Quantum Dots labeling allows detection of the homing of Mesenchymal Stem Cells administered as immunomodulatory therapy in an experimental model of pancreatic islets transplantation

Silvia Mannucci[1,2], Laura Calderan[1], Paola Quaranta[3], Sara Antonini[3], Biancamaria Longoni[3], Pasquina Marzola[2], Federico Boschi[2]

1. Department of Neurological and Movement Sciences, University of Verona, 37134 Verona, Italy

2 Department of Computer Science, University of Verona, 37134 Verona, Italy

3.Department of Translational Research and New Technology in Medicine, University of Pisa 56124 Pisa, Italy

Corresponding authors: Federico Boschi and Silvia Mannucci, Department of Computer Science, University of Verona, Strada Le Grazie 15, I-37134 Verona, e-mail: federico.boschi@univr.it and silvia.mannucci@univr.it

Abstract

Cell transplantation is considered a promising therapeutic approach in several pathologies but still needs innovative and non-invasive imaging technologies to be validated. The use of mesenchymal stem cells (MSCs) draws major interest in clinical transplantation thanks to their regenerative properties, low immunogenicity and their ability to regulate immune responses. In several animal models, MSCs are used in co-transplantation with pancreatic islets (PIs) for the treatment of type I diabetes and their efficacy in supporting graft survival and prolonging normal glycaemia levels is showed. In this study we investigated the homing of systemically administered MSCs in a rat model of pancreatic portal vein transplantation. MSCs labeled with quantum dots (Qdots) were systemically injected by tail vein and monitored by optical fluorescence imaging. The fluorescence signal of the liver in animals co-transplanted with MSCs and PIs was significantly higher than in controls, in which MSCs alone were transplanted. By using magnetic labeling of PIs, the homing of PIs into liver was independently confirmed. These results demonstrate that MSCs injected in peripheral blood vessels preferentially accumulate into liver when PIs had been transplanted in the same organ. Moreover we here proved that bimodal MRI-fluorescence imaging allows differential monitoring of the fate of two types of cells.

Keywords: Fluorescence Imaging, Quantum Dots, transplantation, mesenchymal stem cells, pancreatic islets, MRI

1.Introduction

Cell transplantation is considered a promising therapeutic approach in several pathologies including neurological [1], cancerous [2], musculoskeletal [3], and metabolic diseases [4]. Among cell-based therapies, stem cells evoke great interest due to their ability to self-renewal and to differentiate into multiple cell lineages [5]. In order to be used in safe clinical settings, SCs should demonstrate genomic stability, to be readily harvested and/or expanded and, above all, do not induce teratomas [6]. Embryonic stem cells (ESCs), derived from blastocyst, are pluripotent cells and show genomic stability [7, 8]. Beyond the ethic concerns due to their origin, ESCs can induce teratoma formation *in vivo* [9, 10] as well as have problem of tissue rejection following transplantation patients. The advent of induced pluripotent stem cell, discovered in 2006 from Takahashi and Gurdon [11, 12] opened new perspectives for stem cell therapy. These cells indeed can be obtained by reprogramming differentiated adult cells, so they do not have ethic limits, and can be differentiated in any lineages, but their genomic stability is currently under investigation to be considered clinically safe [13]. MSCs, instead are adult stem cells of mesodermal origin, they are free of ethic concerns, thus they are a promising candidate for cell therapies [14].

Along with multipotency and self-renewal, MSCs lack of the co-stimulatory molecules of the class II major histocompatibility complex (MHC II); further they exhibit immunosuppressive properties thus overall their immunological features and low immunogenicity represent a potential treatment for transplants [15]. The use of MSCs in association with PIs in animal transplants have been largely investigated for the treatment of type I diabetes [16, 17], a pathology characterized by the irreversible autoimmune destruction of pancreatic beta cells [18]. Islet transplantation in the portal vein of patients affected by type 1 diabetes combines minimally invasive surgery and low incidence of peri-operative risks [19] while improving glycemic control and prospective insulin independence [20, 21, 22]. We recently reported that co-transplantation of MSCs and PIs helps prolonging normal glycaemic levels in an experimental model of chemically-induced diabetes in rats [4]. The effect of

