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Original paper

A method for in vivo tracking of mesenchymal stromal cells after intrapancreatic delivery

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Abstract

Background and aim. The intrapancreatic injection of mesenchymal stromal cells may be a viable delivery route for experimental therapy in type 1 diabetes. Adequate *in vivo* cell imaging is important to evaluate the treatment efficiency, the fate of the transplanted cells, and the mechanisms of the effects observed. Here, we present a technique for delivering these cells into the mouse pancreas and tracking them using fluorescent near-infrared quantum dots and *in vivo* imaging.

Methods and results. Bone marrow-derived mesenchymal stromal cells isolated from NOD mice were cultured and labeled with Qdots 800 nanocrystals, before being injected in the pancreas of pre-diabetic mice. *In vivo* analysis (IVIS Spectrum system) showed that the cells were successfully injected and remained localized in the pancreas for at least 24 hours.

Conclusions. Labeling of mesenchymal stromal cells with Qdots 800 nanocrystals is a reliable method for *in vivo* cell tracking, after local delivery in the pancreas.

Keywords Mesenchymal stromal cells, intrapancreatic transplant, *in vivo* tracking, Qdots 800.

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Introduction

Type 1 diabetes (T1D) is an organ-specific autoimmune disease caused by the progressive attack and destruction of insulin producing pancreatic β-cells, resulting in insulin deficiency and subsequent hyperglycemia (ATKINSON, 2014). In humans, as well as in mice, the progression of the disease is mainly mediated by inflammatory T cells. Histologically, the characteristic lesion in T1D is insulitis, consisting of lymphocytic infiltrates around and within the islets (IN'T VELD, 2014; ASKENASY, 2016).

Mesenchymal stromal cells (MSC) based therapies have been considered in the last years as an alternative approach for the treatment of different autoimmune disorders, including T1D (CARLSSON, 2015; FIORINA, 2009; JIANG, 2020; MADEC, 2009). MSC are multipotent cells, with ability to differentiate into different cell lineages, under proper conditions (ROSCA, 2011). Their beneficial effects may be, at least in part, due to their regenerative and immunomodulatory properties (PITTENGER, 2019; PROCKOP, 2012). Thus, MSC may effectively inhibit and diminish the population of self-reactive inflammatory cells responsible for the autoimmune reaction towards the pancreatic β-cells (ABDI, 2008). In addition, MSC secrete cytokines and trophic factors that can provide the right environment for islet recovery by supporting the remodeling of the pancreatic stroma, vascularization and differentiation to produce insulin (RANI, 2015).

Preclinical studies performed in animal models of T1D have employed different strategies for delivering therapeutic MSC in order to prevent or reverse the course of the disease (ASARI, 2011; JUREWICZ, 2010; MURAI, 2017; YAOCHITE, 2015). In many studies, the preferred delivery route is intravenous injection. However, this approach comes with significant side effects resulting from trapping of the cells primary in the lungs, where they can block the capillaries and provoke microemboli (LEE, 2009). In addition, MSC are hardly, if not at all detected within short periods after administration, making it challenging to assess their subsequent fate and the way they impact the target organ or the disease (BRAID, 2018; EGGENHOFER, 2012). Since MSC are endowed with intrinsic capacity to reduce inflammation, their immunomodulatory properties may be enhanced if the cells are delivered locally in the target organ.

In this context, the adequate visualization of MSC delivered *in vivo* is important in order to determine the overall efficiency of the treatment applied, as well as to study the biological mechanisms by which they exert their therapeutic activities. Here, we describe a technique for delivering and tracking of MSC into the mouse pancreas by using near-infrared quantum dots (Qdots) fluorescent labeling and *in vivo* visualization of the transplanted cells.

Materials and Methods

Mice. NOD/ShiLtJ (NOD; Stock No: 001976) mice purchased from The Jackson Laboratory were propagated in our animal facility under specific-pathogen free conditions (12/12-hour light/dark cycle, 21°C and 55% - 60% humidity), with access to food and water *ad libitum*. For transplantation of MSC, we used 12-week-old prediabetic NOD females, as confirmed by blood glucose reading from the age of 10 weeks. All procedures were conducted in accordance with the Directive 2010/63/EU and were approved by the Institutional Ethical Committee of the Institute of Cellular Biology and Pathology "N. Simionescu", and by the National Sanitary Veterinary and Food Safety Authority (authorization no. 296/23.08. 2016).

