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METHODS: ORIGINAL ARTICLE

Robust Long-term Transduction of Common Marmoset Neuromuscular Tissue With rAAV1 and rAAV9

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Profiles of recombinant adeno-associated virus (rAAV)-mediated transduction show interspecies differences for each AAV serotype. Robust long-term transgene expression is generally observed in rodents, whereas insufficient transduction is seen in animals with more advanced immune systems. Non-human primates, including the common marmoset, could provide appropriate models for neuromuscular diseases because of their higher brain functions and physiological resemblance to humans. Strategies to induce pathologies in the neuromuscular tissues of non-human primates by rAAV-mediated transduction are promising; however, transgene expression patterns with rAAV transduction have not been elucidated in marmosets. In this study, transduction of adult marmoset skeletal muscle with rAAV9 led to robust and persistent enhanced green fluorescent protein (EGFP) expression that was independent of the muscle fiber type, although lymphocyte infiltration was recognized. Systemic rAAV injection into pregnant marmosets led to transplacental fetal transduction. Surprisingly, the intraperitoneal injection of rAAV1 and rAAV9 into the neonatal marmoset resulted in systemic transduction and persistent transgene expression without lymphocyte infiltration. Skeletal and cardiac muscle were effectively transduced with rAAV1 and rAAV9, respectively. Interestingly, rAAV9 transduction led to intense EGFP signaling in the axons of the corpus callosum. These transduction protocols with rAAV will be useful for investigating gene functions in the neuromuscular tissues and developing gene therapy strategies.

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Introduction

Adeno-associated virus (AAV) provides a highly promising platform for *in vivo* transduction, due to its physical and chemical stability, broad host range (including nonproliferating cells in the neuromuscular tissues), and lack of its own pathogenicity. In standard recombinant AAV (rAAV) vectors, all of the virus-encoding genes have been removed. Transduction with the resulting vectors results in the persistence of episomal forms in the infected tissue, safe and long-term transgene expression, and comparatively low intrinsic immunogenicity.[1–3]

Several reports on rAAV-mediated transduction in various species have shown interspecies differences in the transduction patterns of each AAV serotype. Whereas rodents generally show robust long-term neuromuscular transgene expression, other animals with more advanced immune systems shown an early decrease in transgene expression. For example, when rAAV was injected both locally and systemically to adult mice, robust transgene expression was observed,[4,5] although a humoral immune response was elicited against the viral capsid. In large animals, such as dogs[6,7] and Old World monkeys,[8–10] rAAV-mediated transduction often led to a strong immune response depending on the transgene.

The common marmoset (*Callithrixjacchus*) is a nonhuman primate (NHP) from South America. It shows several advantages in breeding, such as a small body, short gestation, multiple births, and short sexual maturation.[11] The common marmoset could provide an appropriate model for neuromuscular diseases because of its higher brain function and physiological resemblance to human.[12–14] A green fluorescent protein (GFP) transgenic common marmoset was reportedly developed through gene transfer to the preimplantation embryo by lentiviral vector (rLV); however, this method requires many donor and recipient animals to treat the preimplantation embryo[15] and, therefore, is impractical for generating disease models.

Strategies to induce pathologies in neuromuscular tissues by rAAV-mediated systemic transduction could be promising, if efficient transduction were to be achieved; however, interspecies differences in the transduction patterns with rAAV are expected, even among NHPs. Moreover, data on such differences have not been elucidated in marmosets. Therefore, we studied the transduction and expression patterns of rAAV1 and rAAV9, which are frequently used for neuromuscular research, revealing the robust, long-term, and stable transduction of rAAV in the neuromuscular tissues of marmosets.

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Results

Transduction of adult marmoset skeletal muscle with rAAV9

The tibialis anterior and extensor carpi radialis muscles of an adult marmoset were injected with rAAV9 carrying CAG promoter-driven enhanced green fluorescent protein (EGFP) (rAAV9-CAG-EW, 1.0 × 10^{12} viral genomes (vg)/site). Local intramuscular (i.m.) injection of rAAV9 into the adult marmoset resulted in intense expression by 10 weeks, which lasted for at least 35 weeks (**Figure 1a**). Variegated diminution of EGFP expression observed at 10 weeks after injection seemed to be irrelevant to muscle fiber types (**Figure 1b**). Intense EGFP expression was observed for at least 35 weeks, whereas fibrosis and lymphocyte infiltration were induced by 10 weeks after injection (**Figure 1c**).

