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Citation	Gene therapy, 14(15), 1152-1159 https://doi.org/10.1038/sj.gt.3302970
Issue Date	2007-08
Doc URL	http://hdl.handle.net/2115/34648
Type	article (author version)
File Information	Hydro-ChIP.pdf



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Transient activation of transgene expression by hydrodynamics-based injection may cause rapid decrease in plasmid DNA expression

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Running title: Activation by hydrodynamics-based injection

Summary

The intranuclear disposition of exogenous DNA is quite important for the therapeutic effects of the administered DNA. The expression efficiency from one copy of exogenous DNA delivered by hydrodynamics-based injection dramatically decreases over time, and this ‘silencing’ occurs without CpG methylation. In this study, naked luciferase-plasmid DNA was delivered into mouse liver by hydrodynamics-based injection, and modifications of the histones bound to the plasmid DNA were analyzed by a chromatin immunoprecipitation (ChIP) analysis. In addition, the effects of a second hydrodynamics-based injection on the expression from the plasmid DNA were examined. The ChIP analysis revealed that the modification status of histone H3 remained constant from 4 hr to 4 weeks. Surprisingly, the injection of saline without DNA enhanced the luciferase expression from the preexisting DNA administered 4 and 14 days previously. Our results suggest that histone modification plays no role in the silencing. Instead, our data suggest that the transgene expression is activated by the hydrodynamics-based injection manipulation, and that the return from the activated status causes the silencing.

Keywords: exogenous DNA; histones; intranuclear disposition; silencing; hydrodynamics-based injection

Introduction

Nonviral vectors using plasmid DNA are quite attractive in gene therapy, due to their excellent safety profile. However, transgene expression with nonviral vectors is low in comparison to that with viral vectors.¹⁻⁴ In addition, the transgene expression with nonviral vectors is generally transient.² The intranuclear disposition and the intracellular DNA trafficking are very important for efficient and prolonged transgene expression.⁴ Recently, it was reported that the transience of transgene expression was due to a decrease in the transcription efficiency.⁵⁻¹⁰ The expression from transgenes introduced by a rapid, high-volume injection (hydrodynamics-based injection) is also generally transient,^{5-7,9} although prolonged transgene expression was reported in some cases.^{6,7,11-14}

One possible factor involved in the ‘silencing’ of the exogenous DNA is the methylation of the 5-position of cytosine in the CpG motifs. The methylation of the promoter was reportedly the major mechanism responsible for the decreased expression from adenoviral DNA,¹⁵ although it was also reported that the transcription suppression occurred independently of the methylation.¹⁶ In the case of nonviral vectors, transgene expression was reportedly enhanced when fewer CpG sequences were present in the plasmid DNA,^{13,14} implying that the CpG methylation suppressed transgene expression. Kay and his colleagues reported that minicircles, which lacked the bacterial backbone part containing many

CpG motifs, achieved efficient and prolonged transgene expression.^{6,7} However, we and others found that the silencing of the exogenous DNA proceeded without the DNA methylation.^{8,9} Thus, the involvement of the CpG methylation in the transgene silencing is still debatable.

Alternatively, intranuclear proteins bound to the exogenous DNA might induce the silencing. Histones bind to mammalian chromosomal DNA, and various modifications, such as acetylation, methylation, phosphorylation, ubiquitination, and ADP-ribosylation, of histone tails are believed to be one of the transcription regulation mechanisms (“histone code” hypothesis).¹⁷ For example, it has been reported that the acetylation of histones H3 and H4, the methylation of Lys-4 of H3, and the phosphorylation of Ser-10 of H3 are involved in transcriptional activation (euchromatin formation), and that the trimethylations of Lys-9 on H3 and Lys-20 on H4 play roles in transcriptional repression (heterochromatin formation) in mammals.¹⁸ For integrated DNAs derived from retroviral and plasmid DNAs, the histone modifications were proposed to be the primary events in the silencing.^{19,20} In addition, proteins other than histones, such as heterochromatin protein 1 (HP1), bind to the plasmid DNA integrated within the chromosomal DNA.²¹

Previously, we examined the intranuclear disposition of plasmid DNA delivered into mouse liver by the hydrodynamics-based injection, and found that expression from the plasmid DNA rapidly decreased without

DNA methylation.⁹ In this study, we analyzed the modifications of histones bound to the intranuclear plasmid DNA. In addition, we examined the effects of the hydrodynamics-based injection procedure on the expression from plasmid DNA that was administered 4 and 14 days previously. Our results suggest that histone modification plays no role in the reduced expression. Importantly, activation of the transgene expression by the hydrodynamics-based injection manipulation is strongly suggested.

Results

Time courses of the luciferase activity and the amount of intranuclear DNA.

We previously delivered exogenous DNA (0.5 μg) into mouse liver by a rapid, high-volume injection method (hydrodynamics-based administration), and observed the intranuclear disposition for a week.⁹ Owing to the amount of DNA required for the chromatin immunoprecipitation (ChIP) analysis (see below), 20 μg of the same plasmid DNA (pYK-CMV-luc) was administered to female Balb/c mice (six weeks old) by the hydrodynamics-based method. This method enables the delivery of exogenous DNA into the nuclei of the liver,^{22,23} without the aid of cationic lipids and polymers, which could potentially affect the

quantitation of the intranuclear exogenous DNA. Moreover, we extended the analysis to follow the time course of the disposition up to 28 days.

