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Biochemistry

Evaluation of a D-Octaarginine-linked polymer as a transfection tool for transient and stable transgene expression in human and murine cell lines

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ABSTRACT. Poly(*N*-vinylacetamide-co-acrylic acid) coupled with D-octaarginine (VP-R8) promotes the cellular uptake of peptides/proteins *in vitro*; however, details of the transfection efficacy of VP-R8, such as the cell types possessing high gene transfer, are not known. Herein, we compared the ability of VP-R8 to induce the cellular uptake of plasmid DNA in mouse and human cell lines from different tissues and organs. A green fluorescent protein (GFP)-expression plasmid was used as model genetic material, and fluorescence as an indicator of uptake and plasmid-derived protein expression. Three mouse and three human cell lines were incubated with a mixture of plasmid and VP-R8, and fluorescence analysis were performed two days after transfection. To confirm stable transgene expression, we performed drug selection three days after transfection. A commercially available polymer-based DNA transfection reagent (PTR) was used as the transfection control and standard for comparing transgene expression efficiency. In the case of transient transgene expression, slight-to-moderate GFP expression was observed in all cell lines transfected with plasmid via VP-R8; however, transfection efficiency was lower than using the PTR for gene delivery. In the case of stable transgene expression, VP-R8 promoted drug-resistance acquisition more efficiently than the PTR did. Cells that developed drug resistance after VP-R8-mediated gene transfection expressed GFP more efficiently than cells that developed drug resistance after transfection with the PTR. Thus, VP-R8 shows potential as an *in vitro* or *ex vivo* nonviral transfection tool for generating cell lines with stable transgene expression.

KEYWORDS: gene-delivery, plasmid-derived protein expression, poly(*N*-vinylacetamide-*co*-acrylic acid) bearing D-octaarginine, stable transgene expression, transient transgene expression

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There is an imperative for efficient and safe gene delivery systems, both for basic biological medical as well as veterinary studies and for clinical applications such as gene therapy and regenerative medicine [2, 3, 5, 7–9, 16]. At present, methods to introduce transgenes fall into two categories: nonviral and viral [6]. The viral method utilizes viral vectors such as retrovirus and adenovirus vectors to transfer the gene of interest (GOI) and is currently used in most clinical protocols for gene therapy. Nonviral methods include plasmid-based approaches that can be used not only for transiently expressing exogenous protein from the GOI without integration into the target cell genome, but also for stably expressing exogenous protein from the GOI via random integration or from the GOI inserted into a targeted location on the genome via homologous recombination [17]. In particular, in basic medical/veterinary research such as biochemical or biomedical research, it is very important to maintain stable protein expression in target cells so that the molecular functions of the GOI can be analyzed or to enable large-scale purification of proteins obtained from the GOI.

There are several approaches in place for introducing a plasmid into mammalian cells, for example, mechanical methods such as

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electroporation and microinjection and chemical gene transfer methods involving conjugation of cationic lipids with nucleic acids (e.g., lipofection) or via calcium phosphate–nucleic acid coprecipitation [4, 10]. The choice of the optimal method to provide high gene transfer/transfection efficiency and low cell toxicity depends on the cell type, the purpose, and the experimental setting. In this study, we focused on VP-R8, D-octaarginine (typical cell-penetrating peptide)–linked polymer that we had previously developed and successfully showed has potential for use in chemical transfection [14, 18–20].

VP-R8 is a polymer in which D-octaarginine is immobilized onto a backbone of random copolymers composed of *N*-vinylacetamide and acrylic acid (i.e., poly(*N*-vinylacetamide-*co*-acrylic acid) [PNVA-*co*-AA]; Fig. 1) [14, 16]. This polymer is expected to facilitate efficient cellular uptake of molecules that have poor membrane permeability and are physically mixed with it [20]. We previously found that VP-R8 significantly enhanced peptide penetration through the nasal membrane without cytotoxicity in *in vivo* experiments: nasal administration of an anti-diabetic drug (insulin or exendin-4) with VP-R8 promoted the hypoglycemic effect induced by these drugs in a mouse model [19]. In addition, serum immunoglobulin (Ig) G and mucosal secretory IgA against ovalbumin or influenza virus hemagglutinin vaccines, respectively, were effectively induced when these antigens were nasally coadministered with VP-R8 [18].

We also previously reported enhancement of cellular uptake of molecules that had poor membrane permeability and had been physically mixed with VP-R8 in experiments *in vitro*: VP-R8 enabled dextran (molecular weight: 4 kDa) or 5 (6)-carboxyfluorescein to effectively penetrate the cell membrane of a human colon epithelial cell line (Caco-2) [20]. Furthermore, we found that plasmids or proteins (*β*-galactosidase or bovine serum albumin) premixed with VP-R8 were taken up into a human cervix epithelial cell line (HeLa) [14]. The potential of VP-R8 as an *in vitro* transfection tool for biomolecules has been suggested; however, the intracellular transfer of nucleic acid—such as plasmids—by this polymer has not yet been investigated to the same extent as transfer of peptides and proteins. In particular, many details relating to the transfection efficacy of VP-R8 have not yet been evaluated, such as the cell types that permit high gene transfer, the expression level of transgenes, and capabilities of VP-R8 compares to commercial reagents.

In this study, we compared and evaluated the gene transfer and expression efficiency of VP-R8 with that of a commonly used commercially available polymer-based DNA transfection reagent (PTR) by introducing plasmids into several types of cells and analyzing transient and stable gene expression.

MATERIALS AND METHODS

VP-R8

One production batch of VP-R8, which was reported in our previous study [21], was used in this study. Table 1 shows its physicochemical properties.