MSC isolation, culture and differentiation. Murine MSC were derived from bone marrow harvested from femurs and tibiae of NOD mice aged 6-8 weeks. The aspirate was cultured on plastic dishes at an initial cell density of 2×10^6 cells/cm^2 in normal MSC medium, containing DMEM low glucose (LG) 1g/L glucose (Gibco), 10% FBS suitable for MSC culture (PAN-Biotech) and 1% Penicillin/Streptomycin/Amphotericin. The cells were sub-cultured for several passages at an initial density of 5×10^3/cm^2, with fresh medium added every 3 days. In this study, we used cells between passage 6 and 9. MSC differentiation was performed as previously described (ROSCA, 2011). All images were taken with an Olympus CKX41 microscope equipped with an Olympus XC30 camera.

Flow cytometry analysis. For flow cytometric analysis, 10^5 MSC/sample were incubated with PBS with 2% FBS and 5% mouse serum and stained for 30 minutes on ice with the following antibodies: anti-mouse Sca-1PE (108108), anti-mouse CD105 (120401) followed by anti-rat-FITC (407505), anti-mouse c-kitAPC (105812) or anti-mouse CD45.1PE (110708) (Biolegend). After, the cells were washed in PBS, suspended in PBS containing 2% FBS and 0.5 mM EDTA for analysis. All measurements were done using a CytoFlex flow cytometer

(Beckman Coulter) and the CytExpert v2.1 software (Beckman Coulter).

Intrapancreatic transplantation. The mice were weighed and anesthetized with the appropriate dose of a ketamine/xylazine/acepromazine cocktail (80/20/10 mg/kg). Sterile ocular lubricant ointment (Artelac, Bausch & Lomb) was applied on the eyes to prevent dryness. The left flank region of the mouse was shaved using clippers and the mouse was placed on the right lateral side on a wormed pad. After the skin was disinfected with povidone-iodine (Betadine), the animal was covered with a sterile gauze sponge, previously cut at the center to expose the left flank. The splenic silhouette can be easily seen under the thin layers of skin and muscle, and it served as a visual guide for placing the incision. An incision of about 1 cm was performed at the level of the skin and lateral abdominal muscle layer and then the spleen and the tail of the pancreas were exposed; 50 µl of cellular suspension containing 5 x 10^5 MSC in PBS, previously loaded into a Nanofil-100 syringe (World Precision Instruments) equipped with a 26-G beveled needle, were slowly injected. Afterwards, the syringe was removed carefully from the injection site to avoid leakage. Then, the spleen and the pancreas were gently pushed back into the abdominal cavity, without applying pressure on the organs or the injection site. The muscle layer and the skin were successively closed with interrupted sutures, the wound was cleaned with Betadine and then an ointment containing neomycin sulfate and bacitracin zinc was applied. After the procedure, the animals were hydrated by subcutaneous administration of 300 µl of saline solution, placed for 10 min under an infrared heating lamp, and monitored until recovery from anesthesia. The entire surgical procedure was performed under the hood, in aseptic conditions.

MSC labeling. Fluorescence Qdots labeling was performed using a Qtracker® 800 Cell Labeling Kit (Ex/Em: 405-760/800 nm) (Q25071MP, ThemoFisher), in accordance with the manufacturer's recommendations. Briefly, the cells were detached with trypsin and then incubated with 10 nM Qdot® 800 nanocrystals in a 1.5 mL microcentrifuge tube, for 60 min at 37°C. After labeling, the cells were washed twice with cold PBS and then kept on ice in PBS until transplantation.

Fluorescence confocal microscopy. To evaluate the incorporation of Qdot® 800 nanocrystals in MSC, cells were seeded (at a density of 10 000 cells/cm^2) into Nunc™ Lab-Tek™ II CC2™ Chamber Slides (154917PK, ThermoFisher) and incubated at 37°C, 5% CO_2 overnight. The next day, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature, followed by staining with Fluorescein Phalloidin (F432, ThermoFisher) for 20 min at room temperature and nuclei counterstaining with Hoechst 33258 (H1398, ThermoFisher). The slides were washed, mounted with Prolong antifade reagent (P36930, ThermoFisher) and examined using a TCS-SP5 Leica confocal microscope, with an HC PL APO 10 ×/0.4 NA CS and HC PL APO 40x/1.3 NA oil CS objectives.