The livers were harvested at various time points, from 4 hr to 28 days after the injection, and the luciferase activity was measured. The amount of exogenous DNA at the same time points was examined by quantitative polymerase chain reaction (Q-PCR) after isolation of the nuclei (Figure 1). The luciferase activity peaked at 8 hr post-injection, and then decreased rapidly. The luciferase activities on days 4 and 28 after the injection were 1,700- and 10^4 -fold, respectively, less than that at 8 hr (Figure 2a). On the other hand, the amount of exogenous DNA in the nuclei decreased rapidly within 2 days, and then gradually (Figure 2b). The amounts of exogenous DNA on days 4 and 28 after the injection were 15- and 26-fold, respectively, less than that at 8 hr. The expression efficiencies, the luciferase activities divided by the amounts of the exogenous DNAs at the same time points, peaked at 8 hr, and then rapidly decreased within 4 days. The expression efficiency on day 4 was 110-fold less than that at 8 hr (Figure 2c). The efficiency was similar on days 14 and 28. These results were consistent with those of our previous study,⁹ although we had not analyzed the expression after 7 days. The expression from the exogenous DNA was suppressed over time, and this rapid silencing occurred at an early stage after the hydrodynamics-based administration.

Methylation status of intranuclear DNA

We previously found that the methylation ratio of the exogenous DNA was low, and that the silencing occurred independently of the methylation of the promoter region within a week.⁹ However, the DNA methylation may be involved in the maintenance, rather than the induction, of the silenced state. We calculated the methylation ratio by Q-PCR with methylation-sensitive restriction enzyme treatment (Figure 1). A *Bsa*HI site (5'-GACGTC-3') exists in the CMV (cytomegalovirus) promoter region, and this enzyme cannot cleave DNA when the central cytosine is methylated. Likewise, *Hha*I, which has recognition sites (5'-GCGC-3') within the luciferase gene and the bacterial backbone regions, is sensitive to the methylation of the central cytosine residue. Thus, the exogenous DNA methylated at the target site is resistant to digestion with the corresponding enzyme, and the regions of interest can be amplified in the PCR process. As shown in Figure 2d, the methylation did not increase over time in the promoter, luciferase gene, and bacterial backbone regions. Therefore, DNA methylation was involved in neither the induction nor the maintenance of the silenced state of the exogenous DNA.

Binding and modifications of histones

The results obtained in the previous and present studies suggest that the decrease in the expression efficiency was induced by other factors that

interact with the exogenous DNA. Therefore, we analyzed the binding of the DNA by histones, which have an important function in chromosomal gene regulation. Previously, it was reported that nucleosomes, which are made up of DNA and a histone octamer, are formed on non-integrated plasmids.²⁴ The binding of histones limits the access of transcriptional factors to their recognition sites in the plasmid DNA, and thus, the binding mode of histones to the plasmid DNA could affect transgene expression and explain its decrease. In addition, histone modifications that are important for chromosomal gene regulation might also be involved in the silencing.

We analyzed the binding and modifications of histones by the ChIP assay.^{25,26} This assay was carried out by immunoprecipitating the histones that were crosslinked with DNA (after partial fragmentation of the DNA), followed by de-crosslinking and Q-PCR (Figure 1). An anti-H3 antibody that recognizes histone H3 irrespective of the modifications was used for the analysis of the histone binding to the DNA. Anti-H3 trimethyl K4 and anti-H4 acetyl K5/K8/K12/K16 antibodies, and anti-H3 dimethyl K9 and anti-H3 trimethyl K9 antibodies were employed for the analyses of the active and repressive modifications of histones, respectively. In addition, an anti-HP1 γ antibody was used to analyze the binding of HP1, which reportedly binds to the bacterial backbone region of integrated plasmid DNA to suppress transgene expression.²¹ We expressed the results

of the ChIP analyses as pull-down/input ratios (%), the ratios of the amounts of exogenous DNA precipitated by antibodies to those of the total exogenous DNAs. It should be noted that the ratios shown are not the absolute binding ratios, since the precipitation efficiencies differ amongst each other and are low (data not shown). However, the analyses are valid when the binding kinetics are examined.

As shown in Figure 3a, the pull-down/input ratio increased over time when the anti-H3 antibody was used, suggesting that the ratio of the exogenous DNA bound by histones was augmented. The ratios also followed a similar pattern when other antibodies that recognize the modified histones and HP1 were employed (Figure 3b-f). We did not find any differences in the promoter, luciferase gene, and bacterial backbone regions, except that the binding ratios of HP1 were higher in the luciferase gene and bacterial backbone regions. The pull-down/input ratios were low with the control (nonspecific) IgG antibody (Figure 3g) and we did not consider the nonspecific binding.

We next calculated the amount of exogenous DNA bound with these proteins by multiplying the pull-down/input ratio (Figure 3a-f) by the total amount of exogenous DNA (Figure 2b) at the same time points. The amounts of exogenous DNA bound with these proteins were nearly constant, although the amounts were relatively large at 4 and 8 hr after the administration (Supplemental Figure 1). Thus, the possibility that an

increase in the amount of plasmid DNA bound with histones caused the silencing could be excluded. In addition, the results shown in Figure 3 and Supplemental Figure 1 suggest that the plasmid DNA complexed with histones was retained in the nucleus, although the actual amounts of complexed plasmid DNA remained unknown (see Discussion).

We then calculated the histone modification levels. The pull-down/input ratios for the anti-H3 trimethyl K4, anti-H3 dimethyl K9, and anti-H3 trimethyl K9 antibodies (Figure 3b-d) were divided by that for the anti-H3 antibody (Figure 3a). This calculation indicated that the modification status of histone H3 was constant from 4 hr to 28 days (Supplemental Figure 2), excluding the possibility that the histone modification was the major cause of the transgene silencing.

