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Mouse Embryonic Stem Cell Uptakes of Buforin 2 and pEP-1 Conjugated with EGFP

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생쥐 배아 줄기세포의 Buforin 2 및 pEP-1에 결합된 EGFP의 세포 내 수송

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ABSTRACT : Differentiation of cells can be induced through modulation of endogenous regulators using exogenous factors. Useful transfection systems to transport a specific exogenous regulator into cell have been tried but still there are many obstacles to overcome. In this study, we examined the transfection efficiency of cell permeable peptides (CPPs) in mouse embryonic stem cell under the various conditions. To identify the CPP-mediated translocation of a protein, we employed recombinant CPP-enhanced green fluorescent protein (EGFP). Viability of R1 cells was different between experimental groups depending on the kind of CPP and the concentration of CPP-EGFP. Translocation of CPP-EGFPs into the R1 cells was not detected until 30 min after CPP-EGFPs treatment in all groups. After 1 hr, translocation of pEP-1-EGFP-N was detected, but it could not be detected in the other group. Transfection of pEP-1EGFP-N was independent on its concentration. The time course did not show saturation even after 24 hr in pEP-1-EGFP-N. These results showed that the permeability depended on the kind of CPP and the location of His-tag in the case of examined CPPs, and did not need biological energy. On summary, the efficiency of transfection of CPP-EGFP depends on the CPP sequences but the culture time is not a key factor in transfection for the mouse embryonic stem cell. For the future studies to improve the efficiency of translocation of protein into embryonic stem cells, it is needed to develop modified CPP or mediator. The studies would be very useful to induce the differentiation of embryonic stem cells.

Key words : Cell permeable peptide, Mouse embryonic stem cell.

요 약 : 배아줄기세포는 외부 기인 특정 요소를 이용한 세포 조절물질 활성을 통하여 원하는 세포형태로 분화될 수 있다. 배아줄기세포의 이용성세포침투성단백질(CPP)은 몇 개의 아미노산으로 구성된 작은 펩타이드로 유용한 물질 수송계 중의 하나로 인식되고 있다. 본 연구에서는 몇 종류의 CPP를 이용하여 특정 단백질을 생쥐배아줄기세포(R1)내로 트렌스펙션하는데 있어 그 정도를 결정할 수 있는 요소들의 영향을 분석하였다. CPP인 Buforin II, pEP-1을 강도를 강화한 녹색형광단백질(EGFP)에 부착되도록 하여 살아있는 세포에서 볼 수 있도록 하였다. 다양한 시간대별로 그리고 다양한 농도로 재조합 CPP-EGPF를 생쥐 배아줄기세포에 처리하였으며, 대조군으로 사용한 EGFP는 CPP를 갖고 있지 않았다. 재조합 CPP-EGFP들이 R1 배아줄기세포에 미치는 세포독성은 CPP 종류와 재조합 CPP-EGFP 농도에 의존적이었다. CPP-EGFP가 배아줄기세포의 세포질이나 핵질로 들어간 증거는 처리 후 30분 이내에서는 찾을 수 없었다. 1시간 후, pEP-1-EGFP-N을 처리한 일부 세포에서만 형광단백질이 세포안으로 들어간 것을 발견할 수 있었다. 처리를 24시간까지 하였으나 흥미롭게도 세포안으로 들어가는 정도와는 비례관계가 없었다. 또한, 배아줄기세포의 막을 통과할 수 있는 CPP의 경우, 처리 농도는 세포막을 침투하여 세포내로 이동하는 것과 비례적 관계는 없으나, 매우 높은 농도에서 약간 증가하는 경향을 보였다. 요약하면, CPP-EGFP의 트렌스펙션 효과는 전적으로 CPP에 의존적이며, CPP에 따라서는

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His tag의 위치 또한 트렌스펙션에 영향을 줄 수 있으며, 처리시간은 CPP를 이용하여 배아줄기세포내로 단백질을 수송하는데 있어 중요 변수가 아니다. 또한, CPP-EGFP의 농도는 배아줄기세포막을 가로질러 수송되는데 큰 영향을 주지 않았다. 앞으로의 연구에서, 이들 CPP의 변형 또는 매개자 개발을 통한 배아줄기세포에서 좋은 단백질성 매개 수송자를 만드는 것이 필요하며 이를 이용한 분화 유도는 매우 유용할 것으로 사료된다.

