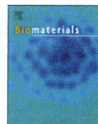




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Quantum dots labeling using octa-arginine peptides for imaging of adipose tissue-derived stem cells

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ABSTRACT

Quantum dots (QDs) have been used to study the effects of fluorescent probes for biomolecules and cell imaging. Adipose tissue-derived stem cells, which carry a relatively lower donor site morbidity, while yielding a large number of stem cells at harvest, were transduced with QDs using the octa-arginine peptide (R8) cell-penetrating peptide (CPP). The concentration ratio of QDs:R8 of 1×10^4 was optimal for delivery into ASCs. No cytotoxicity was observed in ASCs transduced with less than 16 nM of QDs:R8. In addition, >80% of the cells could be labeled within 1 h and the fluorescent intensity was maintained at least for 2 weeks. The ASCs transduced with QDs using R8 could be differentiated into both adipogenic and osteogenic cells, thus suggesting that the cells maintained their stem cell potency. The ASCs labeled with QDs using R8 were further transplanted subcutaneously into the backs of mice or into mice through the tail vein. The labeled ASCs could be imaged with good contrast using the Maestro *in vivo* imaging system. These data suggested that QD labeling using R8 could be utilized for the imaging of ASCs.

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1. Introduction

Quantum dots (QDs) are inorganic probes that consist of CdSe/ZnS-core/shell semiconductor nanocrystals. QDs have several distinctive advantages in comparison to conventional organic labels such as a high luminance, resistance to photobleaching (long time labeling), a range of excitation wavelengths and narrower emission bandwidths [1–4]. According to these characteristics, QDs have recently been investigated as fluorescent probes for biomolecules and live cells, and are expected to be used in medical applications for diagnostics [5,6]. Various approaches have been applied to label cells with QDs, such as microinjection, electroporation, liposome-based transduction and special peptide delivery [7–11].

The transduction of QDs into cells using cell-penetrating peptides (CPPs) has been established and is thought to be a useful

technique because of the low cytotoxicity and high transduction efficiency. Yun Lei et al. showed that Tat peptide conjugated QDs could be transduced into mesenchymal stem cells [12]. Jui-Chih Chang et al. showed that Pep-1 labeled QDs could be transduced into stem cells and remained for about one month [13]. However, few detailed studies have so far explored the appropriate ratio of QDs and peptide, uptake time, cell function *in vitro* and application of *in vivo* imaging.

Regenerative medicine is expected to overcome the shortage of donated organs, donor site morbidity and immune reaction [14]. Many kinds of stem cells have been discovered already and provided numerous applications in regenerative medicine. Mesenchymal stem cells (MSCs) and progenitor cells are detected in various tissues, including umbilical cord blood, bone marrow, periodontal ligament and adipose tissue.

Bone marrow-derived stem cells (BMSCs) have the ability to differentiate into multiple mesenchymal cells such as cardiomyocytes, chondrocytes, osteoblasts and adipocytes.

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Therefore, BMSCs have been considered to be an important source of stem cells for use in regenerative medicine.

Recently, adipose tissue-derived stem cells (ASCs) have been increasingly recognized as multipotent stem cells and as an alternative source of BMSCs [15]. ASCs are easier to obtain in abundance by minimally invasive harvest procedures such as liposuction with local anesthesia. In addition, ASCs possess the ability of self-renewing and differentiating into various mesenchymal cell types, while also secreting significant levels of many potent growth factors and cytokines, such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) [16]. Because of their potential clinical application, it has therefore become important to label the cells for tracing as transplanted cells.

Previous studies have shown that a cationic liposome (Lipofectamine) could transduce negatively charged QDs into ASCs within 4 h and the QDs remained in the cells for more than 2 weeks. However, cytotoxicity was observed at a relatively low concentration, suggesting that the major cause was the liposome (Lipofectamine) [17]. In this study, octa-arginine peptide (R8), which exhibits even greater efficiency in the delivery of several proteins, was utilized as a delivery vector and the characteristics of negatively charged carboxyl QDs transduced using R8 to live ASCs were investigated. In addition, imaging studies in subcutaneous transplantation on the back and intravenous transplantation through the tail vein were conducted.

2. Materials and methods

2.1. Animals

C57BL/6 mice were purchased from SLC Japan. The mice were housed in a controlled environment (12 h light/dark cycles at 21 °C) with free access to water and a standard chow diet before sacrifice. All conditions and handling of animals in this study were conducted with protocols approved by the Nagoya University Committee on Animal Use and Care.

2.2. Isolation and culture of ASCs

The 7–14-month-old female C57BL/6 mice were killed by cervical dislocation; adipose tissue specimens in the inguinal groove were isolated and washed extensively with Hank's balanced salt solution or phosphate-buffered saline (PBS) to remove the blood cells. The isolated adipose tissue specimens were cut finely and digested with 1 mL of 1 mg/mL type I collagenase (Collagenase Type I, 274 U/mg, Koken Co., Ltd., Tokyo, Japan) at 37 °C in a shaking water bath for 45 min. The cells were filtered using 250 µm nylon cell strainers (BD Biosciences) and suspended in Dulbecco's modified Eagle's medium and full-length name of F12 (DMEM/F12) containing 20% fetal bovine serum (FBS; Trace Scientific Ltd., Melbourne, Australia, Uin: 53141 Lot: B01249-500) and 100 U/ml penicillin/streptomycin (culture medium). They were centrifuged at 1200 rpm for 5 min at room temperature and adipose tissue-derived stem cells (ASCs) were obtained from the pellet. They were washed three times by suspension and centrifugation in culture medium and then were incubated overnight in culture medium at 37 °C with 5% CO₂. The primary cells were cultured for several days until they reached confluence and were defined as passage "0". The cells were used for the experiments between passages 2 and 5.

