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A sensitive and reproducible cell-based assay via secNanoLuc to detect neutralizing antibody against adeno-associated virus vector capsid

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Most gene therapy clinical trials that systemically administered adeno-associated virus (AAV) vector enrolled only patients without anti-AAV-neutralizing antibodies. However, laboratory tests to measure neutralizing antibodies varied among clinical trials and have not been standardized. In this study, we attempted to improve the sensitivity and reproducibility of a cell-based assay to detect neutralizing antibodies and to determine the detection threshold to predict treatment efficacy. Application of the secreted type of NanoLuc and AAV receptor-expressing cells reduced the multiplicity of infection (MOI) for AAV transduction and improved the sensitivity to detect neutralizing antibodies with a low coefficient of variation, whereas the detection threshold could not be improved by the reduction of MOI to <100. After human immunoglobulin administration into mice at various doses, treatment with high-dose AAV8 vector enabled evasion of the inhibitory effect of neutralizing antibodies. Conversely, gene transduction was slightly influenced in the mice treated with low-dose AAV8 vector, even when neutralizing antibodies were determined to be negative in the assay. In conclusion, we developed a reliable and sensitive cell-based assay to measure neutralizing antibodies against AAV and found that the appropriate MOI to detect marginal neutralizing antibodies was 100. Other factors, including noninhibitory antibodies, marginally influence *in vivo* transduction at low vector doses.

INTRODUCTION

Adeno-associated virus (AAV) is a promising platform for delivering a transgene that has low pathogenicity and long-term transgenic expression. Clinical studies on gene therapy based on the AAV vector have been extensively developed in recent years and are being touted as the next-generation treatment to cure several inherited and degenerative diseases.[1,2] Although AAVs do not cause any disease in humans, a major limitation of the AAV vector is the presence of pre-existing neutralizing antibodies (Nabs) against the AAV capsid secondary to latent infection. The presence of Nabs is well established to inhibit gene transfer mediated by systemic injection of the AAV vector.[3] Indeed, the seropositivity of Nabs has been the main exclusion criterion for enrolling patients in several clinical trials.[4] The detection of Nabs is important in the application of AAV-mediated gene therapy for the treatment of human diseases.

The methods for measuring Nabs were different among various clinical trials and are currently not standardized.[5–7] Although the prevalence of Nabs relatively varied from 5% to 60% among the AAV serotypes, previous studies were incomparable because of their different assay sensitivities.[8–12] There are two main assays to detect anti-AAV antibodies *in vitro*; these include direct detection of total antibody by ELISA-based capture assays and by a cell-based transduction assay. Total antibody detection is easy to set up; however, it represents all immunoglobulins that bind to the AAV capsid. Therefore, the assay does not necessarily characterize the neutralizing activity that can inhibit virus vector transduction by immunoglobins.[13] Cell-based assays have been widely employed to detect Nabs[14,15] through assessment of the reduction of vector transduction to the cells after incubation of the AAV vectors with the patient's serum. Compared with ELISA-based capture assays, cell-based assays can directly assess the inhibition of vector transduction but are less sensitive.[5,16] There are several reasons for the limited sensitivity of the cell-based assay. First, poor transduction of the cell with several AAV serotypes *in vitro* requires a high vector genome for the assay, resulting in a relatively low detection sensitivity for Nabs.[13] Furthermore, the selection of reporter transgene affects the sensitivity of the assay.[17] For example, the assay based on green fluorescent protein has a limited sensitivity and can lead to false-negative results.[18] Accordingly, highly sensitive and reproducible cell-based assays are warranted for standardization.[19]

In this study, we report an improved cell-based assay to detect Nabs against AAV. Furthermore, we determined the appropriate vector

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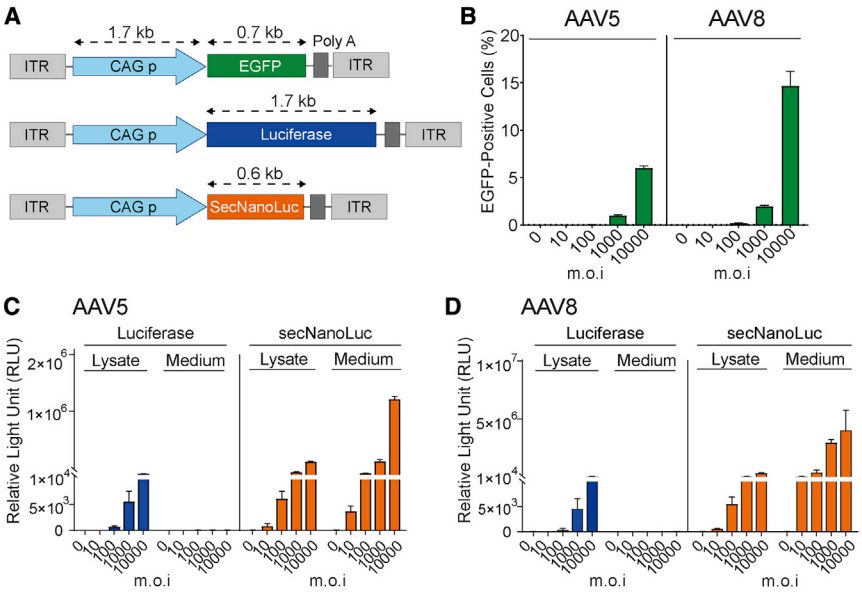

Figure 1. Comparison of the ability to detect protein expression among the reporter genes

(A) Schematic diagram of the AAV vector constructs used in this study. (B) CHO-K1 and Huh-7 cells were transduced with AAV5 and AAV8 vectors harboring the EGFP gene at an indicated MOI. The percentage of EGFP-positive cells was determined by flow cytometry. Values are presented as mean ± SD (n = 3). (C) CHO-K1 cells were transduced with AAV5 vector harboring luciferase or secNanoLuc at an indicated MOI. Transgene expressions in the cell lysate and supernatant were determined by luminescence and expressed as RLU. (D) Huh-7 cells were transduced with AAV8 vector harboring the luciferase gene or secNanoLuc at an indicated MOI. Transgene expressions in the cell lysate and supernatant were determined by luminescence and expressed as RLU. Values are presented as mean ± SD (n = 3). CAGp, CAG promoter; EGFP, enhanced green fluorescent protein; luciferase, firefly luciferase; secNanoLuc, secreting type of NanoLuc; ITR, inverted terminal repeat; RLU, relative light unit.

copy number to detect marginal Nabs *in vitro* and examined whether the results reflected *in vivo* transduction efficacy in mice.

