Fluorescent tag is not a reliable marker for small RNA transfection in the presence of serum

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Chemically synthetic siRNA and miRNA have become powerful tools to study gene function in the past decade. Fluorescent dyes covalently attached to the 5′ or 3′ ends of synthetic small RNAs are widely used for fluorescently imaging and detection of these RNAs. However, the reliability of fluorescent tags as small RNA markers in different conditions has not attracted enough attention. We used Cy3-labelled small RNAs to explore the reliability of fluorescent tags as small RNA markers in cell cultures involving serum. A strong Cy3-fluorescence signal was observed in the cytoplasm of the cells transfected with Cy3-miR24 in the culture medium containing fetal bovine serum (FBS), but qRT-PCR results showed that little miR24 were detected in these cells. Further study demonstrated that small RNAs were degraded in the presence of FBS, suggesting that it was Cy3-RNA fragments, rather than the original Cy3-miR24, diffused into cells. These phenomena disappeared when FBS was replaced by boiled-FBS, further supporting that the Cy3-fluorescence we observed in cells in the presence of FBS could not represent the presence of intact small RNAs. These findings addressed that fluorescent tags are not reliable for small RNA transfection in the presence of serum in culture.

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1. Introduction

Short interfering RNAs (siRNA) are double-stranded RNAs of approximately 21–25 nucleotides that suppress gene expression by triggering posttranscriptional gene silencing (Fire *et al.* 1998; Elbashir *et al.* 2001b). It is reported that chemically synthetic siRNAs can induce gene-specific inhibition of expression in cell lines from humans and mice (Caplen *et al.* 2001; Elbashir *et al.* 2001a). Since then, the chemically synthetic siRNAs have been used as a powerful tool to knock down specific gene expression and study their function in a wide range of cells.

MicroRNAs (miRNAs) are small, noncoding RNAs which regulate gene expression post transcriptionally by partially paring with the 3′UTR sequences of their target mRNAs and causing mRNAs cleavage or translation blockage (Lagos-Quintana *et al.* 2001; Bartel 2004). An increasing number of studies demonstrated that miRNAs were involved in many important biological processes, including cell cycle, differentiation, development and apoptosis (Ambros 2003; Bartel 2004). Guimaraes-Sternberg *et al.* in 2006 presented the chemically synthetic miRNA mimic as a prospective RNA-based tool for leukemia research and therapeutic strategy (Guimaraes-Sternberg *et al.* 2006). This kind of synthetic miRNA mimic then becomes a novel tool for cellular miRNA research.

Chemically synthetic small RNAs have been widely used as experimental approach regulating gene expression *in vitro* and *in vivo*, and their potential therapeutic application has been explored in many researches (Seyhan 2006). One of the major obstacles in small RNAs application is their instability in serum, where they are degraded by RNase. For *in vitro* experiments, serum is essential for most of the cell lines and

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primary cells in culture. For *in vivo* animal experiments, serum widely distributed throughout the whole body is also an inevitable obstacle for efficient small RNA delivery. It is reported that many siRNAs are digested within minutes in mammalian serum (Braasch *et al.* 2003; Chiu and Rana 2003).

Fluorogenic cyanine dyes, such as Cy3 and Cy5, are widely used for fluorescently imaging and detection of chemically synthetic small RNAs by covalently attached to the 5′ or 3′ ends of small RNAs. This is convenient for real-time detection of these molecules without significant loss of RNA interference activity (Grunweller *et al.* 2003). However, much attention should be given to the reliability of fluorescent tags as small RNAs marker in the presence of serum. Here, we aim to address this problem by transfecting cultured HEK 293T cells and adult rat cardiomyocytes with Cy3-labelled siRNA and miRNA mimics. We found that it was the fluorescent dye-RNA fragments instead of intact small RNAs that were uptaken by these cells in the presence of FBS. Furthermore, Cy3-RNA fragments kinetics and localization in cardiomyocytes were also investigated.

2. Materials and methods

2.1 *Ethics statement*

This investigation conformed to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health. Animal experiments were approved by the Institutional Animal Care and Use Committee of Peking University.

