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Persistence of fluorescent nanoparticle-labeled bone marrow mesenchymal stem cells *in vitro* and after intra-articular injection

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Abstract

Mesenchymal stem cells (MSCs) improve the osteoarthritis condition, but the fate of MSCs after intra-articular injection is unclear. We used fluorescent nanoparticles (quantum dots; QDs) to track equine MSCs (QD-MSCs) *in vivo* after intra-articular injection to normal and osteoarthritic joints. One week after injection of QD-MSCs, unlabeled MSCs, or vehicle we determined the presence of QD-MSCs in synovium and articular cartilage histologically. *In vitro*, we evaluated the persistence of QDs in MSCs and whether QDs affected proliferation, immunophenotype or differentiation. In joints injected with QD-MSCs, labeled cells were identified on the synovial membrane, and significantly less often on articular cartilage, without differences between normal and osteoarthritic joints. Joints injected with QD-MSCs and MSCs had increased synovial total nucleated cell count and protein compared to vehicle-injected joints. *In vitro* QDs persisted in non-proliferating cells for up to 8 weeks (length of the study) but fluorescence was essentially absent from proliferating cells within 2 passages (approximately 3 to 5 days). QD-labeling did not affect MSC differentiation into chondrocytes, adipocytes and osteocytes. QD-MSCs had slightly different immunophenotype from control cells, but whether this was due to an effect of the QDs or to drift during culture is unknown. QD-MSCs can be visualized in histological sections one week after intra-articular injection, and are more frequently found in the synovial membrane versus cartilage in both normal and osteoarthritic joints. QDs do not alter MSC viability and differentiation potential *in vitro*. However, QDs are not optimal markers for long-term tracking of MSCs, especially under proliferative conditions.

Keywords

bone marrow; mesenchymal stem cell; quantum dot; cell tracking; intra-articular injection; osteoarthritis

1. Introduction

Mesenchymal stem cells (MSCs) are commonly used clinically for the treatment of musculoskeletal disease in horses (Taylor *et al.*, 2007) and in people (Wakitani *et al.*, 2002). The horse joint is more similar to the human joint in terms of size, volume of synovial fluid, and cartilage thickness (Frisbie *et al.*, 2006) than are joints in more traditional laboratory species such as mice, thus the horse serves as a good model for humans for the study of osteoarthritis. The fact that MSC therapies have been utilized in the horse for the treatment of naturally-occurring injury for over a decade (Broeckx *et al.*, 2014; Ferris *et al.*, 2014; Smith *et al.*, 2013; Taylor *et al.*, 2007) strengthens the utility of the equine model for investigation of regenerative medicine approaches for treatment of OA.

The mechanism by which injected MSCs contribute to tissue healing in the articular environment is not clear. To determine if injected MSCs participate in tissue repair, attempts have been made to label MSCs so they may be tracked after injection. Methods for *in vivo* cell tracking in large animal models include labelling MSCs with compounds that can be imaged using nuclear scintigraphy or magnetic resonance imaging, such as technetium-99 (Dudhia *et al.*, 2015; Spriet *et al.*, 2015) and superparamagnetic iron oxide particles (Burk *et al.*, 2016; Geburek *et al.*, 2016), respectively. In a canine model, MSCs used for intra-articular injection were labeled with iron oxide or with a fluorescent dye and *in vivo* detection of the labeled MSCs was performed using MRI and fluorescent imaging (Wood *et al.*, 2012). The labeled MSCs were shown to remain near the site of the injection for four weeks (the duration of the study). However, locating the label *in vivo* with these modalities demonstrates labeled cell presence within the articular environment, but does not provide information on the contribution of the labeled cells to tissue repair, specifically to articular cartilage.

For post-mortem or histological analysis, intra-articular injection of green fluorescent protein (GFP)-labeled MSCs in mice following cartilage injury allowed detection of GFP expression at the injury site two weeks, but not four weeks, after injection (Mak *et al.*, 2016). In the above-mentioned study in dogs, in which MSCs were labeled with a cellular dye, joint surfaces were directly imaged after euthanasia, and fluorescence indicating persistence of injected cells was detected up to four weeks after injection (Wood *et al.*, 2012).

Another product frequently used for stem cell labeling is quantum dots (QDs) (Wang *et al.*, 2013). Quantum dots are fluorescent nanoparticles which are resistant to metabolic degradation (Collins *et al.*, 2012), have minimal cytotoxic effects and have been shown not to interfere with proliferation of rat bone marrow MSCs in culture (Muller-Borer *et al.*, 2007). Visualization of QDs in labeled rat MSCs is possible in cardiomyocyte co-cultures for up to seven days (Muller-Borer *et al.*, 2007), and QDs have been demonstrated to be present in canine hearts eight weeks following delivery of QD-labeled human MSCs (Rosen *et al.*, 2007). However, to the best of our knowledge, there is no information available on the behavior of QDs after injection of labeled equine MSCs *in vivo*, or in dividing or differentiated equine MSCs *in vitro*.

The initial objective of this work was to (1) Determine if QD-labeled autologous MSCs could be identified one week after intra-articular injection into osteoarthritic and normal joints; (2) Determine their location and whether there were differences in MSC engraftment location due to disease status; (3) Assess their effect on synovial fluid cytology. To explore factors that might be associated with the low persistence of QDs observed *in vivo*, we then performed studies *in vitro* to determine the persistence of QD label in dividing and non-dividing (subjected to chondrogenic differentiation) equine MSCs *in vitro*, and to determine if QD labeling affected the rate of cellular proliferation, the immunophenotype of the cultured cells, or the ability of the MSCs to undergo trilineage differentiation.