***In vivo* fluorescence imaging.** To confirm the transplant procedure and local MSC engraftment, animals were monitored at 15 min and at 24 h after cell transplantation using the IVIS Spectrum *in vivo* imaging system (PerkinElmer). The mice were anesthetized with 1.5% isoflurane (Isoflutek, 710004), placed in the IVIS imaging box and imaged. At the end of the experiment, the animals were sacrificed and individual organs were imaged *ex vivo*. Fluorescence images were captured and analyzed using the Living Image 4.5 software (PerkinElmer). Spectral unmixing analysis was performed to extract the signal from the tissue autofluorescence. Fluorescence signals obtained after unmixing were calculated as total radiant efficiency (p/s/cm^2/sr/µW/cm^2).

Results

Characterization of bone marrow derived-MSC. Bone marrow derived-MSC were isolated from the medullar aspirate harvested from femurs and tibiae of NOD mice. The plastic adherent cells at passage 6 showed specific MSC morphology at confluence (Figure 1, A) and successfully displayed multipotent differentiation potential, under specific conditions. Thus, after a specific induction period of two weeks, the MSC differentiated into adipocytes, as confirmed by Oil Red O staining of lipid droplets accumulated intracellularly (Figure 1, B), or into osteoblasts, as indicated by the calcium deposits highlighted by von Kossa staining (Figure 1, C). After three weeks of incubation under specific conditions, the MSC differentiated into chondrocytes, characterized by the presence of aggrecans and acidic polysaccharides as indicated by Alcian Blue staining (Figure 1, D). Furthermore, the flow cytometry analysis showed that MSC expressed specific markers such as Sca-1 (99.8% positive cells) and CD105 (96.2% positive cells) and were negative for the hematopoietic markers CD45 and cKit (Figure 1, E–H).

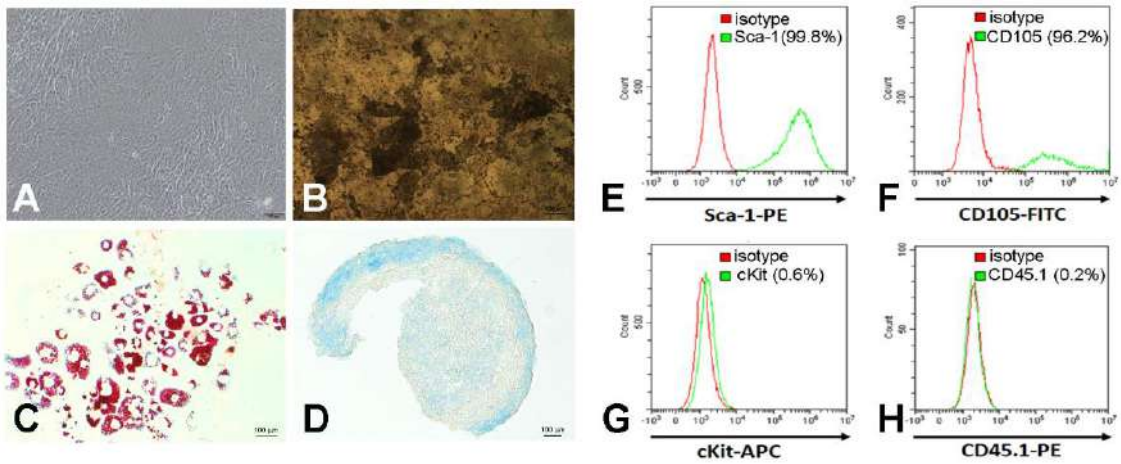

Figure 1. Characterization of bone marrow-derived MSC. (**A**) Phase-contrast microscopy illustrating the morphologic aspect of cells in confluent culture. (**B**–**D**) Analysis of the multipotentcy of MSC by their ability to differentiate into adipocytes, osteocytes, and chondrocytes. Adipogenic differentiation (**C**) was demonstrated by Oil Red staining. Osteogenic differentiation (**B**) was confirmed by von Kossa staining. Chondrogenic differentiation (**D**) was proven by Alcian Blue staining. (**E**–**H**) Cell profile (Sca-1$^+$/CD105$^+$/cKit$^-$/CD45.1$^-$) determined by flow cytometry.