2.2 *Preparation of siRNA and miRNA*

Chemically synthetic siRNA and miRNA mimics were purchased from Ribo Bio Co. Ltd (Guangzhou, China). They are double-strand RNAs (dsRNAs) and their sequences are shown in figure 1. MiRNA used in this paper is the mimic of *rattus norvegicus*-miR24. As a fluorescent tag of small RNAs, Cy3 was conjugated to 5′ ends of siRNA and miRNA.

(A)

miR24 mimic

5′-UGGCUCAGUUCAGCAGGAACAG-3′
3′-ACCGAGUCAAGUCGUCCUUGUC-5′

(B)

siRNA

5′-CUGACGAGCUGCAACUCAA dTdT-3′
3′-dTdT GACUGCUCGACGUUGAGUU-5′

Figure 1. The RNA sequence of small RNAs used in this study: (**A**) miR24 and (**B**) siRNA.

2.3 *Cell culture and transfection*

HEK 293T cells were cultured in DMEM medium with 10% FBS (HyClone) at 37°C. Cells were plated to 80–90% confluence and then transfected with 1.5 μg dsRNAs. Lipofectamine 2000 (Invitrogen) was used in two groups of experiments according to manufacturer's instruction. The other two groups did not use any transfection reagent. Transfection was carried out in the presence or absence of 10% FBS, as indicated in figure 2. Six hours after addition of dsRNAs, the cells were washed out 3 times with PBS, and then placed on the inverted confocal microscope for imaging. Rat ventricular cardiomyocytes were isolated from the hearts of male Sprague–Dawley rats (200–250g), as previously reported (Fu *et al.* 2005). Isolated cardiomyocytes were re-suspended in M199 medium with 5% CO_2. For siRNA and microRNA transfection, the medium was changed into M199 medium with 10% FBS, and 1.5 μg dsRNA were added.

2.4 *RNA isolation and quantitative real-time PCR (qRT-PCR)*

HEK 293T cells were homogenized in Trizol reagent (Invitrogen) 6 h\ after addition of small RNAs. Total RNA was extracted according to manufacturer's instruction. The specific oligonucleotides used for microRNA reverse-transcription were from Ribo Bio Co. Ltd. For assessment of miR24 and U6 quantity, qRT-PCR were carried out using continuous fluorescence detection system Mx3000P (Agilent Stratagene) with SYBR Green QPCR master mix (Agilent Technologies). The specific primers used for miRNA amplification were purchased from Ribo.

2.5 *In vitro small RNAs stability assay*

About 1.5 μg synthetic small RNAs were mixed with 5 μL FBS or heat-treated (100°C, 10 min) FBS. After incubation at 37°C over 0, 0.5, 1, 2, 4 and 6 h, the mixture was electrophoresed with 8% polyacrylamide gel and stained by ethidium bromide (EB). Detection and analysis of dsRNA bands were carried out using Quantity One (Bio-Rad Laboratories). The value of time 0 was set as 100% to normalize the amount of residual intact small RNAs.

2.6 *Imaging*

Six hours after addition of Cy3-small RNAs, HEK 293T cell images were obtained using an inverted confocal microscope