Cell culture

C8-D30 (mouse astrocyte), HeLa (human cervix epithelium), and RAW264.7 (mouse macrophage) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). HEK293 (human embryonic kidney) cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Tsukuba, Japan). HepG2 (human hepatoma) cells were obtained from the RIKEN BioResource Research Center Cell Bank (Tsukuba, Japan). J774.1 (mouse macrophage-like) cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). All cell lines, except J774.1, were cultured in Dulbecco's modified Eagle's medium (DMEM; Fujifilm-Wako, Osaka, Japan) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin/streptomycin (Fujifilm-Wako), and 2 mmol/l L-glutamine (Fujifilm-Wako). J774.1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Fujifilm-Wako) containing 10% heat-inactivated FBS, 100 units/ml penicillin/streptomycin, and 2 mmol/l L-glutamine. All cell lines were maintained in a 5% CO_2 atmosphere at 37°C.

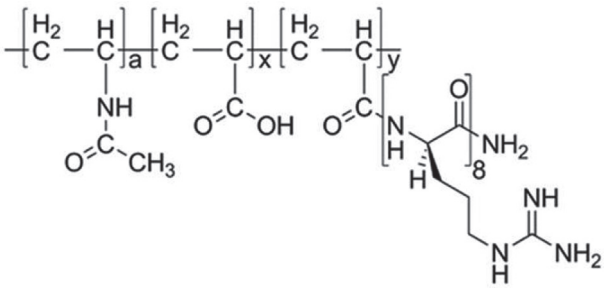

Fig. 1. Chemical structure of VP-R8. Sourced from [14].

Table 1. Characterization of poly(*N*-vinylacetamide-*co*-acrylic acid) bearing D-octaarginine (VP-R8)

Content of the respective units in the backbone of poly(*N*-vinylacetamide-*co*-acrylic acid) [a]			Oligoarginine weight %[b]	Mw (kDa)[c]
N-vinylacetamide	Acrylic acid	Acrylic acid grafting oligoarginines		
70	14	16	75	1,210

[a]Percentage of the number of respective monomer units to the total number of monomer units. [b]Weight percentage of oligoarginines grafted onto polymers (oligoarginines/oligoarginine-linked polymers). [c]Mw of oligoarginine-linked polymers was calculated on the basis of Mw of PNVA-co-AA (350 kDa), Mw of oligoarginines, and the grafting degree. The batch is identical to that used in the previous study [21].

Plasmid

The green fluorescent protein (GFP)-expressing plasmid, pCMV-GFPHA, was constructed by inserting the full-length coding sequence of GFP into pcDNA3-HA, a plasmid that was obtained by inserting an Influenza virus hemagglutinin (HA)-tagged sequence into pcDNA3 (Thermo Fisher Scientific, Waltham, MA, USA). This plasmid contains a neomycin-resistant gene and was provided by Dr. Masaru Murakami (Azabu University, Japan).

Transfection

Cells were seeded in 24-well plates (C8-D30: 1.2×10^5 cells/well; RAW264.7, J774.1, and HEK293: 1.0×10^5 cells/well; HeLa and HepG2: 6×10^4 cells/well) the day before transfection. A mixture of pCMV-GFPHA (0.4 µg) and VP-R8 (4 µg) was prepared using 400 µl serum-free DMEM (for all cell lines except J774.1) or RPMI 1640 (for J774.1 cells) as a solvent. After the mixture was incubated for 15 min at room temperature (approx. 20°C), the culture medium in each well was replaced with the plasmid/VP-R8 mixture and then the plates were incubated at 37°C for 4 or 24 hr. The cells were then washed with serum-free medium and incubated in culture medium at 37°C for a term described in next subsection.

X-tremeGENE™ HP DNA Transfection Reagent (Roche Diagnostics, Basel, Switzerland) is a commercially available, and high-performance PTR that can be used for transfecting transgenes into many cell lines. We used this PTR as the transfection control and the standard by which transgene expression efficiency was compared. For plasmid transfection using the PTR, a mixture of pCMV-GFPHA (0.4 µg) and PTR (1.2 µl) was prepared using 50 µl serum-free medium. After the mixture was incubated for 15 min at room temperature, 450 µl of the culture medium was added and mixed. The culture medium in each well of 24-well plates was replaced with the plasmid/PTR mixture and then incubated at 37°C for a period described in the next subsection.

Drug selection

To generate stable transgene-expressing cells, the cells were subjected to drug selection by the addition of G418 (Nacalai Tesque, Osaka, Japan) to the medium 3 days after transfection. G418 blocks polypeptide synthesis in eukaryotic cells and resistance to G418 is conferred by the neomycin-resistance gene. The cells were exposed to 800 (RAW264.7, J774.1, HEK293, and HeLa), 1,000 (for C8-D30), or 1,200 µg/ml G418 and incubated in a 5% CO_2 atmosphere at 37°C. The G418-containing medium was changed twice a week over a period of 15 days.

Fluorescence detection and analysis

GFP fluorescence was observed at 430–510 nm excitation and 475–575 nm emission with a BZ-X710 fluorescent microscope (KEYENCE, Osaka, Japan), enabling an analysis of transient or stable transgene expression, at 2 days after transfection or at 15 days after drug selection, respectively. Fluorescence images were obtained with a 4×, 10×, or 20× objective (exposure for the GFP: 1/1.7 or 1/3 [only for HeLa] of a second; exposure for the bright field [BF]: 1/80–200, mainly 1/120 of a second).

For flow cytometric analysis, the culture medium was removed, and the cells were washed with PBS. Subsequently PBS was added into the wells, and the cells were harvested by gentle pipetting. The cells were then pelleted by centrifugation at 100 × *g* for 5 min at room temperature, and the cell pellet was resuspended in flow cytometry buffer (PBS containing 1% FBS and 0.1% sodium azide) containing 1 µg/ml propidium iodide (PI). Flow cytometric analysis was performed using a flow cytometry analyzer (EC800; Sony, Tokyo, Japan). Excitation and emission wavelengths were set at 488 and 475–575 nm for GFP, and 488 and 545–645 nm for PI, respectively. The GFP fluorescence in live cells was analyzed after gating out PI-positive dead cells.

