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Evaluation of nuclear transfer and transcription of plasmid DNA condensed with protamine by microinjection: The use of a nuclear transfer score

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Abstract In the present study, the nuclear delivery of a green fluorescence protein (GFP)-encoding pDNA condensed by protamine was investigated in terms of trans-gene expression after cytoplasmic (*E*(cyt)) and nuclear (*E*(nuc)) microinjection. To compare the nuclear transfer process, a novel parameter; the nuclear transfer (NT) score was introduced. The *E*(cyt) value for protamine/pDNA particles increased in a charge ratio-dependent manner. The calculated NT score showed that this increase results from an enhancement in nuclear transfer efficiency, which was also quantitatively confirmed by a recently developed confocal image-assisted three-dimensionally integrated quantification (CIDIQ) method. Moreover, *E*(nuc) for protamine/pDNA particles was significantly higher than that for poly-L-lysine/pDNA particles, suggesting that pDNA, when condensed with protamine, is more accessible to intra-nuclear transcription. Collectively, protamine is an excellent DNA condenser, with bi-functional advantages: improvement in nuclear delivery and efficient intra-nuclear transcription.

Keywords: Protamine; Microinjection; Nuclear delivery; Gene delivery; Plasmid DNA

1. Introduction

To develop a promising non-viral gene vector for clinical applications, an improvement in transfection efficiency is essential. Previous studies have clearly shown that various intracellular barriers, such as lysosomal degradation and nuclear membrane limit trans-gene expression. Lysosomal degradation can be avoided by utilizing various devices such as pH-sensitive fusogenic lipids, consisting of the dioleoyl phosphatidylethanolamine (DOPE) and cholesteryl hemisuccinate (CHEMS) [1–3], polycations that have proton sponge characteristics [4,5], and pH-sensitive membrane lytic peptides [6–9]. However, the nuclear membrane continues to be a barrier to transfection activity, especially in non-dividing cells. It has previously been reported that trans-gene expression is drastically enhanced in the M-phase when the nuclear membrane structure is diminished [10–13]. Therefore, an efficient system for the nuclear delivery of plasmid DNA (pDNA) is highly desired for the development of an artificial gene delivery system.

In an attempt to overcome the nuclear membrane barrier, pDNA is typically condensed with a cationic nuclear targeting signal peptide, such as cationic peptides modified with M9 derived from heterogeneous nuclear ribonucleoprotein-A1 [14], a TAT oligomer [15], a tetramer of the SV40 T-antigen-derived nuclear localization signal (NLS_{SV40}) [16], μ [17–19], and NLS_{SV40}-μ, a chimerical peptide of μ and NLS_{SV40} (Akita et al., submitted). In addition, protamine, a compound that has been approved by the US FDA is widely used as a DNA condenser ([20,21] and Moriguchi et al. submitted). As first proposed by Sorgi and collaborators [20], protamine has four possible NLS-like regions consisting of basic amino acid and proline or serine residues. The pre-condensation of pDNA with protamine actually enhanced the trans-gene expression mediated by cationic liposomes and/or polycations compared with poly-L-lysine (PLL), a commonly used pDNA condenser. However, based on these findings, it cannot be concluded that protamine is a useful device for the nuclear delivery of pDNA, since transfection activity is dependent on multiple intracellular events, including cellular uptake, endosomal escape, intracellular stability, nuclear transfer and intra-nuclear transcription. Cytoplasmic and nuclear microinjection proven to be a powerful technique for understanding the mechanism from the point of view of nuclear transfer and intra-nuclear transcription [22], since it enables the contribution of the efficiency of cellular uptake to be separated from and endosomal escape.

In the present study, we report on a quantitative evaluation of the nuclear transfer efficiency of protamine/pDNA particles in terms of the trans-gene expression of green fluorescence protein (GFP) after the cytoplasmic and nuclear microinjection of GFP-encoding pDNA, compared to that of PLL/pDNA particles, to investigate the potential of the protamine as a nuclear delivery device for pDNA.

