

Establishment of an Adeno-associated Virus-mediated, Locally Inducible Mouse Model of SS18–SSX2-driven Synovial Sarcoma

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Short Report

Keywords: adeno-associated virus, local gene delivery, SS18–SSX2, synovial sarcoma

Posted Date: September 22nd, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10925556/v1>

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Additional Declarations: No competing interests reported.

Abstract

Objective

We generated conditional knock-in mice carrying a canonical human *SS18–SSX2* sequence on a uniform C57BL/6J background and evaluated adeno-associated virus 9 (AAV9)-cytomegalovirus (CMV)-iCre as a new method for direct Cre delivery. AAV9-CMV-iCre was administered intramuscularly to the quadriceps, tibialis anterior, and gastrocnemius muscles.

Results

High-dose administration was associated with mortality, a proliferative brain lesion, and Cre-dependent recombination outside the injected hindlimb. Reducing the dose markedly improved tolerability and induced histologically detectable proliferative lesions in the hindlimbs of four of five mice. Lesions developed in both injected and contralateral hindlimbs, and contained *SS18–SSX2*-positive cells, some of which expressed Ki-67. Within the tissues examined in the low-dose group, no apparent proliferative lesions were detected outside the hindlimbs. These findings demonstrate that AAV9-CMV-iCre can activate a conditional human *SS18–SSX2* allele and induce *SS18–SSX2*-expressing proliferative lesions in adult mice without a cell type-specific Cre driver. By combining a fusion sequence shared with established synovial sarcoma models with AAV9-mediated Cre delivery, this approach maintains genetic continuity with previous models while introducing a distinct method of fusion-gene activation. These findings support AAV9-CMV-iCre-mediated fusion-gene activation as a distinct experimental approach for inducing *SS18–SSX2*-expressing proliferative lesions in adult mouse tissues.

Introduction

Synovial sarcoma (SyS) is an aggressive soft-tissue sarcoma that predominantly affects adolescents and young adults. Previous studies established the t(X;18)(p11;q11) chromosomal translocation as the defining genetic abnormality of SyS and demonstrated that the resulting *SS18–SSX* fusion genes, including *SS18–SSX1*, *SS18–SSX2*, and *SS18–SSX4*, function as the principal oncogenic drivers of the disease [1, 2]. These fusion genes are detected in many patients with SyS and are widely used as diagnostic markers [3].

In 2007, a conditional *SS18–SSX2* knock-in mouse model was reported in which the human *SS18–SSX2* fusion gene was inserted into the *Gt(ROSA)26Sor* locus downstream of a loxP-flanked STOP cassette [4]. In this model, *Myf5–Cre*, *Hprt–Cre*, *Pax3/Pax7–Cre*, and *Myf6–Cre* were used. Subsequently, a model using *Prx1–CreERT2* was reported [5], followed more recently by a model using *Hic1–CreERT2* [6]. Although these cell-specific Cre models are useful for evaluating particular cell populations as candidate cells of origin, the cells expressing *SS18–SSX2* are predefined by the Cre driver used. Therefore, it is difficult to investigate tumor formation without restricting the putative cells of origin.

In contrast, methods using an adenoviral (Ad) vector to deliver the Cre gene (AdCre) or Cre recombinase protein fused to a cell-penetrating TAT peptide (TATCre) have been reported [7]. However, no tumors were detected with AdCre, whereas tumor development following TATCre administration required approximately 8–14 months. These findings demonstrated both the potential to induce SS18–SSX2-expressing tumors through direct delivery of Cre and the challenges associated with this approach.

Therefore, in the present study, we investigated tumor induction using adeno-associated virus (AAV)-Cre. We independently generated a conditional knock-in mouse carrying a human SS18–SSX2 fusion gene similar to that used in previously reported models and administered AAV9-cytomegalovirus (CMV)-iCre into the quadriceps femoris, tibialis anterior, and gastrocnemius muscles. We established an AAV-Cre-induced SS18–SSX2-expressing tumor model and evaluated the anatomical locations of the resulting tumor lesions and SS18–SSX2 expression within these lesions.

Materials and Methods

Animals

Male and female C57BL/6J mice aged 8–10 weeks were maintained at the Laboratory Animal Resource Center of the University of Tsukuba, Ibaraki, Japan, at $23 \pm 2^\circ\text{C}$ and $50 \pm 10\%$ relative humidity under a 12-h light/dark cycle with free access to standard laboratory chow (Oriental Yeast Co., Tokyo, Japan) and filtered water. All animal experiments were conducted in accordance with institutional guidelines and the principles of Replacement, Reduction, and Refinement. The study was reported according to the ARRIVE guidelines.

