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Rac-Mediated Macropinocytosis Is a Critical Route for Naked Plasmid DNA Transfer in Mice

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Abstract: We have recently discovered the potential for in vivo naked plasmid DNA (pDNA) transfer into gastric serosal surface cells in mice. As pDNA are huge molecules, the mechanism of gene transfer without carriers and physical forces is of great biological interest. The endocytic route for naked pDNA transfer into gastric mesothelial cells was not clathrin- or caveolae-mediated endocytosis, but macropinocytosis. Naked pDNA transfer required both actin polymerization and myosin-based contraction. Upstream kinases of Rho family GTPases, Syk, Src family kinases and PI-3K were involved in naked pDNA transfer. Furthermore, the intracellular signaling pathway was not mediated via the Rho pathway, but by the Rac pathway. Downstream molecules of Rac, PAK and WAVE2 co-operated with naked pDNA transfer. Overall, it was demonstrated that the Rac signaling pathway regulated the macropinocytosis of naked pDNA. The information in this study would be helpful to clarify in vivo cell functions and to improve in vivo transfection efficiency.

Keywords: Naked plasmid DNA; mechanism of in vivo gene transfer; macropinocytosis; signaling pathway; Rac

Introduction

Gene therapy is a promising approach to treat acquired, refractory and fatal diseases as well as inherited diseases.[1−4] Gene delivery systems *in vivo* can be categorized as viral and nonviral approaches. Safety in the usage of viral and nonviral vectors for clinical gene therapy is not yet sufficient. Both viral and nonviral vectors have toxic side effects.[5−7] Viral vectors have immunogenicity; consequently, repeated administration is generally limited. Some viral vectors randomly insert foreign genes into the genome; as a result, they have tumorigenicity. On the other hand, nonviral vectors such as cationic liposomes and polymers also have toxic side effects. Cationic liposome/plasmid DNA (pDNA) complex and polyethyleneimine/pDNA complex induce hemagglutination.[8,9] Cationic liposomes induce the apoptosis of macrophages;[10] thus, safety concerns along with the gene delivery system must be resolved prior to clinical use.

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Naked pDNA is the simplest and safest nonviral vector as naked pDNA can be used without considering the cytotoxicity of gene carriers. When naked pDNA was administered *via* the vasculature route, it was rapidly degraded by reticuloendothelial cells (liver Kupffer cells, etc.) and nuclease in the blood;[11] as a consequence, transgene expression after intravenous injection of naked pDNA was negligible.[12] It was previously reported that *in vivo* gene transfer using naked pDNA could be achieved by hydrodynamics-based intravenous injection (high volume injection at high velocity),[13,14] direct injection into tissues,[15] electroporation,[16] and mechanical massage;[17] however, there is great concern about safety because these procedures require physical forces against tissues; consequently, clinical application requiring repeated administration of pDNA is limited.

In contrast, we discovered a less-invasive gene delivery method for simple instillation of naked pDNA onto the liver,[12] kidney,[18] spleen,[19] and gastric serosal surface[20,21] of mice. This method requires neither carriers nor physical forces to achieve efficient gene transfer. As naked pDNA has a high molecular weight (several million), it is biologically important that certain cells actively take up such huge molecules and express the transgene encoded by pDNA. Thus, the mechanism of naked pDNA transfer is of great interest to clarify cell functions. In this study, we elucidated the *in vivo* mechanism of the uptake of naked pDNA, which is required for transgene expression in gastric mesothelial cells in mice.

Materials and Methods

Reagents. Glucose, chlorpromazine hydrochloride, dextran and sodium dextran sulfate were purchased from Nacalai Tesque (Kyoto, Japan). TO-PRO-3, Alexa Fluor 555-conjugated Cholera Toxin Subunit B (recombinant), fixable tetramethylrhodamine (TMR)-dextran, and Alexa Fluor 633-conjugated phalloidin were purchased from Molecular Probes (Invitrogen, Carlsbad, CA). Methyl-β-cyclodextrin, amiloride hydrochloride, polycitidilic acids, polyinosinic acids and bovine serum albumin (BSA, fraction V) were purchased from Sigma-Aldrich (St. Louis, MO). Genistein, latrunculin B, ML-7 hydrochloride, piceatannol, LY294002, PP2, Y-27632, NSC23766, and PAK18 were purchased from Calbiochem (Merck Ltd., Tokyo, Japan). 1-Oleoyl lysophosphatidic acid was purchased from Cayman Chemical (Ann Arbor, MI). Mouse transferrin was purchased from Rockland (Gilbertsville, PA) and labeled with HiLyte Fluor 555 using a kit (Dojindo Laboratories, Kumamoto, Japan). Alexa Fluor 633-conjugated phalloidin was reconstituted with methanol, yielding 200 units/mL. Pharmacological inhibitors were reconstituted in accordance with the manufacturer's protocols.

Antibodies. Rat anti-zonula occludens-one (ZO-1) monoclonal antibody (dilution 1:50), rabbit anti-mesothelin polyclonal antibody (dilution 1:50), rabbit anti-WAVE2 polyclonal antibody (dilution 1:50), goat anti-phospho-myosin light chain (p-MLC, Thr18/Ser 19) polyclonal antibody (dilution 1:50), Texas red-labeled goat anti-rat IgG secondary antibody (dilution 1:100), and rhodamine-labeled bovine anti-rabbit IgG secondary antibody (dilution 1:100) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-RhoA monoclonal, anti-Rac1/2/3 polyclonal, and anti-Cdc42 monoclonal antibodies (dilution 1:50) were purchased from Cell Signaling Technology (Danvers, MA). DyLight 649 conjugated donkey anti-goat IgG (dilution 1:5000) was purchased from Rockland. Antibodies were diluted with PBS containing 1% BSA and centrifuged (15000*g*) before use.

pDNA. pCMV-luciferase was constructed as reported previously.[12] A variant of the *Zoanthus* sp. green fluorescent

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protein, ZsGreen, expressing vector pZsGreen1-N1 was purchased from Clontech Laboratories (Mountain View, CA). pDNA were amplified in the *Escherichia coli* strain DH5α, isolated, and purified using an EndoFree Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). pDNA dissolved in 5% glucose solution was stored at −20 °C prior to experiments. Fluorescein or tetramethyl-rhodamine labeling of pDNA (pCMV-luciferase) was performed using the Label IT Nucleic Acid Labeling Kit (Mirus Co., Madison, WI).