2.3. Preparation and transduction of R8-QDs complex

QDs (Invitrogen; Qdot ITK Carboxyl Quantum Dots) and R8 (Sigma genosys, Japan) were purchased. QDs were made from nanometer-scale crystals of a semiconductor material (CdSe), which are shelled with an additional semiconductor layer (ZnS) to improve their chemical and optical properties. In addition, the polymer coating has COO⁻ surface groups. To determine the optimal concentration ratio of QDs655 (emission at 655 nm)/R8 to transduce into ASCs, QDs655 (2.0 nM) and R8 were mixed for 20 min at room temperature in various ratios of QDs655/R8. Next, ASCs were incubated with the R8-QDs655 complex in a transduction medium (DMEM/F12, 2% FBS, 100 U/ml penicillin/streptomycin) at 37 °C. After 4 h incubation, the transduction efficiency of QDs655 into ASCs was evaluated by conventional fluorescence microscopy and a flow cytometry analysis [18].

2.4. Flow cytometry analysis

ASCs (1.5×10^5 cells) were seeded in 12-well plates (BD Biosciences) with 500 µL of culture medium for 2 h and the cells were transduced with QDs655 using R8 in

transduction medium at 37 °C. After transduction, the cells were washed three times by suspension and centrifugation at 1200 rpm for 3 min. Next, the cells were suspended in 500 µL of PBS and used for flow cytometry analysis on a FACS Caliber (BD Biosciences) flow cytometer using 488 nm laser excitation and 615 nm long pass emission.

2.5. Cytotoxicity of QDs to ASCs

The cells (2×10^4 cells) were seeded in 96-well plates (BD Biosciences) with 100 µL of culture medium for 2 h and then they were replaced with 100 µL of transduction medium at 37 °C. After 4 h incubation, QDs655 (0–24 nM) and R8 were mixed at the optimal ratio of 1:1 $\times 10^4$, and ASCs were transduced with QDs655 using R8 in transduction medium for 24 h. Viable cells were counted using Cell Counting Kit-8 (CCK-8; DOJINDO Laboratories Kumamoto, Japan). CCK-8 reagent (10 µL) was added to each well and the reaction was allowed to proceed for up to 4 h. The absorbance of the sample at 450 nm was measured against a background control using a microplate reader.

2.6. Proliferation of ASCs labeled with QDs

ASCs (1×10^3 cells) were seeded in 96-well plate with 100 µL of culture medium and then transduced with QDs655 using R8 at various concentrations of QDs655 for 4 h. Next, the media was replaced with 100 µL of culture medium. After 0, 2, 4 and 5 days, viable cells were counted using the Cell Counting Kit-8 in the same way.

2.7. Confocal laser scanning microscopy

A confocal laser scanning microscopy (FV1000, OLYMPUS) analysis was conducted to confirm the fluorescent intensity and location of QDs655 in labeled ASCs using R8. Images obtained from the bottom of the coverslip to the top of the cells were recorded and each image was superimposed on PC to qualify the total brightness and pixel area of each region of interest. Before imaging, the cells were washed three times with PBS and replaced with fresh transduction medium [19].

2.8. Transduction of QDs using R8

ASCs were preincubated in the transduction medium with 10 mM sodium azide and 2-deoxy-D-glucose (endocytosis inhibitors) for 1 h at 37 °C, or 3 mM amiloride (macropinocytosis inhibitor) for 10 min at 37 °C. In addition, ASCs were preincubated in the transduction medium for 30 min at 4 °C (both endocytosis and macropinocytosis inhibitors). These cells were then washed three times with PBS followed by the addition of 0.4 nM QDs655 with R8 in fresh transduction medium for 1 h. Thereafter, the cells were observed using confocal laser scanning microscopy.

2.9. Adipogenic differentiation

Adipogenic differentiation was induced by culturing the cells for 3 days in DMEM (high glucose) containing 100 µM indomethacin, 1 µM dexamethasone, 1 µM hydrocortisone, 10 µM insulin (Sigma, I-5500) and 10% FBS. The cells were then further cultured in DMEM (high glucose) containing 10% FBS for 2 weeks and the medium was changed every 3 days. Differentiation was confirmed by conventional microscopic observations of intracellular lipid droplets and Oil Red O staining as an indicator of intracellular lipid accumulation. Briefly, the cells were fixed in 10% solution of formaldehyde in PBS for at least 10 min at room temperature and then were washed with 60% isopropanol. Next, the cells were stained with 2% (w/v) Oil Red O reagent for 10 min at room temperature followed by repeated washing with distilled water and destaining in 100% isopropanol for 1 min.

2.10. Osteogenic differentiation

Osteogenic differentiation was induced by culturing the cells for 2 weeks in DMEM containing 200 µM dexamethasone, 50 µM ascorbate-2-phosphate (Wako Pure Chemical Industries Ltd., 013-12061), 10 mM α -glycerolphosphate (Sigma, G-9891) and 10% FBS. The differentiation was confirmed by staining for any alkaline phosphatase activity and then observing the extracellular matrix calcification using Von Kossa's method. The cells were washed twice with PBS and fixed in 10% formalin for 15 min at room temperature. They were washed and incubated with deionized water for 15 min. Then, they were stained with a solution containing naphthol AS MX-PO₄ (Sigma, N-5000), N,N-dimethylformamide (Wako Pure Chemical Industries Ltd.), Red Violet LB salt (Sigma, F-1625) and Tris-HCl buffer (pH 8.3) for 45 min. Von Kossa staining was carried out with 2.5% silver nitrate solution for 30 min.

