*Original Article*

DOI: 10.5582/ddt.2011.v5.6.286

# A new cell-based reporter system for sensitive screening of nuclear export inhibitors

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**ABSTRACT: Nucleocytoplasmic transport of proteins across the nuclear pore complex (NPC), mediated by the nuclear localization signal (NLS) and the nuclear export signal (NES), is a vital homeostatic process in eukaryotic cells and also in mitogen-activated protein kinase (MEK) signaling molecule in tumor cell proliferation. Some viruses, including the influenza virus and HIV-1, also employ this nuclear export mechanism during their life cycle. Hence, drugs that control nucleocytoplasmic transport of proteins are putative candidate antivirals or anti-cancer agents. Thus, we previously developed a GFP/NES-MDCK reporter cell system for screening novel nuclear export inhibitors. NES signal-conjugated GFP accumulates in the nucleus in the presence of the nuclear export inhibitor leptomycin B (LMB). In this study, a stable GFP/NLS/NES fusion protein-expressing cell line was established, and its potential as a reporter was evaluated. The GFP/NLS/NES-MDCK cell line demonstrates improved nuclear accumulation of GFP in a time-course treatment with LMB. In addition, the dose-response data demonstrated superior sensitivity of GFP/NLS/NES-MDCK over GFP/NES-MDCK cells. As low as 0.01 ng/mL LMB is sufficient to cause accumulation of the GFP fusion protein in the nucleus in GFP/NLS/NES-MDCK cells, while at least 1 ng/mL of LMB is needed for the accumulation of GFP fusion protein in the nucleus of GFP/NES-MDCK cells. These results indicate that the newly established GFP/NLS/NES-MDCK cell line is a potentially powerful tool to screen for novel nuclear export inhibitors.**

***Keywords:*** Reporter system, nuclear export, antiviral drugs

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## 1. Introduction

In eukaryotic cells, proteins and other macromolecules constantly move in and out of the nucleus. The nuclear envelope separates the translational and metabolic machinery of the cytoplasm from the genetic material and transcriptional machinery of the nucleus. The nuclear pore complex (NPC), a 125-MDa macromolecular complex of polypeptides collectively referred to as nucleoporins, with a central diameter of about 10 nm (*1*), is a conduit for the bi-directional movement of molecules between the nucleus and the cytoplasm. Some of these nucleoporins contain arrays of hydrophobic peptide repeats containing phenylalanine and glycine residues, which form the permeability barrier of the NPC. Small molecules (for instance, ions and proteins) up to 50 kDa in size, or less than 10 nm in diameter, can freely diffuse through the NPC without consuming energy. Nucleocytoplasmic shuttling of macromolecules larger than 50 kDa is an active, energy-dependent, and essential process that is mediated by selective sequence-specific motifs, the nuclear localization signal (NLS) and the nuclear export signal (NES) (*2*). Molecules that have to enter the nucleus are carried in by nuclear import receptors called importins (karyopherins) (*3*). Importin α proteins recognize the NLS on cargo proteins (*4*). Once the cargo is bound by importin α, the complex is recognized and bound by importin β that subsequently binds to the fibrils of the NPC and is responsible for the actual translocation. Besides cargo and transport receptors, the signaling protein Ran, which hydrolyzes GTP, is responsible for regulating the interaction of transport receptor and cargo (*5*), and Ran-GDP/Ran-GTP concentration gradients across the nuclear envelope drive nuclear import and export (*6*). Once inside the nucleus, binding of Ran-GTP to importin β elicits a conformational change resulting in cargo release in the nucleus (*7*). By contrast, proteins that transfer cargo out of the nucleus are called exportins. Exportin1, also known as chromosome region maintenance protein 1 (CRM1), mediates the export of the numerous proteins bearing a NES from the nucleus (*8,9*). Ran-GTP stimulates binding of the CRM1 to NES cargo in

