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RESEARCH ARTICLE

Exogenous nucleic acids aggregate in non-P-body cytoplasmic granules when transfected into cultured cells

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Abstract To modulate gene expression in research studies or in potential clinical therapies, transfection of exogenous nucleic acids including plasmid DNA and small interference RNA (siRNA) are generally performed. However, the cellular processing and the fate of these nucleic acids remain elusive. By investigating the cellular behavior of transfected nucleic acids using confocal imaging, here we show that when siRNA was co-transfected into cultured cells with other nucleic acids, including single-stranded RNA oligonucleotides, single and double-stranded DNA oligonucleotides, as well as long double-stranded plasmid DNA, they all aggregate in the same cytoplasmic granules. Interestingly, the amount of siRNA aggregating in granules was found not to correlate with the gene silencing activity, suggesting that assembly of cytoplasmic granules triggered by siRNA transfection may be separable from the siRNA silencing event. Our results argue against the claim that the siRNA-aggregating granules are the functional site of RNA interference (RNAi). Taken together, our studies suggest that, independent of their types or forms, extraneously transfected nucleic acids are processed through a common cytoplasmic pathway and trigger the formation of a new type of cytoplasmic granules "transfection granules".

Keywords small interference RNA (siRNA), nucleic acids, P-body, RNA interference (RNAi), transfection

1 Introduction

Cellular transfection of exogenous genetic materials is generally applied to modulate the expression of endogenous genes, so as to study their functions or to correct abnormal gene expression in gene therapy (Marcusson et al., 1998; Godbey et al., 1999; de Semir et al., 2002; Niidome and Huang, 2002; Wells, 2004; Shimizu et al., 2005). Plasmid DNA (pDNA) and oligonucleotides (antisense DNA and siRNA) constitute the two major categories of genetic material delivered by transfection (Marcusson et al., 1998; Godbey et al., 1999; Shimizu et al., 2005). In general, their relatively large size and hydrophilic nature render it difficult for them to pass through the hydrophobic cell membrane. To achieve efficient trans-membrane delivery of exogenous nucleic acids, various transfection procedures have been developed, such as chemically-induced endocytosis (chemical transfection), ultrasound-mediated membrane permeabilization (sonoporation), electrically-induced membrane pore-formation (electroporation), as well as direct delivery through a glass capillary pipette (microinjection) (Marcusson et al. 1998; Godbey et al., 1999; de Semir et al., 2002; Niidome and Huang, 2002; Wells, 2004; Shimizu et al., 2005). Different from chemical transfection, physical means have been exploited to transiently increase the permeability of the cell membrane in other procedures, allowing delivery of uncoupled nucleic acids.

Due to its great potential in gene therapy, transfection of plasmid DNA has been extensively studied for decades. Even though it is destined to exert its function in the nucleus, studies show that most of the plasmid DNA remain in the cytosol after transfection; only a small portion enters the nucleus to direct gene expression. In the transfection of fluorophore-coupled plasmid DNA, aggregation of fluorophore is observed in microscopically detectable cytoplasmic granules (Godbey et al., 1999; de Semir et al., 2002). In support of these observations, when DNA oligonucleotide is delivered directly into the nucleus by microinjection, Shimizu and colleagues found that some of the DNA oligonucleotides move across the nuclear membrane into the cytoplasm, and concentrate in

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cytoplasmic granules within a few minutes (Shimizu et al., 2005). Regardless of the difference in transfection procedures and entry points, aggregation of these DNAs in cytoplasmic granules suggests that exogenous DNAs are likely processed through a common cytoplasmic pathway (Marcusson et al., 1998; Lukacs et al., 2000; Golzio et al., 2002). However, this processing pathway is poorly understood due to the fact that most of the research interest is focused on enhancing exogenous gene expression in the nucleus.

When siRNAs are transfected into cultured cells, they aggregate in cytoplasmic granules, like plasmid DNA (Chiu et al., 2004; Jakymiw et al., 2005). In light of the fact that siRNA represses its target gene expression within the cytoplasm, these granules are of great interest to RNAi studies from the beginning and thus have been thoroughly investigated. Studies showed that transfected siRNA interacts with components of the RNA-induced silencing complex (RISC) within the granules, suggesting that the siRNA-aggregating granules are the functional sites of RNAi (Sen and Blau, 2005; Lian et al., 2007). Furthermore, RISC components interact with P-body markers such as GW182, DCP1, DCP2 and RCK/p54, as shown by immuno-staining or fusion gene assay (Jakymiw et al., 2005; Chu and Rana, 2006; Jagannath and Wood, 2008). Taken together, these lines of evidence suggest a link between RNAi and P bodies, the well-characterized cytoplasmic mRNA decay site. On one hand, the link between RNAi and P bodies suggests that P bodies are involved in RNAi and very likely serve as its functional site; on the other hand, this link unifies the endogenous RNA decay pathway with mRNA repression mediated by extraneously transfected siRNA. However, there is other evidence which cannot be ignored, showing that the integrity of P bodies is dispensable for efficient RNAi (Eulalio et al., 2007; Serman et al., 2007). Furthermore, a recent study suggested that a significant portion of the on-target siRNA activity does not associate with P-body, while the off-target siRNA activity does (Vickers et al., 2009). These controversial data raise great concern as well as interest in the role of P bodies in RNAi, and in particular, whether the microscopically visible siRNA-aggregating granules are the functional site of RNAi.