INTRODUCTION

In recent years, embryonic stem cells (ES cells) are widely used as a model system to explore the mammalian embryonic development and applied to the cell therapy. Stem cells possess the capability of transforming into many cell types and are good candidates for cell-based therapies in regenerative medicine for diseases (Mathur & Martin, 2004; Webber & Minger., 2004). So far, stem cell biology has been focused on to identify novel pathways such as those that maintaining the pluripotency, inducing specific cell types. However it is not easy to use as medicine or to study about the differential induction to a specific cell type.

To get a specific cell type, it is essential to know the interactions of multiple genes and the associated factors which are involved in differentiation and de-differentiation of ES cells. To meet these requirements, many experimental conditions and gene delivery systems like high performance microinjection (Matsuoka *et al*., 2007), various vector system (Gropp & Reubinoff, 2006), nucleofection (Zaragosi *et al*., 2007), lipofectants and calcium phosphate were used. However, stem cells are resistant to most common transfection methods.

The developments of effective strategies for genetic modification of ES cells have been progressed and have been resulting in improvement the differentiation of the ES cells to a specific cell type. One of the useful methods to modulate the gene expression is transgene delivery. Transgene delivery is a powerful strategy for induction of specific cell types from ES cells since several transcription factors have been demonstrated to regulate stem cell differentiation (Ishizaka *et al*., 2002; Kim & Hebok, 2001; Xian & Gottlieb, 2001). Among the existing approaches for delivery a gene, viral systems is suspected of their potential life-threatening effects of immunogenicity and carcinogenicity whereas non-viral ones possess significant limitation in terms of efficacy as previously mentioned. Besides, genetic modulation has also problem because it should be change the genetic structure of the mammals. In addition, an essential prerequisite for application of ES cells in regenerative medicine is the development of efficient protocols to direct stem cell differentiation into well-defined lineage (Heng *et al*., 2004). So we need various ways to modulate the gene expression, nucleic acids, proteins, chemicals. However, protein delivery or chemical delivery has also limits. Development of a safe as well as an efficient carrier is, therefore, an urgent requirement for effective implementation of stem cells in regenerative medicine or gene therapy.

One of the best candidates is the cell permeable peptides (CPP; protein transduction domains, membrane translocating sequences); it is known as small peptides that are able to ferry much larger molecules, nucleic acids, protein, chemical, into living cells. However, it is know that the efficiency is controversy and that the permeability depends on the kinds of CPPs and cell types. In this study therefore to get a good vehicle for transfection of proteins, chemicals or nucleic acids to the differentiating mES cell, we designed two synthetic CPPs, expressed CPP-EGFPs and analyzed the transfection modulating factors.

MATERIALS AND METHODS

1. Expression and Purification of CPP fusion EGFP

All used buffer and chemicals were purchased from Aldrich-Sigma, and restriction enzymes (BamH1 and NdeI) were got from New England Biolab. CCP genes were syn-

Table 1. Cell permeable protein transduction domains

CPP	Amino acid sequence	Net charge(+)
Buforin 2	TRSSRAGLQFTPVGRVHRLLRK	7
pEP-1	KETWWETWWTEWSQPKKKRKV	6

thesized as 60～70 mer oligonucleotide based on the amino acid sequences (Table 1) and got full length sequence using overlapPCR. For getting CCP- EGFP proteins, we used pET15b and pET20b(+) (Merck) and linked to a EGFP construct, and then transformation into *E. coli* DH5 α or BL21 (DE3). pET15b has sequences for His-tag in N terminal (depicted as -N) and pET20b in C terminal (depicted as -C) (Fig. 1). DNA sequencing was done in Solgent In. C. (Korea). Recombinant CCP-EGFPs were purified using Ni-NTA agarose resin (Quagen) according to the manual of manufacture. Protein concentration was measured using SDS-PAGE and standard protein.