Animals. Male ddY mice were housed in a cage in an air-conditioned room and maintained on a standard laboratory diet (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and water *ad libitum*. All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of Nagasaki University.

Gastric Serosal Instillation of pDNA. Five-week-old male ddY mice (24.4−33.1 g) were anesthetized with sodium pentobarbital (40−60 mg/kg, intraperitoneal administration). Laparotomy was performed, and the stomach was exposed. Naked pDNA (1.5 μg/10 μL for fluorescein-labeled pDNA and 15 μg/10 μL for pZsGreen1-N1) was instilled onto the gastric serosal surface using a micropipet (PIPETMAN; Gilson Inc., Villiers-le-Bel, France). For experiments using an endocytosis marker, fluorescein-labeled pDNA solution contained each endocytosis marker at concentrations of 0.4 μg/μL, 0.03 μg/μL or 2.5 μg/μL for HiLyte555-transferrin, Alexa555-cholera toxin B, or TMR-dextran, respectively. After instillation, the peritoneum was sutured and then the mice were freed into the cage. At appropriate time intervals after naked pDNA administration, mice were subjected to immunohistochemistry.

Immunohistochemistry. At the indicated time after gastric serosal surface instillation of pDNA, mice were killed under anesthesia and the stomach was removed. Imprints of the gastric mesothelial cells were prepared by a modified method which was reported previously.[22] Briefly, the stomach was washed twice with saline and dried for 4−5 min at room temperature. Imprints of the gastric mesothelial cells were obtained on MAS-coated microslide glasses (SUPERFROST S-9441; Matsunami Glass Ind. Ltd., Osaka, Japan). Imprints were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilized for 5 min with PBS containing 0.2% Triton X-100. Nonspecific staining was reduced with an Image-iT FX signal enhancer (Invitrogen, Carlsbad, CA) before incubating the imprints with antibodies. Imprints were incubated with primary and secondary antibodies for 1 h to each antibody in a humidified chamber. After staining with antibodies, nuclei or F-actin were stained with TO-PRO-3 (dilution 1:2000 with PBS) or Alexa633-phalloidin (dilution 1:40 with PBS containing 1% bovine serum albumin) by 1 h incubation in a humidified chamber. SlowFade Gold antifade reagent (Invitrogen) was applied to imprints before mounting.

Laser Scanning Microscopy for Imprints. Imprints were analyzed by confocal laser scanning microscopy using LSM 510 META (Plan-Apochromat 63× NA 1.4 oil immersion objective lens; Carl Zeiss Microimaging Inc., Thornwood, NY). Laser lines used were 488 nm, 543 nm, and 633 nm to excite fluorescein or ZsGreen1; HiLyte555, Alexa555, TMR, or Texas red; and TO-PRO-3, Alexa633, or DyLight 649, respectively. Each dye was scanned in sequential mode to prevent fluorescence cross-talk. Acquisition software was ZEN 2007 (Carl Zeiss Microimaging Inc.). Each image is expressed in an *xy* plane except for Figure 3S in the Supporting Information panel A3 (*z*-stack).

Fluorescent Stereomicroscopy for the Stomach. Fifteen micrograms of pZsGreen1-N1 (10 μL) was instilled onto the gastric serosal surface in ddY mice under anesthesia. Twenty hours after administration, transgene expression (ZsGreen1) in the stomach was observed by fluorescent stereomicroscope (MZ-16F with Plan-apo 1× NA 0.141 objective lens; Leica Microsystems GmbH, Wetzlar, Germany). The camera was DFC300FX (Leica Microsystems GmbH). Acquisition software was Leica Application Suit (Leica Microsystems GmbH).

Inhibition of Transgene Expression by Pharmacological Inhibitors. Five-week-old male ddY mice (23.1−36.1 g) were anesthetized with sodium pentobarbital (40−60 mg/kg, intraperitoneal injection). Laparotomy was performed and a cylindrical glass diffusion cell (i.d. 6 mm, effective area 28 mm^2) was attached to the gastric serosal surface with a thin film of surgical adhesive (Aron Alpha; Sankyo Co. Ltd., Tokyo, Japan).[20] Each pharmacological inhibitor (90 μL in 5% glucose solution) was added directly to the diffusion cells. Concentrations of pharmacological inhibitors were as follows; chlorpromazine (100 μg/mL), genistein (100 μg/mL), methyl-β-cyclodextrin (10 mM), amiloride hydrochloride (10 mM), latrunculin B (25 μM), ML-7 hydrochloride (500 nM), piceatannol (25 μM), LY294002 (50 μM), PP2 (30 nM), Y-27632 (50 μM), NSC23766 (100 μM), and PAK18 (50 μM). Thirty minutes after administration of a pharmacological inhibitor, naked pDNA solution (15 μg/10 μL in 5% glucose solution) was mixed with the pharmacological inhibitor. The top of the diffusion cell was sealed with a piece of aluminum foil to prevent evaporation of the pDNA solution. After 120 min, the pDNA solution was removed from the diffusion cell and the gastric serosal surface within the diffusion cell was washed with 5% glucose solution (100 μL) five times. After the diffusion cell was removed from the gastric serosal surface, the peritoneum was sutured and then mice were freed into the cage. Six hours after the addition of pDNA, the mice were killed under anesthesia, and the stomach was removed. The site of diffusion cell attachment (i.d. 9 mm, area 0.64 cm^2) was removed from the other side of the stomach and subjected to luciferase assay.