RESULTS

Comparison of transduction efficiency among different AAV serotypes in several cell lines

We first attempted to select the cell lines that had high transduction efficiency for each AAV serotype. Because 10,000 relative light units (RLU) of luciferase has been reported to be important for reproducible Nab assays,[15] the multiplicity of infection (MOI, vector genomes [vg]/cell) of the AAV vector genome that can obtain 10,000 RLU was considered the minimal threshold for transduction to detect Nabs. We generated several AAV vectors expressing luciferase, and the transduction efficiency of each serotype was examined in HEK293 cells. We found that luciferase expression was efficient by transduction with AAV1, AAV2, and AAV6 but was less efficient by transduction with AAV5 and AAV8 (Figure S1A). To find an efficient cell line for AAV5 and AAV8 transduction, we employed other cell lines, such as CHO-K1, HT1080, and Huh-7 cells. AAV5 and AAV8 showed better gene transfer efficiency in CHO-K1 cells and Huh-7 cells, respectively (Figures S1B and S1C). We used CHO-K1 and Huh-7 cells for further cell-based assays for Nabs against AAV5 and AAV8, respectively.

Comparison of the ability to detect protein expression among the reporter genes

Next, we attempted to further increase the transduction sensitivity by AAV5 and AAV8, whose transduction efficacies were inefficient *in vitro*, to alter the reporter genes. We compared the threshold of the AAV vector genome to detect transduction among enhanced green fluorescent protein (EGFP), luciferase, and the secreting type of NanoLuc (secNanoLuc). NanoLuc has been demonstrated to offer >150-fold RLU than firefly luciferase or Renilla luciferase, *in vitro* and *in vivo*.[20,21] Moreover, NanoLuc conjugated with an interleukin (IL)-6 signal peptide (secNanoLuc) was shown to enable the detection of protein expression in the supernatant.[22–24] We generated the AAV5 and AAV8 vectors harboring EGFP, luciferase, or secNanoLuc reporters under the control of the CAG promoter (Figure 1A) and transduced the CHO-K1 or Huh-7 cells at different MOI values (Figures 1B–1D). We detected only 5%–15% of EGFP-positive cells even in the high vector genome (MOI of 10,000) (Figure 1B). Efficient EGFP expression could not be obtained at an MOI of <1,000 (Figure 1B). The application of luciferase resulted in efficient reporter expression in the cytoplasm at an MOI of 1,000 (Figures 1C and 1D). No luciferase reporter was expressed in the supernatant; however, >10-fold higher secNanoLuc activity was noted in the supernatant (Figures 1C and 1D). The sensitivity of secNanoLuc in the supernatant was 10- to 100-fold higher than that of luciferase, and significant expression was detected even at an MOI of 10 (Figure 1D). The signal of secNanoLuc was stably detected within 30 min, whereas that for luciferase was more unstable (Figure S2).

Influence of the AAV vector genome number on the detection of Nabs

Next, we examined whether differences in MOI affected the detection of AAV Nabs. The AAV5 or AAV8 vector harboring luciferase or secNanoLuc was incubated with a serial dilution of human immunoglobulin or anti-AAV monoclonal antibody (mAb); this mixture was then added to the cell culture. The titer of Nabs was expressed as the dilution ratio that obtained 50% inhibition of transgene expression (ND_{50}) (Figure 2). At the same dilution rate, the detection of Nabs was better with a lower MOI; ND_{50} increased more at an MOI of 100 than at an MOI of 1,000 (Figure 2). Both luciferase and secNanoLuc yielded similar results (ND_{50} is higher at an MOI of 100), but the

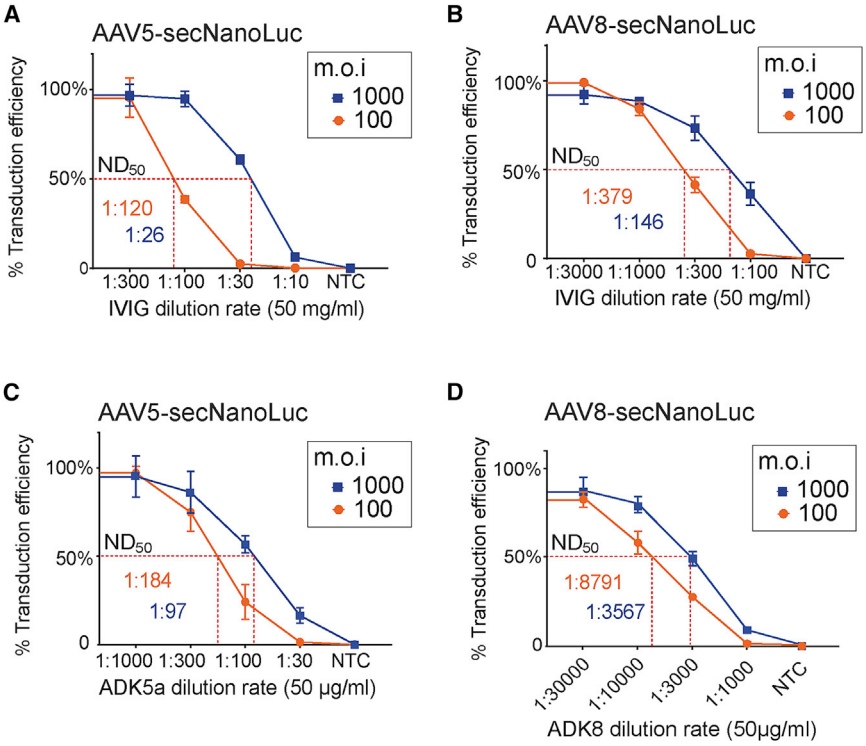

Figure 2. Sensitivity in detecting neutralizing antibody at different MOI values

(A–D) The AAV vector (AAV5 [A and C] or AAV8 [B and D]) expressing secNanoLuc was incubated with an indicated concentration of human immunoglobulin (IVIG) (A and B) or anti-AAV monoclonal antibody (mAb) (C and D) for 1 h. CHO-K1 cells for AAV5 and Huh-7 cells for AAV8 were transduced with the AAV vector at an MOI of 100 (orange) or 1,000 (blue). Transgene expressions in the supernatant and cell lysate were determined by luminescence and expressed as transduction efficiency (%). Complete transduction (100%) was defined based on the result of the RLU obtained from incubation of the AAV vector with fetal bovine serum. Inhibition of vector transduction by neutralizing antibody is expressed as the percentage of transduction. ND_{50} values were calculated as the dilutions needed to neutralize 50% vector transduction. Values are presented as mean ± SD (n = 3). NTC, no transduction control.

detection system using secNanoLuc had better interassay and intraassay % coefficient of variation (CV) (Tables 1 and 2). In addition, secNanoLuc had a consistently high RLU of >10,000 (Figure 1).