Cell transplantation into the pancreas. We set up an experimental design in order to assess if the fluorescent labeling of MSC using Qdots 800 is an efficient tool for tracking the cells after their local delivery into the pancreas of pre-diabetic mice (Figure 2, A). After the incision of the skin and lateral abdominal muscle layer, the spleen and the underlying region of the pancreas were partially exposed outside of the peritoneal cavity by using a pair of blunt-tip forceps and taking care not to touch the pancreas or injure the spleen (Figure 2, B). The cell suspension was slowly injected into the pancreatic parenchyma (Figure 2, C), taking special care to avoid the damaging of the pancreatic blood vessels and circumvent organ hemorrhage. The formation of a fluid-filled region within the pancreatic parenchyma visually confirmed the successful delivery of the cells inside the organ (Figure 2, D). Afterwards, the organs were carefully placed back into the abdomen and sutures were applied to the muscle and skin (Figure 2, E). Some animals have been monitored post-operative for up to one month and indicated that the procedure is safe with all the animals recovering well after the surgery (Figure 2, F).

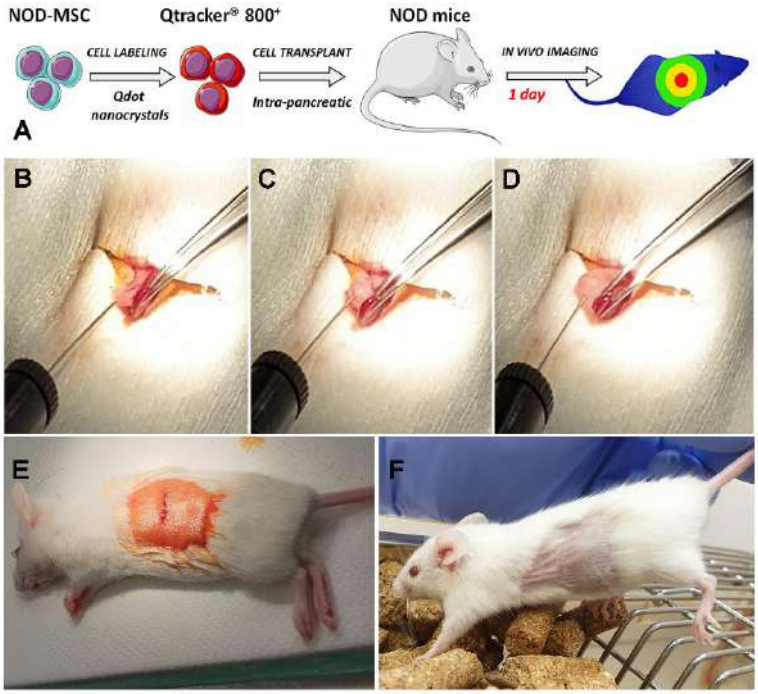

Figure 2. Intrapancreatic transplantation of MSC. Diagram showing the experimental set-up for cell labelling and *in vivo* tracking (**A**). After the surgical incision of the upper left lateral skin and muscle layer, the spleen and the sub-adjacent pancreas were exposed (**B**). The suspension of fluorescently-labelled MSC was injected into the splenic region of the pancreas (**C**), as visually confirmed by the inflation of the pancreatic parenchyma (**D**). After cell transplantation, the skin and the muscle layer were sutured (**E**). A representative image of an animal two weeks post-surgery is presented, showing complete healing of the surgical wound (**F**).

Efficient *in vitro* labelling and *in vivo* tracking of transplanted MSC. Prior transplantation, the cells were labelled with near-infrared Qdots 800. First, MSC were imaged using a confocal fluorescence microscope in order to evaluate the intracellular distribution of the Qdots 800 nanocrystals. After the incubation period, the Qdots labelled homogenously all the cells (Figure 3, A). The nanocrystals were detected in the cytosol, mostly adjacent but not inside the nucleus, appearing to have a vesicular localization (Figure 3, B). In order to assess whether there is a direct correlation between the cell number and the fluorescence signal from the Qdots 800 nanocrystals, increasing numbers of labeled MSC (from 10^4 to 4 x 10^5 cells) were imaged *in vitro*, using the IVIS Spectrum imaging system. The Qdots signal was measured and the correlation coefficient R2 between the number of cells and the intensity of the fluorescence signal was calculated. The results showed a linear increase of the fluorescence intensity with the number of cells, ensuring that the fluorescent signal can be used also for quantitative studies (Figure 3, C).