2. Materials and Methods

2.1 Experiment 1: Identification of MSCs after intra-articular injection

2.1.1 Experimental design—This study was approved by Cornell University's Animal Care and Use Committee. Bone marrow was harvested from 16 skeletally-mature horses that had been retired from athletic performance and donated for lameness due to osteoarthritis of a metacarpophalangeal and/or femoropatellar joint. Recovered MSCs were isolated and expanded in culture.

The 16 horses were divided into two groups: MSC injection (n = 10) or vehicle (n = 6). The MSCs injected were autologous to the horse receiving them. The metacarpophalangeal (MC) and femoropatellar (F) joints of the horses were characterized as normal or osteoarthritic (OA) based on clinical and radiographic examinations. Joints were injected with either QD-labeled MSCs (QD-MSC) or unlabeled MSCs (uMSC). For the QD-MSC injection group, 11 normal joints (4 MC and 7 F) and 11 OA joints (7 MC and 4 F) were injected. For the uMSC injection group, 6 normal joints (3 MC and 3 F) and 1 OA F joint were injected. One week after injection the horses were euthanized and the joints were evaluated for synovial fluid cytology, characterization of QD-MSC distribution within the joint via fluorescent microscopy, and characterization of articular cartilage injury via routine microscopy. For the vehicle group, 12 joints (6 OA and 6 normal) were injected with a similar volume of cell-free medium. One week after injection, synovial fluid was aspirated from joints of live horses in this group, and synovial fluid cytology was assessed.

2.1.2 Stem cell isolation and labeling—Bone marrow aspirates were obtained from the sternum of each horse under local anesthesia and light sedation. Bone marrow biopsy needles (Jamshidi, VWR Scientific, Bridgeport, NJ) were used to aspirate bone marrow. Marrow was collected into four 60-mL syringes each containing 10 mL of 1,000 IU/mL heparin (APP Pharmaceuticals, LLC; Schaumburg, IL). Each 50 mL of bone marrow was collected from a separate site. At each site, the Jamshidi needle was advanced after each 15 mL of marrow had been drawn. Each 60-mL of heparinized bone marrow aspirate was mixed with 60 mL of ammonium chloride solution (7.7 mg/mL NH₄Cl buffered with 206 mg/mL TRIS (hydroxymethane-aminomethane), pH 7.2), to induce lysis of red blood cells, then centrifuged at 300 x g for 5 min. The supernatant was discarded and the remaining volume, containing nucleated marrow cells, was suspended in 240 mL of standard MSC culture medium (Dulbecco's modified Eagles' media; 1,000 mg/L glucose; 2 mM L-glutamine; 100

units/mL penicillin-streptomycin; 1 ng/mL basic fibroblast growth factor (Sigma-Aldrich); and 10% stem cell-tested fetal calf serum) and this suspension was plated to T-175 tissue culture flasks (60 mL per flask). Once colony formation was evident on microscopic evaluation, adherent cells were passaged using trypsin and replated at 20,000 cells/cm². Medium was replaced every other day. When cell growth was 80–90% confluent, cultures were passaged a second time. When Passage 2 cultures were 80–90% confluent, adherent MSCs were either labeled with QDs or left unlabeled and prepared for joint injection.

Cell labeling with red (625 nm) or green (525 nm) fluorescent QDs was performed per manufacturer instructions. Briefly, 1 µL each of the supplied reagents (A and B; Qtracker kit®, ThermoFisher Scientific; Invitrogen; Carlsbad, CA) were mixed in a 50-mL conical tube and incubated at room temperature for 5 min. This mixture was vortexed for 30 sec then diluted 1:100 with cell culture medium. The medium was aspirated from MSC culture flasks containing approximately 7.5×10^6 adherent MSCs, and 3 mL of the labeling solution were added. The culture flasks with the labeling solution were incubated for 60 min at 37 °C. After this, the labeling solution was aspirated and labeled adherent cells were washed twice with culture medium and then prepared for injection.

2.1.3 Joint injection—For injection, the cells were collected from the flasks by trypsinization, washed once in phosphate buffered saline and washed twice in Modified Eagles' Medium (MEM). Cells to be injected into the MC and F joints were prepared as 3×10^6 and 5×10^6 MSC aliquots, respectively. Cells were diluted to 1×10^6 cells/mL in MEM containing 40 µg/mL gentamicin immediately prior to injection so that MC joints received a total injection volume of 3 ml and F joints received a total of 5 ml. The gentamicin concentration used in the final MEM diluent is similar to the typical working concentration of gentamicin (25 to 50 µg/ml) used when supplementing eukaryotic cells in culture. When horses were injected bilaterally with QD-MSC, different wavelength QDs were used in each joint (e.g. right metacarpophalangeal joint received 625 nm QDs (red) and left metacarpophalangeal received 525 nm QDs (green)). Horses in the vehicle group did not receive MSCs; MC and F joints in these horses were injected with a similar volume of cell-free MEM/gentamicin.

Injections were performed under sedation. Injection to the MC joint was made through the lateral collateral sesamoidean ligament and those to the F joint between the middle and medial patellar ligaments. Following injection, the limb was taken through passive range of motion for 20 joint flexions and extensions to distribute injectate. Horses were administered phenylbutazone at the time of injection (4.4 mg/kg, IV) and this was repeated once daily for two days (2.2 mg/kg, PO) after joint injection. Metacarpophalangeal joints were maintained under a clean bandage for 3 days after joint injection. Horses were housed individually in box stalls without forced exercise until one week after joint injection, at which time euthanasia was performed using an overdose of pentobarbital. Vehicle-injected horses were not euthanized and had synovial fluid collected from the injected joints one week after injection.

