Long-term in vivo gene expression in mouse kidney using ϕC31 integrase and electroporation

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Keywords: Kidney, transfection, plasmid DNA, electroporation, gene therapy, ϕC31 integrase, in vivo long-term gene expression.

Abstract

Background. Achieving long-term gene expression in kidney will be beneficial for gene therapy of renal and congenital diseases, genetic studies constructing animal disease models and the functional analysis of disease-related genes.

Purpose. To develop an *in vivo* long-term gene expression system in murine kidney using ϕC31 integrase.

Methods. Gene expression in cultured RENCA, TCMK-1 and HEK293 cells was assessed. Long-term *in vivo* gene expression system in the kidney was achieved by co-transfecting 5 μg of pORF-luc/attB as donor plasmid and 20 μg of pCMV-luc as helper plasmid into the right kidney of mice by electroporation. Luciferase expression levels were measured to determine longevity of expression.

Results. Significantly high luciferase expression levels were observed in cultured RENCA, TCMK-1 and HEK293 cells over one month compared with controls (non-integrase system). The luciferase cDNA sequence was integrated at a pseudo attP site termed mpsL1. *In vivo* luciferase expression levels in the integrase group were sustained and significantly higher than those in the control group over 2 months. Furthermore, ϕC31 integrase transfected cells had less genomic DNA damage caused by integrase expression.

Discussion and Conclusion. These results demonstrated the ϕC31 integrase system could produce long-term (2 months) *in vivo* gene expression in mouse kidney.

Introduction

In vivo long-term gene expression system is a promising tool for a wide variety of biomedical studies, such as the functional analysis of disease-related genes, construction of animal disease models and gene therapy. However, there are a number of obstacles to achieving long-term gene expression. Retroviral vectors have high integration efficiency but randomly integrate into genomic DNA that might cause malignancies by deregulation of cancer genes (Nienhuis et al., 2006; Okita et al., 2008). In contrast, plasmid DNA (pDNA) is commonly used for gene therapy as it has a low frequency of random integration and therefore has a lower risk for malignancy (Capecchi, 1989). Homologous recombination can induce site-specific integration, but this also occurs at a low frequency (Liang et al., 1998). Consequently, the use of site-specific recombinases, such as Cre and Flp are expected to be a potential method to obtain long-term gene expression (Rodríguez et al., 2000; Iwatate et al., 2003). However, their main utility is for creating deletions due to the reversibility of the enzyme reactions (Kulkarni et al., 1999; Wu et al., 2007).

Recent studies revealed that the *Streptomyces* bacteriophage ϕC31 integrase can achieve long-term gene expression because of its site-specific and unidirectional integration reaction (Kuhstoss and Rao, 1991; Groth et al., 2000). In nature, ϕC31 integrase mediates site-specific recombination of the phage genome into the bacterial chromosome through a unidirectional

reaction between the phage attP site and bacterial attB site (Thorpe et al., 1998). Recently, Thyagarajan et al. reported that integrases can function in cultured mammalian cells to efficiently recombine pDNA including an attB site into a pseudo-attP site in mammalian genomic DNA (Fig. 1) (Thyagarajan et al., 1998). More recently, we and other groups demonstrated the successful use of ϕC31 integrase for prolonged gene expression in the murine liver, lung and skeletal muscle (Olivares et al., 2002; Bertoni et al., 2006; Aneja et al., 2007; Umemoto et al., 2012). pDNA expression in muscle tissue in a model of muscular dystrophy was sustained at a high level up to 540 days (Bertoni et al., 2006). However, a similar expression profile of the integrase system was not observed in other organs when using different transfection methods. Therefore, the optimization of transfection conditions including pDNA dose and a ratio of donor plasmid and helper plasmid is a prerequisite for successful long-term gene expression *in vivo*.

Intractable diseases in the kidney such as chronic kidney failure, IgA nephropathy and Alport syndrome are major world health problems with poor diagnosis and uncertain therapeutic outcomes require to the development of new innovative treatments (Galla, 1995; Kashtan and Michael, 1996). Long-term expression of therapeutic genes in the kidney is one approach to this problem. However, few studies have reported sustained gene expression using the ϕC31 integrase system in the kidney.

In this study, we used donor pDNA encoding luciferase cDNA as a reporter gene and

helper pDNA that expressed an integrase protein. Using these pDNAs, we demonstrated long-term gene expression in kidney cell lines (RENCA, TCMK-1 and HEK-293 cells). To obtain a high expression level of ϕC31 integrase *in vivo*, the direct injection of pDNA solution followed by delivering an electric pulse in the kidney was examined. Site-specific integration by ϕC31 integrase in the genomic DNA was analyzed in these cell lines and murine kidneys.

Materials and methods

Cell cultures

Three kinds of cell lines were obtained for this experiment: RENCA, TCMK-1 and HEK293, which are derived from mouse renal cortical adenocarcinoma, mouse renal tubular epithelial cells and human embryonic kidney cells, respectively. RENCA cells were cultured in RPMI 1640 medium and TCMK-1 cells and HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (D-MEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (Equitedch-Bio, Kerrville, TX), 0.15% $NaHCO_3$, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 2 mM L-glutamine at 37 °C in humidified air containing 5% CO_2. Opti-MEM I® was purchased from Gibco BRL (Grand Island, NY).

Animals

Female ICR mice (5 weeks, 18–22 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were performed in accordance with the Principles of Laboratory Animal Care as adopted and propagated by the U.S. National Institutes of Health and the Guidelines for Animal Experiments of Kyoto University.

pDNA construction

ϕC31 integrase expressing plasmid, pENTR11-int C-NLS and pZerofC31attB3xP3EGFP containing an attB site were kindly provided by Dr. T. Kusakabe, Kyushu University Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu, Japan. The construction of helper pDNA, pCMV-int (Fig. 1), was reported previously (Umemoto et al., 2012). The plasmid pORF-mIL2 contains a murine IL-2 coding sequence driven by the Elongation Factor 1-α (EF1-α) promoter (Invitrogen, Carlsbad, CA, USA). The pORF-luc plasmid was generated as follows. The luciferase coding sequence was amplified from pCMV-luc (Nomura et al., 1999) using primers (5’-GAGATCACCGGCGAGCCACCATGGAAGACGCCA-3’ and 5’-CATGTCGAGCTAGCTTACACGGCGATCTTTCCG-3’) followed by restriction enzyme digestion (*Nco*I and *Nhe*I). The digested product was ligated into a plasmid where the mIL2 coding sequence from pORF-mIL2 was excised by *Nco*I and *Nhe*I. The donor plasmid

pORF-luc/attB (Fig. 1) was generated from the pORF-luc and attB sequences. The attB sequence was amplified from pZerofC31attB3xP3EGFP using primers (5'-ATATGGATCTCGAGAACCGTGCGATCAAACAAAC-3' and 5'-GCGGCCGCTCGAGGCATCAAGCTAATTCTGCAGA-3') followed by restriction enzyme digestion (*Xho*I). This digested fragment was ligated into the *Xho*I site of pORF-luc.

In vitro-transfection experiments

In vitro transfection was performed using Lipofectamine 2000® reagent (LF2000, Invitrogen). LF2000 was used according to the recommended procedures with a 4 h-incubation for all experiments. For long-term gene expression, cells were cultured in 24-well plates and transfected with 0.2 μg of pORF-luc/attB and either 0.6 μg of pCMV-int or pcDNA3.1. At 24 h post-transfection, plates for long-term expression were split at 1:2 and incubation continued for another 24 h. One plate was used for luciferase measurement and the other was incubated and cells were split regularly (2–3 days) to obtain cells at different time points. The cells were removed from plates by scraping and suspended in lysis buffer (0.25% Triton X-100, 2 mM EDTA, 0.1 M Tris, pH 7.8). Then, the cell suspension was centrifuged at 10,000 × g, at 4 °C for 10 min. The supernatant was mixed with luciferase assay buffer (Picagene, Toyo Ink Co. Ltd., Tokyo, Japan) and luciferase activity was measured by luminometer (Lumat LB 9507, EG&G Berthold, Bad Wildbad, Germany).