Luciferase Assay. Tissue samples were washed twice with saline and homogenized with lysis buffer consisting of 0.1

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M Tris/HCl buffer (pH 7.8) containing 0.05% Triton X-100 and 2 mM EDTA.[20] The volume of lysis buffer added was 8 μL/mg tissue. Homogenates were centrifuged at 15000*g* for 5 min. Twenty microliters of the supernatant was mixed with 100 μL of luciferase assay substrates (PicaGene; Toyo Ink Mfg Co. Ltd., Tokyo, Japan), and the light produced was immediately measured using a luminometer (MiniLumat LB9506; Berthold Technologies, Bad Wildbad, Germany). Luciferase activity is indicated as the relative light units (RLU) per gram of tissue.

Determination of Cellular Association of pDNA. Tetramethyl-rhodamine-labeled pDNA (1.5 μg/10 μL) was added into a cylindrical glass diffusion cell attached to the gastric serosal surface in five-week-old male ddY mice (24.5−30.6 g) 30 min after administration of a pharmacological inhibitor mentioned above (90 μL). Two hours after pDNA addition, the remaining amount of tetramethyl-rhodamine-labeled pDNA in the diffusion cell was determined using a spectrofluorophotometer (excitation 546 nm, emission 576 nm) (RF-1500, Shimadzu, Kyoto, Japan). Cellular association of pDNA was calculated by subtraction of remaining amount in the diffusion cell from total amount of pDNA.

Statistical Analysis. Statistical comparisons were performed by the Mann−Whitney U test (Figures 2−6 and cellular association) or by the Steel test (Figure 5S in the Supporting Information).

Results

Transgene Expression in Gastric Mesothelial Cells. Instillation of naked pDNA onto the gastric serosal surface in mice resulted in efficient transgene expression, as detected using fluorescent proteins (see Figure 1S in the Supporting Information). To identify the cell type which takes up naked pDNA and expresses the transgene, tight-junction-associated proteins, zonula occludens-one (ZO-1) were immunohistochemically stained after naked pDNA instillation onto the gastric serosal surface (Figure 1A,B). Fluorescently labeled pDNA was taken up by cells surrounded by a tight junction (Figure 1A). In addition, tight-junction-surrounding cells expressed transgene products (Figure 1B). Taking the shape of these cells into consideration, these results suggested the involvement of gastric mesothelial cells in transgene expression on the gastric serosal surface. Involvement of gastric mesothelial cells in transgene expression was confirmed by immunostaining with anti-mesothelin antibodies (Figure 1C,D).

Macropinocytosis Is Required for Transgene Expression. There are several endocytic routes, i.e., clathrin- and caveolae-mediated endocytosis, macropinocytosis, phagocytosis, and other endocytosis. To determine the endocytic route required for naked pDNA transfection, we used various