2.1.4 Joint tissue processing and analysis—In horses receiving MSCs, synovial fluid was collected from injected joints after euthanasia. The fluid was analyzed for total

nucleated cell count, differential cell count, protein quantification (by refractometry), and presence of QDs. The differential cell count was performed by a Board-certified Veterinary Pathologist on smears of the fluid. Presence of QD-labeled cells was determined on examination of synovial fluid cytopins under fluorescent microscopy using filter sets for visualization of 535 nm or 625 nm QDs.

After the joints were opened, gross articular cartilage lesions were mapped and the articular surfaces were photographed. Specimens of articular cartilage and synovial membrane from different regions of the joint (see below) were collected and prepared for frozen sectioning by embedding in OCT medium. For MC joints, articular cartilage specimens were obtained from the sagittal ridge of the distal metacarpus, dorsomedial proximal phalanx, palmaromedial distal metacarpal condyle, and medial sesamoid; and synovial membrane specimens were obtained from the palmarolateral, palmaromedial, dorsomedial, and dorsolateral aspects of the joint. For the F joints, articular cartilage specimens were obtained from the lateral trochlear ridge, medial trochlear ridge, and distal patella; and synovial membrane specimens were obtained from the lateral cul-de-sac, medial cul-de-sac, and proximal pouch. For MC joints, osteochondral specimens were collected from the palmaromedial distal metacarpal condyle and the dorsomedial proximal phalanx. For the F joints, osteochondral specimens were collected from the lateral and medial trochlear ridges. Osteochondral specimens were fixed in 4% paraformaldehyde, decalcified in 10% EDTA, and embedded in paraffin. Presence (y/n) of a gross lesion on collected cartilage and osteochondral specimens was recorded at the time of collection. One histological section was mounted from each SM biopsy specimen. Two to four histological sections were mounted per cartilage specimen. Frozen sections were stained with a nuclear stain (propidium iodide for 525 nm QDs or Hoechst for 625 nm QDs) and examined for the presence of QD-labeled cells using both a 525- and a 625-nm filter set. Tissue specimens were considered positive for QD-labeling if any of the mounted sections from the specimen were positive. Osteochondral sections were stained with hematoxylin and eosin, then examined and photographed (50 X magnification).

2.1.5 Statistical analysis—Statistical analysis was performed using Statistix 9. Cytology parameters were reported as a median with a 95% confidence interval, and were tested for differences with Wilcoxon's Rank Sum test. The number of specimens positive for QD-labeled MSCs was reported as a percentage of all specimens evaluated. Differences in the proportion of tissue specimens that were positive for QD-labeled MSCs were evaluated using Fisher's Exact test. For all tests a $P \leq 0.05$ was considered significant. Synovial fluid cytology was compared between QD-MSC and uMSC-injected joints, between MSC-injected and Vehicle-injected joints, and between MSC-injected OA joints and MSC-injected normal joints. The location of QD-MSCs (synovial membrane vs cartilage sections) was visually compared using fluorescence microscopy between joint type (MC vs F) and between normal and OA joints.

2.2 Experiment 2: Persistence of QDs in dividing and non-dividing MSCs

2.2.1 Experimental design—All procedures were approved by the Institutional Animal Care and Use Committee of Texas A&M University. Bone marrow aspirates were obtained

from three horses and MSCs were propagated and frozen, then thawed and plated. Half of the cells were labeled with QDs. Labeled and unlabeled (control) cells were then placed in monolayer culture (dividing cells) or were induced to differentiate into chondrogenic pellets (non-dividing). The persistence of QDs was determined by fluorescent imaging daily in the dividing cells, and once every two weeks in the chondrogenic pellets; separate cultures were used for each assessment point. To determine whether labeling with QDs affected the differentiation ability or immunophenotype of the MSCs, QD-labeled cells were induced to differentiate into adipocytes, chondrocytes, and osteocytes, and were immunophenotyped by flow cytometry. Three replicates (one replicate with MSCs from each of the three different MSC donors) were performed for each endpoint.

2.2.2 Stem cell isolation and labeling—Bone marrow was harvested aseptically from the sternum of three horses as described above, with the exception that only two 60-mL syringes with 10 mL of heparin were collected and that following red blood cell lysis, the cells were suspended in 120 mL of MSC culture medium containing 1% antibiotic-antimycotic (10,000 U penicillin, 10 mg streptomycin, 25 µg amphotericin B/mL; GIBCO; Invitrogen) in place of the penicillin-streptomycin mixture. Cells from P2 were trypsinized, resuspended in Dulbecco's phosphate-buffered saline (DPBS; BioWhittaker; Lonza, Walkersville, MD), then centrifuged again and the pellet resuspended in cryopreservation medium containing 95% autologous serum and 5% dimethylsulfoxide at a concentration of 10×10^6 cells/mL. Cryovials containing this suspension were frozen by placing them in a Cryo 1 °C Freezing Container (Thermo Scientific; VWR, Radnor, PA) for 24 hr at -80 °C before transfer to liquid nitrogen where they were stored until used.