2. ES Cell Culture

Undifferentiated mouse ES cells (mES cells, R1) were maintained as described previously (Lee *et al*., 2000; Schoonjans *et al*., 2006). Briefly, undifferentiated mES cells were grown on gelatin-coated tissue culture plates in the mES medium, Dulbecco's modified eagles medium (DMEM) supplemented with 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, and 10% fetal calf serum. mES medium contained the 1,000 U/ml leukemia inhibitory factor (LIF; Chemicon, Temecula, CA). The ES cells were passed every 3 or 4 days onto freshly prepared gelatin-coated plates, and fed every day with mES medium.

3. Import Assay

mES cells were plated on the gelatin-coated slide glass (Nalgene, Rochester, NY) and grown to 2 or 3 days until 70～80% confluency at 37℃ with 5% CO_2 in mES medium supplemented with 10% fetal bovine serum (FBS) and 1000 U/ml LIF. To asses the concentration effects of CPP-EGFP fusion proteins, R1 was treated with CPP-EGFPs with 5 μg/mL, 50 μg/mL and 500 μg/mL for 12 hr. To know whether is there time dependent procedure, R1 mouse embryonic stem cells were overlaid with preformed CPP-EGFP fusion proteins (50 μg/mL) in mES medium and the cells were cultured for 5 min, 30 min, 60 min, or 24 hr. Confocal microscopy was performed on unfixed condition after 3 times washing with PBS as described in detail elsewhere (Lundberg *et al*., 2003).

4. Dye-exclusion Assay

To assay the viability of the CPP-EGFPs, R1 cells were washed with PBS, and incubated with 0.4% trypan blue (Sigma-Aldrich, Stl Louis, MO) for 3 min. The dye was thereafter rinsed off with PBS and observed viability was analyzed under the inverted microscope (IX 70, Olympus).

RESULTS

1. Toxicity of CPPs on Mouse Embryonic Stem Cell

To know the effects of CPP-EGFPs on viability of R1 stem cell, R1 cells were exposed to CPP-EGFPs for 12 hr with various concentrations. The viability was analyzed using trypan blue import assay. The toxicity was detected and it was dependent on the kind of CPPs. pEP 1-EGFP-N and pEP 1-EGFP-C showed cytotoxicity at 500 μg/mL dose. In the case of pEP-EGFPs, that cytotoxicity was correlated with concentration. However Buforn-2 was not much toxic to R1 cells compared with pEP-1-EGFPs (Fig. 2).

2. Concentration Effects on Transfection

To analyze the concentration effects on CPP-EGFPs transfection, CPP-EGFPs were treated as mentioned in Materials and Methods. R1 was treated with CPP-EGFPs, 5 μg/mL, 50 μg/mL and 500 μg/mL for 12 hr, and observed using confocal microscope. The permeability of CPP-EGFPs depended on the kinds of CPPs but not on their doses. Transfection of EGFP and Bufroin 2-EGFP was not detected after 12 hr (Fig. 3). Buforin 2 could not translocate