the nucleus, and the complex is then exported to the cytoplasm where Ran-GTP is hydrolyzed to Ran-GDP. Thereafter, Ran undergoes a conformational change, causing the complex to dissociate and the exportins and Ran-GDP to shuttle back to the nucleus leaving the cargo behind in the cytoplasm. CRM1 is the normal exportin for proteins, RNAs, and ribonucleoproteins complexes (*10*). Leptomycin B (LMB), an antibiotic with antifungal and antitumor activity, has recently become an important tool for elucidating nuclear localization and trafficking in eukaryotic cells. The export substrates of CRM1 possess a leucine-rich NES. A protein containing both NES and NLS is supposed to shuttle between nucleus and cytoplasm, but instead accumulates preferentially in the nucleus of cells treated with LMB (*11*). LMB specifically and irreversibly inhibits CRM1 by covalently binding to a cysteine residue in a central domain of CRM1 (*12*), confirming that CRM1 is a crucial factor for nuclear export of proteins in eukaryotes. The NES has also been identified in viral proteins such as type A influenza virus NS2 (*13*), HIV-1 Rev (*14*), and in host factors like cAMP-dependent protein kinase A inhibitor (PKI-α) (*15*), heat shock cognate protein 70 (Hsc70) (*16*), and MAP kinase kinase (MEK) (*17*).

A virus is a unique pathogen that utilizes host cell environment and proteins for its propagation. Therefore, nuclear export proteins can serve as targets for new and potent antiviral drugs since some viruses, including HIV-1 and influenza virus utilize the nuclear export machinery of the host cell. Development of antiviral drugs is one of the best ways to treat these infectious diseases. However, newly developed antiviral drugs are likely to be ineffective on some viruses in short time, especially RNA viruses, which develop resistance through mutation. For instance, a globally high prevalence of more than 90% was reported for oseltamivir-resistant influenza A (H1N1) in 2009 compared to a lower rate in 2008 (*18*). Similarly, HIV can also develop drug resistance, and therefore, a multi-drug combination therapy protocol termed HAART (highly active anti-retroviral therapy) is now used to treat HIV patients (*19*).

We have focused on identifying novel nuclear export inhibitors that confer selective inhibition of viral propagation with minimal toxicity to host cells. To achieve this, a MDCK cell-based reporter system based on an enhanced GFP fused with a NES domain was established (*20*). GFP-fused protein accumulates in the nucleus in the presence of LMB in this cell line. Using this cell line, we previously reported 2 possible nuclear export inhibitors, namely, ACA and valtrate (*21*). Nonetheless, the cell line was not sufficiently sensitive to screen for nuclear export inhibition activity, possibly because of the absence of NLS in its construct. In this report, we describe a newly established stable cell line that expresses a GFP/NLS/NES fusion protein, and thus, is more sensitive to nuclear export inhibitors as evidenced by the significant nuclear GFP fluorescence.

## 2. Materials and Methods

### 2.1. *Chemicals*

Mardin-Darby canine kidney cells (MDCK) were grown in minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (unless otherwise stated) at 37°C in a 5% $CO_2$ incubator. Geneticin (G418) was dissolved in water at 10 mg/mL and stored at −20°C. LMB was purchased from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA, USA) and was dissolved in 100% ethanol at a concentration of 10 µg/mL and stored at −20°C. Paraformaldehyde (Wako, Osaka, Japan) was prepared as 4% (w/v) in PBS and stored at 4°C. Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA) was stored as a 10 mM stock solution in water at −20°C.

