Faint electric treatment-induced rapid and efficient delivery of extraneous hydrophilic molecules into the cytoplasm

Mahadi Hasan[1], Akinori Nishimoto[1], Takashi Ohgita[1], Susumu Hama[1], Hiromu Kashida[2], Hiroyuki Asanuma[2], Kentaro Kogure[1,3]*

[1]Department of Biophysical Chemistry, Kyoto Pharmaceutical University, Nakauchi-cho 5, Misasagi, Yamashina-ku, Kyoto, 607-8414, Japan

[2] Department of Molecular Design and Engineering, Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603, Japan

[3]Department of Pharmaceutical Health Chemistry, Institute of Biomedical Sciences, Tokushima University, Graduate School, Shomachi 1, Tokushima, 770-8505, Japan

*Correspondence:

Kentaro Kogure, Ph. D.

Department of Pharmaceutical Health Chemistry, Institute of Biomedical Sciences

Tokushima University, Graduate School

Shomachi 1, Tokushima, 770-8505, Japan

Tel.: +81-88-633-7248

E-mail: kogure@tokushima-u.ac.jp

Abstract

Effective delivery of extraneous molecules into the cytoplasm of the target cells is important for several drug therapies. Previously, we showed effective *in vivo* transdermal delivery of naked siRNA into skin cells induced by faint electric treatment (ET) iontophoresis, and significant suppression of target mRNA levels (Kigasawa et al., Int. J. Pharm., 2010). This result indicates that electricity promoted the delivery of siRNA into cytoplasm. In the present study, we analyzed the intracellular delivery of naked anti-luciferase siRNA by faint ET, and found that the luciferase activity of cells expressing luciferase was reduced by *in vitro* ET like *in vivo* iontophoresis. Cellular uptake of fluorescent-label siRNA was increased by ET, while low temperature exposure, macropinocytosis inhibitor amiloride and caveolae-mediated endocytosis inhibitor filipin significantly prevented siRNA uptake. These results indicate that the cellular uptake mechanism involved endocytosis. In addition, voltage sensitive fluorescent dye DiBAC4 (3) penetration was increased by ET, and the transient receptor potential channel inhibitor SKF96365 reduced siRNA uptake, suggesting that faint ET reduced membrane potentials by changing intracellular ion levels. Moreover, to analyze cytoplasmic delivery, we used in-stem molecular beacon (ISMB), which fluoresces upon binding to target mRNA in the cytoplasm. Surprisingly, cytoplasmic ISMB fluorescence appeared rapidly and homogeneously after ET, indicating that cytoplasmic delivery is markedly enhanced by ET. In conclusion, we demonstrated for the first time that faint ET can enhance cellular uptake and cytoplasmic delivery of extraneous molecules.

Key words: Faint electric treatment, Cellular uptake, Cytoplasmic delivery

1. Introduction

Over the last decade, the delivery of extraneous hydrophilic molecules across the plasma membrane has been a subject of intense study. For effective therapies, small chemical molecules or large biological medicines, such as functional nucleic acids, must be delivered into the cytoplasm or nucleus of target cells. The lipophilic plasma membrane presents a dynamic barrier that restricts entry of extraneous hydrophilic or charged molecules into cells, while inefficient endosomal escape is another obstacle to effective delivery of macromolecular medicines [1, 2]. To overcome these cellular barriers, various strategies, including development of nanocarriers, cell penetrating peptides, and physical methods such as electroporation, have been studied [3, 4]. However, given the fundamental challenges of intracellular delivery, the versatile application of these systems remains unsatisfactory due to issues with delivery efficiency and toxicity of the carrier [5, 6]. Therefore, safer and more effective methods to deliver extraneous hydrophilic molecules into cells are needed.

Iontophoresis has recently attracted the attention of researchers studying drug delivery systems. Iontophoresis is the promising non-invasive transdermal drug delivery technology [7-9]. Iontophoresis facilitates transdermal delivery of water soluble and ionized molecules by application of small electrical current (0.5 mA/cm^2 or less) with electrodes on the skin surface. Thus, electric treatment of the skin provides the driving force for transdermal delivery of drug molecules. Previously, we demonstrated non-invasive *in vivo* transdermal delivery of naked siRNA induced by iontophoresis that significantly suppressed levels of target mRNA [10]. This result indicates that hydrophilic siRNA molecules could be delivered non-invasively into the cytoplasm of skin cells by faint electric treatment (ET) without the need for any modification of the siRNA to promote effective cellular association and endosomal escape. A mechanistic

analysis of the transdermal penetration of this macromolecule revealed that ET induced the dissociation of intracellular junctions via activation of cell signaling pathways mediated by protein kinase C (PKC) [11]. However the mechanism of cellular uptake and cytoplasmic delivery of siRNA and other hydrophilic molecules are still unclear. Here we explored whether faint ET could enhance cellular uptake and cytoplasmic delivery of extraneous hydrophilic molecules by affecting cellular physiology.

We examined the effect of faint ET in the presence of siRNA targeting luciferase in cells stably expressing luciferase. Uptake mechanisms were analyzed by assessing the effect of low temperature and endocytosis inhibitors such as the clathrin-mediated endocytosis inhibitor hypertonic sucrose [12], the caveolae-mediated endocytosis inhibitor filipin [13], and the macropinocytosis inhibitor amiloride [14], on the cellular uptake of fluorescent labeled-siRNA. In addition, the effect of ET on membrane potential and the effect of a transient receptor potential (TRP) channel inhibitor on fluorescent labeled-siRNA uptake were also examined. We also analyzed the cytoplasmic delivery using a functional oligonucleotide in-stem molecular beacon (ISMB), which fluoresces upon specific binding with target mRNA in the cytoplasm [15]. The present study offers ET as an effective and safe technology for cytoplasmic delivery of functional hydrophilic molecules and describes its mechanism.

2. Materials and Methods

2.1 Materials

In-stem molecular beacon (ISMB) against luciferase (5'-C(Cy3:Y_D)TGG(Y_D)GTTGGCACCAGCAGCGCAC(Nitromethylred:N_m)(N_m)CCA(N_m)(N_m)G -3': ISMB-Luc) and ISMB against GFP (5'-G(Y_D)GTT(Y_D)GAAGAAGATGGTGCGCTCTC(N_m)(N_m)AAC(N_m)(N_m)C-3': ISMB-GFP) were synthesized by Tsukuba Oligo Service, Inc. (Tsukuba, Japan). Calcein, amiloride hydrochloride hydrate and filipin were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). SKF 96356 and sucrose were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). $DiBAC_4(3)$ was obtained from Dojindo Molecular Technologies, Inc. (Rockville, MD, USA). Lipofectamine 2000, anti-luciferase siRNA (21-mer, 5'-GCGCUGCUGGUGCCAACCCTT-3', 5'-GGGUUGGCACCAGCAGCGCTT-3': anti-Luc) and anti-GFP siRNA (21-mer, 5'-GCUGACCCUGAAGUUCAUCTT-3', 5'-GAUGAACUUCAGGGUCAGCTT-3': anti-GFP) were obtained from Invitrogen Life Technologies (Carlsbad, CA, U.S.A). Cell lysis buffer was purchased from Promega Corporation (Madison WI, USA). The mouse melanoma cell line B16-F1 was obtained from Dainippon Sumitomo Pharma Biomedical Co, Ltd. (Osaka, Japan), and stable transformants of B16-F1 cells expressing luciferase (B16-F1-Luc) were established in our laboratory [16]. These cells were cultivated in DMEM supplemented with 10% FBS at 37 ^oC in 5% CO_2.

