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# EGF domain peptide of Developmentally regulated endothelial locus1 facilitates gene expression of extracellularly applied plasmid DNA

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## ABSTRACT

**Background:** The successful development of messenger RNA vaccines for SARS-CoV-2 opened up venues for clinical nucleotide-based vaccinations. For development of DNA vaccines, we tested whether the EGF domain peptide of Developmentally regulated endothelial locus1 (E3 peptide) enhances uptake of extracellularly applied plasmid DNA.

**Methods:** DNA plasmid encoding lacZ or GFP was applied with a conditioned culture medium containing E3 peptide to cell lines *in vitro* or mouse soleus muscles *in vivo*, respectively. After 48 h incubation, gene expression was examined by  $\beta$ -galactosidase ( $\beta$ -gal) assay and fluorescent microscope, respectively.

**Results:** Application of E3 peptide-containing medium to cultured cell lines induced intense  $\beta$ -gal activity in a dose-dependent manner. Intra-gastrocnemius injection of E3 peptide-containing medium to mouse soleus muscle succeeded in the induction of GFP fluorescence in many cells around the injection site.

**Conclusions:** The administration of E3 peptide facilitates transmembrane uptake of extracellular DNA plasmid which induces sufficient extrinsic gene expression.

## 1. Introduction

Several different strategies have been utilized to develop a vaccine against the global pandemic of COVID-19 [1]. In December 2020, the U. S. Food and Drug Administration authorized the emergency use of two mRNA vaccines for the prevention of COVID-19. Due to the fundamental vulnerability of mRNA itself and mRNA-encapsulated lipid nanoparticles, the mRNA vaccines are unstable at room temperature and require ultra-low temperature freezers for storage and transportation. The requirement of the non-household use freezers and huge electric supply prevents the prevailing of the vaccine in rural regions and developing countries [2]. A vaccine based on adenoviral vectors was also proposed. Although the adenoviral vectors are more stable at room temperature than the mRNA vaccine, adverse effects such as the formation of blood clots and inflammations have been reported [3,4]. As one of the alternative strategies, DNA vaccines against COVID-19 also developed [5,6]. The DNA vaccines are relatively stable at room temperature, and less immunogenic. However, the delivery of DNA requires specialized devices such as *in vivo* electroporation devices as well as

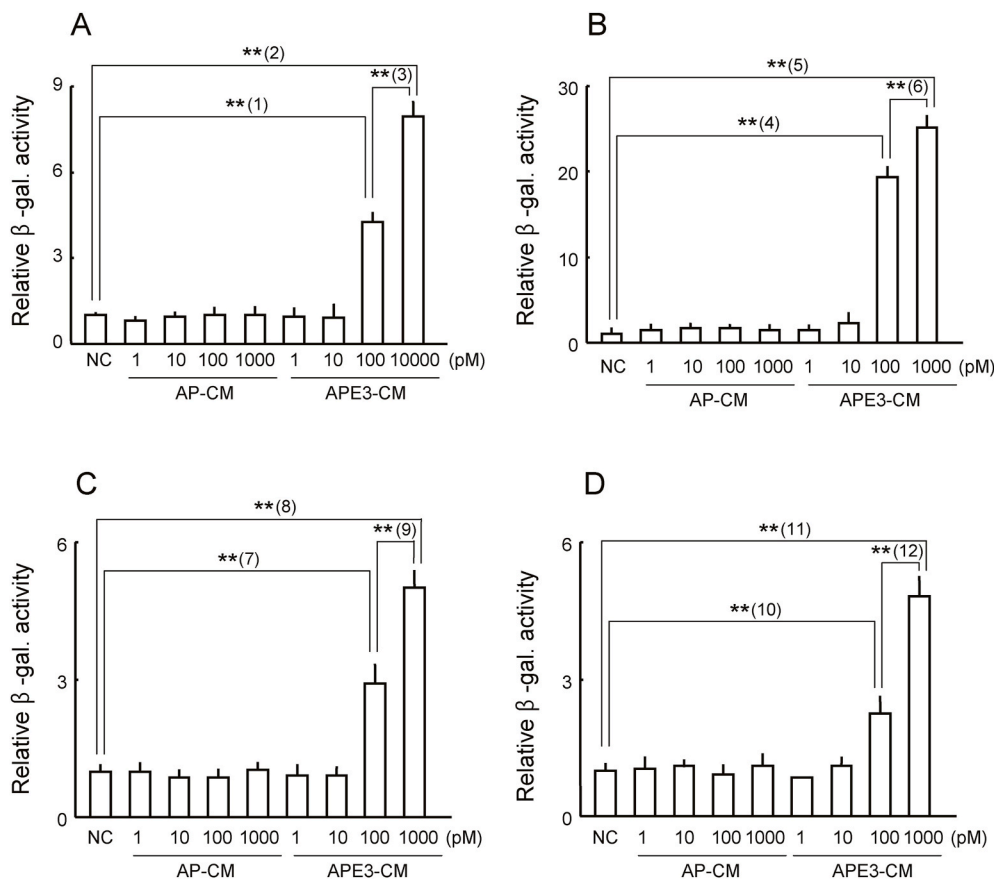
chemical reagents, such as polycationic lipids. These limitations require extra efforts in supplying the electric devices to the locations of the vaccine administration, on relieving pain at the voltage-mediated gene transfer, and invalidating the toxicity and immune response that may be occurred by the intravital administration of transfection reagents. Taken together, a safer and easier DNA gene transfer method conquering these problems is desired for the development of next-generation nucleotide-based vaccines.