### 2.2. *Construction of pEGFP-NLS-NES plasmid*

To generate a plasmid harboring GFP/NLS/NES, the previously established pEGFP-NES (*22*) was modified to add the NLS coding region from the SV40 large T antigen (Figure 1). First, 2 oligonucleotides, 5'-GATCTCCAAAAAAGAAGAGAAAGGTACA-3' and 5'-AGCTTGTACCTTTCTCTTCTTTTTTGGA-3' encoding the NLS sequence (PKKKRKV), were annealed by incubation at 90°C for 15 min, followed by gradual cooling at room temperature. The resulting oligonucleotide segment was subsequently ligated between the GFP and NES coding sequence of pEGFP-NES by using *Hin*dIII and *Bgl*II restriction sites with a ligation mix (Nippon Gene, Tokyo, Japan). The ligation product was transformed into ultra-competent *Escherichia coli* Mach1 cells and grown on a Luria-Bertani agar plate containing 30 µg/mL kanamycin. Plasmids were isolated from a single cultured positive colony by the alkaline lysis method. Verification of NLS sequence in the plasmid was confirmed with an Applied Bio 3100-Avant Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). To increase the amount of pEGFP-NLS-NES plasmid, a 100-mL culture suspension of *E. coli* cells harboring the plasmid was grown, and the plasmid was purified by Nucleobond® Xtra Midi-prep kit (Machery-Nagel, Duren, Germany).

### 2.3. *Establishment of GFP/NLS/NES fusion protein expressing cells*

MDCK cells were seeded into 12-well plate at a density of $1 \times 10^5$ cells/well in MEM medium supplemented with 10% FBS without antibiotics and incubated for 24 h at 37°C in a 5% $CO_2$ incubator. After 1 day, plasmid DNA was introduced into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Briefly, 1.6 µg of

