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Identification of a cell-penetrating peptide applicable to a protein-based transcription activator-like effector expression system for cell engineering

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ABSTRACT

Cellular reprogramming is a promising technology in regenerative medicine, but most studies have been performed by using expression vectors. For future clinical applications, it is necessary to establish a system in which cell engineering can be manipulated without any risk of damaging the genome. Here, we identified a cell-penetrating peptide composed of 10 amino acids (RIFIHFRIGC) with nuclear trafficking activity and found that it was significantly more potent than a Tat-derived peptide or polyarginine peptide (R11). We named the peptide "nuclear trafficking peptide" (NTP) and applied it to a protein-based artificial transcription factor (NTP-ATF), which was composed of a transcription activator-like effector and transcription domain (VP64). An NTP-ATF designed to the proximal promoter region of the microRNA-302/367 cluster efficiently induced endogenous RNA expression at an extremely low concentration (0.25 nM), and repetitive treatment of mouse embryonic fibroblasts with NTP-ATF generated induced pluripotent stem-like cells, which gave chimeric mice. Together with the observation that recombinant NTP-ATF protein did not induce any apparent cytotoxicity, we propose that NTP-ATF is a promising system for cellular reprogramming applicable to regenerative medicine.

1. Introduction

Due to recent progress in regenerative medicine, cells of interest can be engineered by the ectopic expression of transcription factors [1]. Since Takahashi and Yamanaka demonstrated that the expression of 4 defined gene products in somatic cells converted them to pluripotent cells [2], a variety of cells have been engineered from different somatic cells by the ectopic expression of transcription factors These engineered cells include hepatic cells [3,4], cardiomyocytes [5,6], neuronal cells [7,8], and pancreatic β-cells [9]. These findings suggest that cell fate can be manipulated by replacing the core transcription network, further implying that cellular reprogramming is a promising technology for regenerative medicine [1]. For clinical applications, however, it is strictly required to exclude the risk of introducing genomic mutations into processed cells. As most studies on reprogramming experiments have been performed using expression vectors, the next step is to develop a cell engineering system that is free of the possibility of damaging the genome.

Transcription activator-like effector (TALE), a molecule of the AvrBs family in the plant pathogenic bacterial genus *Xanthomonas*, activates transcription in plant cells and gives pathogenic effects to bacteria expressing this protein [10–13]. The DNA-binding domain of TALE is composed of 34 amino acid repeats, the 12th and 13th residues of which are polymorphic and called repeat-variable di-residues (RVDs) [10,11]. Together with the

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amino (N-) and carboxy (C-) terminal domains, an RVD in each repeat module recognizes a single nucleotide [10,11]. For efficient trans-activator activity, Boch et al. identified that the first repeat of TALE should recognize the nucleotide "T," and 10.5 repeats are sufficient [10]. Considering that TALE is more designable and less toxic than the zinc-finger system, which was a first-generation genome editing system [13], it has a variety of applications (e.g., as a site-specific nuclease and transcription activator or repressor) [14,15]. Notably, an artificial transcription factor (ATF), which is composed of TALE and a transcription domain, is an attractive tool for cellular reprogramming, because a gene of interest can be activated when the DNA-binding domain of TALE is well designed [14,15].

Viral protein R (Vpr) is a 96-amino-acid virion-associated nuclear protein of human immunodeficiency virus type 1 (HIV-1) [16]. Vpr has been shown to function as a positive factor for the viral infection of resting macrophages [17,18]. Vodica et al. proposed that Vpr is involved in the nuclear import of the preintegration complex of HIV-1 [19]. Interestingly, when a recombinant Vpr protein is added to the culture medium of cells, it enters the cells and induces cell cycle abnormalities [20] and DNA double strand breaks (DSBs) [18,21]. Notably, Coeyytaux et al. reported that Vpr-derived peptides could permeabilize the cell membrane and transduce plasmid DNA into cells [22,23]. In 2004, we found that C45D18, a peptide composed of 27 amino acids encompassing amino acids 52–77 of Vpr, functioned as a cell-penetrating peptide (CPP) [24]. When β-galactosidase that was chemically conjugated to C45D18 was added to the culture medium of HeLa cells, β-galactosidase activity was detected in the nuclei of the cells [25]. Moreover, an exogenous gene could be expressed in resting macrophages when plasmid DNA was transduced with a C45D18-conjugated cationic polymer of poly(*N*,*N*-dimethylaminopropylacrylamide)-*block*-oligo(4-aminostyrene) [25]. These findings suggested that C45D18 is a CPP that can be used as a nuclear cargo of macromolecules.

CPP-based cell engineering is a promising approach for cellular reprogramming [26]. Among the more than 100 CPPs identified to date, TAT-derived peptide (Tat) and polyarginine peptides (R9 or R11) have mostly been utilized [26]. Induced pluripotent stem cells (iPSCs) have been established from somatic cells by fusing reprogramming factors to R9, R11, and Tat [27–29]. Moreover, these peptides have also been applied to genome editing systems such TALE-based nuclease (TALEN) [30,31] and the clustered, regularly interspaced, short palindromic repeat (CRISPR)-associated (Cas9) system [32]. Although these trials were successful, an extremely high amount of recombinant proteins was required: 50–100 nM was used to establish iPSCs [27,29], whereas 2–3 μM was used for genome editing with TALEN [30,31] and Cas9 [32].