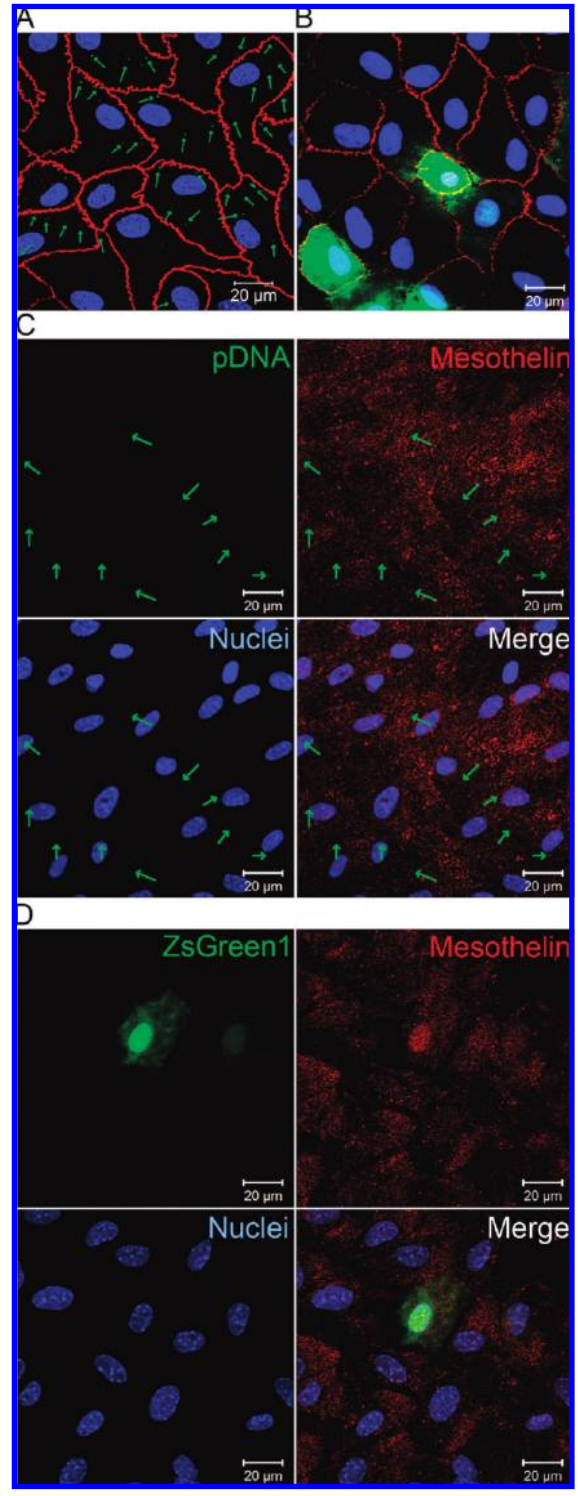

Figure 1. Uptake of naked pDNA and transgene expression in gastric mesothelial cells. (A, C) Fluorescein-labeled pDNA (green) was taken up by gastric mesothelial cells. Two hours after administration. Green arrows indicate pDNA. (A) Red: ZO-1 (tight junction). Blue: nuclei (TO-PRO-3). (C) Red: Mesothelin. (B, D) Green fluorescent protein ZsGreen1 (green) expression in gastric mesothelial cells. Twenty-four hours after administration. (B) Red: ZO-1 (tight junction). (D) Red: Mesothelin.

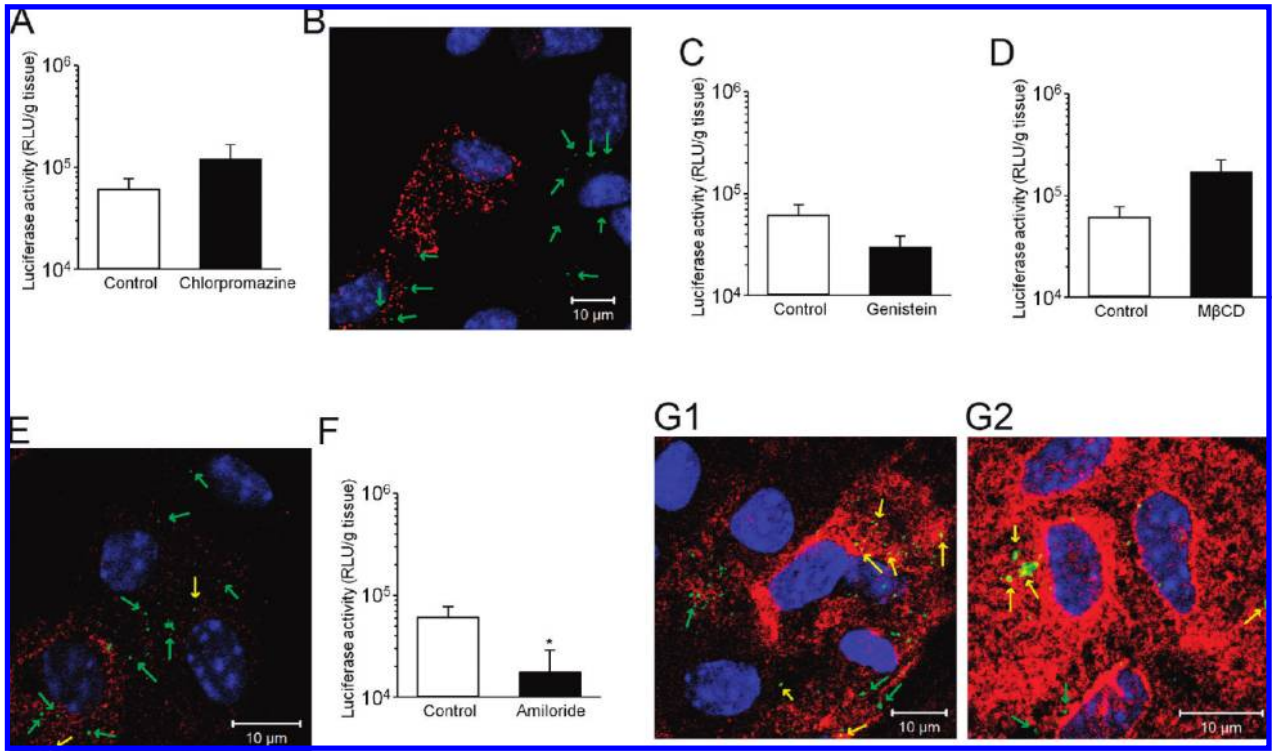

Figure 2. Macropinocytosis is required for transgene expression. (A, C, D, F) Effect of treatment with endocytosis inhibitors on transgene expression. Bar represents the mean + SEM of at least 14 mice. Only amiloride exhibited significant inhibitory effect (* $P < 0.05$). For inhibition experiments; pretreatment with pharmacological inhibitors, 30 min prior to pDNA addition; pDNA treatment, 2 h; luciferase determination, 6 h after pDNA addition. (B, E, G) Intracellular localization of pDNA and endocytosis markers. Green: fluorescein-labeled pDNA. Red: HiLyte555-transferrin (B), Alexa555-cholera toxin B (E), TMR-dextran (G1, G2). Blue: nuclei (TO-PRO-3). Green and yellow arrows indicate pDNA alone and pDNA colocalized with endocytosis markers, respectively. Imprints of mesothelial cells were taken 10 min after instillation of naked pDNA onto the gastric serosal surface. Panels G1 and G2 represent imprints of two individual mice. Panel G2 has high magnification to indicate colocalization of pDNA and dextran clearly.