2. Material and methods

2.1. General

HeLa cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). To prepare the reporter gene vector for the pDNA, an insert fragment encoding the EGFP was obtained by digesting *Eco*RI/*Not*I digestion of pEGFP-N1 (Clontech, Palo Alto, CA, USA) with *Eco*RI/*Not*I, followed by ligation to *Eco*RI/*Not*I digested site of pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). pDNA samples were

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purified with a Qiagen (Valencia, CA) EndFree Mega kit. Protamine sulfate was obtained from Calbiochem (Ishikari, Japan) in purified form. Before use, protamine solution was filtered through the cellulose acetate filter (DISMIC-13cp: 0.2 μm pore size) obtained from Advantech (Chiba, Japan). PLL (MW = 27400) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Tetramethylrhodamine-labeled dextran (RhoDex: MW: 70000) was purchased from Molecular probe (Eugene, OR, USA).

2.2. Preparation of the polycation/pDNA complex

For the condensation of pDNA, 20 μl of a pDNA solution (0.1 μg/μl in H_2O) was added dropwise to 100 μl of polycation solution under vortex for five times (totally 100 μl) at approximately 20-s intervals. The concentration of the protamine and PLL ($C_{\text{polycation}}$) at various charge ratios (+/−) was calculated using the following equation.

$$\text{Charge ratio} = \{C_{\text{polycation}} \times n_{\text{cation}}/\text{MW}_{\text{polycation}}\}/(C_{\text{DNA}}/\text{MW}_{\text{DNA}}),$$

where n_{cation} denotes the number of lysine and arginine residues in PLL and protamine, respectively. $\text{MW}_{\text{polycation}}$ and MW_{DNA} denote the molecular weight of the polycation (protamine: 4250, PLL: 27400) and one nucleotide (average: 308), respectively. C_{DNA} denotes the concentration of pDNA (0.1 μg/μl). As demonstrated previously, the polycation/pDNA complex was readily aggregated when condensed under a charge ratio of approximately 1.5 [23]. Therefore, charge ratio-dependent trans-gene expression was analyzed at the charge ratio above 2. The hydrodynamic diameter was measured by quasi-elastic light scattering by means of an electrophoresis light scattering spectrophotometer (ELS-8000, Otsuka electronics, Japan).

2.3. Microinjection study

The microinjection study was carried out, as described in the previous report [24] with minor modifications. Cells were seeded on the glass base dish 1 day (IWAKI, Osaka, Japan) before the microinjection. In this procedure, a semiautomatic injection system (Eppendolf transjector 5246, Hamburg, Germany) was attached to the Eppendolf micromanipulator 5171. Cytoplasmic and nuclear microinjections were performed under conditions of P_i = 50–70 hPa, P_c = 30 hPa and an injection time of 0.2 s. Just prior to injection, the pDNA, condensed with polycations, was diluted to 3.32 fmol/μl and 33.2 amol/μl with a 0.5% RhoDex/H_2O solution for cytoplasmic or nuclear microinjection, respectively. Under these conditions, 1000 copies and 10 copies of pDNA, respectively, were injected. At 24 h post-injection, GFP-expression was monitored by fluorescence microscopy, and the ratio of cells expressing GFP to RhoDex positive cells were calculated. In the nuclear microinjection, the rhodamine-positive cells were counted afterward, to avoid a situation in which the RhoDex diffuses into the cytosol during the 24 h incubation, which would lead to an underestimation of the number of nucleus-injected cells.

2.4. Quantitative evaluation of the nuclear delivery of protamine/pDNA particle by confocal images

For visualization of the pDNA after cytoplasmic injection, pDNA was labeled with rhodamine by the Label IT reagent (Panvera Corporation, Madison, WI, USA). At 1 h post-injection of the rhodamine-labeled pDNA condensed with protamine at charge ratios of 2 and 9, cells were incubated with 0.5 μM SYTO24 for 15 min to stain the nucleus. The cells were then washed three times with culture medium including 10% fetal calf serum. Fluorescence and bright field images were captured using a Zeiss Axiovert 200 inverted fluorescence microscope equipped with Achroplan 63×/0.95N objective (Carl Zeiss Co. Ltd.; Jena, Germany).