For use, frozen MSCs were thawed and re-plated in MSC culture medium at a density of 5 to 8×10^3 cells/cm² and cultured at 37 °C. When the cells reached 70% confluence, the cells were trypsinized and divided into two groups, QD and control. Cells in the QD treatment were labeled as described above, with red (625 nm) QDs, with the modification that cells were labeled in suspension after trypsinization. Briefly, 2 µL of the QD-labeling solution were added per 0.2 mL of medium containing 5×10^6 cell/mL in suspension. The cells were incubated at 37 °C for 60 min, after which they were washed twice with serum-free MSC culture medium. One hour after labeling, cells in the QD treatment were imaged using a fluorescent inverted microscope (excitation 405–585 nm) to ensure uptake of QDs, as evidenced by the presence of red fluorescent particles in the cytoplasm of the cells. The cells in both QD-labeled and control groups were placed in either standard monolayer culture conditions (dividing cells) or were induced to differentiate into chondrogenic pellets (non-dividing).

2.2.3 Monolayer culture—QD-labeled and control MSCs were plated in cell culture flasks in standard MSC culture medium and cultured at 37 °C in a humidified atmosphere of 5% CO₂ in air. The cells within the culture flasks were evaluated every other day until QDs were no longer detected. Multiple flasks were prepared and each flask was imaged only once, to eliminate the effect of photobleaching. The medium was replaced every other day and the flasks were passaged at 70–80% confluence throughout the study. At every passage, the cell concentration was determined using a hemocytometer and a sample of MSCs was

cryopreserved in 95% autologous serum and 5% DMSO at a concentration of 10×10^6 cells/mL. Cryopreserved cells were later thawed and evaluated by flow cytometry to determine changes in mean fluorescence intensity (MFI; measures the mean fluorescence intensity of a population of cells) as well as the proportion of viable, labeled cells over time. Viability was assessed by adding 5 μ L of 7-aminoactinomycin (7-AAD; Biolegend, San Diego, CA) to the cell suspensions. This compound has a strong affinity for DNA but does not readily penetrate intact cell membranes; therefore, it can be used to determine cell viability as only cells with compromised membranes will stain with 7-AAD.

The proportion of cells showing QD-associated fluorescence was detected using a Beckman Coulter MoFlo Astrios high-speed cell sorter (Beckman Coulter, Brea, CA). The 405-nm laser and the 620/29 bandpass filter were used and the PMT voltage was set to 390 volts. Dead cells labeled with 7-AAD were detected using the 488-nm laser and the 664/22 bandpass filter; the PMT voltage was set to 575 volts. A minimum of 20,000 live cell events were collected per sample.

2.2.4 Chondrogenic differentiation—QD-labeled MSCs and control MSCs were induced to differentiate into chondrogenic pellets as previously described (Mitchell *et al.*, 2015). Briefly, 2.5×10^5 cells were suspended in 1 mL of chondrogenesis-induction medium (DMEM with 4.5 g/L glucose, supplemented with 1% FBS, 2.5% HEPES buffer, 1% antibiotic-antimycotic, 10 ng/mL transforming growth factor beta (TGF- β 3; Life Technologies), 0.6 μ g/mL dexamethasone (Sigma Aldrich), 50 μ g/mL L-ascorbic acid, 40 μ g/mL proline (Sigma Aldrich), and 1% ITS premix (VWR)) in 15-mL conical tubes and centrifuged at $300 \times g$ for 10 min. After centrifugation, the supernatant was aspirated and replaced with 1 mL of fresh chondrogenic media. The centrifuged preparations (pellets) were maintained in static culture at 37 °C for 21 days with medium changes every other day. On day 21, for evaluation of chondrogenic differentiation, the pellets were removed from the culture tube and fixed with 4% paraformaldehyde (PFA; Sigma Aldrich) at room temperature for 10 min, after which they were embedded in paraffin and histological sections were prepared. Sections were stained with Toluidine Blue to identify cartilaginous extracellular matrix, which stains purple; and fibrous tissue, which stains blue. Photographs of the sections were taken with a phase-contrast microscope immediately after staining.

For detection of QDs, 12 additional pellets were prepared and three pellets were removed from culture every two weeks for eight weeks for evaluation. The pellets were fixed and histological sections were prepared as described above. These sections were stained with the nuclear dye 4',6-diamidino-2-phenylindole (DAPI) and imaged with a fluorescent microscope (Ex/Em 405–585/625 nm) to determine the presence of QDs.

2.2.5 Adipogenic differentiation—Adipogenic differentiation was induced as previously described (Mitchell *et al.*, 2015). Briefly, QD-labeled and control MSCs were seeded onto 10-cm plates at a density of 1000 cells/cm² in MSC culture medium. Once the cells reached 70% confluence, the medium was exchanged for adipogenic induction medium consisting of DMEM/F12 supplemented with 3% FBS, 1% antibiotic-antimycotic, 5% rabbit serum (Life Technologies), 33 μ M biotin (Sigma-Aldrich), 17 μ M/L pantothenate (Sigma-Aldrich), 1 μ M/L insulin (Sigma-Aldrich), 1 μ M/L dexamethasone, 225 μ L

isobutylmethylxanthine (Sigma-Aldrich) and 89 μ L rosiglitazone and cultured for 72 hr. After 72 hr the medium was exchanged for adipogenic maintenance media (adipogenic induction medium without isobutylmethylxanthine and rosiglitazone) for an additional 72 hr. Cells in induced plates were stained with Oil Red O (Sigma-Aldrich) to detect lipid droplets within the cells, which stain red.