Construction of the Conditional Gt(ROSA)26Sor^{em5(CAG–floxed EGFP–SS18–SSX2)Utr} Allele

A 20-nt sequence located within intron 1 of Gt(ROSA)26Sor (5'-CGCCCATCTTCTAGAAAGAC-3') was selected as the CRISPR target. A synthetic crRNA containing the target sequence was purchased from Integrated DNA Technologies (Coralville, IA, USA). In the pROSA-LEL-SS18–SSX2 donor plasmid vector, a cassette containing the CAG promoter, loxP, EGFP, rabbit β -globin polyadenylation signal, loxP, human SS18–SSX2 fusion cDNA, and rabbit β -globin polyadenylation signal was inserted between the 5' and 3' homology arms. The human SS18–SSX2 fusion cDNA comprised bp 1–1137 of the coding sequence of human SS18 (RefSeq: NM_005637.4), and bp 331–567 of the coding sequence of human SSX2 (RefSeq: NM_175698.4). The SS18 sequence used in the donor vector contained two synonymous variants, c.510A > G and c.699A > G, relative to NM_005637.4. The 5' and 3' homology arms in the donor vector corresponded to the genomic regions chr6:113,054,156–113,053,074 and chr6:113,052,991–113,050,021, respectively, based on the mouse reference genome assembly GRCm39/mm39. The CRISPR-Cas9 ribonucleoprotein complex and donor vector were microinjected into C57BL/6J mouse zygotes (Jackson Laboratory Japan, Kanagawa, Japan) according to a previous report [8]. The microinjected zygotes were transferred into the oviducts of pseudopregnant ICR females obtained from Jackson Laboratory Japan, Inc. (Kanagawa, Japan), and newborn mice were obtained. Founder mice

were screened by conventional PCR and green fluorescent protein (GFP) fluorescence detection in tail or toe tissues.

AAV Vector Construction and Production

AAV vectors were generated by triple-plasmid transfection of HEK293T cells using polyethyleneimine (PEI MAX; Polysciences, Warrington, PA, USA). Briefly, $1-1.5 \times 10^7$ cells were seeded in 15-cm culture dishes 16–24 h before transfection. For each dish, 30 µg each of the AAV transfer plasmid, packaging plasmid, and helper plasmid were mixed and transfected using PEI MAX. AAV isolation and purification were performed using a previously published two-step purification method with minor modifications [9].

Plasmid Cloning

The pAAV–CMV–iCre plasmid was generated by cloning the *iCre* gene, which was amplified from the pBS–GSGP2A–iCre–rGpA plasmid into the pAAV–CMV backbone (Takara, Shiga, Japan), using the In-Fusion Cloning Kit (Takara). The pHelper plasmid was purchased as part of the AAVPro Helper Free System (Takara). The pAAV2/9 plasmid was a gift from James M. Wilson (Addgene plasmid #112865; Addgene, Watertown, MA, USA).

Intramuscular Injection of AAV

Eleven-week-old homozygous *ckI-SS18-SSX2* mice with a C57BL/6J background and littermate control mice lacking the floxed allele were randomized, anesthetized with isoflurane, and intramuscularly injected with recombinant AAV particles. AAV–CMV–iCre was injected into the quadriceps femoris, tibialis anterior, and gastrocnemius muscles of the left hindlimb. In the high-dose experiments, mice received 1×10^{11} vg in 100 µL. In the reduced-dose experiments, mice received 1×10^{10} vg in 100 µL. Injections were performed using a 29-gauge insulin syringe under sterile conditions.

Tissue Collection and Histological Analysis

Mice were euthanized at the ages indicated in Tables 1 and 2 after AAV injection. Mice were monitored until the development of severe clinical signs, including respiratory distress, marked weight loss, impaired mobility, or moribund condition, at which point they were euthanized and subjected to necropsy. Animals that survived without severe symptoms were euthanized at the experimental endpoint. The animals were deeply anesthetized with isoflurane and euthanized via cervical dislocation. Tissues were fixed in 10% neutral-buffered formalin (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) at 4°C for 24 h, and embedded in paraffin. Serial sections (3–5 µm thick) were prepared and stained with hematoxylin and eosin (H&E). For histological analysis, paraffin-embedded tissues were sectioned at 2-µm thickness. All images were captured using a Biorevo BZ-9000 microscope (Keyence Corporation, Osaka, Japan).