2 Materials and methods

2.1 Oligonucleotides

DNA oligonucleotides were purchased from Invitrogen. Un-labeled and FAM-labeled siRNAs were from RiboBio Inc. (Guangzhou, China) and Genepharma Inc. (Shanghai, China), respectively. Alexa-647-labeled siRNA was from Qiagen (Germany). Plasmid DNAs were extracted using a mini-purification kit (Promega) and labeled by YoYo-1 (Invitrogen).

2.2 GFP-fusion reporter vector

GFP-fusion Ago1, Ago2 and DCP1 reporter vectors were prepared by inserting the respective coding sequence into a pEGFP-C3 expression vector (Clontech). Full-length coding sequences were obtained by RT-PCR from total RNA of HEK293 cells, and sub-cloned into the expression vector between the *Eco*R I and *Sac* II restriction sites. Primers used are shown in Table 1.

2.3 Confocal imaging

Cells were cultured in 35 mm dishes with glass coverslips on the bottom. At the indicated time points after transfection, the distributions of the nucleic acids were examined and recorded in living cells by confocal microscopy (LSM510, Zeiss). Imaging processing programs were coded in Interactive Data Language. The fluorescence density was calculated based on (average fluorescence intensity $\times$ spots)/cell area.

2.4 RNAi assay

Human embryonic kidney (HEK293) cells were grown in DMEM culture medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Gibco), and seeded into 24-well plates at a density of 1×10^5 cells/well one day before transfection. siQuant vector (0.17 μg/well) carrying the specific target site of the tested siRNA was transfected into cultured cells at approximately 50% confluence, together with pRL-TK control vector (0.017 μg/well), with or without the siRNA (13 nmol/L). Transfections were performed using three transfection reagents (Lipofectamine 2000, Invitrogen; siPORT, Ambion; INTERFERin, Polyplus) according to their product manuals. The activities of both luciferases were determined by a fluorometer (Synergy HT, BioTek, USA) before the *firefly* luciferase activity was normalized to *renilla* luciferase for each well. Silencing efficacy of each siRNA was calculated by comparison with a sample without siRNA treatment. All of our experiments were performed in triplicate and repeated at least twice.

3 Results

3.1 Localization of transfected siRNA in non-P-body granules

To address these concerns, fluorophore-conjugated siRNA (Alexa-647-siRNA-1) was transfected into cultured cells according to a documented method (Jakymiw et al., 2005; Pauley et al., 2006). By laser confocal microscopy imaging, the cellular distribution of the siRNA was assessed and the results showed that, in agreement with

Table 1 Oligonucleotides in the study

oligonucleotides	sequence
siRNA-1	5′-UUCUCCGAACGUGUCACGUtt
(sense 3′-Alexa-647)	ttAAGAGGCUUGCACAGUGCA-5′
siRNA-2	5′-GCGACUCCAGAAGUUGUAAtt
(sense 5′-FAM)	ttCGCUGAGGUCUUCAACAUU-5′
siRNA-3	5′-GCGGAUCUGUGUUGCUCAUtt
(sense 5′-FAM)	ttCGCCUAGACACAACGAGUA-5′
siRNA-4	5′-CCCUGCAGUACAACUCCAUtt
	ttGGGACGUCAUGUUGAGGUA-5′
ssRNA-1 (5′-FAM)	5′-UCGGGAAAUUUCUCUAUUAtt
ssRNA-2 (5′-FAM)	5′-GCAGCACGACUUCUUCAAGtt
dsDNA-1	5′-GGAGTGTAACGATTACATCTT
(upper 5′-FAM)	TTCCTCACATTGCTAATGTAG-5′
dsDNA-2	5′-GTAATGGATGATTATGGAATT
(upper 5′-FAM)	TTCATTACCTACTAATACCTT-5′
ssDNA-1 (5′-FAM)	5′-GGAGTGTAACGATTACATCTT
ssRNA-2 (5′-FAM)	5′-GTAATGGATGATTATGGAATT
Ago1 upstream primer	5′-CTGGCAAGAATTCTATATGGGATGGAAGCGGGAC
Ago1 downstream primer	5′-TGCCTCACCGCGGTCCAGTGAGGTAACAGCGTTCTG
Ago2 upstream primer	5′-CTGGCAAGAATTCGCGCCACCATGTACTCGGGAGC
Ago2 downstream primer	5′-TGCCTCACCGCGGGAATCCCACTCGGTACACAATCG
DCP1 upstream primer	5′-CTGGCAAGAATTCGATTCAAGATGGAGGCGCTGAGT
DCP1 downstream primer	5′-TGCCTCACCGCGGTGGGGCTCTGCCTTTAGACTTA

previous reports (Jakymiw et al., 2005; Pauley et al., 2006), the siRNA aggregated in discrete cytoplasmic granules. To visualize RISC in living cells, a GFP-fused RISC component gene (GFP-Ago1 or GFP-Ago2) was constructed in a mammalian expression vector, and transfected into cultured cells together with fluorophore-conjugated siRNA (Fig. 1a). Similarly, GFP-fused DCP1 (a P-body component) was constructed and co-transfected into cultured cells with siRNA, displaying the spatial relationship between transfected siRNA and P bodies. In contrast to studies showing that transfected siRNA co-localizes with RISC and P bodies (Liu et al. 2005a,2005b; Berezhna et al., 2006; Pauley et al., 2006; Jakymiw et al., 2007), confocal imaging as well as quantitative analysis in the present study revealed that the siRNA aggregated into cytoplasmic granules that are distinct from RISC or P bodies (Fig. 1).