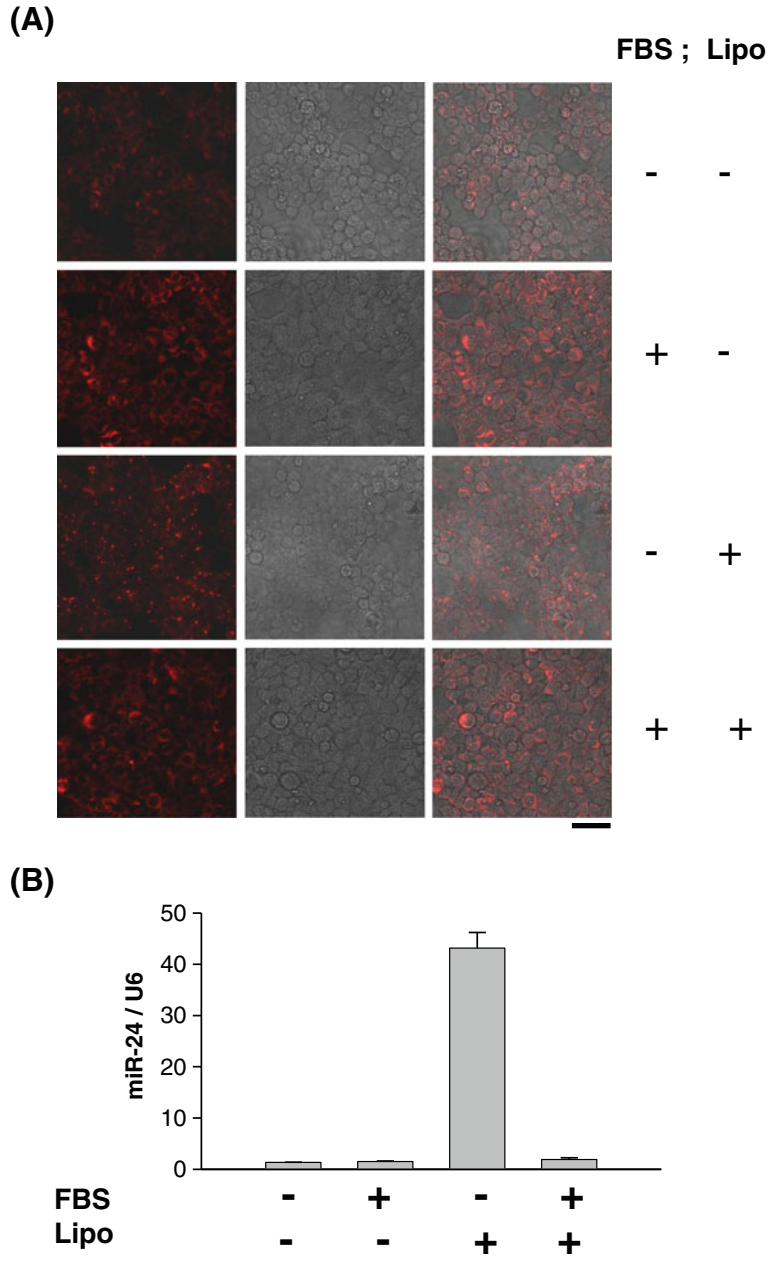

Figure 2. Cy3, but not miR24, diffused into 293T cells in the presence of FBS. (**A**) Confocal imaging of 293T cells in different experimental conditions. Left: Cy3; middle: transmitted light; right: merge. Scale bar: 50 μm. (**B**) Results of qRT-PCR measurement of miR24 relative to U6.

(LSM-510, Carl Zeiss, Germany) equipped with helium/neon (543 nm) laser, as well as a 40, 1.3 N.A. oil immersion objective.

Mitochondria of cardiomyocytes were localized by 1 μM MitoTracker Far-Red (Invitrogen) using 633 nm excitation (LSM-710, Carl Zeiss, Germany). Cy3 fluorescence was measured with excitation at 543 nm.

For real-time recording of live cell images, cardiomyocytes grown on coverslips were placed in the microscope chamber perfused with 5% CO_2 at 37°C. The cell images were captured frame by frame at 5 min intervals with a DeltaVision system (Applied Precision,Issaquah, WA) following Cy3-miR24 addition. For imaging, a 60×, 1.42 N.A. lens was used. Images were processed using DeltaVison SoftWoRx 5.5 software.

2.7 *Statistics*

All data are presented as mean±SEM as indicated and compared by Student's *t*-test for normal test-past data. A *P*-value of < 0.05 was considered significant.