Developmentally regulated endothelial locus1 (Del1) is an extracellular matrix protein and expressed in the mesoderm during embryogenesis, angioblasts, and endothelial cells in the heart, lung, kidney, eye, and hypertrophic chondrocytes after birth [7–9]. Del1 is composed of three epidermal growth factor (EGF) domains on the N-terminal side and two discoidin domains on the C-terminal side [10,11]. *In vitro* experiments showed that the third EGF domain, E3 peptide, enhances endocytosis [12] and promotes the polycationic lipid-mediated plasmid DNA transfection. The sequence motif of amino acids containing CXDXXXXYXCXC in the EGF domain is responsible for the assistive effect of E3 peptide on transfection [13]. However, whether the E3 peptide

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**Fig. 1.** *In vitro* gene transfer by E3 peptide. Relative  $\beta$ -galactosidase ( $\beta$ -gal) activity in the cultured cells was quantified after application of lacZ expression plasmid with E3 peptide-containing conditioned medium (APE3-CM) or control medium (AP-CM). SCCKN (A), CHO (B), HUVEC (C) or COS-7 (D) cells were used. Concentrations of E3 peptides were indicated in each horizontal axis. "NC" represents negative control cells which are incubated with lacZ expression plasmid dissolved in the conditioned medium from mock-transfected CHO cells. The values relative to NC were shown as means  $\pm$  SEM (n = 4). p-values are: 0.00024 for (1); 0.000051 for (2); 0.000033 for (3); 0.000053 for (4); 0.000021 for (5); 0.0011 for (6); 0.0033 for (7); 0.00028 for (8); 0.000043 for (9); 0.001 for (10); 0.00043 for (11); 0.0036 for (12).

itself can enhance plasmid DNA transfection remains unclear. In the current study, we evaluated the transfection efficiency of plasmid DNA which was coapplied with E3 peptide.

## 2. Methods

### 2.1. Cell culture

Human oral squamous cell carcinoma (SCCKN) cells [14], a gift from Dr. Hayashido (Hiroshima University, Hiroshima, Japan), were grown in RD medium [a mixture of 45% Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA), 45% RPMI 1640 medium (Invitrogen), and 10% fetal bovine serum]. Chinese hamster ovary (CHO) [15] and COS-7 [16] cells were grown in  $\alpha$ -minimum essential medium supplemented with 10% fetal bovine serum (FBS) and 0.2  $\mu$ M  $\beta$ -mercaptoethanol. Human umbilical vein endothelial cells (HUVEC) [17] were grown in Medium 200 (Invitrogen) with a Low-serum Growth Supplement kit (Invitrogen). All cells were cultured in 5% CO<sub>2</sub> at 37 °C.

### 2.2. DNA constructs

To quantify the amount of E3 peptide, E3 cDNA was C-terminally fused to heat-stable placental alkaline phosphatase (AP) which contains a signal peptide for secretion in the N-terminal region. This plasmid was constructed by PCR extraction of the E3 cDNA from a plasmid containing mouse Del1 cDNA (pcDNA3-Del1; a kind gift from Dr. Quertermous) using primers (forward: tgtgaagctgagccttcgagaatggcgga, reverse: tataactgttaaagcagggtatattaaga), and then, inserted into the pAptag-4 vector (GenHunter, Nashville, TN).