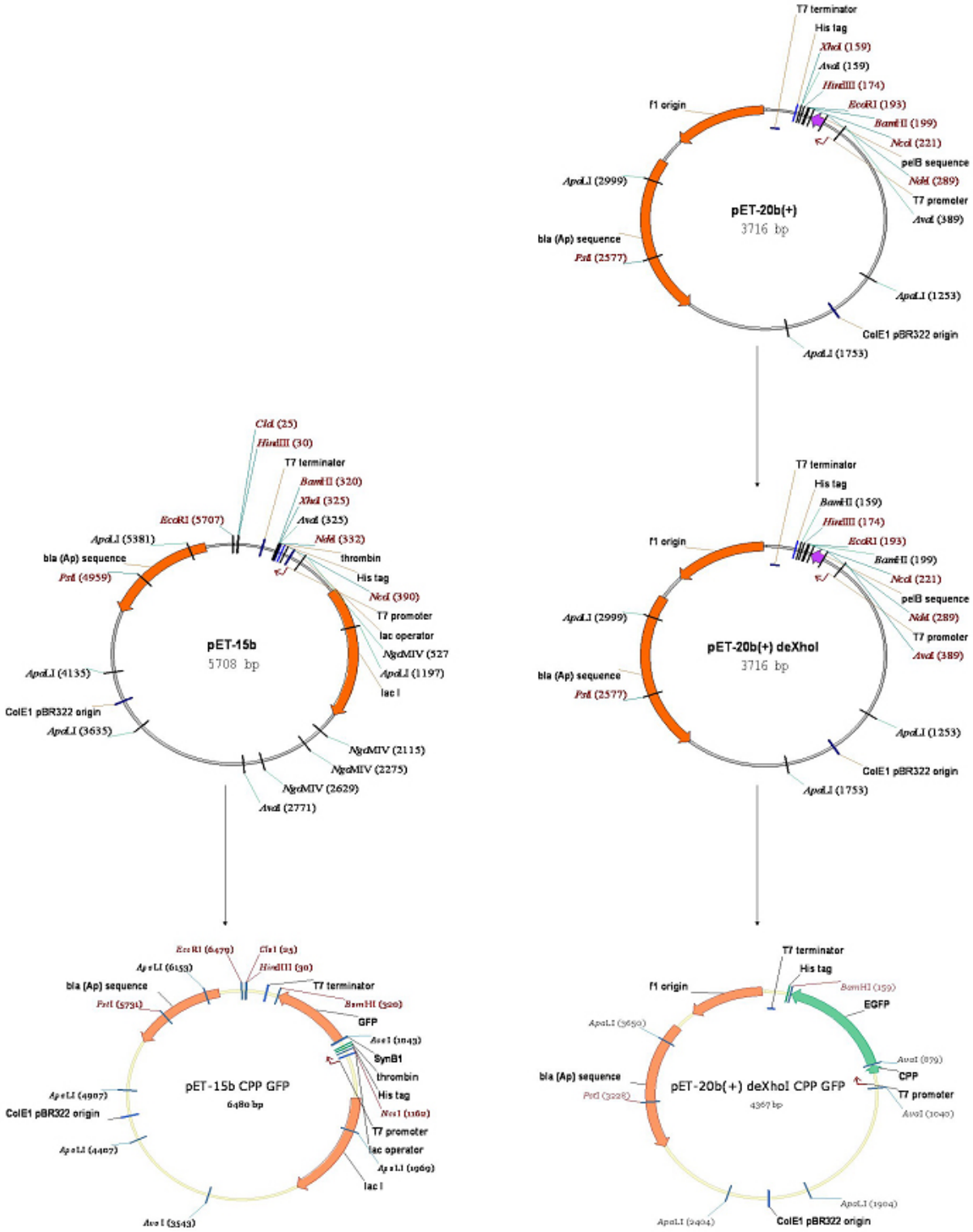

Fig. 1. Vector map of constructed. pEP20b (+) was modified to make the pEP20b-deXho1. CPP and EGFP were arranged as depicted in the panel.

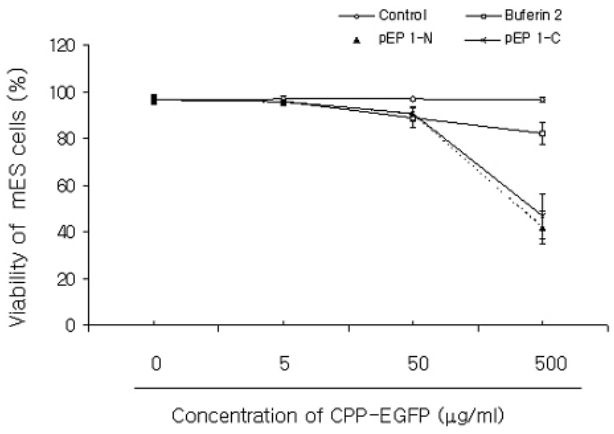

Fig. 2. Cytotoxicity of CPP-EGFPs on the R1 mouse embryonic stem cell. Cells were treated with CPP-EGFPs for 12 hr and checked viability using trypan blue dye exclusion mentioned in Materials and Methods.

