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Improved transfection efficiency of CS/DNA complex by co-transfected chitosanase gene

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Abstract

Previously, we had demonstrated that insufficient intracellular unpacking of exogene from its chitosan carrier contributes towards the restricted transfection efficiency of CS/DNA complex. In order to enhance intracellular unpacking and thus improve the transfection efficiency, our present work has addressed a novel strategy of chitosanase gene (*csn*) co-transfection. An *Aspergillus fumigatus csn* gene was semi-synthesized and cloned into a prokaryotic expression vector, plasmid pGEX-3X, meanwhile a mutant *csn* gene encoding an inactive Asp129-Asn chitosanase was generated by site-directed mutagenesis. Both active *csn* (acCSN) and inactive *csn* (inCSN) genes were expressed in bacteria cells and chitosan degradation activities of those purified recombinant proteins were tested. These *csn* genes were further subcloned into an eukaryotic expression vector, plasmid pTracer-CMV/Bsd, containing a *gfp* reporter gene. Recombinant plasmid pTracer-ac*csn* or pTracer-in*csn* was co-transfected with plasmid pTracer/Bsd/LacZ, which contains an additional *lacZ* reporter gene, into C2C12 myoblast cells by CS/DNA complex. The expression of *gfp* reporter gene was determined by fluorescence microscope, while the expression of *lacZ* reporter was evaluated quantitatively by β-galactosidase activity. All together, findings indicate that during the exogene being delivered into mammalian cells by CS/DNA complex, the *csn* co-transfection is beneficial for the exogene expression.
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Keywords: Chitosan; Chitosanase gene; Co-transfection

1. Introduction

In recent years, the potential of chitosan as a non-viral gene carrier has been extensively considered by several research groups. Chitosan/DNA (CS/DNA) microparticles containing reporter genes or therapeutic genes are being used for the transfection of mammalian cells both *in vitro* and *in vivo* (Corsi et al., 2003; Sato et al., 2001; Iqbal et al., 2003). However, the transfection efficiency of CS/DNA microparticles reported to date is lower as compared with liposome or other non-viral gene delivery vectors, and just this relatively low transfection efficiency obstructs its further use as a perfect gene therapy vector (Prabaharan and Mano, 2005). A variety of strategies had been employed, such as coupling deoxycholic acid (Kim et al., 2001), galactose (Kim et al., 2004), poly(vinyl pyrrolidone) (Park et al., 2004), urocanic acid (Kim et al., 2003), glycol (Yoo et al., 2005), PEG (Yun et al., 2005) and alkyl chains (Liu et al., 2003) to chitosan. Although, several reports suggest that the modification of chitosan could really increase the transfection efficiency of CS/DNA microparticles to some extent (Chen et al., 2004; Zhang et al., 2006; Mansouri et al., 2004, 2006). But still is not comparable with liposome reagent or virus gene carrier (Aral and Akbuga, 2003; Romoren et al., 2003; Ozgel and Akbuga, 2006; Wong et al., 2006).

To the best of our knowledge, much work has been focused on improving the cell membrane permeability of CS/DNA complex via conjugation of various ligands. This increased transmem-

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brane ability, could let chitosan deliver more DNA into host cell and hence augment its tranfection. No doubt, cell membrane permeation is the precondition of transfection, but after being transported into the cell the unpacking of DNA from its chitosan carriers may also play an equal or even more important role in determining the transfection efficiency (Liang et al., 2006). In our previous experiments, a radioactive P^{32} labeled plasmid containing *lacZ* reporter gene was transfected into C2C12 myoblast cells and some other cell lines by CS/DNA complex or liposome reagent. The radioactivity of cell lysate representing the amount of DNA being transferred into cells and β-galactosidase activity representing the level of exogene expression were detected quantitatively. It was interesting to find that in comparison with lipofectamine 2000, CS/DNA had higher cell permeability but significant lower exogene expression. So we hypothesized that the strong electrostatic interaction between chitosan and DNA might prevent exogene unpacking from its carrier and unfavorably hinder its expression. To support this hypothesis, we pre-delivered chitosanase which can degradate chitosan specially and effectively into the cells prior to CS/DNA transfection. As a result, the pre-delivered chitosanase can really increase the transfection of CS/DNA to a high level exceeding lipofectamine 2000.

