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Visualizing of the Cellular Uptake and Intracellular Trafficking of Exosomes by Live-Cell Microscopy

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ABSTRACT

Cells release exosomes to transfer various molecules to other cells. Exosomes are involved in a number of physiological and pathological processes. They are emerging great potential utility for diseases diagnosis and treatment recently. However, the internalization and intracellular trafficking of exosomes have not been described clearly. In this work, exosomes were isolated from the culture medium of PC12 cells, labeled by lipophilic dye and amino-reactive fluorophore, incubated with resting PC12 cells. The results of live-cell microscopy indicated that exosomes were internalized through endocytosis pathway, trapped in vesicles, and transported to perinuclear region. Particle tracking fluorescent vesicles suggested that the active transport of exosomes may be mediated by cytoskeleton. The proteins on exosome membrane were found to be released from exosomes and trapped in lysosome. The inverted transport of lipophilic dye from perinuclear region to cell peripheries was revealed, possibly caused by recycling of the exosome lipids. This study provides new sight into the mechanisms of exosome uptake and intracellular fate. J. Cell. Biochem. 111: 488–496, 2010.

KEY WORDS: EXOSOME; INTERNALIZATION; INTRACELLULAR TRAFFICKING; LIVE-CELL MICROSCOPY

Cells generate vesicles that are secreted into extracellular medium. These vesicles named as microparticle, microvesicle, exosome, enlargeosome, ectosome, iccosome, prostasome, and prominosome are still poorly defined and have not been understood clearly [Cocucci et al., 2008; Simpson et al., 2008]. Exosome, a membrane vesicle secreted from many cell types including red blood cells, platelets, lymphocytes, dendritic cells, epithelial cells, and tumor cells, draws most attention in recent years [Lakkaraju and Rodriguez-Boulan, 2008]. Exosomes are 40–100 nm in diameter, 1.13–1.19 g/ml in density, and released by multivesicular body fusing with plasma membrane [Simons and Raposo, 2009]. They are involved in various cellular functions and disease states. Exosomes can act as toxin [Zhang et al., 2009], retroviruses [Wiley and Gummuluru, 2006], or prion shuttlers [Fevrier et al., 2005], and may transfer protein, soluble factor, RNA, and miRNA between cells [Smalheiser, 2007; Skog et al., 2008]. For example, human keratinocytes externalize stratifin protein via exosomes to stimulate the expression of matrix metalloproteinase-1 in fibroblasts [Chavez-Muñoz et al., 2008]. The exosomes derived from mature dendritic cells transfer functional MHC–peptide complexes to other dendritic cells [Segura et al., 2005]. Emerging preclinical evidence points their potential utility for cancer treatment [Viaud et al., 2010]. Moreover, new mouse proteins were detected in the recipient cells after transfer of mouse exosome RNA to human mast cells [Valadi et al., 2007]. Although the interaction between exosomes and cells has been verified and many important functions of exosomes are being revealed, how exosomes enter cells and their trafficking in cells are still unclear. Several hypotheses have described the mechanisms of interaction between exosomes and cells. Due to its large size, endocytosis and fusion can be the two most possible mechanisms of exosome uptake. It was proposed that exosomes were internalized by dendritic cells through endocytosis [Morelli et al., 2004]. Recent study indicated that the exosome uptake by phagocytic cells was through phagocytosis, a special mode of endocytosis [Feng et al., 2010]. Alternatively, exosomes could be internalized by melanoma cells through fusion [Parolini et al., 2009]. Finally, the intracellular trafficking of exosome components was less studied.

In this study, exosomes from PC12 cells (rat pheochromocytoma) were isolated, labeled, and internalized by resting PC12 cells. Vesicle markers were applied to study the intracellular distribution of exosomes. Temperature and actin were indicated to be involved in

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the cellular uptake of exosomes. Several results indicated that exosomes may enter cells through endocytic pathway, while fusion was against. From live-cell imaging, that exosomes were trapped in vesicles and transported to perinuclear region were observed. Furthermore, trajectories of vesicles contained exosomes trafficking in cells were described by single particle tracking (SPT), and classified. An interesting finding was that the exosome proteins left from exosome and was targeted to lysosome. The exosome lipids may be recycled to plasma membrane, leading to the inverted transport of lipophilic dye from perinuclear region to cell peripheries. These results provided new sights into the mechanisms of exosomes uptake and intracellular trafficking.

MATERIALS AND METHODS

CELL CULTURE

PC12 cell line (Shanghai Cellular Research Institute, China) was cultured in Dulbecco's modified eagle's medium (DMEM; HyClone Laboratories, USA) and 10% fetal bovine serum (FBS; ExCell Biology, USA) in a 5% CO_2 humidified atmosphere at 37°C. Cells were detached from culture dish using 0.25% trypsin and 1 mM EDTA. For exosomes purification, cells were cultured for 4 days with DMEM and FBS deprived of bovine microvesicles by ultracentrifugation (4 h at 200,000*g*). For light microscopic analysis, PC12 cells and 1.5 ml culture medium were placed in 35 mm glass-bottom culture dishes (NEST Biotech, China) coated by 0.1 mg/ml poly-L-lysine (Sigma, USA).