Immunofluorescence Staining

Paraffin sections were deparaffinized in xylene. Heat-mediated antigen retrieval was performed using Target Retrieval Solution (pH 6.0; Dako, Agilent Technologies, Glostrup, Denmark) at 121°C for 20 min in an autoclave. The sections were washed with PBS and blocked with PBS containing 5% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) for 1 h, at approximately 25°C. The following antibodies were used: rabbit monoclonal anti-SS18-SSX fusion protein antibody (clone EPR24159-81, ab254406; Abcam, Cambridge, UK; dilution 1:100) and Alexa Fluor 594-conjugated rat monoclonal anti-Ki-67 antibody (clone 11F6, cat. no. 151213; BioLegend, San Diego, CA, USA; dilution 1:100). Sections were incubated with the antibodies overnight at 4°C. After washing with PBS, SS18-SSX was detected using Alexa Fluor 488-conjugated anti-rabbit IgG secondary antibody (1:500; Life Technologies, Carlsbad, CA, USA). The nuclei were counterstained with Hoechst 33342 (Molecular Probes, Eugene, OR, USA). The sections were mounted using Fluoromount (Diagnostic BioSystems, Pleasanton, CA, USA). Fluorescence images were captured using a Bioevo BZ-9000 microscope (Keyence Corporation, Osaka, Japan). Sections from non-injected mice were used as negative controls.

Results

Generation of C57BL/6J-*Gt(ROSA)26Sor^{em5(CAG-floxed EGFP-SS18-SSX2)Utr}* Mice

To establish the model on a defined genetic background, we generated genome-edited mice carrying the *Gt(ROSA)26Sor^{em5(CAG-floxed EGFP-SS18-SSX2)Utr}* allele on a C57BL/6J background, thereby enabling Cre-dependent expression of the human SS18-SSX2 fusion gene. The SS18-SSX2 sequence was based on a previously reported canonical SS18-SSX2 fusion [10]. The construct consisted of a CAG promoter followed by a loxP-flanked EGFP-polyA cassette, with the SS18-SSX2 sequence positioned downstream (Fig. 1A). Successful generation of knock-in mice was confirmed by tail GFP fluorescence (Fig. 1B) and PCR using primers designed within the SS18-SSX2 region (4,663 bp; forward, ATGGGAACTATGGTCCCAGACCAAATA; reverse, AGGATAGTGCAGGGAAACCCAAAGAAGT: Fig. 1C, and 5926 bp; forward, TCTTTTCTGTTGGACCCTTACCTTGACC; reverse, TGACCTTGTCCTGTGGGTAGTTAGGAT: Fig. 1D). These analyses confirmed the successful generation of C57BL/6J-*Gt(ROSA)26Sor^{em5(CAG-floxed EGFP-SS18-SSX2)Utr}* mice, referred to as conditional knock-in SS18-SSX2 (*cKI-SS18-SSX2*) mice.

Mortality and Cre-Dependent Recombination Beyond the Injected Limb Following High-Dose AAV9-CMV-iCre Administration

AAV9-CMV-iCre (1×10^{11} vg) was administered into the left quadriceps, tibialis anterior, and gastrocnemius muscles of *cKI-SS18-SSX2* mice. Five of the seven mice developed respiratory dysfunction and died approximately 15–20 weeks after administration. A proliferative lesion was observed in the brain of one of these mice (Table 1). In contrast, no apparent proliferative lesions were identified in the examined hindlimb tissues. H&E staining of the brain lesion revealed the proliferation of

atypical cells within a hypercellular region demarcated from the surrounding normal brain tissue (Fig. 2A). Immunofluorescence staining detected SS18–SSX2-positive cells within the lesion, a subset of which was positive for Ki-67 (Fig. 2B). These findings demonstrated the presence of proliferative cells expressing SS18–SSX2 within the brain lesion. To determine whether Cre-dependent recombination was confined to the injected limb, homozygous *cKI-SS18–SSX2* mice were administered either saline or AAV9-CMV-iCre, and GFP fluorescence was evaluated in the injected and contralateral hindlimb muscles. GFP fluorescence was retained in saline-treated mice, whereas loss of the GFP signal was observed in both the injected and contralateral hindlimbs of AAV-treated mice (Fig. 2C).

Thus, administration of 1×10^{11} vg of AAV9-CMV-iCre was associated with mortality in five of seven mice, and Cre-dependent recombination was not confined to the injected hindlimb. A proliferative lesion containing SS18–SSX2-positive cells was observed in the brain of one of the seven mice, whereas no apparent proliferative lesions were identified in the hindlimbs.