Transplacental transduction of fetus after systemic rAAV injection into pregnant marmoset

To determine which stages of development were affected by rAAV-mediated transduction, pregnant marmosets were intraperitoneally (i.p.) injected with rAAV1 or rAAV9 expressing EGFP (1.0 × $10^{11–13}$ vg/body) at 41, 43, 49, 50, and 122 days of gestation (gd). Whereas rAAV injection until 50 gd resulted in fetal resorption or abortion, the marmoset that received a rAAV injection at 122 gd delivered two live births and one stillbirth at full term (**Table 1**). In the case of abortion after injection at 43 gd, the transgene in the rAAV genome was detected by PCR analysis in both the fetus and placenta. After injection at 122 gd, the rAAV genome was detected in all live birth, stillbirth, and afterbirth samples, including the placenta, umbilical cord, liver, kidney, brain, limb muscle, diaphragm, and ear (**Supplementary Figure S1**).

Long-term and effective skeletal muscle transduction after systemic rAAV1 injection into the neonatal marmoset

A neonatal marmoset was i.p. injected with EGFP-expressing rAAV1 (rAAV1-CAG-EW, 1.0 × 10^{13} vg/body) at birth. Necropsy was performed at 20 months after the injection. Although a single i.p. injection into the neonate resulted in persistent skeletal muscle transduction, robust EGFP expression did not induce lymphocyte infiltration (**Figure 2a**). Abundant EGFP expression was found in the liver (**Figure 2b**). Detectable EGFP expression was seen in the heart, kidney, ovary (but not the oocytes), and lung (**Figure 2c–e**, **Supplementary Figure S2a**). Scattered EGFP expression was observed in the spleen (**Supplementary Figure S2b**). Thin line-shaped EGFP signals surrounded the hair root (**Supplementary Figure S2c**). In the brain tissue, scattered EGFP expression was observed in mature neurons, astrocytes, and oligodendrocytes (**Figure 2f–h**). Percentage of NeuN, GFAP, and OLIG2 positive cells co-expressing EGFP were 44, 39, and 17% respectively. Dense EGFP-positive cell populations were scattered in the pons (**Supplementary Figure S2d**). The grade of EGFP-positive cells in each organ is summarized in **Table 2**. Lymphocyte infiltration was not obvious in any of the organ tissue samples examined.

Long-term and effective brain and cardiac muscle transduction after systemic rAAV9 injection into the neonatal marmoset

rAAV9-expressing EGFP (rAAV9-CAG-EW, 1.0 × 10^{12} vg/body) was i.p. injected into a neonatal marmoset at 1 day after birth. Necropsy was performed at 10 months after the

