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Rapid communication

# Dual imaging of mRNA and protein production: An investigation of the mechanism of heterogeneity in cationic lipid-mediated transgene expression

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ABSTRACT

Heterogeneity of transgene expression is a severe disadvantage in the use of cationic lipid-mediated gene vectors. We previously demonstrated that heterogeneity of the post-nuclear delivery process, as well as intracellular trafficking (i.e. nuclear delivery) is a major determinant in the overall heterogeneity in gene expression, when plasmid DNA (pDNA) is transfected to HeLa cells using a lipoplex. In this study, we explored the mechanism underlying this heterogeneity in a post-nuclear transport process by the dual imaging of mRNA and its encoded protein (histone H2B-tagged mTFP1; mTFP1-H2B) in a single cell. To establish a highly sensitive imaging system for mRNA, we used fluorescence in situ hybridization (FISH) combined with tyramide signal amplification (TSA) and a semiconductor quantum dot (QD) probe. The mRNA expression and protein production were quantified by counting the total pixel intensity in the region of interest (r.o.i.) surrounding single cells. As a result, the correlation was poor in a scattered plot of mRNA expression versus protein production in individual cells. These findings demonstrate that cell-to-cell differences in the translation process are also a key factor in heterogeneous gene expression.



Heterogeneity of transgene expression on a cell-by cell basis is a crucial factor that hampers the use of non-viral vectors (Cohen et al., 2009; Tseng et al., 1997; Wilke et al., 1996). Numerous studies have reported that the transfection efficiencies of various types of non-viral vectors are substantially enhanced when the cells cycle progresses through the M phase, where the nuclear membrane structure is temporarily disrupted (James and Giorgio, 2000; Mortimer et al., 1999; Tseng et al., 1999). Thus, it is likely that this heterogeneity can be attributed to the nuclear delivery process, which, in turn, arises from the cell-to-cell differences in cell cycle status. Meanwhile, we reported on the development of a dual imaging system to monitor the nuclear delivery of plasmid DNA (pDNA) and the expression of its encoded marker protein in individual cells that had been transfected with Lipofectamine PLUS (LFN) (Akita et al., 2007). In this study, nuclear pDNA-positive cells were observed in only 22.5% of the total cells, while the cellular uptake of plasmid DNA occurred in 100% of the cells. Furthermore, protein expression was detected in only 32.6% of the nuclear pDNA-positive cells. These data indicate that post-nuclear delivery processes (transcription and/or translation), as well as intracellular trafficking (the nuclear delivery) are key factors in heterogeneous gene expression. The importance of post-nuclear delivery processes in the development of a non-viral vector is also evident from a quantitative comparison of intracellular trafficking between adenovirus and pDNA transfected with LFN. A comparison of the nuclear delivery of DNA revealed that 3- to 4-orders of magnitude more pDNA gene copies were required to be delivered to the nucleus when LFN was used to achieve a transfection activity comparable to that of adenovirus (Hama et al., 2006). Further quantification of the mRNA products revealed that the three orders of magnitude difference in the post-nuclear-delivery process was due to a one order of magnitude difference in transcription efficiency and a two orders of magnitude difference in translation efficiency (Hama et al., 2007). The above data prompted us to examine the contribution of the translation process to cell-to-cell heterogeneity in the post-nuclear delivery process.

In an attempt to address this issue, the relationship between mRNA expression and protein production was investigated at the single cell level. We used streptavidin-conjugated quantum dot (QD) as an imaging probe, in an attempt to improve the sensitivity of mRNA detection. QD is generally recognized as an ideal probe for imaging the intracellular trafficking of macromolecules (Algar and Krull, 2008; Pinaud et al., 2006) and mRNA (Tholouli et al., 2006; Wu et al., 2006) owing to their high degree of brightness, and bio- and photo-stability. As a marker protein for visualizing gene expression, we used a histone H2B-tagged fluorescence protein. A use of nucleus-targeting protein is rational since the nuclear accumulation of marker proteins can facilitate the visual