Next, we wanted to evaluate if the labelling of MSC with Qdots 800 is a reliable method for tracking these cells *in vivo*, more specifically after transplantation in the pancreas of pre-diabetic NOD mice. For this, 50 µl of cell suspension containing 5 x 10^5 Qdots 800-labelled MSC were transplanted at a single site into the splenic region of the pancreas, as described above. The animals were imaged at 15 min and 24 hours following cell transplantation. The fluorescent signal indicated that the cells were successfully injected into the pancreas, where they remained localized, at least for the period monitored. We did not detect any leakage from the transplant site into the abdominal cavity immediately after the surgical procedure or 24 hours later (Figure 3, D and E). This was confirmed also by the analysis of the major individual organs (pancreas, spleen, mesenteric and pancreatic lymph nodes, lungs, liver and skin adjacent to the surgical site) using *ex vivo* fluorescence imaging, at 24 after surgery (Figure 3, F). Our analysis revealed that, except for the pancreas, none of the analyzed organs showed any detectable fluorescent signal.

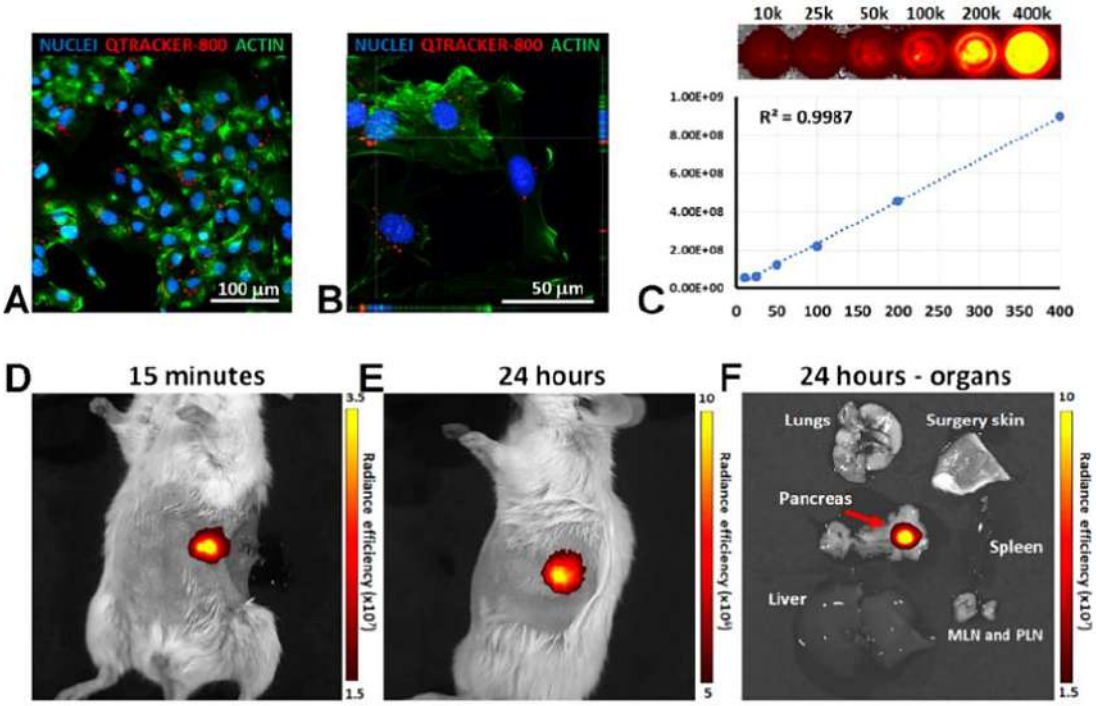

Figure 3. Labelling of MSC with Qdots® 800 nanocrystals and *in vivo* tracking. MSC were incubated with Qdots 800, which labelled uniformly all cells in culture (**A**) and were confined inside the cytoplasm of the cells (**B**). A linear correlation between the number of cells stained and the intensity of the signal was established by imaging increasing MSC numbers (**C**). After intra-pancreatic injection, the cells remained localized at the transplant site (**D**), engrafted into the pancreas (**E**) and did not migrate in other organs (**F**), as seen by *in vivo* whole body and *ex vivo* organ imaging.