2.11. RNA extraction and real-time RT-PCR

Total RNA was extracted from cells by using RNeasy[®] reagent (QIAGEN) according to the manufacturer's instructions. cDNA synthesis and amplification via PCR were performed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany). The cDNA was used for PCR by LightCycler[®] FastStart DNA Master PLUS SYBR Green I (Roche). The PCR reaction mixture was made up in 20 µL of 10 µM targeted gene oligonucleotide primers. Using LightCycler[®] (Roche), heat denaturation at 95 °C for 10 min and 40 cycles of PCR

Table 1

Primer sequences used for real-time RT-PCR.

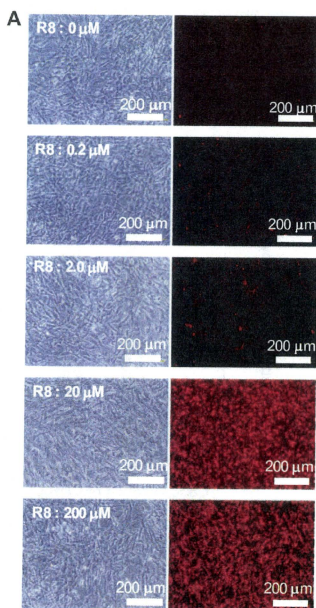
Gene	Accession	Sense primer	Antisense primer
ALP	NM_007431	GTTGCCAAGCTGGGAGAACAC	CCCCCCCCGCTATTCCAAAC
β -Actin	NM_007393	TATGGAATCTCTGTGCATCC	CTTCTGCATCTCTGCAGCA

with denaturing at 95 °C for 10 s, annealing temperature 55 °C for 10 s and extension at 72 °C for 10 s were performed ALP and β -Actin. The sequences of all primers are shown in detail in Table 1.

2.12. *In vivo* fluorescent imaging of ASCs labeled with QDs

QDs655, QDs755 or QDs800 were transduced into ASCs using R8 in the same way. ASCs (0.5×10^6 and 3×10^6 cells) labeled with QDs were subcutaneously transplanted with 50 μ L PBS into the back of C57BL/6 mice. Images were taken using the Maestro *in vivo* imaging system (CRI Inc., Woburn, MA; excitation filter: 575–605 nm, emission filter: 645 nm long pass). The detection was set to capture images automatically at 10-nm increments from 630 nm to 850 nm with constant 1 s exposure. The resulting TIFF images were loaded into the vendor's software and analyzed.

On the other hand, for the *in vivo* imaging of ASCs transplanted into mouse through the tail vein, ASCs (5×10^5 cells) labeled with QDs800 were injected with 0.15 mL saline. Images were taken using the Maestro *in vivo* imaging system in the same way after 10 min. Moreover, for the histological analysis, ASCs (5×10^5 cells) labeled with QDs655 were injected into the mouse. The mouse was then sacrificed after 10 min and major organs (heart, lung, liver, spleen and kidney) were harvested and washed three times with PBS to remove retained erythrocytes. The collected tissue specimens were then fixed by formalin and embedded in paraffin. To examine the histological QDs uptake and distribution of transplanted ASCs, the tissue specimens were cut into 10 μ m thick sections and then were observed by fluorescent microscopy.



2.13. Statistical analysis

Numerical values are presented as the mean \pm SD. Each experiment was repeated three times. Statistical significance was evaluated using unpaired Student's *t*-test for comparisons between the two groups; *p*-values < 0.05 were considered to be statistically significant. All statistical analyses were performed using the SPSS software package.

3. Results

3.1. Transduction of QDs into ASCs using R8

QDs655 (2.0 nM) were mixed with concentrations of R8 (0, 0.2, 2.0, 20 and 200 μ M) for 20 min respectively and they were tested for their ability to transduce into ASCs using conventional fluorescent microscopy and flow cytometry analysis. Red fluorescence derived from QDs655 was strongly observed at the ratio of 20 μ M R8 to 2.0 nM QDs655. Increasing the R8 peptide concentration beyond 20 μ M did not increase the QDs655 internalization. This result suggests that the concentration ratio of QDs:R8 of 1:1 $\times 10^4$ is optimal for delivery into ASCs (Fig. 1A).

3.2. Cytotoxicity to ASCs

QDs655 was transduced into ASCs using R8 at various concentrations in transduction medium for 24 h at 37 °C. Significant cytotoxicity was observed in ASCs transduced with more than

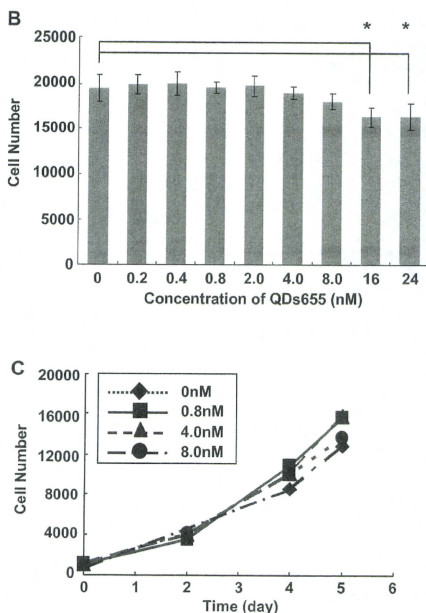


Fig. 1. Optimal formation of R8-QDs and the cytotoxicity to ASCs. A: QDs655 (2.0 nM) were mixed with various concentrations of R8 for 20 min and the complex were transduced into ASCs for 4 h to determine the optimal concentration ratio of QDs/R8. B: ASCs were transduced with QDs655 using R8 at various concentrations for 24 h. The survival of ASCs transduced with QDs655 using R8 is compared with non-transduction ASCs. C: The proliferation rate is shown in the nontoxic range of QDs655. The number of cells was estimated at 0, 2, 4, and 5 days after transduction as described in Materials and Methods. The data, all in triplicate, are shown as the mean \pm SD values. **p* < 0.05.