Application of the AAV receptor-expressed cell line

To obtain maximum transduction efficacy, we generated HEK293 cells expressing the AAV receptor (AAVR). HEK293 cells were transduced with the plasmid vector harboring the AAVR cDNA by controlling the EF1α promoter, followed by selection of the transduced cells by the addition of G418 (Figure 3A). We obtained HEK293 cells that expressed AAVR at a high level after limited dilution (Figure 3A).

Next, we examined whether the expression of AAVR could enhance AAV vector transduction. AAV1, AAV2, and AAV5 had enhanced transduction efficacy at higher vector doses but not at an MOI of 100 (Figure S3). Conversely, transduction with AAV8 was significantly enhanced by the expression of AAVR in all vector doses (Figure S3). To further enhance the transduction efficacy, we employed secNanoLuc instead of luciferase. When the HEK293 cells expressing AAVR were transduced with the AAV8 vector expressing secNanoLuc, significant expression of the transgene could be detected even at an MOI range of 0.1–1.0 (Figure 3B). Nevertheless, the ND_{50} values at MOI levels of 1, 10, and 100 did not differ in the HEK293 cells expressing AAVR (Figures 3C and 3D). Because the assay CV tended to become worse as the amount of the vector genome decreased (data not shown), an MOI of 100 was the appropriate vector genome to detect the marginal Nab *in vitro*. Nab titers assessed by the HEK293 cells expressing AAVR seemed similar to those assessed by Huh-7 cells (see Figure 6), but showed a better CV (Table S1).

Influence of empty capsid in the detection of Nabs

Next, we examined whether the existence of empty capsid affected the detection of Nabs. We purified AAV8 vectors using two methods, i.e., the commercially available purification kit (AAVpro purification kit, Takara Bio, Shiga, Japan) and the cesium chloride-based centrifugation method. We examined the quality of each AAV8 vector by sedimentation velocity analytical ultracentrifugation. The additional peak for empty capsid was observed for AAV8 vectors purified using the purification kit (11.6% and 16.9% at optical density $[OD]_{260}$, 20.3% and 25.6% at OD_{280} in two independent vectors), but not for those purified using the chloride-based centrifugation method (Figure 4A). We compared Nab titers assessed by both vectors and found that the purification method did not affect Nab detection at an MOI of 100 in Huh-7 and HEK293 cells expressing AAVR (Figure 4B).

Prediction of AAV vector transduction *in vivo*

We further investigated whether the detection of Nabs by the cell-based assay would predict *in vivo* AAV8 vector transduction. We employed AAV8 to assess the effect of Nabs on systemic administration of the vector *in vivo* because of high transduction efficiency in the mouse liver. The transduction efficiency of mouse liver with AAV5 is 100-fold lower than that with AAV8 (data not shown). We intravenously administered human immunoglobulin or anti-AAV8 mAbs into mice to obtain AAV8 Nabs in the blood (Figure S4A). We extracted blood at 1 h after the injection and measured Nabs using a cell-based assay (Figure S4A). As expected in previous experiments, the sensitivity to detect Nabs was poor at an MOI of 1,000 (Figure S4B). Nabs in mice could be detected after injection of >0.5 mg

Table 1. Interassay CV in ND_{50} at an MOI of 100

AAV5			
Nab	Reporter	ND_{50} (mean ± SD)[a]	Interassay CV (%)[a]
IVIG	luciferase	87.82 ± 15.62	17.79
	secNanoLuc	120.23 ± 16.91	14.07
mAb	luciferase	278.45 ± 170.98	61.40
	secNanoLuc	184.23 ± 64.23	34.86
AAV8			
Nab	Reporter	ND_{50} (Mean ± SD)	Interassay CV (%)
IVIG	luciferase	458.70 ± 252.61	55.07
	secNanoLuc	379.87 ± 32.48	8.55
mAb	luciferase	8,791.33 ± 1,763.29	20.06
	secNanoLuc	8,041.00 ± 1,523.33	18.94

IVIG, human immunoglobulin; mAb, monoclonal antibody; SD, standard deviation; CV, coefficient of variation.
[a]The data were derived from a mean of three independent experiments.

of human immunoglobulin or 30 ng of mAbs but not after injection of 0.15 mg of human immunoglobulin or 10 ng of mAbs at an MOI of 100 (Figures S4C and S4D).

To examine whether the presence of Nabs in blood inhibited transduction after systemic injection of the AAV8 vector, we administered the AAV8 vector harboring secNanoLuc after the injection of human immunoglobulin or mAbs (Figure 5A). After administration of the AAV8 vector at a high dose (1×10^{13} vg/kg), AAV vector transduction was marginally inhibited in mice treated with 1.5 mg of human immunoglobulin (ND_{50} 1:7.9 at an MOI of 100) (Figure S4C; Figure 5B). However, after administration of the AAV8 vector at a low dose (0.5×10^{12} vg/kg), transduction was completely abolished in mice treated with 0.5 mg of immunoglobulin (ND_{50} 1:2.28 at an MOI of 100) or 100 ng of mAbs (ND_{50} 1:2.74 at an MOI of 100) (Figures S4C and S4D; Figures 5C and 5D). The detection of Nabs at 1:1 serum dilution in mice treated with immunoglobulin (0.5 mg) or mAbs (30 ng) showed a significant decrease in vector transduction *in vivo* (Figures 5C and 5D). In mice treated with 0.15 mg of immunoglobulin, we observed a statistically significant reduction of vector transduction, but we could not detect Nabs in their blood (Figures 5C). We compared the results between the cell-based assay for Nabs and the ELISA-based capture assays for total antibody. The ELISA-based capture assays could detect immunoglobulin levels that could not be detected using the cell-based Nabs assay (Figure S5).