the intravenous administration of MSCs was assessed both in an allogeneic and a syngeneic model. PIs were transplanted in the portal vein and they engraft into the liver as demonstrated by *in vivo* imaging studies [23, 24]. When MSCs are alternatively infused alone by portal vein they migrate for more than 70% in the lungs while less than 10% remained in the liver. Several papers showed that when MSCs are systemically injected they preferentially distribute in a wide variety of organs i.e. spleen and lungs (a cell entrapment is triggered by MSCs dimensions) [25]. This effect cause a loss of therapeutic efficacy. Therefore a further investigation of the stem cell homing is required to improve the beneficial effect of cell therapies. Currently, non-invasive imaging techniques as magnetic resonance imaging (MRI), optical imaging (OI) or positron emission tomography (PET) have been widely used to visualize cells *in vivo* in intact organisms after labeling cells with magnetic [26, 27], fluorescent [28] or radioactive [29] probes, prior to their transplantation. We reported that PIs can be labeled with superparamagnetic iron oxide particles (SPIOs) and visualized in the liver of the recipient subject by MRI with high sensitivity [23, 24]. Furthermore, several articles have shown that stem cells can also be labeled with superparamagnetic particles without relevant cytotoxic effects and detected with high sensitivity *in vivo* by MRI. In the present study, MSCs were inoculated and PIs transplanted within the same experimental procedure and imaged separately. PIs are easily detectable using SPIO labeling and MRI as demonstrated in our previous work [4]. In order to image MSCs we decided to use a fluorescent probe as Quantum Dots (Qdots) that are specifically designed for cell labeling. Qdots emitting in the near infrared range i.e. inside the transparency window of living tissue were chosen. Due to the thickness of rat's tissue we were forced to work *ex vivo* by acquiring fluorescence images of excised organs rather than *in vivo*. Despite this limitation, our data clearly demonstrate that MSCs, inoculated immediately after PIs transplantation during the same experimental procedure, were driven into the liver to a greater extent than in controls, in which only MSCs were inoculated. This paper provides the proof of concept that bimodal labeling (and bimodal imaging) can be used to unveil the cellular homing when two type of cells are transplanted.

2.Materials and methods

2.1 Animals

Inbred male Lewis (L) (n=36) and Wistar Furth (WF) (n=20) rats, weighing 275-300 g, were purchased from Charles River Laboratories (Italy). The animals, fed on standard rodent chow (Rieper, Italy) and water ad libitum, were kept under a 12 h light/dark cycle. In our experimental model, WF rats were used as donors and L rats as recipients of PIs. All experimental procedures were carried out with the approval of the Ethical Committee for Animal Experimentation of the University of Pisa.

2.2 MSCs isolation and culture

MSCs and bone marrow cells were collected from both tibias and femurs of L rats following the Dobson's procedure [30]. Total nucleated cells were cultured in Dulbecco's modified culture medium (DMEM) (Sigma-Aldrich, Italy) supplemented with 10% Fetal Bovine Serum (FBS) (Eurobio, Italy), 1% L-glutamine (Sigma-Aldrich, Italy), penicillin (50µg/mL, Eurobio,Italy), streptomycin (50µg/mL, Eurobio, Italy), amphoterycin B (0,2µg/mL, Sigma-Aldrich, Italy), and incubated at 37°C in fully humidified atmosphere containing 95% air and 5% carbon dioxide. After 7 days, half of the culture medium was changed. On reaching confluence, the adherent cells were detached by 0.05% trypsin and 0.02% EDTA for 5–10 min at 37°C, harvested and washed with HBSS and 10% FBS and finally re-suspended in complete medium (primary culture, P0). Cells were re-seeded at 10^4 cells/cm^2 in 100mm dishes (P1) for both *in vitro* differentiation assessment and further cellular expansion which was achieved by successive cycles of trypsinization and re-seeding. The frequency of Colony Forming Units-Fibroblasts (CFU-F) was measured using the method of Castro-Malaspina [31]. Visible colonies with 50 or more cells (the conventional value for defining a colony) were counted and referred to 10^6 plated cells (no. of CFU-F/10^6 TNC).