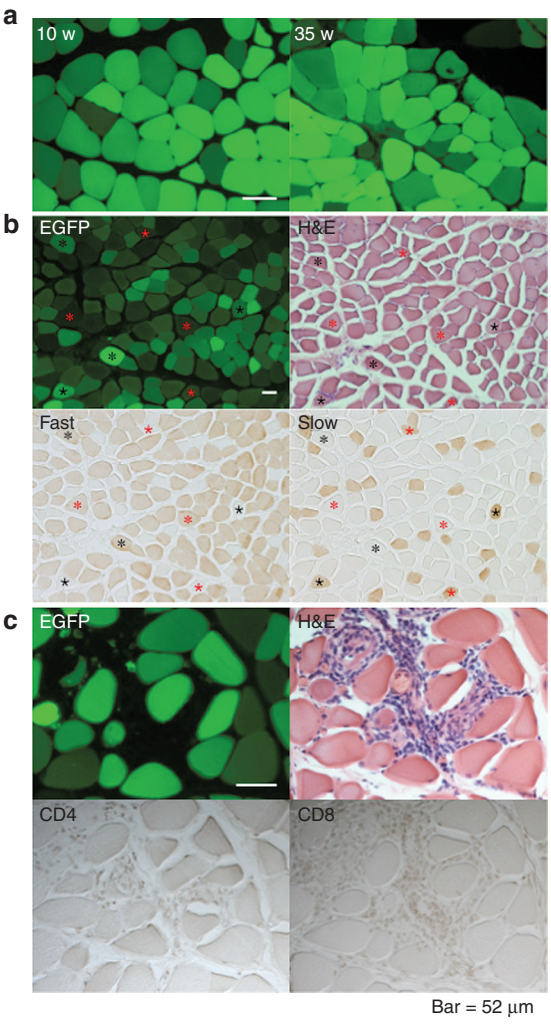

Figure 1 Local rAAV9 injection in adult marmoset muscle leads to robust transduction and a cellular immune response. (**a**) Representative photomicrographs of EGFP expression after injection of rAAV9-CAG-EW into the adult marmoset muscles. Intense expression was observed by 10 weeks (ECR muscle) and lasted for at least 35 weeks (TA muscle). (**b**) Diverse diminution of EGFP expression observed at 10 weeks after injection was irrelevant to muscle fiber types (ECR muscle). Representative photomicrographs of serial muscle sections showing EGFP expression, H&E, fast-type MyHC, and slow-type MyHC. Red star, EGFP-positive slow fiber; red asterisk, EGFP-negative slow fiber; black star, EGFP-positive fast fiber; black asterisk, EGFP-negative fast fiber. (**c**) Fibrosis and lymphocyte infiltration were induced by 10 weeks after injection. Representative photomicrographs of serial muscle sections showing EGFP expression, H&E staining, $CD4^+$, and $CD8^+$ T-cell immunostaining. Scale bar indicates 50 µm. ECR, extensor carpi radialis; EGFP, enhanced green fluorescent protein; H&E, hematoxylin& eosin; MyHC, myosin heavy chain; rAAV9, recombinant adeno-associated virus-9; TA, tibialis anterior.

Table 1 Summary of transplacental gene transduction experiments

Marmoset ID	Sex	rAAV serotype	Vector dose	Route	Transgene	Gestational age		Vector genome	
						Injections	Abortion	Fetus/neonate	Placenta
M04029F2	F	1	1×10^{11}	i.p.	EGFP	E41	E90	NA	–
7028	F	1	1×10^{13}	i.p.	EGFP	E43	E85	+	+
I3594	F	1	1×10^{13}	i.p.	EGFP	E49	Resorbed	NA	NA
8058	F	1	1×10^{11}	i.p.	EGFP	E50	Resorbed	NA	NA
90989	F	9	1×10^{12}	i.p.	EGFP	E122	+[a]	+	

NA, not available.
[a]The marmoset that received a rAAV injection at 122 gestational days delivered two live births and one stillbirth at full term. The rAAV genome was detected in organs of the live birth (ear) and the stillbirth (umbilical cord, liver, kidney, brain, limb muscle, diaphragm, and lung).