Although chitosanase modification can really benefit CS/DNA transfection, however, it is not feasible to pre-deliver chitosanase into host cells prior to each transfection. So in the present work, we tried a new method of chitosanase gene (*csn*) co-transfection instead of pre-delivering a large molecule of chitosanase. We hope the slightly expressed chitosanase can degrade chitosan carrier, help exogene releasing and improve the transfection efficiency effectively. In this paper, the strategy of *csn* gene co-transfection is critically evaluated.

2. Materials and methods

2.1. Materials

Chitosan (CS) was purchased from Haihui Bioengineering Com. (Qingdao China) in molecular mass = 5000 Da (degrees of deacetylation approximate to 80%). Lipofectamine2000 and DMEM (Dubelco's Eagle Modified Medium) cell culture medium powder were purchased from Invitrogen Corporation (Invitrogen Co., USA). Plasmid pGEX-3X and GST protein purification system were purchased from Amersham Com. (Amersham Pharmacia, USA). Plasmid pTracer-CMV/Bsd contains a Human cytomegalovirus (CMV) promoter, located upstream of MCS (multi-clone site), regulates the expression of inserted gene. Another Human EF-1α promoter present in plasmid controls *gfp* reporter gene expression. Plasmid pTracer-CMV/Bsd/LacZ is the control plasmid of pTracer-CMV/Bsd, has a *lacZ* reporter gene inserted in the MCS. Activity of β-galactosidase, product of *lacZ*, can be determined quantitatively by a β-galactosidase activity assay kit. While, GFP can be observed directly under a fluorescence microscope. These two plasmids were purchased from Invitrogen Com. Chitosanase powder and aminoglucose (NAG) were purchased from Sigma (Sigma–Aldrich Co. USA). β-Galactosidase activity assay kit and plasmid purification kit were purchased from Promega Com. (Promega, USA). Site-directed mutagenesis kit was purchased from Stratagene (Stratagene, USA).

2.2. Semi-synthesis and PCR amplification of an entire chitosanase gene

According to published *Aspergillus fumigatus* chitosanase gene sequence (Genbank AJ607393), eight DNA single strands (a1–a4 and b1–b4) each of around 100 bp and four oligonucleotide primers (L1–L4) were synthesized. *A. fumigatus csn* was designed taking the advantage of the degeneracy of genetic codes, wherein some codes were modified to fit the bias of mammalian cells. By two steps of PCR, these eight DNA single strands were assembled to an entire chitosanase gene. The schematic diagram was shown in Fig. 1. DNA strands and primers were diluted in ddH_2O to a final concentration of 0.5 and 5 μM, respectively. Two separate PCR systems (PCR A and PCR B) were set up to generate DNA fragment A and B. PCR A, total volume of 50 μL, consists of 0.5 μM primer L1 and primer L2, 0.25 mM dNTPs, 1 U *Taq* DNA polymerase (Gibco), 0.001 μM strands a1–a4, PCR buffer and ddH_2O. In PCR B, L3 and L4 substituted for L1 and L2 while a1–a4 were replaced by b1–b4.

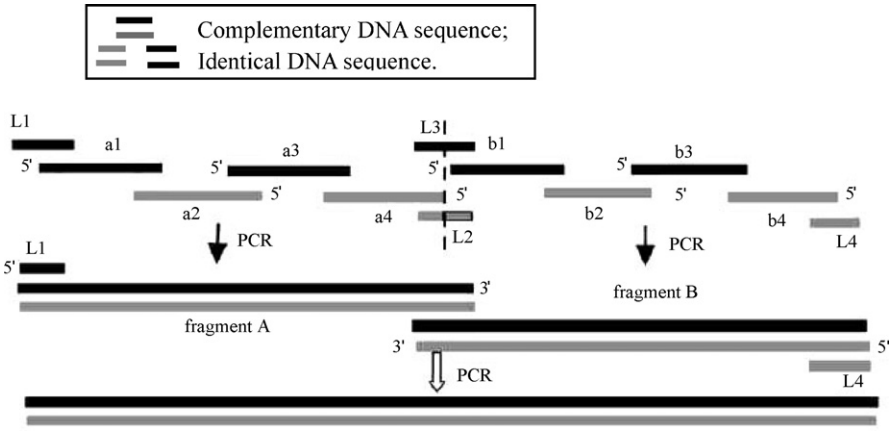

Fig. 1. Schematic representation of PCR connection of the entire chitosanase gene.

After denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s were followed by a 5 min extension. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide. DNA fragment A and B, purified from the gel and diluted to the concentration of 0.5 μM, were subjected to another PCR with L1 and L4 as primers. Sequence of L1 and L4 are listed below, restriction endonuclease BamHI and EcoRI (shaded in primers) were introduced into primer L1 and L4 separately, L1: 5'-CA*ggATCC*ACAAgggAAAATgTTCCAAg-3' and L4: 5'-ggg*AATTC*TCACTATgCTTTCAAACCAgC-3' .

2.3. Cloning and site mutagenesis of Csn gene

PCR product of the entire *csn* gene was recovered from agarose gels. Plasmid pGEX-3X was amplified and extracted from *E. coli* DH5α transformant. BamHI/EcoRI digested *csn* gene was ligated to EcoRI and BamHI predigested plasmid pGEX-3X by T4 DNA ligase. Competent cell *E. coli* DH5α (transformation efficiency of 10^6 to 10^8) was transformed with ligated DNA, recombinant plasmid pGEX-ac*csn* was screened out by BamHI/EcoRI digestion. DNA sequence and open reading frame (ORF) of the insert was verified by DNA sequencing.

Site-directed mutagenesis for the mutation of Asp^{129} to Asn was performed according to the QuikChange method (Stratagene, USA). The basic procedure involved PCR amplification with pGEX-ac*csn* as the template and two synthetic oligonucleotides containing the desired mutation as the primer: D129N(+) 5′-GGAATCTGGGGTAACACCAACGGTG-3' ; D129N(−) 5′-CACCGTTGGTGTTACCCCAGATTCC -3' . The desired mutations were confirmed by DNA sequencing of the full gene. The recombinant plasmid containing this mutational inactive *csn* (in*csn*) gene was named as pGEX-in*csn*.

2.4. Expression and purification of the recombinant chitosanase

The transformed *E. coli* DH5α cells were grown in LB (Luria-Bertani) (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0) medium with ampicillin (50 μg/mL) for 16 h (22 °C, 250 rpm). After the optical density (OD_{600}) of the culture approached 0.6, IPTG was added to a final concentration of 0.1 mmol/L for induced expression, followed by 3 h culturing at 25 °C on a shaker at 250 rpm. When OD_{600} of the culture reached 3.0, bacterial cells where harvested by centrifuge (4 °C, 7700 g, 10 min). The harvested cells were lysed by cycles of ultrasonic in 10 mL phosphate buffer [20 mg/L lysozyme, 5 mmol/L dithiothreitol (DTT), 1 mmol/L phenylmethyl-sulfonyl fluoride (PMSF), pH 7.3]. Supernatant and pellet of cell lysate were separated by centrifuge (4 °C, 12,000 g, 15 min) and the supernatant was introduced to one-step affinity purification, using Glutathione Sepharose 4B, followed the instruction (Glutathione Sepharose 4B Instructions). Purified recombinant fusion proteins GST-acCsn and GST-inCsn were analyzed by SDS-PAGE, staining at room temperature using Coomassie brilliant blue (CBB) R-250.

2.5. Chitosan degradation activity of the recombinant GST-chitosanase

After dialysis against 0.01 mol/L PBS buffer, protein concentration of these purified recombinant proteins were determined with a BCA Protein Assay kit (Pierce Corp., USA) and diluted with PBS to 20.0 μmol/L. 50 μL GST-acCsn and GST-inCsn solutions were subjected to chitosan degradation assay. Briefly, protein samples were added into 1 mL 0.5% chitosan solution (acetic buffer, pH 5.4), incubated for 15 min at 37 °C then boiled for 5 min to stop the reaction. Amount of reduced sugar were determined by modified Schales method. Aminoglucose solution of different concentrations was used as standard. Schales solution, 0.1% potassium ferricyanide ($K_3Fe(CN)_6$) in 0.5 mol/L Na_2CO_3, was prepared. 50 μL samples or standard NAG solution were transferred into 1 mL Schales solution, boiled for 10 min. After cooling down, the absorbance at 420 nm (A420) of each sample was detected. Chitosan degradation ratio was calculated according to the equation below. As the controls, chitosan degradation activities of lysozyme and purchased recombinant chitosanase (Sigma, USA) of equal molar quantities were also evaluated.