EGFP into the cytoplasm, even in 500 μg/mL (Fig. 3C). In the case of pEP-1, however, it could translocate the EGFP-N in to the cytoplasm of R1 cells throw plasma membrane. Efficiency in transfection of pEP-1 was not correlated with the concentration (Fig. 3D). However, if His-tag was moved to the C-terminal of EGFP, pEP-1 could not translocate the EGFP through the plasma membrane. pEP-1-EGFP-C was mainly localized around the plasma membrane of R1 cells (Fig. 3E).

3. Treatment Time on the Transfection Efficiency

In order to explore the treatment-time efficiency on CPPs-facilitate-delivery, we examined cellular uptake of the recombinant CPP-EGFPs, after 5 min, 30 min, 1 hr and 24 hr incubation of mES cells with EGFP, Buforin 2-EGFP,

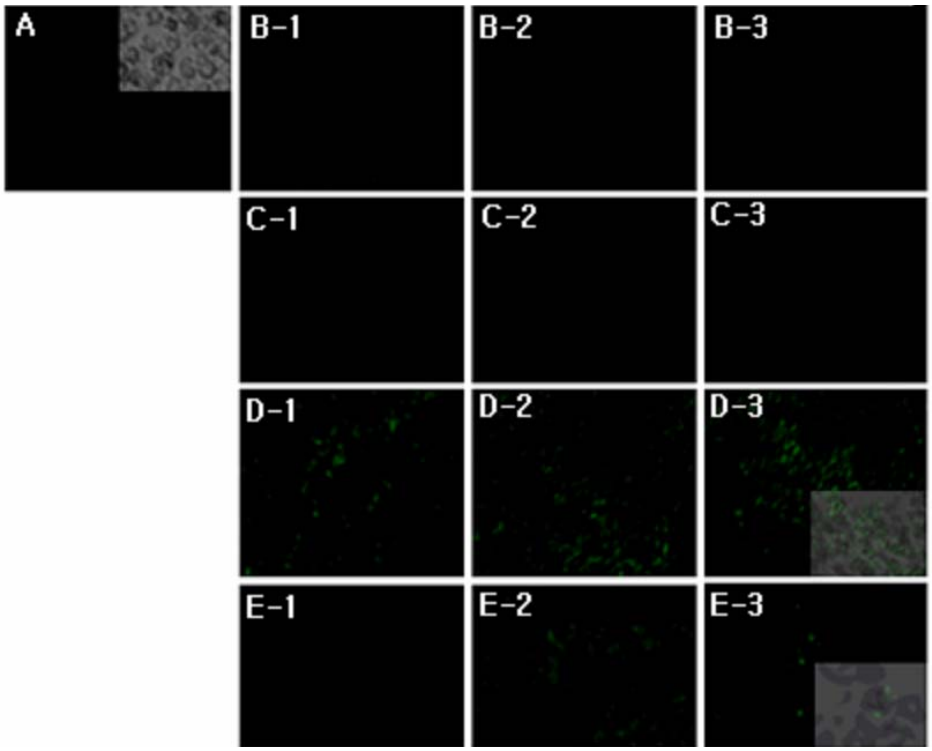

Fig. 3. Efficiency of transfection depends on the CPP. CPP-EGFPs were treated with various concentration (-1; 5 μg/mL, -2; 50 μg/mL, -3; 500 μg/mL) and observed after 12 hr. Vehicle (A), EGFP (B), Buforin 2-EGFP (C), pEP-1-EGFP-N (D), pEP-1-EGFP-C (E) were exposed to R1 embryonic stem cell and transfection was detected using confocal microscope. Confocal images illustrate clear intracellular localization of pEP-1-EGFP-N (D-1, D-2, and D-3). In the case of pEP-1-EGFP-C it was mainly localized around the cells. Overlays show where localization of EGFP (D-3, E-3). Magnification = 200×(box: 400×).