120 **2.3 *In vitro* experiments**

121 MSCs were labeled with Qdots (Qtracker® 800 kit cell labelling, Invitrogen^{TM} Milan, Italy)
122 following the datasheet instructions. Briefly, 10 nmoles of Qdots were added to MSCs in T75 flask.
123 After one hour, the adherent cells were detached by 0.05% trypsin/EDTA (Life technology, Italy),
124 counted, centrifuged at 1200 rpm, washed three times in Phosphate Buffered Saline (PBS) and
125 resuspended in 300 µl of DMEM without FBS. After labeling, the fluorescence signal of MSCs was
126 measured *in vitro*. Different amounts of labeled cells (range 50-10^5 cells) were suspended in non-
127 fluorescent wells and acquired by OI in the fluorescence modality using the same experimental
128 setup as in excised organs acquisition protocol (see below).

129 **2.4 Islet isolation and culture**

130 PIs were isolated from WF rats (275-300 g) by collagenase P (Roche Diagnostics, Italy) perfusion
131 and purified by continuous-density Ficoll gradient as previously described [4]. Briefly, the pancreas
132 was distended by bile duct injection of 15 mL of 4°C-cold collagenase P (1 mg/mL; Roche
133 Diagnostics, Italy) diluted in HEPES-buffered Hank's balanced salt solution (HBSS Sigma-Aldrich,
134 Italy), and then it was excised and minced. Islets were digested at 37°C for 20 min under constant
135 stirring. Islets were separated from exocrine tissue by centrifugation on a Histopaque (Sigma-
136 Aldrich, Italy) discontinuous gradient, removed from the interface of the layers, washed in HBSS
137 and finally resuspended in 10 mL of RPMI (Eurobio, Italy) supplemented with 10% fetal calf serum
138 (Eurobio, Italy), 1% L-glutamine, 10mM glucose (Sigma-Aldrich, Italy), penicillin (50U/ml,
139 Eurobio, Italy), streptomycin (50µg/ml, Eurobio, Italy), amphoterycin B (0,2 µg/ml, Eurobio, Italy)
140 and 1% HEPES buffer (Sigma-Aldrich, Italy) in free floating culture flask. Islets were handpicked
141 under an inverted microscope under sterile conditions and purity was assessed by Dithizone staining
142 (Sigma-Aldrich, Italy). For each graft, the total islet mass, expressed as the 150 µm diameter islet
143 equivalent number which was calculated on volumetric assumptions. PIs were incubated at 37°C
144 (95% air and 5% CO2), for 1-2 days before transplantation. The amount of PIs needed to transplant

in n=2 recipients was labelled with a MRI contrast agent (Endorem®, Guerbet France) according to previously published methods [24].

2.5 Transplantation of pancreatic islets and MSCs

The effect of MSCs intravenous administration was assessed in an allogeneic graft using Wistar Furth rats as donors and Lewis rats as recipients of PIs. Lewis rats were divided into 4 groups: group 1 (n=6) received saline intravenously (i.v.), group 2 (n=6) received 5 x 10^5 unlabelled MSCs i.v.; group 3 received 5 x 10^5 MSCs labelled with Qdots 800 i.v. (n=12; n=6 rats were sacrificed 5 days and n=6 rats 10 days after i.v. injection). Group 4 received 700 IE islets in portal vein and 5 x 10^5 MSCs labelled with Qdots i.v. (n=12; n=6 were sacrificed 5 days and n=6 rats 10 days after i.v. injection).