Here, we identified a peptide of 10 amino acids as a minimal stretch of C45D18 and named it "nuclear trafficking peptide" (NTP). We found that the trafficking activity of NTP was more potent than that of Tat and R11, and applied it to a protein-based ATF (NTP-ATF) composed of TALE and VP64 [14,15,33]. Strikingly, NTP-ATF efficiently induced the mRNA expression of an endogenous target gene at extremely low concentrations (0.25 nM). Moreover, repetitive treatment with NTP-ATF, which targeted the microRNA (miR)-302/367 cluster [34–36], could convert mouse somatic cells into immature pluripotent cells, which could generate chimeric mice. Taken together with the observations that NTP-ATF was not toxic to human cells and recombinant NTP-ATF proteins can be prepared in a few days, we propose that NTP-ATF could function as a versatile platform for a protein-based cellular reprogramming system that is applicable to regenerative medicine.

2. Materials and Methods

2.1. Cells

HeLa cells, HT1080 cells (human fibrosarcoma cell line), and MRC-5 cells (human fibroblast cell line) were acquired from the RIKEN Cell Bank (Ibaraki, Japan). Mouse embryonic fibroblasts (MEFs) were prepared from embryonic day 13.5 embryos of Nanog-green fluorescent protein (GFP) transgenic mice, which were derived from C57BL/6 mice expressing an exogenous *GFP* gene introduced into the 3′ region of the Nanog promoter [37] (RIKEN Cell Bank). The human cell lines and MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) under humidified conditions with 5% CO_2. Human peripheral blood was donated by healthy volunteers who gave informed consent. Peripheral blood mononuclear cells (PBMCs) were prepared from these samples using Ficoll-Hypaque (PBMCs, 5.0×10^5 cells/well, at the second passage), and were maintained in a dish coated with 10 μg/mL purified NA/LE mouse anti-rat CD3 (BD Biosciences, USA), suspended in KBM550 medium (KOHJIN BIO, Japan).

2.2. Conjugation of peptides to magnetic nanoparticle and measurement of the trafficking activity of Vpr-derived peptides

As an initial screening of the minimum stretch of C45D18-derived CPP, we used carboxymethyl dextran magnetite (CMDM; Meito Sangyo Co., Ltd., Aichi, Japan), a dextran-coated superparamagnetic iron oxide, which was conjugated to the C45D18-derived peptides through the SH residue (peptide-CMDM), according to a method described previously [38]. HeLa cells were treated overnight with each peptide-CMDM, and intracellular magnetic intensity was measured by time-domain nuclear magnetic resonance (TD-NMR; Minispec MQ20; Bruker, Massachusetts, USA). The detailed procedures for peptide conjugation to CMDM and measurement analysis of intracellular magnetic intensity are described in the Supplementary Methods and Supplementary Fig. 1.

2.3. Purification of enhanced GFP and flow cytometry analysis

Enhanced GFP (EGFP) proteins were over-expressed in *Escherichia coli* BL21(DE3) carrying plasmid DNA that included pET-His-EGFP, pET-His-NTP-EGFP, pET-His-C45D18 L/A-EGFP, pET-His-LR20-EGFP, pET-His-EGFP-NTP, pET-His-Tat-EGF, pET-His-R11-EGFP, and pET-His-EGFP-R11. The bacteria were grown to $OD_{600} = 0.4$ at 37 °C, and IPTG was added to a final concentration of 0.5 mM. After growth for 16 h at 15 °C, the cells were collected by centrifugation and suspended in buffer D (10% glycerol, 20 mM HEPES-NaOH pH 7.8, 250 mM NaCl, 10 mM mercaptoethanol, 10 mM imidazole, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) containing 1.5 mg/mL lysozyme and incubated for 30 min on ice. The cell lysate was sonicated and soluble protein was collected by centrifugation. The supernatant was loaded onto an Ni-NTA column and protein was eluted with buffer D containing 250 mM imidazole. Ammonium sulfate (4 M) was added to the solution to a final concentration of 500 mM. After centrifugation, the supernatant was subjected to hydrophobic interaction chromatography using HiTrap Butyl HP (GE Healthcare, USA) with a linear gradient of 500 mM–0 mM ammonium sulfate in buffer E (10% glycerol, 20 mM HEPES–NaOH, pH 7.8, 50 mM NaCl, 0.1 mM dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid [EDTA]). EGFP proteins were eluted as a single peak with the use of the ÄKTA Prime System (GE Healthcare). For high-level purification, EGFP proteins were subjected to size exclusion chromatography using Superdex 75 10/300 GL (GE Healthcare). Both proteins were eluted