ISOLATION AND LABELING OF EXOSOMES

Culture medium from PC12 (1×10^8 cells) was collected, filtered by 0.22 μm syringe filters (Millipore, USA) to exclude cell debris, and then centrifuged at 20,000*g* for 30 min to remove microvesicles further. 32.4 ml resultant supernatant was ultracentrifuged at 200,000*g* for 2 h using a Type 70 Ti rotor in a L-80 XP ultracentrifuge (Beckman Coulter, USA). The exosome pellet was resuspended by 5 μg/ml DiD (Biotium, USA) in 1 ml phosphate-buffered saline (PBS), incubated for 10 min, washed once in PBS with 200,000*g* centrifugation, and resuspended in 500 μl PBS to be used as the original purified solution. For simultaneously labeling exosome proteins, 200 μl original purified exosome solution was added to 1 ml 0.1 M sodium bicarbonate buffer (pH ~8.3) contained 100 μg TAMRA-NHS (carboxytetramethylrhodamine succinimidyl ester, Biotium). Reaction was performed for 1 h at room temperature. The unincorporated TAMRA-NHS was removed by using a 100 kDa ultrafiltration tube (Millipore) and washing once in PBS with 200,000*g* centrifugation. The concentrated solution was collected and diluted to 200 μl by PBS.

ATOMIC FORCE MICROSCOPY (AFM)

AFM images were recorded at room temperature using a NanoWizard II AFM (JPK Instruments, Germany). SiN tips (OMCL-TR400PSA; Olympus, Japan) with a spring constant of 0.08 N/m were employed to obtain AFM images in contact mode. The samples were prepared by applying a drop of the exosome solution (50× dilution of the original purified solution in PBS) onto mica, incubating for 10 min, and blowing dry with N_2. Images were processed and analyzed by using SPM control software v. 3.3.16 (JPK).

LIVE-CELL FLUORESCENCE MICROSCOPY

A spinning disk confocal system (Revolution XD, Andor Technology, Northern Ireland) was built on the left port of Ti-E inverted microscope (Nikon, Japan). The confocal images were collected by an electron-multiplying CCD (EMCCD) iXon DV885 (Andor Technology) with 1,004 × 1,002 pixels. A back-illuminated EMCCD iXon DU897 (Andor Technology) with 512 × 512 pixels was built on the back port of the microscope for wide-field fluorescence imaging. 405, 491, 532, and 640 nm solid-state lasers modulated by AOTF (acousto-optic tunable filter) were used as the illumination sources for Hoechst 33342, FITC/FM4-64, TAMRA, and DiD, respectively. Fluorescence emission was collected by 60× or 100× oil-immersion objective (Apo, NA = 1.49, Nikon), passed through EM 452/45, EM 520/15, EM 585/40, EM 685/40, or 710/40 emission filters (Semrock, USA). A stage top incubator (INUB-PI-F1, Tokai Hit, Japan) was equipped to provide temperature and CO_2 for keeping cells alive. All the images were acquired and processed by iQ v. 1.10 software (Andor Technology).

EXOSOME UPTAKE ASSAY

One hundred microliters of original purified exosome solution was added to cells and both wide-field fluorescence and Z-stack confocal imaging were performed. Excessive exosomes were washed by culture medium at 3 h. To quantify the cellular uptake of exosomes, the experiment was repeated three times and for each individual experiment more than 200 cells were imaged. All the settings of imaging and processing were kept constant, and the relative fluorescent intensities were calculated. To co-localize exosomes and vesicles, 5 μM FM4-64 (Biotium) was incubated with cells for 10 min, and washed excessively for subsequently exosomes adding. To co-localize exosomes and lysosome, 0.3 mg/ml FITC-dextran (70 kDa, Sigma) was incubated with cells for 2 h and washed excessively 3 h before exosomes adding. To co-localize exosomes and nucleus, 5 μg/ml Hoechst 33342 (Sigma) was added to living cells 10 min before imaging. Two hundred microliters dual labeled exosomes (DiD and TAMRA) were added to cells for studying the fate of exosomes after cellular uptake. Excessive exosomes were washed 3 h afterward.

UPTAKE INHIBITION STUDIES

To study the temperature influence on exosome uptake, two 100 μl original purified exosome solutions were added to cells, respectively, and subsequently kept at 4 or 37°C for 3 h prior to imaging. To study the role of cytoskeleton during exosome uptake, another 100 μl original purified exosome solution was added to cells pretreated by 25 μM cytochalasin D (Sigma) for 30 min at 37°C. Imaging was performed after 3 h incubation. The exosomes used in the above parallel experiments were all from once exosome purification, and the imaging settings were kept constant. Calcein AM (Biotium) and trypan blue (Sigma) were applied to test cell viability after 4 h treatment of cytochalasin D.

SINGLE PARTICLE TRACKING

During intracellular trafficking of the vesicles contained exosomes, T-series imaging at two frames per second was selected applied. Fluorescence particles in these consecutive images were tracked using PolyParticleTracker, a Matlab (MathWorks, USA) program written by Rogers et al. [2007]. All sequence numerical analyses were carried out using custom-designed Matlab routines.