Discussion

In the present study, we employed fluorescent Qdots to label MSC and we assessed the efficacy of this labeling method in the context of intrapancreatic transplantation in pre-diabetic NOD mice. For this, syngeneic MSC from NOD mice were isolated and fully characterized by assessing their specific surface markers and their trilineage differentiation potential. Our results showed that NOD-derived MSC expressed specific molecular markers and generated adipocytes, osteoblasts and chondrocytes when cultured under appropriate conditions.

In order to track MSCs *in vivo*, cells were labeled with intracellular Qdots. Structurally, Qdots are nanocrystals composed of a semiconductor core treated to allow incorporation of ionizable functional groups and coupling of different biomolecules such as proteins, target peptides, oligonucleotides etc. As compared to other cellular dyes and labels, Qdots have a long fluorescence half-life and high resistance to photobleaching, meaning that they can be imaged for long periods with a minimal loss of the signal. In addition, Qdots have wide excitation and narrow emission spectra and are commercially available with different defined emission wavelengths extending from green to the near-infrared (JAISWAL, 2003; KINGETER, 2009). Qdots are biocompatible and have no cytotoxic effect. These properties make Qdots very reliable bio-imaging tools.

Previous studies have determined that MSC are efficiently labeled by Qdots (MULLER-BORER, 2007; SELEVERSTOV, 2006). In our study, we used near-infrared fluorescent Qdots with a maximal emission of 800 nm (Qdots 800) to label bone marrow-derived murine MSC and to follow them *in vivo*, after intrapancreatic transplantation. We used confocal microscopy to evaluate the efficiency of the labeling *in vitro*. Our results showed that murine bone marrow-derived MSC had efficiently uptaken Qdots 800 and that the nanocrystals were confined into the cell cytoplasm, in an apparent vesicular distribution in the vicinity of the nucleus.

There are several considerations to be made regarding the administration route of MSC and how it influences the efficacy of the therapy. First, it is well documented the fact that the large majority of the intravenously injected MSC are trapped and subsequently cleared from the lungs, and only a fraction of the cells reaches the target organs (BRAID, 2018; EGGENHOFER, 2012). In addition, the relatively large size of these cells and their tendency to form cellular aggregates represent a serious risk for pulmonary embolization and death (LEE, 2009). Second, MSC work at least partially by direct contact cell-cell in order to act as immune regulators (FAN, 2020). From this point of view, local delivery of these cells may have superior therapeutic outcomes than systemic delivery. Third, most studies report that the MSC are not detected anymore within short periods after administration, making it difficult to evaluate their fate after inoculation and to assess the way they impact the target organ or disease (ULLAH, 2019).

In the context of type I diabetes, the intrapancreatic injection of MSC has been used as an alternative route to deliver the cells to the injured pancreas, in an attempt to avoid complications related to systemic delivery (MURAI, 2017; YAOCHITE, 2015). One may argue that due to the increased local pressure caused by the intra-pancreatic injection and because the mouse pancreatic tissue is extremely thin, the cell suspension could easily extravasate from the injection site, resulting in intra-abdominal spillage. To avoid leakage into the abdominal cavity, the injection was performed slowly and the needle was not immediately retracted from the injection site. *In vivo* imaging analysis of the animals at 15 min after the closure of the surgical procedure demonstrated that the transplant was conducted successfully.

To determine whether MSC migrated from the site of transplantation, the mice were sacrificed 24 h after transplantation and the organs were harvested and imaged *ex vivo*. Our analysis revealed that none of the analyzed organs showed any detectable fluorescent signal, except for the pancreas. The signal remained localized at the site of injection, suggesting the local engraftment of the cells. In addition, the lack of any detectable fluorescent signal in the organs analyzed *ex vivo* suggests that MSC did not migrate from the injection site immediately or within the first 24 hours after transplantation. Subsequent studies are necessary to determine the fate of the cells transplanted into the pancreas and to establish if they migrate to other regions or whether they are removed by other cells.

Conclusions

Our study shows that labeling of MSC using fluorescent near-infrared Qdots is a reliable method for *in vivo* cell tracking, after local delivery in the pancreas. This method can facilitate a better understanding of how MSC function after transplantation and how they can be applied for the treatment of type 1 diabetes.

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Acronyms and abbreviations

T1D – type 1 diabetes; MSC – mesenchymal stromal cells; Qdots – quantum dots; FBS – fetal bovine serum; PBS – phosphate-buffered saline.

Conflict of Interest

The authors have no conflict of interest to declare.

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