Luciferase activity was normalized to the protein content of the cells. The protein concentration was determined using a Protein Quantification Kit (Dojindo Molecular Technologies, Inc., Tokyo, Japan).

In-vivo-gene expression experiments in mouse kidney

Five week-old ICR female mice were anesthetized by intraperitoneal injection of sodium pentobarbital. Then, a midline incision was made in the abdomen followed by direct injection of pDNA in 0.9% saline into the right kidney. Immediately, electric pulses were applied to the right kidney using a pair of 1-cm^2 forcep-type electrodes connected to a rectangular direct current generator (CUY-21, Nepagene, Chiba, Japan). The parameters of the electric pulses were 5 ms/pulse, 12 pulses, 2 Hz, and 250 V/cm. At predetermined times after injections, mice were sacrificed and the kidneys were collected. For the luciferase assay, kidneys were homogenized in lysis buffer at 4 ml/mg kidney. Then the luciferase activity assay was performed.

Quantification of donor pDNA integration by real-time PCR

The donor pDNA was transfected with the helper pDNA or the control pDNA into kidney cell lines. At 14 days after transfection, genomic DNA and plasmid DNA were extracted from the transfected cells using a GenElute™ mammalian genomic DNA extraction

Kit (Sigma-Aldrich Japan Co., Tokyo, Japan). For the measurement of the intact donor pDNA and luciferase cDNA sequences, real-time PCR was performed with the extracted DNA using a LightCycler instrument (Roche Diagnostics, Basel, Switzerland). Amplified products were detected online via intercalation of the fluorescent dye SYBR green (Takara Bio Inc. Shiga, Japan). Amplifications were performed using 1 ml of template DNA for 35 cycles of 95 °C for 5 s, and 60 °C for 20 s. Gene expression was normalized to the RAD52 gene expression.

Detection and characterization of mpsL1 integration sites

At three days before PCR analysis, pDNAs were transfected to the right kidney by electroporation. Genomic DNA from kidneys was prepared using a GenElute™ mammalian genomic DNA extraction Kit. Then, semi-nested PCR was performed. Primary amplifications used two mpsL1 integration site–specific primers: mpsL1 forward (5'-TCTGAGTAGTACCCTGGCTT-3') and mpsL1 reverse-1 (5'-CGTGAAGGAGAGATGCGAGC-3'), which were designed from the mpsL1 sequence and donor plasmid sequence respectively. A total of 400 ng of genomic DNA was used for 30 cycles of PCR with the following conditions: 98 °C for 10 s, 60 °C for 30 s, and 68 °C for 40 s followed by 68 °C for 10 min. Primers were removed from the primary reactions using a GenElute™ PCR Clean-up Kit (Sigma-Aldrich Japan Co.), and 1 μl of the elution was re-amplified with nested primers, mpsL1 forward (5'-TCTGAGTAGTACCCTGGCTT-3')

and mpsL1 reverse-2 (5’-GGAGCCAGTACACGACATCAC-3’) with the following conditions: 30 cycles of 98 °C for 10s, 60 °C for 30 s, and 68 °C for 30 s followed by 68 °C for 10 min. Finally, 10 μl of product from the semi-nested PCR was subjected to agarose gel electrophoresis.

Analysis of cell viability by MTT assay

As a positive control for cytotoxicity, cells were exposed to 10 μM camptothecin for 4 h. Each group of cell was seeded to 96 well plates at a density of 10,000 cells per well. The time point of performing MTT assay was configured at 0, 24, 48 and 72 h. At designated time, 25 μL of MTT solution (5 mg/mL in PBS) were added to each well and the plate was incubated at 37 °C for 4 h in the dark. The crystals were then dissolved by addition of SDS in water and were sonicated. The absorption wavelength 570 nm of each well was determined with a reference wavelength of 690 nm. The mitochondrial dehydrogenase (MD) activity in each group was calculated as relative values using the absorption at 0 h.

Analysis of cytotoxicity by immunostaining

Before immunostaining, cells were incubated with pDNA/LF2000 for 48 h at 37 °C. Cells exposed to 10 μM camptothecin for 4 h were used as a positive control. Then, cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 min and

permeabilized with 0.25% Triton X-100 in PBS for 30 min. After blocking with 1% bovine serum albumin containing PBS-Tween 20, cells were incubated with anti-γ-H2A.X (phosphor S139) antibody (Abcam, Tokyo, Japan) for 1 h at room temperature. Secondary antibodies were conjugated to Texas Red. Cells were mounted using DAPI-containing Vectashield (Vector Laboratories) before image capture.

Statistical analysis

Results were expressed as the mean standard deviation of more than three experiments. Statistical analysis was performed using analysis of variance and the Turkey–Kramer test for multiple comparisons between groups or Student's t-test for two-group comparisons. $P < 0.05$ was considered statistically significant.

Results

ϕC31 integrase-mediated gene expression in vitro

First, we determined whether ϕC31 integrase could mediate long-term gene expression in murine and human kidney cell lines. We performed co-transfection of the luciferase coding pDNA including the attB site (pORF-luc/attB) and a plasmid expressing the integrase (pCMV-int) into cell lines using LF2000. Then, we measured the luciferase expression level over time. There was a statistically significant increase in long-term gene

expression in human HEK293 cells and murine RENCA and TCMK-1 cells when the gene was transfected using pORF-luc/attB in the presence of the integrase (Fig. 2 (A), (B) and (C)). Sustained luciferase expression was observed over 4 weeks. However, expression levels dropped over time in the absence of pCMV-int. These results are consistent with the integrase-mediated integration of the luciferase gene into genomic DNA. The dependence of the reaction on integrase and attB sites suggested that the pseudo attP sites in human and murine genomic DNA are recognized by ϕC31 integrase.

Next, we evaluated whether the steady gene expression in these cell lines was derived from episomal pDNA or integrated pDNA. Real-time PCR was performed to measure the amounts of episomal pDNA in the cellular nucleus and total luciferase cDNA sequences. Genomic DNA and pDNA were extracted from cells at 14 days post-transfection. Controls receiving donor pDNA and control pDNA showed no episomal pDNA or luciferase cDNA sequences by quantitative PCR (Fig. 2 (D), (E)). However, in the integrase group, luciferase cDNA sequences were detected and were higher than that of the control group, although episomal pDNA was not detected in the integrase group (Fig. 2 (D), (E)). Thus, the luciferase cDNA sequence may exist in genomic DNA of the host cells.

DNA damage and effects of proliferation caused by integrase expression

Because ϕC31 integrase can cause transient genomic DNA cleavage at pseudo attP

sites, integrase transfected cells have a risk of mutagenesis or death. Therefore, the rate of cell proliferation was examined by MTT assay to assess the cytotoxic effect of integrase expression (Fig. 3 (A), (B)). The cell growth curves in each group revealed a similar behaviour up to 72 h after transfection except for the camptothecin treated group. These results indicate that cell proliferation was not affected by integrase expression in the host cells. Phosphorylation of histone γ-H2A.X is a marker of double strand breaks in genomic DNA (20). Genomic double strand breaks were additionally assessed in a more detail by evaluating integrase expression and enzymatic reactions. The presence of γ-H2A.X was investigated using an antiγ-H2A.X antibody. Low levels of double strand breaks were observed in pORF-luc/attB and pCMV-int treatment groups compared to camptothecin treatment group (positive control) (Fig. 3 (C)).