2.2 Electric treatment of the cells

For *in vitro* ET, cells were cultivated in 35 mm dishes. The number of cells used is mentioned in each section below. After 18 hr of cultivation, cells were washed with PBS, and

then 1 ml serum free DMEM containing 0.5 μg ISMB-Luc, 25 μM calcein, 100 pmol anti-Luc siRNA or anti-GFP siRNA was added to the cells. Ag-AgCl electrodes with 2.5 cm^2 surface area (3M Health Care, Minneapolis, MN, USA) were placed into the dish, and cells were treated with a constant current of 0.34 mA/cm^2 for 15 min.

2.3 Transfection and measurement of luciferase activity

B16-F1-Luc cells were cultivated at a density of $1x10^4$ cells in 35 mm culture dishes. After 18 hr of incubation, cells were washed with PBS, and 1 ml serum free DMEM containing 100 pmol anti-luc siRNA was added before the cells were treated with electricity as described above. After 3 hr of ET, 1 ml DMEM containing 10% FBS was added, and the cells were further incubated for 45 hr. After the incubation, the cells were lysed with Reporter Lysis Buffer (Promega) according to the manufacturer's protocols. The luciferase assay substrate (Promega) was added to cell lysates, and chemiluminescence intensity was measured by a luminometer (Luminescensor-PSN, ATTO). The total protein concentration was measured with BCA protein assay kit (Thermo Scientific).

2.4 Cytotoxicity assay of ET treated cells

For determination of cytotoxicity, $4x10^5$ B16-F1-Luc cells were cultivated in 35 mm culture dishes. After 18 hr of cultivation, cells were washed by PBS and added 1ml of serum free DMEM medium followed by ET for 15 min. Since the cytotoxicity by electricity-based delivery system, such as electroporation, had been observed at immediately after the treatment [17], we evaluated the cell viability at immediately after ET in this study. Immediate after ET, cells were collected from the dish by trypsin treatment, and taken 10 μl of cell suspension into a micro tube. An equal volume (10 μl) of 0.4% trypan blue solution (Wako Pure Chemical Industries, Ltd.

Osaka, Japan) was added to the cell suspension, and the mixture was incubated for 2 min. Then, the numbers of stained and non-stained cells were counted. The percentage of viability was calculated by the formula (100 x number of non-stained cells/ total number of cells).

2.5 Treatment with low temperature or pharmacological inhibitors and measurement of fluorescence intensity

For mechanistic studies, B16-F1 cells were seeded at a density of $1x10^5$ cells in 35 mm culture dishes. After 18 h of cultivation, cells were washed with PBS. For low temperature experiments, 1 ml serum free DMEM containing 100 pmol rhodamine-labeled siRNA was added into the dish, and ET was then performed on ice. For pharmacological inhibitor experiments, 1 ml serum free DMEM containing either 0.45 M sucrose, 1 mM amiloride, 0.5 μg filipin, or 25 μM SKF96365 was added into the dish, and the dish was incubated for 15 min. After the incubation, rhodamine labeled siRNA (100 pmol) was added to the dish, and the cells were treated with electricity as described above. After the ET, the cells were incubated for 1 hr at 37 °C. The cells were then lysed with reporter lysis buffer (Promega) according to the manufacturer's protocols. Fluorescence intensity of the cell lysates was measured with a microplate reader Infinite 200 (Tecan Group Ltd., Männedorf, Switzerland) at excitation and emission wavelengths of 546 nm and 576 nm, respectively.

2.6 Membrane potential measurement

To measure membrane potential, $5x10^4$ cells were seeded in 35 mm culture dishes. After 18 hr, cells were washed with PBS, and 2 ml DMEM containing 5 μM $DiBAC_4(3)$ dye was added before the cells were incubated for 30 min at room temperature. After incubation the cells

were treated with electricity for 15 min. ET was performed on ice in case of low temperature exposure. Then the cells were washed with PBS. The cells were lysed with reporter lysis buffer (Promega) according to the manufacturer's protocols. Fluorescence intensity of the cell lysate was measured using the microplate reader.

2.7 Confocal laser scanning microscopic observation of the cells after electric treatment

For evaluation of intracellular delivery of ISMB-Luc, $5x10^4$ cells were seeded on 0.002% poly-L-lysine coated 35 mm glass bottom dishes. After 18 hr, cells were washed with PBS and treated with ET (0.34 mA/cm^2 for 15 min). After ET, the cells were incubated for 1 hr at 37 °C in 5% CO_2. After incubation, the cells were observed with a confocal laser scanning microscope A1R+ (Nikon Co. Ltd., Japan). For time-lapse imaging, observation was performed immediately after ET.

2.8 Measurement of fluorescent intensity of the cells treated with ISMB

For the comparison of ET based delivery with LFN based delivery of ISMB, B16-F1 cells were cultivated at a density of $0.5x10^5$ cells in 35 mm culture dishes. After 18 hr of cultivation, cells were washed with PBS. For ET, 800 μl of serum free DMEM containing 0.5 μg ISNB-Luc added into the dish. For LFN treatment, 800 μl of serum free DMEM containing ISMB-Luc/LFN lipoplex prepared with 0.5 μg ISNB-Luc and 1 μl LFN (according to the manufacturer guide line) was added into the dish. The cells were washed with PBS after ET (0.34 mA/cm^2, 15 min) with ISMB-Luc or addition of LFN/ISMB-Luc lipoplex at various time points, and lysed cell with reporter lysis buffer (Promega) according to the manufacturer's protocols. Fluorescence intensity of the cell lysates was measured by a microplate reader Infinite

200 (Tecan Group Ltd., Männedorf, Switzerland) at excitation and emission wavelengths of 525 nm and 580 nm, respectively.