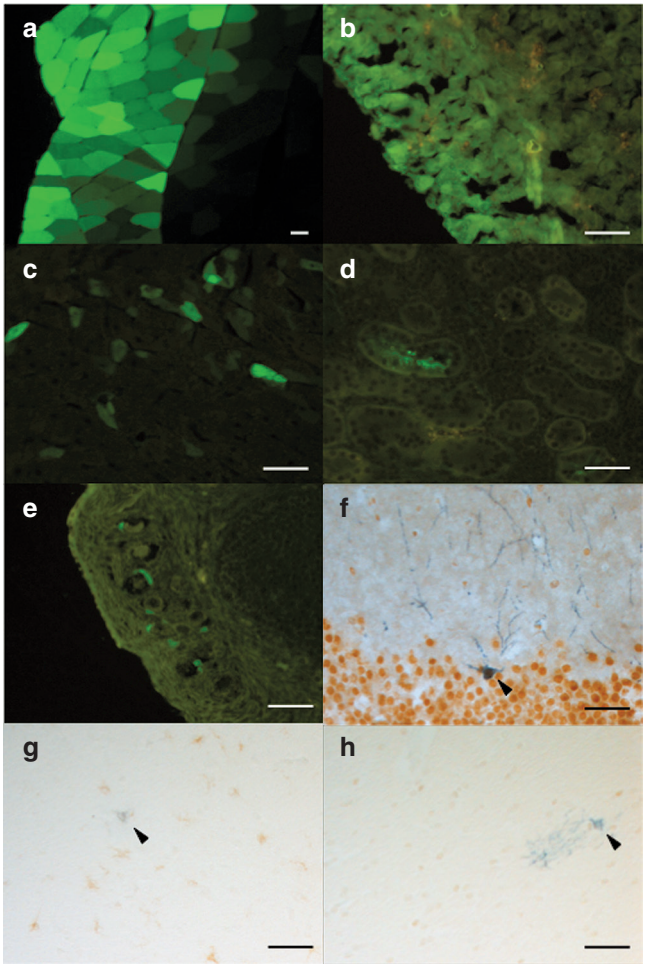

Figure 2 Representative photomicrographs of EGFP expression within assorted organs at 20 months after a single rAAV1 intraperitoneal injection in the neonatal marmoset. Among the tissues examined, GFP expression was most abundant in the (**a**) quadriceps and (**b**) liver. Detectable EGFP expression was seen in the (**c**) heart, (**d**) kidney, (**e**) ovary (but not the oocytes). In the brain tissue, double immunohistochemical staining for cell type-specific markers (DAB, brown) and EGFP (vector SG, dark blue) was performed. EGFP expression was observed in (**f**) NeuN-positive mature neurons, (**g**) GFAP-positive astrocytes, and (**h**) OLIG2-positive oligodendrocytes. Arrowhead indicates double-positive cells. Scale bar indicates 50 μm. EGFP, enhanced green fluorescent protein; rAAV1, recombinant adeno-associated virus-1.

injection. In the case of rAAV9, a single i.p. injection into the neonate resulted in robust cardiac muscle transduction and persistent EGFP expression, as seen in both the cross-sectional view (**Figure 3a**) and the longitudinal view (**Supplementary Figure S3a**). Detectable EGFP expression was not seen in the quadriceps muscle. Intense EGFP expression in the liver was seen in the rAAV9-transduced animal (**Figure 2b**). Detectable EGFP expression was seen in the diaphragm, kidney, and ovary (but not in the oocytes) (**Figure 3c–e**). Scattered EGFP-positive cells were observed in the dermis layer of the skin, lung, myometrium of the uterus, and sclera (**Supplementary Figure S3b–e**). The spleen did not show detectable EGFP expression (**Supplementary Figure S3f**).

In the brain tissue, robust EGFP expression was observed in neurofilament subunit M-positive axons, especially in the corpus callosum (**Figure 3f**, **Supplementary Figure S3g**). The EGFP expression was observed in a few oligodendrocytes (**Figure 3g**), but not in astrocytes (**Figure 3h**). Percentage of NeuN and OLIG2 positive cells co-expressing EGFP were 73 and 1% respectively. The grade of EGFP-positive cells in each organ is summarized in **Table 2**. As was demonstrated in the rAAV1-transduced animal, lymphocyte infiltration was not remarkable in any organ.

Discussion

To our knowledge, this is the first study to administer a rAAV to marmosets by several routes and at various stages of maturation. When EGFP-expressing rAAV9 was administrated to the skeletal muscles of an adult marmoset, intense EGFP expression was observed for at least 35 weeks in both slow- and fast-type myofibers. Fibrosis and lymphocyte infiltration were induced by 10 weeks after injection. Although the i.p. injection of EGFP-expressing rAAV into the pregnant marmoset until 50 gd resulted in fetal resorption or abortion, rAAV was able to transduce the fetus transplacentally in the marmoset. The marmoset that received a rAAV injection at 122 gd gave birth at full term. The i.p. injection of EGFP-expressing rAAV into the neonatal marmoset resulted in systemic transduction and persistent transgene expression that was unaccompanied by lymphocyte infiltration. In particular, rAAV1 and rAAV9 efficiently transduced the skeletal and cardiac muscle, respectively. Additionally, EGFP expression might be characteristic in neurofilament subunit M-positive axons in the rAAV9-transduced animal.