$$\text{CS degradation ratio (\%)} = \frac{\text{Concentration of reducing sugar (mg/ml)} \times 161.16}{\text{Primary concentration of chitosan (mg/ml)} \times 179.17} \times 100\%$$

2.6. Cloning csn genes into mammalian expression plasmid pTracer-CMV/Bsd

Another pair of primers were synthesized, sense: 5′-CACGAATTCACGATGGGAAAATGTTCCAAGATCC -3′ include EcoRI digestion site and initial codon; antisense: 5′-GGTTCTAGATCACTATGCTTTCAAACCAGC -3′ include XbaI digestion site and stop codon. Both ac*csn* and in*csn* genes were amplified by PCR with plasmid pGEX-ac*csn* or pGEX-in*Csn* as template. PCR condition was same as above. The EcoRI/XbaI fragment of PCR products were ligated with EcoRI/XbaI pre-digested plasmid pTracer-CMV/Bsd. After transformation, the recombinant plasmids of pTracer-ac*csn* and pTracer-in*csn* were screened out in *E. coli* JM109 by EcoRI/XbaI digestion. DNA sequence and ORF (open reading frame) was verified by DNA sequencing.

2.7. Csn gene and reporter gene co-transfention

Plasmids pTracer-ac*csn*, pTracer-in*csn* and pTracer/Bsd/LacZ were extracted and CS/DNA complexes were prepared following previously described method (Liu et al., 2003). Particle size of CS/DNA complexes were measured as previously described (Liang et al., 2006). The mean diameter was evaluated by the Stokes–Einstein relationship. As shown in Table 1, four kinds of CS/DNA complexes were prepared, their transfection efficiency were evaluated with three other transfection methods as controls.

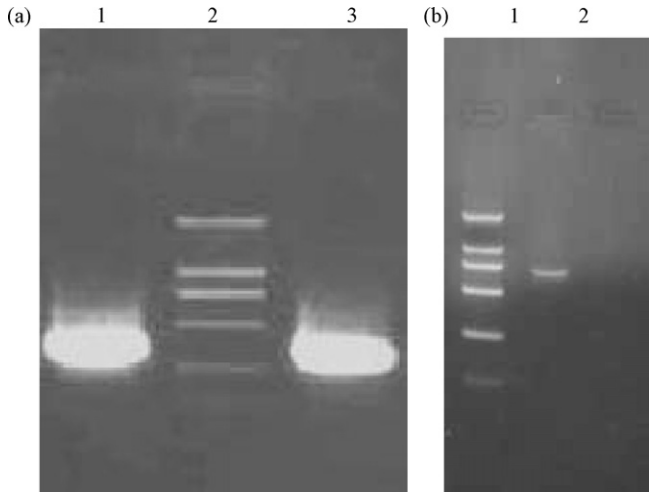

Fig. 2. Agarose gel electrophoresis of PCR product. (a) PCR product of fragment A and B. Lane 1: fragment A; lane 2: DNA marker DL2000 (2000, 1000, 750, 500, 250 bp); lane 3: fragment B. (b) PCR product of the entire *Csn* gene. Lane 1: DNA marker DL2000; lane 2: entire *Csn* gene.

Fig. 3. SDS-PAGE of purified proteins expressed in bacteria. Lane 1: purified GST from naked plasmid pGEX-3X; lane 2: protein MW marker (96, 67, 43, 31, 20, 14.4 kDa); lane 3: purified GST-Csn; lane 4: purified GST-inCsn.

C2C12 mouse myoblast cells were seeded at a density of 5×10^5 mL^{-1} on 24-well microplates in DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS (fetal bovine serum). Cells were cultured at 37 °C under 5% CO_2 atmosphere. When the cells were grown to half confluence, culture medium was extracted and the cells were rinsed three times with serum-free DMEM. CS/DNA complexes were diluted with 1 mL serum-free DMEM and added into corresponding wells. After 4 h incubation, the complexes were removed and the medium were replaced by fresh FBS-containing DMEM medium and incubated for another 48, 72 or 96 h. Based on the composition of plasmids and transfection reagents, seven experimental groups were established. The detailed composition of each group was shown in Table 1. In order to insure equal reporter genes, both *gfp* and *lacZ*, were transfected and evaluated. β-Galactosidase activity was assayed in group A to E, while GFP was investigated in group A, B, C, F and G.