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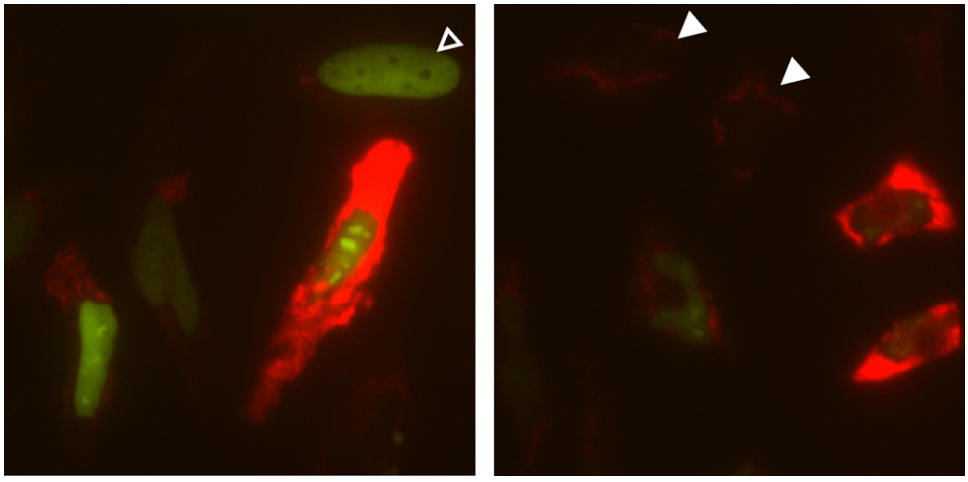

**Fig. 1.** Typical images for dual imaging of mTFP-H2B mRNA and protein. HeLa cells were transfected with pDNA encoding mTFP-H2B by Lipofectamine PLUS for 24 h, and the mRNA was then stained for FISH with TSA. Closed triangles represent cells in which mTFP-H2B expression was under the detection limit, but mRNA expression was detected. Open triangles represent cells in which mTFP-H2B expression was clearly detected, even in cells with low mRNA expression. Fluorescence signals derived from mTFP-H2B and mRNA were pseudocolored in green and red, respectively.

discrimination of fluorescence signals derived from marker fluorescence proteins from those of mRNA, which is mainly localized in the cytoplasmic region. Detail materials and methods in following studies are described in Supplemental Information.

After the transfection of pDNA encoding mTFP-H2B by LFN for 24 h, the cells were fixed, and mRNA was thereafter detected by FISH combined with TSA. Preliminary studies demonstrated that mTFP-H2B expression is little, if any, just above the detection limit at the shorter time incubation (i.e. 6 h and 12 h). While the half-life of the mTFP-H2B is not available in previous works, it is plausible is that the proteins mainly observed in the present study (24 h incubation) is not a remaining proteins which were biosynthesized at shorter time. The hybridization of used probes to the *in vitro* translated mRNA encoding mTFP1-H2B was preliminarily confirmed by gel electrophoresis (Supplemental Fig. 1). To minimize spectral overlap between mTFP and mRNA signals, we used QD705 as a near-infrared probe. Fig. 1 shows typical images for the dual imaging of mRNA (red) and mTFP-H2B (green). The mRNA and mTFP-H2B product were largely detected in cytoplasmic regions and the nucleus, respectively (For interpretation of the references to color in this text, the reader is referred to the web version of the article.). Of note, mTFP-H2B expression was under the detection limit in some cells, while mRNA expression was clearly detected (indicated as closed triangles). On the other hand, prominent mTFP-H2B expression was detected, even in cells with low mRNA expression (indicated as open triangles). These images suggest that the level of mRNA expression is not a determining factor for its encoded protein.

To observe the correlation between protein expression and mRNA expression, total cellular regions were defined as the region of interest (r.o.i.), and total pixel intensities derived from the mTFP-H2B signals and mRNA signals in a single optical slice were counted in each of 36 cells (Fig. 2A). Regarding mRNA expression, 3 out of 36 cells showed high mRNA signals (>$5.0 \times 10^5$ arbitrary units).