2.2.6 Osteogenic differentiation—Osteogenic differentiation was induced as previously described (Mitchell *et al.*, 2015). Briefly, QD-labeled and control MSCs were seeded onto 10-cm plates at a density of 1000 cells/cm² in standard MSC culture medium. Once the cells reached 70% confluence, the medium was exchanged for osteogenic induction medium consisting of DMEM/F12 supplemented with 10% FBS, 1% antibiotic-antimycotic, 10 μ M/L β -glycerophosphate (Sigma-Aldrich), 20 nM/L dexamethasone, and 50 μ g/mL L-ascorbic acid. The plates were maintained in culture for 21 days. After 21 days, the plates were stained with 2% Alizarin Red (Sigma-Aldrich) to identify the presence of calcified extracellular matrix and bone nodules, which stain red. Photographs of cells in each plate were taken under a phase-contrast microscope within 2 hours of staining.

2.2.7 Immunophenotype analysis by flow cytometry—Control and QD-MSCs at P3 were immunophenotyped by assessing immunoreaction using mouse anti-horse antibodies to MHCII (Bio-Rad, Raleigh, NC), CD44 (Bio-Rad), CD29 (Beckman Coulter, Brea, CA), CD90 (VMRD Inc., Pullman, WA), and CD45RB (VMRD Inc.) as previously described (Mitchell *et al.*, 2015). The antibodies against MHC II, CD44 and CD29 were fluorescently labeled with fluorescein isothiocyanate (FITC). The antibodies against CD90 and CD45 were unlabeled and a secondary goat anti-mouse IgG antibody, labeled with phycoerythrin (PE), was used for visualization.

All labeled cell suspensions had 7-AAD added immediately prior to analysis for assessment of viability. A sample of MSCs without antibody, and MSCs with only with secondary antibody were also analyzed by flow cytometry to determine negative control staining.

Acquisition of the cell surface marker data was performed using a Becton Dickinson FACS Caliber flow cytometer using Cell Quest Version 3.3 (BD San Jose, CA). FITC, PE and 7-AAD were detected using a 488 nm laser with a 515/30 bandpass filter, 585/42 bandpass filter, and 650 Long Pass filter, respectively. At least 10,000 live events were collected. Data analysis was performed using FlowJo 9.8 Mac version (TreeStar Corp., Ashland, OR).

2.2.8 Statistical analysis—Data were analyzed using JMP Pro ver. 11 for Windows (SAS Institute Inc, Cary, NC). Data were log-transformed prior to analyses to minimize interactions between means and variances. Paired t-tests were performed to determine differences in MFI between P1 and P2 and between P2 and P3. Differences were considered significant when $P < 0.05$.

3. Results

3.1 Experiment 1: MSC distribution in tissue after intra-articular injection

A total of 22 joints (11 normal and 11 OA (2 mild, 4 moderate, and 5 severe)) were injected with QD-MSCs and 7 joints (6 normal and 1 severe OA) were injected with uMSCs. Twelve joints were injected with vehicle only (6 normal and 6 OA (3 mild, 2 moderate and 1 severe)). There were no significant differences ($P > 0.1$) in synovial cytology parameters between joints injected with QD-MSCs and joints injected with uMSCs. There was a significant increase in total nucleated cell count and total protein in joints injected with MSCs compared to vehicle-injected joints (Table 1). Cytospins of synovial fluid collected from injected joints were positive for fluorescently-labeled cells from all 22 QD-MSC-injected joints, and none were positive from the seven unlabeled MSC-injected joints or from the 12 vehicle-injected joints. In the 6 cases in which two joints from the same horse were injected with QD-MSCs, no QDs from the contralateral joint (different color fluorescence QD) were identified from any joint.

Tissue specimens from uMSC-injected joints were negative for QD-MSCs. In QD-MSC-injected joints, synovial membrane specimens were positive for QDs significantly more often (54/77, 70%) than were cartilage sections (17/77, 22%; $P < 0.0001$). Furthermore, in QD positive sections, there appeared to be a greater number of QD-labeled cells per field on synovial membrane sections than on cartilage sections (Figure 1). When comparing QD-MSC-injected joints by joint type, there were no differences in the proportion of QD-positive sections between MC joints and F joints for synovial membrane sections ($p > 0.05$) or cartilage sections ($p > 0.05$). There was no difference in the proportion of QD-positive sections between OA and normal joints, for either synovium or cartilage ($P > 0.5$; Figure 2).

3.2 Experiment 2: *In vitro* assessment of QD-labeled MSCs

3.2.1 Persistence of QDs in proliferating and non-proliferating MSCs—In dividing MSC cultures, flow-cytometric analysis showed a rapid decrease in the percentage of QD-labeled cells, as well as in the MFI, as passage number increased (Table 2). The initial MFI at P1 varied among trials, between 154 and 1796; however the percentage of cells labeled with QDs at P1 was consistently high (between 98.1 and 99.9%). Both the proportion of labeled cells and the MFI dropped precipitously between P1 and P2, and again between P2 and P3. The proportion of labeled cells and the MFI then stayed at fairly consistent low levels for 2 to 5 more passages. For P2 and P3, the MFI measured by flow cytometry was less than the MFI expected due to dilution associated with cell division, calculated based on the MFI of the previous passage, the number of cells seeded and the number of cells present at confluence (Table 3).

Visual assessment of cell cultures under fluorescent microscopy supported the flow-cytometric results; the proportion of cells labeled with QDs as well as the number of QDs per cell decreased from P1, when an estimated 100% of the cells were labeled with QDs to P2, when an estimated 50% of cells were labeled, and continued to decrease rapidly thereafter (Figure 3).