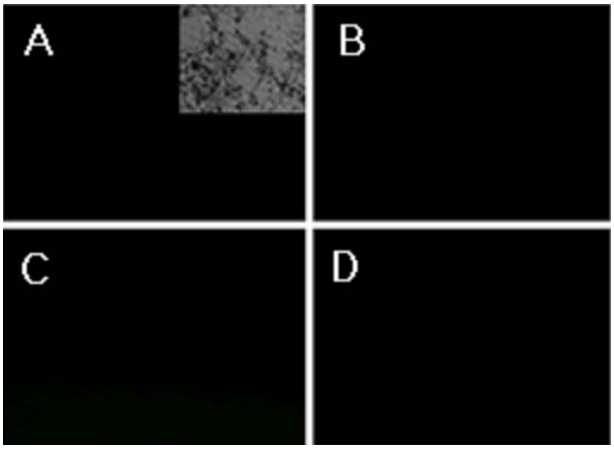

Fig. 4. Transfection of EGFP (A), Buforin 2-EGFP (B), pEP-1-EGFP-N (C) and pEP-1-EGFP-C (D) into R1 mES cells at 5 min after treatment. 50 μg/mL CPP-EGFPs overlaid on the R1 cells for 5 min and washed with PBS three times. Evidence of transfection could not detect in all group (Large box figure; fluorescence field, small box; overlapped image with bright field). Magnification = 200×.

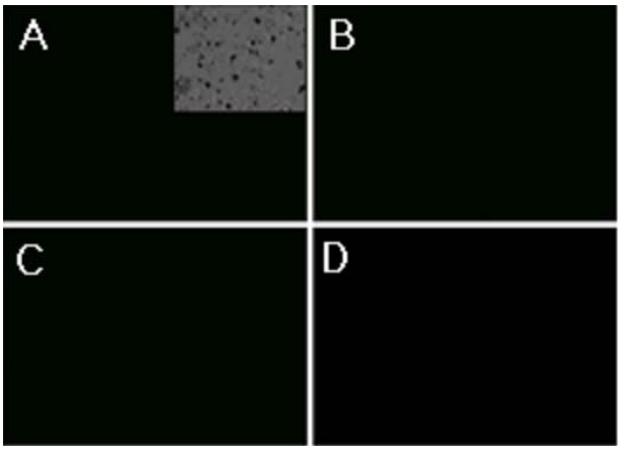

Fig. 5. Transfection of EGFP (A), Buforin 2-EGFP (B), pEP-1-EGFP-N (C) and pEP-1-EGFP-C (D) into R1 mES cells at 30 min after treatment. 50 μg/mL CPP-EGFPs overlaid on the R1 cells for 30 min and washed with PBS three times. Evidence of transfection could not detect in all group (Large box figure; fluorescence field, small box; overlapped image with bright field). Magnification = 200×.

pEP-1-EGFP-N and pEP-1-EGFP-C. After treatment the R1 cells were observed without fixation under the confocal microscope. Until 30 min, translocation of recombinant CPP-EGFPs was not detect in all groups (Fig. 4, 5).

pEP-1-EGFP-N was detected in the cytoplasm of R1 cell at 1 hr post treatment (Fig. 6). However, translocalization of Buforin 2-EGFP and pEP-1-EGFP-C through plasma membrane or nuclear membrane was not observed in R1 cells at 1 hr post treatment. Besides the signals was not detected even after 24 hr treatment (Fig. 7). pEP-1-EGFP-C could not be transfected into the R1 cell but localized mainly around the R1 plasma membrane (Fig. 7).