RESULTS

EXOSOMES WERE TRAPPED IN VESICLES AND DELIVERED TO PERINUCLEAR REGION

Figure 1A shows AFM images of purified exosomes from PC12 cells on mica. Although exosomes deformed on the substrate [Dimitrievski, 2010], the isolation was verified successful. Because their sizes were below diffraction limit, DiD-labeled exosomes on glass were little spots in wide-field fluorescence images (Fig. 1B). To study the cellular uptake and the intracellular trafficking of exosomes at early time, PC12 cells were incubated with DiD labeled exosomes for a 3-h period. At least 200 cells were recorded at each time points by time-lapse fluorescence microscopy. At early time points of incubation, the fluorescent intensity of DiD in cells was very weak. Thus, wide-field microscopy was employed to record images with good sensitivity. The typical images are shown in Figure 2A–E. Exosomes were detected in a small part (~5%) of cells at 5 min. At 30 min, more exosomes were internalized by cells and some exosomes appeared at perinuclear region. From 30 min to 3 h, exosomes accumulated near nucleus and brighter and larger spots emerged. The dynamic curve of exosome uptake is shown in Figure 2F by determining the fluorescent intensity quantitatively. Moreover, the intracellular distribution of exosomes was studied by co-localization test with the markers of cellular compartments after 3 h incubation. As shown in Figure 3J,K, most exosomes were internalized into the endocytic-derived vesicles marked by FM4-64. And little co-localization was observed between exosomes and the lysosome marker dextran (Fig. 3L,M), suggesting that exosome may not be targeted to lysosome. The nuclear staining experiment demonstrated the exosome lipids did not enter the nucleus (Fig. 4A). The results indicated exosomes were trapped into vesicles, transported to perinuclear region actively, and accumulated in large organelles. Moreover, the exosome lipids stained by DiD were observed in cells and targeted to vesicles with a big amount, and were not incorporated into plasma membrane. This result suggested that the exosome internalization may occur through endocytosis, while fusion was against.

For further researching the fate of exosomes after internalization, cells were excessively washed by culture medium to remove exosomes in solution at 3 h. Confocal imaging was performed and typical maximum intensity projection (MIP) images are shown in Figure 3A–H. Although vesicles transported to perinuclear region continually, not all the fluorescent vesicles accumulated at perinuclear region by 24 h. The result indicated the active transport of vesicles contained DiD was bidirectional. Two possible mechanisms could explain the vesicles transporting from cell inward to peripheries. One was that the exosome components were sorted at perinuclear region and DiD dye returned back following lipid recycling. Or exosomes were transported back on purpose to be expelled from cells. The dynamic curve of relative fluorescent intensity is shown in Figure 3I. During the 21 h culture in normal culture medium after 3 h uptake of exosomes, the fluorescent intensity in cells decreased markedly. The reason can be that the DiD dye diffused into the plasma membrane by lipid recycling or be expelled by exocytosis. For judging the fate of exosomes, only staining their membrane was not enough and further studies would be carried out.

TEMPERATURE AND ACTIN WERE INVOLVED IN EXOSOME UPTAKE

To understand the key factor depending exosome uptake, two inhibition studies were carried out. First, cells were incubated at 4°C to block all active processes. The condition led to almost no

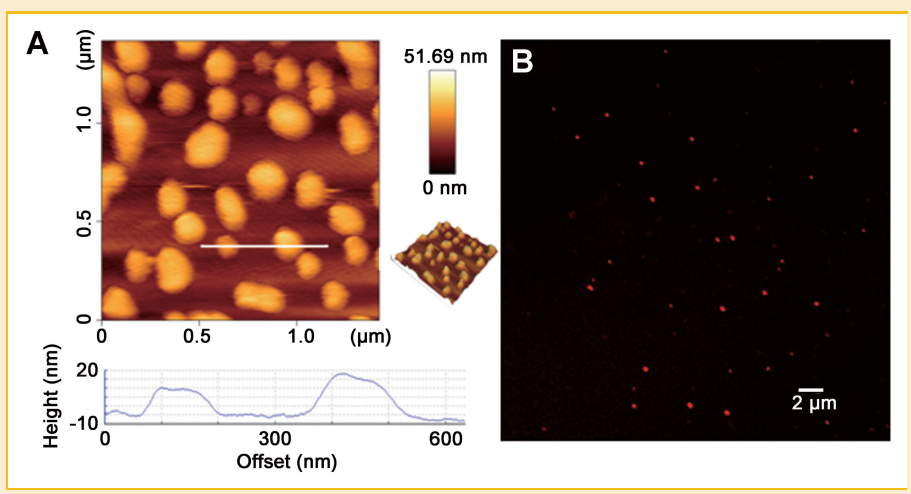

Fig. 1. A: AFM image of exosomes on mica, with 3D topography inserted. Curve below is the cross section following the line in the image. B: Wide-field fluorescence image of individual exosome stained by DiD on glass. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