3.2 The siRNA silencing activity does not correlate with the formation of cytoplasmic granules

This alerting discrepancy led us to re-examine the role of the siRNA-aggregating granules in RNAi. Based on immuno-staining and fusion gene assay, it was proposed that RNAi silencing occurs mainly in the siRNA-aggregating cytoplasmic granules. If this is the case, we should expect a positive correlation between the amount of siRNA aggregating in granules and the gene silencing activity. In other words, more granule-aggregated siRNA should result in better gene silencing. To this end, a dilution assay was performed to assess this correlation in cultured cells. A series of 2-fold diluted labeled or unlabeled siRNA were transfected into HEK293 cells, and then the amount of siRNA aggregating in the granules and the gene silencing efficacy were quantified by confocal imaging and a reporter-based assay. A fluorophore-labeled siRNA (FAM-siRNA-2) was used to monitor the granule-aggregation of transfected siRNA, while gene silencing efficacy was evaluated with an unlabeled siRNA (siRNA-4) targeting a firefly luciferase fusion gene. These two siRNAs were diluted and transfected into HEK293 cells with the same experimental setting and the amount of granule-aggregated siRNA and gene silencing efficacy were quantified. Results showed that although potent gene silencing occurred at siRNA concentrations as low as 0.4 nmol/L (Fig. 2b), the fluorescent signal of siRNA-aggregating granules was barely detectable at 1.6 nmol/L (Fig. 2a). Furthermore, the expected correlation was not found in our study. Similar results were obtained when another set of siRNAs was examined in HEK293 cells.

3.3 Co-localization of transfected plasmid DNA and siRNA

Taken together with the finding in Fig. 1, we concluded

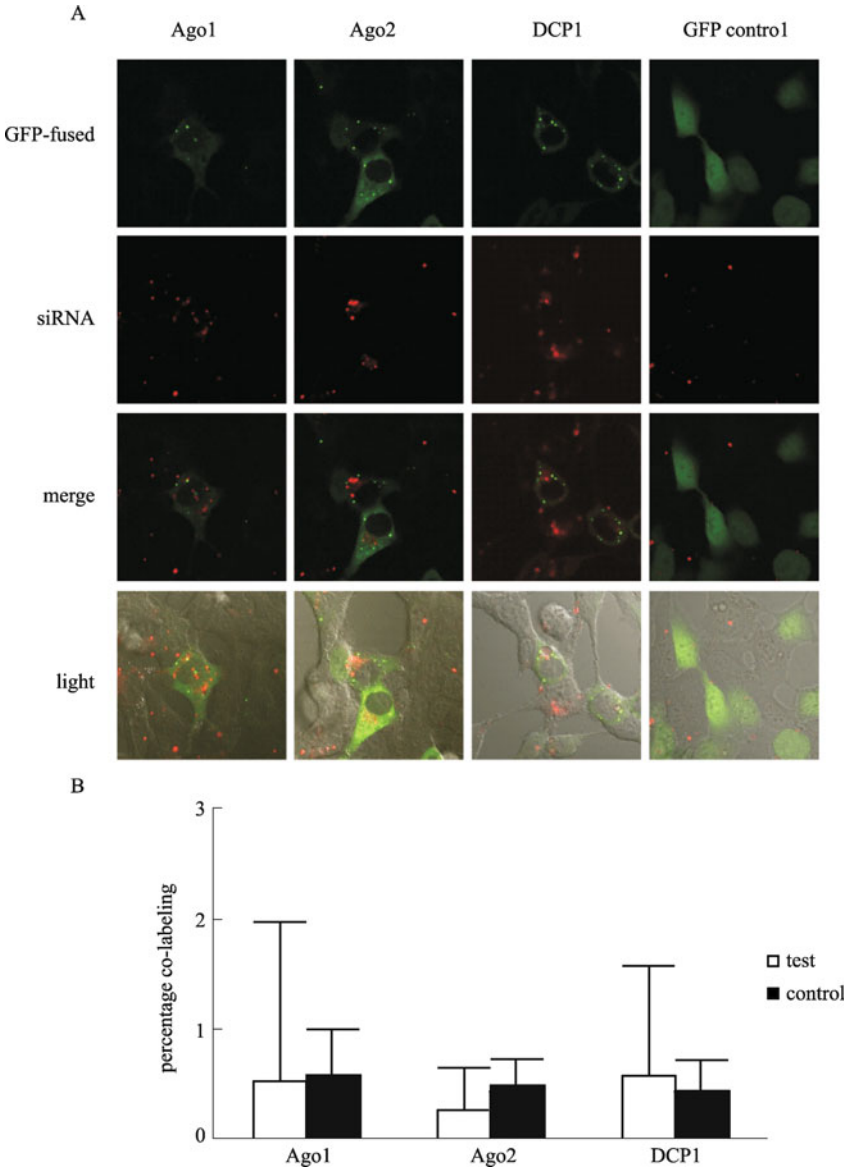

Fig. 1 Transfected siRNA aggregates in cytoplasmic granules which are not colocalized with RISC or P body components. Fluorescently labeled siRNA (Alexa-647-siRNA-1) was co-transfected into cultured HEK293 cells with a vector expressing the GFP-fused DCP1, Ago-1 or Ago-2 gene. An original GFP expressing plasmid was included as control. A: Distributions of siRNA and fusion genes in living cells 24 h after the transfections, using laser confocal microscopy. B: siRNA was transfected at a final concentration of 13 nmol/L and fusion reporter vectors were transfected at a final concentration of 0.33 ng/μL. All of the transfections were performed using Lipofectamine 2000. The area covered by random overlapping of the two types of fluorescence was set as control, and the area covered by co-labeling was compared to control to quantitatively analyze the significance of co-aggregation.