3. Results

3.1 *Cy3, but not intact miRNA, entered 293T cells in the presence of FBS*

To investigate the reliability of fluorescent tag as small RNAs marker in different conditions, Cy3-labelled miR24 mimics (Cy3-miR24) were used to transfect HEK 293T cells. Confocal image of 293T cells were acquired 6 h after Cy3-miR24 addition. As a control group, we added Cy3-miR24 mimics into culture medium without FBS. Weak fluorescence was detected in 293T cells from the control group (figure 2A). But in the group with FBS, 6 h after we added Cy3-miR24 into culture medium supplemented with 10% FBS, considerably more Cy3 fluorescent spots were found diffusely distributed in the cytoplasm of 293T cells (figure 2A). To confirm whether miRNA mimics entered cells, we harvested the cells and quantified the levels of miR24 using quantitative real-time PCR (qRT-PCR). Quantitative analysis showed that little miR24 was detected in 293T cells in both control and FBS groups (figure 2B). Lipofectamine 2000 (Lipo) is a widely used transfection reagent that functions by forming complexes with nucleic acid molecules and fusing with the cell membrane to effectively mediates the entry of the nuclei acids (Dalby *et al.* 2004). In the group with Lipo, Cy3-miR24 mimics were found encapsulated in liposome and formed fluorescent particles, and Cy3 fluorescence observed in these cells was less intense than that of the FBS group (figure 2A). But qRT-PCR results showed that much more miR24 were detected in Lipo group compared with those of FBS group (figure 2B), indicating that it was Cy3 but not miR24 diffused into cells in the FBS group. In the group with Lipo and FBS, similar phenomena as in the FBS group were observed (figure 2A and B), further confirming that the Cy3 fluorescence we observed in the presence of FBS did not indicate the presence of miR24, which had possibly been degraded by RNase from the FBS. When we transfected cells with siRNA instead of miRNA, results similar to those in figure 2A were obtained (supplementary figure 1).

3.2 *Small RNAs were degraded by FBS*

To confirm whether small RNAs were digested by FBS and only Cy3-RNA fragments entered the cells, we examined the integrity of small RNAs in the presence of FBS *in vitro*. Cy3-labelled small RNAs were mixed with FBS and then incubated for various durations (0, 0.5, 1, 2, 4, 6 h) at 37°C. The small RNAs were separated in polyacrylamide gels and visualized in the gels by EB-staining. The densitometric analysis of residual small RNAs in different time was performed subsequently. We found that the small RNAs in FBS became progressively shortened fragments over time (figure 3A). About 60% miR24 were degraded within half an hour, which was the earliest time point assayed (figure 3A and B). There are dTdT overhangs at the 3′ ends of siRNA (figure 1B), which are beneficial for reconstitution of the RNAi nuclease complex and may be required for high-affinity binding of the short RNA duplex to the protein components (Elbashir *et al.* 2001b). It was found that siRNA used in present study was more resistant towards degradation in comparison with miR24 mimics. Approximately 40% siRNA were degraded in half an hour (figure 3A and C). To determine whether the observed degradation of small RNA resulted from the presence of RNase in FBS, we incubated small RNAs with heat-denatured (100°C, 10min) FBS. We found that degradation of siRNA and miR24 was almost fully eliminated, indicating that they had significantly greater stability in boiled FBS (figure 3). Of note, there were still a little small RNAs digested by RNase which was hard to be completely denatured. Besides, when we added Cy3-miR24 mimics into culture medium with 10% boiled-FBS, little fluorescence was detected in 293T cells 6 h later. These data suggested that RNase in FBS degraded small RNAs, and elimination of RNase activity strongly increased the stability of small RNAs in FBS.

3.3 *Cy3-RNA fragments translocated into adult cardiomyocytes and enriched in mitochondria*

To study whether similar phenomena can be observed in primary cell, we used Cy3-labelled small RNAs to transfect adult rat cardiomyocytes. They are big rod-shaped cells whose size is about 20×100 μm (data not shown), which makes it also a perfect candidate for observing molecule movement. Cy3-labelled small RNAs were added into 10% FBS-M199 culture medium with cardiomyocytes attached on the dish bottom. As shown in previous results, small RNAs were digested by RNase in FBS continuously, leaving Cy3 conjugated with small RNA fragments. We followed the change in fluorescence intensity of Cy3 spots in living cardiomyocytes. A HeNe laser (543 nm) was used for observation of Cy3. Cy3-miR24 was not detectable as fluorescent spots in the culture medium because of Brownian movement in solution. The background auto-fluorescence of cells before addition of Cy3-miR24 was low. Two hours after the addition of Cy3-miR24, fluorescent spots appeared first at the two ends of the cells. Then the fluorescence diffused into the whole cell and the intensity increased with