In contrast to the rapid decrease in fluorescence seen in dividing MSC cultures, non-dividing MSCs (chondrogenic pellets) remained labeled with QDs, without an apparent decrease in intensity, for the duration of the study (up to 8 weeks; Figure 4). However, the fluorescence was not present in cells on the periphery of the pellets.

3.2.2 Effect of QD-labeling on cell proliferation and trilineage differentiation

—The time between passages and the number of cells present at each passage was similar for QD-MSCs and control cells (Table 4), indicating that QD labeling did not cause increased cell death or slow cell division.

Labeling MSCs with QDs did not affect the cells' ability to undergo trilineage differentiation into adipogenic, osteogenic and chondrogenic lineages as assessed by visualizing positive staining for Oil Red O, Alizarin Red, and Toluidine Blue, respectively (Figure 5). There were no qualitative differences observed between adipogenic, osteogenic and chondrogenic preparations for control vs QD- MSCs.

3.2.3 Effect of QD labeling on immunophenotype—On immunophenotyping, both QD-MSCs and unlabeled MSCs maintained a single population for all markers (Figure 6). The expression of CD29, CD44, and CD90 varied among control (unlabeled) MSCs, whereas CD45 was low. (Table 5). QD-MSCs differed in that the percentage of cells expressing CD29 was high from all three MSC donors. The differences between control and QD-labeled MSCs of the same MSC donor were not repeatable among MSC donors (e.g. for donor A, CD44 was expressed in 86% of control cells vs 23% of cells with QDs; whereas for donor B, CD44 was expressed in 2% of control cells vs. 30% of cells with QDs). All MSCs in both control and QD treatments showed low expression of MHC II.

4. Discussion

Interest in using MSCs in regenerative therapies continues to grow; however, little is known about the fate and distribution of the cells after delivery, hindering understanding of their mechanism of action. This study examined for the first time the use of QDs to label and track equine MSCs after injection. Labeling of cells with QDs did not change the inflammatory response to the cells after injection, as there were no differences in differential synovial fluid cell counts between joints injected with QD-MSCs and joints injected with uMSCs. It was possible to detect QD-labeled cells in the joints one week after injection. In contrast to what we expected, MSCs did not engraft on articular cartilage, but rather populated the synovial membrane of both OA and normal joints after intra-articular injection.

The low persistence of QD-labeled cells in cartilage in Experiment 1 raised questions regarding the persistence of the label in cells over time, and the effects of the label on cell viability and differentiation potential. To address this, we evaluated the persistence of QD label in proliferating and non-proliferating (subjected to chondrogenic differentiation) equine MSCs *in vitro* and its effect on cell proliferation and differentiation.

We found that in monolayer culture conditions, QD-labeled MSCs continued to divide normally. However, they did not retain sufficient QD label to provide an effective method for cell tracking after the first two passages (approximately 3 to 5 days of *in vitro* culture) and the QDs were essentially not detectable after 8 days of culture. Similar results have been found with rat and human QD-labeled MSCs, with loss of labeling after 1 to 7 days of culture (Muller-Borer *et al.*, 2007; Seleverstov *et al.*, 2006; Z.-G. Wang *et al.*, 2016). The rapid loss of QD label that we observed appears not to be directly related to cell proliferation, as loss was much higher than that calculated for simple dilution due to cell division. The striking loss of fluorescence in the first passage could be due to death of QD-labeled cells or to ejection of QDs by the cells during cellular division; further research is needed in this area.

In contrast to the loss of QD label seen in cells in monolayer culture, non-proliferating MSCs, i.e. MSCs from the central 2/3s of chondrogenic pellets, retained QDs without an apparent decrease in label for up to 8 weeks (the length of the experiment). These results are similar to those of Ohyabu *et al.* (Ohyabu *et al.*, 2009), who reported that chondrocytes, adipocytes and osteocytes (which also do not proliferate rapidly in culture) differentiated from human, rabbit, rat and monkey MSCs retain strong QD label for 28 days *in vitro* and that chondrocytes differentiated from rat and rabbit MSCs retain QD label for eight weeks after *in vivo* transplantation into experimentally-induced osteogenic defects. This suggests that QDs would be an effective method to track differentiated non-proliferating MSCs injected into lesions. It is also possible that QD label would allow for tracking of MSCs which, if they were retained, would differentiate into non-dividing tissue types such as cartilage, adipose tissue or bone, or cells with limited capacity for division, such as cardiac muscle. The loss of QD label in the periphery of the chondrogenic pellets *in vitro* is a complicating factor. In our experience, chondrogenic pellets will commonly form a fibrotic layer that does not stain for cartilage matrix as the central 2/3s of the pellets does, which we believe is due to cellular proliferation on the outer surface of the pellet (Figure 5). As for cells in monolayer culture, loss of QDs from the periphery of the pellet could be due to proliferation of cells on the pellet periphery while forming this new layer and ejection of QDs during proliferation; if this occurred in injected MSCs before their differentiation into non-proliferating cells within a lesion, the label would be lost.

Another requirement for use of QDs to label and track MSCs is that they must not affect the cells' characteristics. In support of this, we found that the ability of equine MSCs to differentiate into chondrocytes, adipocytes, and osteocytes was not affected by labeling with QDs. These results are similar to those reported previously for human, rabbit, rat, and monkey MSCs (Ohyabu *et al.*, 2009; Ranjbarvaziri *et al.*, 2011; Tautzenberger *et al.*, 2010).