ϕC31 integrase-mediated gene expression in vivo

ϕC31 integrase activity was further evaluated in murine kidney. To obtain high levels of integrase gene expression in the kidney, electroporation was used for naked gene transfection. It was previously reported that prominent gene expression was obtained in murine kidney by electroporation and gene expression was observed at a definite site (Thanaketpaisarn et al., 2005). First, transfection conditions for long-term *in vivo* gene expression were optimized. Donor pDNA driven by an EF1-α promoter, rather than the CMV

promoter, was selected because of its continued luciferase gene expression (Fig. 4 (A)). ϕC31 integrase expression is thought to have a high integration efficiency. The CMV promoter induces higher levels of integrase protein production compared with the EF1-α promoter, with a saturated expression at 20 μg of pDNA (Fig. 4 (B)). Thus, the combination of pORF-luc/attB as a donor pDNA and pCMV-int as a helper pDNA is efficient for long-term gene expression in the kidney. Moreover, we investigated the ratio of donor pDNA and helper pDNA to determine the most efficient integration ratio. As shown in Fig. 4 (C), the gene expression level at day 7 was maintained for half of day 1 when 5 μg of donor pDNA and 20 μg of helper pDNA were co-transfected into the kidney by electroporation. Using this ratio, luciferase expression profiles were assessed over time. There was a high level of luciferase expression at 24 h post-transfection in both the integrase transfected group and control group. However, luciferase expression in the integrase transfected group was maintained over 60 days whereas expression in the control group slowly returned to background values by 60 days after transfection (Fig. 5 (A)). The weight ratio of the right kidney and left kidney in the integrase transfected group at 60 days was not significantly different from that of control and non-treatment groups. The result of sustained gene expression is consistent with integrase-mediated integration of the luciferase gene into genomic DNA. However, pDNA remained in murine kidneys and luciferase expression was maintained at a low level in the control group. Therefore, the difference in gene expression over 10 days was smaller *in vivo*

compared with *in vitro*. Next, we tried to determine the integration reaction at pseudo attP sites in murine kidneys.

Confirmation of integration in mouse kidney and analysis of integration sites

To examine whether the integrase reaction occurred correctly in murine kidney, genomic DNA was extracted at 3 days post-transfection of pDNAs by performing semi-nested PCR. The PCR reaction detected a hot spot of integration at mpsL1 in mouse chromosome 2, band H3, in three of four integrase-transfected mice (Fig. 6). In contrast, there was no PCR product specific for the hot spot in control mice (Fig. 6). The same result was previously observed in the liver using a hydrodynamic transfection method (Umemoto et al., 2012).

Discussion

Long-term gene expressions in tissues such as liver, lung and muscle have been reported (Olivares et al., 2002; Bertoni et al., 2006; Aneja et al., 2007); however, the transfection conditions for each organ need to be optimized (Chalberg et al., 2005). In this study, we demonstrated the effectiveness of ϕC31 integrase using integrase expression pDNA in murine kidney. We initially tried to construct pDNAs, which are adequate for long-term gene expression. Promoter silencing is a major obstacle to sustained *in vivo* gene expression

from integrated pDNAs (Ochiai et al., 2007). However, the EF-1α promoter can mediate sustained gene expression because it encodes a house keeping gene and can escape promoter silencing (Gopalkrishnan et al., 1999). Therefore, we selected the EF-1α promoter to drive donor pDNA (pORF-luc/attB) gene expression. The CMV promoter, which can promote high gene expression levels within a few days post-transfection, is often selected for helper pDNA (pCMV-int) because integration completes within a few days (Kawano et al., 2007). Based on these previous studies we investigated the development of a long-term gene expression system using ϕC31 integrase in the murine kidney.

First, we studied long-term gene expression in kidney cell lines using the constructed donor and helper pDNA. Long-term gene expression was obtained in the integrase transfected groups in three kinds of cell lines and significantly high gene expression levels were maintained. At 14 days post-transfection, luciferase sequences in the nucleus of the host cells were detected in the integrase transfected groups despite the absence of external donor pDNAs in host cells (Fig. 2 (D), (E)), suggesting that the integration reaction in the genomic DNA of the host cells is conferred by ϕC31 integrase expression. Thus, long-term gene expression was obtained using these pDNAs and strongly suggested that the reaction occurs at a hot spot in host genomic DNA in the cultured kidney cell lines. In addition, we obtained the sustained gene expression in both murine and human kidney cell lines. This result showed that the integrase system might be applicable not only to murine kidney but

also to human kidney.

Then, we optimized the long-term *in vivo* gene expression system in murine kidney. The high efficiency of *in vivo* transfection is important for the long-term gene expression system using ϕC31 integrase, because it is essential to transfect both donor pDNA and helper pDNA into the nucleus of the host kidney cells. In this study, the donor pDNA was co-transfected into the kidney with helper pDNA by electroporation because of its high transfection characteristics at the injection site (Thanaketpaisarn et al., 2005). We obtained long-term gene expression for up to 60 days in the kidney (latest time point studied) (Fig. 5).

We observed that a high ratio of helper pDNA to donor pDNA (4:1) was required to obtain sustained gene expression in the kidney. Recently, Aneja et al. and Olivares et al. reported a ratio of helper pDNA to donor pDNA of 1:1 was sufficient in the liver (Olivares et al., 2002) and in the lung (Aneja et al., 2007). This discrepancy may be explained as, i) integration frequency is influenced by variables that favour transcriptional activity, such as chromatin accessibility in determining the locations where integrations occur in mammalian genomes (Groth and Calos, 2004), and ii) different integration sites are present in different tissues based on their accessibility (the kidney contains the mpsL1 site) (Maucksch et al., 2008). Further studies are needed to clarify this phenomenon.

In this study, *in vivo* experiments demonstrated the need for helper pDNA for high expression in the kidney. We used the electroporation method for transfection to the kidney,

but this method may not be directly applied to human tissues from the point of view of physical burden. Additionally, direct injection does not regulate the active targeting of pDNAs because of the limitation of tissue diffusion. pDNAs should be delivered into target organs without any carriers to reduce toxicity. Therefore, novel safe and effective transfection methods are needed before these vectors can be used in the clinic. Recently, we reported a tissue pressure- or suction-mediated transfection method for the kidney using naked pDNA in mice (Mukai et al., 2008; Mukai et al., 2010; Shimizu et al., 2012a; Shimizu et al., 2012b). In addition, we developed kidney-selective gene transfection using anionic bubble lipopolyplexes with renal ultrasound irradiation in mice (Kurosaki et al., 2014). The combination of these *in vivo* transfection methods and use of a long-term gene expression vector will be of value to future biomedical studies in mice.

There have been few reports of *in vivo* experiments to demonstrate whether aberrant events are caused by ϕC31 integrase. Because of the nature of recombination, ϕC31 integrase might cause negative effects such as genomic DNA double strand breaks in host cells. However, cytotoxicity of ϕC31 integrase was not observed in terms of cell proliferation and double strand breaks (Fig. 4). These results are in agreement with a previous report where ϕC31 integrase mediated integration reactions that occurred at hot spots in mammalian genomic DNA (Groth et al., 2000). This suggests ϕC31 integrase is safer than other recombinases or virus vectors. However, these findings are insufficient to demonstrate the

safety of the ϕC31 integrase system, because the long-term effect of integration reactions was not determined in this study. Chalberg et al. reported that about 1.9% of integration reactions are exonic, which might induce aberrant events including intrachromosomal deletions and inter-chromosomal rearrangements in cultured cell (Chalberg et al., 2006). The toxicity of ϕC31 integrase is related to its transfection characteristics (i.e., efficacy and duration) and transfected cell types used. Therefore, toxicity studies using various *in vivo* transfection methods are required.

In conclusion, we demonstrated long-term *in vivo* gene expression in murine kidney by ϕC31 integrase expression pDNA. Although this system needs further improvement in many aspects, such as integration frequency, safety issues, optimization of promoters for specific organs and pDNA delivery methods, the findings indicate the ϕC31 integrase method will be of use for biomedical studies and gene therapy of chronic and congenital kidney diseases.