Table 1

occurrence in SS18–SSX2 flox/flox mice following adeno-associated virus (AAV) administration

Sex	Survival (Weeks After AAV)	Tumor
Male	9	No
Male	21	No
Male	42	No
Male	49	No
Female	14	No
Female	14	No
Female	18	Yes

a) Data represent individual SS18–SSX2 flox/flox mice following AAV administration.
b) Tumor incidence: 1/7 (14.3%).

SS18–SSX2-Expressing Proliferative Lesions in the Hindlimbs Following Reduced-Dose AAV9-CMV-iCre Administration

The dose of AAV9-CMV-iCre was reduced to 1×10^{10} vg and administered into the left quadriceps, tibialis anterior, and gastrocnemius muscles using the same procedure. No apparent respiratory dysfunction was observed in the reduced-dose group, and one of the five mice died. Gross hindlimb swelling was observed in three of the five mice, and well-demarcated lesions were identified on cross-sectional examination in four of the five mice (Fig. 3A). Histological evaluation confirmed hypercellular proliferative lesions in the hindlimbs of these four mice (Fig. 3B). Proliferative lesions distinguishable

from the surrounding tissues were also histologically identified in the contralateral, non-injected hindlimbs of all four mice (Fig. 3C). However, the basic architecture of the surrounding skeletal muscle outside the lesions was largely preserved, with no evidence of widespread muscle degeneration or tissue destruction.

Immunofluorescence staining detected *SS18–SSX2*-positive cells within the lesions on the injected side, a subset of which were positive for Ki-67 (Fig. D). Cells positive for both Ki-67 and *SS18–SSX2* were also detected within the lesions in the contralateral, non-injected hindlimbs (Fig. 3E). Although lesion formation was not confined to the injected limb, these findings demonstrated that reduced-dose AAV9-CMV-iCre administration induced *SS18–SSX2*-expressing proliferative lesions associated with Cre-dependent recombination.

Overall, we generated *cKI-SS18–SSX2* mice that express human *SS18–SSX2* in a Cre-dependent manner and evaluated lesion induction following intramuscular administration of AAV9-CMV-iCre. High-dose administration was associated with mortality and recombination beyond the injected limb, whereas reduced-dose administration was not accompanied by apparent respiratory dysfunction and induced bilateral hindlimb proliferative lesions expressing *SS18–SSX2* in four of the five mice. These results indicate that we established a mouse model in which AAV9-CMV-iCre induces *SS18–SSX2*-expressing proliferative lesions without the use of cell type-specific Cre drivers.

Table 2

development in *SS18–SSX2* flox/flox mice following adeno-associated virus (AAV) administration

Sex	Necropsy (Weeks After AAV)
Male	30
Male	37
Male	49
Female	39
Female	49

- a) Hindlimb tumor incidence: 4/5 (80%).
- b) No gross hindlimb swelling was observed in two mice; however, the presence of a tumor was confirmed in one of these animals.

Discussion

An important strength of this model is the evaluation of an AAV9 vector as a new method for introducing Cre activity. Most previous models have used cell type-specific Cre drivers [4–6]. These models are useful for identifying the cell lineages and developmental stages permissive for tumor formation; however, the cells expressing *SS18–SSX2* are predefined by the Cre driver used. Direct introduction of Cre activity into tissues using AdCre or TATCre has also been reported [7]; however, challenges related to the latency to lesion development and the frequency of lesion formation remained. In contrast, the present study identified *SS18–SSX2*-expressing proliferative lesions following administration of AAV9-CMV-iCre. Although the lesion induction efficiency cannot be directly compared with those of AdCre and TATCre, under the experimental conditions used in the present study, lesions developed in four of five mice following low-dose AAV9-CMV-iCre administration. These findings suggest that AAV9-mediated Cre delivery may improve the prolonged latency and low lesion formation frequency observed with previous methods of direct Cre introduction.

Furthermore, because this model was generated on a uniform C57BL/6J background while maintaining genetic continuity with previously reported models [4–6], AAV-Cre-mediated lesion induction could be evaluated under a defined genetic background. Genetic background can influence the phenotypes, tumor incidence, growth kinetics, and host responses of genetically engineered mouse models [11]. Although the C57BL/6J background itself is not a unique feature of the present study, expression of the same human *SS18–SSX2* used in previous models under a defined genetic background using AAV9-CMV-iCre is important for comparing lesion formation among different Cre delivery methods. This importance is further supported by reports that the tumor microenvironment and immune-cell composition can differ among mouse strains, even when tumors are induced by the same oncogenic driver [12]. Thus, this model may provide a foundation for future comparisons of tumor immune microenvironments and therapeutic responses under the same genetic background [13, 14, 15].