2.9 Statistical analysis

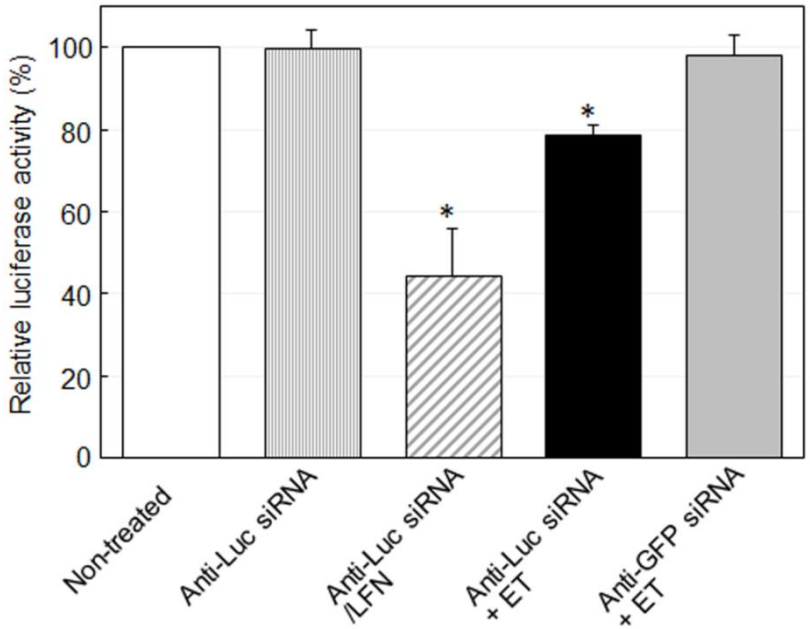
Statistical analysis was determined using one-way ANOVA followed by Turkey-Kramer HSD test. P values <0.05 were considered to be significant.

3. Results

3.1 Effect of *in vitro* electric treatment on luciferase expression of cells with anti-luciferase siRNA

To evaluate *in vitro* faint electric treatment (ET) on cellular uptake, the effect of ET with anti-luciferase (Luc) siRNA on luciferase activity of B16-F1-Luc cells stably expressing luciferase was examined. ET with anti-Luc siRNA significantly suppressed luciferase activity, which was similar to our previous report of iontophoresis of anti-interleukin 10 siRNA *in vivo* [10], although the RNAi effect was weaker than that for positive control cells incubated with Lipofectamine 2000/anti-Luc siRNA lipoplexes (Fig. 1). No suppression effect was observed for cells exposed to anti-GFP siRNA, indicating that the suppression effect was specific for anti-Luc siRNA. The cytotoxicity was evaluated immediately after ET. No cytotoxicity was recognized even after ET (Supplemental Fig. 1). This result indicates that ET did promote delivery of siRNA into the cytoplasm without the need for any modification of siRNA or other functional devices.

Figure 1. Effect of anti-Luc siRNA on luciferase activity in the presence of *in vitro* electric treatment (ET)



Luciferase activity of cells expressing luciferase was measured after 48 hr of ET (0.34 mA/cm^2, for 15 min) in the presence of naked siRNA solution. Lipofectamine 2000/anti-Luc siRNA lipoplex was used as a positive control. LFN: lipofectamine 2000, ET: electric treatment. Data are expressed as mean±SD. *p<0.05.

3.2 Effect of low temperature and pharmacological inhibitors on ET-induced cellular uptake of siRNA

To study the mechanism of ET-mediated intracellular delivery, the effects of low temperature (0-4 °C) and endocytosis inhibitors on cellular uptake of rhodamone-labeled siRNA were examined. The fluorescence intensity of cellular lysates was significantly increased by ET at room temperature (Fig. 2), indicating that cellular uptake of rhodamone-labeled siRNA was

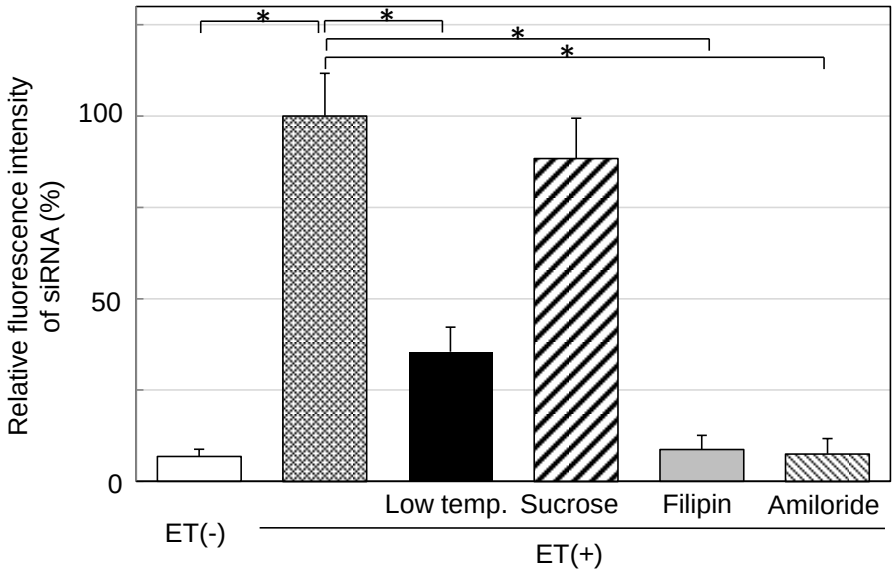


Figure 2. Effects of low temperature and endocytosis inhibitors on cellular uptake of rhodamine-labeled siRNA
For low temperature treatment, ET of cells was performed on ice. To examine the effect of inhibitors, cells were pre-treated for 15 min with inhibitors prior to the exposure to electricity (0.34 mA/cm^2, for 15 min) in the presence of siRNA solution. After 1 hr of ET, the cells were lysed, and the fluorescence intensity of the lysate was measured. Data are expressed as mean±SD. *p<0.05.

enhanced by ET. Low temperature (0-4 °C) treatment is commonly used to nonspecifically inhibit energy-dependent cellular uptake [18]. Thus, we assessed the effect of low temperature on ET-mediated cellular uptake by treating cells with electricity in the presence of rhodamone-labeled siRNA on ice. As a result, low temperature treatment significantly reduced the fluorescence intensity, which implies that cellular uptake of siRNA was inhibited (Fig. 2). This result suggests that ET activated biological processes involving energy-dependent pathways.

To specify the cellular uptake pathway induced by ET, effects of the macropinocytosis inhibitor amiloride, the caveolae-mediated endocytosis inhibitor filipin, and the clathrin-mediated endocytosis inhibitor hypertonic sucrose on fluorescent-labeled siRNA uptake were examined. Clathrin-mediated endocytosis inhibitor hypertonic sucrose slightly inhibited siRNA uptake, although the inhibition was not statistical significant. Furthermore, macropinocytosis inhibitor amiloride and caveolae-mediated endocytosis inhibitor filipin showed significant reduction of fluorescent labeled-siRNA uptake.