### 2.3. Conditioned medium

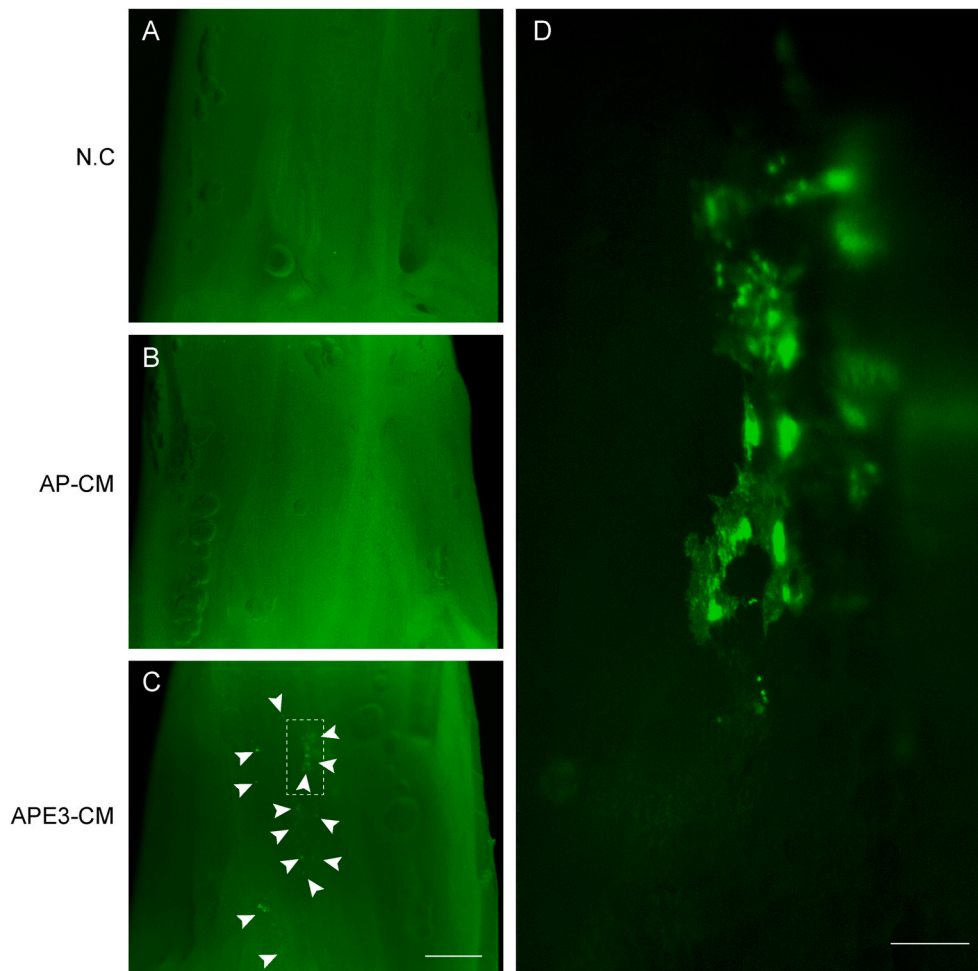
To obtain conditioned media (CM) containing the AP fused E3 peptides, CHO cells were transfected with plasmid DNAs using Lipofectamine 3000 (Invitrogen). After 4 h, cells were washed with phosphate-buffered saline (without Calcium and Magnesium) and incubated with fresh media for 48 h. The collected CM was stored at 4 °C and used for experiments within several weeks. The AP activity in 20  $\mu$ l CM was measured in a 96-well plate. The plate was heated at 65 °C for 30 min to inactivate endogenous AP. The enzyme reaction was initiated by adding 200  $\mu$ l of substrate [1 mg/ml p-nitrophenyl phosphate (Sigma, St Louis, MO, USA) in 1 mM MgCl<sub>2</sub> and 1 M diethanolamine, pH 9.8] to each well, and the absorbance at 405 nm was measured after 30–60 min with a microplate reader (Benchmak Plus, BIO-RAD Laboratories, Hercules, CA, USA).

### 2.4. $\beta$ -galactosidase assay

The CMs from CHO cells expressing AP-E3 (APE3-CM) or APtag-4 (AP-CM; as control) was prepared as described above. 0–1000 pM APE3-CM or AP-CM were added with plasmid DNA encoding LacZ cDNA (pcDNA3-LacZ) to SCCKN, CHO, HUVEC, or COS-7 cells. For the dilution of APE3-CM, the medium of each cell was used. After 48 h, the  $\beta$ -galactosidase ( $\beta$ -gal) activity was measured using a  $\beta$ -gal enzyme assay system (Promega, Madison, WI, USA) with a microplate reader (Benchmak Plus, BIO-RAD Laboratories).