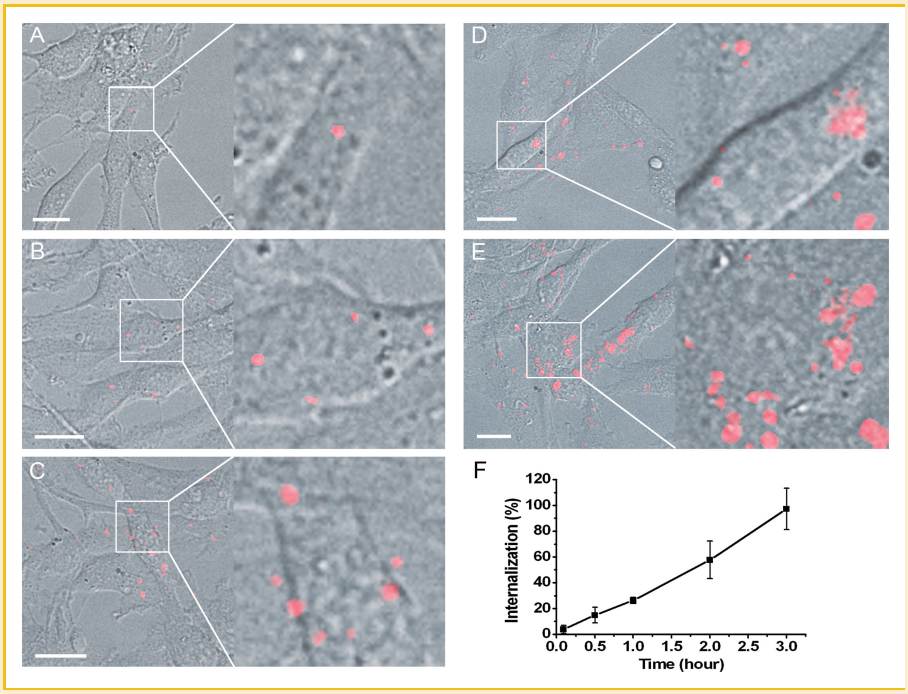

Fig. 2. A–E: Time-lapse images of PC12 cells incubated with DiD-labeled exosomes (red) within 3 h. Merge of bright field with wide-field fluorescence images at 5 min (A), 30 min (B), 1 h (C), 2 h (D), and 3 h (E) after exosomes adding, respectively. Scale bar, 15 μm. F: Curve of exosome uptake dynamics by determining the fluorescent intensity. Mean ± SD of three independent experiments are shown. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

fluorescence detected in cells (Fig. 4B), indicating it was an energy-dependent process of exosome uptake. Second, actin-disturbing drug was applied to determine whether actin was involved. Cells were demonstrated alive after 4 h treatment of cytochalasin D (Supplementary Fig. S2). As shown in Figure 4C, drug-treated cell was deformed because of actin disruption. And the fluorescent intensity decreased markedly compared to which in normal cells (Fig. 4D). The two results above suggested energy and actin-dependent endocytosis was involved in exosome uptake, and fusion did not dominate in the process.

ANALYSIS OF EXOSOME TRAFFICKING BY SPT

Dynamic imaging was carried out to reveal the intracellular trafficking of exosomes in detail. Figure 5A shows *x*–*y*–*t* MIP image from 60 s imaging of three cells. The image was useful in the T-dimension for highlighting feature tracks and the motions of most vesicles were vibratory. One hundred trajectories were generated from 16 cells at different time points after DiD stained exosomes application. All the trajectories can be distinguished into four phases by their conformation and direction. In the first phase, vesicles moved along cell peripheries possibly on actin filaments (Fig. 5B; Supplementary Fig. S1A and Mov. S1). It can be the stage waiting for transporting inward cells [Lakadamyali et al., 2003]. The second phase was classified by particles motion from the cell peripheries to perinuclear region straight (Fig. 5C; Supplementary Fig. S1B and Mov. S2). The motion was possibly along microtubules [Burgess et al., 2003]. The average speed of the trajectories was calculated to be about 1.0 μm/s, corresponding to the value of dynein-dependent transport along microtubules [Mallik et al., 2004]. The third phase was differed in confined random motion, shown by trajectories without arrow indicating in Figure 5. Most particles in this phase intermittently moved at perinuclear region, resulting from the exosome trapped in large vesicles. In addition, vesicles not near nucleus in the phase were also observed. They were possibly confined by actin filaments and in an intermediate stage [Apodaca, 2001]. The three phases above met a three-stage transport process involved in endocytic pathway of influenza virus reported by Lakadamyali et al. [2003]. The last phase was observed at later time points. The vesicles moved directly from perinuclear region to cell peripheries (Fig. 5D; Supplementary Fig. S1C and Mov. S3), meeting a reported motion along microtubules mediated by kinesins [Schliwa and Woehlke, 2003]. The motion can be involved in recycling lipids to plasma membrane.

STUDY OF EXOSOME FATE BY DUAL-LABELING

The RNA in exosomes and the proteins inner or at the surface of exosomes were the active components to transfer information between cells, while the lipids were just structural components for keeping them. It was reported that some of the proteins at exovesicle surface took part in the interaction between exovesicles and plasma membrane, and can determine the processes of endocytosis or fusion [Obregon et al., 2006, 2009]. However, the fate of the surface proteins after uptake was less known. If the proteins played specific roles in cells, they should be released from the exosome membrane. To verify the hypothesis and further research the intracellular trafficking of exosomes, an uptake assay was performed with the exosomes dual stained by lipophilic DiD and amine-reactive TAMRA-NHS. Figure 6 shows time-lapse wide-field fluorescence