Quantification of the confocal images to evaluate the nuclear transfer efficiency was demonstrated by the recently developed confocal image-assisted three-dimensionally integrated quantification (CIDIQ) method [25]. 20 Z-series images were obtained from the bottom of the coverslip to the top of the cells and recorded by the Zeiss LSM510 on a PC. Each 8-bit TIFF image was transferred to Image-Pro Plus ver. 4.0 (Media Cybernetics Inc., Silver Spring, MD) to quantify the total brightness and pixel area of each r.o.i.. For the data analysis, the pixel areas of each cluster in the cytosol; s_i(cyt) and nucleus s_i(nuc) were separately summed in each XY plane, and are denoted as $S'_{Z=j}$(cyt) and $S'_{Z=j}$(nuc), respectively. The values of $S'_{Z=j}$(cyt) and $S'_{Z=j}$(nuc) in each X–Y plane were further summed through the all of Z-series of images, and are denoted as S(cyt) and S(nuc), respectively. These values represent the total amount of pDNA in the cytosol and the nucleus in an individual cell. The fractions of nuclear pDNA in the nucleus to the totally injected one; F(nuc), which represents the nuclear transfer efficiency were calculated using the equation below:

$$F(\text{nuc}) = \frac{S(\text{nuc})}{S(\text{cyt}) + S(\text{nuc})}.$$

3. Results

3.1. Physicochemical characterization of the polycation/pDNA particles

The particle sizes and Z-potentials of the pDNA/polycation particles condensed at charge ratios of 2 and 9 were characterized (Table 1). In the cases of both protamine and PLL, the particle sizes were approximately 65–90 nm at both charge ratios. The Z-potential of these particles increased depending on the charge ratios. In addition, the Z-potentials of the PLL/pDNA particles were significantly higher than for the protamine/pDNA particles.

3.2. Cytoplasmic and nuclear microinjections

We first evaluated the percent of GFP-positive cells obtained after cytoplasmic microinjection (E(cyt)). As shown in Fig. 1(a), the E(cyt) for protamine/pDNA particles at the high charge ratio of 9 (41.8% ± 4.9) was significantly higher than that at the low charge ratio of 2 (16.1% ± 3.8). Between the charge ratios of 2 and 9, E(cyt) was also increased in a charge ratio dependent manner (24.7% ± 4.5 and 38.8% ± 4.6 at a charge ratio of 4.5 and 6, respectively). At higher charge ratio of 12, E(cyt) was lower (33.5% ± 3.9) than that at a charge ratio of 9. In contrast, the E(cyt) values in PLL were not affected by the charge ratio (Fig. 1(a)). Therefore, the charge ratio-dependent increase in E(cyt) was specific for protamine/pDNA particles.

Since the maximum and minimum E(cyt) values occurred at charge ratios of 9 and 2, respectively, the percent of GFP-positive cells after nuclear microinjection (E(nuc)) were further compared at these charge ratios, in order to compare the intra-nuclear transcription efficiency. As shown in Fig. 1(b), the E(nuc) values for both protamine and PLL were not affected by the charge ratio. It is noteworthy that the E(nuc) values for the protamine/pDNA particles were higher than for PLL, suggesting that pDNA, when condensed with protamine, is more efficiently subject to the intra-nuclear transcription compared with that condensed with PLL.

Table 1
Physicochemical characteristics of polycation/pDNA particles

Sample name	Charge ratio	Size (nm)	Zeta potential (mV)
Protamine/pDNA	2.0	88.6 ± 9.0	8.9 ± 4.8
	9.0	76.3 ± 8.5	17.7 ± 4.0
Poly-L-lysine/pDNA	2.0	71.2 ± 3.5	27.6 ± 6.1
	9.0	65.7 ± 5.1	36.2 ± 9.3

Sizes and Z-potentials were measured an electrophoresis light scattering spectrophotometer. Data are represented as means ± S.D. of triplicate experiments.