DISCUSSION

Genetic manipulation of ES cells is essential to elucidate gene function. Through genetic manipulation, we can direct the differentiation of ES cells to specific lineages, use the differentiated cell types from mixed populations of ES cell derivatives, use the differentiated derivatives of ES cells as a vehicle for gene therapy, and modulated immune response to transplanted ES cell derivatives. For therapeutic application in transplantation medicine, controlled modification of specific genes could be useful for purifying specific ES cell-derived and differentiated cell type from a mixed population, and for giving cells new properties to combat specific diseases.

To manipulate the genes, homologous recombination and electrophoration protocols have been used with mouse ES cells and human ES cells. Nanoparticles are also used as a mediator to transfection the gene constructs or proteins into the eukaryotic cell. However it is needed help of the component of target cell to quick transport such as fibronectin or E-cadherin in stem cells (Kutsuzawa *et al*., 2006). To use as a medicine, there are overwhelming safety concerns with regards to the application of genetic manipulation in human clinical therapy, uncontrolled expression or suppression, cellular viability, and unpredictable physiological consequences (Chou *et al*., 2004; Dunbar & Emmons, 1994). Therefore it has been asked to develop a

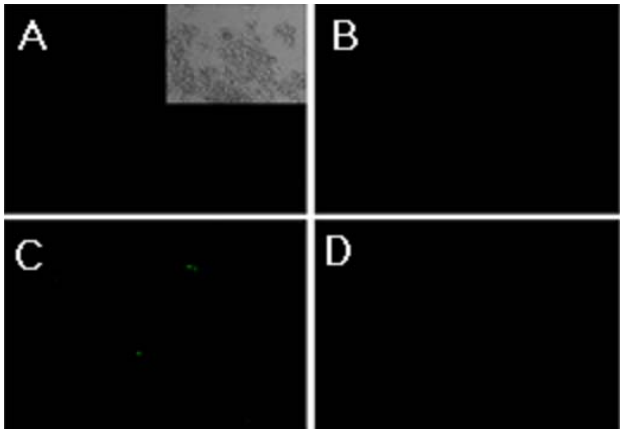

Fig. 6. Transfection of EGFP (A), Buforin 2-EGFP (B), pEP-1-EGFP-N (C) and pEP-1-EGFP-C (D) into R1 mES cells at 1 hr after treatment. 50 μg/mL CPP-EGFPs overlaid on the R1 cells for 1 hr and washed with PBS three times. Evidence of transfection could detect in pEP-1-EGFP-N but signal was weak (C). Large box figure is fluorescence field and small box is overlapped image with bright field. Magnification = 200×.

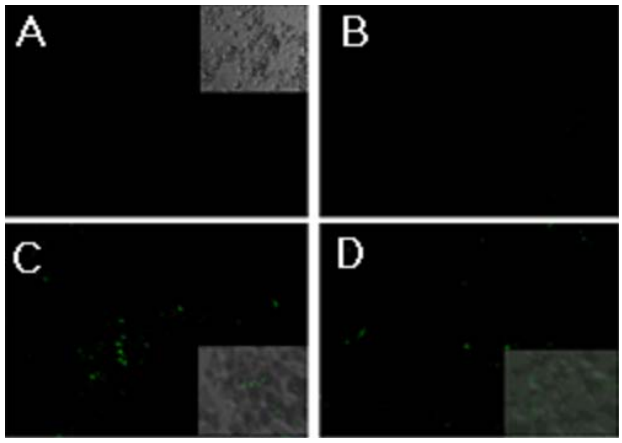

Fig. 7. Transfection of EGFP (A), Buforin 2-EGFP (B), pEP-1-EGFP-N (C) and pEP-1-EGFP-C (D) into R1 mES cells at 24 hr after treatment. 50 μg/mL CPP-EGFPs overlaid on the R1 cells for 24 hr and washed with PBS three times. Evidence of transfection could detect both in pEP-1-EGFP-N and pEP-1-EGFP-C. Signal was mainly detected inner cell area in pEP-1-EGFP-N but it was mainly observed around the cells in pEP-1-EGFP-C. Large box figure is fluorescence field and small box is overlapped image with bright field. Magnification = 200× (box: A; 200×, C & D; 400×).

methodology which is non-genomic modulation.