3.3 Evaluation of the effect of ET on membrane potential

Since ET was previously suggested to affect intracellular ion balance [11], ET may also promote altered membrane potentials that could enhance the cellular uptake of extraneous materials. Thus, to evaluate the effect of ET on membrane potential, cells were treated with electricity in the presence of the voltage sensitive fluorescent dye $DiBAC_4(3)$ [19, 20]. After ET, the fluorescence intensity of cells treated with ET was higher than the control cells (Fig. 3), indicating that ET decreased the membrane potential of the cells through a process that may involve the influx of cations such as Ca^{2+}. In quantitative measurements of fluorescence intensity, intracellular amounts of $DiBAC_4(3)$ increased by 13 % (Fig. 3). In order to confirm the

possibility of physical penetration of DiBAC4(3), the effect of low temperature on cellular staining with DiBAC4(3) dye was examined. As a result, the fluorescent intensity depending on DiBAC4(3) dye was almost the same as that of control cells (Fig. 3), indicating that the membrane potential was not changed at low temperature even after ET. This result suggested

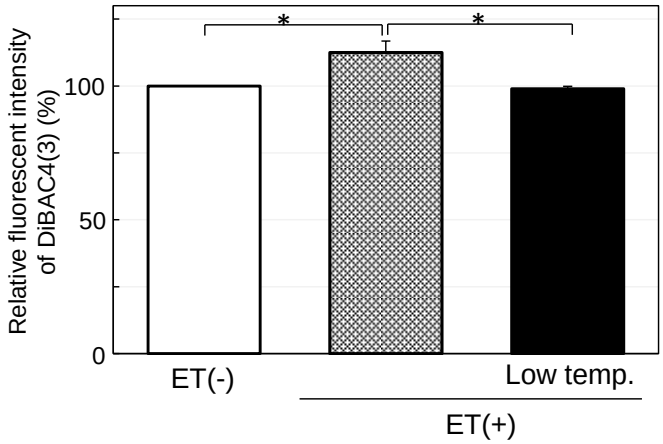


Figure 3. Evaluation of DiBAC4(3) cellular penetration in the presence of faint ET

Cells were incubated for 30 min with $DiBAC_4(3)$ followed by ET (0.34 mA/cm^2) for 15 min. For low temperature exposure, ET was performed on ice. After ET, cells were lysed and the fluorescence intensity of the lysate was measured. Data are expressed as mean ± SD. *$p<0.05$.

that ET-induced activation of cation channels, such as transient receptor potential (TRP) channels, could have resulted in the observed alterations in membrane potential. Thus, to analyze the role of TRP channels in ET-mediated cellular uptake, the effect of the TRP channel blocker SKF96365 [21] on the fluorescence intensity of cells treated with ET in the presence of fluorescent-labeled siRNA was examined. ET-mediated cellular uptake of siRNA was significantly reduced by SKF96365 (Fig. 4). Nonetheless, this result suggests that activation of cation channels, including TRP channels, may have some involvement in ET-mediated cellular uptake.

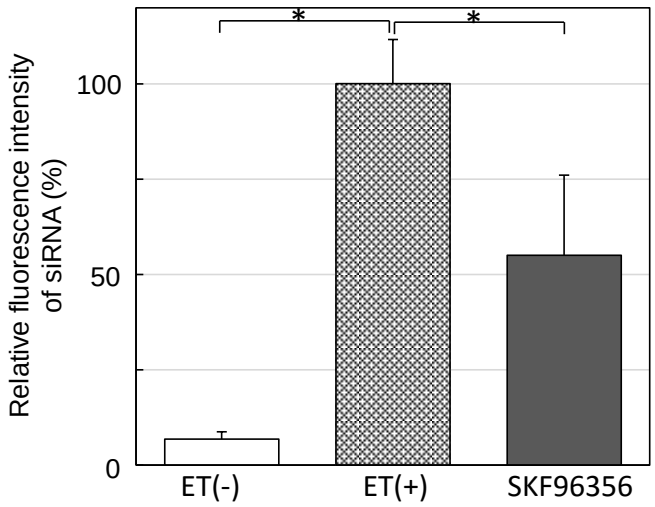


Figure 4. Effect of TRP channel inhibitor SKF96365 on cellular uptake of rhodamine-labeled siRNA
Cells were pre-treated for 15 min with SKF96365 before being treated with electricity (0.34 mA/cm^2, for 15 min) in the presence of rhodamine-labeled siRNA solution. After ET, cells were lysed and the fluorescence intensity of the lysate was measured. Data are expressed as mean ± SD. *$p<0.05$.

3.4 Evaluation of ET-mediated cytoplasmic delivery processes

As mentioned above, ET could induce cytoplasmic delivery during the uptake of extraneous materials. Thus, the materials must overcome membranous barriers, such as plasma membrane and endosomal membrane, after ET-mediated cellular uptake. To analyze cytoplasmic delivery processes after ET-mediated cellular uptake, cytoplasmic delivery of in-stem molecular beacon (ISMB), which can show potent fluorescence signals upon association with target mRNA (here, luciferase, ISMB-Luc), into B16F1-Luc cells was evaluated by confocal laser scanning microscopy. One hour after ET, all cells showed potent red fluorescence signals, although no fluorescence signals were observed in the cells without ET, even in the presence of ISMB-Luc

(Fig. 5). On the other hand, no fluorescence signal was seen in the cells after 1 hr ET in the presence of ISMB against GFP (ISMB-GFP). These results indicated that ISMB-Luc efficiently

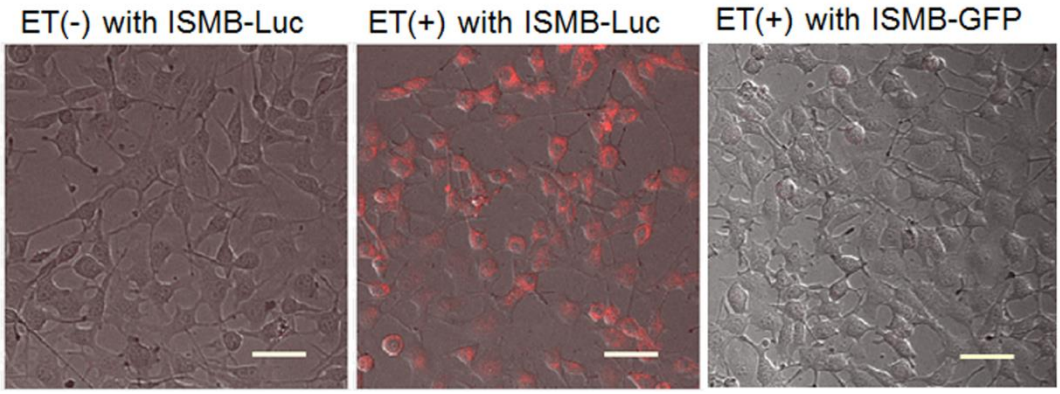


Figure 5. Cytoplasmic delivery of in-stem molecular beacon (ISMB) induced by faint ET
Cells were treated with faint electricity (0.34 mA/cm^2, 15 min) in the presence of ISMB solution. After 1 hr, the cells were observed with a confocal laser scanning microscope. Left and middle panel images are cells incubated in medium containing ISMB against luciferase mRNA (ISMB-Luc) with or without ET. The image in the right panel shows cells incubated in medium with ISMB against GFP mRNA (ISMB-GFP) in the presence of ET. The scale bars indicate 50 μm.