Crystal violet staining

Crystal violet staining was performed as previously described [15]. Fifteen days after drug selection, the culture medium was removed, and the cells were washed with PBS. Subsequently, they were stained with 0.05% crystal violet for 10 min at room temperature, washed with distilled water, and air-dried. The air-dried plates were photographed using a camera phone (Xperia SO-20J; Sony).

Statistical analysis

The data were statistically analyzed using one-way analysis of variance, followed by Tukey's multiple comparison tests; the analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Data have been presented in terms of mean ± standard deviation values. Statistical significance was set at $P<0.05$.

RESULTS

Transient expression of transgenes in cell lines using VP-R8

We first transfected pCMV-GFPHA using VP-R8 or the PTR into three mouse cell lines (C8-D30, RAW264. 7, and J774.1). Two days after plasmid transfer, GFP expression was measured by fluorescence microscopy and flow cytometry. When pCMV-GFPHA was transfected into C8-D30 cells using the PTR, GFP fluorescence was detected by fluorescence microscopy and flow cytometry. However, GFP fluorescence levels or the frequency of GFP-positive cells were relatively low (Fig. 2a). On the other hand, no GFP fluorescence levels or very few GFP-positive cells were detected when plasmid was transfected into cells using VP-R8 (Fig. 2a). When pCMV-GFPHA was transfected into RAW264.7 cells using the PTR, GFP fluorescence was observed by fluorescence microscopy (Fig. 2b). Flow cytometry showed that approximately 31% of cells were GFP-positive (Fig. 2b). In contrast, when the

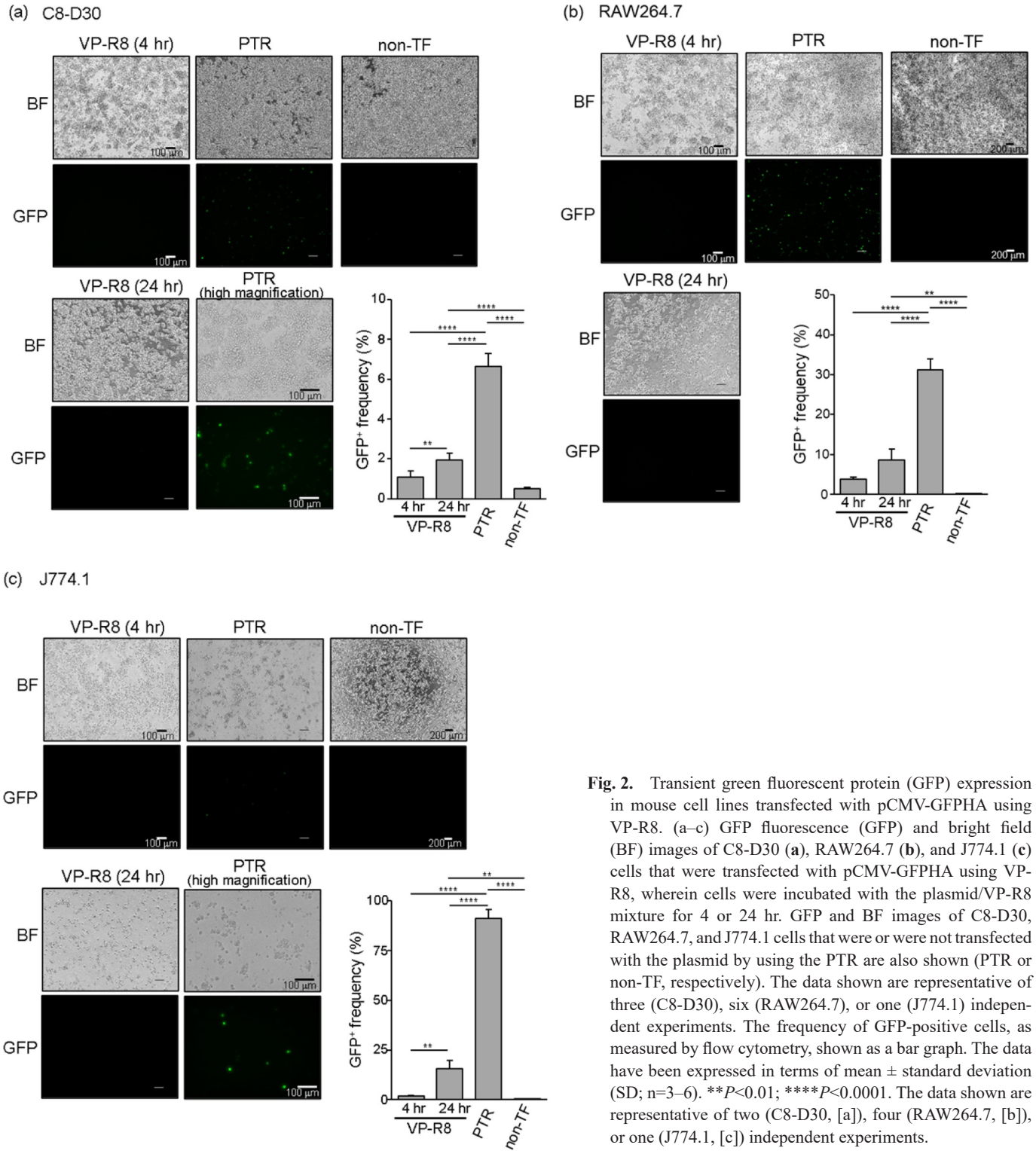

Fig. 2. Transient green fluorescent protein (GFP) expression in mouse cell lines transfected with pCMV-GFPHA using VP-R8. (a–c) GFP fluorescence (GFP) and bright field (BF) images of C8-D30 (**a**), RAW264.7 (**b**), and J774.1 (**c**) cells that were transfected with pCMV-GFPHA using VP-R8, wherein cells were incubated with the plasmid/VP-R8 mixture for 4 or 24 hr. GFP and BF images of C8-D30, RAW264.7, and J774.1 cells that were or were not transfected with the plasmid by using the PTR are also shown (PTR or non-TF, respectively). The data shown are representative of three (C8-D30), six (RAW264.7), or one (J774.1) independent experiments. The frequency of GFP-positive cells, as measured by flow cytometry, shown as a bar graph. The data have been expressed in terms of mean ± standard deviation (SD; n=3–6). ***P*<0.01; *****P*<0.0001. The data shown are representative of two (C8-D30, [a]), four (RAW264.7, [b]), or one (J774.1, [c]) independent experiments.