2.8. Expression level of two transfected reporter genes

At different timepoints (48, 72 or 96 h) after the transfection, β-galactosidase activity in cell lysates was detected by an E-2000 β-galactosidase activity assay kit (Promega, USA) in group A to E. In group A, B, C, F and G intracellular GFP was investigated under fluorescent microscope every 24 h. Maximum GFP expression was observed 72 h after transfection, shown in Fig. 4. Besides C2C12 mouse myoblast cell line, there were three other cell lines of 3T3 mouse fibroblast cell line, 2T3 mouse osteoblast cell line and MG63 human osteosarcoma cell line were also analyzed by same transfection and detection patterns. Since only *lacZ* reporter gene expression was evaluated in this three cell lines, so transfection conditions of group F and G in Table 1 were not applied.

3. Results and discussion

For this experiment, we need a *csn* gene firstly. Chitosanase are widely expressed in bacteria and fungus. In the present study we cloned is an *A. fumigatus* chitosanase gene whose product can degrade chitosan with high activity and specificity (Zhang et al., 2001). Long strand DNA synthesis and linkage PCR were adopted to obtain this *csn* gene. There were two reasons to choose this method instead of RT-PCR. One reason is that it is difficult to obtain an *A. fumigatus* strain with thoroughly

Table 1
Plasmid and transfection reagent composition of each experimental group

	pTracer/Bsd/LacZ	pTracer-ac*csn*	pTracer-in*csn*	Transfection method
Group A	2.0 μg	–	–	Naked DNA
Group B	2.0 μg	–	–	CS/DNA
Group C	2.0 μg	–	–	Lipofectamine 2000
Group D	2.0 μg	2.0 μg	–	CS/DNA
Group E	2.0 μg	–	2.0 μg	CS/DNA
Group F	–	2.0 μg	–	CS/DNA
Group G	–	–	2.0 μg	CS/DNA

Table 2
Chitosan degradation activity of different samples

	lysozyme	GST-acCSNase	GST-inCSNase	chitosanase
Degradation ratio	0.4%	23.2%	0.8%	25.9%

known genetic background, since there are numerous kinds of *Aspergillus* in the nature. The other reason is the biased utilization of genetic code between mammalian cell and fungus. For a certain amino acid, mammalian always have their own favorite codes and which might be seldom used in other organisms. In the present study, we cloned this specific *A. fumigatus csn* gene which was expected to be highly expressed in mammalian cells further. In order for the adapt to be expressed in mammalian cells, some mutations were introduced into the wild type *csn* gene during DNA synthesis, according to a genetic codes utilization frequency chart (mouse/rat NG108-15 [gbrod]), without any change of amino acid composition. This code-modified *csn* gene was registered on Genbank (AY789050).

Table 3
Time course β-galactosidase activity in transfected C2C12 cell line (U/mg protein)

	48 h ($n=4$)	72 h ($n=4$)	96 h ($n=4$)
Cell itself	21.4 ± 2.6	22.8 ± 3.2	19.5 ± 3.6
Group A	28.4 ± 3.8	33.3 ± 4.9	30.4 ± 6.2
Group B	38.3 ± 5.2	42.4 ± 7.2	35.6 ± 4.7
Group C	183.5 ± 26.3	134.1 ± 19.2	65.4 ± 6.6
Group D	36.7 ± 5.8	143.3 ± 21.6	86.3 ± 13.6
Group E	32.4 ± 3.6	37.3 ± 4.1	29.5 ± 5.1

By linkage PCR, these 8 DNA single strands were linked together, formed a DNA fragment about 650 bps in accordance with the length of entire *csn* gene (Fig. 2b). To confirm the chitosan degradation activity of the product of this *csn* gene, it was cloned into a prokaryotic expression plasmid pGEX-3X firstly. An inactive *csn* gene was generated by site-directed mutagenesis as a control, wherein, a reserved amino acid Asp^{129} was mutated to Asn (Cheng et al., 2006). Recombinant proteins of