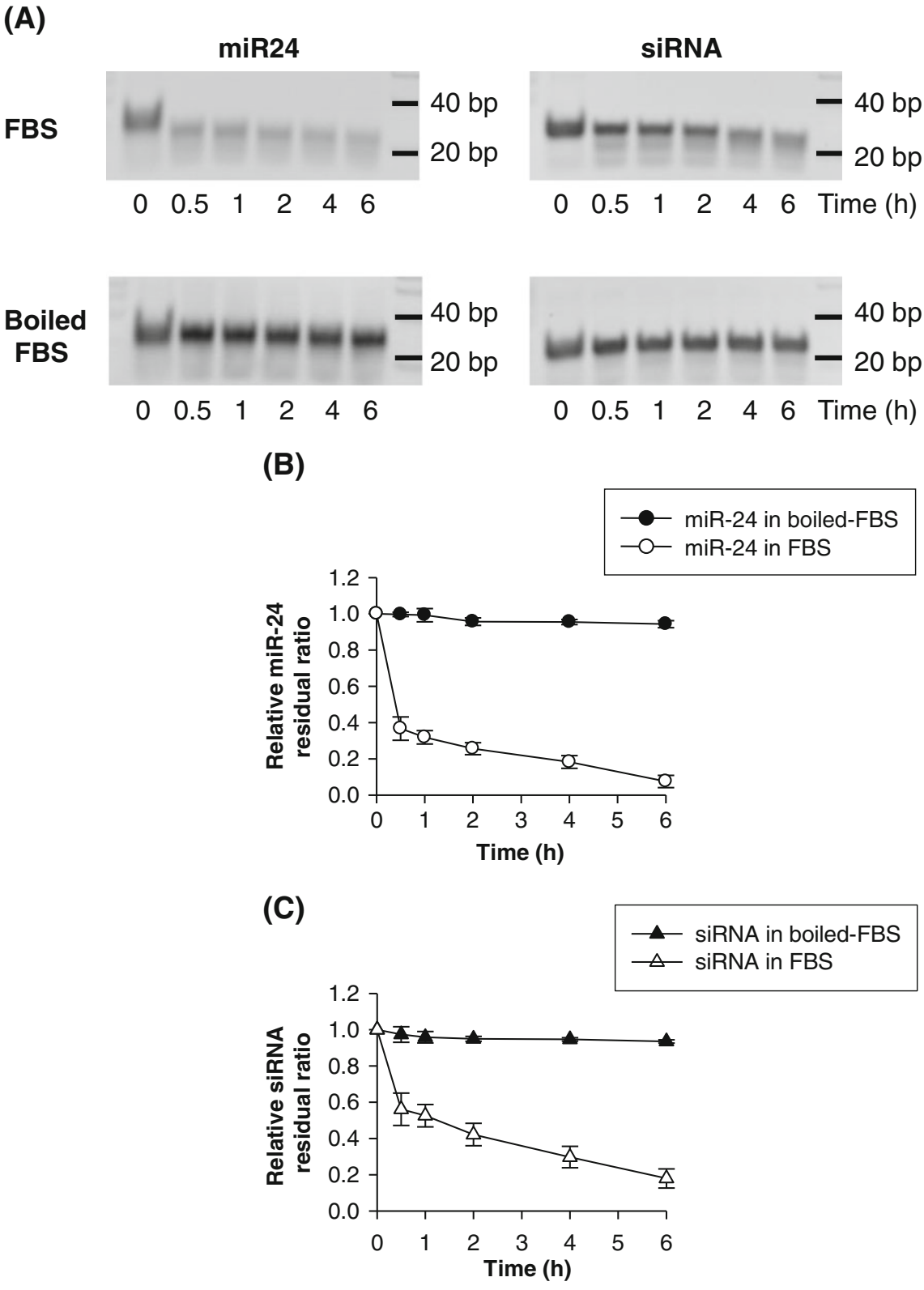

Figure 3. Synthetic small RNAs were degraded by RNase in FBS. (**A**) The stability of siRNA (left) and miRNA (right) in FBS (top) and boiled-FBS (bottom). Incubations were performed for the indicated duration at 37°C (0, 0.5, 1, 2, 4, 6 h). (**B**) Stability analysis of siRNA in FBS and boiled-FBS. (**C**) Stability analysis of miR24 in FBS and boiled-FBS. Residual small RNAs quantities at different time points were calculated by dividing the total counts of small RNAs by the 0 h input RNAs.