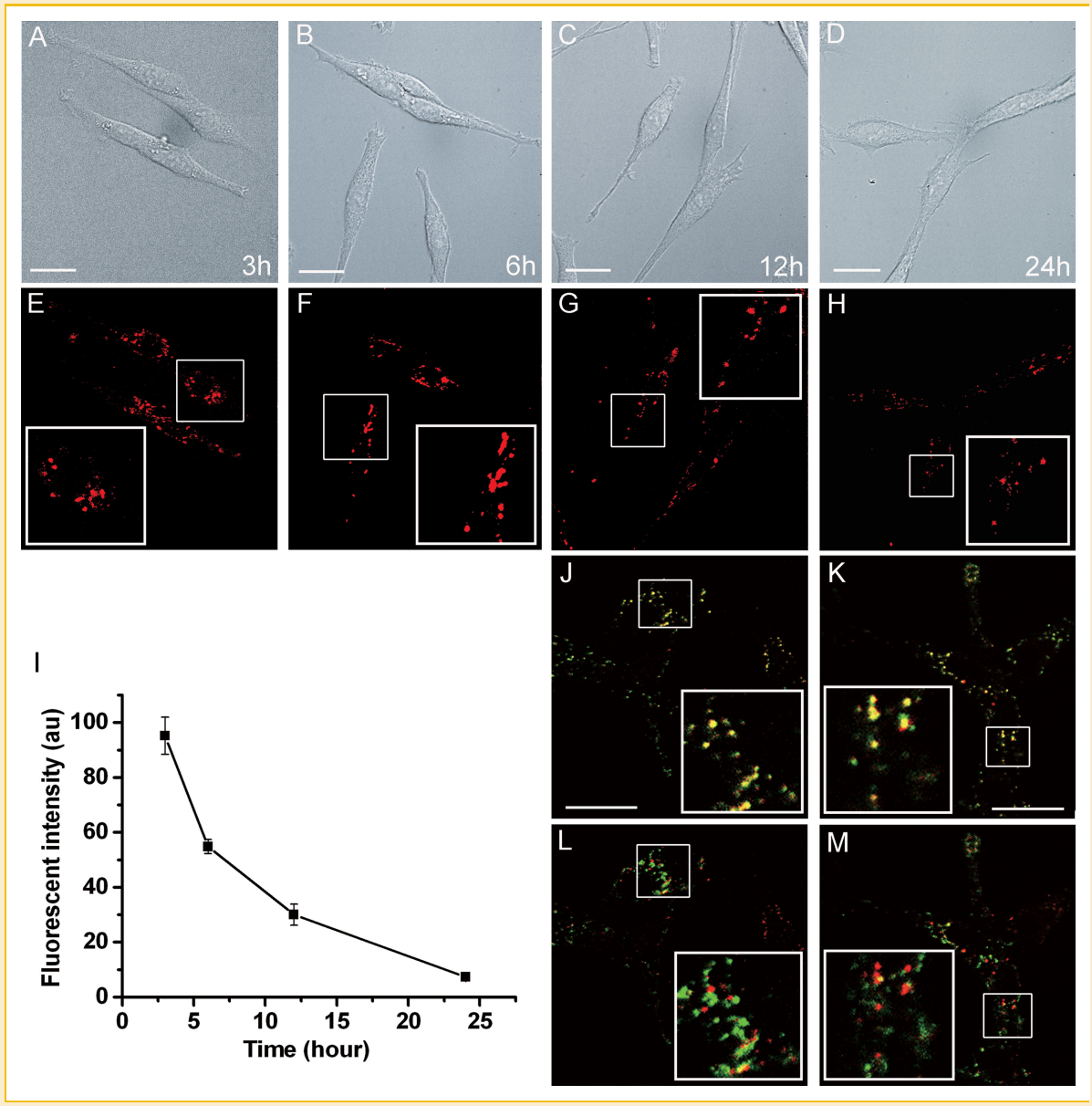

Fig. 3. A–H: Time-lapse images of PC12 cells after 3 h uptake of DiD-labeled exosomes (red). Bright field (A–D) and corresponding confocal images (E–H) 3, 6, 12, and 24 h after exosomes added, respectively. Scale bar, 15 μm. I: Curve of relative fluorescent intensity in cells versus time. Mean ± SD of three independent experiments are shown. Co-localization images of DiD-labeled exosomes (red) with FM4-64 (green) (J,K) or FITC-dextran (green) (L,M) by merging the confocal images after 3 h incubation of PC12 cells with exosome. Scale bar, 15 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

images for a 24-h period. At 1 h, most exosome proteins co-localized with the lipids. After 3 h incubation, excessive exosomes were washed and bright fluorescent vesicles appeared at perinuclear region. Some vesicles had been dominated by only one dye, suggesting the separation between exosome proteins and lipids occurred. At 6 h, a number of vesicles were observed at cell peripheries through active transport from cell inward, meeting the results of single labeling experiment above. But DiD was delivered back more than exosome proteins. The result demonstrated the release of exosome proteins from membrane. From 6 to 24 h, the amount of DiD stained vesicles was obviously reduced, corresponding to the results of single labeling experiment above again. Finally, at 24 h incubation, the signal of DiD vanished in a part of cells and the proteins did not only localize at perinuclear area. To study the target compartments of the two components, vesicle markers were imaged at 12 h. As shown in Figure 7A–C, most DiD co-localized with FM4-64 but not with dextran, suggesting that exosome lipids did not enter lysosome. Co-localizations of TAMRA and FM4-64 or dextran are shown in Figure 7E,F. Much protein was trapped in lysosome after releasing from exosome membrane. Based on the results above, it can be deduced that the proteins were started separating from exosome membrane after endocytosis within 3 h,