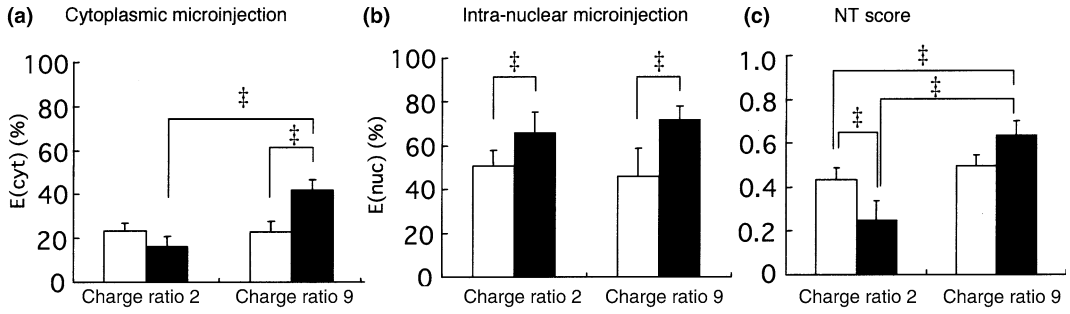

Fig. 1. GFP-expression after cytoplasmic and nuclear microinjection, and the calculated NT scores for protamine/pDNA particles and PLL/pDNA particles. After cytoplasmic (a) and nuclear (b) microinjection of the pDNA condensed with protamine (closed bar) and PLL (open bar) at a charge ratio of 2 and 9, 1000 copies of the pDNA, mixed with 0.5% RhoDex were injected to the cytoplasm of HeLa cells. At 24 h post-injection, the ratio of GFP-positive cells to RhoDex-positive cells were calculated. Vertical bars indicate the standard deviation of triplicate experiments. NT scores (c) were calculated as the percent of GFP-expression after cytoplasmic microinjection (E(cyt)) divided by that after nuclear microinjection (E(nuc)). The statistical differences were determined by one-way ANOVA followed by Fisher's PLSD test. $^{‡}P < 0.05$.

3.3. Evaluation with a nuclear transfer score (NTscore)

The E(cyt) values were highly dependent on intra-nuclear transcription efficiency as well as nuclear transfer efficiency. In contrast, E(nuc) exclusively represents the efficiency of nuclear transcription. Therefore, we define a parameter of nuclear transfer score (NT score), denoted as E(cyt) divided by E(nuc) to compare the nuclear transfer process. The calculated NT scores are summarized in Fig. 1(c). In protamine/pDNA particles, the NT score significantly increased depending on the charge ratio, whereas it was unaffected in PLL/pDNA particles, indicating that the improvement in nuclear transfer efficiency is mainly due to the protamine/pDNA-specific increase in E(cyt). Surprisingly, these scores indicate that the NT scores of the protamine/pDNA particles and PLL/pDNA particles are only a slightly different from each other, which is inconsistent with the general assumption that protamine is a more profitable polycation than PLL from the point of view of the potential to deliver pDNA to the nucleus [20].

3.4. Quantitative evaluation of the nuclear delivery of rhodamine-labeled pDNA with CIDIQ

The enhanced nuclear delivery of pDNA was further confirmed by the fluorescence images captured by confocal laser microscopy. Rhodamine-labeled pDNA was condensed with protamine at charge ratios of 2 and 9 and injected to the cytoplasm. At both charge ratios, pDNA signals were detected in the cytoplasm and nucleus as a cluster (Fig. 2(a) and (b)). In contrast, when naked DNA was injected, pDNA diffused through the cytoplasm and was barely detected as a cluster in the nucleus (Fig. 2(c)). These data suggest that pDNA, when condensed with protamine, is able to translocate through the nuclear membrane in a condensed form.

To confirm the improvement in nuclear transfection efficiency, the confocal images were quantified by a recently developed CIDIQ method as mentioned in Section 2. As a result, nuclear protamine/pDNA particles to the totally injected ones (F(nuc)) at a charge ratio of 9 were higher than at a charge ratio of 2 (Fig. 3), which is in agreement with the NT scores shown in Fig. 1(c).