Acknowledgements

We are grateful to Dr. T. Kusakabe (Laboratory of Silkworm Science, Kyushu University Graduate School of Bioresource and Bioenvironmental Sciences) for the ϕC31 integrase expression plasmid, pENTR11-int C-NLS and pZerofC31attB3xP3EGFP containing an attB site. The authors thank Yoshiaki Umemoto for his skilful technical support in the in vitro experiments.

Declaration of interest

This research was supported in part by a grant-in-aid for Challenging Exploratory Research (26670082, S.K.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by JFE (the Japanese Foundation for Research and Promotion of Endoscopy) Grant (S.K.). The authors alone are responsible for the content and writing of the paper.

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FIGURE LEGENDS

Figure 1. Schematic representations of DNA plasmids used. The donor plasmid pORF-luc/attB integrates into genomic DNA with ϕC31 integrase. The helper plasmid pCMV-int expresses ϕC31 integrase. pcDNA3.1 was used as a control plasmid.

Figure 2. Stable long-term expression in the presence of integrase expression plasmid. The line chart shows the time course of luciferase expression after transfection with pORF-luc/attB with or without integrase expression plasmids, pCMV-Int, at a 1:3 ratio into (A) RENCA, (B) TCMK-1 and (C) HEK293 cells. Each data point represents the mean of three independent replicates with standard deviation. Differences between the control and the integrase group are significant after 14 days in RENCA, 7 days in TCMK-1 and 12 days in HEK293 cells (* $P < 0.05$, ** $P < 0.01$). Confirmation of integration into genomic DNA in host cells (D), (E). The relative amounts of (D) luciferase cDNA or (E) intact pORF-luc/attB in the nucleus. pORF-luc/attB was transfected into cells, RENCA or TCMK-1 with pCMV-int or a control plasmid, pcDNA3.1. After 14 days post-transfection, genomic DNA and plasmid DNA were extracted from the nucleus. Luciferase cDNA sequences in extracted DNA were detected and quantified by real-time PCR.

Figure 3. Cytotoxicity of ϕC31 integrase expression *in vitro*. Cell viability was measured by

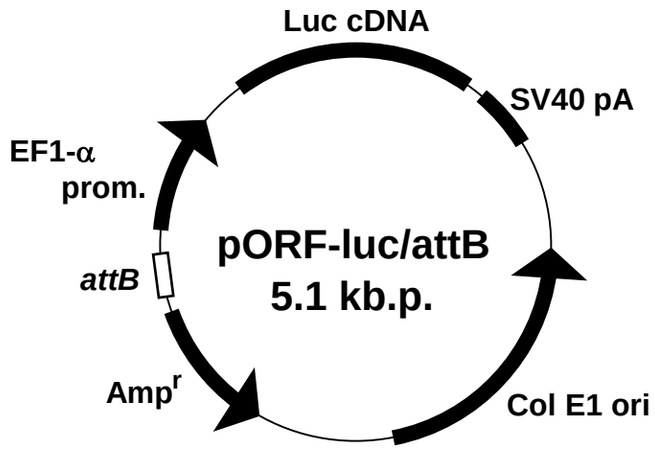
MTT assay after transfection of pDNAs in (A) RENCA and (B) TCMK-1 cells. MTT assay was performed at 0, 24, 48 and 72 hours after transfection. The mitochondrial dehydrogenase (MD) activity in each group is shown as relative values based on 0 hours. (C) The assessment of double strand breaks by γ-H2A.X immunostaining. pDNAs were transfected with Lipofectamine 2000 and immunostaining was performed at 48 hours after transfection. Nuclei were stained by DAPI (blue) and phosphorylated γ-H2A.X was stained by anti-γ-H2A.X antibodies.

Figure 4. Optimization of ϕC31 integrase system in mouse kidney. pDNAs were transfected into the right kidney by electroporation. (A) The effect of promoters with respect to gene expression and duration. Luciferase gene expression levels were compared between pCMV-luc with pORF-luc over 7 days. (B) Effect of the promoters with respect to mRNA expression. Integrase mRNA expression levels were compared between pCMV-int and pORF-int at each dose. (C) Effect of pCMV-int dose on long-term gene expression. Five mg of pORF-luc/attB and various doses of pCMV-int were transfected into the kidney and a luciferase assay was performed at 1 or 7 days after transfection. Differences between day 1and day 7 were significant in pCMV-int dose of 0 and 50 μg (* $P < 0.01$).

Figure 5. (A) Stable long-term expression in the presence of integrase expression plasmid.

The line chart shows the time course of luciferase expression after transfection of pORF-luc/attB with or without integrase expression plasmids, pCMV-Int, at a 1:4 ratio, into the right kidney. Each data point represents the mean of five independent replicates with standard deviation. Differences between the control and integrase groups was significant after 30 days and 60 days (* $P < 0.05$). (B) Right kidney/left kidney weight ratio at 60 days after transfection. pDNAs were transfected by electroporation into the right kidney. Right and left kidneys of three groups of mice (integrase, control and non-treatment groups) were measured. Each data bar represents the mean of five independent replicates with standard deviation.

Figure 6. Ethidium bromide–stained gel shows integration at mpsL1 by semi-nested PCR. Genomic DNA was extracted from the right kidney at 48 hours post-transfection. The integrated donor plasmid was detected by semi-nested PCR using two sets of primers designed from mpsL1 and donor plasmid sequences and the expected band size was 450 bp (left panel). The bands are shown in circles and the *RAD52* gene was used as an internal standard.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prom.
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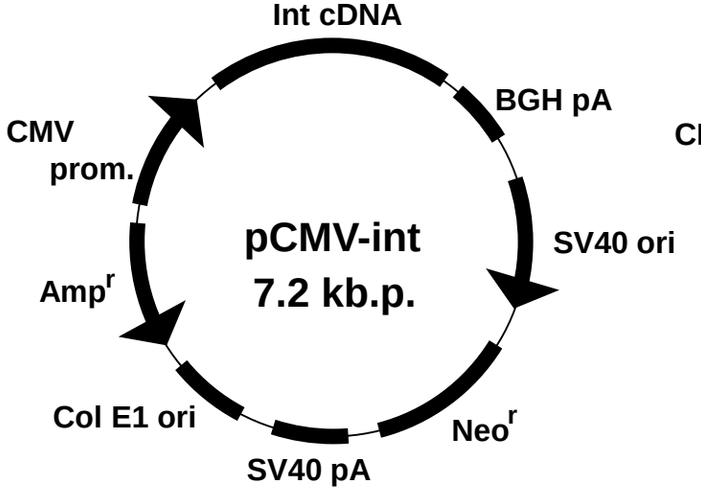
attB
Amp^r
Col E1 ori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CMV
prom.
pCMV-int
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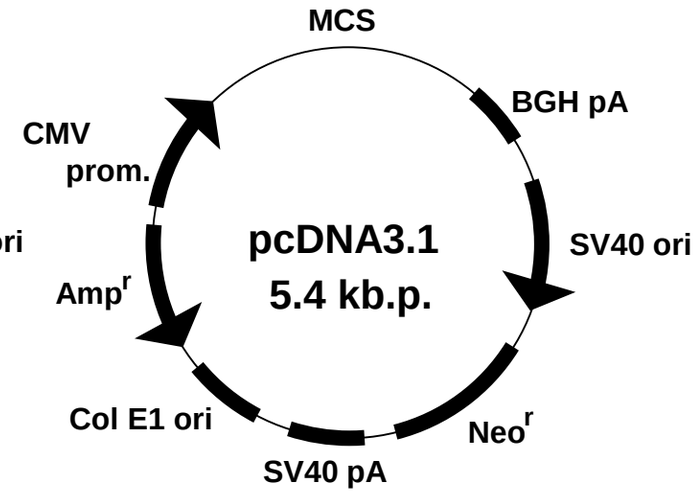
SV40 ori
Amp^r
Col E1 ori
Neo^r
SV40 pA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BGH pA
CMV
prom.
pcDNA3.1
5.4 kb.p.
SV40 ori
Amp^r
Col E1 ori
Neo^r
SV40 pA