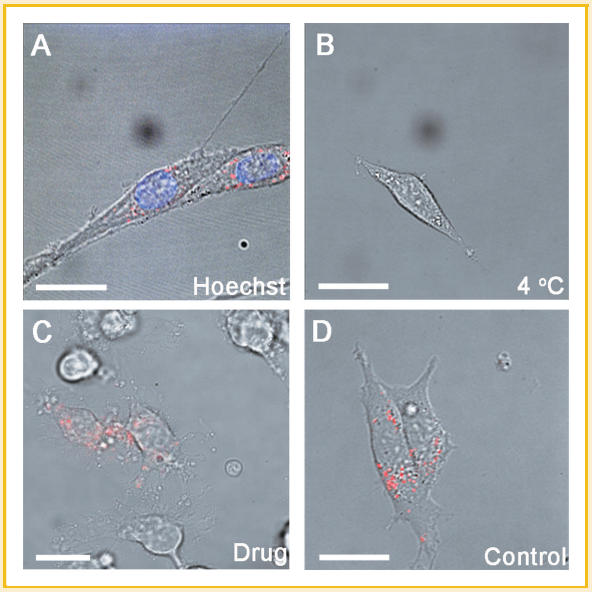

Fig. 4. Exosomes and nucleus colocalization assay and uptake inhibition studies. A: Confocal images of DiD stained exosomes (red) and Hoechst 33342 stained nucleus (blue) after 3 h incubation of PC12 cells with exosomes. B: Fluorescence images of exosomes incubated with PC12 cells at 4°C. C: Fluorescence images of exosomes incubated with PC12 cells pretreated with cytochalasin D. D: Exosomes and PC12 cells co-cultured in the absence of drugs at 37°C for 3 h as control. The imaging parameters were kept the same in (B), (C), and (D). Scale bar, 15 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

possibly by fusion of exosomes to endosome or acidified environment in late endosome. Then, the proteins may be sorted into lysosome mostly, stored or utilized at there. At the same time, the exosome lipids may join in the lipid recycling to plasma membrane through the other independent pathway.

DISCUSSION

This work imparted a series of results on exosome uptake and intracellular delivery by tumor cells. Due to its large size, endocytosis and fusion can be the two most possible mechanisms of exosome uptake. That exosome internalizing via endocytosis or fusion was controversial over the past decade [Morelli et al., 2004; Parolini et al., 2009]. This study suggested that the PC12-derived exosomes enter resting PC12 cells through endocytic pathway. The hypothesis was based on the four results below. First, the exosome lipids stained by DiD were observed in cells and targeted to vesicles with a big amount, and were not incorporated into plasma membrane. The result was similar to the evidence of epithelial cells endocytosing exovesicles reported by Obregon et al. [2006, 2009]. If fusion dominated in exosome uptake, Plasma membrane would contain fluorescent lipids after co-culture of exosomes and cells. Second, the exosome proteins were co-localized with the exosome lipids in vesicles after internalization. The result supported endocytosis and was against fusion, because fusion would cause the diffusion and the separation of the exosome lipids and proteins at

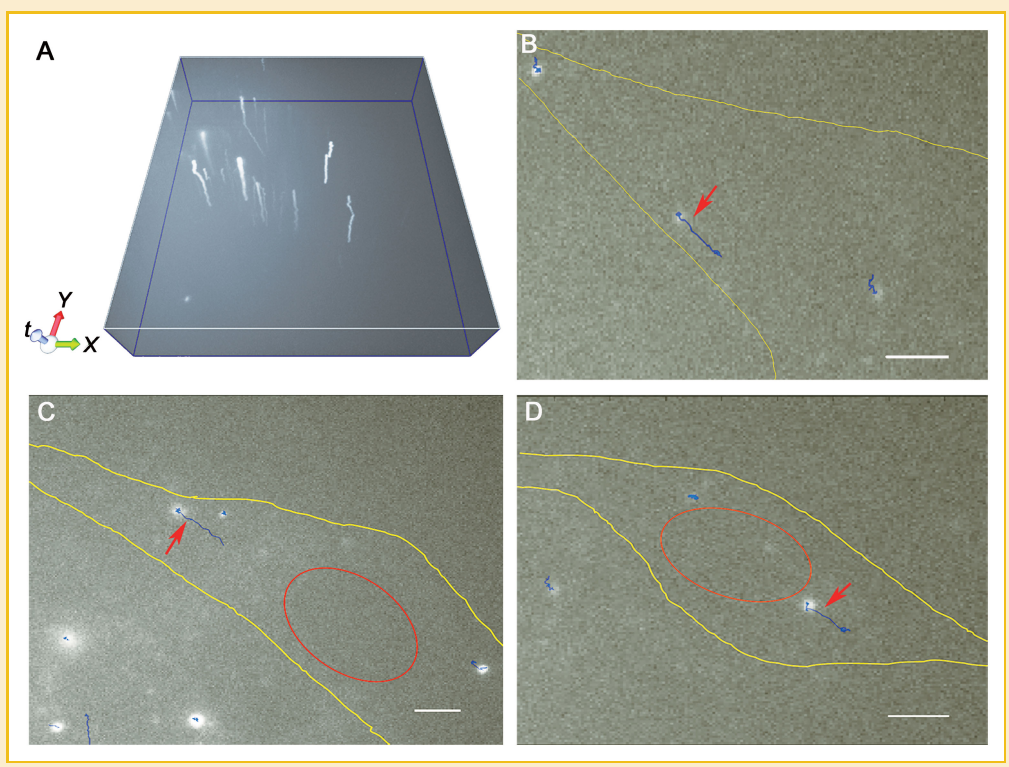

Fig. 5. A: *x–y–t* maximum intensity projection image from 60 s imaging of exosomes in three cells. B,C,D: Trajectories of exosomes transport in PC12 cells (blue lines). The yellow lines show the boundary of cells and the red lines show the nucleus boundary. The red arrows denote the trajectories of the motion along the cell peripheries (B), from the cell peripheries to nuclear region (C), and from nuclear region to the cell peripheries (D). The trajectories without arrow pointing shows confined random motion. Scale bar, 5 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