Effects of hydrodynamics administration

We hypothesized that the major cause of the rapid decrease in the expression was the transient transcriptional activation induced by the administration method itself. This prompted us to examine the effects of a second hydrodynamics-based injection on the expression from the plasmid DNA delivered by the first injection. The second injection was carried out 4 days after the first injection, and the luciferase activity was examined at 8 hr after the second injection.

When the same plasmid DNA (pYK-CMV-luc) was injected, the luciferase activity was increased, as expected (Figure 4a). Remarkably, the injections of plasmid DNAs without the luciferase gene and of saline without DNA also enhanced the luciferase expression (Figure 4a and b). The luciferase activity was 150-fold when only saline was administered, as compared to the untreated group. The enhancement occurred when the saline was injected 14 days (Figure 4b) and 3 months (data not shown) after the plasmid administration.

The luciferase activity decreased 490- and 110-fold from 8 to 96 hr after the second injection, when it was conducted at 4 and 14 days, respectively, after the first administration (Figure 4b). The amount of exogenous DNA was not affected by this second injection (Figure 4c). The decrease in the expression efficiency, the luciferase activity divided by the amount of the exogenous DNA at the same time point, followed a similar pattern to that of the initial administration (Figure 4d). These results suggest that the transience of the transcription activation due to the hydrodynamics-based injection manipulation cause the decreased transgene expression.

We analyzed the binding and modifications of histones after the second injection. As also seen with the first injection, no evident alterations were observed (data not shown).

Discussion

Hydrodynamics-based administration has been used as a simple and effective *in vivo* DNA introduction method.^{22,23,27,28} The expression from transgenes introduced by this method is generally transient, and the expression efficiency per single DNA molecule decreases rapidly.^{5-7,9} Previously, we found that this silencing is independent of DNA methylation.⁹ In this study, we analyzed the modification of histones, which interact with chromosomal DNA and play important roles in transcription regulation.

In contrast to our hypothesis, we did not obtain any evidence that the binding and modifications of histones were involved in the silencing. The amounts of exogenous DNA bound by histones were similar during the 28 days after the administration (Supplemental Figure 1). This result suggests that the binding of the exogenous DNA with histones occurred at an early stage. In addition, the time-dependent decrease and increase of histone modifications, associated with active and inactive chromatin formation, respectively, were not detected (Figure 3 and Supplemental Figure 2). Thus, histones did not seem to play a major role in the silencing of the transgene.

As described above, the amounts of exogenous DNA bound with histones were similar during the 28 days after the administration

(Supplemental Figure 1). This result suggests the relatively stable retention of the exogenous DNA-histone complex, although the actual amount of the complexed DNA was unclear, due to the unknown efficiency of the precipitation by the anti-H3 antibody. In a previous paper, we observed that the amount of the full-length plasmid DNA was less than that of the total exogenous DNA until 72 hr after the injection, suggesting the presence of fragmented DNA.⁹ The degraded DNA could be produced by intranuclear proteins with endonuclease and/or exonuclease activities. Based on these results, it would be reasonable to speculate that the binding of the exogenous DNA and histones occurs at an early stage after the administration, and that the “free” DNA molecules disappeared by degradation/excretion. The histones seem to bind to the exogenous DNA randomly, since similar patterns were observed for the promoter, luciferase gene, and bacterial backbone regions (Figure 3, and Supplemental Figures 1 and 2).

We then examined the effects of the hydrodynamics-based method itself on transgene expression. We found that the second hydrodynamics-based injection of saline on day 4 after the first injection enhanced the luciferase expression 150-fold (Figure 4a and b). This result suggests that the higher expression from plasmid DNAs delivered by the hydrodynamics-based administration at the early time points was due to activated transcription and/or translation. When we carried out similar

experiments with luciferase plasmid DNA containing the β -actin promoter instead of the CMV promoter, a 13-fold increase in the luciferase expression was observed with the second injection on day 4 (data not shown). In addition, a promoter-dependent reduction in the luciferase expression was demonstrated by Al-Dosari *et al.*²⁹ They reported that the decline was rapid for the CMV, α_1 -antitrypsin, heat shock protein 70, and nuclear factor κ B (NF κ B) promoters. Collectively, the activation by the hydrodynamics-based administration seemed to occur at the transcription level, suggesting the involvement of transcription factors. Thus, altered intranuclear disposition of exogenous DNA would be involved in the promoter-dependent reduction in the transgene expression. The transcription activation reported in this study, together with studies on the impacts of hydrodynamics-based injection,^{27,28,30-34} have revealed the various effects of this administration method on the cellular trafficking, and the intracellular and intranuclear dispositions of plasmid DNA.

Transgene expression was reportedly enhanced and maintained when minicircles that lacked the bacterial backbone part, containing many CpG motifs, and the plasmid DNA with fewer CpG sequences were used in the hydrodynamics-based injection.^{6,7,13,14} These results implied that the presence of the CpG motifs caused transgene suppression. This interpretation suggests, together with our present observation, that the 'activation' of the exogenous DNAs is dependent on CpG as well as the

promoter. In addition, long-term transgene expression was achieved by the hydrodynamics-based injection, using plasmid DNAs containing the full genomic human α_1 -antitrypsin gene with its natural promoter.^{11,12} Similar prolonged expression was reported for the plasmid DNA delivered with liposomes and for adenoviral vector.^{35,36} Moreover, the presence of intron and 3'-untranslated region contributed to long-term expression of a cDNA sequence.³⁷ Thus, functional sequences in the expression cassette other than promoters are also suggested to be important for the intranuclear disposition. These experimental findings might provide a key for understanding the silencing mechanism observed in various studies.