that these siRNA-aggregating granules are unlikely to serve as the functional site of RNAi, as proposed in previous studies (Sen and Blau, 2005; Chu and Rana, 2006; Lian et al., 2007; Jagannath and Wood, 2008); instead, these granules are more likely to function as the processing site of exogenous nucleic acids, including siRNA. To test this hypothesis, co-transfections were carried out with a siRNA (Alexa-647-siRNA-1) and other nucleic acids, single-stranded DNA oligonucleotide (FAM-ssDNA-1), double-stranded DNA oligonucleotide (FAM-dsDNA-1), single-stranded RNA oligonucleotide (FAM-ssRNA-1) or long double-stranded plasmid DNA (YoYo-1-pDNA) (Fig. 3a and Table 1). Similar to siRNA transfection, when these nucleic acids were transfected alone, they aggregated in cytoplasmic granules, as shown by confocal imaging. However, when they were co-transfected with siRNA, all these nucleic acids aggregated in the same cytoplasmic granules together with the siRNA (Fig. 3b). Co-localization was further confirmed by quantitative analysis (Fig. 3c). To demonstrate that this phenomenon was independent of the cell type and the sequence of the nucleic acids used in the experiments,

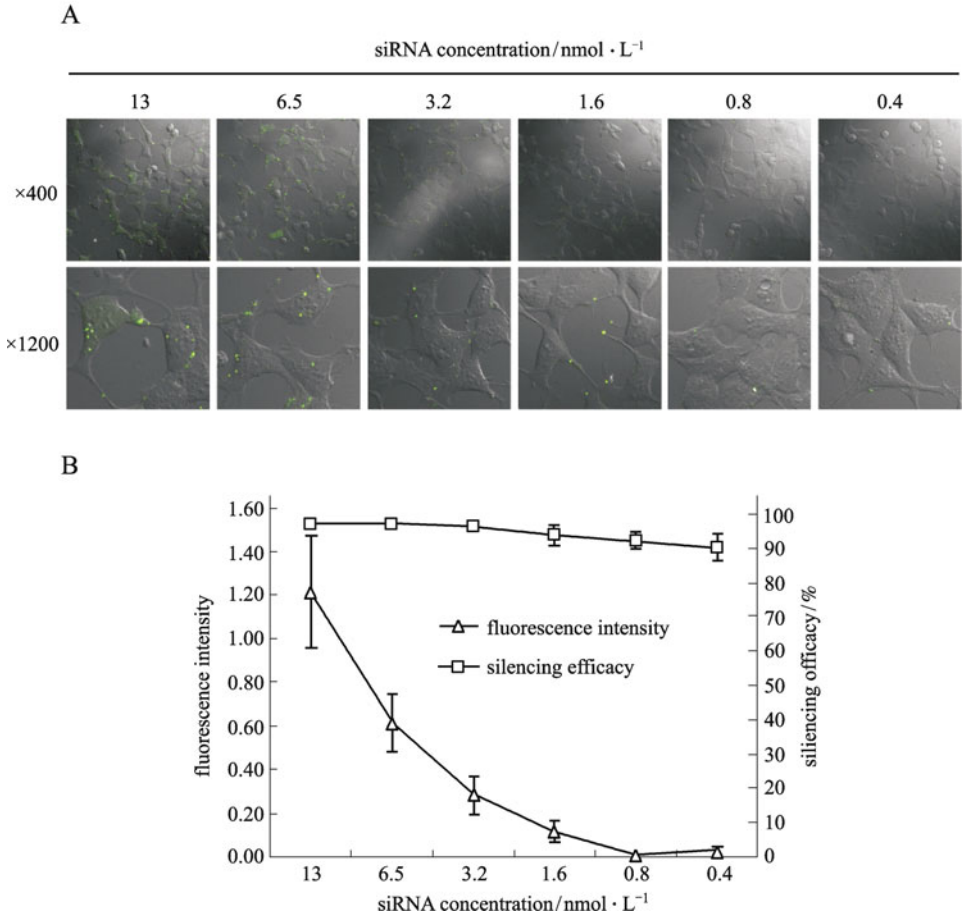

Fig. 2 No correlation was found between granule-aggregated siRNA and gene silencing efficacy. A fluorescently labeled siRNA (FAM-siRNA-2) and an effective non-labeled siRNA (siRNA-4) targeting a *firefly* luciferase fusion gene were individually diluted and transfected into HEK293 cells, together with the target reporter vector. To keep the siRNA concentration constant, an irrelevant siRNA was added into the dilutions. A: Distributions of the labeled siRNA 4 h after the transfections. B: Granule-aggregated siRNA was quantified by measuring the fluorescence intensity of the siRNA-aggregating granules, and was compared with the gene silencing efficacy of the unlabeled siRNA.

primary rodent astrocytes as well as another set of nucleic acids (ssRNA-2, dsDNA-2 and ssDNA-2) were further tested, giving similar results. Therefore, our data showed for the first time that transfected siRNA and other nucleic acids co-localized in cytoplasmic granules, indicating that these granules are the common processing site for exogenous nucleic acids.