Limitations

The present study has a few limitations. First, the effects of AAV9-CMV-iCre were not restricted to the injected hindlimb. Following intramuscular administration of AAV9, AAV DNA has been detected not only in and around the injected muscle but also in multiple distant tissues, including the brain, and this dissemination has been reported to be dose-dependent [16]. Therefore, given the delivery of AAV9 to distant tissues through the circulation, this model cannot be regarded as hindlimb-specific or as a strictly localized induction model.

Conversely, in the low-dose group, although the experimental conditions allowed the possibility of distant AAV9 dissemination, no apparent proliferative lesions were identified outside the hindlimbs within the tissues examined. SyS commonly arises around the major joints of the extremities and in deep soft tissues adjacent to bone [17]. Tumor formation and progression are also influenced by interactions with the surrounding microenvironment, including stromal cells, immune cells, vasculature, and the

extracellular matrix [18, 19]. The present findings suggest that adult hindlimb tissues may represent one environment permissive for *SS18–SSX2*-dependent lesion formation. Therefore, together with future quantitative analyses of the relationship between AAV biodistribution and lesion location, optimization of the tissue selectivity of Cre-dependent recombination may enable this model to serve as an experimental system that complements existing cell-of-origin models.

Second, it is unclear whether the lesions formed in this study can be classified as SyS. Detailed histopathological assessments and transcriptomic analyses were not performed in the present study. Furthermore, the central nervous system is a rare primary site of SyS.

Nevertheless, *SS18–SSX2* is a major oncogenic driver that characterizes SyS, and previous genetically engineered mouse models have demonstrated the formation of SyS following expression of the same human *SS18–SSX2* fusion gene [4–7]. The conditional knock-in allele generated in the present study incorporated the previously reported canonical human *SS18–SSX2* fusion sequence [10]. EGFP expression was confirmed in the absence of Cre, and dsRed, which supported Cre-mediated removal of the EGFP-polyA cassette, was also detected. Thus, while retaining the human *SS18–SSX2* fusion sequence and Cre-dependent expression mechanism shared with previous models, the present study used the previously untested AAV9-CMV-iCre approach to activate the fusion gene and induce *SS18–SSX2*-expressing proliferative lesions.

Abbreviations

AAV
adeno-associated virus
CMV
cytomegalovirus
GFP
green fluorescent protein
H&E
hematoxylin and eosin
SyS
synovial sarcoma.

Declarations

Ethics approval and consent to participate

All animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Tsukuba and were approved by the Institutional Animal Care and Use Committee of the University of Tsukuba (approval number: 25–152). All mice used in this study were generated and maintained at the University of Tsukuba specifically for the present study. No privately owned animals from another institution, individual, or farm were used; therefore, informed consent from

animal owners was not applicable. All procedures involving recombinant DNA were approved by the Institutional Biosafety Committee of the University of Tsukuba (approval number: 260085). All animal experiments were performed in accordance with the principles of Replacement, Reduction, and Refinement.

Consent for publication

Not applicable.

Conflict of Interest

The authors declare no conflicts of interest.

Funding

This study was supported by AMED-CREST [grant number JP23gm171008h], the Japan Agency for Medical Research and Development (AMED) under the Fast-track program [grant numbers JP22ama121047, JP23ama121047, and JP24ama121047] and its subsequent funding [grant numbers JP25ama121047 and JP26ama121047], the Japan Science and Technology Agency (JST) [grant number JPMJPF2017], and MEXT KAKENHI [grant number 22H04922].

Author Contribution

MF conceived and designed the study, performed the experiments, analyzed and interpreted the data, and drafted the manuscript. SM contributed to the experimental design and provided the plasmid used for AAV vector construction. KI, TH, and KT contributed to the interpretation of the data and critically reviewed the manuscript. ST supervised the study, contributed to the interpretation of the data, and critically revised the manuscript. All authors read and approved the final manuscript.

Acknowledgement

We would like to thank Editage (www.editage.com) for the English language editing.