The immunophenotypic results in the present study were unexpected. According to the panel of immunophenotypic markers proposed by De Schauwer *et al.* (De Schauwer *et al.*, 2012) and Schnabel *et al.* (Schnabel *et al.*, 2014) for equine MSCs, these cells tend to be positive for CD29, CD44 and CD90, negative for CD45, and heterogeneous (positive or negative) for MHC II. In the present study, all the cells used had the expected low expression of CD45 and, interestingly, of MHC II; however, the MSCs varied in expression of CD29, CD44 and CD90. Notably, MSCs cultured with QDs showed different immunophenotypes from

unlabeled-MSCs of the same donor, and the effect of QDs was not consistent among donors. Whether this was due to an effect of QDs or to drift of MSC immunophenotype over the passages in separate cultures is not clear, and further studies are needed in this area. While molecular markers continue to be used to characterize stem cell populations, recent studies with human MSCs indicate that cells isolated from different donors (Szepesi *et al.*, 2016), or from different sites from the same donor (Hatakeyama *et al.*, 2017; Sacchetti *et al.*, 2016), and cells treated with different enzymatic digestion methods for detachment (Tsuji *et al.*, 2017) can have different surface antigen expression, and even MSCs with identical cell surface phenotype from different tissues and donors have distinct transcriptomic signatures and differentiation capacities, likely due to the broad overlap of cells with MSC markers with other cell populations (Assoni *et al.*, 2016; Sacchetti *et al.*, 2016). Thus, the immunophenotypic characterization of MSCs remains unclear and MSCs are currently best defined functionally.

We used a relatively low dose of MSCs for intra-articular injection, which was elected to minimize inflammatory reaction in injected joints (Carrade *et al.*, 2011; Pigott *et al.*, 2013). It is possible that higher cell doses may be associated with different MSC persistence within the joint.

In conclusion, we found that QD-MSCs were detectable seven days after injection into both normal and OA joint *in vivo*, and that QD-labeling does not affect the ability of MSCs to proliferate and differentiate *in vitro*. We also found that the large majority of QD-labeled cells persisted in the synovial membrane as compared to the articular cartilage, without differences between normal and osteoarthritic joints. However, QDs as applied in this study do not appear to be a good option for long-term tracking of equine MSCs *in vivo* for most indications, because the percentage of labeled cells and the MFI of the cells decreased rapidly in proliferating cells. This makes the low proportion of QD-labeled specimens, such as was found in Exp. 1 of this study, difficult to interpret- it cannot be determined if the cells were eliminated, migrated elsewhere, or proliferated *in situ* and lost their label.

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Figure 1. Distribution of 625 nm QD-labeled MSCs (red) to synovial membrane (A, B) and articular cartilage (C, D) with a nuclear counter-stain (compound 33258, Hoechst; Sigma-Aldrich).

Sections are from a severe OA metacarpophalangeal joint. QD-labeled MSCs are abundantly present in the intimal layers of the synovial membrane villi, and a single labeled cell is evident in the fibrotic surface of the OA cartilage. Images were taken at (A) 200 X and (C) 400 X magnification. (B, D) Areas of expanded images from A and C denoted by the white boxes. Bar = 200 μ m in original images.

Figure 2. Proportion of tissue sections positive for QD fluorescence in synovial membrane and articular cartilage.

Dark grey bars represent the number of sections that were positive for any amount of QD fluorescence and light grey bars represent sections negative for QD fluorescence. Within tissue type, there was no significant difference in the proportion of positive sections between OA and normal joints. The synovial membrane had a significantly higher proportion of positive sections than did the articular cartilage.

Figure 3. Dividing QD-labeled MSC cultures.

Overlays of phase contrast and fluorescent images showing a rapid decrease in detected QD-labeled cells; QDs appear as red dots. A) 24 hr post-QD label; B) 7 d (P5) post-QD label; C) 26 d (P7) post QD-label. Scale bar = 100 μ m.

Figure 4. Non-dividing QD-labeled MSCs (chondrogenic pellets). Overlays of DAPI and fluorescent images showing persistence of QDs (red dots) in chondrogenic pellets. A) 2 wk post QD-label; B) 4 wk post-QD label; C) 6 wk post-QD label; D) 8 wk post-QD label. Scale bar = 200 μ m.

Figure 5. Trilineage differentiation of QD-labeled (A, B, C) and control (D, E, F) MSCs.
A, D) chondrogenic- purple staining of cartilaginous extracellular matrix with Toluidine Blue (Scale bar = 100 μ m) B, E) adipogenic- red staining of lipid droplets with Oil Red O (Scale bar = 200 μ m) C, F) osteogenic- red staining of calcified extracellular matrix with 2% Alizarin Red (Scale bar = 200 μ m).

Figure 6. Flow cytometry histograms of QD-labeled and control MSCs from Donors A, B and C. MSCs were stained with antibody for the designated markers (red histogram), secondary antibody only when applicable (black histogram) or were unstained (grey histogram).

Table 1.

Synovial fluid analysis including total nucleated cell count, differential cell count based on smears, and protein quantification between MSC-injected and Vehicle-injected joints.