16 nM of QDs655, however, >80% of the cells were still alive. In addition, no remarkable cytotoxicity was observed with less than 8.0 nM of QDs655 (Fig. 1B). The morphology and fluorescent images were confirmed by conventional fluorescent microscopy.

Moreover, the influence on the proliferation rate was examined within the non-cytotoxic range of concentrations. The cells were confirmed to exhibit a logarithmic growth rate that was nearly equal to normal ASCs. There were no significant differences in these concentrations (Fig. 1C). These data suggest that ASCs could be labeled within an 8.0 nM concentration of QDs using R8.

3.3. Transduction efficiency of QDs using R8

To determine the transduction efficiency of QDs into ASCs, ASCs were transduced with QDs655 using R8 for 1 h at 0, 0.8, 2.0 and 8.0 nM concentrations of QDs655. The internalization of QDs655 was estimated and compared using flow cytometry analysis. The transduction efficiency of QDs655 after 1 h transduction was $81.4 \pm 5.4\%$ at 0.8 nM, $90.8 \pm 2.8\%$ at 2.0 nM and $95.2 \pm 1.9\%$ at 8.0 nM (Fig. 2A). More than 80% of the cells could be labeled within 1 h (Fig. 2B).

In addition, the location and fluorescent intensity of QDs655 was analyzed using confocal laser scanning microscopy at a concentration of 0.4 nM QDs655 after 1 h of transduction. The successive location of QDs655 in the ASCs was observed in 1 h, 8 h, 1 day, 7 days, 10 days and 14 days after transduction. QDs655 were widely dispersed in the cell cytoplasm during the early time points, however, aggregation of QDs655 was observed as time passed, QDs655 could not be observed in the nuclei at any time (Fig. 3A). The fluorescent intensity level of QDs655 was approximately equal during 14 days (Fig. 3B).

3.4. Transduction of QDs using R8 by macropinocytosis

The internalization of QDs655 using R8 with sodium azide and 2-deoxy-D-glucose, or amiloride, or at 4 °C was compared to that of normal transduction. The fluorescence of QDs655 could be observed on the face of the ASCs only and not in the cytoplasm (Fig. 4A–C). On the other hand, QDs655 were incorporated into the cytoplasm of ASCs after no application of inhibitors (Fig. 4D). These results suggested that the internalization of QDs655 using R8 occurs mainly by macropinocytosis.

3.5. Differentiation of ASCs transduced with QDs using R8

To examine the influence on the pluripotency of ASCs, ASCs were transduced with 0.4 nM of QDs655 using R8 and cultured with adipogenic and osteogenic medium for 2 weeks. Following adipogenic differentiation, intracellular lipid droplets in ASCs labeled with QDs655 were observed in the same frequency as the control ASCs and the red fluorescence derived from internalized QDs655 was confirmed (Fig. 5A–a). Oil Red O staining confirmed that intracellular droplets were lipid (data not shown). On the other hand, after incubation in osteogenic medium, ASCs labeled with QDs655 became osteocyte-like in morphology with cuboidal shapes (Fig. 5A–b). The cells were positive for von Kossa's staining in the same frequency as the control ASCs (data not shown). Red fluorescence of QDs655 was clearly observed for more than 2 weeks using confocal laser scanning microscopy in both of the differentiated cell types. However, the fluorescence of QDs655 was only slightly observed in the ASCs cultured with normal culture medium (Fig. 5A–c).

Moreover, to estimate the influence on osteogenic differentiation ability of ASCs at the mRNA level, ALP activity was measured by real-time RT-PCR. ALP activity of ASCs transduced with QDs655

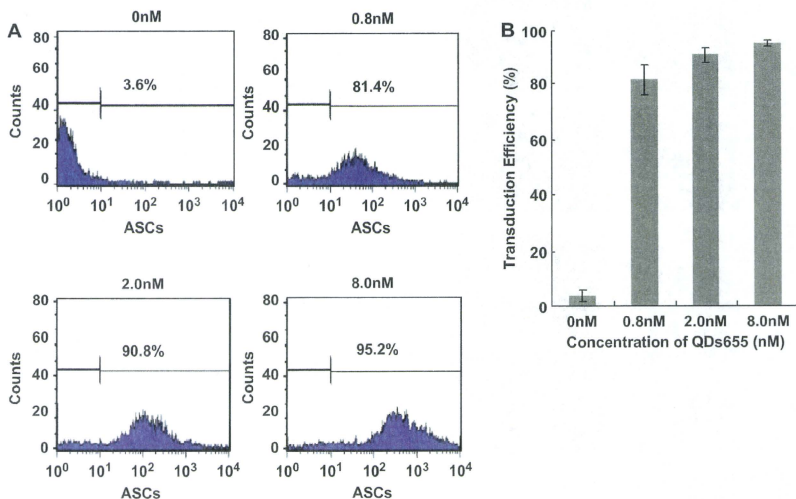


Fig. 2. Transduction efficiency of QDs using R8 quantified by flow cytometry. A: The transduction efficiency of QDs655 into ASCs using R8 after 1 h of incubation was quantified by flow cytometry. The mean fluorescent units are the average of 10,000 units. B: The bar graph shows the transduction efficiency of QDs655 using R8 after 1 h of transduction.