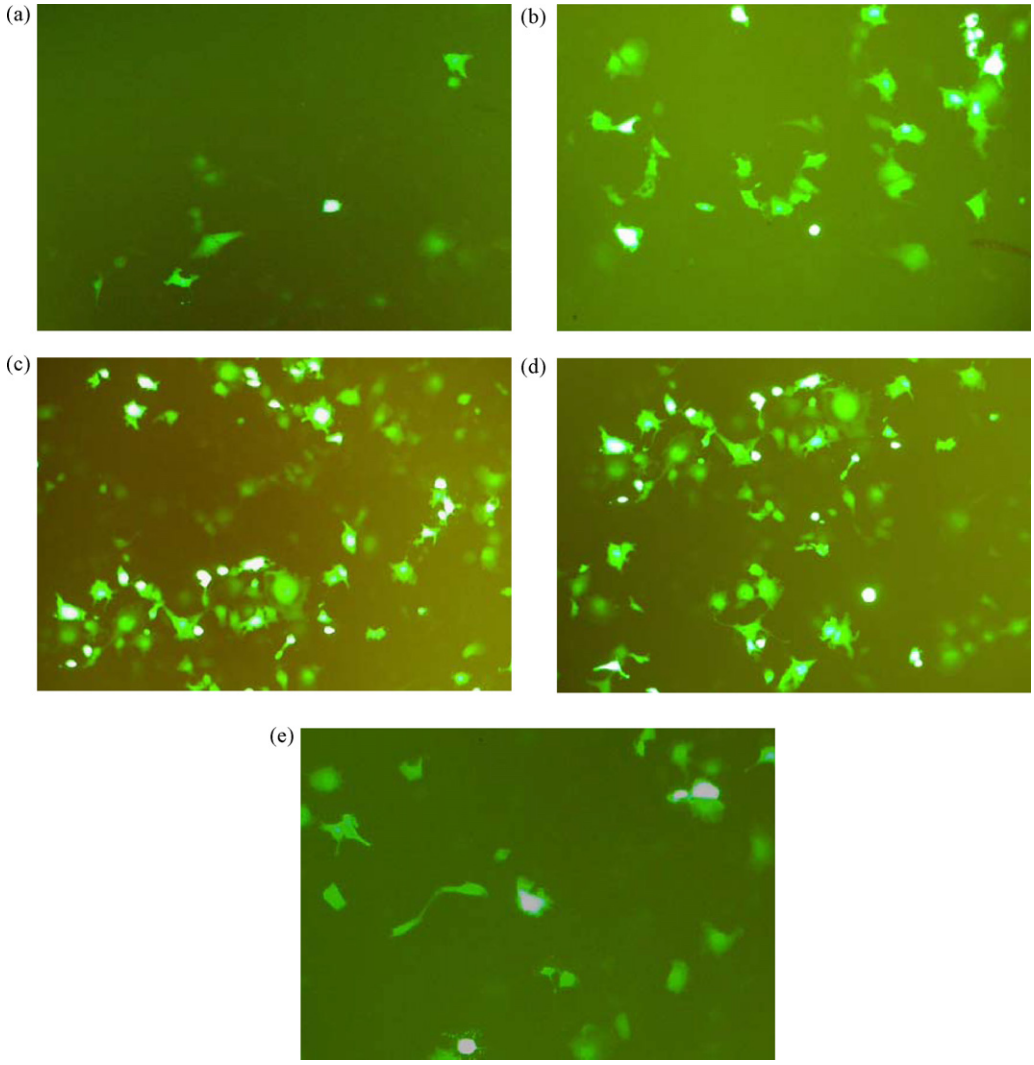

Fig. 4. GFP expression in C2C12 cell, 72 h after transfection. (a) Transfected by naked plasmid pTracer-CMV/Bsd/LacZ; (b) plasmid pTracer-CMV/Bsd/LacZ transfected by CS/DNA; (c) plasmid pTracer-*Csn* transfected by lipofectamine 2000; (e) plasmid pTracer-*Csn* transfected by CS/DNA; (d) plasmid pTracer-in*Csn* transfected by CS/DNA.