endocytosis inhibitors and markers. Chlorpromazine inhibits clathrin-mediated endocytosis;[23] however, chlorpromazine did not inhibit transgene expression (Figure 2A). Figure 2B shows that fluorescently labeled pDNA was not colocalized with a marker of clathrin-mediated endocytosis, i.e., transferrin.[24] These results suggested that naked pDNA would not be taken up *via* clathrin-mediated endocytosis by gastric mesothelial cells. A general tyrosine kinase inhibitor, genistein, inhibiting caveolae-mediated endocytosis,[25,26] did not significantly inhibit transgene expression (Figure 2C). Caveolae-mediated endocytosis requires lipid rafts and is sensitive to cholesterol depletion;[27] however, transgene expression was not decreased, but slightly enhanced by treatment with a cholesterol-depleting reagent, methyl-β-cyclodextrin (MβCD) (Figure 2D). Figure 2E shows that the majority of fluorescently labeled pDNA was not colocalized with the lipid raft marker cholera toxin B;[26] therefore, naked pDNA transfer did not require caveolae-mediated endocytosis. On the other hand, the Na^+-H^+ exchanger inhibitor amiloride, inhibiting macropinocytosis,[28] significantly inhibited transgene expression (Figure 2F). Also, treatment with amiloride significantly inhibited cellular association of tetramethyl-rhodamine-labeled pDNA (0.634 ± 0.092 μg for control group ($n = 5$) and 0.245 ± 0.061 μg for amiloride group ($n = 6$), $P < 0.05$).

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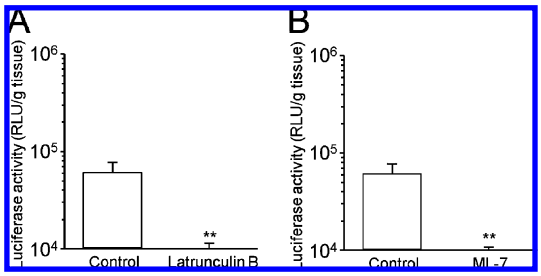

Figure 3. Involvement of actin dynamics in naked pDNA transfer. (A, B) Effect of treatment with actin dynamics inhibitors on transgene expression. For inhibition experiments; pretreatment with pharmacological inhibitors, 30 min prior to pDNA addition; pDNA treatment, 2 h; luciferase determination, 6 h after pDNA addition. The bar represents the mean + SEM of at least 18 mice. Both drugs exhibited significant inhibitory effects (**$P < 0.01$).

Furthermore, Figure 2G shows that the majority of fluorescently labeled pDNA was colocalized with the macropinocytosis marker dextran.[29] Thus, macropinocytosis would be required for naked pDNA transfer into gastric mesothelial cells.

Intracellular Signaling Regulates Naked pDNA Transfer. Macropinocytosis is regulated by intracellular signaling causing actin dynamics, such as the reorganization of actin cytoskeleton[30] and myosin-based contractile activity.[31] To confirm the involvement of actin dynamics, we inhibited actin polymerization and myosin-based contraction. Both the actin polymerization inhibitor, latrunculin B,[32] and myosin light chain kinase (MLCK) inhibitor, ML-7,[33] strongly inhibited transgene expression (Figure 3A,B), suggesting the involvement of actin dynamics in naked pDNA transfer. Actin dynamics, i.e., stress fiber formation, lamellipodia and filopodia, are controlled by Rho family GTPases Rho, Rac and Cdc42, respectively.[34] Pharmacological inhibitors, inhibiting upstream molecules of Rho family GTPases (Piceatannol for Syk,[35] LY294002 for phosphoinositide 3-kinase (PI-3K),[36] and PP2 for Src family kinases[37]), significantly reduced transgene expression (Figure 4A,B,C). To investigate the involvement of the Rho pathway, we inhibited ROCK, a downstream molecule of Rho, by Y-27632.[38] There was no significant difference between control and Y-27632 treatment groups (Figure 5A). When filamentous actin (F-actin) was stained by fluorescently labeled phalloidin, there were no stress fibers during the test time (30−120 min) (Figure 5B,C,D), while the treatment with lysophosphatidic acid as a positive control resulted in the formation of stress fibers (see Figure 2S in the Supporting Information). Therefore, the Rho pathway would not be necessary for naked pDNA transfer into gastric mesothelial cells. In contrast, the Rac pathway was significantly involved in naked pDNA transfer into gastric mesothelial cells as follows. Both the Rac inhibitor NSC23766[39] and p21-activated kinase (PAK, downstream molecule of Rac) inhibitor PAK18[40] inhibited transgene expression significantly (Figure 6A,B). Moreover, cellular association of tetramethyl-rhodamine-labeled pDNA in gastric mesothelial cells was significantly inhibited by the treatment with NSC23766 (0.634 ± 0.092 μg for control group ($n = 5$) and 0.400 ± 0.048 μg for NSC23766 group ($n = 5$), $P < 0.05$). Immunostaining of Rho family GTPases 10 min after instillation of fluorescently labeled pDNA showed that a small proportion of pDNA was colocalized with Rac, whereas pDNA was not colocalized with Rho and Cdc42 (see Figure 3S in the Supporting Information). Since pDNA was not always colocalized with Rac, it is unclear whether naked pDNA transfer requires assembly of Rac around pDNA. Thirty minutes after administration of fluorescently labeled pDNA, pDNA was colocalized with the Rac downstream molecule Wiskott−

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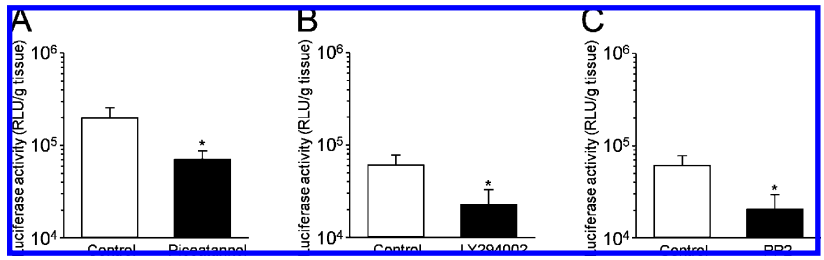

Figure 4. Upstream signaling molecules of Rho family GTPases regulates naked pDNA transfer. (A, B, C) Effect of treatment with kinase inhibitors on transgene expression. For inhibition experiments; pretreatment with pharmacological inhibitors, 30 min prior to pDNA addition; pDNA treatment, 2 h; luciferase determination, 6 h after pDNA addition. Bar represents the mean + SEM of at least 15 mice. All of these drugs exhibited significant inhibitory effects (*$P < 0.05$).