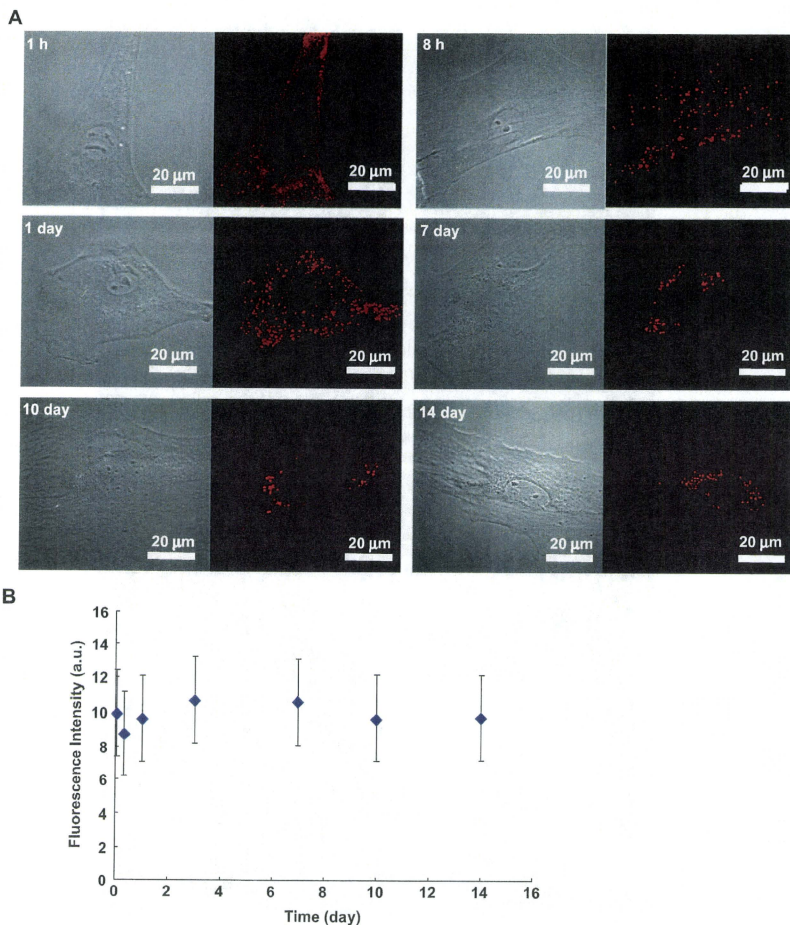


Fig. 3. Intracellular distribution and fluorescence intensity of QDs. **A:** The intracellular distribution of QDs655 in 1 h, 8 h, 1 day, 7 days, 10 days and 14 days after transduction were obtained by confocal laser scanning microscopy. Internalized QDs655 are shown by the red fluorescence. **B:** The fluorescence intensity was measured by confocal laser scanning microscopy. The data, in each triplicate, are shown as the mean \pm SD values.

using R8 in osteogenic differentiation was observed at the same level as that of the non-transduced ASCs (Fig. 5B and C). These data suggested that the differentiation ability of ASCs was not inhibited by QDs655 transduction using R8.

3.6. Detection sensitivity for *in vivo* imaging

ASCs (0.5×10^5 , 1.0×10^5 and 3.0×10^5 cells) labeled with QDs655 using R8 were injected subcutaneously with saline into the

back of the mice. The Maestro Optical Imaging System (excitation filter: 575–605 nm, emission filter: 645 nm long pass) was used to observe the mice 1 h after transplantation. Moreover, images were obtained 1, 2, 5 and 7 days after transplantation (Fig. 6A) and the intensity was evaluated (Fig. 6B). The results suggested that ASCs labeled with QDs655 could be detected up to 7 days following subcutaneous transplantation.

On the other hand, one of the advantages of QDs is their ability for multiplex imaging. Multiplex imaging may be applied for