Data Availability

The datasets supporting the findings of this study are available in the Figshare repository at <https://doi.org/10.6084/m9.figshare.33424840>

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Figures

Figure 1

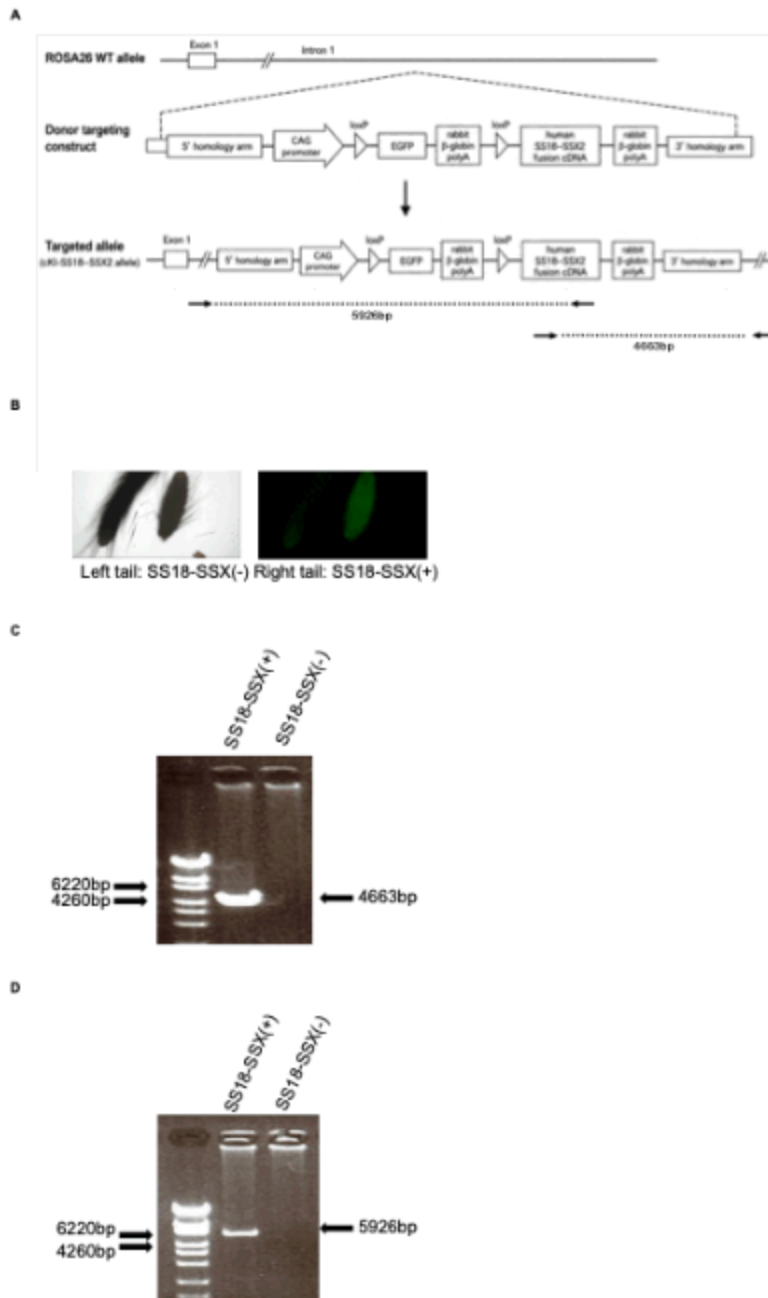


Figure 1

Generation and validation of the conditional *SS18-SSX2* knock-in mouse allele. (A) Schematic representation of the conditional *SS18-SSX2* knock-in construct. The construct consists of a promoter followed by a loxP-flanked EGFP-STOP cassette placed upstream of the *SS18-SSX2* fusion gene. Cre-mediated recombination excised the STOP cassette and activated *SS18-SSX2* expression. (B) Detection of green fluorescent protein (GFP) fluorescence in the tail or digit tissue of homo *cKI-SS18-SSX2* mice. Fluorescence imaging was performed to confirm the presence of the reporter cassette associated with conditional alleles. (C) PCR genotyping strategy for identifying the *SS18-SSX2* allele. Genomic DNA isolated from mouse tail or digit tissue was subjected to PCR using primers designed within the *SS18-*

SSX2fusion region. The expected PCR product corresponding to the SS18–SSX2 allele was 4663 and 5926 bp. Representative images from n = 3 mice are shown. Scale bars: 100 μm.