Figure 2

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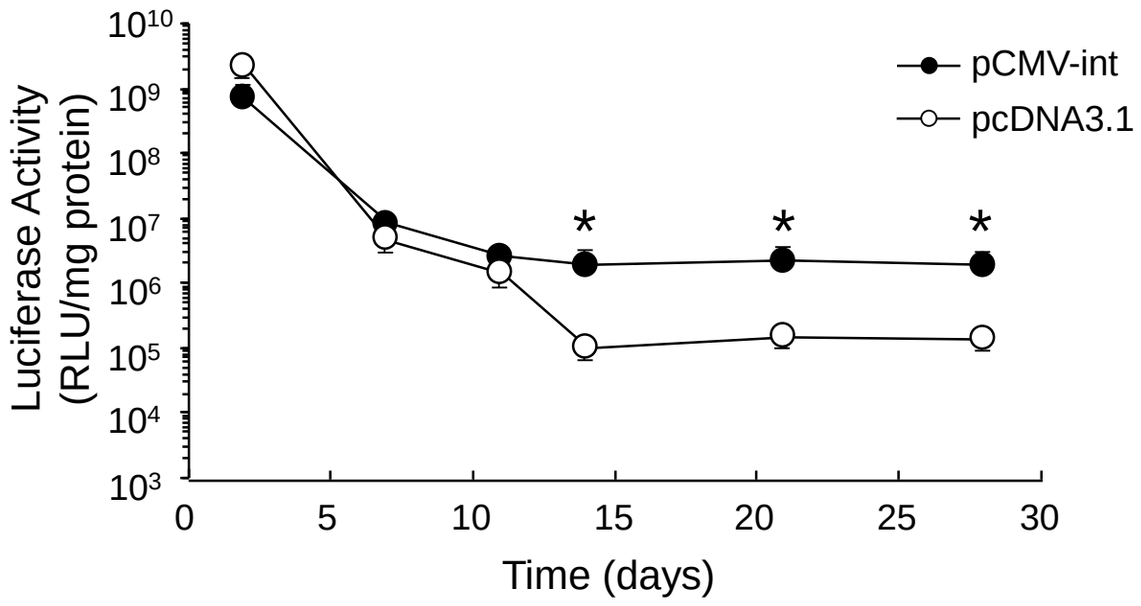
(A)



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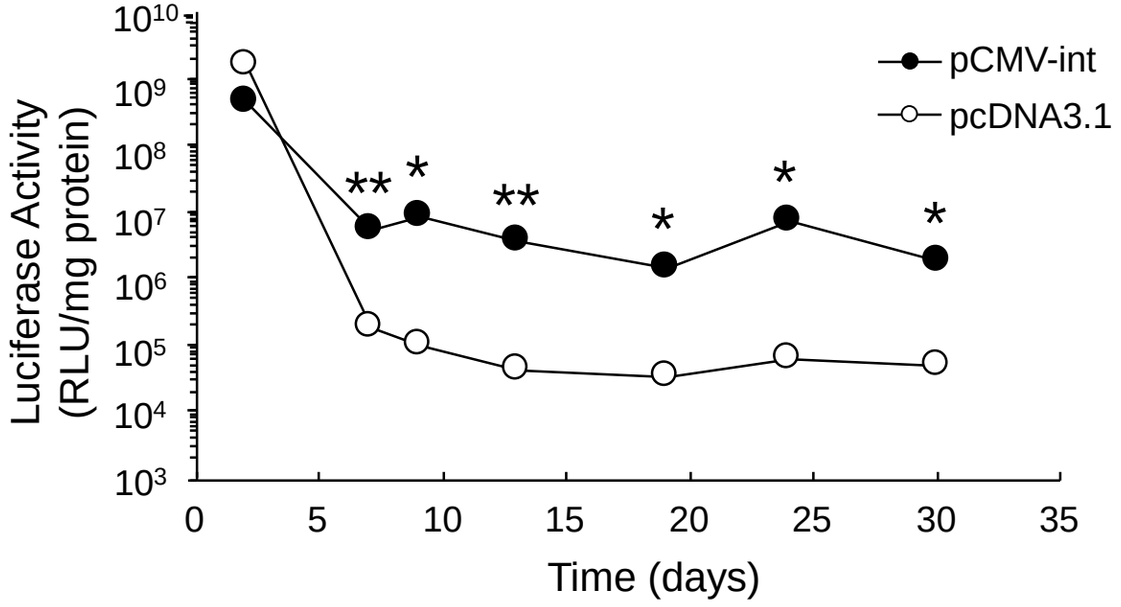

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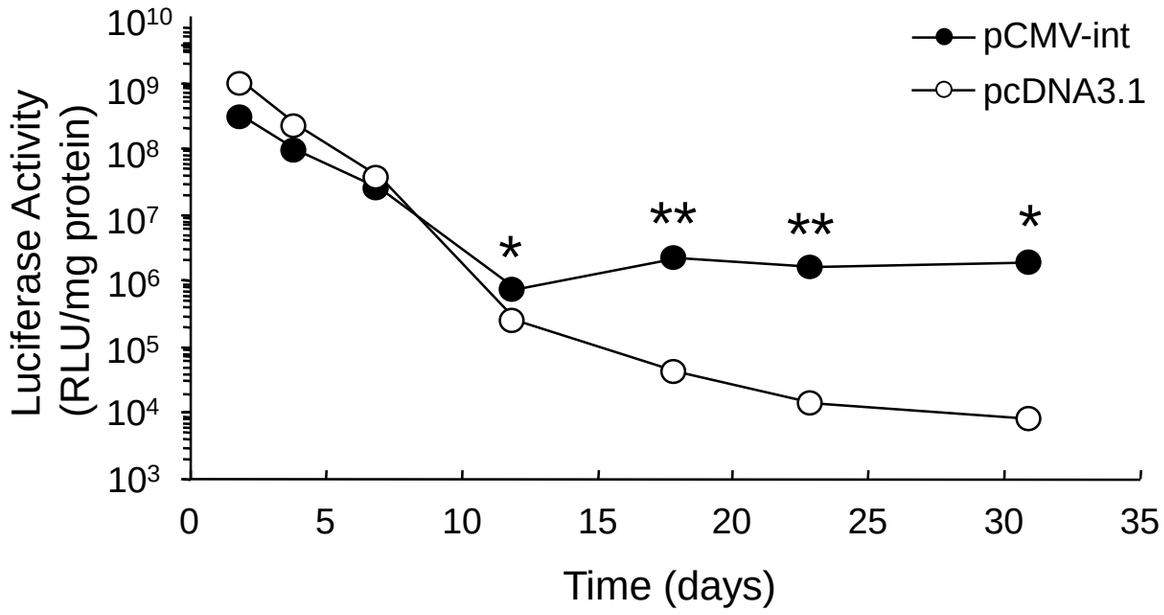

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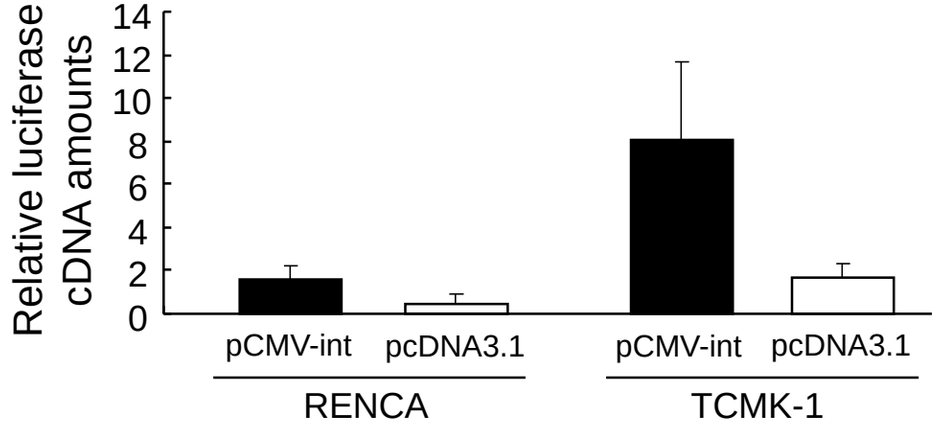