We found no evidence that histones were involved in the silencing of the exogenous DNA. However, this result does not deny the importance of controlling the interactions between exogenous DNA and histones. For example, the location of a left-handedly curved sequence with high histone affinity affects the transgene expression.^{38,39} Thus, the control of the histone interactions is crucial for controlling the intranuclear disposition of the exogenous DNA.

In this study, we found that the major cause of the rapid decline in transgene expression after the hydrodynamics-based injection, under our experimental conditions, was suggested to be the return from the activated state induced by the injection procedure. However, the silencing has been observed in the cases of lipoplex delivered to the lung⁸ and naked plasmid

DNA introduced into cultured cells by electroporation.¹⁰ Thus, our findings do not exclude the actual silencing of exogenous DNA. Moreover, our data on effects of second injection could be interpreted as transient reactivation of silenced transgene. Further studies are necessary to interpret the data obtained in this study and others. In addition, elucidation of the mechanisms involved in the promoter-dependent (re)activation of the exogenous DNA will help us to design DNA for its controlled intranuclear disposition.

Materials and methods

Materials

Oligodeoxyribonucleotides were purchased from Sigma Genosys Japan (Ishikari, Japan) in purified forms. Balb/c mice (female, 6 weeks old) were from Japan SLC (Shizuoka, Japan).

Salmon Sperm DNA/Protein A Agarose-50% Slurry (catalog number: 16-157), anti-H3 dimethyl K9 (07-441), anti-H3 trimethyl K9 (07-442), anti-H4 acetyl K5/K8/K12/K16 (06-866), anti-HP1 γ (05-690) and anti-H3 (07-690) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti-H3 trimethyl K4 antibody

(ab8580) was from Abcam (Cambridge, UK). Control IgG antibody (sc-2027) was from Santa Cruz Biotechnologies (Santa Cruz, CA, USA).

Plasmid

The pYK-CMV-luc plasmid containing the CMV promoter and the luciferase gene⁹ was amplified in the *E. coli* DH-5 α strain, and was purified with a Qiagen EndoFree plasmid Mega Kit (Qiagen, Hilden, Germany). The absorbance at 260 nm and the Hoechst 33258 fluorescence were measured to quantify the DNA concentrations.

Hydrodynamics-based injection

The hydrodynamics-based injection was performed according to the method of Liu *et al.* and Zhang *et al.*^{22,23} Plasmid DNA (20 μ g), in 2 ml of saline, was injected into female six week-old Balb/c mice via the tail vein within 5 sec. For the second injections, 2 ml of saline (without plasmid DNA) was injected via the tail vein within 5 sec, 4 or 14 days after the first injection. Mice were killed and the livers were harvested at various time points. The luciferase activity and the amount of intranuclear plasmid DNA in the livers were measured as described previously.⁹

DNA methylation analysis

The methylation analysis was performed as described previously⁹ with some modifications. Briefly, the DNAs recovered from the injected mice were digested with *Pst* I. After phenol/chloroform extraction and ethanol precipitation, the DNA was treated with *Bsa*H I or *Hha* I. These restriction enzymes cleave 5'-G(A/G)C*G(C/T)C-3' and 5'-GC*GC-3' sequences, respectively, but cannot cut the sequences when the C* residue is methylated. Thus, only the methylated DNA was amplified by quantitative PCR (Q-PCR) after the digestion.

Q-PCR was performed using an Applied Biosystems 7500 real time PCR system (Foster, CA, USA). To detect the DNA methylated in the *Bsa*H I site (promoter region) by Q-PCR, the TaqMan chemistry was employed, by using the following oligodeoxyribonucleotides: CMV pro (+), 5'-GCGGAGTTGTTACGACATTTTG; CMV pro (-), 5'-CGTGGATAGCGGTTTGACTION; CMV pro (TaqMan probe), 5'-FAM-CTCCCATTGACGTCAAT-MGB (Figure 1). Similarly, the SYBR Green system was used for detection of DNA methylation at the *Hha* I sites (the luciferase gene and backbone regions), and the following primers were used: Luc gene (+), 5'-GTAAGACCTTTCGGTACTTCG; Luc gene (-), 5'-ATCGTGGATTACGTGCCAG and Backbone (+), 5'-CGGCGAGAAGCAGGCCATTAT; Backbone (-), 5'-AAGGCCATCCAGCCTCGCGT. Total luciferase DNA was also determined by Q-PCR using the following primers: Luc (+),

5'-GGTCCTATGATTATGTCCGGTTATG; Luc (-),
5'-ATGTAGCCATCCATCCTTGTCAAT, and no *Bsa*I and *Hha*I sites were present in this amplified region. The methylation ratio was calculated by determining the amounts of methylated DNA relative to the total DNA. Under our experimental conditions, the calculation by this procedure provided reasonable methylation ratios when mixtures of unmethylated and *in vitro*-methylated plasmid DNAs were tested (data not shown).