L rats (230-250 g) were anaesthetized (Zoletil 100: tiletamin 90 mg/Kg and zolazepam 10 mg/Kg, Virbac s.r.l., by intraperitoneal injection) and the portal vein was exposed through a midline incision parallel to the spine. PIs suspended in 200µl of PBS solution were injected and re-flushed 2-3 times into the portal vein. Two additional rats, treated as the animals belonging to group 4, were injected with PIs labeled with a magnetic contrast agent (Endorem®, Guerbet, France) in order to monitor them by MRI.

An amount of 5 x 10^5 MSCs labeled with QDots (or the same amount of unlabeled MSCs) were transplanted in L rats by i.v. injection in the tail vein. After 5 or 10 days, the animals were sacrificed and perfused with PBS. The following organs were excised: liver, lungs, spleen, kidneys and pancreas.

2.7 Imaging *in vivo* and *ex vivo* experiments

MRI acquisitions were acquired with the same protocol described in Longoni B et al (2010) [4].

Fluorescence images were acquired using a VivoVision Systems, IVIS® 200 Series, for small laboratory animals (Caliper, Alameda, USA). The system is made of a camera sensor back thinned, back illuminated, grade CCD 1 (2.7 x 2.7cm, -90°C), with a minimal image pixel resolution of 20 µm (pixel dimension 13.5 µm, imaging pixels 2048 x 2048), quantum efficiency >85% between 500 and 700 nm, and > 30% between 400 and 900 nm. For the experiments we used a fluorescent modality with Cy5.5 (615-665 nm) excitation filter and ICG (810-875 nm) emission filter. Images were acquired with binning factor = 8, field of view = 12.8 cm, exposure time = 1 s, opening of diaphragm (f/stop) = 2.

Fluorescence images of excised organs were acquired and analysed by using Living Image 4.1 software (Caliper, Alameda, USA). According to Yukawa and co-workers [28] the quantification of fluorescence emission of different excised organs was performed by considering the parameter RFI (ratio of fluorescence intensity) that was defined as follows: RFI= (Fluorescence Intensity of the organ)/(Total Fluorescence Intensity of the five excised organs of the same animal: liver, kidneys, lungs, spleen, pancreas). Data analysis and statistics were carried out using routines written in Matlab 7.1 (The MathWorks, Inc., USA). A t-test analysis was performed with a significance level of $p \leq 0.05$.

3. Results

3.1 MSCs characterization

Rat bone marrow-derived MSCs were purified by plastic adherence. After the fifth passage, the cells grew exponentially, requiring weekly passages. MSCs treated with osteogenic medium formed small deposits of hydroxyapatite intensely red stained with Alizarin S (Fig. 1A). Treatment with adipogenic medium differentiated MSCs towards adipogenic lineages with Oil Red-O staining (Fig. 1B). Cytofluorimetric analysis showed the existence of a homogeneous population of adherent cells (after 4-5 passages), positive for CD90, CD44, CD54, CD73 and

CD106. There was no significant contamination of hematopoietic cells, as flow cytometry was negative for markers of hematopoietic lineage, including CD11b and CD45(data not shown).

3.1 *In vitro* experiment

The MSCs labeling was observed *in vitro* by optical imaging used in the fluorescence modality. Images of wells containing different amounts of labeled or unlabeled cells are shown in Fig 1C. The dependence of fluorescence signal intensity on the cell number is shown in Fig. 1D for labeled and unlabeled cells. Good correlation was found between signal intensity of wells containing labeled cells and the number of cells itself (r^2=0.84). The fluorescence signal acquired in non-fluorescent wells containing pure DMEM or unlabeled cells was significantly lower than the signal acquired in wells containing labeled-cells (Fig. 1). Even at the lowest number of cells investigated (n=50), the fluorescence signal (1.14±0.03 x 10^8 photon/s) was substantially higher compared to pure DMEM (2.47±0.33 x 10^7 photon/s) or 50 unlabeled cells (2.20±0.42 x 10^7 photon/s). This result shows that, our detection limit *in vitro* is about 50 cells.