3.4 Cellular assembly of siRNA granules

To gain more insights into the cellular behavior of these granules, their formation and intracellular trafficking were further studied by time-lapse confocal imaging. After transfection, the fluorescent signal was first diffusely distributed in the cytoplasm, then gradually concentrated and eventually formed microscopically visible granules (Fig. 4a). Later, active translocations were observed for a small fraction of the granules (Fig. 4b). When two siRNAs labeled by different fluorophores were co-transfected or sequentially transfected into cells, a significant distribution pattern appeared (Fig. 4c). In co-transfection, all the granules were double-labeled by both dyes, indicating that both siRNAs aggregated in the same granules when they were transfected at the same time; whereas in sequential transfection, no granules were co-labeled. Each granule was specifically labeled by only one dye, indicating that the two sequentially transfected siRNAs never aggregated in the same granule.

The existence of granules in which only the first transfected siRNA aggregated indicated that formation of these granules was a transient process. Once these granules were formed in the first transfection, aggregation of siRNA was terminated; so siRNA introduced in the second transfection could not be further recruited to these granules. While the occurrence of granules in which only the second transfected siRNA aggregated indicated that these granules did not exist before the second transfection, otherwise siRNA introduced in the first transfection should

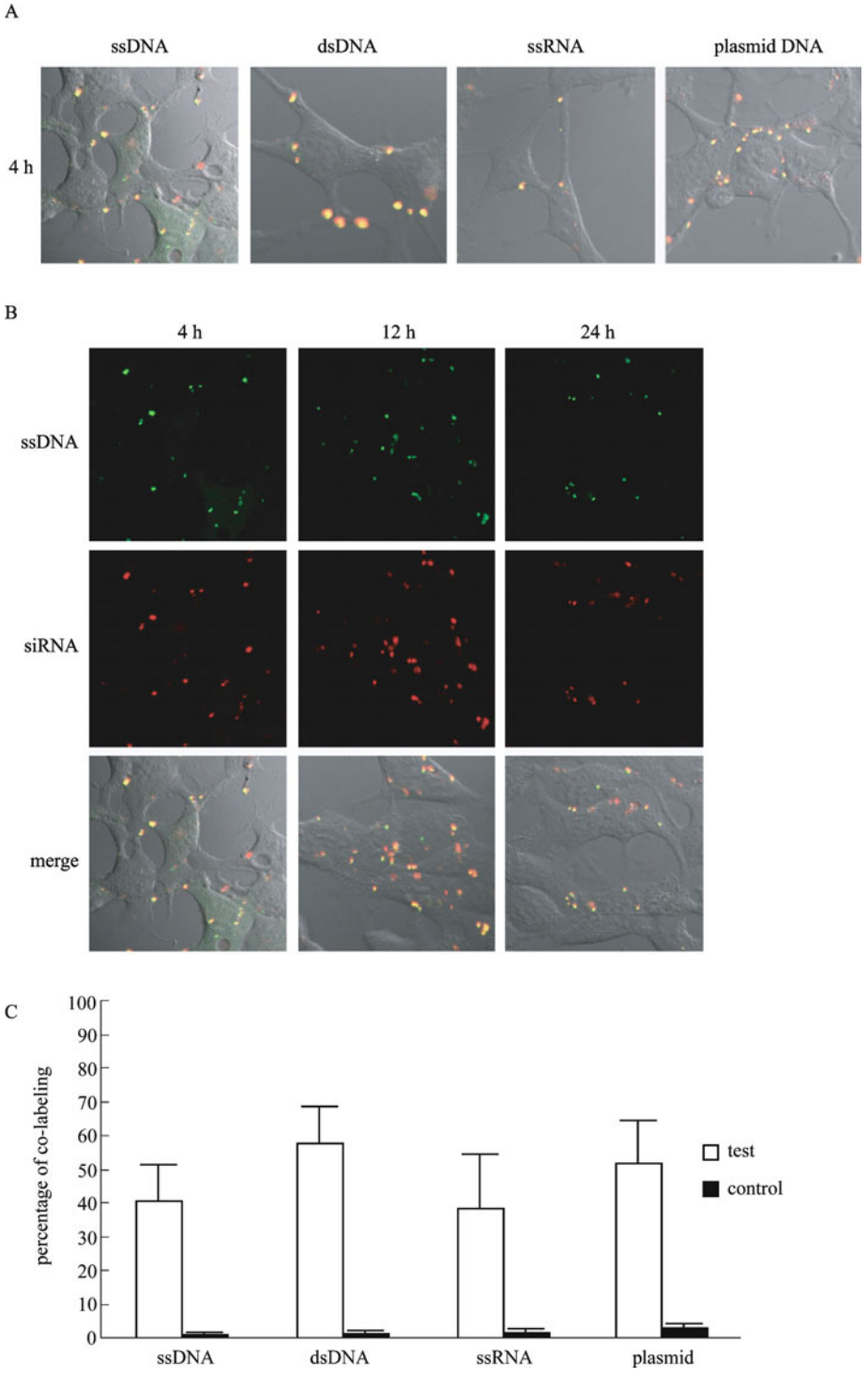

Fig. 3 Aggregation of various exogenous nucleic acids in the same cytoplasmic granules. Single-stranded DNA oligonucleotide (FAM-ssDNA-1), double-stranded DNA oligonucleotide (FAM-dsDNA-1), single-stranded RNA oligonucleotide (FAM-ssRNA-1) as well as a plasmid DNA (YoYo-1-pDNA, an asymmetric cyanine dye from Molecular Probes) was individually transfected into HEK293 cells together with Alexa-647-siRNA-1. A: Distribution of the nucleic acids 4 h after the