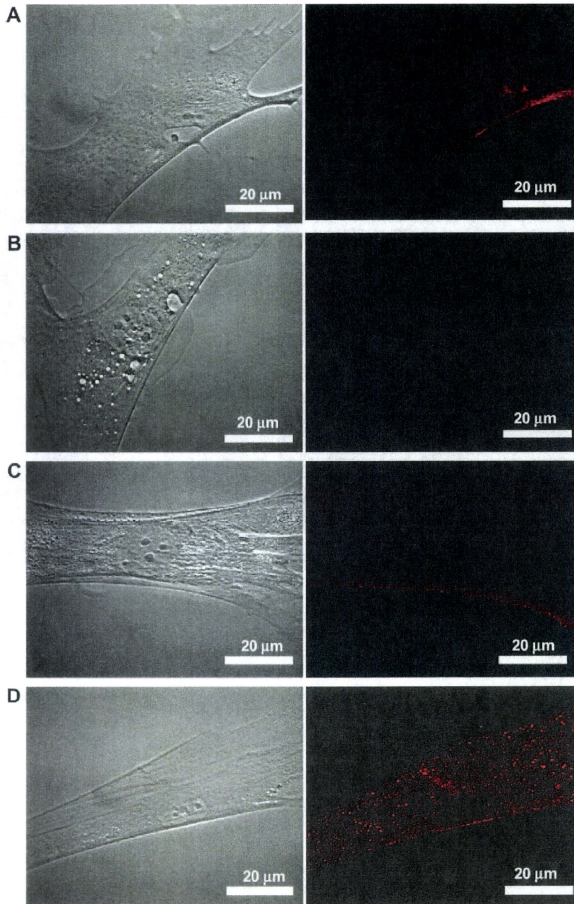


Fig. 4. Transduction mechanism of QDs using R8 into ASCs. A: ASCs were preincubated in the transduction medium with 10 mM sodium azide and 2-deoxy-D-glucose (endocytosis inhibitors) for 1 h at 37 °C. B: ASCs were preincubated in the transduction medium with 3 mM amiloride (macropinocytosis inhibitor) for 10 min at 37 °C. C: ASCs were preincubated in the transduction medium for 30 min at 4 °C (both endocytosis and macropinocytosis inhibitors). These cells were washed three times with PBS followed by addition of 0.4 nM QDs655 using R8 in the fresh transduction medium for 1 h. D: ASCs were incubated with 0.4 nM QDs655 with R8 under the normal transduction condition. These cells were observed using confocal laser scanning microscopy.

tracing different cell populations at the same time. Almost all kinds of QDs can be excited by the same wavelength and a different wavelength of light is emitted depending on the size of the QDs. In this study, ASCs (1×10^5 cells) were labeled with QDs655, 705 and 800 using R8 respectively and subcutaneously transplanted into the back of the mice and the multiplex image in 1 h was obtained in 1 h after transplantation (Fig. 6C).

Furthermore, ASCs labeled with QDs800 using R8 were transplanted through the tail vein of a mouse to examine whether the fluorescence of QDs800 from the ASCs could be detected and demonstrate the distribution of transplanted ASCs. When the mouse was sacrificed after 10 min, the fluorescence of QDs800 could be detected in the lung only by the Maestro *in vivo* imaging system (Fig. 7A). On the other hand, a histological analysis showed that the

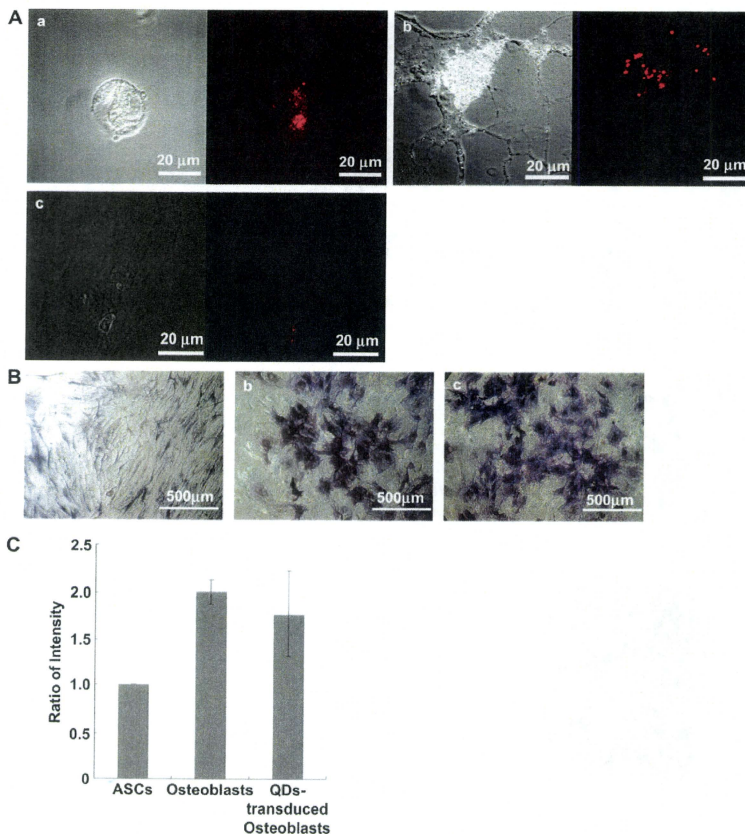


Fig. 5. Differentiation of ASCs transduced with QDs using R8. A: ASCs transduced with QDs655 (0.4 nM) using R8 were differentiated to lipocytes (a) or osteocytes (b) for 2 weeks. ASCs transduced with QDs655 (0.4 nM) using R8 were incubated in the culture medium without differentiation (c). These figures were obtained using confocal laser scanning microscopy. Phase contrast (left) and QDs655 fluorescent (right) were shown. B: Negative ALP staining of normal cultured (non-differentiated) ASCs (a), positive ALP staining of osteogenic induced ASCs (b) and osteogenic induced ASCs transduced with QDs655 using R8 (c) were confirmed. C: The quantification of ALP mRNA levels was compared in three groups. There was no significant difference between non-transduced ASCs and ASCs transduced with QDs655 using R8 ($p > 0.05$), but significantly more ALP activity was expressed than in the control ASCs ($p < 0.05$).

presence of ASCs could be confirmed in slices of the lungs only using conventional fluorescent microscopy (Fig. 7C). The heart and kidney data were not shown.

4. Discussion

Several studies have reported that some cells could passively internalize QDs in the culture medium without carrier or micro-injection, electroporation and liposome-based transduction [7,11]. ASCs could not be transduced with QDs only without the help of carriers (data not shown). A cationic liposome "Lipofectamine" was

used for the transduction of QDs into ASCs without further modification [17]. However, cytotoxicity was observed at a comparatively low concentration of >2.0 nM. In addition, the transduction time was relatively long (4 h at 0.8 nM of QDs655). Recently, CPPs mediated transduction has attracted more and more attention for the efficient cellular membrane delivery and labeling [12,20,21].