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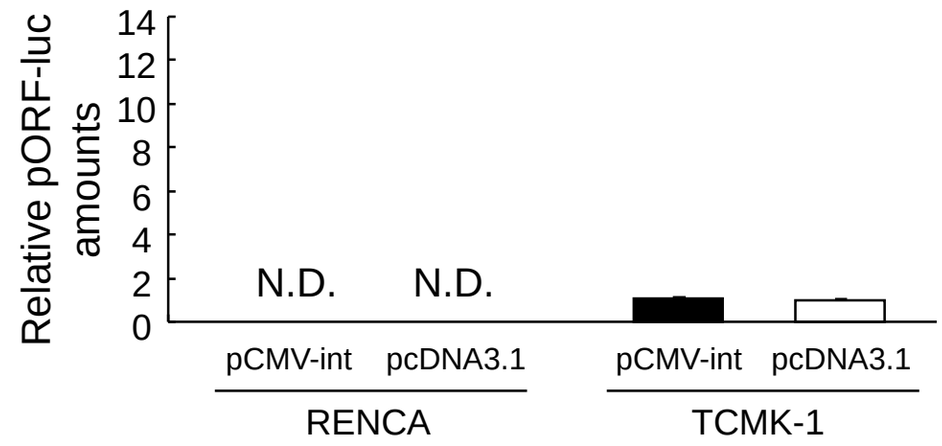

Figure 3

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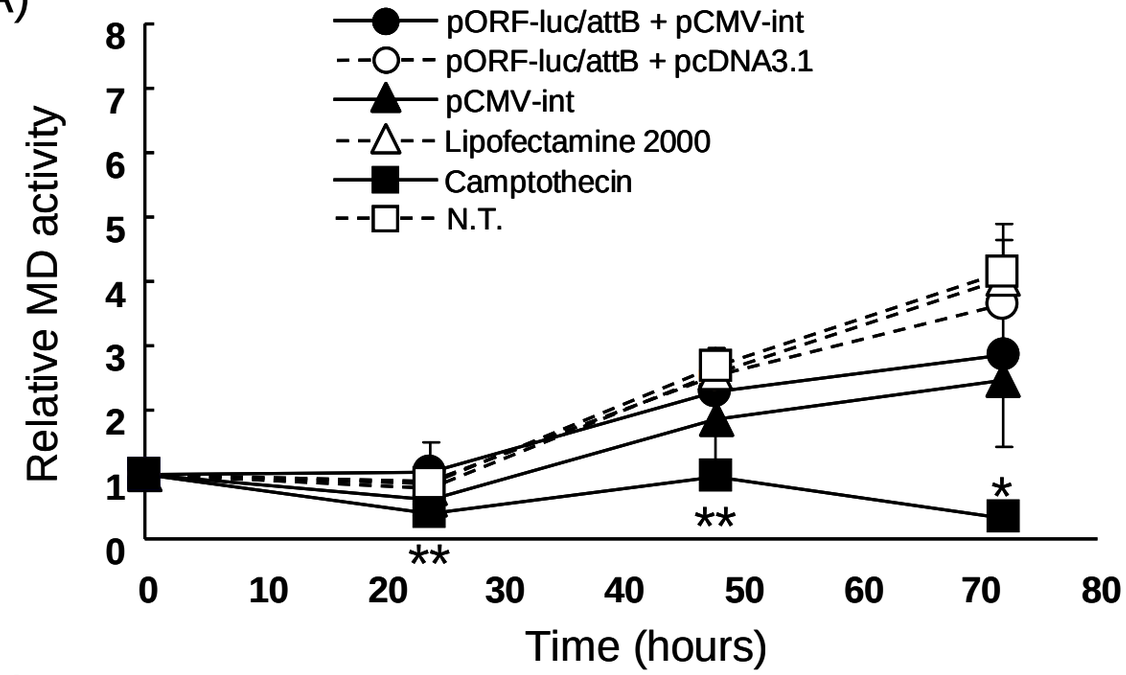
(A)



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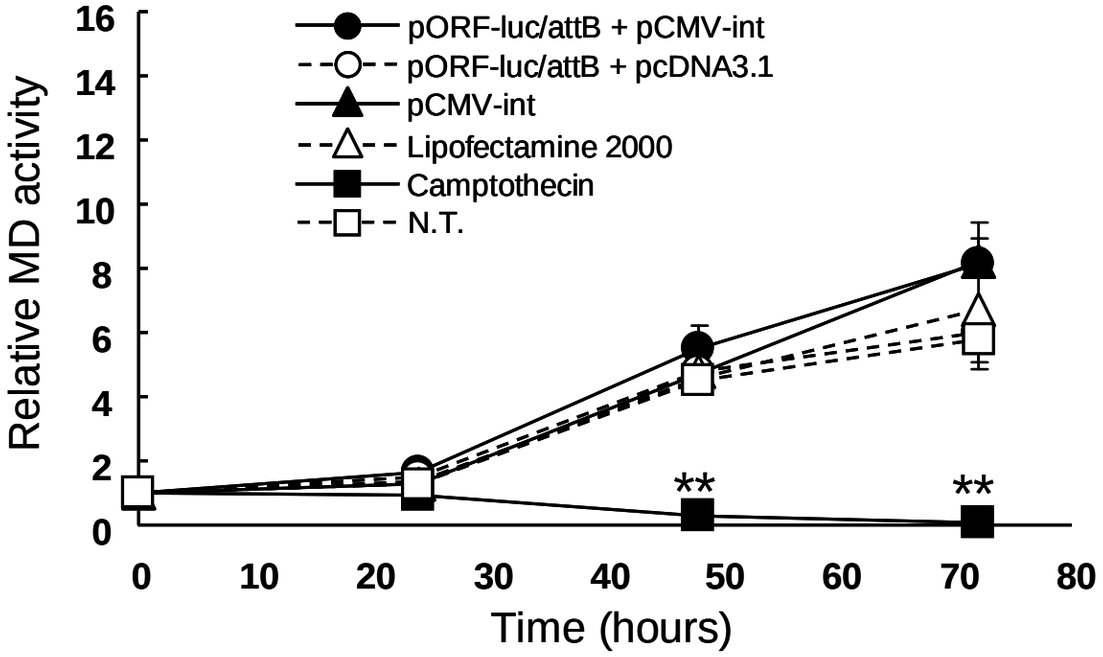