Detection of Nabs in human sample

Finally, we assessed whether our assay accurately selects eligible patients for a clinical trial. We used serum samples obtained from a cynomolgus monkey successfully treated with AAV8 vector expressing coagulation factor IX,[25] as a control. We detected extremely high Nab titers in the serum after vector administration, but not before the administration (Figure S6). Next, we assessed Nabs against AAV5 and AAV8 in commercially available human samples (n = 10, Precision for Medicine, Chevy Chase, MD, USA). When samples were assessed with the method using luciferase at an MOI of 1,500, three samples were evaluated as negative for Nabs (nos. 6, 8, and 10 in Figures 6A and 6B). One negative sample at an MOI of 1,500 (no. 8) became positive as assessed by the assay using secNanoLuc at an MOI of 100 (Figures 6C and 6D).

DISCUSSION

The presence of Nabs is an important factor in determining the success of gene therapy with AAV vectors.[26] Cell-based assays have been mainly performed to detect Nabs, but they are not standardized and are less sensitive, compared with the direct detection of total antibody using ELISA-based capture assays.[5,6,13] In addition, the required assay sensitivity to predict the success of an AAV-mediated gene therapy remains to be elucidated. In this study, we maximized the sensitivity of cell-based Nab assays and found the appropriate vector genome for the detection of marginal Nabs. Furthermore, we clarified how antibody titers affected the actual transduction of AAV vectors *in vivo*.

We have developed a sensitive and reproducible cell-based Nab assay. Using the highly sensitive reporter secNanoLuc and cells that were easy to transduce, we could reduce the number of AAV vector genomes required for transduction. Although decreasing the vector dose increased the sensitivity of the assay, we identified an MOI of 100 as the appropriate vector copy number to detect Nabs with a high CV. The reason why the assay sensitivity does not improve with decreasing the MOI to <100 may be explained by the dissociation constant. If the dissociation constant between AAV and Nabs is kept consistent, the binding rate of Nabs to AAVs will deteriorate with the decrease in the concentration of AAV and Nabs in the solution. A lower MOI and Nab resulted in weaker Nab inhibition, and the Nab detection might reach the limitation. The secNanoLuc simplified the test and was expected to improve CVs because AAV infection can be detected by direct measurement of the cell supernatant. The employment of secNanoLuc increased the sensitivity of the Nab assays with AAV5 and AAV8, whose transduction could not be efficiently detected *in vitro*.[27,28] The combination with AAVR-expressed cells further enhanced the transduction and allowed evaluation even at a very low concentration of AAV8 vectors. The assay for the detection of Nabs against AAV5 and AAV8 reportedly utilized a relatively high number of vector genomes to detect reporter expression *in vitro*. A previous report that employed an MOI of 30,000 for the Nab assay against AAV8 suggested the requirement for a more sensitive assay because the presence of a very low Nab below the detection limit impeded the transduction efficacy *in vivo*.[14] Clinical trials on AAV5 employed a MOI of 25,000 for Nab assays of HEK293 cells.[5] Another nonhuman primate study on AAV5 used an MOI of 347, but only 1,000 RLU was obtained for the positive control in the HEK293 cells.[18,29]

The association between the treatment vector dose and the Nab titers was found to be important in this study. Compared with low vector doses, relatively high doses of the AAV vectors could evade the inhibitory effect of Nabs. This was consistent with the results of our previous experiments on pigs, in which high doses of the AAV8 vectors

Table 2. Intraassay CV in cell-based Nab assay at an MOI of 100

Nab	Reporter	Dilution rate	% Transduction efficiency (mean ± SD)[a]	Intraassay CV (%)[b]
AAV5				
IVIG	luciferase	1:300	108.08 ± 4.61	22.99
		1:100	66.48 ± 21.80	35.6
		1:30	3.63 ± 2.40	74.72
	secNanoLuc	1:300	95.65 ± 15.64	11.81
		1:100	38.57 ± 2.50	21.44
		1:30	2.49 ± 1.28	55.20
mAb	luciferase	1:1,000	89.84 ± 24.05	19.91
		1:300	65.23 ± 25.99	19.64
		1:100	28.12 ± 19.03	32.46
	secNanoLuc	1:1,000	97.06 ± 5.35	16.98
		1:300	73.51 ± 13.56	9.80
		1:100	24.18 ± 14.00	18.84
AAV8				
IVIG	luciferase	1:3,000	87.31 ± 10.24	20.56
		1:1,000	79.28 ± 17.57	18.57
		1:300	46.44 ± 18.42	22.53
	secNanoLuc	1:3,000	99.02 ± 1.37	16.73
		1:1,000	84.08 ± 3.04	14.12
		1:300	41.45 ± 3.54	25.79
mAb	luciferase	1:30,000	91.44 ± 6.87	25.12
		1:10,000	62.08 ± 1.86	29.88
		1:3,000	16.57 ± 7.73	12.33
	secNanoLuc	1:30,000	83.57 ± 5.76	18.19
		1:10,000	57.89 ± 9.10	12.58
		1:3,000	27.17 ± 2.55	18.81

[a]The data were derived from triplicate samples in one experiment.
[b]The data were derived from three independent experiments composed of triplicate samples.

prevented the inhibition by Nabs.[30] Clinical trials and preclinical studies on monkeys using AAV5 vectors showed a therapeutic effect even in the presence of Nabs, suggesting that AAV5 is effective even in Nab-positive patients.[18] Although precise mechanisms including a serotype-specific property have not yet been fully understood, high vector doses (1×10^{13} vg/kg or higher[4,31]) in an AAV5 clinical trial may evade Nabs. For clinical trials that required high vector administration, a sensitive assay to detect Nab may not be necessarily required.[32] Cumulatively, the therapeutic effect of systemic gene therapy in clinical practice is determined according to the AAV vector dose, serotype's propensity, and Nab titer in the serum. We should carefully determine the specific threshold value for the inclusion criteria of eligible patients based on the vector type and dosage in each clinical trial.