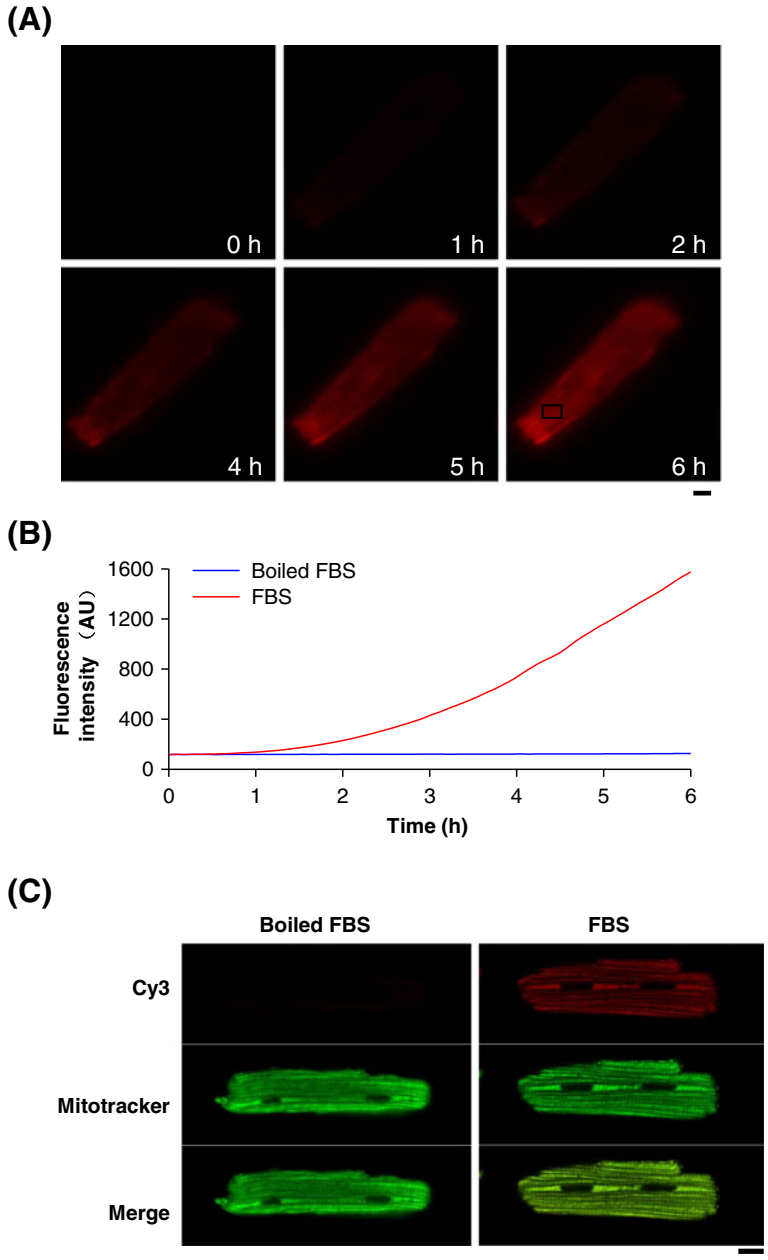

Figure 4. Degraded Cy3-RNA fragments translocated into cardiomyocytes and were localized to mitochondria. (**A**) Real-time imaging of Cy3-RNA fragments in cardiomyocytes. Cells were observed with a DeltaVision elite imaging system. Scale bar: 10 μm. (**B**) Real-time assessment of Cy3 fluorescence intensity in the cardiomyocytes incubated in FBS-M199 medium (red line) or boiled-FBS-M199 medium (blue line). The images were obtained frame by frame at an interval of 5 min. (**C**) Colocalization of Cy3-RNA fragments with mitochondria marker, MitoTracker (right). Scale bar: 10 μm.

time (figure 4A and 4B). The changes of Cy3 fluorescence intensity in the region of interest (as indicated in figure 4A) were measured frame by frame (12 frames every hour) and plotted against time (figure 4B, red line). However, when we added Cy3-miR24 into culture medium supplemented with pre-boiled FBS instead of FBS, little Cy3 fluorescence was observed in cardiomyocytes (figure 4B and C, and the supplementary movie), further indicating that it was the Cy3-RNA fragments that enriched in cardiomyocytes in the presence of FBS. Cy3-labelled small RNA did not enter the cardiomyocytes in the absence of FBS. The velocity of Cy3-dsRNA fragments translocation into cells was positively

related to the concentration of FBS (supplementary figure 2). Cy3 movement in cardiomyocytes over time is shown in the supplementary movie. It seems that Cy3-RNA fragments enriched in mitochondria after translocating into cardiomyocytes. To define the localization of Cy3-RNA fragments more precisely, we loaded the cells with mitochondrial marker, MitoTracker Far-Red. As expected, Cy3 fluorescence in FBS group shows specific mitochondrial localization, overlapping completely with MitoTracker (figure 4C).