transfection, using laser confocal microscopy. Only merged images are shown. B: Prolonged observations for ssDNA transfection. C: Quantitative analysis of co-aggregation of siRNA with other nucleic acids. All the transfections were performed using Lipofectamine 2000 at an oligonucleotide concentration of 13 nmol/L, and the plasmid DNA was transfected at a concentration of 0.33 ng/μL. Co-aggregation by chance was calculated as the control.

also have aggregated into the granules. Formation of these granules was likely triggered by the second transfection. Taken together, these results suggested that although the formation of the granules is a continuous process,

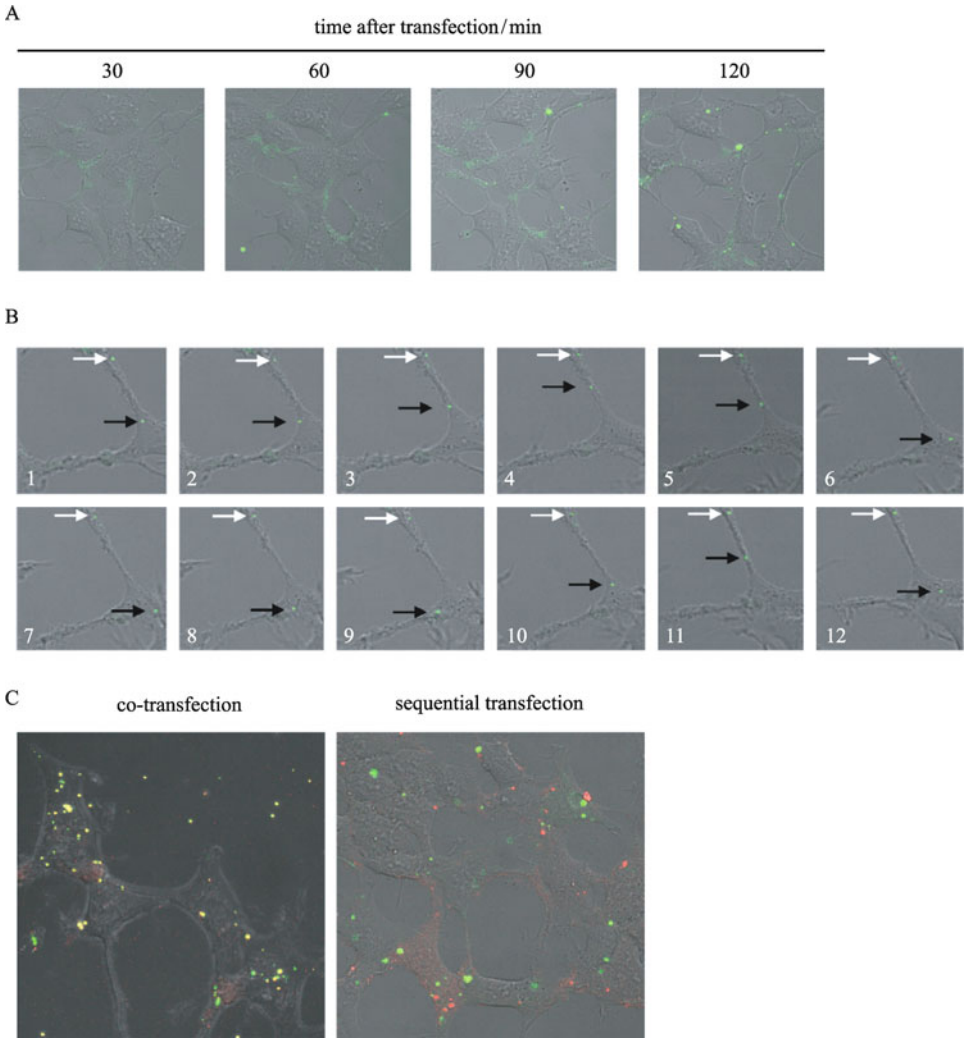

Fig. 4 Formation of siRNA-aggregating granules is triggered by nucleic acid transfection. Time-lapse microscopy imaging was used to study the formation and cellular trafficking of the siRNA-aggregating granules, following transfection of a fluorescently labeled siRNA (Alexa-647-siRNA-1). A: Formation of the transfection granules. Distribution of the siRNA at different time points after transfection, showing the assembly process of the transfection granules. B: Time-lapse images showing the cellular trafficking of the granules. The time interval between frames is 1 min. The white arrows indicate a static transfection granule during the observation, while the black arrows indicate an actively trafficking granule. The size and fluorescence intensity of the granules remained constant during the observations. C: Two siRNAs labeled by different fluorophores, Alexa-647 (siRNA-1) or FAM (siRNA-3), were co-transfected or sequentially transfected into HEK293 cells. In sequential transfection, the second siRNA was transfected 2 h after the first one. Distributions of the siRNAs 4 h after the co-transfection or the second transfection in sequential transfection. All the transfections were performed with Lipofectamine 2000 at a concentration of 13 nmol/L.

assembly of an individual granule is a transient process. These transfection-triggered cytoplasmic granules are thus named “transfection granules”.