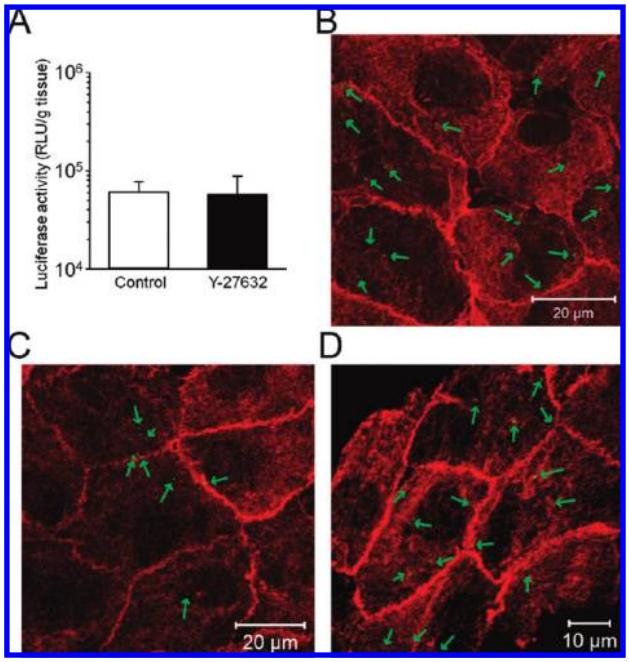

Figure 5. Naked pDNA transfer does not require Rho pathway. (A) Effect of treatment with a ROCK inhibitor on transgene expression. For inhibition experiments; pretreatment with pharmacological inhibitors, 30 min prior to pDNA addition; pDNA treatment, 2 h; luciferase determination, 6 h after pDNA addition. Bar represents the mean + SEM of 23 mice. The difference was not significant. (B−D) Actin morphology in gastric mesothelial cells. Green: fluorescein-labeled pDNA (green arrows). Red: F-actin (Alexa633-phalloidin). Imprints of mesothelial cells were taken (B) 30 min, (C) 60 min and (D) 120 min after instillation of naked pDNA onto the gastric serosal surface.

Aldrich syndrome protein family verprolin-homologous protein (WAVE) 2, whereas most pDNA signals were not colocalized with the phospho-myosin light chain (pMLC) (Figure 6C). Figure 4S in the Supporting Information shows that the cells that took up fluorescently labeled pDNA contained more pMLC signals than the cells that did not take up fluorescently labeled pDNA, suggesting that the phosphorylation of MLC was required for naked pDNA transfer but occurred at a site distant from pDNA. Overall, it was demonstrated that the Rac signaling pathway regulated the macropinocytosis of naked pDNA, which was required for transgene expression in gastric mesothelial cells in mice.

Discussion

The stomach is a digestive organ that is essential for nutrient intake. An acidic environment is required in the stomach for the digestive system, and a barrier function against acidic pH is necessary. Alcohol, drugs, stress, and *Helicobacter pylori* reduce this barrier function and cause gastritis and gastric ulcer. Moreover, gastric cancer is one of the most common malignant tumors in the world. Investigations into gene therapy for stomach diseases are actively proceeding for gastric cancer.[4] We have also developed a novel, safe and stomach-selective gene delivery method of instilling naked pDNA onto the gastric serosal surface in mice.[20,21] The proportions of transgene-expressing cells differed markedly among individuals (see Figure 1S in the Supporting Information). In individuals and at sites which exhibited low transfection efficiency, there is room to improve transfection efficiency, for which a study of the mechanism of transfection is appropriate. Here, the transfection efficiency of naked pDNA in cell cultures is generally low.[41] In general, cell culture lacks a network among different cell types, and consequently may explain the differences in transfection efficiency in cell cultures and in animals. Thus, we evaluated the *in vivo* mechanism of naked pDNA transfer into gastric mesothelial cells in mice.

This study indicated that naked pDNA would be taken up *via* macropinocytosis in gastric mesothelial cells in mice (Figure 2). The signal transduction required for the macropinocytosis of naked pDNA was mediated by the Rac pathway (summarized in Figure 7). Here, we could not rule out the possible involvement of phagocytosis for naked pDNA transfer to gastric mesothelial cells because macropinocytosis and phagocytosis share a similar intracellular signaling pathway.[42] Both upstream