4. Discussion

The nuclear membrane is a strict intracellular barrier, which prevents effective gene transfection. Since Sorgi et al. [20] first reported that the pre-condensation of pDNA with protamine enhanced the transfection activity by lipoplex to a greater extent than PLL, many investigators recognize protamine as a nuclear-targeting pDNA condenser based on the NLS-like amino acid sequence. However, the condensation of pDNA

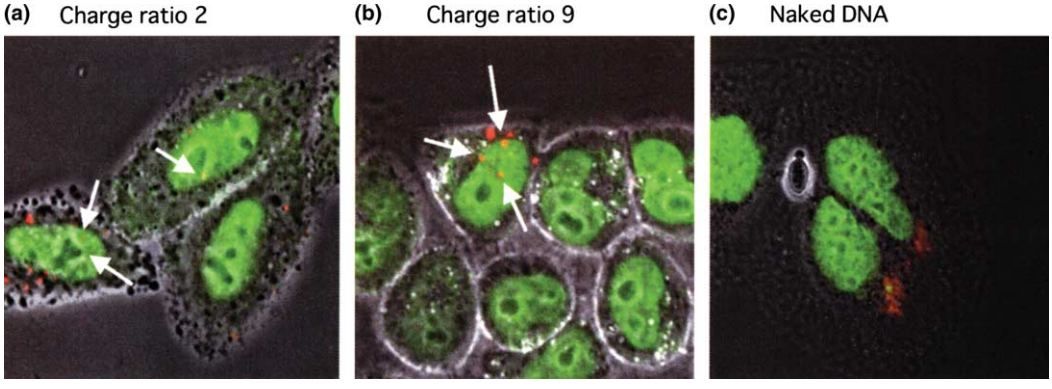

Fig. 2. Intracellular distribution of rhodamine-labeled pDNA after the cytoplasmic microinjection. Rhodamine-labeled pDNA condensed with protamine at a charge ratio of 2 (a) and 9 (b), were injected into the cytoplasm. As a control, naked pDNA were also injected (c). At 1 h post-injection, the cells were incubated with 0.5 μM SYTO24 for 15 min to stain the nucleus. Dots, corresponding to rhodamine-labeled pDNA condensed with protamine, were detected in both of the cytoplasm and nucleus (indicated by arrows) as dot shapes.

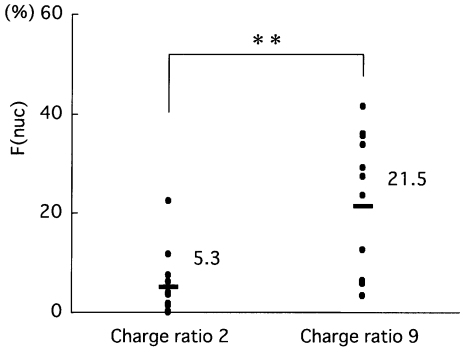

Fig. 3. Quantitative evaluation of nuclear transfer efficiency based on confocal images. The nuclear fractions of protamine/pDNA particles condensed at charge ratios of 2 and 9 relative to the total amounts injected were quantified by CIDIQ, as described in Section 2. 12 individual cells were analyzed in each group. ** indicate significant differences determined by the Mann–Whitney test ($P < 0.05$).

may affect various intracellular trafficking processes and/or the stability of the pDNA. Therefore, an evaluation of transfection activity is not sufficient to conclude that protamine is potent in delivering pDNA to the nucleus.

In the present study, we investigated the utility of the protamine as a nuclear-targeting device by microinjection, in an attempt to evaluate the nuclear transfer and intra-nuclear transcription process [22]. In the case of cytoplasmic microinjection, the *E*(cyt) of protamine/pDNA particles increased up to a charge ratio of 9 (Fig. 1(a)). In contrast, the *E*(cyt) values for the PLL/pDNA particles were independent of the charge ratio. These results are in good agreement with the results of a previous transfection study [20]. At a charge ratio of 2, it is calculated that 1.3 μg of protamine or 0.83 μg of PLL are used as a condensation per 1.0 μg pDNA. Under these conditions, trans-gene expression after the lipofection of precondensed particle by protamine was only slightly higher than that by PLL [20]. When the charge ratio was increased, the transfection activity for protamine-condensed pDNA was drastically enhanced, whereas it was unaffected in the PLL-condensed pDNA [20].