R8 which exhibits even greater efficiency in the delivery of several proteins was employed in order to overcome these problems. Moreover, compared with other articles [4,7,8], the transduction efficiency of R8 seems to be similar to CLO-Tat, CHPNH2 and pep-1. On the other hand, the molecular length of R8 is shorter

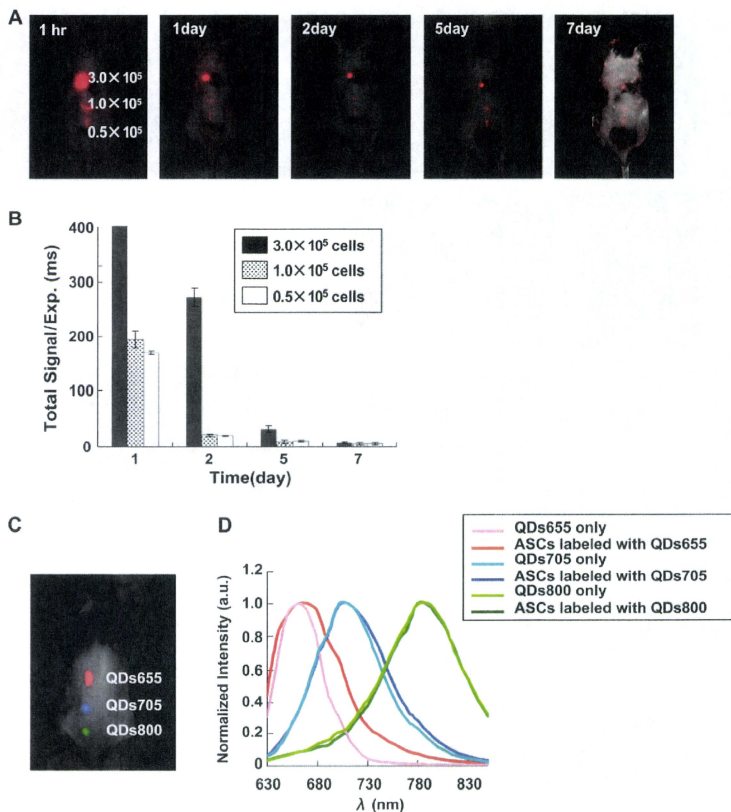


Fig. 6. Detection and multiplex imaging capability of QDs in subcutaneous transplantation. **A:** ASCs (0.5×10^5 , 1.0×10^5 and 3.0×10^5 cells) labeled with QDs655 (0.8 nm) using R8 were subcutaneously transplanted into the backs of the mice. These images were taken 1 h, 1, 2, 5 and 7 days past injection (excitation filter 575–605 nm, emission filter 645 nm long pass). **B:** The bar graph shows the change of fluorescence intensity of QDs655 for 7 days at the number of ASCs labeled with QDs655. **C:** ASCs (1.0×10^5 cells) labeled with QDs655, 705 and 800 were subcutaneously injected on the back of the mice. The image was taken 10 min after transplantation with a single excitation light source. **D:** The graph was the fluorescent spectra of ASCs labeled with QDs655, QDs705 and QDs800.

than the others, thus the cost of synthesis is lower than the others. The cytotoxicity was greatly reduced by using R8. No cytotoxicity was observed in ASCs transduced with less than 16 nM of QDs655, and no drastic decrease in the number of cells was observed with more than 8.0 nM of QDs655. These results suggested that the effect of internalized QDs on the cell cytotoxicity was dependent on surface coating rather than on the total amount of internalized QDs. Furthermore, as observed in a previous report, the current results indicate that ASCs labeled with QDs using R8 had no adverse effects on cell proliferation, measured by the CCK-8 assay [7]. On the other hand, the QDs transduction mechanism using R8 into ASCs was investigated by incubation at 4 °C, with inhibitors of endocytosis, such as sodium azide, and macropinocytosis, such as amiloride

(Fig. 4). The QDs internalization was suppressed by these inhibitors, thus suggesting that the transduction of QDs into ASCs occurred through macropinocytosis.

The transduction time and maintenance of stem cell potency after transduction of QDs are very important, when QDs are employed clinically for regenerative medicine. In this study, the fluorescence intensity of internalized QDs reached almost its peak by 1 h. The transduction time of QDs using R8 was markedly decreased in comparison to Lipofectamine [17]. On the other hand, the CPPs delivery of QDs rescued the cells from the negative effects caused by the internalized QDs to osteogenic and chondrogenic-associated lineage markers [7]. ASCs transduced with QDs655 using R8 cultured with adipogenic and osteogenic

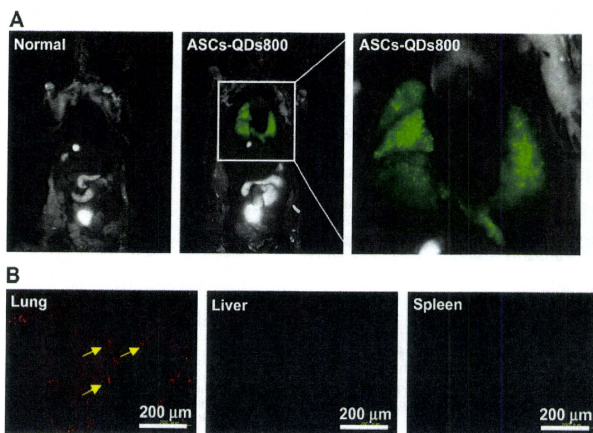


Fig. 7. *In vivo* imaging of ASCs labeled with QDs after intravenous injection. A: ASCs (5.0×10^5 cells) labeled with QDs800 (0.8 nm) using R8 were transplanted through the tail vein into mouse. The images were taken 10 min after injection (excitation filter 575–605 nm, emission filter 645 nm (long pass)). B: The red fluorescence of QDs655 was detected in the lung only, with little or no QDs655 accumulation in the liver or spleen.