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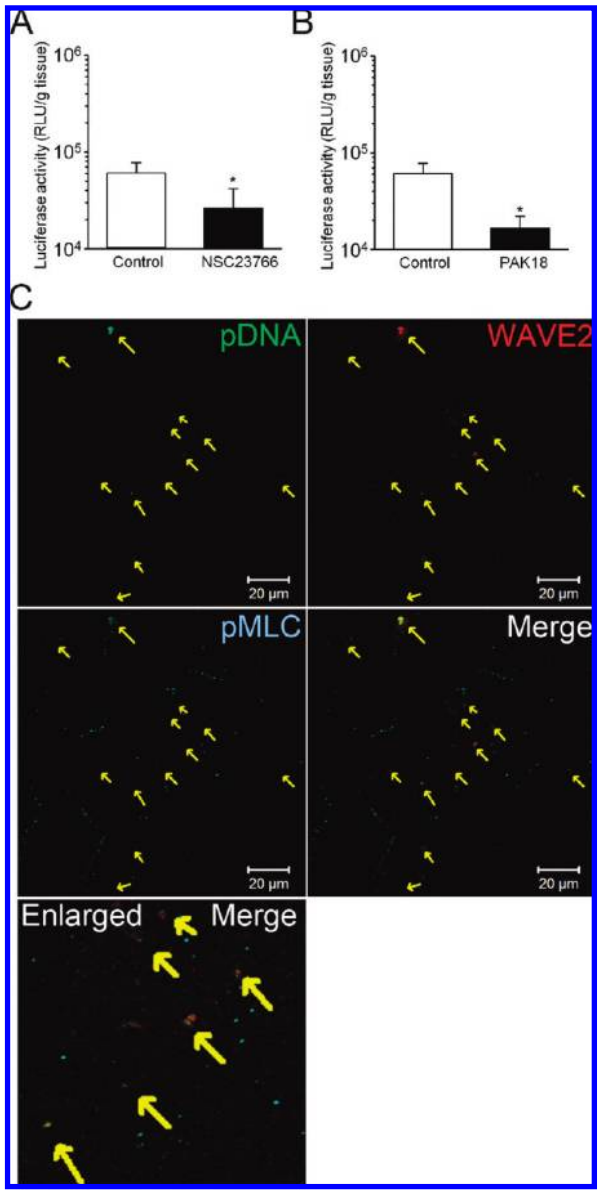

Figure 6. Rac pathway is required for naked pDNA transfer. (A, B) Effect of treatment with Rac pathway inhibitors on transgene expression. For inhibition experiments; pretreatment with pharmacological inhibitors, 30 min prior to pDNA addition; pDNA treatment, 2 h; luciferase determination, 6 h after pDNA addition. Bar represents the mean + SEM of at least 16 mice. Both drugs exhibited significant inhibitory effects ($^*P < 0.05$). (C) Colocalization of pDNA with WAVE2. Green: fluorescein-labeled pDNA. Red: WAVE2. Cyan: phospho-MLC. Yellow arrows indicate pDNA colocalized with WAVE2. A center area of the merged picture was enlarged in a separate panel. Imprints of mesothelial cells were taken 30 min after instillation of naked pDNA onto the gastric serosal surface.

(Figure 4) and downstream (Figure 6B,C) molecules of the Rac pathway would act cooperatively to induce actin dynamics (Figure 3) for the macropinocytosis of naked pDNA. As for upstream of Rac, all of Syk, Src family kinases and PI-3K were involved in naked pDNA transfer into gastric mesothelial cells. It was also reported that these three kinases were also involved in Fc gamma receptor (FcγR)-mediated phagocytosis, but not in FcγR-mediated endocytosis.[43] Both Syk and Src family kinases activate PI-3K.[44,45] PI-3K is required for the completion of macropinocytosis/phagocytosis rather than their initiation.[46] Thus, the completion of macropinocytosis might be required for naked pDNA transfer into gastric mesothelial cells and subsequently, pDNA might enter macropinosomes. The mechanism of how naked pDNA escapes from macopinosomes into the cytosol remains to be elucidated. Among Rho family GTPases, Rac and Cdc42 are required for FcγR-mediated phagocytosis, while Rho is required for complement receptor-mediated phagocytosis.[47,48] Also, Rho regulates the phagocytosis of apoptotic cells.[49] Thus, the Rho family pathways that regulate phagocytosis are dependent on the substances taken up. For naked pDNA in gastric mesothelial cells, this did not involve the Rho pathway (Figure 5), but the Rac pathway (Figure 6). Although pDNA was not colocalized with Cdc42 (see Figure 3S in the Supporting Information), we could not completely rule out involvement of Cdc42 in naked pDNA transfer since we did not perform inhibition experiments for the Cdc42 pathway. Rac is required for constitutive macropinocytosis.[50] At this moment, however, it is unclear whether naked pDNA is taken up *via* constitutive macropinocytosis or whether the binding of naked pDNA to certain receptors, such as integrins, triggered the intracellular signaling pathway for macropinocytosis of naked pDNA. In macrophages, the uptake of naked pDNA was inhibited by inhibitors of scavenger receptor class A, such as dextran sulfate and polyinosinic

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acids;[51] however, transgene expression after the administration of naked pDNA onto the gastric serosal surface was not inhibited by excess polyinosinic acids, whereas dextran sulfate significantly inhibited transgene expression (see Figure 5S in the Supporting Information); therefore, the uptake mechanism might be dependent on cell types. PAK, a downstream molecule of Rac, was also involved in platelet-derived growth factor-stimulated macropinocytosis of dextran in fibroblasts;[52] PAK inhibits MLC phosphorylation.[53] Both MLCK inhibitor and PAK inhibitor decreased transgene expression (Figures 3B and 6B), suggesting that both the phosphorylation and dephosphorylation of MLC would be required for naked pDNA uptake. WAVE is also involved in the phagocytosis of amyloid-β in rat microglia.[54] WAVE activates the Arp2/3 complex, inducing actin polymerization.[55] Thus, PAK and WAVE2 would cooperate in naked pDNA transfer by inducing myosin-based contraction of the cytoskeleton and actin polymerization. It was reported that lipid rafts were involved in macropinocytosis of HIV,[56] eosinophil cationic protein,[57] and the phagocytosis of *Pseudomonas aeruginosa*.[58] In contrast, the macropinocytosis of naked pDNA in gastric mesothelial cells was lipid raft independent (Figure 2D). Since the transgene expression was increased by treatment with MβCD, it is possible that the structure of lipid rafts might interfere with certain processes required for naked pDNA transfer, such as endosomal escape. Since it was reported that protein kinase C (PKC) and extracellular signal-regulated kinase (ERK) were involved in