Table 2 Summary of neonatal systemic gene transduction experiments

Marmoset ID	Sex	Age	rAAV serotype	Vector dose	Route	Transgene	Necropsy	Lymphocyte invasion[a]	Presense of vector genome[a]					
									Brain	Kidney	Liver	Heart	Muscle	Ovary
10034	F	0d	1	1×10^{13}	i.p.	EGFP	1 year 8 months	–	1+	±	3+	2+	3+	1+
11095	F	1d	9	1×10^{12}	i.p.	EGFP	10 months	–	1+	1+	1+	3+	±	1+

[a]EGFP positive cells per 10 field of view: –, 0; ±, <10; 1+, <100; 2+, <1,000; 3+, >1,000.

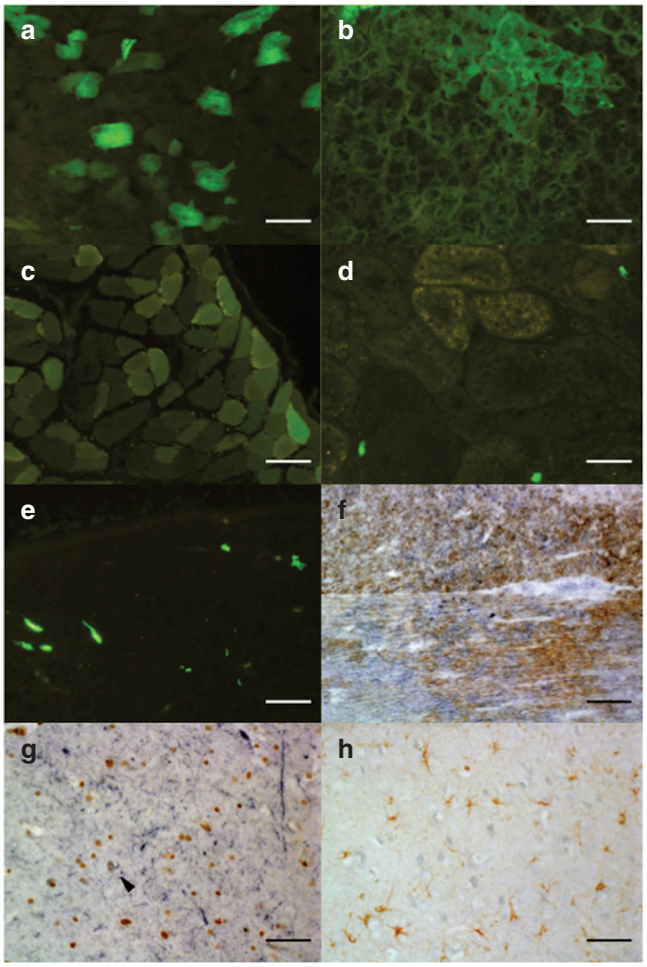

Figure 3 Representative photomicrographs of EGFP expression within assorted organs at 10 months after a single rAAV9 intraperitoneal injection in the neonatal marmoset. Among the examined tissues, EGFP expression was most abundant in the (**a**) heart and (**b**) liver. Detectable EGFP expression was seen in the (**c**) diaphragm, (**d**) kidney, and (**e**) ovary (but not the oocytes). In the brain tissue, double immunohistochemical staining for cell type-specific markers (DAB, brown) and EGFP (vector SG, dark blue) was performed. EGFP expression was observed in (**f**) neurofilament M-positive axons and (**g**) OLIG2-positive oligodendrocytes. EGFP expression was not detected in (**h**) GFAP-positive astrocytes. Arrowhead indicates double-positive cells. Scale bar indicates 50 μm. EGFP, enhanced green fluorescent protein; rAAV9, recombinant adeno-associated virus-9.