ChIP assay

The ChIP assay was carried out according to the methods of Eberhardy *et al.*²⁵ and Scribner and McGrane,²⁶ with the following modifications. Briefly, dissected livers were minced with scissors and crosslinked with 1 % formaldehyde for 30 min at room temperature. The crosslinking reaction was stopped by the addition of 0.125 M glycine. After centrifugation at 3,000 rpm for 5 min at 4°C, the pellet was washed with PBS. The crosslinked liver was then Dounce homogenized with a Digital Homogenizer (Iuchi, Osaka, Japan). After centrifugation at 3,000 rpm for 5 min at 4°C, the pellet was resuspended in lysis buffer (50 mM Tris-HCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS), 1 mM phenylmethane sulfonyl fluoride (PMSF), 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, pH 8.0). The cell lysate was diluted with ChIP dilution buffer (16.7 mM Tris-HCl, 167 mM NaCl, 1.2 mM

EDTA, 1.1% Triton X-100, 0.11% deoxycholate, 1 mM PMSF, 1 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ pepstatin A, pH 8.0) and was sonicated on ice with a Digital Sonifier (Branson, Danbury, Connecticut, USA) for twenty 30-s pulses (output, 20%). After centrifugation at 10,000 rpm for 10 min at 4°C, the chromatin was pre-cleared for 30 minutes at 4°C with rotation, using the Salmon Sperm DNA/Protein A Agarose-50% Slurry. The pre-cleared chromatin was aliquoted and stored at -80°C.

Immunoprecipitation was performed overnight at 4°C with rotation, by using either 2 μg of anti-H3 trimethyl K4, anti-H3 dimethyl K9, anti-H3 trimethyl K9, anti-HP1 γ or control IgG antibodies, or 5 μl of anti-H3 or anti-H4 acetyl K5/K8/K12/K16 antibodies. After the overnight incubation, 30 μl of Salmon Sperm DNA/Protein A Agarose-50% Slurry were added, and the mixture was incubated for 2 hours at 4°C with rotation. The beads were washed twice with low salt RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 0.1% deoxycholate, pH 8.0), once with high salt RIPA buffer (20 mM Tris-HCl, 500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 0.1% deoxycholate, pH 8.0), once with LiCl wash solution (10 mM Tris-HCl, 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% deoxycholate, pH 8.0), and finally twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The immune complexes were eluted with elution buffer (10 mM Tris-HCl, 300 mM NaCl, 5 mM EDTA, 0.5% SDS, pH 8.0) and were incubated for 6

hr at 65 °C to reverse the crosslinking. After a treatment with proteinase K, the DNA was purified by phenol/chloroform extraction and ethanol precipitation.

The amounts of immunoprecipitated DNA were quantified by Q-PCR. CMV pro (+), CMV pro (-), and CMV pro (TaqMan probe) were used for quantification of the CMV promoter region, and Luc (+) plus Luc (-) and Backbone (+) plus Backbone (-) were used for the luciferase gene and backbone regions, respectively (Figure 1). The precipitation ratio (pull-down/input) was calculated by determining the amounts of precipitated DNA relative to the input DNA.

Acknowledgments

This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Japan Society for the Promotion of Science.

Supplementary Information accompanies the paper on Gene Therapy website (<http://www.nature.com/gt>)

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Figure legends

Figure 1. Locations of the DNA regions amplified in Q-PCR, the ChIP assay, and the methylation analysis. The recognition sites of the methylation-sensitive restriction enzymes used in this study are also shown. CMV, cytomegalovirus promoter.

Figure 2. (a) Expression of the luciferase gene, (b) amounts of total luciferase DNA in the nucleus, (c) expression efficiencies per single copy of DNA, and (d) methylation ratio of the intranuclear exogenous DNA. (a-b) The pYK-CMV-luc plasmid (20 μ g) was injected into the tail vein of female Balb/c mice (six weeks old), as described in the Materials and Methods section. The livers were harvested, and the luciferase activities and the amounts of luciferase DNA were measured. (c) The values of the luciferase activities in panel a were divided by the DNA amounts in panel b at the same time points. (d) The values were calculated by Q-PCR after digestion with BsaHI and HhaI. Black columns, luciferase gene; gray columns, CMV promoter; white columns, bacterial backbone. The values represent the averages of at least three independent experiments. Bars indicate SD (standard deviation). RLU, relative light unit.

Figure 3. ChIP analysis of luciferase DNA in the nucleus. The pYK-CMV-luc plasmid (20 μg) was injected into the tail vein of female Balb/c mice (six weeks old), and the ratios of the amounts of exogenous DNAs precipitated by the antibodies indicated in each panel to those of the total exogenous DNAs were calculated, as described in the Materials and Methods section. Black columns, luciferase gene; gray columns, CMV promoter; white columns, bacterial backbone. The values represent the averages of three independent experiments. Bars indicate SD (standard deviation).

Figure 4. Enhancement of luciferase expression by the second injection. The pYK-CMV-luc plasmid (20 μg) was injected into the tail vein of female Balb/c mice (six weeks old), and plasmid DNAs (20 μg) in saline and only saline were injected 4 and 14 days after the first injection. (a) Expression of the luciferase gene at 8 hr after the second injection when the second injection was performed at 4 days after the first injection. The values represent the averages of two independent experiments. (b) Expression of the luciferase gene, (c) amounts of total luciferase DNA in the nucleus, and (d) expression efficiencies per single copy of DNA after the second saline injection. Arrows indicate the second injection. Closed circles, without the second injection; open circles, after the second injection.

The values represent the averages of three independent experiments. Bars indicate SD (standard deviation). RLU, relative light unit.