3.2 Imaging *in vivo* and *ex vivo* experiments

Fig. 2 shows fluorescence images of excised organs of representative rats sacrificed 5 (Fig. 2A) and 10 (Fig. 2B) days after injection with unlabeled MSCs (group 2), labeled MSCs (group 3), labeled MSCs plus transplantation of PIs (group 4). It is clearly evident that the fluorescence signal emitted from liver and kidneys is substantially higher with respect to the other organs in all experimental groups. Fluorescence images acquired in organs excised from rats injected with MSCs labeled and sacrificed 10 days after MSC injection and PI transplantation are shown in Fig. 2B (group 4). Due to the low fluorescence emission *ex vivo* images of group 1 (saline) are not shown. Fig. 2C reports the mean fluorescence signal emitted in the different organs of the experimental groups considered, expressed through the RFI parameter.

The presence of PIs into the liver can be independently monitored by MRI after labeling with SPIOs [24]. Fig. 2D shows a representative MRI of excised liver from rat transplanted with PIs labeled with SPIOs (Endorem®, Guerbet France). The presence of several dark spots corresponding to PIs (or groups of PIs) are clearly evident. MRI acquisitions showed PIs in the hepatic parenchyma (Fig 2D) confirming data described in Longoni B *et al* 2010 (4) .

4. Discussion

In the past years MSCs were the main topic of the studies on the use of cell therapy regenerative medicine. They were largely applied to tissue regeneration models, to transplantation and for the treatment of several degenerative pathologies. The numerous data derived from these studies showed that MSCs are a safe and a feasible therapy for clinical applications. Strictly related to this issue there was also the need of monitoring the fate of transplanted cells and several related works investigating the homing and biodistribution of MSCs in animal models. Nevertheless MSCs were often implanted in association with other cell types thus it is very important to develop a double imaging technique to follow the fate of implanted cells.

In the present work, we investigated the homing of MSCs by bi-modal imaging techniques in an animal model of PIs transplantation The fluorescence signal emitted by different excised organs 5 or 10 days after injection of labeled MSCs, is at the baseline level (i.e., approximatively equal to the fluorescence emitted in the animal receiving unlabeled MSCs) (Fig. 2).

Fig. 2 (A, B) shows that the fluorescence signal emitted by different excised organs 5 or 10 days after injection of labeled MSCs, is at the baseline level (i.e., approximatively equal to the fluorescence emitted in the animal receiving unlabeled MSCs). It has been reported that 10 min after stem cells i.v. injection in mice, stem cells localize preferentially into lungs [28]. However, the same authors reported that 24 h after stem cells i.v. injection, the fluorescence of the lung is strongly decreased. We accordingly found that the signal of the lung is at the baseline level 5 days

after i.v. injection of MSCs labeled with Qdots. Fluorescence emission in the liver and kidneys in group 4 is significantly higher than in the remaining groups, while this difference is not significant in other organs. These results and the good linear correlation observed *in vitro* between the number of labeled MSCs and fluorescence signal (see Fig. 1), demonstrate that when MSCs are i.v. injected after transplantation of PIs they show preferential homing into the liver. Moreover in excised organs 10 days after PIs transplantation and MSCs i.v. injection the signal is decreased compared to 5 days. In the experimental group 4, 5 and 10 days after PIs transplantation and MSCs i.v. injection, significant fluorescence signal was observed in the kidneys, probably indicating urinary elimination of QDots [32]. Yukawa and co-workers [28] have recently studied the homing of adipose-tissue derived stem cells (ASCs) in an experimental model of acute liver failure by using QDots labeling and fluorescence imaging in mice. They found an increase of the fluorescence signal in the kidneys 2 days vs. 10 min after transplantation [28].

The high presence of MSCs in liver observed when animals are transplanted with PIs can be correlated with inflammation and eventually graft rejection of allogeneic islets. Indeed it is well known that during an inflammation process (or a tissue damage) MSCs are recruited by means of chemokines into the site of inflammation to suppress immune response and promote regeneration (33). The immunomodulatory effect of the MSCs. is demonstrated in many *in vitro* studies which found that MSCs suppress T-Cell proliferation indicating an immunosuppressive properties and reduced inflammation [34, 35].