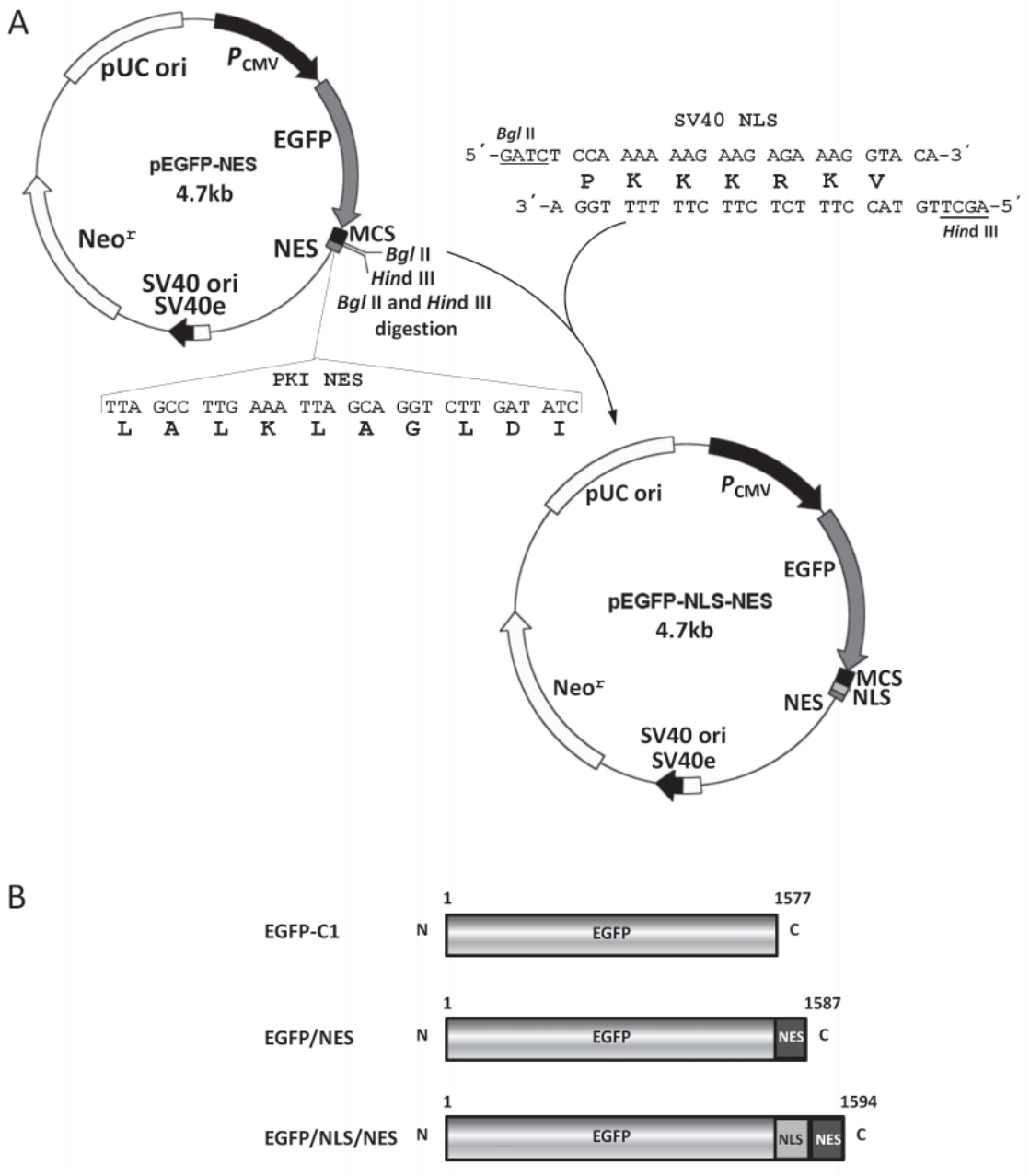


**Figure 1. Schematic depiction of GFP-NLS-NES plasmid construction. (A)** An insert DNA fragment encoding a PKKKRKV polypeptide from the SV 40 large T antigen was cloned into a *Hin*dIII and *Bgl*II digested pEGFP-NES plasmid. **(B)** Schematic representations of wild-type GFP-C1 and its derivatives.

plasmid DNA was mixed with 8 μL of Lipofectamine 2000 and incubated for 20 min at room temperature to allow DNA-Lipofectamine 2000 complexes to form. The transfection mixture was added to each well containing cells and medium, mixed gently by rocking the plate and incubated for 5 h after which the growth medium was replaced. Twenty-four hours after transfection, the cells were passaged onto a 90-mm dish and incubated at 37°C in a 5% $CO_2$ incubator. Cells transfected with pEGFP-NLS-NES were selected for neomycin resistance by culturing in a selection medium containing 600 μg/mL of G418 for 2 weeks. The G418-resistant clones (GFP/NLS/NES-MDCK) were then collected and expanded.

2.4. *Characterization of established cell line*

GFP-MDCK, GFP/NES-MDCK, and GFP/NLS/NES-MDCK cells were separately cultured on 15-mm glass coverslips in a 24-well plate at a density of $6 \times 10^4$ cells/well in 500 μL of MEM medium supplemented with 5% FBS and 1% penicillin/streptomycin. After 24 h, the cells were fixed for 10 min with 4% (w/v) paraformaldehyde in 1× PBS at room temperature, followed by staining with 10 μM Hoechst 33342 for 10 min at room temperature. The coverslips were then inverted onto microscope slides, and the morphology and the GFP fluorescence distribution pattern of each cell line was observed using an Axiophot fluorescence microscope (Carl Zeiss, Jena, Germany).

2.5. *Time-course nuclear translocation assay*

GFP-MDCK, GFP/NES-MDCK, and GFP/NLS/NES-MDCK cells were independently seeded on glass coverslips in a 24-well plate at a concentration of $6 \times 10^4$ cells/well in 500 µL of MEM medium containing 5% FBS and 1% penicillin/streptomycin and incubated at 37°C in 5% $CO_2$ incubator. After 24 h, each cell line was treated with 10 ng/mL LMB and further incubated for 1 h and 3 h. Cells at representative timepoints were washed twice with 1× PBS and fixed with 4% (w/v) paraformaldehyde in 1× PBS for 10 min, and subsequently stained with 10 µM Hoechst 33342 for 10 min. The coverslips were then mounted onto glass slides, and the nucleocytoplasmic distribution of GFP-fusion protein was observed under an Axiophot microscope (Carl Zeiss).