plasmid was transfected with VP-R8 into RAW264.7 cells, no GFP-fluorescent cells were observed by fluorescence microscopy regardless of the incubation time (4 or 24 hr, Fig. 2b) and very low levels of GFP fluorescence were detected by flow cytometry. The percentage of GFP-expressing cells was considerably lower than that obtained using the PTR (Fig. 2b). In J774.1 cells, a decrease in the number of survival cells and deformation of cells were observed when the plasmid was transfected using either VP-R8 or the PTR. In particular, incubation of cells with a mixture of plasmid and VP-R8 for 24 hr strongly induced these phenomena. When the plasmid was transfected using PTR, slight GFP fluorescence was observed by fluorescence microscopy; although flow

cytometry showed only a few propidium iodide (PI)-negative cells (viable cells), most of them expressed GFP (Fig. 2c). When the plasmid was transfected using VP-R8, particularly when VP-R8 was applied for 4 hr, no or almost no GFP fluorescence was detected by fluorescence microscopy or flow cytometry, respectively (Fig. 2c). When VP-R8 was applied for 24 hr, some of the small amount of PI-negative cells showed GFP expression; however, the percentage of GFP-positive cells was much lower than that obtained using the PTR (Fig. 2c). These results showed that transient transgene expression in J774.1 cells was more efficiently induced by PTR than by VP-R8; however, the findings suggested that both transfection agents could not enable transient transgene expression in J774.1 cells as efficiently as they could for the other cell lines used in this study because of their cytotoxic effect on J774.1 cells.

We next examined whether VP-R8 efficiently induced transient transgenes expression in human cell lines HEK293, HeLa, and HepG2. Among the cell lines used in this study, the highest GFP expression was detected in HEK293 cells using either VP-R8 or the PTR (Fig. 3a). In particular, fluorescence microscopy showed extremely strong GFP fluorescence when the plasmid was transfected into HEK293 cells by using the PTR, and flow cytometry showed that almost all cells expressed GFP (Fig. 3a). Strong GFP fluorescence was also observed by fluorescence microscopy even when plasmid transfer was performed using VP-R8 regardless of the incubation time (4 or 24 hr); however, the population of cells expressing strong GFP fluorescence was much lower than that obtained using the PTR (Fig. 3a). Flow cytometry showed that the percentage of GFP-expressing cells was also much lower than that obtained using the PTR (Fig. 3a). When pCMV-GFPHA was transfected into HeLa cells using the PTR, strong GFP fluorescence was observed by fluorescence microscopy (Fig. 3b). Flow cytometry showed that the frequency of GFP-positive cells was approximately 62% (Fig. 3b). In contrast, when the plasmid was transfected with VP-R8 into HeLa cells, regardless of the incubation time (4 or 24 hr), no GFP-fluorescent cells were observed by fluorescence microscopy (Fig. 3b) and very low levels of GFP fluorescence were detected by flow cytometry. The percentage of GFP-expressing cells was considerably lower than that obtained with the PTR (Fig. 3b). In HepG2 cells transfected with the plasmid using the PTR, relatively strong GFP fluorescence was detected by fluorescence microscopy (Fig. 3c). However, the transfection efficiency for these cells was lower than obtained when plasmids were introduced into HEK293 or HeLa cells by using the PTR. Flow cytometry also showed that the percentage of GFP-positive cells for HepG2 cells was lower than that for HEK293 and HeLa cells transfected with the plasmid using the PTR (Fig. 3c). When the plasmid was transfected into HepG2 cells using VP-R8, only a few cells expressed GFP-fluorescence regardless of the incubation time (Fig. 3c) and the percentage of GFP-expressing cells analyzed by flow cytometry was much lower than that using the PTR (Fig. 3c).

These results indicate that, compared to the PTR, VP-R8 was less efficient in inducing transient intracellular transgene expression in the mouse and human cell lines we assayed.