Notably, although a high-dose vector could evade the inhibitory effect of Nabs, it had a tendency to be associated with hepatotoxicity and other serious adverse events.[33–36] In addition, cases of death secondary to hepatotoxicity from high-dose AAV8 (3×10^{14} vg/kg) were reported.[37] Considering the safety of AAV-mediated gene therapy, the amount of AAV vectors required to achieve a therapeutic effect should be minimal. Conversely, transduction with relatively low vector doses can be easily affected by the presence of Nabs. Moreover, the existence of marginal antibodies or other unknown factors may slightly inhibit vector transduction *in vivo*, even if the Nab is determined to be negative on cell-based assays. The therapeutic effects of AAV vectors are known to vary among individuals, and the presence of these factor(s) may be linked to individual differences after vector administration. Binding of antibodies that do not have neutralizing activity may promote antibody-dependent complement activation, which can affect viral clearance from the blood[38] and result in deceased transduction. Meanwhile, serum nonantibody-based neutralizing factors that cannot be detected in a total antibody assay may influence transduction efficiency.[39] From these perspectives, inhibition of transduction efficiency *in vivo* may vary. Some reports suggested that a Nab assay was more sensitive *in vivo* than *in vitro*.[7,40] Indeed, we could identify the inhibitory effect of marginal antibodies even in the *in vivo* Nab assay in mice (Figure S7). In future clinical trials, we should carefully discuss the requirement for an *in vivo* assay from the point of view of animal welfare as well as the complexity of the procedure and standardization.

The detection threshold of our method met the criteria of the US Food and Drug Administration recommendation of at least 100 ng/mL assay sensitivity to detect antidrug antibodies in biological samples,[41] albeit whether the guidance should be adapted for gene therapies remains to be decided.[42] Approximately 100 ng/mL antibodies has been reported to completely prevent disease, and 10 ng/mL provided considerable protection after vaccination against tetanus and diphtheria.[43] Although the clinical desired sensitivity to detect Nabs against AAV has not been revealed, the detection threshold in our present cell-based assay was 50 ng/mL mAbs against AAV. A total antibody assay was reported to detect <100 ng/mL mAbs against AAVs,[5–7,18] whereas the previous cell-based Nab assay was less sensitive at 180 ng/mL (low quality control) and 3,000 ng/mL (high quality control).[44,45] Our data suggested that an *in vitro* cell-based assay at a MOI of 100 detects the desired level of a mAb and mostly predicted the success of gene therapy.

This study had several limitations that should be addressed in future work. First, we did not assess the influence of clinical sample-related validation (e.g., hemolytic, lipemic, and jaundiced serum). The impact of these validations on the test value should be examined. Second, we could not evaluate the effect of inhibitory factors other than immunoglobulin because we only assessed the effect of purified human immunoglobulin and mAb. We should clarify and carefully assess the importance of serum substances that can inhibit AAV transduction. Furthermore, we assessed only 10 human serum samples in the cell-based assay and did not evaluate whether increasing the sensitivity altered the prevalence of Nabs in a specific population. We previously examined the prevalence of Nabs in Japanese patients

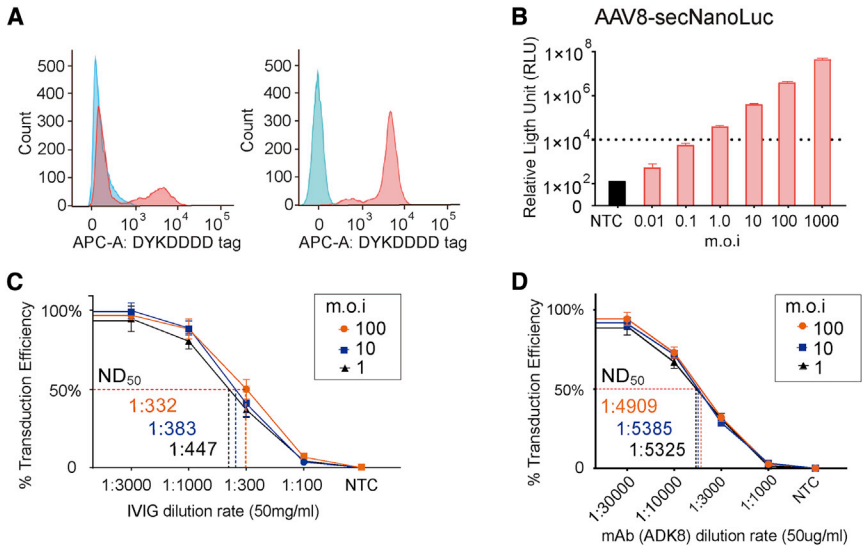

Figure 3. Threshold to detect AAV-neutralizing antibody among different MOI values in HEK293 cells expressing AAVR

(A) AAVR expression after transduction of HEK293 cells with the linearized pBApo-EF1 plasmid containing AAVR was determined by intracellular fluorescent staining with anti-FLAG antibody using flow cytometry (left). The clone with a high expression of AAVR after limited dilution was selected (right). Histograms represent the degree of FLAG expression (blue, isotype-matched control; red, anti-FLAG antibody). (B) HEK293 cells expressing AAVR were transduced with the AAV8 vector harboring the NanoLuc gene at an indicated MOI. Transgene expressions in the supernatant were determined by luminescence and expressed as a RLU. Values are presented as mean ± SD (n = 3). (C and D) The AAV8 vector expressing secNanoLuc was incubated with an indicated concentration of IVIG (C) or anti-AAV mAb (D) for 1 h. HEK293 cells expressing AAVR were transduced with the AAV8 vector at an MOI of 1 (black), 10 (blue), or 100 (orange). Transgene expression in the supernatant was determined by luminescence and expressed as transduction efficiency (%). Complete transduction (100%) was defined based on the result of RLU obtained from incubation of the AAV vector with fetal bovine serum. Inhibition of vector transduction by neutralizing antibody is expressed as the percentage of transduction. ND_{50} values were calculated as the dilutions needed to neutralize 50% vector transduction. Values are mean ± SD (n = 3).

with hemophilia and found that 27.4%–39.7% were positive for Nabs against several AAV serotypes.[10] Our previous examination employed an MOI of 1,000 for AAV5 and AAV8.[25] We are now conducting clinical research to examine the prevalence of Nabs in healthy volunteers (expected number for recruitment = 100) and patients with hemophilia (expected number for recruitment = 240) in Japan (UMIN-CTR: UMIN000039069). The study will reveal the real prevalence of Nabs in patients with hemophilia and the percentage of patients eligible for AAV-mediated gene therapy in Japan. Finally, we have shown data only for AAV8 in *in vivo* mouse experiments in this paper. It is necessary to verify whether the same phenomenon is observed in other serotypes.