2.6. *Dose-course nuclear translocation assay*

The 3 different cell lines, namely, GFP-MDCK, GFP/NES-MDCK, and GFP/NLS/NES-MDCK were separately cultured for 24 h on 15-mm glass coverslips in a 24-well plate at a concentration of $6 \times 10^4$ cells/well in 500 µL of MEM medium containing 5% FBS and 1% penicillin/streptomycin and incubated at 37°C in a 5% $CO_2$ incubator. After 24 h, each cell line was subjected to a varying concentration of LMB (10, 1, 0.1, 0.03, and 0.01 ng/mL) for 3 h. After removing the LMB-containing MEM, cells were rinsed twice with 1× PBS, fixed with 4% paraformaldehyde in 1× PBS for 10 min, and stained with 10 µM Hoechst 33342 for 10 min. The coverslips were then mounted onto glass slides, and the nucleocytoplasmic distribution of GFP-fusion protein was observed under Axiophot microscope (Carl Zeiss).

## 3. Results

3.1. *Establishment of a cell line expressing a GFP/NLS/NES fusion protein*

To establish a cell line stably expressing the GFP/NLS/NES fusion protein, MDCK cells were transfected with pEGFP-NLS-NES (Figure 1), and colonies were selected in the presence of geneticin (G418) sulfate. One clone out of the 3 individual clones isolated demonstrated relatively high fluorescence and was preferentially selected and designated GFP/NLS/NES-MDCK.

3.2. *General properties of the established cell line*

To evaluate the general characteristics of the cell lines established, GFP-MDCK, GFP/NES-MDCK (GES5) (*20*), and GFP/NLS/NES-MDCK cell lines were cultured for 24 h, and the nucleocytoplasmic distribution of the GFP-fusion protein was observed under a fluorescence microscope (Figure 2). GFP fluorescence is detected in both the nucleus and the cytoplasm of GFP-MDCK cells, although the fluorescence is slightly higher in the nucleus. GFP/NES-MDCK cells demonstrate significantly higher GFP fluorescence accumulation in the cytoplasm, while the nucleus appears dark due to the lack of GFP fluorescence. By contrast, GFP fluorescence in GFP/NLS/NES-MDCK cells is distributed in both the nucleus and cytoplasm, but the fluorescence intensity is slightly higher in the nucleus than in the cytoplasm. This GFP fluorescence distribution in both the nucleus and the cytoplasm indicates that the NLS in the GFP/NLS/NES-MDCK is functional in the cell. All GFP/NLS/NES-MDCK cells had extensive bright green fluorescence that facilitates microscopic observation. Although these cell lines were fundamentally MDCK, the observed size of the nucleus in GFP/NLS/NES-MDCK cells was slightly smaller compared to other counterparts.

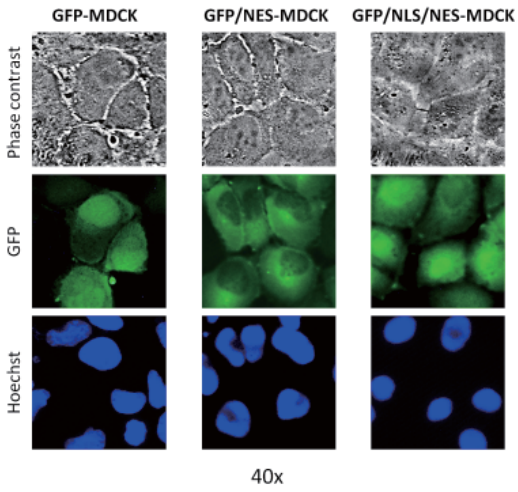


**Figure 2. Distribution of sub-cellular fluorescence in established cell lines.** Three different cell lines, namely, GFP-MDCK, GFP/NES-MDCK, and GFP/NLS/NES-MDCK were independently cultured, fixed with 4% paraformaldehyde and successively stained with 10 µM Hoechst 33342. The nucleocytoplasmic distribution of GFP fusion protein was observed using a fluorescence microscope (40×).