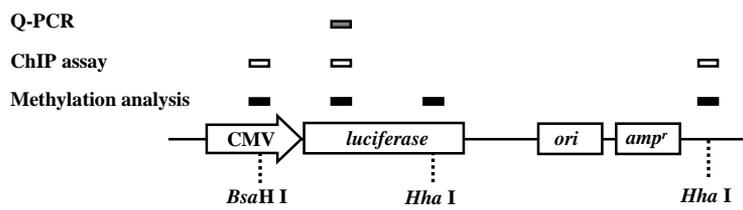


Figure 1

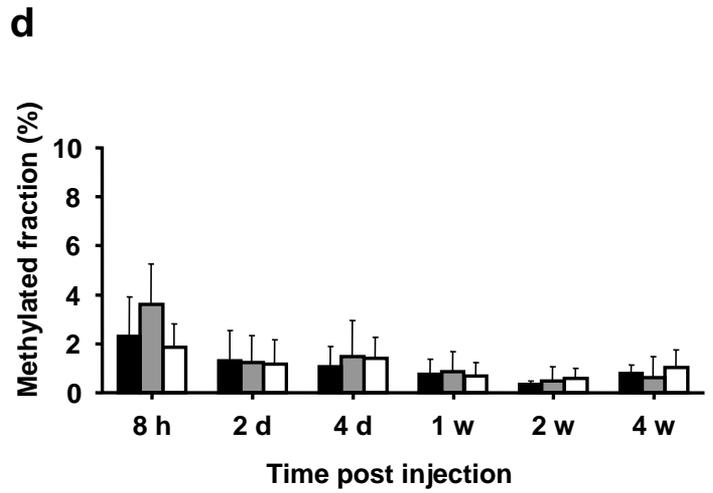
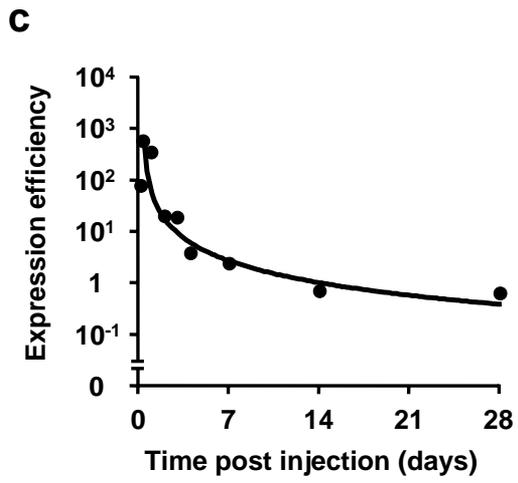
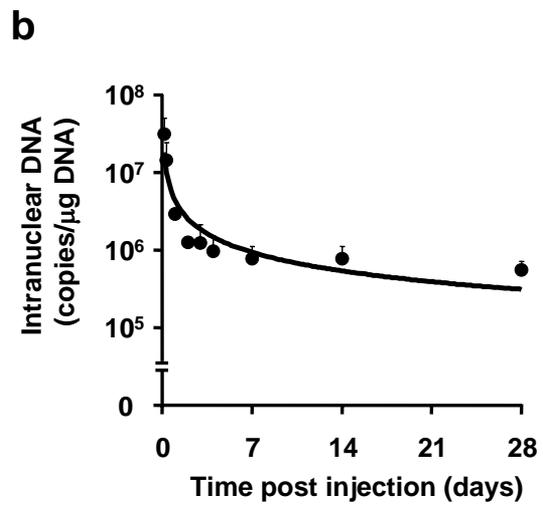
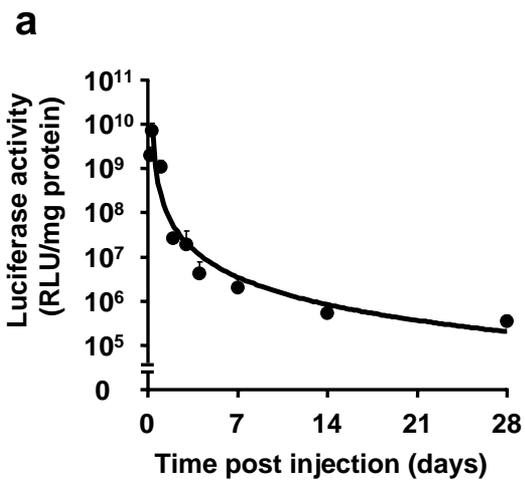