In conclusion, we developed a reliable and sensitive cell-based assay for the determination of Nabs against AAV and found an MOI of 100 as the appropriate vector copy number to detect the existence of marginal Nabs. Accurate measurement of Nabs against AAVs can predict the success of systemic AAV vector-mediated gene therapy. Further validation of the assays is required for future clinical application as a laboratory test. In addition, factors other than Nabs may affect the individual differences in treatment response. These factors that influence the therapeutic efficacy of AAV vectors should be clarified to avail the benefits of gene therapy to many patients with refractory diseases.

MATERIALS AND METHODS

Cell culture

HEK293 cells (JRCB Cell Bank, Osaka, Japan), AAVpro 293T cells (Takara Bio), Huh-7 cells (JRCB Cell Bank), and CHO-K1 cells (JRCB Cell Bank) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA) and 2 mM L-glutamine (Thermo Fisher Scientific). HT1080 cells (JRCB Cell Bank) were cultured in DMEM supplemented with 10% FBS, 2-mM L-glutamine, and 1% nonessential amino acids (Wako Pure Chemical, Osaka, Japan).

Generation of AAVR-expressing stable cell line

A codon-optimized human AAVR gene (KIAA0391L: NM_024874) conjugated with FLAG tag at the C terminus was synthesized at GenScript (Piscataway, NJ, USA). The cDNA sequence was cloned into the pBApo-EF1 plasmid (Takara Bio). The linearized pBApo-EF1 plasmid containing AAVR (pBApo-EF1-AAVR) was transfected into HEK293 cells using Lipofectamine 2000 (Thermo Fisher Scientific, CA, USA). To select the transfected cell clones, G418 (Nacalai Tesque, Kyoto, Japan) was added in the culture medium; culture was continued for selection with limited dilution in 96-well plates. We selected the clone that had a high expression of AAVR on flow cytometry.

Plasmid construct and AAV vector production

The cDNA of NanoLuc containing the IL-6 signal peptide (secNanoLuc) was synthesized at GenScript. Firefly luciferase cDNA was derived from pGL3 basic plasmid (Promega, Madison, WI, USA). A cDNA fragment that comprised a chimeric CAG promoter (CMV enhancer, chicken β-actin promoter and splice donor, and rabbit β-globin intron with splice acceptor), a transgene (EGFP, secNanoLuc, or luciferase), and the SV40 polyadenylation signal was introduced between the inverted terminal repeats in the pAAV2 plasmid.

The genes were packaged by triple plasmid transfection of AAVpro 293T cells (Takara Bio) to generate the AAV vector (helper-free system), as described previously.[46] A plasmid-expressing Rep/Cap (serotypes 1, 2, 5, and 6) and pHelper were purchased from Takara Bio.

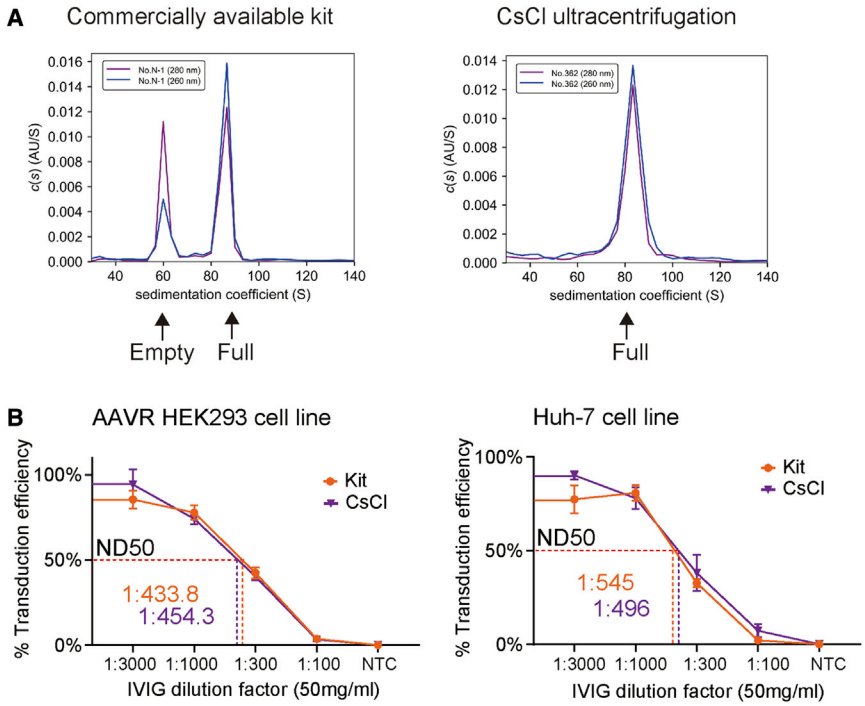

Figure 4. Effect of empty capsid on the detection of neutralizing antibody

AAV8 vectors expressing secNanoLuc under the control of CAG promoter were produced by helper-free plasmid transfection. AAV vectors were purified using a commercially available purification kit (AAVpro purification kit, Takara Bio) or CsCl density gradient combined with ultracentrifugation. (A) AAV purity was determined by analytical ultracentrifugation. Representative results of two independent vectors are shown. Left, commercially available kit; right, CsCl-based purification. (B) The AAV8 vector expressing secNanoLuc was incubated with an indicated concentration of IVIG for 1 h. Huh-7 cells or HEK293 cells expressing AAVR were transduced with the AAV8 vector at an MOI of 100. Transgene expression in the supernatant was determined by luminescence and expressed as transduction efficiency (%). Complete transduction (100%) was defined based on the result of RLU obtained from incubation of the AAV vector with fetal bovine serum. Inhibition of vector transduction by the neutralizing antibody is expressed as the percentage of transduction. ND_{50} values were calculated as the dilutions needed to neutralize 50% vector transduction. Values are presented as mean ± SD (n = 3).

The capsid sequence of serotype 8 was synthesized at GenScript. AAV vectors were purified from the transfected cells after 72 h using the AAVpro purification kit (Takara Bio) or the ultracentrifugation method, as previously described.[30] Titration of recombinant AAV vectors was performed by quantitative PCR, as described previously.[46] The quality of each AAV8 vector was examined by sedimentation velocity analytical ultracentrifugation at the Gene Therapy Research Institution (Kanagawa, Japan).