3.3. *Effect of LMB on nuclear accumulation of GFP in stable cell lines*

To compare the distribution and intensity of GFP fluorescence in the presence of nuclear export inhibitor, the 3 cell lines were treated with 10 ng/mL of LMB treatment for specified length of time and observed under fluorescence microscope (Figure 3). As previously reported, the GFP fusion protein is primarily detected in the nucleus of the GFP/NES-MDCK cell line, whereas the GFP fluorescence in GFP-MDCK did not change after 1 or 3 h of LMB treatment (*20*). By contrast, at both 1 h and 3 h timepoints, fluorescence accumulation was significantly higher in the nucleus of GFP/NLS/NES-MDCK cells when compared to the fluorescence of GFP/NES-MDCK cells. These results demonstrate a more prominent response to LMB treatment in the newly established GFP/NLS/NES-MDCK cells than in the GFP/NES-MDCK cells.

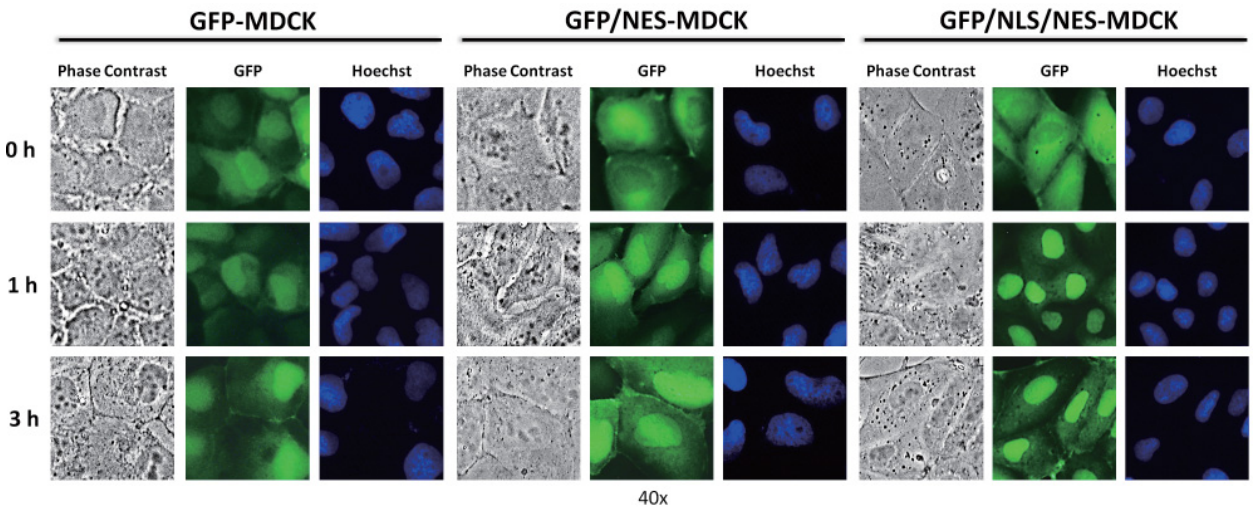


**Figure 3. Time-course of nuclear translocation.** GFP-MDCK, GFP/NES-MDCK, and GFP/NLS/NES-MDCK cells were independently cultured for 24 h, and each cell line was treated with 10 ng/mL LMB for 1 and 3 h. Cells from representative timepoints were then fixed with 4% paraformaldehyde and stained with 10 μM Hoechst 33342 and observed under a fluorescence microscope (40×).