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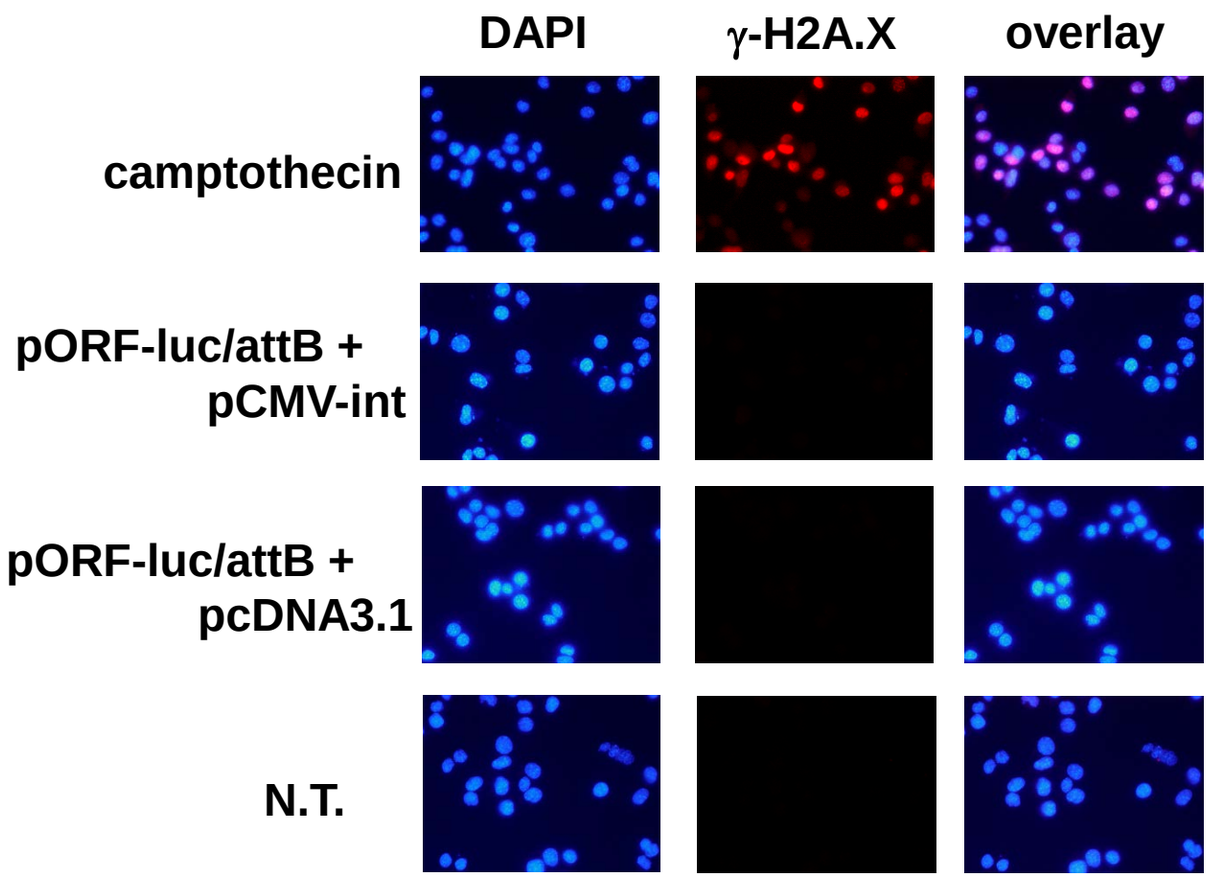

Figure 4

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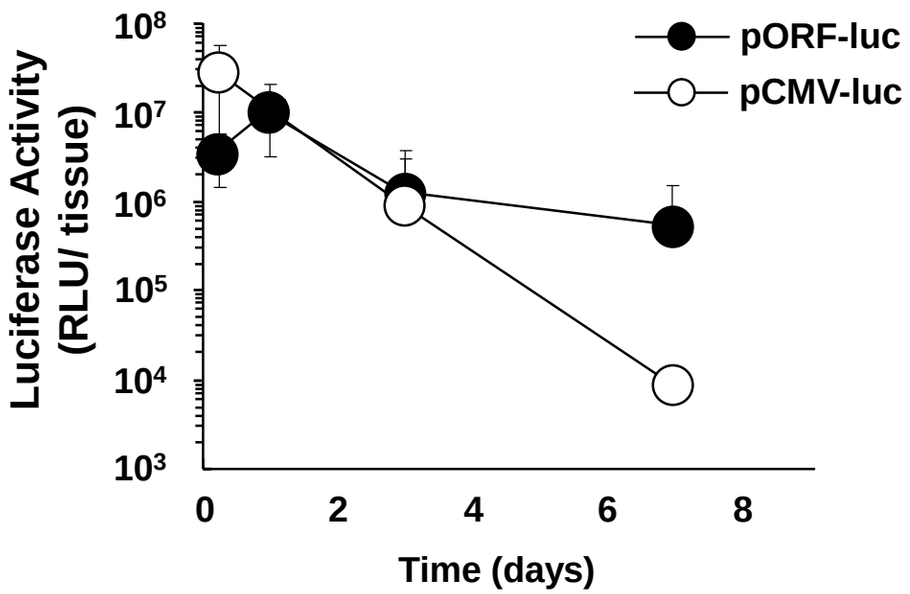
(A)



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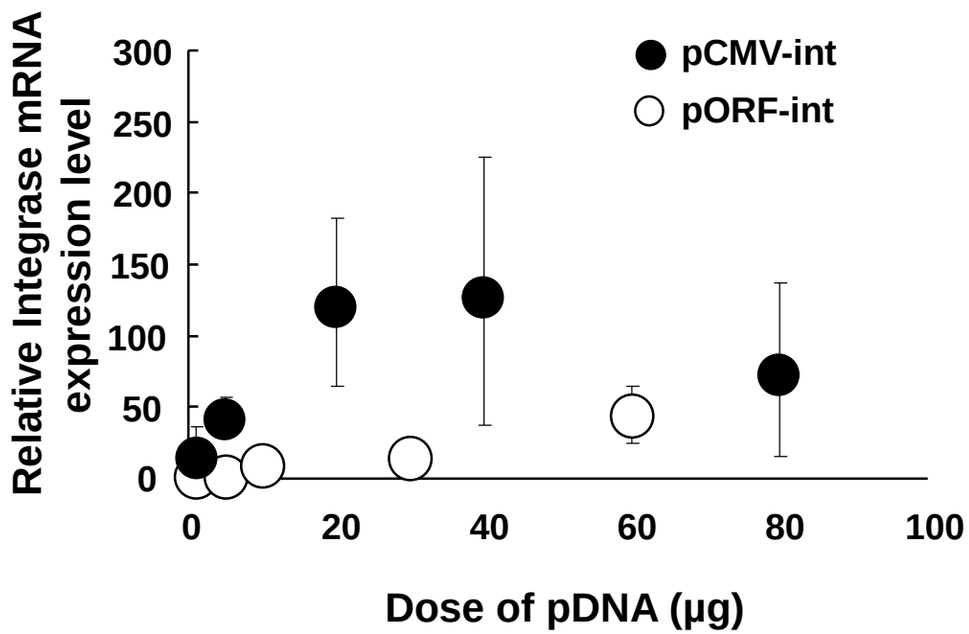

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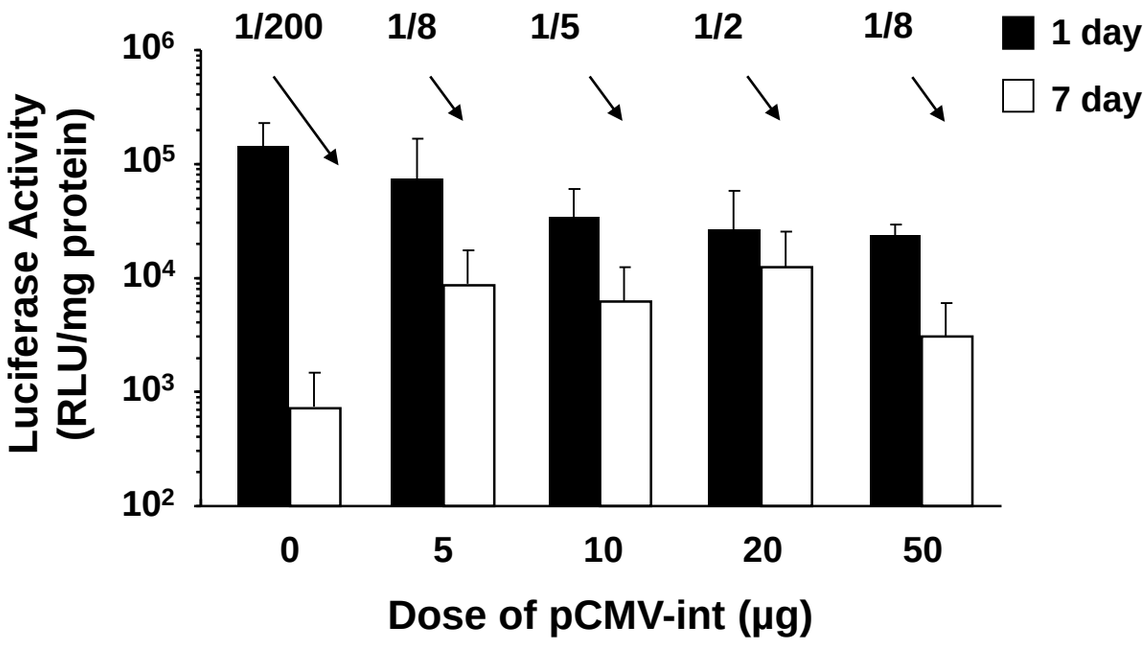