4. Discussion

Chemically synthetic small RNAs are proving to be a valuable tool for analysing gene and protein function in many researches. The reliability analysis of small RNA marker may be of great importance for a future application of small RNAs and may improve the development of feasible strategies to evaluate small RNAs transfection efficiency precisely. In this study, we used Cy3-labelled siRNA and miRNA to explore the possible role of fluorescent tag as small RNAs marker in different conditions. Cy3 was covalently linked to the 5′ end of small RNAs.

The present study shows that FBS significantly decreased the amount of intact Cy3-labelled small RNAs in short time, and Cy3 fluorescence observed in the cells can not indicate the presence of original small RNAs. Of note, FBS is same as fetal calf serum (FCS), which is also widely used by researchers. So we recommend that during the process of small RNAs transfection *in vitro,* serum-free medium should be used. Besides, it is noticeable that serum is hard to fully remove – there may be a little serum attached on the surface of cells. This part of serum makes the quantification of small RNAs by fluorescent tag a little higher than actual value. In addition, the FBS we used in present study was heat-inactivated by manufacturer before delivery. Due to the different efficiency of heat treatment, there might be batch-to-batch variation in the proposed RNase activity. Chemically synthetic small dsRNAs are increasingly important molecular tools, as they are often used by researchers to elucidate the function of a gene. It requires a better understanding of the factors interfering small RNA stability and some improving strategies to increase the efficiency of small RNA transfection.

The data of the present study indicate that siRNA and miRNA used in this research had different degradation kinetics in FBS. According to previous report, this can be attributed to their sequence differences (Haupenthal *et al.* 2006). To improve the serum resistance of small RNAs, various chemical modifications significantly stabilizes small RNAs without reducing their knockdown efficiency were studied. Synthetic siRNA molecules with specific 2′-O-methyl, 2′-*F*, and locked nucleic acid (LNA) modifications are protected against serum-derived nucleases and are tolerated without significant loss of RNA interference activity (Braasch *et al.* 2003; Czauderna *et al.* 2003; Harborth *et al.* 2003; Layzer *et al.* 2004; Choung *et al.* 2006). MiRNAs have been found in the serum and plasma of humans and other animals, and they are resistant to RNase digestion and other harsh conditions (Chen *et al.* 2008). It is reported that they are protected against RNase either by encapsulated in exosomes and microvesicles or through association with protein complexes (Arroyo *et al.* 2011).

The successful execution of RNAi experiments depends upon multiple factors. The following requirements must be met: effective and stable small RNAs, successful delivery of small RNAs into the cells, and a detection system to measure the suppression of expression of target gene (Duxbury and Whang, 2004; Choung *et al.* 2006). Molecular detection methods, such as qRT-PCR and Western blot, are necessary to confirm the efficiency of gene silencing. The fluorescent tag, a real time marker of small RNAs, can combine with these methods to estimate the transfection and silencing efficiency of small RNAs. For the detection of miRNA delivery, qRT-PCR of corresponding miRNA can also be used to measure the quantity of miRNA incorporated into cells, as shown in figure 2B. Furthermore, our finding that Cy3-RNA fragments enriched in mitochondria after entering the cells also help to discriminate Cy3-RNA fragments from Cy3-intact small RNAs.

In summary, our findings demonstrate that synthetic small RNAs were degraded by RNase in serum and isolated Cy3-RNA fragments entered cells and concentrated in mitochondria subsequently. In this case, fluorescent tag is not a reliable marker for small RNA transfection. These findings could be of great importance for a future application of small RNA and may result in feasible strategies to evaluate small RNA transfection efficiency precisely.

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