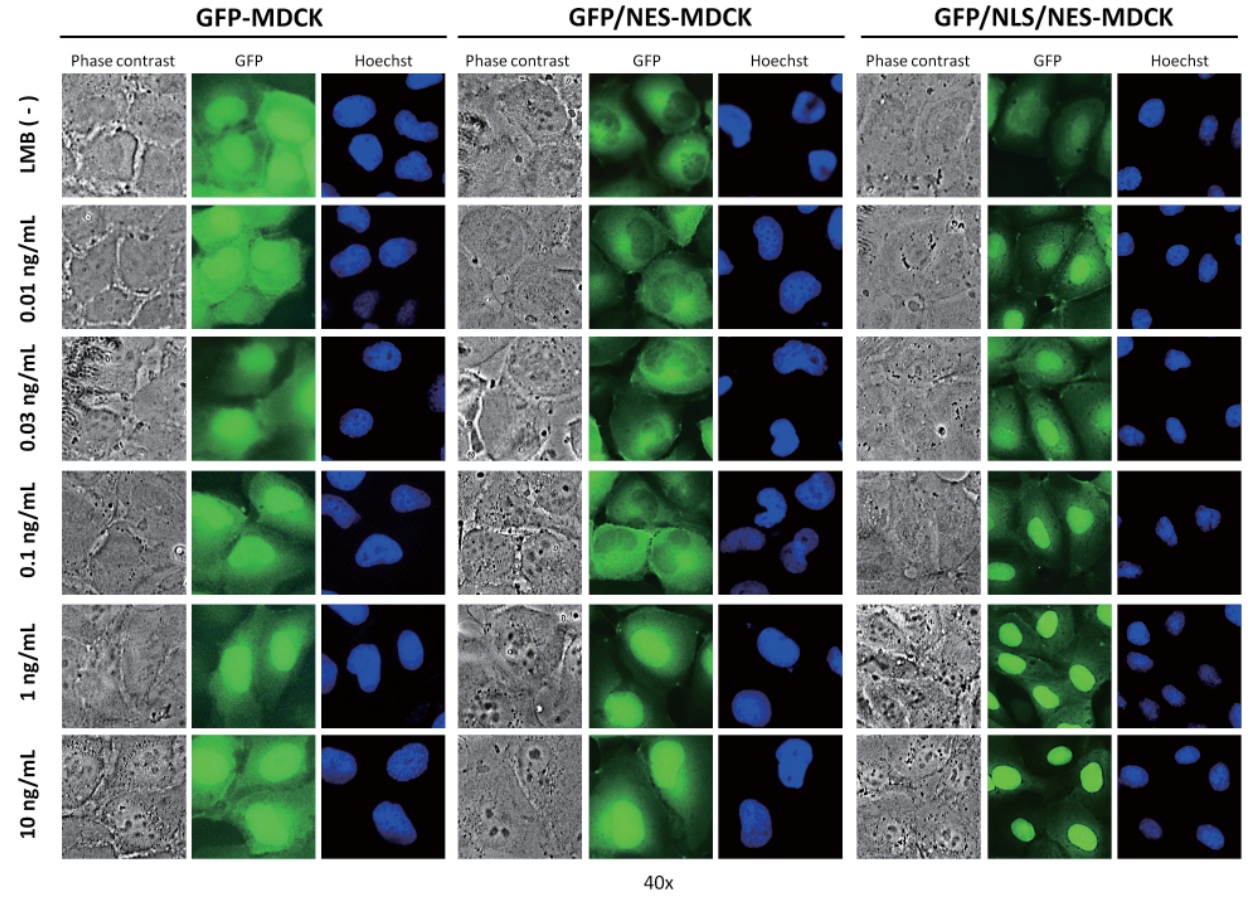


**Figure 4. Titration of LMB.** GFP-MDCK, GFP/NES-MDCK, and GFP/NLS/NES-MDCK cells were cultured independently for 24 h before each cell line was treated with varying concentrations of LMB for 3 h. The cells were fixed with 4% paraformaldehyde, stained with 10 μM Hoechst 33342, and the nucleocytoplasmic fluorescence distribution and intensity was observed under a fluorescence microscope (40×).

3.4. *Sensitivity of the GFP/NLS/NES-MDCK cells to the nuclear export inhibitor*

To demonstrate the sensitivity of GFP-MDCK, GFP/NES-MDCK, and GFP/NLS/NES-MDCK cells to different concentrations of LMB, these cells were treated with varying concentrations of LMB. As shown in Figure 4, a steady dose-dependent increase in nuclear fluorescence accumulation is observed in both GFP/NES-MDCK and GFP/NLS/NES-MDCK cells.