reach to the cytoplasm after ET-mediated cellular uptake and specifically bound with target luciferase mRNA in the cytoplasm.

To evaluate cytoplasmic delivery processes, we next performed time-lapse imaging of cells immediately after ET. Surprisingly, ISMB fluorescence signals appeared in the cytoplasm immediately after ET, and the fluorescence intensity rapidly increased for 15 min (Supplemental Fig. 2). In contrast, no fluorescence signals were observed in cells without ET even after 15 min. This result confirmed that endosomal escape of extraneous materials after ET-mediated cellular uptake was very rapid and effective. Furthermore, to evaluate the speed of cytoplasmic delivery of ET method, fluorescent intensities of the cells treated by electricity in the presence of ISMB-Luc at various time points were compared with the cells treated by LFN/ISMB-Luc lipoplex. As a result, the significant fluorescent intensity was observed in the cells of ET method even at 15

min. The fluorescent intensity of ET was over 4 fold higher than that of LFN at 30 min. (Supplemental Fig. 3) Furthermore the cells after ET with ISMB-Luc or transfection of LFN/ISMB-Luc were observed by confocal laser scanning microscopy. As a result, red fluorescent signals were observed in all cells treated with electricity, although heterogeneous distribution of red fluorescent signals recognized in the cells after transfection of LFN. (Supplemental Fig. 4)

4. Discussion

Cytoplasmic delivery of hydrophilic molecular medicines, such as functional nucleic acids, is difficult to achieve due to membranous barriers, including the plasma membrane and endosomal membrane [22]. Thus, ineffective delivery is an important concern that must be addressed in order to improve drug delivery methods. Recently, we found that *in vivo* faint electric treatment (ET) iontophoresis can deliver functional siRNA into the cytoplasm of skin cells [10]. Based on this finding, we hypothesized that faint ET could enhance cellular uptake and cytoplasmic delivery of extraneous hydrophilic molecules by affecting cellular physiology.

In this study, we first confirmed that the significant RNAi effect by faint ET in vitro (Fig. 1). Although the suppression effect was lower than that of Lipofectamine 2000/siRNA lipoplexes, this result indicates that siRNA was delivered into the cytoplasm by electricity and without any modification of siRNA to promote effective cellular association and endosomal escape. The reduced effect of ET in facilitating RNAi may be due to the amount of siRNA that reached the cytoplasm. Although lipoplexes can deliver much higher amounts of siRNA as condensed particles into cells, ET induced cellular uptake of a homogenous siRNA solution rather than concentrated bodies. Thus, delivery efficiency could be improved with additional studies.

We next found that the cellular uptake of siRNA was enhanced by ET in the presence of fluorescent-labeled siRNA (Fig. 2). The ET-enhanced cellular uptake of labeled siRNA was significantly reduced by low temperature treatment (Fig. 2), indicating that ET-mediated cellular uptake processes likely involved energy dependent pathways. In order to further characterize the uptake process, we used a wide range of endocytosis inhibitors, including the clathrin-mediated endocytosis inhibitor hypertonic sucrose [12], the caveolae-mediated endocytosis inhibitor filipin [13] and the macropinocytosis inhibitor amiloride [14]. Partial inhibitory effect was induced by

hypertonic treatment with sucrose, while significant inhibitory effects were obtained by amiloride and filipin treatment (Fig. 2). It was suggested that endocytosis, such as macropinocytosis or caveolae-mediated endocytosis, would be involved in ET mediated uptake of siRNA.

The voltage clamping membrane potential was previously shown to vary between -25 mV and -90 mV for BICR/MIR-k cells, and the fluorescence response of the voltage sensitive dye $DiBAC_4(3)$ was found to be about 1% per mV [20]. In the present study, we observed that ET significantly increased the fluorescence intensity of $DiBAC_4(3)$ by 13 % over that of control cells (Fig. 3). Thus, this result indicated that ET induced a membrane potential decrease in approximately 13 mV. The fluorescent intensity did not increase at low temperature. It was suggested that membrane potential change by ET would be due to biological mechanism, not physical factors such as membrane leak. Moreover, activation of non-specific cation channels- known as TRP channels- is known to alter cellular membrane potentials [23]. Here, cellular uptake of fluorescent-labeled siRNA was significantly reduced by the TRP channel blocker SKF96365 (Fig. 4), indicating that cationic channels would be involved in ET-mediated cellular uptake processes. Together these findings implicate membrane potential changes via activation of cationic channels, such as TRP channels, in contributing to ET-mediated cellular uptake of extraneous materials. Previously we reported that Ca^{2+} influx-mediated PKC activation could be responsible for iontophoresis-induced opening of intercellular junctions in the skin [11]. Therefore, combining the previous *in vivo* findings and the *in vitro* findings of the present study, faint ET appears to regulate physiology of tissues and cells by promoting cationic ion influx.

Breakthrough of membranous barriers is also an important step for cargo molecules to be delivered to the cytoplasm following cellular uptake [24]. We monitored the cytoplasmic

delivery process using ISMB-Luc. The ISMB probe is a hairpin-shaped oligonucleotide that contains fluorophores and quenchers. In the absence of target mRNA, the fluorescence emission of the probe is quenched by interactions between the fluorophores and quenchers. In the presence of target mRNA, the ISMB hairpin structure opens, thus releasing the fluorophore to result in fluorescence emission [15]. Interestingly, we observed that all cells showed strong red fluorescence after 1 hr of ET with ISMB-Luc (Fig. 5), which supports that ET induced efficient cytoplasmic delivery of ISMB-Luc, and that ISMB-Luc could associate with luciferase mRNA in the cytoplasm. Surprisingly, fluorescence signals appeared immediately after ET, and the intensity of the signal increased rapidly for a short period (Supplemental Fig. 2), indicating that cytoplasmic delivery was indeed enhanced by faint electric treatment.