Several papers have investigated the immunomodulatory effect of MSCs in the transplantation of PIs but at the best of our knowledge, the homing of MSCs has not been investigated in this model. Over the last 10 years, a number of studies have investigated *in vivo* the homing of stem cells in a wide variety of other experimental diseases [6, 36, 37, 38]. Most papers were based on MRI, but recently attention was devoted to optical techniques in bioluminescence or fluorescence modality [28]. Although many attempts were done to reveal MSC fate for several surgery approach within

PIs transplants, this topic needs to be further analyzed. In conclusion, we here transplanted PIs and injected MSCs in one single step in a single recipient by a double imaging approach that could allow for differential monitoring of cellular homing. We used a bi-modal acquisition system: fluorescence detection by OI to explore the homing of MSCs, and MRI to investigate the fate of PIs. A limit of this approach is that OI suffers from relatively small penetration depth in living tissues that prevented us to work *in vivo* in rats. This paper represents the proof of concept that the joint application between MRI and OI can allow simultaneous monitoring of transplanted cells fate.

5. Conclusions

We herein investigated the homing of MSCs labeled with QDots and i.v. injected in an animal model of PIs transplantation [4]. When unlabeled MSCs were injected, the fluorescence signal of the liver was not different from the controls (where either saline or unlabeled MSCs were injected). When MSCs were injected immediately after PIs transplantation, the fluorescence signal of the liver was significantly higher than the control showing that MSCs preferentially accumulate into the liver. This paper demonstrate that the bimodal MRI and fluorescence imaging allows differential monitoring of the fate of two types of transplanted cells. Moreover it shows that i.v. injected MSCs accumulate into liver when PIs have been transplanted in the same organ.

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Caption to Figures

Figure 1. Differentiation of MSCs. (A) Osteocytes, with visible deposits of hydroxyapatite intensely stained red by means of Alizarin S, (B) orange–red stained lipid vacuoles of the cytoplasm of MSCs treated with adipogenic medium with Oil-Red O. (C) Fluorescence images of non-fluorescent wells containing varying amounts of MSCs labeled with Qdots 800: a) Control, no cells, b) 50 cells, c) 100 cells, d) 500 cells, e) 1000 cells, f) 2.5 x 10^3 cells, g) 5 x 10^3 cells, h) 10 x 10^3 cells, i) 20 x 10^3 cells, l) 40 x 10^3 cells, m) 80 x 10^3 cells, n) 100 x 10^3. (D): Signal intensity of fluorescence images of non-fluorescent wells containing varying amounts of Qdots-labeled (blue squares) or unlabeled (red squares) cells. Acquisition modality: fluorescence with filters ex/em= Cy5.5 (615-665 nm)/ ICG (810-875 nm).

Fig. 2. Fluorescence images of excised organs for representative rats injected with MSCs and sacrificed 5 (A) and 10 (B) days after PIs transplantation. A: group 2 rat inoculated i.v. with unlabeled-MSCs, 5 days after injection; group 3 rat inoculated i.v. with MSCs labeled, 5 days after injection; group 4 rats inoculated i.v. with MSCs labeled and PIs, 5 days injection. B: group 2 rat inoculated i.v. with unlabeled-MSCs, 10 days after injection; group 3 rat inoculated i.v. with MSCs labeled, 10 days after injection; group 4 rats inoculated i.v. with MSCs labeled and PIs, 10 days injection, Acquisition modality: fluorescence with filters ex/em= Cy5.5 (615-665 nm)/ ICG (810-875 nm). Organs are showed here with different magnification with respect to the real size. C) Average signal emitted in different organs (RFI). Data are reported as mean ± SD over the different experimental groups. D) *Ex vivo* Magnetic Resonance Imaging of liver excised from a rat to which pancreatic islets labeled with SPIOs were transplanted.