Since the protamine/pDNA particle appears to be transported to the nucleus in a condensed form, as shown in Fig. 2, intra-nuclear decondensation is necessary for achieving efficient transcription. In this sense, the *E*(cyt) values (Fig. 1(a)) should be affected by intra-nuclear transcription as well as nuclear transfer. To compare the nuclear transfer process, we investigated the efficiency of intra-nuclear transcription by nuclear microinjection (*E*(nuc): Fig. 1(b)), and defined the parameter, an NT score (Fig. 1(c)), which is denoted as *E*(cyt) divided by *E*(nuc). It should be noted that the NTscore could be affected by the decondensation efficiency of pDNA in the cytoplasm as well as nuclear transfer efficiency, since it may be different depending on the DNA condenser used [26]. However, when rhodamine-labeled pDNA, condensed with PLL was injected into the cytoplasm, pDNA was also dominantly observed as dot shaped forms, similar to protamine/pDNA particles (Akita et al., unpublished observation), Therefore, NTscores were used as an index of the nuclear transfer efficiency, based on the hypothesis that pDNA is mainly present as the condensed form in the cytoplasm, whereas the extent to which pDNA is actually released from polycations remains unknown at this time. As a result, the NT score for protamine/pDNA particles significantly increased depending on the charge ratio, whereas it was unaffected in the case of PLL/pDNA particles. Improved nuclear transfer was also confirmed by the quantification of confocal images by a recently developed CIDIQ method [25], in which the pixel areas of the pDNA clusters are used as an index of the amount of pDNA (Fig. 2). A proposed mechanism for the charge ratio-dependent improvement of the nuclear transfer is shown in Fig. 4. It is possible that the protamine molecule possess heterogenic functions: one of which is a condenser of pDNA via its arginine residues, and other of which is a nuclear localization signal. At a low charge ratio, all of the basic amino acids of protamine may be consumed in the condensation of pDNA, and, as a result, the recognition of the nuclear localization signal by the nuclear transport-associating proteins (i.e., importins) would be limited. By increasing the charge ratio, the protamine displayed on the surface of particles functions as a nuclear localization signal, allowing the particles to be recognized by nuclear transport-associating proteins.

However, this hypothesis is inconsistent with the generally accepted theory that the maximum size of the macromolecules for the passage of nuclear pore complex (NPC) is limited to up to ~39 nm as estimated by experiments using gold particles [27]. Similarly, the nuclear transfer of pDNA as a condensed form with a polycation (e.g., lactosylated PLL) was also reported previously [28]. As opposed to the gold particle, a polycation/pDNA particle may be flexible in shape, and could possibly pass through the limited size of the NPC by changing its form. When the charge ratio was further increased from 9 to 12, the *E*(cyt) value decreased. Above a charge ratio of 9, free protamine may inhibit the nuclear transfer of pDNA by competing with the recognition of protamine/pDNA particles by the nuclear transport-associating proteins, or the NPC-dependent transfer process.

Unexpectedly, the NT score for protamine/pDNA particles was not significantly higher than that for PLL/pDNA particles. This is not in agreement with the widely accepted opinion that protamine is superior to PLL in delivering the pDNA into the nucleus. Considering that the nuclear membrane consists of the negative charged lipids such as cardiolipin, phosphatydylinositol, phosphatidic acid and phosphatidylserine [29], positively charged particles should be efficiently drawn close to the nucleus. Presumably, physicochemical characteristics, such as high *Z*-potentials (Table 1) of PLL/pDNA particles are driving forces for the nuclear delivery, which can complement the lack of biologically active nuclear transport.