A novel alterative would be look at the possibility of incorporating CPP within transcription factors. Recent reports show that the usefulness of the cell permeable proteins as a delivery mediator. Recently using a recombinant cell permeable Cre protein successfully conditional knock-in efficiently was induced recombination at defined stages of neural differentiation. Cre treatment has no overt side effects on proliferation and neural differentiation (Haupt *et al*., 2007).

In HeLa cell, Buforin-2 and GFP fused Buforin-2 could enter into the cytoplasm. Syn B1 could effectively transfect peptide through blood-brain barrier and into the nervous system (Rousselle *et al*., 2003). In human ES cells import efficacy of PDX1 is very poor compared with TAT-PDX1 in the human ES cells (Kwon *et al*., 2005; Noguchi *et al*., 2003). In addition, from this study we know that Buforin-2 is a poor transfection mediator in R1 embryonic stem cells. However, pEP-1 is a good candidate as a mediator for protein transfection into the embryonic stem cells. Based on them, it is clear that the composition of CPP is a key factor for the high efficiency of transfection and the cell types in effect of the transfection mediated with CPP. It is suggested that translocation of CPP is partially under the control by the composition of target membrane.

So far the mechanism of CPP mediated transport is controversy. Because in the previous studies, CPP-mediated translocation occurred even at low temperatures, and does not have strong cellular specificity, it is likely to be independent of endocytotic mechanisms, transmembrane protein channels, and protein receptor binding (Vive *et al*., 1997). On the other hand, the other groups suggested that any effects of CPP were due to an enhanced physical-chemical interaction but not uptake of the fusion peptide (Holm *et al*., 2006; Falnes *et al*., 2001). pEP-1-EGFP-N binding to the cell membrane in R1 cells was not observed at 5 min or 30 min after treatment. From 1 hr after treatment, cell-membrane-binding pEP-1-EGFP-N was observed and translocalized pEP-1-EGFP-N was detected in the

cytoplasm. Besides the tranlocalization of pEP-1EGFP-N was not depend on the concentration. This transfection pattern is similar with the Buforin peptide in the HeLa cells (Takeshima *et al*., 2003). Put together it is suggested that pEP-1-EGFP transfection follows the concentration-independent passive mechanism.

It is also known that there is dependence of translocation of CPP on the dose, time, temperature, and energy (Takeshima *et al*., 2003). The Buforin-2 peptide is moved into the cytoplasm within 10 min by a temperature-independent and less concentration-dependent passive mechanism, and the magainin peptide show cooperative concentration dependence of uptake (Haupt *et al*., 2007; Kwon *et al*., 2005; Morris 1997; Takeshima *et al*., 2003). CPPs used in this study, Buforin 2 and pEP-1 also were not showed dependence on the time and dose. It is suggested that the transfer of CPP is dependent on their chemical characteristics in embryonic stem cells.

Recently the techniques for protein transfection has been developed and used in studies such as nanoparticle (Tran *et al*., 2007). Until, however, it is limited to apply them on various cell types same as other techniques such as DNA or RNA transfection (Kutsuzawa *et al*., 2006). It has been suspected that CPP mediate transfection of protein, and it can be supported from our results. On summary, translocation of CPPs is under the control through the composition of target membrane and through on their chemical characteristics. pEP-1-EGFP transfection is follow the concentration-independent passive mechanism. In conclusion our data provide an asking other CPP for apply to the embryonic stem cells. Our data showed a possible application to protein transfection into the ES cells. For the future studies, modified CPP or mediator based on this study is needed to develop a better construct for translocation of target protein in embryonic stem cell.

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