Figure 2

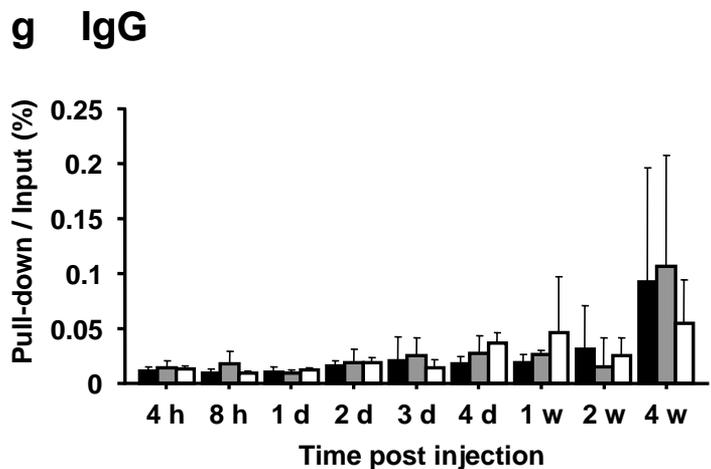
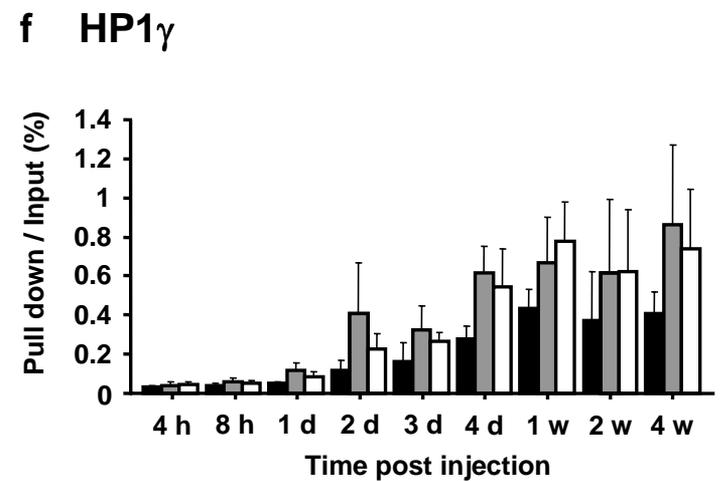
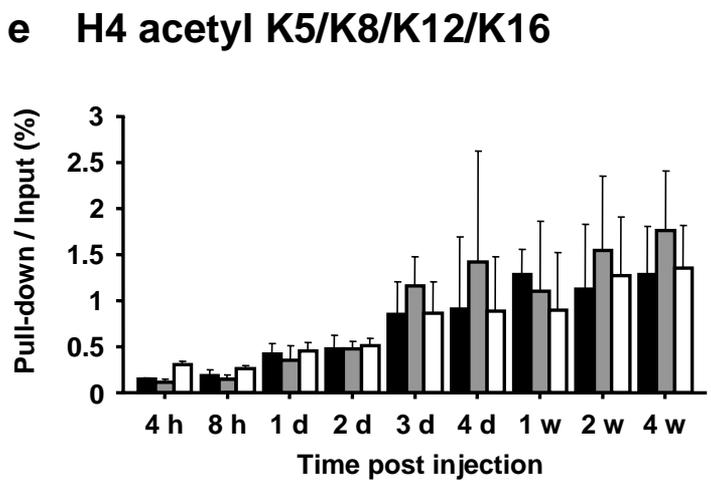
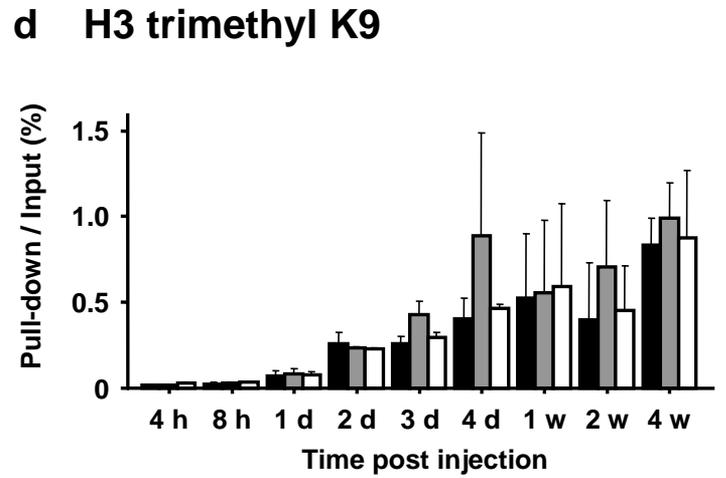
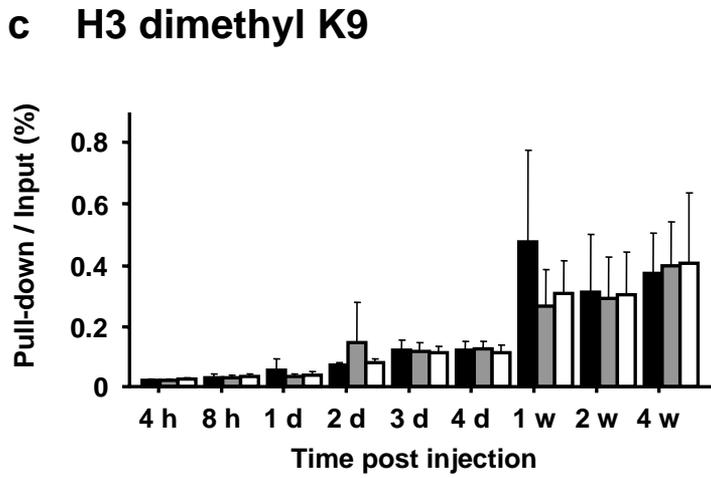
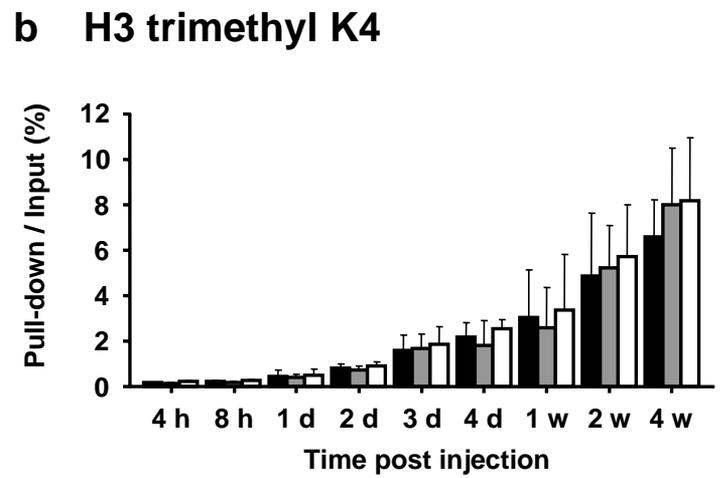
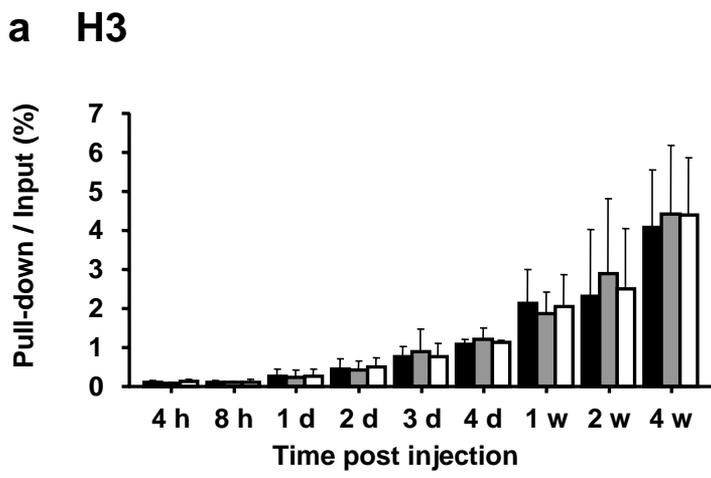