Although GFP transgenic common marmosets have been generated via gene transfer to the preimplantation embryo by rLV, in this method, many donor and recipient animals are needed to treat the preimplantation embryo.[15] The phenotypes of transgenic animals produced by this approach could be diversified depending on the integration site, due to position-effect variegation and transcriptional interference.[16] Moreover, in species that require several years to reach sexual maturation, huge costs are required to produce such transgenic animals and their phenotypically expected posterity. By contrast, the induction of pathologies by rAAV-mediated transduction has already been achieved in several species, including NHPs.[17–20] Although it is desired that this technology be applied to a wide range of diseases and species, the transgene expression patterns with rAAV transduction remain unclear, particularly in marmosets.

Robust transgene expression was found after local and systemic rAAV injection in adult mice,[4,5] although a humoral immune response was elicited against the viral capsid. In dogs[6,7] and Old World monkeys,[8–10] rAAV-mediated transduction has often been shown to elicit a strong immune response depending on the transgene. A recent phase I trial concerning Duchenne muscular dystrophy[21] revealed potential cellular immune responses to the transgene. In this trial, one of the two subjects with a positive ELISpot assay was responsive to the microdystrophin protein before exposure to AAV expression dsytrophin. In the present study in marmosets, lymphocyte infiltration was observed by 10 weeks after injection, and robust transgene expression persisted until at least 35 weeks after injection. Therefore, the immune response of the marmoset might be weaker than that of animals with more advanced immune systems.

The rAAV-mediated transduction of the fetus by systemic injection to the pregnant animal is useful for the induction of tolerance to the viral capsid and transgene product, especially in small animals such as marmosets. The systemic infection of wild-type AAV into pregnant mice through 7 gd was shown to inhibit fetal development.[22] Infection of mice after 13 gd allowed the delivery of wild-type AAV-positive neonatal mice at full term.[23] In the marmoset, the detection of the vector genome from the fetus indicated that rAAV-mediated transplacental transduction had occurred by rAAV injection until 50 gd. Isolated feeding of the animals in early pregnancy after rAAV injection might affect the fetal loss because marmosets are very sensitive to their ambient environmental change. The developmental stage of 50 gd in marmoset corresponds to before 9 gd in mouse.[24,25] Conversely, rAAV-mediated transduction throughout conceptus did not affect

fetal development at the stage corresponding to after 14 gd in mouse. Our transplacental experiments in this study would provide the fundamental data aiming to induce tolerance more easily for a small non-human primate in the future. However, the fetal loss by EGFP gene transduction in early pregnancy evokes the fact that embryos transduced with GFP-expressing rLV showed lower implantation and pregnancy rates than nontreated embryos.[15]

There are several reports of persistent systemic transgene expression and escape from cellular immunity via intravenous rAAV injection into neonates in several species.[26,27] In marmoset, the intraperitoneal injection of rAAV into the neonate resulted in persistent systemic transduction without obvious lymphocyte infiltration, although moderate inflammatory events at earlier time points might be missed. Remarkably, different rAAV transduction patterns were found between marmoset and macaque. Systemic injection of rAAV9 into the neonate macaque resulted in robust skeletal muscle and weak cardiac muscle transduction,[26] but injection into marmoset resulted in the opposite findings. On the other hand, rAAV1 appeared to be useful for skeletal muscle transduction in marmoset.

Interspecies differences between marmoset and macaque were also seen in the brain tissue. In macaque, the brain cell type that was predominantly transduced with rAAV1 was the oligodendrocyte.[28] In marmoset, scattered rAAV1 transduction was observed throughout the brain, with similar levels of transduction in mature neurons, astrocytes, and oligodendrocytes. In macaque, intravascular rAAV9 delivery transduced mostly glia throughout the brain[29] and a few motor neurons.[26] Conversely, in marmoset, the rAAV9-mediated transduction of glia was not remarkable. Our observations support the findings of a previous report on the marmoset brain transduction patterns with local rAAV9 injection,[13] although the studies differed in their administration routes. Furthermore, we demonstrated that the systemic transduction of the neonate marmoset with rAAV9 resulted in intense expression in axons of the corpus callosum.

The AAVS1 locus-targeted integration assisted by Rep protein[30] might be useful as a reproductive technology to resolve phenotypic diversity in heritable transgenic animals; however, at present, rAAV-mediated *in vivo* transduction seems to be a more feasible approach to generate a neuromuscular disease model in marmoset.