Although the knockdown effect by siRNA treated with ET was weaker than that with LFN, the ET method homogenously and very rapidly delivered functional nucleotide ISMB into cytoplasm of all cells (Fig. 5 and Supplemental Figs. 2 and 4). Even 15 min after ET, red fluorescent signals were observed in cytoplasm (Supplemental Figs. 2 and 3), indicating that ISMB rapidly reached and bind with a target mRNA. Furthermore, from the results of comparison of ISMB cytoplasmic delivery between ET method and LFN, it was suggested that ISMB was delivered rapidly and homogeneously into cytoplasm by ET method, and the delivery speed into cytoplasm of ET method was faster than LFN (Supplemental Figs. 3 and 4). Thus, the rapid and homogenous delivery of functional nucleotides into cytoplasm is the advantage of ET, although the delivery efficiency should be improved in the future. Since functional nucleic acid medicines are easily degraded by degradation enzymes at the extracellular environment and inside of endosome, long stay in such situation reduce activity of the medicines. Thus, rapid cellular uptake and cytosolic delivery would be useful for effective therapy due to avoiding the

decrease in activity of nucleic acids. Furthermore, for effective therapy, medicines should be delivered into all target cells. Since heterogeneous delivery is the cause of inefficient RNAi effect [25], siRNA should be delivered into all cells for effective regulation of target genes. Thus, homogenous delivery would be useful for effective therapy. As mentioned above, the reason for lower RNAi effect by ET method than that of LFA would be due to less cellular uptake amount of siRNA. Thus, the cooperative effect by combination of ET method with cationic non-viral delivery system is expected to improve delivery efficiency of ET method. The accumulation of cargo on the cell surface would increase by cationic carriers, and cellular uptake and cytosolic delivery would be enhanced by ET method. Thus, we expected that ET method would be improved by combination with other delivery methods, such as cationic non-viral delivery systems. To our knowledge, this study represents the first report of rapid and effective cytoplasmic delivery of extraneous hydrophilic materials induced by faint ET, although the underlying mechanism for this rapid delivery into the cytoplasm await further investigation.

5. Conclusion

In the present study we found that faint electric treatment can enhance cellular uptake and rapid cytosolic delivery by inducing energy-dependent pathways along with the activation of cationic channels. Thus, faint electric treatment could be useful for effective and safe delivery of extraneous hydrophilic molecules, such as siRNA, miRNA and decoy-oligo DNA, into the cytoplasm of target cells.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas ‘‘Nanomedicine Molecular Science’’ (No. 2306) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

6. References

[1] A. I. Khalil, K. Kogure, H. Akita, H. Harashima, Uptake pathways and subsequent intracellular trafficking in non-viral gene delivery, Pharmacol. Rev. 58 (2006) 32-45.

[2] S. Xiang, H. Tong, Q. Shi, J.C. Fernandes, T. Jin, K. Dai, X. Zhang, Uptake mechanism of non-viral gene delivery, J. Control. Release 158 (2012) 371-378.

[3] L. Chou, K. Ming, W. Chan, Strategies for the intracellular delivery of nanoparticles, Chem. Soc. Rev. 40 (2011) 233-245.

[4] A.J. Mellott, M.L. Forrest, M.S. Detamore, Physical non-viral gene delivery methods for tissue engineering, Ann. Biomed. Eng. 41 (2013) 446-68.

[5] L. Xu, T.J. Anchordoquy, Drug delivery trends in clinical trials and translational medicine: challenges and opportunities in the delivery of nucleic acid based therapeutics, J. Pharm. Sci. 100 (2011) 38-52.

[6] X. Guo, L. Huang, Recent advances in non-viral vectors for gene delivery, Acc. Chem. Res. 45 (2012) 971-979.

[7] C.M. Schoellhammer, D. Blankschtein, R. Langer, Skin permeabilization for transdermal drug delivery: recent advances and future prospects, Expert Opin. Drug Deliv. 11 (2014) 393-407.

[8] T.W. Wong, Electrical, magnetic photomechanical and cavitation waves to overcome skin barrier for transdermal drug delivery, J. Control. Release 193C (2014) 257-269.

[9] T. Gratieri, I. Aiberti, M. Lapteva, Y.N. Kalia, Next generation intra and transdermal therapeutic system: using non- and minimally invasive technologies to increase drug delivery into and across the skin, Eur. J. Pharm. Sci. 50 (2013) 609-622.

[10] K. Kigasawa, K. Kajimoto, S. Hama, A. Saito, K. Kanamura, K. Kogure, Noninvasive delivery of siRNA into the epidermis by iontophoresis using an atopic dermatitis-like model rat, Int. J. Pharm. 383 (2010) 157-160.

[11] S. Hama, Y. Kimura, A. Mikami, K. Shiota, M. Toyoda, A. Tamura,Y. Nagasaki, K. Kanamura, K. Kajimoto, K. Kogure, Electric stimulus opens intracellular space in skin, J. Biol. Chem. 289 (2014) 2450-2456.

[12] J.E. Heuser, R.G.W. Anderson, Hypertonic media inhibit receptor mediated endocytosis by blocking clathrin-coated pit formation, J. Cell Biol. 108 (1989) 389-400.

[13] J. Rejman, A. Bragonzi, M. Conese, Role of clathrin and caveolae-mediated endocytosis in gene transfer mediated by lipo and polyplex, Mol. Ther. 12 (2005) 468-474.

[14] J.T.H. Wang, R.D. Teasdale, D. Liebl, Macropinosome quantitation assay, MethodsX 1 (2014) 36-41.

[15] H. Kashida, T. Osawa, K. Morimoto, Y. Kamiya, H. Asanuma, Molecular design of Cy3 derivative for highly sensitive in-stem molecular beacon and its application to the wash-free FISH, Bioorg. Med. Chem. 23 (2015) 1758-1762.

[16] A. Mitsueda, Y. Shimatani Y, M. Ito, T. Ohgita, A. Yamada, S. Hama, A. Gräslund, S. Lindberg, U. Langel, H. Harashima, I. Nakase, S. Futaki, K. Kogure, Development of a novel nanoparticle by dual modification with the pluripotential cell-penetrating peptide PepFect6 for cellular uptake, endosomal escape, and decondensation of an siRNA core complex, Biopolymers 100 (2013) 698-704.

[17] K.J. Stacey, I.L. Ross, D.A. Hume, Electroporation and DNA-dependent cell death in murine macrophages, Immunol. Cell Biol. 71 (1993) 75-85.

[18] C.C. Chang, M. Wu, F. Yuan, Role of specific endocytic pathways in electrotransfection of cells, Mol. Ther. Methods Clin. Dev. 1 (2014) 14058.