We also found that the *E*(nuc) values of protamine/pDNA particles are significantly higher than that of PLL/pDNA particles. Considering the previous report showing that arginine-rich polycation condenses pDNA more tightly than a lysine-rich one [30], it would be expected that arginine-rich protamine would inhibit intra-nuclear transcription more severely than PLL by interfering with the binding of transcriptional factors. However, the result was completely different and unexpected. At fertilization, nucleoplasmin and related chaperone proteins are responsible for the decondensation of the paternal genome DNA from the protamine, leading to remodeling with histones to form the diploid genome with maternal genomic DNA [31,32]. Furthermore, in the remodeling, paternal genomic DNA is efficiently remodeled with acetylated histones, and rapidly demethylated for DNA activation [33]. Whereas mammalian orthologs of nucleoplasmin are exclusively expressed in

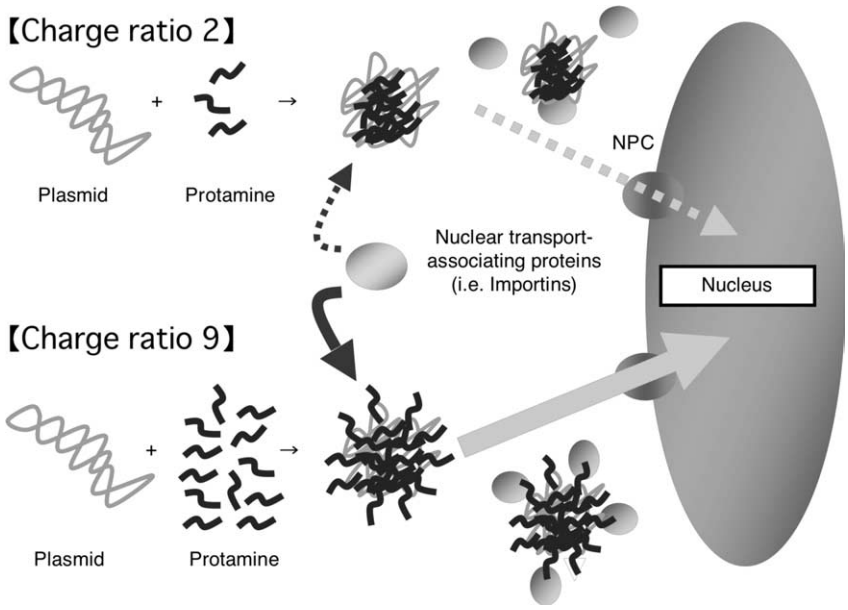

Fig. 4. Schematic diagram illustrating the possible mechanism for the improved nuclear transfer of pDNA by protamine. A possible mechanism for the improved transfer of pDNA is illustrated. At a low charge ratio, the recognition of the nuclear localization signal by the nuclear transport-associating proteins is limited since all of the basic amino acids (arginines) of protamine may be consumed in the condensation of pDNA. When the charge ratio is increased, single protamine exhibits heterogenic functions: a partial domain involves in the pDNA condensation, and other domain is displayed on the particle allowing them to be recognized as a nuclear localization signal.

ova [34], various chaperone proteins have been found in somatic cells, including HeLa cells [35]. One such example includes the nuclear template-activating factor (TAF) family of proteins, which play an important role in remodeling of the adenovirus DNA/core protein complexes during adenovirus infections [36]. Therefore, nuclear chaperone proteins may also be involved in the remodeling of the protamine/pDNA complex, allowing them to be subject to effective transcription. Alternatively, protamine may regulate the intra-nuclear localization towards the nuclear matrix-rich regions, where it plays an important role in gene translation [37,38].

Collectively, we succeeded in demonstrating the potential of protamine to deliver pDNA to the nucleus in a charge ratio dependent manner, as evidenced by a microinjection study. Furthermore, we also found that pDNA, when condensed with protamine, is more efficiently subject to transcription in the nucleus than that condensed with PLL. Therefore, protamine is an excellent pDNA condenser, which possesses bi-functional advantages: improvement in nuclear targeting and efficient intra-nuclear transcription. This report provides the first demonstration of the mechanism underlying the improved transfection activity of pDNA, when condensed with protamine, from the point of view of nuclear delivery and intra-nuclear transcription.

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