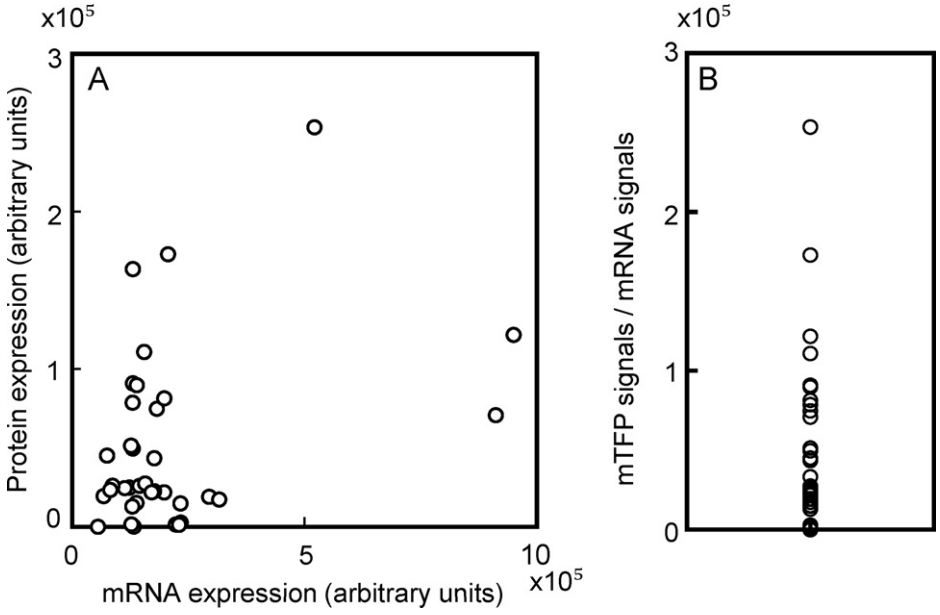


**Fig. 2.** Correlation between mTFP-H2B production and mRNA expression. (A) Scattered plot of mTFP-H2B production and mRNA levels in individual HeLa cells (single optical slice) after transient transfection using lipoplex. (B) Plots of mTFP production normalized by mRNA.

However, in the majority of cells, the cell-to-cell variation in mRNA expression fell into the dynamic range of 1 order of magnitude (from $5.5 \times 10^4$ to $3.1 \times 10^5$ arbitrary units). This range appears to be narrow compared with those obtained from reporter genes that were integrated into the cell's genome (>2 order of magnitude range) (Raj et al., 2006). Raj et al. proposed a stochastic model of gene activation and inactivation, in which genes that are integrated into the genome are transcribed in short but intense bursts. According to their model, the elevated expression of transcription factors would result in an increase in the size of the bursts, but not their frequency. In addition, the mRNA levels transcribed from genes that were integrated in different genome loci were poorly correlated, suggesting that these gene expressions were independently regulated. Thus, in stable cell lines in which a low copy number of genes are integrated into the genome, the stochastic regulation of each integrated gene might be directly reflected by cell-to-cell variation of mRNA expression. In contrast, in transient gene transfection, large number of plasmid DNA molecules enter the nucleus (>500 copies) (Cohen et al., 2009; Hama et al., 2006, 2007). Thus, the stochastic regulation of each intra-nuclear pDNA might be averaged, resulting in a decrease in heterogeneous mRNA expression.

Another important interpretation of Fig. 2A is that the expression levels of mRNA and mTFP-H2B were not correlated (correlation coefficient; −0.043). In addition, the values for mTFP-expression normalized by mRNA were highly varied (coefficient of variation; 1.13) as shown in Fig. 2B. Collectively, these data indicate that the translation process is not the rate-limiting process. In stably genome-integrated cells, cell-to-cell variation in protein expression was relatively small compared with that of mRNA expression. In other words, the burst of mRNA expression could be buffered at the protein level by a slow rate of protein degradation (Raj et al., 2006). In contrast to these previous observations, the variation in mTFP-H2B expression (coefficient of variation = 1.06) was larger than the corresponding values for mRNA expression (coefficient of variation = 0.39) in the transient expression system. Thus, the poor correlation between mRNA expression and the corresponding protein production cannot be explained by a balance between pulses and bursts of mRNA expression and a slow rate of protein degradation. While the mechanism remains to be clarified in the future, one possible mechanism is electrostatic interactions between the cationic component of a lipoplex and mRNA. Hama et al. previously reported that the *in vitro* mRNA translation process was drastically inhibited in the presence of cationic liposomes, whereas this process is not inhibited by adenovirus, a neutral particle (Hama et al., 2007). Therefore, heterogeneity in the cellular uptake of cationic components could induce cell-to-cell heterogeneity in translation efficacy.

In summary, the dual imaging of mRNA expression and protein production revealed that the translation process contributes to the heterogeneous gene expression in lipoplex-mediated transfections. Taking previous information into consideration, heterogeneity in the cellular uptake of a cationic component might be the reason for heterogeneous translation efficiency. The development of vectors that are less cationic which can compensate for the intracellular trafficking process (i.e. cellular uptake and nuclear delivery) might be a key technology for overcoming the observed heterogeneity.

## Conflict of interest

No potential conflicts of interest are disclosed.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.05.051.

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