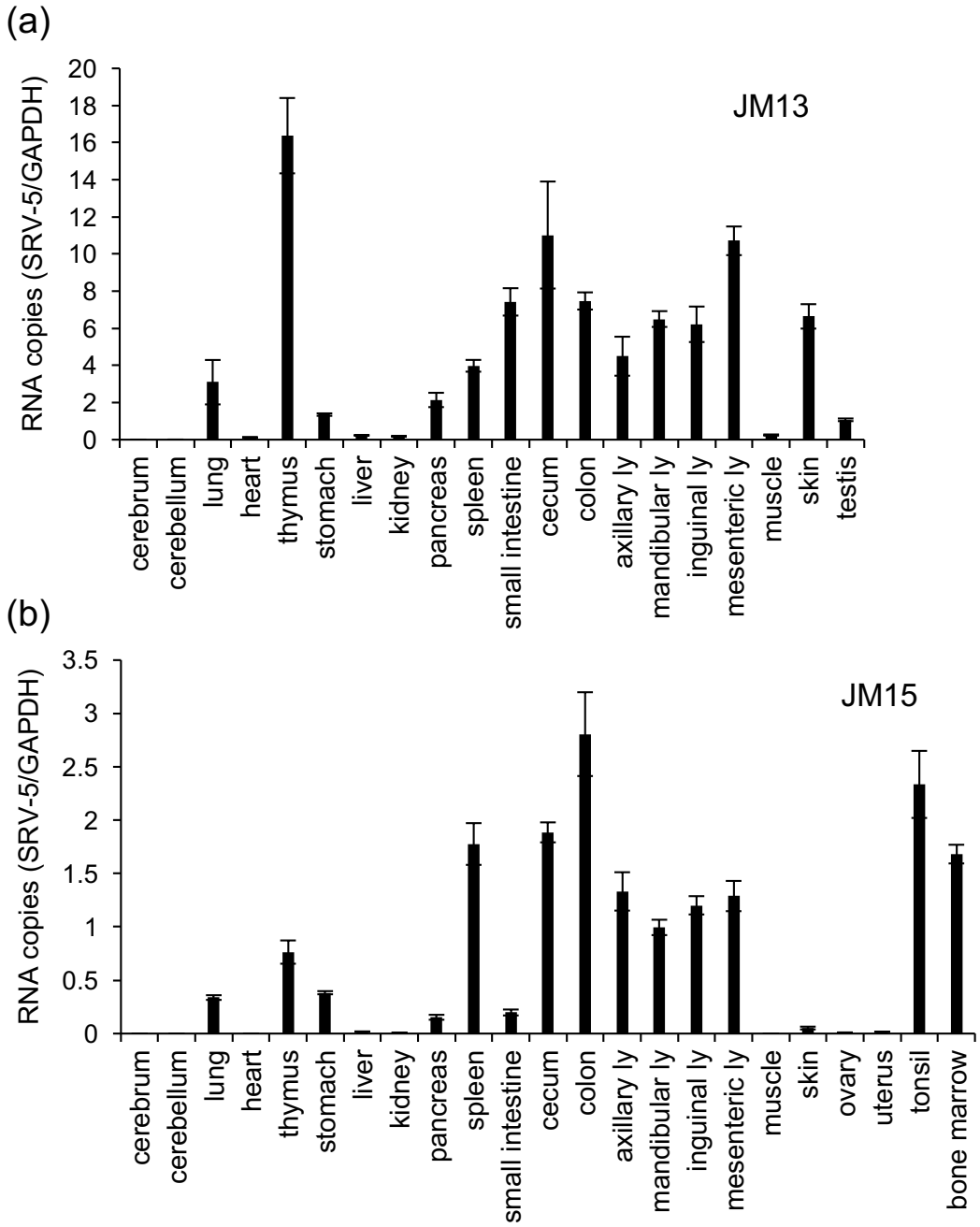
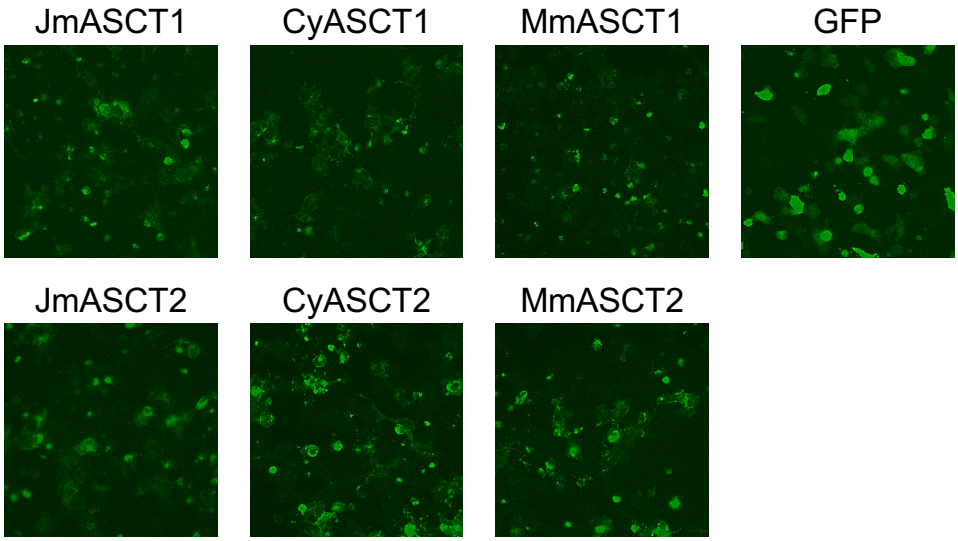