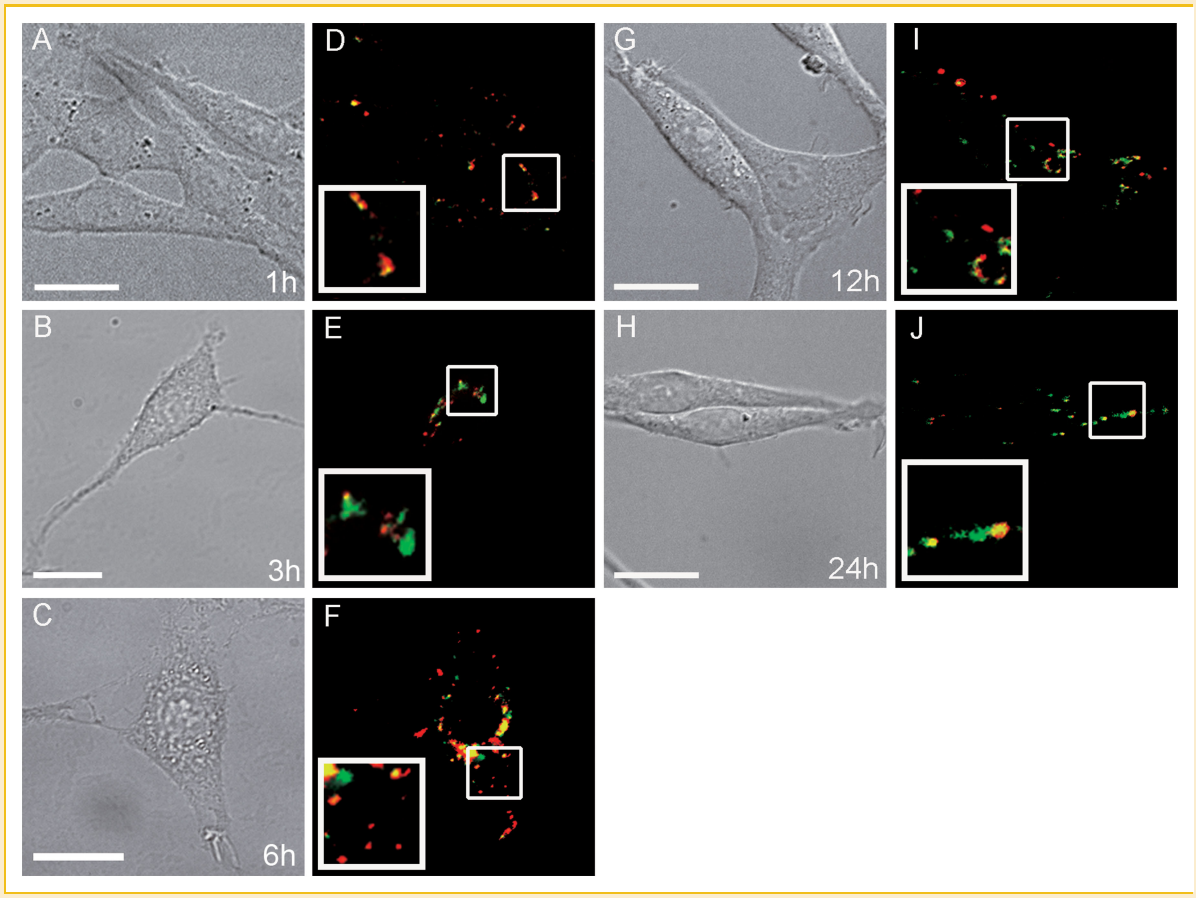

Fig. 6. PC12 cells were incubated with exosome lipids and proteins components stained by DiD and TAMRA-NHS for 3 h and washed excessively. Bright field images (A–C,G,H) and corresponding fluorescence images (D–F,I,J) of DiD (red) and TAMRA (green) in cells at 1, 3, 6, 12, and 24 h after exosomes adding are shown. Scale bar, 15 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

plasma membrane. Third, actin was involved in exosomes internalization. Actin polymerization was required in the initial stage of most endocytosis pathways including phagocytosis, macropinocytosis, and clathrin-dependent endocytosis [Robertson et al., 2009]. Conversely, membrane fusion was a physical mechanism independent of actin. Fourth, exosomes internalization was blocked under 4°C. Low temperature inhibited all active processes including endocytosis [Huth et al., 2006]. Fusion, a process independent of energy, was not influenced by temperature so much. Recent study provided solid results of exosomes existing in phagosome-like compartments by electron microscopy [Feng et al., 2010], demonstrating exosomes entered phagocytic cells via endocytosis pathway. On the other hand, several reports indicated exosomes can fuse with plasma membrane [Parolini et al., 2009]. The possible explanation was that the uptake pathway depended on the interaction between surface molecules of exosomes and receptors on plasma membrane. Different origin of exosomes and the kinds of recipient cells can lead to different interaction modes. Until now, direct real-time observation of exosomes internalization by live-cell microscopy was still lacking. Simultaneity labeling of the exosome membrane and contents can provide more direct evidence for determining its internalization mechanism in the future.