medium for 2 weeks differentiated into adipogenic and osteogenic cells, respectively, in the current study. ALP activity of ASCs transduced with QDs655 using R8 following osteogenic differentiation was exhibited at the same level as that for the non-transduced ASCs. Moreover, Jui-Chih Chang et al. checked the influence to the cell surface of ASCs with QDs using cell-penetrating peptide (CPP). In mesenchymal stem cells (MSCs), cell surface receptors such as CD29, CD44, CD73, CD90, CD105, etc. are accredited the prototypical markers. According to the reference, it was found that CPP delivery preserved CD29, CD44, CD73, CD90 and CD105 expressions [7]. These data suggested that ASCs transduced with QDs using R8 maintained their stem cell potency. However, Jui-Chih Chang et al. reported that Pep-1, a CPP, coated QDs, affected the gap junctions of human mesenchymal stem cell [13]. It will be necessary to ascertain the influence of R8 on gap junctions of ASCs in future studies. The fluorescence intensity in differentiated ASCs transduced with QDs655 was sustained at least for 2 weeks. On the other hand, undifferentiated ASCs transduced with QDs655 in the culture medium, showed markedly reduced fluorescence intensity after 2 weeks of culture. The differences may be due to their proliferative activity [5]. However, the QDs655 signal was confirmed, thus suggesting that QDs could be available for labeling of ASCs for 2 weeks.

QDs exhibit several advantages in comparison to conventional organic labels, such as a high luminance, resistance to photobleaching, a range of excitation wavelengths and narrow emission bandwidths. Therefore, QDs have a potential application for *in vivo* imaging. ASCs labeled with QDs655 using R8 were subcutaneously injected with saline into the backs of the mice. ASCs labeled with QDs655 could be detected up to 7 days following subcutaneous transplantation, but the fluorescence intensities were drastically decreased. This reduction might be due to the rapid division of ASCs as suggested in a previous report [22]. When the ASCs labeled with QDs655 were incubated with DMEM/F12 medium containing 2% FBS, the fluorescence of QDs was maintained for 2 weeks at almost the same label. This result was suggested that internalized QDs were diluted by cell proliferation in the culture medium and

incorporated into the cytoplasm of ASCs after escape from the lysosome fraction system [23]. Another advantage of QDs is their ability to for multiplex imaging of different QDs at the same time [22]. When ASCs transduced with QDs655, 705 and 800 respectively were transplanted into the back of mouse subcutaneously at the different points, their fluorescence of all three species could be observed at the same time 1 h after transplantation.

On the other hand, QDs fluorescence below the wavelength range 650 nm was not used often for sensitive imaging of deeper tissues in recent papers [22,24,25]. Deep tissue imaging (millimeters to centimeters) requires the use of far red and near infrared light in the spectral range 650–900 nm [26], because these regions eliminated overlap with major absorption peaks of blood and water *in vivo*. When ASCs labeled with QDs800 were transplanted through the tail vein into a mouse, the fluorescence could be detected in the lung. ASCs labeled with QDs655 and 705 could not be observed *in vivo* because of the strong autofluorescence of liver tissue. Besides, it was reported that QDs fluorescence could be detected in other organs such as the liver and spleen in other papers [12]. In this study, mice were killed 10 min after ASCs transplantation and this suggested that almost all ASCs stayed in the lung. Therefore, *iv*. ASCs transplantation may be useful treatment modality for lung disease in the future [24].

As for the fate of QDs using R8 *in vivo*, Neera V. Gopee et al. showed that when QDs were injected intradermally (ID) on the right dorsal flank of the female SFH-1 hairless mice, the QDs could be observed moving from the injection sites apparently through the lymphatic duct system to regional lymph nodes within minutes. Residual QDs remained at the site of injection until necropsy at 24 h. Moreover, using inductivity coupled plasma mass spectrometry (ICP-MS), showed accumulation in the liver, regional draining lymph nodes, kidney, spleen, and hepatic lymph node [27].

On the other hand, Hak Soo Choi et al. showed that when QDs leaked into the vascular in the intravenous injection, the QDs were accumulated in the liver. And then, the QDs were transported to the kidney and cleared by urinary excretion [28].

5. Conclusion

ASCs possess the ability of self-renewing and differentiating into various mesenchymal cell types. Because of their potential clinical application, it has therefore become important to label the cells for tracing as transplanted cells. The concentration ratio of QDs:R8 of 1.1×10^4 was optimal for delivery into ASCs and the cells could be labeled within an 8.0 nM concentration of QDs using R8. In addition, ASCs could be labeled within about 1 h and the fluorescent intensity level of QDs was during 14 days at least. The differentiation ability of ASCs was not inhibited by QDs transduction using R8. Moreover, ASCs labeled with QDs could be detected following subcutaneous transplantation and injection through tail vein *in vivo*. This study suggested that QDs labeling using R8 could be utilized for the imaging of ASCs.

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Appendix

Figures with essential color discrimination. All figures of this article are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.01.134.

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