(a)
JM13
RNA copies (SRV-5/GAPDH)
20
18
16
14
12
10
8
6
4
2
0
cerebrum
cerebellum
lung
heart
thymus
stomach
liver
kidney
pancreas
spleen
small intestine
cecum
colon
axillary ly
mandibular ly
inguinal ly
mesenteric ly
muscle
skin
testis
(b)
JM15
RNA copies (SRV-5/GAPDH)
3.5
3
2.5
2
1.5
1
0.5
0
cerebrum
cerebellum
lung
heart
thymus
stomach
liver
kidney
pancreas
spleen
small intestine
cecum
colon
axillary ly
mandibular ly
inguinal ly
mesenteric ly
muscle
skin
ovary
uterus
tonsil
bone marrow

Fig. 6

(a)


JmASCT1
CyASCT1
MmASCT1
GFP
JmASCT2
CyASCT2
MmASCT2

(b)

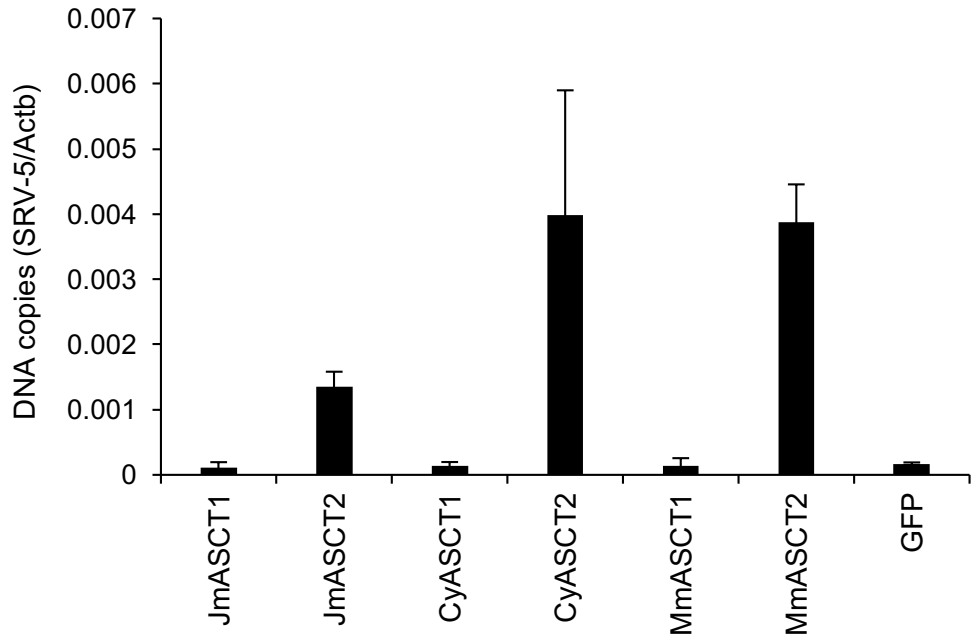

DNA copies (SRV-5/Actb)
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
JmASCT1
JmASCT2
CyASCT1
CyASCT2
MmASCT1
MmASCT2
GFP

Fig. 7