AAV vector transduction *in vitro*

The cell lines were seeded at a density of 10^4 cells in each 96-well dish coated with poly-L-lysine (Peptide, Osaka, Japan). A vial of vector stock was thawed and diluted in DMEM containing 5% FBS immediately before the transduction experiment and was directly added into each well. AAV vector transduction was measured in triplicate, and the mean value of three measurements was considered as one experiment.

Flow cytometry

To examine EGFP expression, the cells were directly resuspended in stain buffer containing 7-aminoactinomycin D (7-AAD). The expression of EGFP was analyzed on an LSRFortessa (BD Biosciences, Franklin Lakes, NJ, USA). The cells were gated by side scatter (SSC) and forward scatter (FSC), and dead cells were removed on the basis of 7-AAD staining. To examine the surface expression of AAVR, the cells were fixed, permeabilized with Cyto-Fast Fix/Perm buffer (BioLegend, San Diego, CA, USA), and stained with anti-DYKDDDDK mAb conjugated with allophycocyanin (BioLegend). The expression of the FLAG tag was analyzed on an LSRFortessa. FCS files were obtained using FACSDiva software and reanalyzed with FlowJo software (BD Biosciences).

Measurement of firefly luciferase and secNanoLuc expressions

Cells were lysed with 100 μL of lysis reagent (Promega) for luciferase assays and were then immediately stored in a deep freezer. To measure secNanoLuc activity, 50 μL of supernatant was directly collected and immediately stored in a deep freezer. After thawing frozen samples at room temperature, 10 μL of lysates or supernatants was added into a 96-well plate (Berthold Technologies, Germany). The 96-well plate was placed in a luminometer (Centro LB 960, Berthold Technologies), and 50 μL of luciferase assay reagent (Promega) or Nano-Glo luciferase assay reagent (Promega) was injected in each well using an automatic injector. The delay and measurement times were set for 2 and 10 s, respectively. All measurements were performed at room temperature and completed within 15 min. The luminescence of the negative sample (cell lysate or supernatant not transduced with AAVs) was approximately 50 RLU and saturated at 1×10^8 RLU. To simplify the method, we did not usually measure protein concentrations in each well. We measured protein concentrations of cell lysate in 10 independent wells and found that the concentration of each well seemed to be identical in each well (Figure S8).

Nab assay

We used the following two types of positive control to inhibit AAV transduction: human immunoglobulin (Gammagard, Baxalta, CA, USA) and mAb against AAV (ADK8 against AAV8; ADK5a against AAV5) (Progen, Biotechnik, Heidelberg, Germany). Human immunoglobulin was diluted at a concentration of 50 mg/mL and stored

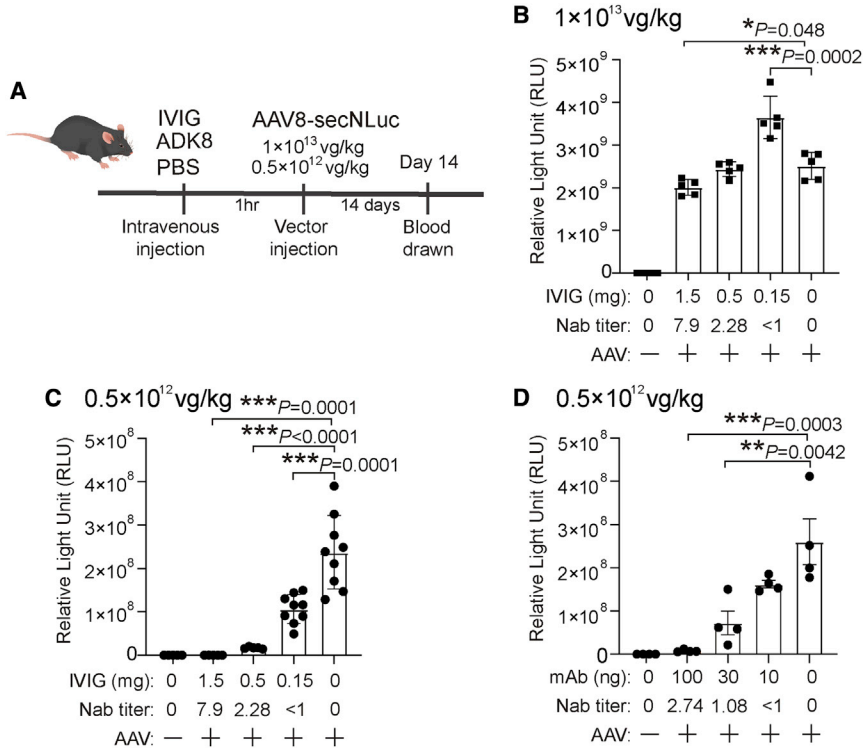

Figure 5. Transduction efficacy in mice passively immunized by the AAV8 vector *in vivo*

(A) Schematic diagram for the experiment. (B–D) An indicated dose of IVIG (B and C) or anti-AAV mAb (D) was intravenously administered into C57BL6/J mice. Phosphate-buffered saline (PBS) was used as a control. The AAV8 vector expressing secNanoLuc (B, 1.0×10^{13} vg/kg; C and D, 0.5×10^{12} vg/kg) was injected into mice at 1 h after the IVIG or mAb injection. Blood was drawn at 14 days after the administration, and serum secNanoLuc expression was determined by luminescence and expressed as RLU. Values are presented as mean ± SD (n = 5 for B, n = 5–9 for C, n = 4 for D). *p < 0.05, **p < 0.01, ***p < 0.001, ***p < 0.001 (one-way ANOVA, post hoc Turkey test).

at −80°C for future use. mAb was adjusted at a concentration of 50 μg/mL and stored at −80°C for future use.

A Nab assay was essentially performed, as described previously.[14,15] Briefly, the cells were seeded at 1×10^4 into 96-well culture plates. On the day of transduction, 20 μL of human immunoglobulin or anti-AAV-mAb (ADK8 or ADK5a) was serially diluted with FBS and incubated with 20 μL of the AAV vector at 37°C for 1 h. A portion (7.5 μL) of the mixture was then added into three culture wells. After 48 h, vector transduction was examined based on the expression of luciferase or secNanoLuc. The mean value of the triplicate samples was considered as one experiment. Inhibition of vector transduction by Nabs was expressed as the percentage of transduction; 100% meant no inhibition, whereas 0% meant no transduction.