Figure 5

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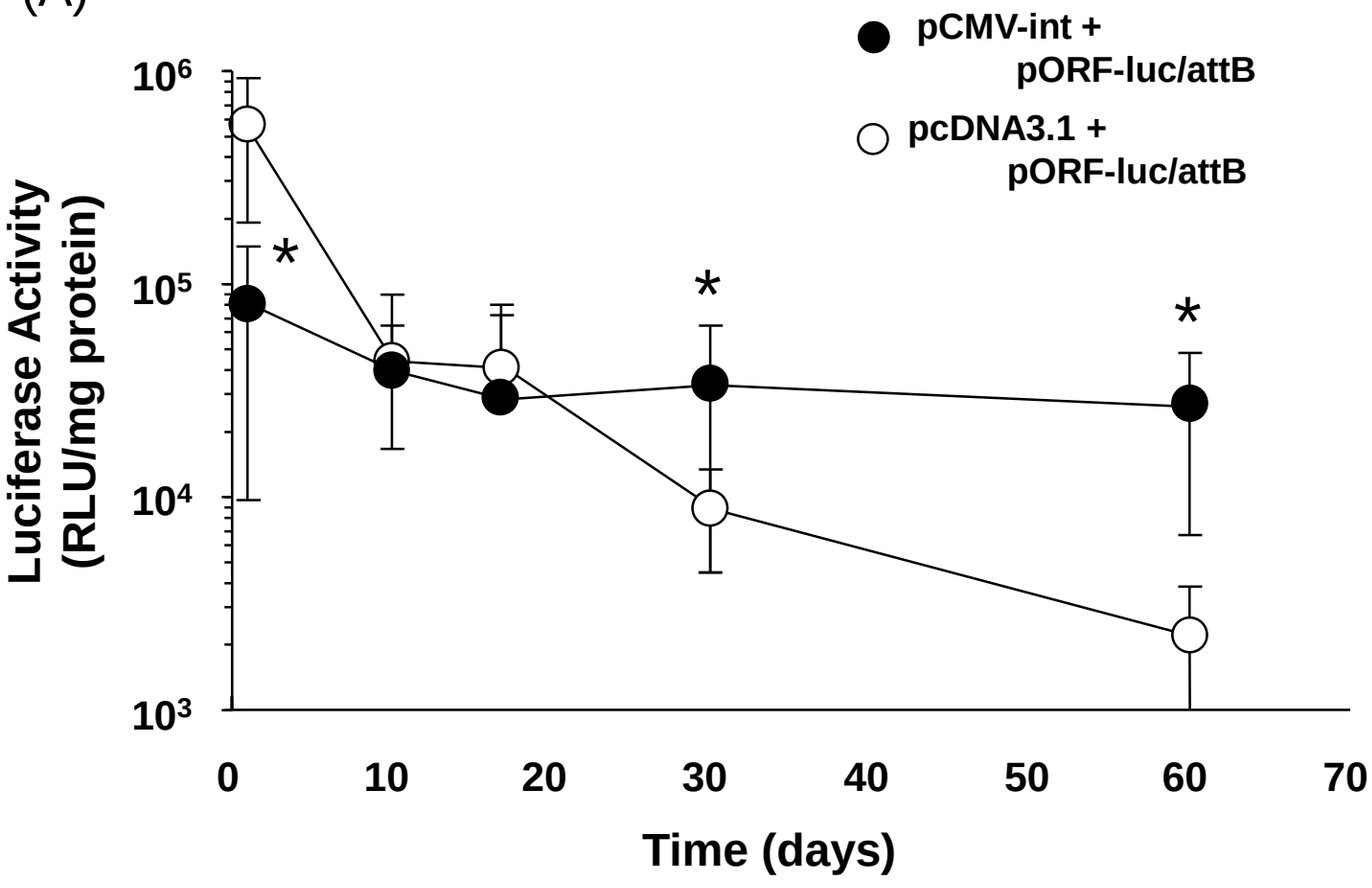
(A)



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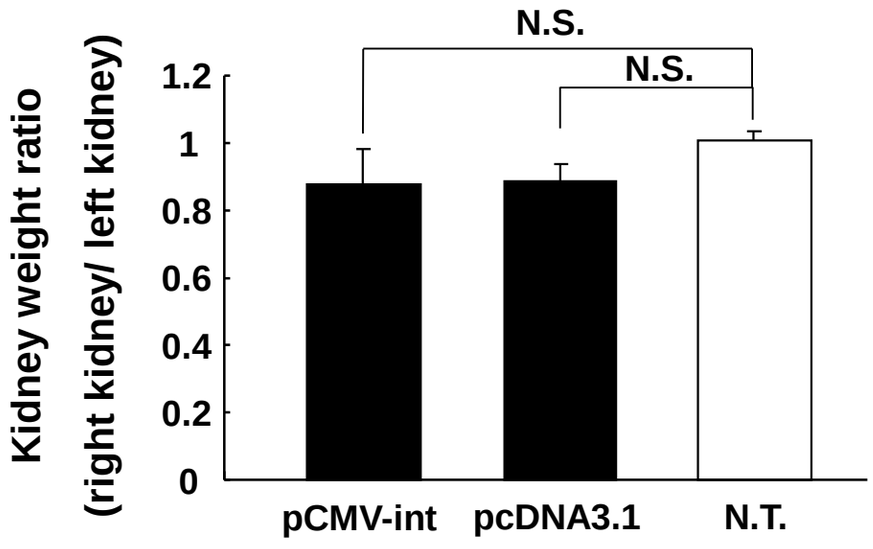
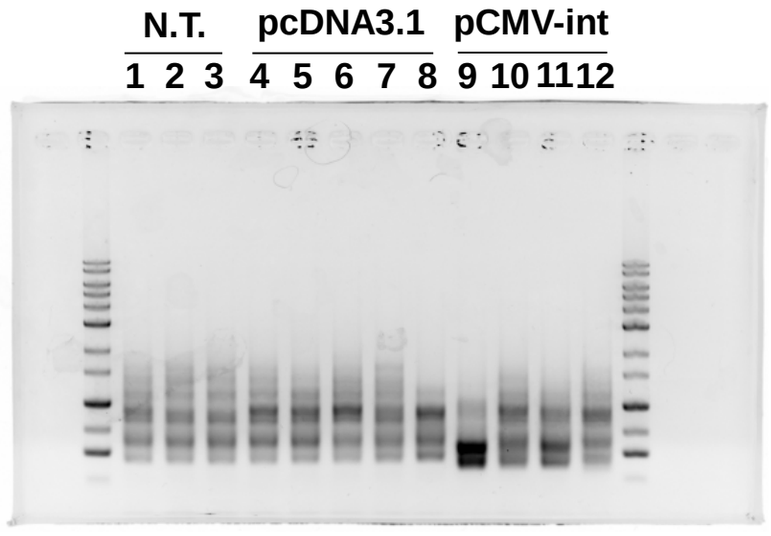


Integrated pDNA



RAD52