Figure 2

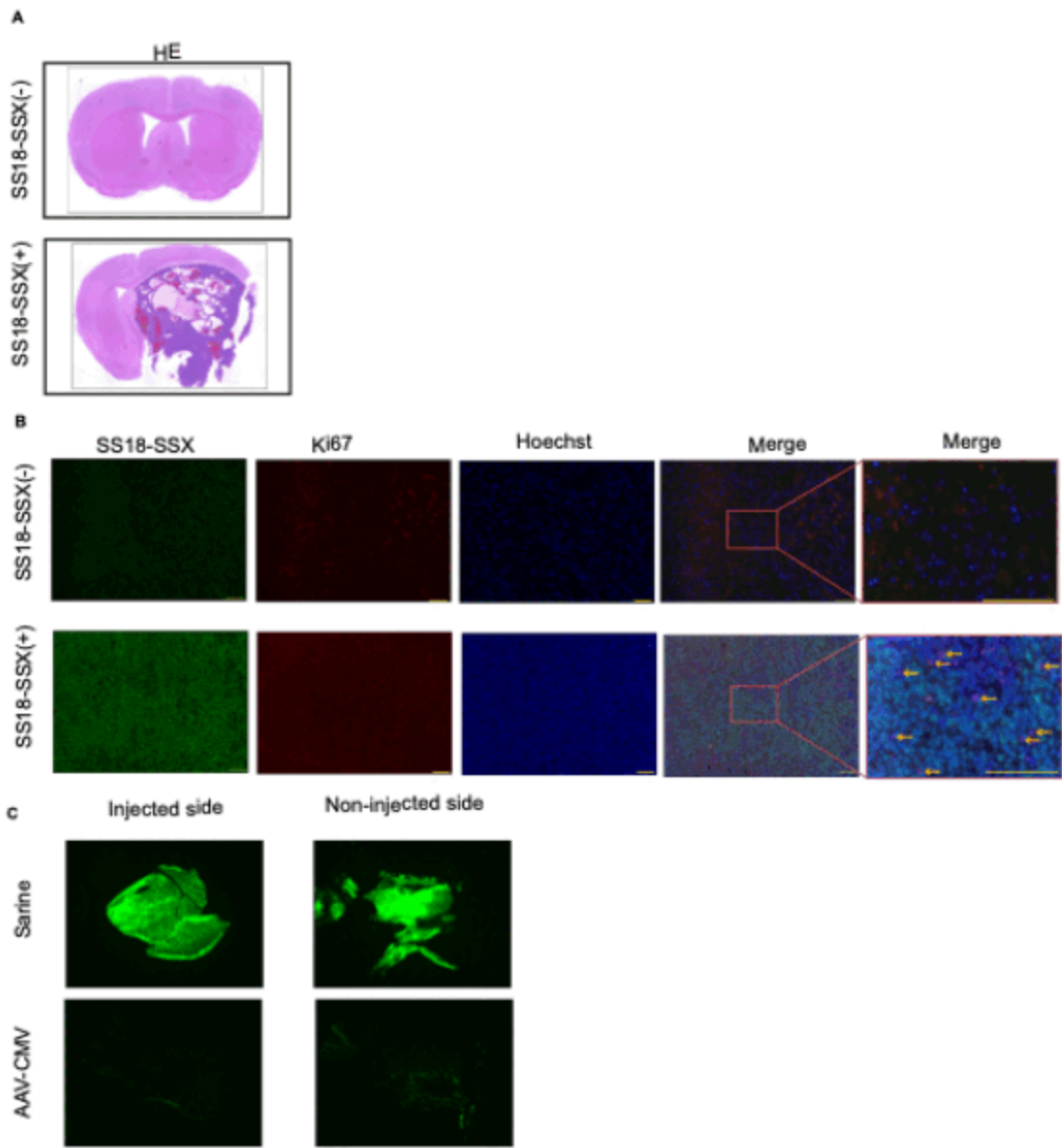


Figure 2

Histological and immunofluorescence analyses following adeno-associated virus (AAV)-mediated gene delivery. Eleven-week-old homo *cKI-SS18–SSX2* mice were injected with AAV–cytomegalovirus (CMV)–iCre (1×10^{11} vg) into the quadriceps, tibialis anterior, and gastrocnemius muscles. (A) Histological analysis of the brain tissue. The sections were stained with hematoxylin and eosin (H&E). (B) Immunofluorescence staining for SS18–SSX2 and Ki-67 in the brain tissue sections. The nuclei were counterstained with Hoechst. Orange arrows indicate cells that are positive for both SS18–SSX2 and Ki-67. (C) Green fluorescent protein (GFP) fluorescence imaging of the skeletal muscle tissue from the

injected and contralateral hindlimbs. Representative images from n = 5 mice are shown. Scale bars: 100 μ m.