[19] K.R. Konrad, R. Hedrich, The use of voltage-sensitive dyes to monitor signal induced changes in membrane potential-ABA triggered membrane depolarization in guard cells, Plant J. 55 (2008) 161-173.

[20] T. Brauner, D.F. Hulser, R.J. Strasser, Comparative measurement of membrane potentials with microelectrodes and voltage sensitive dyes, Biochim. Biophys. Acta. 771 (1984) 208-216.

[21] A. Singh, M. Hildebrand, E. Garcia, T. Snutch, The transient receptor potential channel antagonist SKF96365 is a potent blocker of low-voltage-activated T-type calcium channel, Br. J. Pharmacol. 160 (2010) 1464-1475.

[22] M. Elsabahy, A. Nazarali, M. Foldvari, Non-viral nucleic acid delivery: key challenges and future directions, Curr. Drug Deliv. 8 (2011) 235-244.

[23] J. Zheng, Molecular mechanism of TRP channels, Compr. Physiol. 3 (2013) 221-242.

[24] A.K. Varkouhi, M. Scholte, G. Storm, H.J. Haisma, Endosomal escape pathways for delivery of biologicals, J. Control. Release 151 (2011) 220-228.

[25] A.A. Chen, A.M. Derfus, S.R. Khetani, S.N. Bhatia, Quantum dots to monitor RNAi delivery and improve gene silencing, Nucleic Acids Res. 33 (2005) e190.

Figure legends

Figure 1. Effect of anti-Luc siRNA on luciferase activity in the presence of *in vitro* electric treatment (ET)

Luciferase activity of cells expressing luciferase was measured after 48 hr of ET (0.34 mA/cm^2, for 15 min) in the presence of naked siRNA solution. Lipofectamine 2000/anti-Luc siRNA lipoplex was used as a positive control. LFN: lipofectamine 2000, ET: electric treatment. Data are expressed as mean±SD. *$p<0.05$.

Figure 2. Effects of low temperature and endocytosis inhibitors on cellular uptake of rhodamine-labeled siRNA

For low temperature treatment, ET of cells was performed on ice. To examine the effect of inhibitors, cells were pre-treated for 15 min with inhibitors prior to the exposure to electricity (0.34 mA/cm^2, for 15 min) in the presence of siRNA solution. After 1 hr of ET, the cells were lysed, and the fluorescence intensity of the lysate was measured. Data are expressed as mean±SD. *$p<0.05$.

Figure 3. Evaluation of DiBAC4(3) cellular penetration in the presence of faint ET

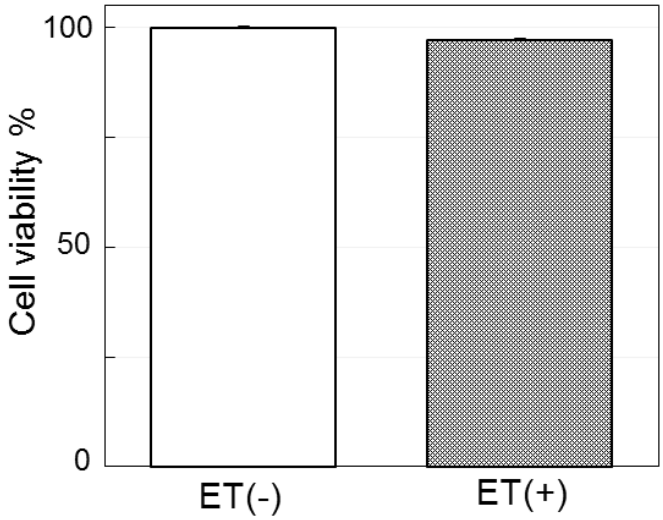
Cells were incubated for 30 min with DiBAC4(3) followed by ET (0.34 mA/ cm^2) for 15 min. For low temperature exposure, ET was performed on ice. After ET, cells were lysed and the fluorescence intensity of the lysate was measured. Data are expressed as mean ± SD. *$p<0.05$.

Figure 4. Effect of TRP channel inhibitor SKF96365 on cellular uptake of rhodamine-labeled siRNA

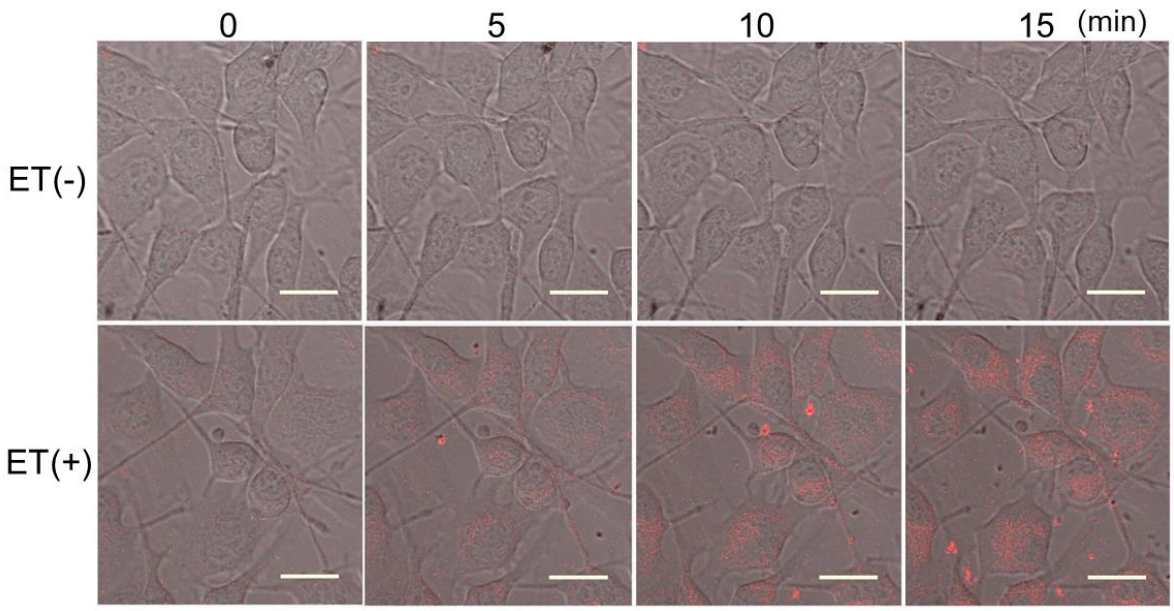
Cells were pre-treated for 15 min with SKF96365 before being treated with electricity (0.34 mA/cm^2, for 15 min) in the presence of rhodamine-labeled siRNA solution. After ET, cells were lysed and the fluorescence intensity of the lysate was measured. Data are expressed as mean ± SD. *$p<0.05$.