Furthermore, at 3-h post-LMB treatment, significant nuclear localization of GFP fluorescence is observed in GFP/NLS/NES-MDCK at an LMB dose as low as 0.01 ng/mL. By contrast, GFP/NES-MDCK does not appear to respond to LMB doses below 1 ng/mL. These results suggest that the newly established GFP/NLS/NES-MDCK cell line, but not the GFP/NES-MDCK cell line, is hypersensitive to LMB.

## 4. Discussion

The aim of this study was to establish a cell line with improved sensitivity of GFP distribution to nuclear export inhibitors as a tool for preliminary screening of nuclear export inhibitors as antiviral lead compounds. To achieve this goal, we developed a cell line stably expressing a GFP/NLS/NES fusion protein to monitor the localization of GFP fluorescence in the nucleus in the presence of putative nuclear export inhibitors. In general, the GFP/NLS/NES fusion protein is distributed in both the nucleus and the cytoplasm of GFP/NLS/NES-MDCK cells; however, the fluorescence intensity in the nucleus was slightly higher than that in the cytoplasm of these cells (Figure 2). GFP fluorescence is observed in both the nucleus and cytoplasm of GFP/NLS/NES-MDCK cells because of the action of NLS and NES signals that enable the GFP-fusion protein to be transported to and from the nucleus. Nuclear fluorescence intensity is slightly higher than the cytoplasmic fluorescence intensity of GFP/NLS/NES-MDCK cells possibly because the NES of PKI is slightly weaker than the NLS of SV40 large T antigen (*15*). By contrast, GFP/NES-MDCK cells demonstrate substantially higher GFP fluorescence in the cytoplasm, while the nucleus is devoid of green fluorescence.

The LMB time-course response data (Figure 3) demonstrate that the GFP-fused protein localizes mainly in the nucleus of GFP/NES-MDCK cells, as previously reported (*20*). Despite the addition of LMB, no variation in sub-cellular GFP fluorescence distribution was observed in GFP-MDCK cells. By contrast, a GFP with both NLS and NES – expected to shuttle between the nucleus and cytoplasm – extensively accumulates in the nucleus of the LMB-treated cells. At 1-h and 3-h timepoints, GFP fluorescence was significantly more conspicuous in the nucleus of GFP/NLS/NES-MDCK cells than in the nucleus of GFP/NES-MDCK cells. This remarkable nuclear accumulation response of the newly established GFP/NLS/NES-MDCK cell line in the presence of nuclear export inhibitor is crucial in the efficient detection of novel nuclear export inhibitors.

Dose-response assays demonstrate considerable nuclear fluorescence accumulation in GFP/NLS/NES-MDCK cells at LMB concentrations as low as 0.01 ng/mL, whereas previously established GFP/NES-MDCK cells (*20*) do not exhibit nuclear accumulation of GFP-fusion protein at less than 1 ng/mL of LMB. Thus, there is almost a hundred-fold increase in LMB sensitivity in the GFP/NLS/NES-MDCK cell line when compared to the GFP/NES-MDCK cell line. Moreover, the effective working concentration of 0.01 ng/mL LMB in GFP/NLS/NES-MDCK cells is significantly lower than the working concentrations (5-10 ng/mL) employed in other nuclear export inhibition studies (*22-24*). This improved sensitivity underscores the utility of this new cell line as a tool for screening potentially novel nuclear export inhibitors even at very low concentrations. The newly established cell line is also safer because it eliminates the need for the use of live influenza or HIV-1 viruses when screening for antivirals with nuclear export inhibition activity. Furthermore, recent studies have demonstrated that MEK contains NES (*17*) and it shuttles between the cytoplasm and nucleus (*25*) and thus inhibitors of nuclear transport may also present novel opportunity for the development of anti-cancer drugs. This cell line is easily maintained with standard media and serum, and hence, it is fairly cost-effective for high-throughput screening. We are currently using this cell line to screen for novel nuclear export inhibitors.

## Acknowledgments

This work was supported in part by the Global Center of Excellence Program of Nagasaki University and Grant-in-Aid for Young Scientists (H.K) of Ministry of Education, Culture, Sports, Science and Technology.

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***(Received November 16, 2011; Accepted November 28, 2011)***