Stable transgene expression in cell lines using VP-R8

We next examined whether VP-R8 efficiently induced stable transgene expression in cell lines. At first, pCMV-GFPHA was introduced into three mouse (C8-D30, RAW264.7, and J774.1) cell lines using VP-R8 or the PTR, and drug selection was performed for 15 days, starting 3 days after plasmid transfer. After drug selection, cell colonization of drug-resistant cells was detected by crystal violet staining, and GFP expression in drug-resistant cells was measured by fluorescence microscopy and flow cytometry. In the absence of plasmid transfection, cell colony formation and cell monolayer formation were not observed in all cell lines after drug selection (Fig. 4). When the plasmid was transfected into C8-D30 cells using VP-R8, regardless of the incubation time (4 or 24 hr), many small cell colonies and monolayer cells were noted upon performing crystal violet staining (Fig. 4a). Fluorescence microscopy and flow cytometry showed that most of these cell colonies exhibited high GFP fluorescence levels (Fig. 4a). In contrast, we could not efficiently obtain drug-resistant cell colonies or even monolayer cells when the transfection was performed using PTR (Fig. 4a), in contrast to the results obtained when the transfection was performed using VP-R8. Furthermore, on performing fluorescence microscopy, GFP fluorescence was not observed in many cell colonies or monolayer cells obtained after drug selection of C8-D30 cells transfected using the PTR (Fig. 4a). Flow cytometry detected GFP fluorescence in a few drug-resistant cells; however, the GFP fluorescence level in these cells was significantly lower than that in drug-resistant cells obtained after drug selection of C8-D30 cells transfected using VP-R8 (Fig. 4a). In RAW264.7 cells, many small cell colonies and monolayer cells were observed upon staining with crystal violet when the plasmid was transfected into cells using VP-R8 regardless of the incubation time (4 or 24 hr, Fig. 4b). These drug-resistant cells had high GFP levels (Fig. 4b). Cell colony formation was also observed after drug selection of cells in which plasmids had been introduced using the PTR (Fig. 4b); however, fluorescent microscopy showed that most of the cell colonies did not have detectable GFP levels. Flow cytometry also showed that the GFP fluorescence level in drug-resistant cells was higher when VP-R8 was used (Fig. 4b). In J774.1 cells, when the plasmid was transfected into J774.1 cells using the PTR, cell colony formation, monolayer cells, and GFP fluorescence were not detected by fluorescent microscopy or flow cytometry (data not shown). Although a small number of viable cells were observed after drug selection of cells in which the plasmid was transfected using VP-R8, GFP fluorescence was not observed upon fluorescence microscopy or flow cytometry (data not shown).

We next transfected pCMV-GFPHA by using VP-R8 or the PTR into human cell lines HEK293, HeLa, and HepG2 and performed drug selection as described above. In the absence of plasmid transfection, cell colony formation and cell monolayer formation were not observed in all cell lines after drug selection. (Fig. 5). We detected many cell colonies and monolayer cells by crystal violet staining when the plasmid was transfected into HEK293 cells using VP-R8 regardless of the incubation time (4 or 24 hr, Fig. 5a). When transfection was performed using the PTR, the numbers of cell colonies and monolayer cells were similar to or lower than those obtained after drug selection of cells for which the plasmid had been transfected using VP-R8 (Fig. 5a).

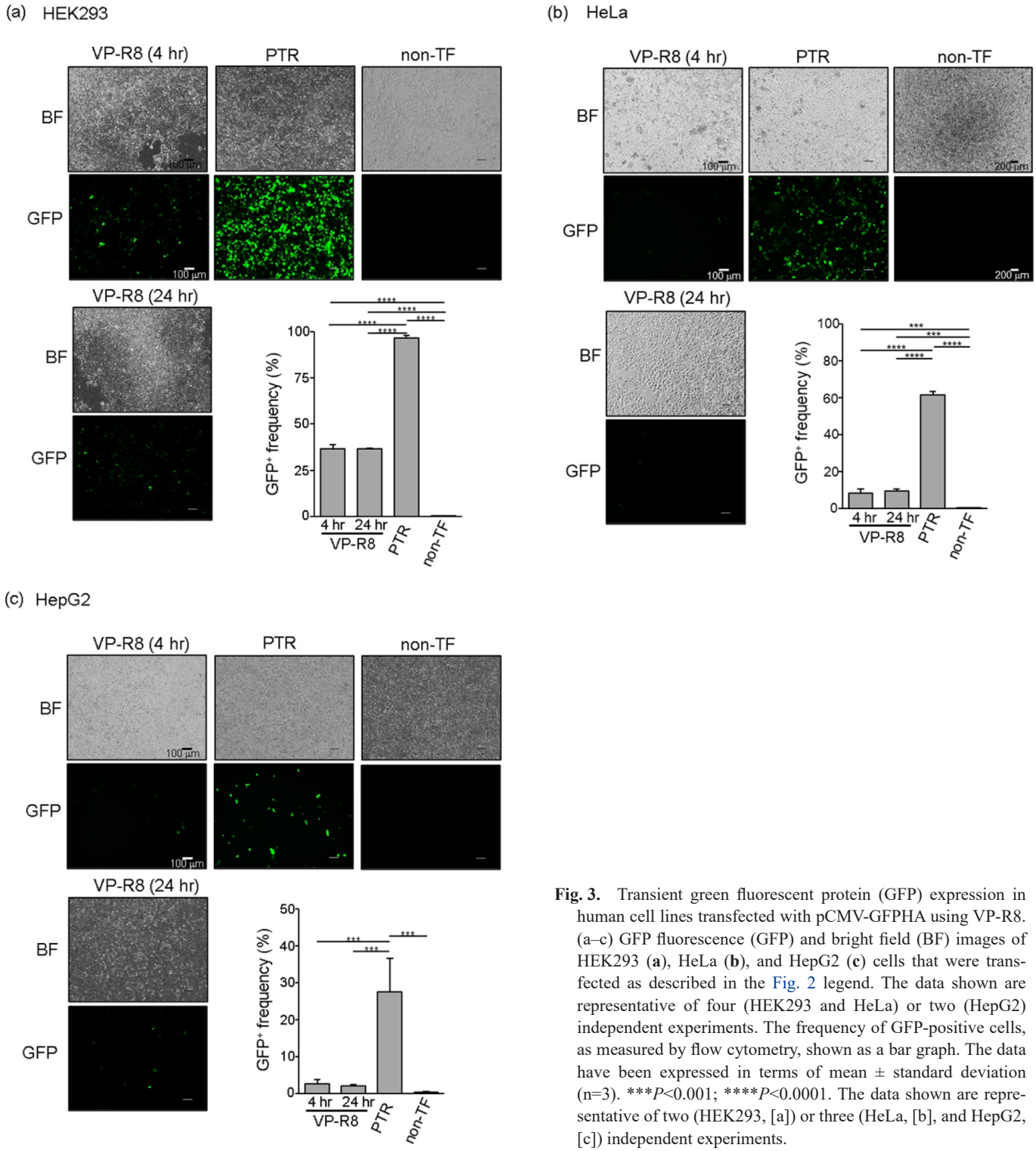

Fig. 3. Transient green fluorescent protein (GFP) expression in human cell lines transfected with pCMV-GFPHA using VP-R8. (a–c) GFP fluorescence (GFP) and bright field (BF) images of HEK293 (**a**), HeLa (**b**), and HepG2 (**c**) cells that were transfected as described in the Fig. 2 legend. The data shown are representative of four (HEK293 and HeLa) or two (HepG2) independent experiments. The frequency of GFP-positive cells, as measured by flow cytometry, shown as a bar graph. The data have been expressed in terms of mean ± standard deviation (n=3). ***P<0.001; ****P<0.0001. The data shown are representative of two (HEK293, [a]) or three (HeLa, [b], and HepG2, [c]) independent experiments.