Figure 5. Cytoplasmic delivery of in-stem molecular beacon (ISMB) induced by faint ET

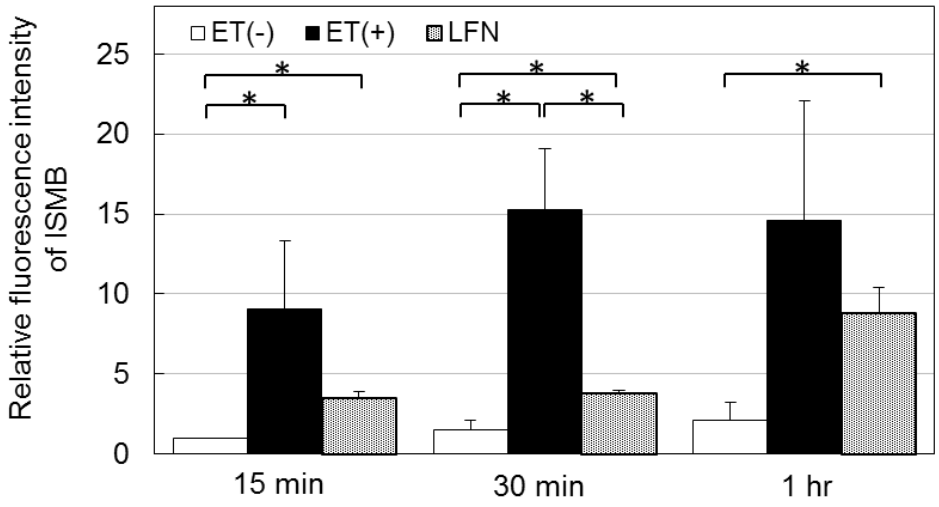
Cells were treated with faint electricity (0.34 mA/cm^2, 15 min) in the presence of ISMB solution. After 1 hr, the cells were observed with a confocal laser scanning microscope. Left and middle panel images are cells incubated in medium containing ISMB against luciferase mRNA (ISMB-Luc) with or without ET. The image in the right panel shows cells incubated in medium with ISMB against GFP mRNA (ISMB-GFP) in the presence of ET. The scale bars indicate 50 μm.



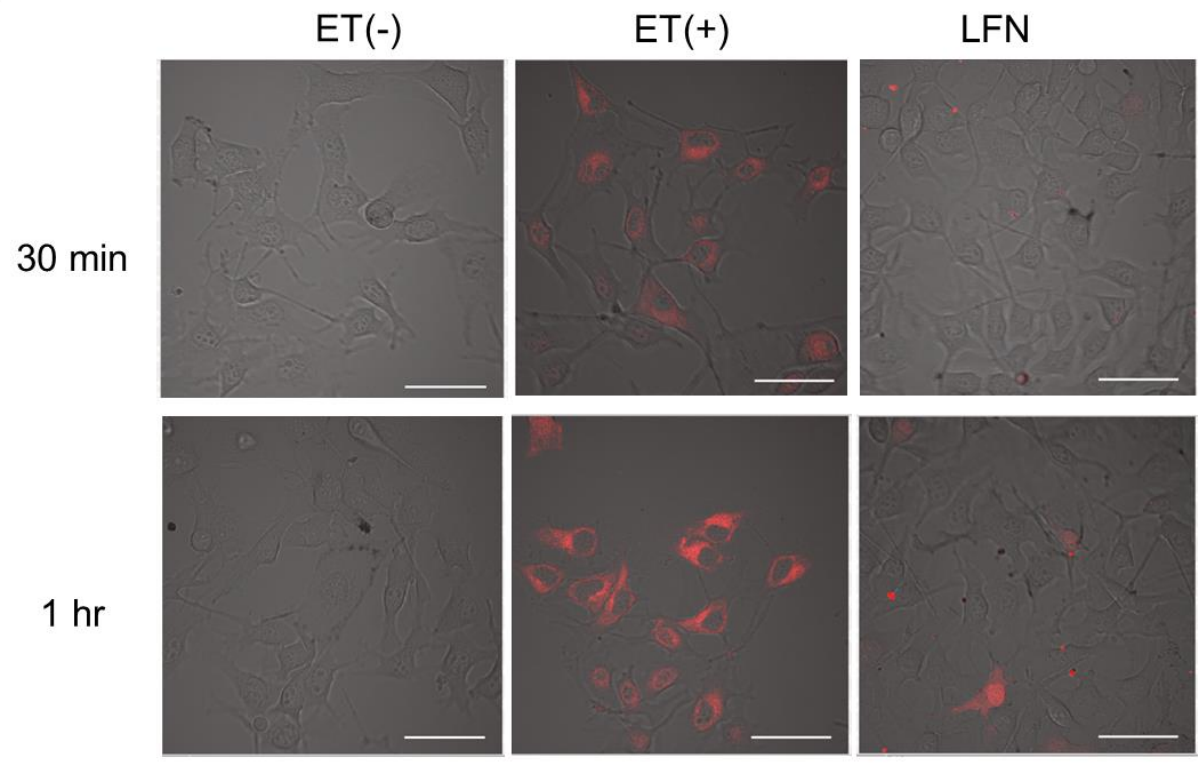
Supplemental Figure 1. Cell viability of cells treated by electricity Immediate after 15 min of ET (0.34 mA/cm^2, 15 min), cells were stained with trypan blue solution. The numbers of stained and non-stained cells were counted. The percentage of viability was calculated by the formula (100 x number of non-stained cells/ total number of cells). Data are expressed as mean ± SD.



Supplemental Figure 2. Time-lapse observation of ET-mediated cytoplasmic delivery of ISMB-Luc
Cells were treated with faint electricity (0.34 mA/cm^2, 15 min) in the presence of ISMB-Luc solution, and time-lapse images were taken immediately after ET and at 5 min intervals until 15 min. The scale bars indicate 20 μm.



Supplemental Figure 3. Time-dependent change of fluorescent intensity of in-stem molecular beacon (ISMB)-Luc delivered into cytoplasm
Cells were treated with faint electricity (0.34 mA/cm^2, 15 min) in the presence of ISMB solution or LFN/ISMB lipoplex. After 15 min, 30 min and 1 hr from the start of ET or LFN treatment, fluorescent intensity of the cells were measured. Data are expressed as mean ± SD. *$p<0.05$.



Supplemental Figure 4. Cytoplasmic delivery of in-stem molecular beacon (ISMB) induced by faint ET
Cells were treated with faint electricity (0.34 mA/cm^2, 15 min) in the presence of ISMB-Luc solution or transfected with LFN/ISMB-Luc lipoplex. At the time points at 30 min or 1 hr from the start of ET or LFN transfection, the cells were observed with a confocal laser scanning microscope. The scale bars indicate 50 μm.