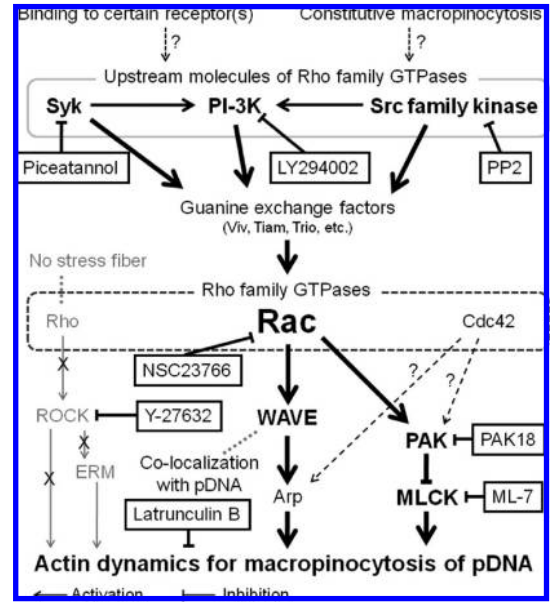

Figure 7. Schematic representation of intracellular signaling pathway for naked pDNA transfer in gastric mesothelial cells in mice. Each pharmacological inhibitor is indicated in the box.

oridonin-enhanced phagocytosis of apoptotic bodies,[59] the PKC/MEK/ERK pathway might also be involved in naked pDNA transfer to gastric mesothelial cells. PKC inhibitor BIM[60] significantly inhibited transgene expression (Fumoto et al., unpublished result). Involvement of the PKC/MEK/ERK pathway should be elucidated in a future study.

There are several routes for endocytosis, such as clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis and phagocytosis. It was reported that clathrin- and caveolae-mediated endocytosis was involved in gene transfer by lipoplex and polyplex, respectively.[61] Thus, using carriers, the internalization routes of pDNA could be changed. Differences in endocytic routes may explain the difference in the transfection efficiency of the vectors, which highly depends on cell types. In keratinocytes, pDNA was also taken up *via* macropinocytosis, and bound to several proteins, such as ezrin and moesin.[62] Ezrin and moesin are members of ezrin/radixin/moesin (ERM), and ERM are downstream of the Rho

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pathway.[63,64] In this study, however, involvement of the Rho pathway in naked pDNA transfer to gastric mesothelial cells was negligible (Figure 5). Involvement of the Rho pathway might depend on cell types and might explain the difference in transfection efficiency. It was also reported that pDNA was internalized through proteoglycan-dependent macropinocytosis when pDNA was incubated with conditioned medium.[65] The conditioned medium contains several proteins, including histones; as a consequence, histones might play a crucial role in transfection, as reported previously.[66] However, in our experiments, naked pDNA was taken up without conditioned medium, suggesting that naked pDNA would be transferred without carrier proteins, such as histones, in gastric mesothelial cells in mice. In mice, a complicated signaling network is maintained among different cells, and a signaling network with other cells may be important in naked pDNA transfer. In dendritic cells, constitutive macropinocytosis was downregulated upon the activation of dendritic cells;[50] therefore, transfection efficiency might be dependent on the cell condition. On the other hand, it was unclear whether transgene expression by naked pDNA was specific to mesothelial cells. If naked pDNA transfer was possible *via* other administration routes, it would be valuable. In intravenous injection, degradation by nuclease in the blood and uptake by macrophages may prevent *in vivo* naked pDNA transfer. Thus, naked pDNA transfer *via* intravenous injection may be possible by interfering with the degradation by nuclease and uptake by macrophages.

The biological significance of naked pDNA transfer remains unclear. The mesothelium covers internal organs and has a protective function. Moreover, mesothelial cells are antigen-presenting cells;[67] thus, the most likely explanation for naked pDNA transfer is protection against infection. The fate of cells that took up naked pDNA was also unclear. PI-3K, involved in naked pDNA transfer, is also involved in cell survival through the Akt pathway.[68] In neutrophils, however, apoptosis occurs upon phagocytosis.[69] Currently, it is unclear whether cross-talk occurs among cell functions, such as macropinocytosis/phagocytosis, cell survival, proliferation and apoptosis; thus, the fate of cells that took up naked pDNA should be clarified in the future study.

Treatment with macropinocytosis/phagocytosis enhancers may improve the *in vivo* transfection efficiency of naked pDNA into gastric mesothelial cells in mice. There are several endogenous and exogenous enhancers of macropinocytosis/phagocytosis (see Table 1S in the Supporting Information), among which phorbol 12-myristate 13-acetate (PMA) activates macropinocytosis through PKC activation;[70] however, transgene expression was not significantly changed by treatment with PMA (Fumoto et al., unpublished result). As for endogenous enhancers of macropinocytosis, we have already tested fetuin and epidermal growth factor (EGF). Preadministration of EGF could enhance transgene expression in the stomach after gastric serosal surface instillation of naked pDNA in mice, while fetuin apparently had no effect on transgene expression (Fumoto et al., unpublished result). Since it was not always seen that macropinocytosis/phagocytosis enhancers could improve transfection efficiency, there may be critical factors to determine transfection efficiency, such as the endosomal escape of naked pDNA. Furthermore, some macropinocytosis/phagocytosis enhancers have tumorigenicity or tumor promotion activity (such as PMA and EGF), or induce inflammation (such as Zymosan and Leukotriene B_4), so selection of a safe enhancer is important for the future clinical use of naked pDNA.

Conclusion

We elucidated that macropinocytosis is required for naked pDNA transfer into gastric mesothelial cells in mice. Macropinocytosis of naked pDNA was regulated by the Rac pathway. The information in this study would be helpful to clarify *in vivo* cell functions and to improve *in vivo* efficiency of naked pDNA transfer.

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Supporting Information Available: Supplementary Figures 1S−5S and Table 1S as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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