Table 4
β-Galactosidase activity in other cell lines (U/mg protein) ($n = 4$)

	2T3			3T3			MG63		
	48 h	72 h	96 h	48 h	72 h	96 h	48 h	72 h	96 h
Cell itself	26.2 ± 2.3	23.4 ± 3.4	27.4 ± 3.3	19.5 ± 1.3	24.2 ± 4.6	21.5 ± 3.7	16.7 ± 3.1	18.7 ± 3.2	15.6 ± 1.9
Group A	30.4 ± 2.5	34.3 ± 3.8	28.4 ± 3.8	26.5 ± 1.9	28.6 ± 3.1	25.2 ± 1.9	25.2 ± 2.5	25.3 ± 4.1	21.3 ± 2.4
Group B	45.3 ± 5.2	41.4 ± 5.4	30.3 ± 5.8	39.7 ± 3.6	33.6 ± 4.9	28.7 ± 3.0	38.1 ± 3.4	27.6 ± 1.3	28.5 ± 2.9
Group C	183.7 ± 27.1	123.1 ± 26.4	69.4 ± 21.6	108.7 ± 23.8	71.1 ± 16.4	66.7 ± 13.2	164.7 ± 25.2	85.7 ± 23.6	66.5 ± 3.2
Group D	32.3 ± 7.3	98.6 ± 17.2	85.3 ± 11.9	43.4 ± 9.1	87.2 ± 22.1	65.2 ± 17.3	42.5 ± 5.8	87.3 ± 12.1	54.1 ± 13.4
Group E	41.3 ± 10.3	34.5 ± 7.7	34.5 ± 3.4	35.7 ± 6.3	34.2 ± 4.0	33.7 ± 6.3	32.1 ± 5.7	32.6 ± 4.3	29.5 ± 5.2

GST (glutathione *S*-transferase)-fused acCSNase and inCSNase were purified from bacteria cell lysate by affinity chromatography. As shown in Fig. 3, proteins with molecular mass about 48 kDa could be detected, which presented the fusion proteins of GST-acCSNase and GST-inCSNase. Their molecular mass were in accordance with GST (25 kDa) + CSNase (23 kDa, encoded by a 624 bp DNA fragment). Herein, the chitosan degradation activity of GST-acCSNase and GST-inCSNase were evaluated as shown in Table 2. In terms of chitosan degradation ratio, it was confirmed that our ac*csn* gene can generate a recombinant chitosanase with high chitosan degradation activity, while the mutation of D129N made it to completely lose its activity.

After the formation of DNA/CS complexes the diameter of each complex was determined. They were all around 112 ± 16 nm. Thus, it is reasonable to consider that this particle size is feasible for the complex to transfer into host cells.

β-Galactosidase is the product of *lacZ* reporter gene, its activity can be detected quantitatively by a chemical illumination kit. While another *gfp* reporter gene in the construct gave us a convenient way to visualize the expression of exogene. Table 3 and Fig. 4 showed the expression levels of these two reporter genes. In comparison with only reporter genes transfected group (group B), co-transfected ac*csn* gene could significantly increase the transfection efficiency of CS/DNA complex, as much more green fluorescence could be seen and markedly increased β-galactosidase activity could be detected. While in*csn* brought no change to the expression of these two reporter genes. Therefore, it is rational to consider that after *csn* gene co-transfection slightly expressed chitosanase could facilitate the degradation of chitosan carrier. Hence the exogenes is readily dissociated from their vectors, accordingly increasing the expression of reporter gene. Only following *csn* gene expression, exogene can unpack from its chitosan carrier, so it is reasonable to accept that why the highest β-galactosidase activity was detected with some delayed time as compared with lipofectamine 2000.

In order to prove whether this protocol can be extended to other cell lines, we performed the transfection on 3T3, 2T3 and MG63 cells using same strategy. From Table 4, it was seen that although transfection efficiencies were different among these cell lines, the co-transfected ac*csn* gene increases the expression of *lacZ* reporter gene markedly without exception.

4. Conclusion

The quantity of DNA ferried into the cell was an important factor influencing the expression of the exogenous gene. But the release of DNA from its carrier may play an equal or even more important role. We had demonstrated that for chitosan the endocellular unpacking issue maybe the predominant one. Previously we incorporated chitosanase into the host cell which in turn degraded chitosan and promoted gene unpacking from its carrier, so the level of gene expression was considerably raised.

In our present study, we have replaced the incorporated protein of chitosanase by co-transfected chitosanase gene. Just as we expect, the co-transfected *csn* gene also increased the transfection efficiency to a high level, although a delayed expression pattern was observed. Compare with chitosanase incorporation, co-transfecting a *csn* gene is more practicable and applied easily. In further study we want to determine whether introducing such a *csn* gene into the cell would bring any effect to cell proliferation and cell cycle. Maybe in the future, a plasmid with pre-inserted *csn* gene could be constructed acting as a perfect gene assembling vector for chitosan transfection.

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