### 2.5. Intravital injection

All animal experiments performed in this study were conducted in accordance with the animal husbandry regulations of Nihon University. This study was approved by the ethics committee of Nihon University



**Fig. 2.** *In vivo* gene transfer by E3 peptide-After 48 h from the injection of GFP expression plasmid with E3 peptide-containing conditioned medium (APE3-CM; C), control conditioned medium without E3 (AP-CM; B) or saline (N.C.; A) to mouse soleus muscles, green fluorescence images around the injection site are shown. Arrowheads indicate the cells with intense GFP expression. (D) High-magnification image in a region highlighted by dashed rectangle box in (C). Scale bars, 1 mm (C) and 100  $\mu$ m (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(AP18MED022-4). C57BL/6 mice were kept in a specific pathogen-free environment. Each mouse was injected with 10  $\mu$ l saline containing AP-CM (1 nM) or APE3-CM (1 nM) and plasmid DNA encoding GFP cDNA (pCIS-GFP) in mouse soleus muscles. After 48 h of injection, mice were sacrificed by cervical dislocation under deep anesthesia with isoflurane, and fluorescence images around injection sites were captured using a dissection fluorescent microscope (M205FA, Leica microsystems, Germany) equipped with an sCMOS camera (DFC9000GT, Leica microsystems).

## 2.6. Statistical analysis

Values in Fig. 1 are expressed as mean  $\pm$  SEM. Dunn's tests or a Wilcoxon's test was used for statistical analysis. Statistical significance was set at \*\*:  $p < 0.01$  and \*:  $p < 0.05$ .

## 3. Results

In this study, we utilized conditioned media containing the E3 peptide which is N-terminally fused to AP for quantification of the total amount of E3 peptide [14]. After the application of lacZ reporter plasmid with 100 or 1000 pM APE3-CM, SCCKN cells showed 4.27 or 7.95-fold increase in  $\beta$ -gal activity, respectively. In contrast, 1 or 10 pM APE3-CM failed to show  $\beta$ -gal activity, which is comparable to that in negative control groups of NC and AP-CMs (Fig. 1A). A 10-fold increase in APE3-CM concentration from 100 to 1000 pM resulted in 1.86-fold increase in  $\beta$ -gal activity, suggesting the dose-dependent increase of transfection efficiency. We obtained similar results in the experiments

using the other three types of cell lines. 100 or 1000 pM APE3-CM induced  $\beta$ -gal activity with 19.33 or 25.17-fold increase in CHO (Figs. 1B), 2.91 or 5.0-fold increase in HUVEC (Fig. 1C), and 2.24 or 4.81-fold increase in COS-7 (Fig. 1D) cells, respectively. These results indicate that E3 peptide enhances the *in vitro* plasmid internalization without cationic liposomes in a dose-dependent manner.

Next, we tested the E3-mediated transfection enhancement *in vivo*. The green fluorescent intensity in mouse soleus muscles was evaluated after 48 h of injection of the GFP expression plasmid with or without E3 peptides co-administration. A higher GFP signal was observed in more cells of the APE3-CM injected group (Fig. 2C and D), whereas the control groups (Fig. 2A and B) revealed lower intensity signal in fewer cells. A total of three individual experiments were performed to confirm the efficacy of E3 peptides that enhanced co-injected DNA plasmid internalization and the gene expression *in vivo*.

## 4. Discussion

Our current study showed that the E3 peptide of Del1 enhanced uptake of plasmid DNA, which induces expression of the external gene *in vitro* (Fig. 1) and *in vivo* (Fig. 2). The mechanism of the plasmid uptake is assumed to be due to enhanced endocytosis of extracellular plasmids by the EGF motif in the E3 peptide [12]. Further studies are required to clarify this mechanism.

The conditioned culture medium containing E3 peptide used in the current study can be stored at 4  $^{\circ}$ C for several weeks. Hence, the materials for gene transduction containing E3 peptides and DNA plasmids do not require ultra-low temperature freezers. Furthermore, the

conditioned culture medium containing E3 peptide can be replaced with a solution of synthesized E3 peptide which can be prepared immediately before use [13]. In addition, because E3 peptide is an intrinsic and ubiquitous peptide in mammals [18], it is unlikely that E3 peptide itself activates host immune response upon intravital administration. Actually, in our knowledge, sudden death was observed in the mice with extremely mass intravenous of E3 peptide [19], but, no detrimental phenotypes, including local and systemic inflammation and anaphylactic shock, were observed in the mice under over expression of E3 peptide [20]. Therefore, clinical applications of E3 peptides are safe and able to reduce the time and cost for *in vivo* study to validate potential adverse effects on intravital injection of artificial reagents, like polycationic lipids.

In conclusion, E3-mediated gene transfer has significant advantages on the stability of materials, simplicity of protocols, and high affinity to an organism, which may contribute to developing an easy-to-handle DNA vaccine for COVID-19.

### Declaration of competing interest

The authors have no conflict of interest or research funding to declare.

### Acknowledgments

None to declare.

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