3.5 The function of nucleic acid is uncoupled from the assembly of the granules

Triggering of granule formation by nucleic acid transfection, a challenge artificially imposed on the cells, reminded us of the formation of stress granules in response to internal or external stress (Kedersha and Anderson, 2007; Anderson and Kedersha, 2008). Similarly, to deal with other environmental challenges such as ultraviolet irradiation, heat shock and oxidative stress, or physiological changes including oogenesis or neuronal cell development, cells develop mechanisms to form various kinds of RNP granules so as to maintain a stable internal environment and/or protect vital cellular components (St Johnston, 2005; Anderson and Kedersha, 2006; Bhattacharyya et al., 2006; Arimoto et al., 2008). A common feature shared by these granules is that mRNAs stored within them are dormant in terms of performing their normal cellular functions. In light of these phenomena, we speculated that to deal with the huge amounts of nucleic acids introduced

in the transfections, cells develop a mechanism to process the over-loaded exogenous nucleic acids by packing them into specialized transfection granules, so as to keep the internal environment stable. We further speculated that, similar to stress-induced and other cytoplasmic granules, nucleic acid materials that accumulated in the transfection granules were dormant.

To this end, co-transfection of an Alexa 647-labeled siRNA and YoYo 1-labeled pDNA was performed, using three common transfection reagents, Lipofectamine 2000 (Invitrogen), siPORT (Ambion) and INTERFERin (Polyplus). Our results showed that both fluorophores colocalized in Lipofectamine 2000 transfection (Fig. 5a, right column), while only the plasmid fluorescence was observed in siPORT transfection (Fig. 5a, middle column). Similar to Lipofectamine 2000 transfection, both siRNA and plasmid fluorescence signals were observed in INTERFERin transfection (Fig. 5a, left column), indicating that both were efficiently transfected into the cells.