Figure 3

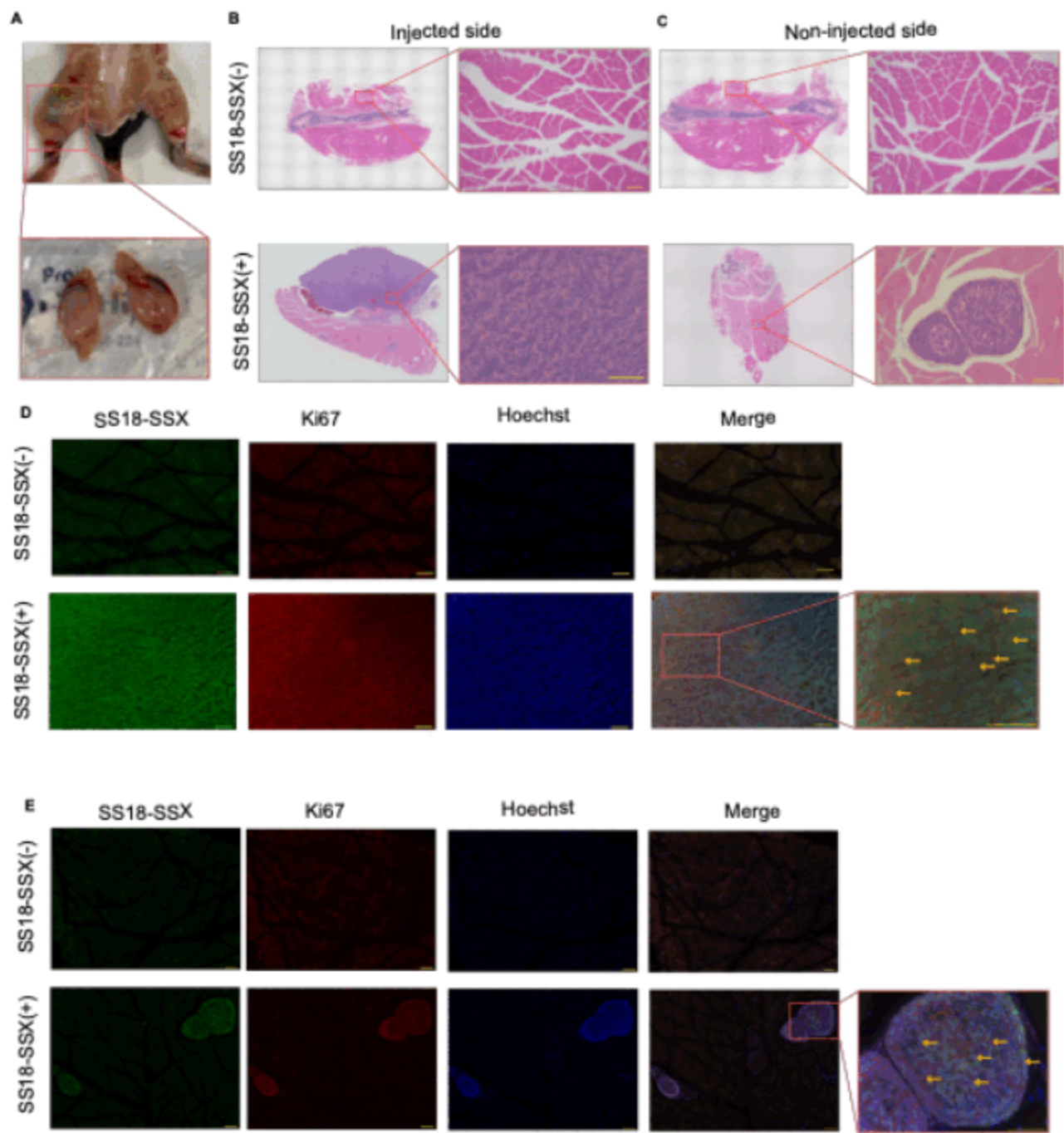


Figure 3

Tumor analysis of hindlimb tissues following reduced-dose adeno-associated virus (AAV) administration. Homozygous *cKI-SS18-SSX2* mice were injected with AAV–cytomegalovirus (CMV)–iCre (1×10^{10} vg) into the quadriceps, tibialis anterior, and gastrocnemius muscles. (A) Gross morphology of the hindlimb after AAV administration. Histological analysis of hindlimb tissue via hematoxylin and eosin

(H&E) staining on the injection side (B) and contralateral non-injected side (C). Immunofluorescence staining for *SS18-SSX2* and Ki-67 in hindlimb tissues from the injection side (D) and contralateral non-injected side (E). Orange arrows in (D) and (E) indicate cells that are positive for both *SS18-SSX2* and Ki-67. Representative images from n = 5 injected mice are shown. Scale bars: 100 μ m.