Fluorescence microscopy analysis of drug-resistant cells showed that when the plasmid was introduced using VP-R8, some GFP fluorescence was observed; however, GFP fluorescence was seen in some cells in each colony, not the whole colony (Fig. 5a). A similar GFP-fluorescence pattern was observed in drug-resistant cells in which the plasmid was introduced using the PTR; the number of GFP-fluorescent cells seemed to be slightly less than that obtained with VP-R8 (Fig. 5a). The GFP fluorescence level did not significantly differ between drug-resistant cells transfected with the plasmid by using VP-R8 and those transfected with the PTR (Fig. 5a). In HeLa cells, many large cell colonies and monolayer cells were observed by crystal violet staining when the plasmid was transfected into cells using VP-R8 regardless of the incubation time (4 or 24 hr, Fig. 5b). Microscopy analysis showed

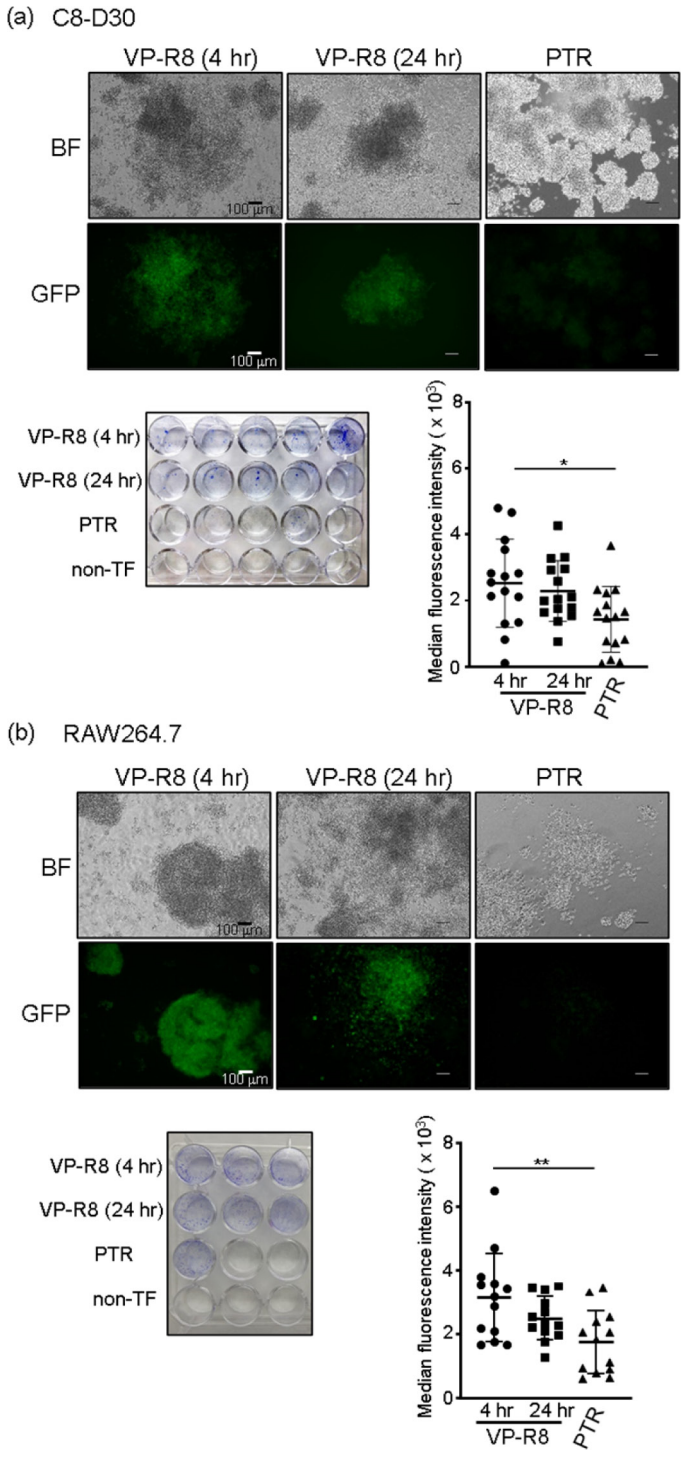

Fig. 4. Stable green fluorescent protein (GFP) expression in mouse cell lines transfected with pCMV-GFPHA using VP-R8. (a and b) Top: GFP fluorescence (GFP) and bright field (BF) images of drug-resistant C8-D30 (**a**) and RAW264.7 (**b**) cells after selection with G418. Cells were transfected with pCMV-GFPHA using VP-R8, wherein cells were incubated with the plasmid/VP-R8 mixture for 4 or 24 hr, followed by drug selection 3 days after the transfection. GFP and BF images of C8-D30 cells after drug selection are also shown; these cells had been transfected with the plasmid using the PTR. The data shown are representative of three (C8-D30) or four (RAW264.7) independent experiments. Bottom left: crystal violet staining of drug-resistant C8-D30 (a) and RAW264.7 (b) cells after drug selection. The data shown are representative of five independent experiments. Bottom right: The median GFP fluorescence intensity expressed in drug-resistant C8-D30 (a) and RAW264.7 (b) cells after selection, as measured by flow cytometry. The data have been expressed in terms of mean ± standard deviation (n≥13) of three (C8-D30) or four (RAW264.7) independent experiments. **P*<0.05; ***P*<0.01.