AAV vector transduction *in vivo*

All animal experiments were approved by the Institutional Animal Care and Concern Committee of Jichi Medical University and were conducted in accordance with the committee's guidelines. For *in vivo* studies, 6- to 8-week-old C57BL6/J mice (SLC, Shizuoka, Japan) were used. Anesthetized mice were passively immunized by intravenous administration of 50 μL of immunoglobulin or anti-AAV mAb. Blood was drawn from the right jugular vein at an indicated time after the injection. The serum was isolated, heat-inactivated for 30 min at 56°C, and used for the determination of Nab titer *in vitro*, as described above. For the transduction of the AAV vector *in vivo*, the mice received 150 μL of the AAV8 vector expressing secNanoLuc (0.5×10^{12} or 1×10^{13} vg/kg) 1 h after the immunoglobulin or mAb injection. To examine vector transduction *in vivo*, 100 μL of blood was drawn from the jugular vein. The expression of secNanoLuc in serum was determined, as described above.

Detection of total antibody by ELISA-based capture assay

The 96-well microplates were coated with 1×10^9 vg of AAV vector in phosphate buffer (pH 9.0) and incubated at 4°C overnight. After washing with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T), the wells were blocked with 5% casein in PBS-T. After three washes with PBS-T, 100 μL of serially diluted human immunoglobulin or mAb was added into each well, which was incubated for 1 h. After washing with PBS-T, 100 μL of horseradish peroxidase-conjugated anti-human immunoglobulin G (IgG) (Proteintech Group, Rosemont, IL, USA) or anti-mouse IgG (Proteintech Group) was added, and the mixture was incubated for 1 h. Antibody binding was detected using ABTS peroxidase substrate (KPL Protein Research Products, Washington, DC, USA). The OD of each well was measured at 405 nm.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8.4.3 (GraphPad, CA, USA). Antibody titers that reached ND_{50} by human immunoglobulin or anti-AAV-mAb were estimated by nonlinear regression (inhibitor versus normalized response, variable slope). A Student's t test was used to compare firefly luciferase and secNanoLuc expressions in the AAV serotypes on several cell lines. One-way analysis of variance (ANOVA) with a post hoc Turkey test was used for calculations that involved two-group comparisons in the *in vivo* experiments. CV was calculated as (standard deviation/ mean) × 100 (%). Intraassay CV and interassay CV indicated variations of simultaneous values within one assay and variation of values obtained in three independent experiments, respectively. A p value of <0.05 was considered statistically significant.

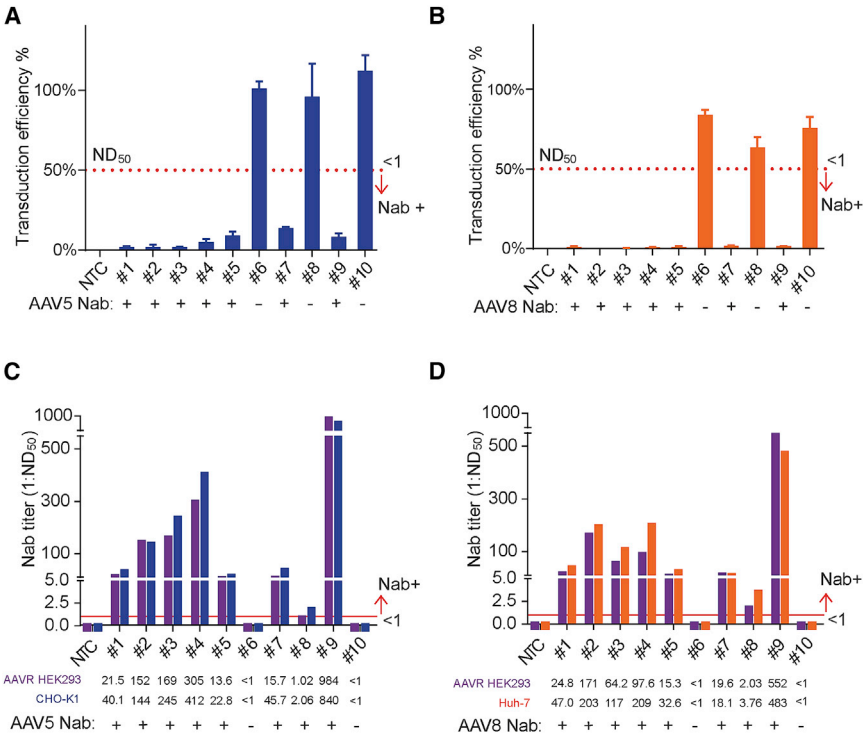

Figure 6. Detection of neutralizing antibody against AAV5 and AAV8 in human serum samples

(A and B) Human serum was diluted with fetal bovine serum at a 1:1 ratio and then incubated with AAV vector expressing luciferase for 1 h. The mixtures were subsequently added to the cells at an MOI of 1,500. Transgene expression in the lysate was determined by luminescence and expressed as transduction efficiency (%). Complete transduction (100%) was defined based on the result of RLU obtained from the incubation of the AAV vector with fetal bovine serum. Values are presented as mean ± SD of triplicate samples. Neutralizing antibodies in the serum were expressed as negative (<1) when transgene expression was >50% of control experiment. (A) CHO cell experiments using AAV5. (B) Huh-7 cell experiments using AAV8. (C and D) Human sera were serially diluted with fetal bovine serum and then incubated with AAV vector expressing secNanoLuc for 1 h. The mixtures were subsequently added to the cells at an MOI of 100. The Nab titer was expressed as the dilution ratio that achieved 50% inhibition of transgene expression (ND_{50}). Values are presented as a mean of triplicate samples. (C) CHO cells (blue bar) and HEK293 cells expressing AAVR experiments (violet bar) using AAV5. (D) Huh-7 cells (orange bar) and HEK293 cells expressing AAVR (violet bar) experiments using AAV8.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2021.06.004.

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AUTHOR CONTRIBUTIONS

N.B. designed the study, performed the experiments, analyzed the data, and wrote the manuscript. Y.K. designed the study, performed the experiments, and revised the manuscript. M.H., N.K., T.H., and H.M. designed the study, analyzed the data, and revised the manuscript. T.O. designed the study, analyzed the data, and wrote the manuscript. All authors approved the final version of the manuscript.

DECLARATION OF INTERESTS

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