Gene expression and silencing efficacy assays were

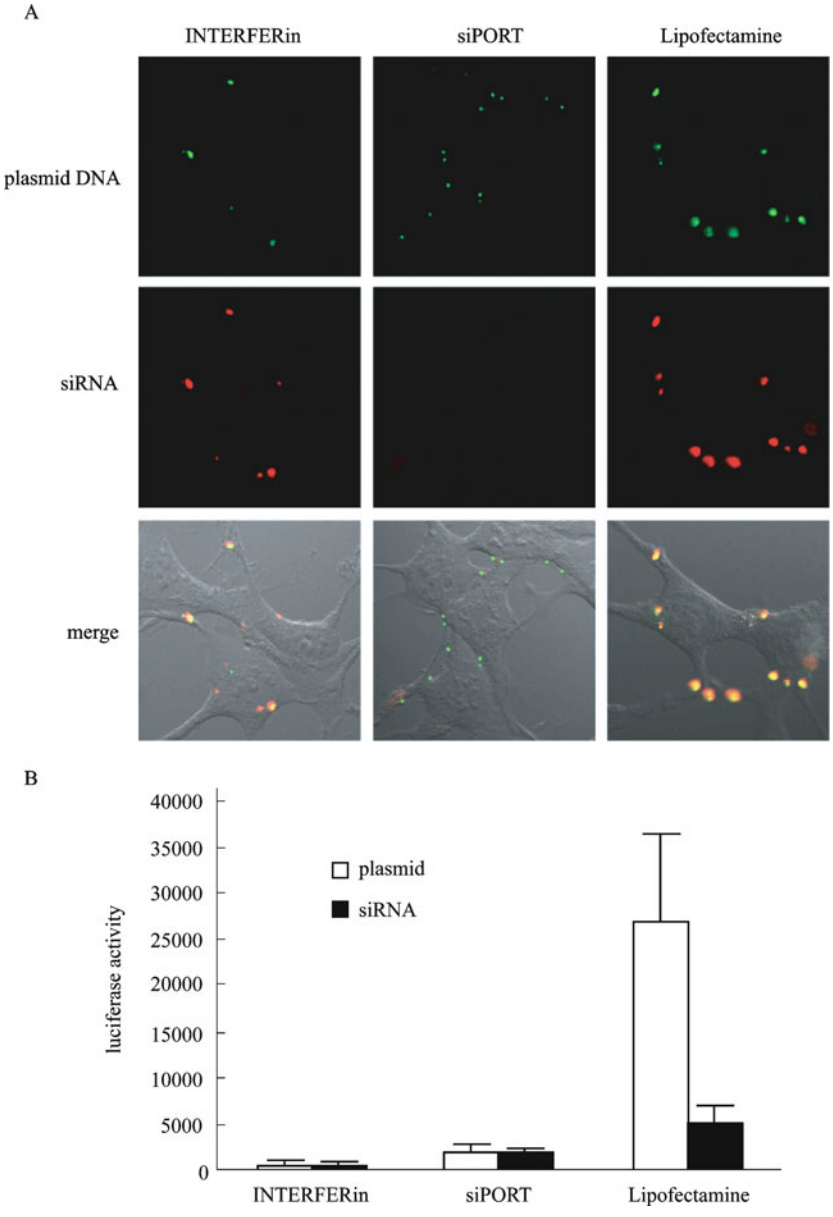

Fig. 5 Granule-aggregation of plasmid DNA represses reporter gene expression. A: A fluorescently labeled siRNA (Alexa-647-siRNA-1) and a labeled plasmid DNA (a fusion *firefly* luciferase reporter vector, YoYo-1) were co-transfected into HEK293 cells using INTERFERin, siPORT or Lipofectamine 2000. The distributions of siRNA and plasmid DNA 4 h after the transfections are shown. B: An effective siRNA (siRNA-4) targeting a *firefly* luciferase fusion gene was co-transfected into HEK293 cells together with its target reporter vector using INTERFERin, siPORT or Lipofectamine 2000. The gene silencing efficacies were measured 24 h after the transfections by luciferase assay. The transfections were performed according to the respective product manual. The plasmid DNA was transfected at a concentration of 0.33 ng/μL, and the siRNA was transfected at a concentration of 13 nmol/L.

similarly performed using these transfection reagents. A luciferase reporter vector and a siRNA targeting luciferase gene were individually or co-transfected into cultured cells, and then the expression level of the luciferase gene and the silencing efficacy of the siRNA were quantified by dual-luciferase assay (Fig. 5b). Strong reporter gene activity and potent RNAi silencing were observed in Lipofectamine 2000 transfection, which was in agreement with confocal imaging showing that both the reporter vector and siRNA were effectively transfected into the cells. In siPORT transfection, we observed a lower level of luciferase activity, which was not further knocked down by co-transfection of luciferase-targeting siRNA (Fig. 5b). This indicated that only the reporter vector, but not the siRNA, was transfected into the cells by siPORT. Accordingly, no siRNA fluorescence was observed in microscopy observation (Fig. 5a, middle column). However, in INTERFERin transfection, only background levels of luciferase activity were detected (Fig. 5b), although confocal imaging indicated that the reporter vectors were actually transfected into the cells (Fig. 5a, left column). These data suggest that in INTERFERin transfection, most if not all of the reporter vector are sequestered in the transfection granules and therefore become inactive in reporter gene expression, demonstrating the dormant of granule-aggregated nucleic acids. During prolonged culture, no reporter gene expression was observed.

4 Discussion

In summary, several lines of evidence strongly suggest that the siRNA-aggregating granules likely serve as a common storage/processing site for extraneously transfected nucleic acids, but are not the function site of RNAi. First, formation of granules is likely triggered by nucleic acid transfection; second, in addition to siRNA, other forms of nucleic acid aggregate in the granules after transfection; third, the amount of siRNA aggregating in the granules is not correlated with its gene silencing activity; and the last, granule aggregation of plasmid DNA leads to its inactivity.

In addition to P bodies, a few distinct RNP granules have been shown to contain dormant mRNAs in eukaryotic cells under stress, during oogenesis or in neuronal cells (St Johnston, 2005; Anderson and Kedersha, 2006). A common feature of these granules is that they store non-translating mRNAs. For example, stress granules form in response to environmental stresses such as ultraviolet irradiation, heat shock and oxidative stress. These abnormal conditions inhibit the translation of bulk mRNAs, which subsequently aggregate in the stress granules. Like stress granules, siRNA-aggregating granules are triggered by nucleic acid transfection. siRNA, and other forms of nucleic acid, all aggregate in the granules after transfection. Once they have aggregated in the granules, the expression of plasmid-encoded genes is inhibited, a remarkable feature of stress and other mRNP granules. This repression in many cases is reversible, and mRNPs can exit from the repressed state and re-enter into active translation (Brengues et al., 2005; Coller and Parker, 2005; St Johnston, 2005). In our studies, sequestration of the plasmid DNA in the granules seems to be irreversible, leading us to the hypothesis that the transfection granules are formed to collect over-loaded extraneous nucleic acids, keeping intra-cellular environment free of unneeded material. Further evidence are that gene silencing activity was not correlated with the amount of siRNA aggregating in the granules, indicating that the granules are not the function site in RNAi. An alternative functional site is the cytoplasm itself. Given the large volume of cytoplasm relative to P bodies, it might play an important role in RNAi.

In previous studies, depletion of RISC and P-body components was commonly used (Bhattacharyya et al., 2006; Chu and Rana, 2006). siRNA targeting key components of RISC and P body, for example, Ago1, Ago2 or GW182, was transfected into cells to determine its effects on gene silencing. Depletion is a well-established method to investigate gene function as overexpression. However, it is not suitable for RNAi pathway studies because internal RNAi pathway is compromised when the key component is depleted. And more seriously, due to our limited knowledge of RNAi, the influence of depleting the RNAi component is largely unpredictable. In the present study, interference with normal RNAi processes was avoided by manipulating only the nucleic acid to be transfected or the concentration of transfected siRNA. Therefore, our observations more likely reflect the real situation in normal RNAi processes.

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