Exosome trafficking in PC12 cells was shown in the present work through both time-lapse and dynamic microscopy. The results indicated the entrance of exosomes into vesicles. The active transporting of vesicles contained exosomes in cells possibly mediated by cytoskeleton was observed and tracked. The transport mode was similar to that of virus [Lakadamyali et al., 2003] or nanoparticles [Ruan et al., 2007]. Besides, the lipid dye transported back to cell peripheries and exosome proteins were sorted into lysosome. This finding indicated the separation of exosome lipids and proteins. Consistent with our results, Parolini et al. [2009] reported that the exosome proteins were preferentially targeted to endosome and lysosome. It was hypothesized that exosomes may fuse with endocytic-derived vesicles and released their surface proteins and contents. The exosome lipids were abundant in sphingomyelin which tend to recycling back to plasma membrane [Koivusalo et al., 2007]. Thus, most DiD was speculated to transport to plasma membrane following the exosome lipids. The decrease of fluorescence intensity was caused by the dilution of DiD in plasma membrane. Further, a part of DiD can be expelled into medium by microvesicle shedding from plasma membrane. At the same time, the exosome proteins, originated from PC12 cells, contained many proteins the same to those existed in endosome and lysosome of recipient cells. Thus, the proteins tended to be utilized or

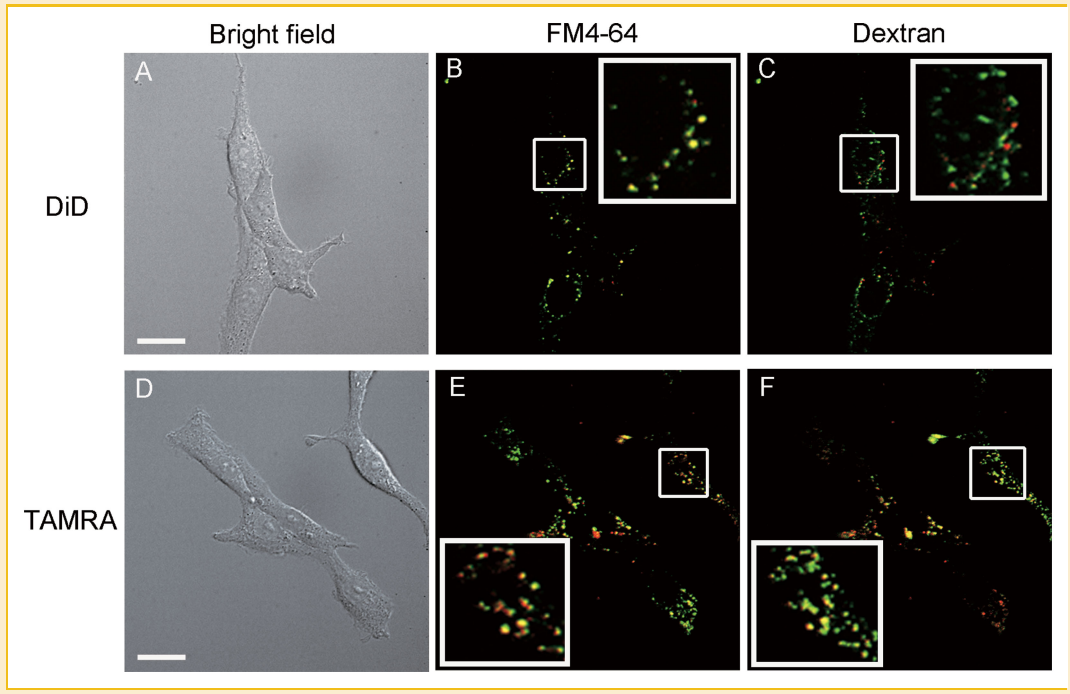

Fig. 7. PC12 cells were incubated with exosomes stained by DiD (for lipid component) and TAMRA-NHS (for membrane proteins) for 3 h and washed excessively. A,D: Bright field images at 12 h after exosomes adding. Scale bar, 15 μm. Co-localization images of DiD (red) with FM4-64 (green) (B) or FITC-dextran (green) (C) by merging the confocal images corresponding to (A). Co-localization images of TAMRA (red) with FM4-64 (green) (E) or FITC-dextran (green) (F) by merging the confocal images corresponding to (D). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

stored at vesicles surface or in their lumen. The exosome proteins from various cell types were quite different, leading to various fates after internalization. More diverse cells derived exosomes should be examined in the future.

In summary, exosomes were isolated from the culture medium of PC12 cells, labeled by lipophilic dye and amine-reactive fluorophore, and co-cultured with resting PC12 cells. The uptake and intracellular trafficking of exosomes were analyzed by live-cell fluorescence microscopy and SPT. The evidence of that exosomes were internalized through endocytosis pathway were provided. The intracellular exosomes were found to be trapped in vesicles and active transported. Exosome proteins may separate from exosome membrane in endosome within 3 h after internalization. Outward transport of lipophilic dye from perinuclear region to cell peripheries in a big amount was shown. One hypothesis was that the exosome lipids were abundant in sphingomyelin leading to recycling back to plasma membrane. On the other hand, exosome proteins were trapped in lysosome, which can be regarded as an evidence of transferring proteins between tumor cells. These results provided new insights into the mechanisms of exosome internalization and intracellular trafficking.

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