Figure 3

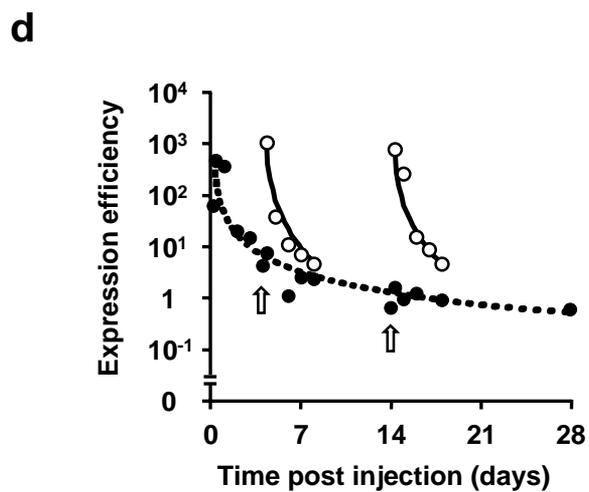
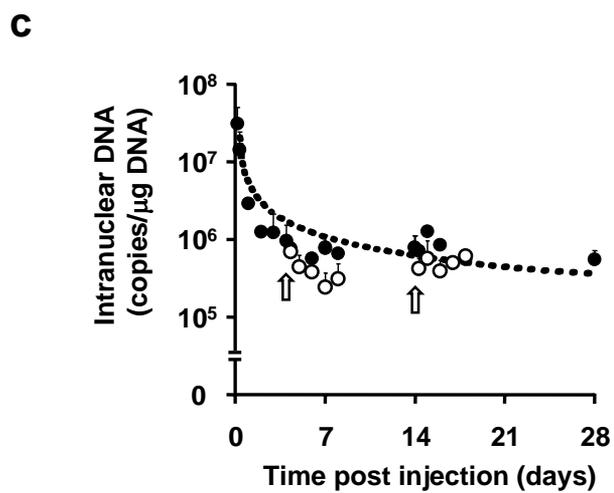
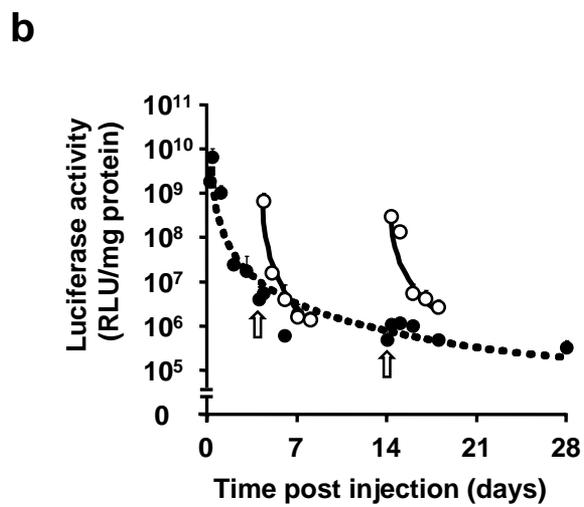
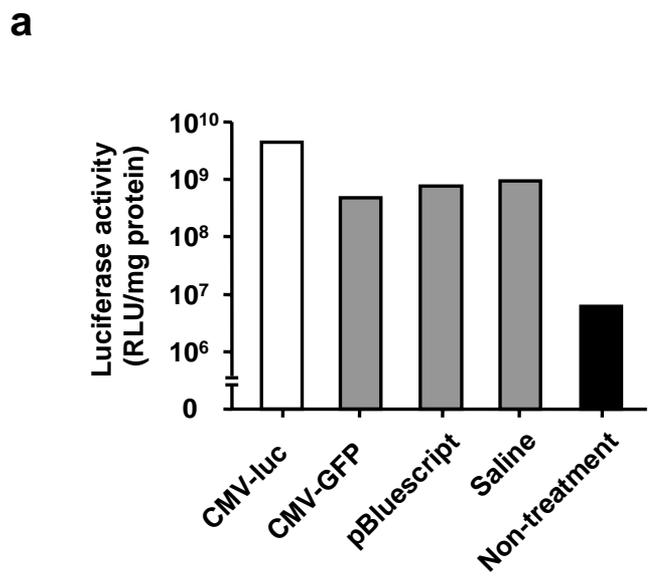


Figure 4