strong GFP fluorescence from these drug-resistant colonies (Fig. 5b). High GFP levels were also detected by flow cytometry (Fig. 5b). When transfection was performed using the PTR, the number of cell colonies after drug selection was lower than that after drug selection of cells in which the plasmid had been transfected using VP-R8 (Fig. 5b). The GFP fluorescence level did not significantly differ between drug-resistant cells transfected with the plasmid using VP-R8 and those transfected using the PTR (Fig. 5b); however, drug-resistant cells transfected with the plasmid using VP-R8 tended to show higher GFP fluorescence than those transfected using the PTR (Fig. 5b). When the plasmid was transfected into HepG2 cells using VP-R8, although drug-resistant cells were partially detached during the washing steps of crystal violet staining, we detected many large cell colonies (Fig. 5c). Fluorescence microscopy and flow cytometry showed that most of these cell colonies exhibited high GFP fluorescence levels (Fig. 5c). When transfection was performed using the PTR, the number of cell colonies after drug selection and the GFP expression level in these colonies were lower than those after drug selection of cells in which the plasmid had been transfected using VP-R8

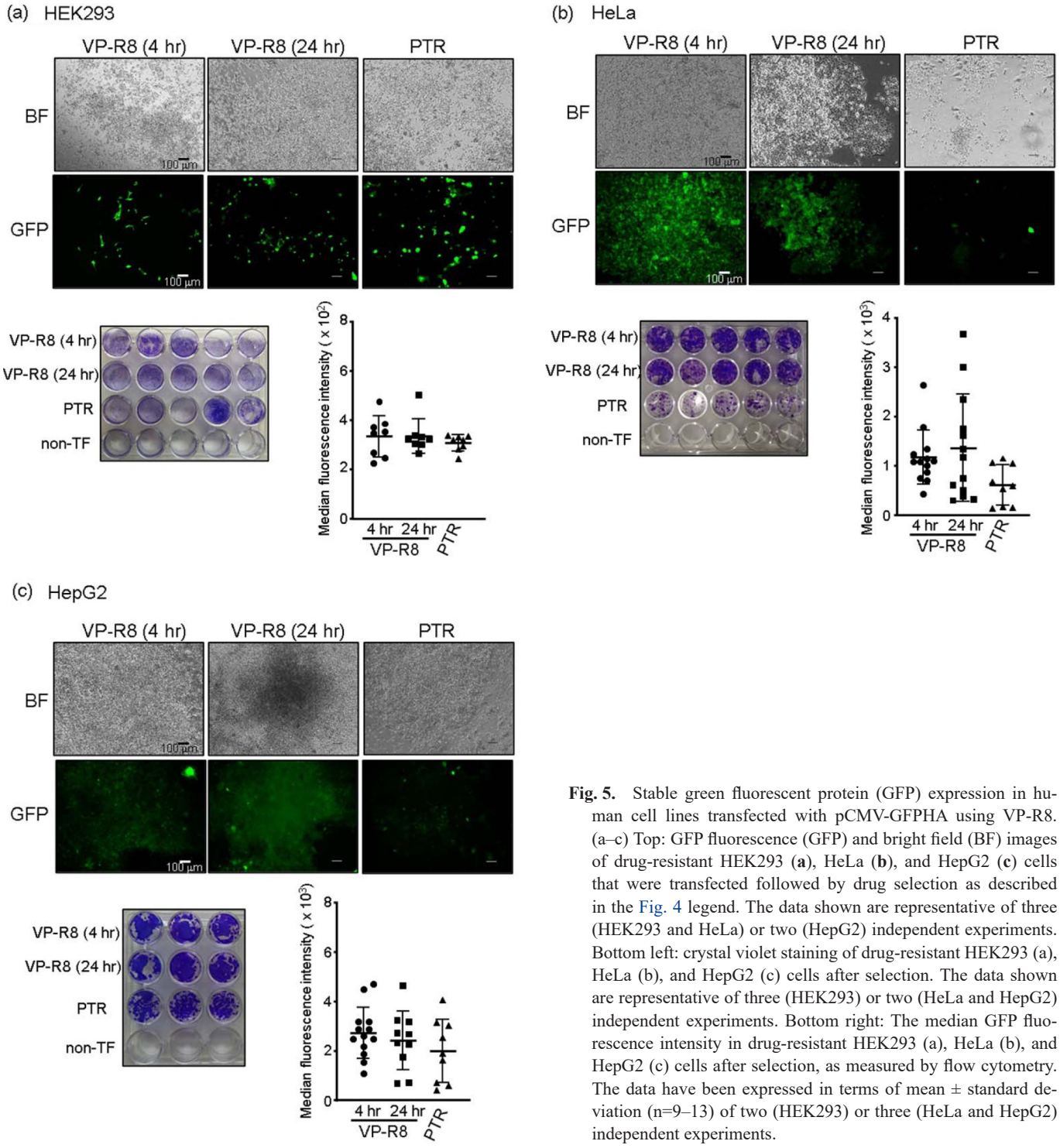

Fig. 5. Stable green fluorescent protein (GFP) expression in human cell lines transfected with pCMV-GFPHA using VP-R8. (a–c) Top: GFP fluorescence (GFP) and bright field (BF) images of drug-resistant HEK293 (**a**), HeLa (**b**), and HepG2 (**c**) cells that were transfected followed by drug selection as described in the Fig. 4 legend. The data shown are representative of three (HEK293 and HeLa) or two (HepG2) independent experiments. Bottom left: crystal violet staining of drug-resistant HEK293 (a), HeLa (b), and HepG2 (c) cells after selection. The data shown are representative of three (HEK293) or two (HeLa and HepG2) independent experiments. Bottom right: The median GFP fluorescence intensity in drug-resistant HEK293 (a), HeLa (b), and HepG2 (c) cells after selection, as measured by flow cytometry. The data have been expressed in terms of mean ± standard deviation (n=9–13) of two (HEK293) or three (HeLa and HepG2) independent experiments.