In conclusion, the neuromuscular tissues of a primate (common marmoset) were strongly, persistently, and stably transduced with rAAV. This transduction protocol would be useful for investigating various gene functions in the neuromuscular tissues and developing gene therapy strategies.

Materials and methods

Proviral plasmid construction and rAAV vector production. The AAV2 vector proviral plasmids harboring EGFP cDNA, the woodchuck hepatitis virus post-transcriptional regulatory element, and the CAG promoter, a modified chicken actin promoter with a cytomegalovirus immediate early enhancer (CAG-EW), were propagated. The vector genome was packaged into the pseudotyped AAV1 or AAV9 capsid in HEK293 cells. A large-scale cell culture method with an active gassing system was used for transfection.[31] The vector production process involved triple transfection of a proviral plasmid, a chimeric helper plasmid encoding either AAV2 rep/AAV1 cap genes or AAV2 rep/AAV9 cap genes (pAAV2/1, pAAV2/9, respectively, gifts from James M Wilson),[32–34] and an adenovirus helper plasmid pHelper (Stratagene, La Jolla, CA). All of the viral particles were purified by CsCl gradient centrifugation, followed by dual ion-exchange procedures with high-performance membrane adsorbers.[35] Viral titers were determined by quantitative PCR, with SYBR-green detection of PCR products in real time with the MyiQ single-color detection system (Bio-Rad, Hercules, CA) and the following primer set for EGFP: forward primer 5′-GTGAGCAAGG GCGAGGAG-3′, and reverse primer 5′-GTGGTGCAGA TGAACTTCAG G-3′.

Animals. One adult male, five pregnant female, and two neonatal common marmosets were used for this study. The body weights of adults ranged from 250 to 350 g, and those of neonates were 25 to 30 g. The animals were maintained in rooms under controlled conditions of temperature (27–29 °C), humidity (40–60%), and light cycle (12 hours light/12 hours dark). Animals were allowed free access to water and were fed a diet of monkey chow and supplemental vitamins daily. They were housed in a cage measuring 100 cm wide × 60 cm deep × 80 cm high, in accordance with the Guidelines of the National Center of Neurology and Psychiatry (NCNP). All experiments were conducted in accordance with protocols approved by the Ethics Committee for Primate Research of the National Institute of Neuroscience, NCNP, Japan.

Direct administration of rAAVs into skeletal muscle. Animals were initially anesthetized with 15–22 mg/kg of i.m. ketamine. The tibialis anterior and extensor carpi radialis muscles of marmosets were injected with 200 and 100 µl, respectively, of rAAV9-CAG-EW (1×10^{12} vg/site).

Neonatal systemic delivery of rAAV. Neonatal marmosets (postnatal day 0–1) were positioned by hand, and rAAV1-CAG-EW or rAAV9-CAG-EW was administered i.p. with a 29-gauge hypodermic needle and disposable syringe. Each marmoset received a total volume of 500 µl containing 1×10^{12} to 1×10^{13} vg.

Transplacental delivery of rAAVs into the fetus. Pregnant female marmosets were initially i.m. anesthetized with 15–22 mg/kg of ketamine. One milliliter of rAAV1-CAG-EW or rAAV9-CAG-EW was administered i.p. with a 29-gauge hypodermic needle and disposable syringe.

Biopsy of transduced muscles. Biopsy was conducted under aseptic conditions. Animals were i.m. anesthetized with 15–22 mg/kg of ketamine. Anesthesia was maintained with inhalation of sevoflurane (1.5–2.5% in oxygen). The tibialis anterior or extensor carpi radialis muscles of i.m. or systemically transduced marmosets were sampled. The muscle was postfixed at 4 °C with 4% paraformaldehyde.

Necropsy. Animals were sacrificed with an intravenous pentobarbital overdose. The animals were perfused with phosphate-buffered saline and ice-cold 4% paraformaldehyde, and samples collected for histological analysis were postfixed at 4 °C with the same fresh fixative.