(Fig. 5c). Although the GFP fluorescence level did not significantly differ between drug-resistant cells transfected with plasmid via VP-R8 and those transfected using PTR (Fig. 5c), the use of VP-R8 seemed to enable stable transgene expression in HpG2 cells relatively more efficiently than the PTR.

These results showed that, compared to the PTR, VP-R8 was more efficient in enabling stable transgene expression in the mouse cell lines (except J774.1) and the human cell lines studied.

DISCUSSION

In this study, we evaluated the gene transfer and expression efficiency of VP-R8 under comparison with that of the PTR by introducing plasmids into several types of cells and analyzing transient and stable gene expression. We used three mouse (C8-D30, RAW264. 7, and J774.1) and three human (HEK293, HeLa, and HepG2) cell lines in this study. Mouse macrophage RAW264.7 and macrophage-like J774.1 cells are frequently used to identify molecular mechanisms involved in innate immunity, such as the production of inflammatory cytokines. Mouse astrocyte C8-D30 cells can secrete inflammatory chemokines upon LPS/IFNγ stimulation and are helpful for research in neuroinflammatory reactions in the central nervous system [12]. Human embryonic kidney HEK293 cells are most commonly used for expressing proteins exogenously and analyzing their interactions or effects in signal transduction. Human cervix epithelium HeLa cells and hepatoma HepG2 cells are commonly used to elucidate the molecular mechanisms of cancer and to identify target molecules for therapy. Gene transfer is essential for *in vitro* experiments using any of these cells to measure transcriptional activity based on the expression level from reporter genes, analyze intracellular molecular interactions, and evaluate cell survival, growth inhibition, and morphological changes.

We showed that gene transfections performed using VP-R8 induced stable protein expression from transgenes more efficiently than did those performed using the PTR. Similar results obtained in this study using VP-R8 have not been reported so far with other transfection compounds. Stable protein expression from transgenes via lipofection is known to be difficult in the case of nervous system cells, including astrocytes such as C8-D30 cells, which have been shown to be difficult to transfect by lipofection [13]. Polyethyleneimine (PEI), a cationic polymer, is a cost-effective and convenient transfection compound. We tried to introduce the GFP gene into a mouse astrocyte cell line using polyethyleneimine: PEI MAXTM (Polysciences, Warrington, PA, USA). However, it exerted considerable cytotoxicity against cells. In addition, the efficiency of GFP gene transfection was also quite low, and even if the GFP gene was successfully introduced into the cells, these cells subsequently died. If VP-R8 can help induce highly stable expression in other types of nervous system cells as well, it would likely prove to be useful in elucidating the functions of nervous system cells. We have not yet established the mechanism(s) by which VP-R8 induces efficient and stable gene expression. It is thought that VP-R8 triggers macropinocytosis or endocytosis-mediated gene transfer via recognition of cell-penetration peptide branches (D-octaarginine) in the polymer backbone on the membrane surface of cells exposed to transgene mixtures [20]. However, it is also possible that the complexes of VP-R8 plus plasmid DNA are taken up by cells. One of the factors responsible for the success or failure of induction of transient or stable expression could be the tightness of VP-R8 binding with the plasmid complex because gene expression requires dissociation of the plasmid from the complex. Even if the VP-R8 plus plasmid complex is located in the nucleus, the complex binding might be tight, leading to slow dissociation and absence of transient expression. A plasmid that successfully dissociates from VP-R8 might be more likely to integrate into the cell genome, resulting in high and stable transgene expression.

In the case of transient transgene expression, VP-R8 was less efficient in inducing transient intracellular transgene expression than the PTR. The escape from endosomes after endocytic uptake of plasmids mixed with VP-R8 into the cell might also contribute to the success or failure of induction of transient expression. If plasmids remain in the endosomes for a long time, the endosomes eventually fuse with the lysosomes, and plasmids inside presumably undergo degradation. We could not detect transient and stable transgene expression when the plasmid was introduced into J774.1 cells using VP-R8. This might be because plasmids mixed with VP-R8 taken up into J774.1 cells predominantly undergo degradation via the endosome-lysosome degradation pathway. We are currently conducting experiments to examine this hypothesis. In addition, to improve transient expression efficiency and further improve stable expression efficiency, more studies to elucidate the mechanisms for gene transfer and expression associated with VP-R8 transfection are required.

At present, viral vectors are widely used in gene therapy because they enable highly efficient gene introduction and expression. VP-R8 enabled excellent stable gene expression in this study; therefore, it is a potential new gene transfer agent, similar to retroviral or lentiviral vectors with highly stable expression efficiencies. However, several further studies are required to validate this. The experiments performed in this study only involved transgene expression analysis *in vitro*. For utilization in gene therapy, the transgene mixed with VP-R8 would have to be administered to the organism, followed by gene expression analysis performed *in vivo*. Assessment of side toxicity would be indispensable. Gene therapy includes the following: (a) *in vivo* methods for gene transfer involving direct administration of the gene to the patient's body; (b) *ex vivo* methods for collecting the target cells from the patient, such as CAR-T therapy, which is a type of cancer immunotherapy, performing gene transfer *in vitro* followed by injecting the established stable-expression cells into the patient [1, 11]. If stable-expression cells, which are established using VP-R8, are administered to the organism, and beneficial results are obtained, the application of VP-R8 to gene therapy in *ex vivo* methods wherein such highly stable expression efficiency is required will also be possible.

CONFLICT OF INTEREST. None of the authors have any conflict of interests.

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