Histology and immunohistochemistry. Tissues postfixed in 4% paraformaldehyde were transferred to 30% sucrose in 0.1 mol/l phosphate buffer (pH 7.4) for cryoprotection and stored at 4 °C overnight. The tissues were briefly rinsed with phosphate-buffered saline before freezing. Muscle tissues were immediately frozen in liquid nitrogen-cooled isopentane. Other tissues were placed in specimen molds containing Tissue-Tek optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan) and frozen in liquid nitrogen. The GFP expression was examined in tissue cryosections by direct fluorescence. Histological examination was performed through hematoxylin and eosin staining.

Immunohistochemical procedures were performed as previously described, with a few modifications.[12,36] Antigens were retrieved by incubating the sections in 10 mmol/l sodium citrate buffer (pH 6.0) at 93 °C for 20 minutes in an autoclave apparatus. For peroxidase staining, endogenous peroxidase activity was blocked by incubating the cryosections with 3% hydrogen peroxide for 30 minutes before the application of primary antibody. Sections were preincubated for 1 hours with TBS containing 5% normal calf serum and 2% Block Ace (Dainipponseiyaku, Osaka, Japan; Cat. No. UK-B80) at 4 °C. The sections were incubated with primary antibodies in TBS with 5% normal calf serum, 2% Block Ace, and 2% Triton X-100 at 4 °C overnight. Antibodies against the following cell lineage marker and reporter proteins were used: neuron-specific nuclear protein (NeuN; mouse IgG, 1:100; Millipore, Billerica, MA), tyrosine hydroxylase (TH; mouse IgG, 1:1,000; Sigma-Aldrich, St Louis, MO), glial fibrillary acidic protein (GFAP; rabbit IgG, 1:1,000; Dako, Carpinteria, CA), oligodendrocyte transcription factor 2 (Olig2; rabbit IgG, 1:20; Millipore, Billerica, MA), neurofilament M (NF-M; rabbit IgG, 1:1,000; Millipore), CD4 (mouse IgG, 1:50; BD Biosciences, San Jose, CA), CD8 (mouse IgG, 1:25; Serotec, Oxford, UK), myosin slow-type heavy chain (MHCs; mouse IgG, 1:20; Leica Biosystems, Richmond, IL), myosin fast-type heavy chain (MHCf; mouse IgG, 1:10; Leica Biosystems), and EGFP (EGFP; rabbit IgG, 1:10; TaKaRa Bio, Otsu, Shiga, Japan).

The sections were rinsed with TBS and incubated with secondary antibodies in TBS, 5% normal calf serum, 2% Block Ace, and 2% Triton X-100 at 4 °C overnight. The following secondary antibodies, which were directed against the species in which the primary antibody was raised, were used in each case: horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (1:200; Bio-Rad) and HRP-labeled goat anti-rabbit IgG (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, or 1:200; Bio-Rad). The sections were rinsed with TBS and developed with the DAB (Vector Laboratories, Burlingame, CA) or VECTOR SG (Vector Laboratories) peroxidase substrate kit, according to the manufacturer's instructions. In the case of double staining, antibodies were removed by incubating the cryosections with 0.1 mol/l glycine buffer (pH 2.2) for 1 hours. Finally, the sections were cover-slipped for light microscopy.

rAAV genome detection. Total DNA was extracted with the Illustra Tissue and Cells genomic Prep Mini Spin Kit (GE Healthcare Life Sciences, New York, NY), according to the manufacturer's instructions. The rAAV genome in each tissue was PCR-amplified with primer sets for EGFP by using TaKaRa Ex Taq (Takara). Primers to 18S rRNA (Life technologies) were run in parallel as a positive control. The amplicon sizes were verified by electrophoresis on a 3% agarose gel.

Supplementary material

Figure S1. Agarose gel electrophoresis shows EGFP and 18S specific PCR amplification in samples of live birth, stillbirth, and afterbirth after rAAV9 injection at day 122 of gestation.
Figure S2. Photomicrographs of EGFP expression within assorted organs at 20 months after single adeno-associated virus-1 (rAAV1) intraperitoneal injection in neonatal marmoset.
Figure S3. Photomicrographs of EGFP expression within assorted organs at 10 months after single adeno-associated virus-9 (rAAV9) intraperitoneal injection in neonatal marmoset.

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Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (http://www.nature.com/mtna)