	<u>MSC-injected (n = 22)</u>		<u>Vehicle-injected (n=12)</u>		<u>P-value</u>
	<u>Median</u>	<u>95% CI</u>	<u>Median</u>	<u>95% CI</u>	
TNCC (x 10 ³ cells/ μ L)	2.8	2.6 – 4.2	0.85	0.5 – 1.4	< 0.001
Macrophages (%)	64	51 – 60	42	30 – 60	0.05
Lymphocytes (%)	32	27 – 41	34	28 – 48	0.5
Neutrophils (%)	0.05	–1.3 – 13	7	5 – 29	0.12
Total protein (g/dL)	2.8	< 2.5 – 2.9	< 2.5	< 2.5 – < 2.5	0.01

There was a significant increase in total nucleated cell counts (TNCC) and total protein in MSC-injected joints compared to Vehicle-injected joints.

Table 2.

Percentage of QD-labeled cells and measured MFI for cells in monolayer culture conditions.

P	Donor A		Donor B		Donor C		MFI Mean $\pm$ s.d.
	Labeled cells (%)	MFI	Labeled cells (%)	MFI	Labeled cells (%)	MFI	
1	99.9	651.0	99.6	1796.0	98.1	156.0	867.7 ^a $\pm$ 847.2
2	51.5	31.6	78.4	83.9	82.0	38.0	51.2 ^b $\pm$ 28.5
3	11.0	4.4	20.1	5.4	24.4	2.9	4.2 ^c $\pm$ 1.3
4	2.1	4.4	5.8	4.9	8.5	2.2	3.8 $\pm$ 1.5
5	0.3	3.0	5.5	5.4	1.9	2.1	3.5 $\pm$ 1.7
6	0.1	4.7	13.0	6.9			5.8 $\pm$ 1.5
7	0.1	3.9					3.9

Cells from a different horse were used for each replicate. Within the Mean column, values with different superscripts differ significantly ($P < 0.05$).

Table 3.

Expected MFI for cells in monolayer culture conditions.

P	Donor A			Donor B			Donor C		
	Initial # of cells	Final # of cells	Exp MFI	Initial # of cells	Final # of cells	Exp MFI	Initial # of cells	Final # of cells	Exp MFI
1	1.02×10 ⁷		651.0	1.02×10 ⁷		1796.0	5.40×10 ⁶		156.0
2	1.02×10 ⁷	2.04×10 ⁷	325.5	1.02×10 ⁷	5.76×10 ⁷	318.0	5.40×10 ⁶	1.23×10 ⁷	68.5
3	1.10×10 ⁷	3.03×10 ⁷	11.5	1.15×10 ⁷	4.08×10 ⁷	23.6	4.30×10 ⁶	5.60×10 ⁶	29.2
4	1.98×10 ⁷	4.28×10 ⁷	2.1	8.75×10 ⁶	2.07×10 ⁷	2.3	4.60×10 ⁶	2.01×10 ⁷	0.7
5	4.38×10 ⁶	7.40×10 ⁶	2.6	8.75×10 ⁶	2.28×10 ⁷	1.9	5.00×10 ⁶	8.60×10 ⁶	1.3
6	1.55×10 ⁶	5.50×10 ⁶	0.8	4.38×10 ⁶	3.20×10 ⁵	74.2			
7	4.75×10 ⁶	6.60×10 ⁶	3.4						

Cells from a different horse were used for each replicate. Expected (Exp) MFI was calculated based on the measured MFI of the previous passage (Table 2), the number of cells seeded (initial # of cells) and the number of cells present when the cells were passaged (final # of cells). Exp MFI was higher than the MFI measured by flow cytometry for passages 2 and 3 (Table 2).

Table 4.

Number of cells and time between passages for control and QD-labeled cells.

P	Donor A			Donor B			Donor C		
	Control	QD-labeled	CT	Control	QD-labeled	CT	Control	QD-labeled	CT
1	1.01×10 ⁷	1.02×10 ⁷		1.00×10 ⁷	1.02×10 ⁷		5.20×10 ⁶	5.40×10 ⁶	
2	1.98×10 ⁷	2.04×10 ⁷	4	5.74×10 ⁷	5.76×10 ⁷	6	1.25×10 ⁷	1.23×10 ⁷	4
3	2.98×10 ⁷	3.03×10 ⁷	4	4.10×10 ⁷	4.08×10 ⁷	4	5.40×10 ⁶	5.60×10 ⁶	2
4	4.20×10 ⁷	4.28×10 ⁷	4	2.05×10 ⁷	2.07×10 ⁷	4	2.00×10 ⁷	2.01×10 ⁷	4
5	7.40×10 ⁶	7.40×10 ⁶	5	2.25×10 ⁷	2.28×10 ⁷	4	8.40×10 ⁶	8.60×10 ⁶	4
6	5.30×10 ⁶	5.50×10 ⁶	4	3.00×10 ⁵	3.20×10 ⁵	3	1.96×10 ⁷	1.95×10 ⁷	4
7	6.50×10 ⁶	6.60×10 ⁶	6						

The time between passages (CT- culture time in days) and the number of cells present at each passage was similar for QD-labeled and control cells. Cells from a different horse were used for each replicate.

Table 5

Cell surface markers of unlabeled and QD-labeled MSCs determined by flow cytometry.

	Control (unlabeled)					QD-labeled				
	CD29	CD44	CD90	CD45	MHC II	CD29	CD44	CD90	CD45	MHC II
Donor A	99.2	85.5	5.25	5.23	3.61	99.8	22.8	82.8	1.89	2.57
Donor B	99.8	1.81	54.5	1.15	1.06	99.7	29.5	35.6	5.41	1.11
Donor C	1.7	16.8	41.4	1.99	6.23	78.7	0	0.16	0	0

P4 cells were used from three different horses. Data are presented as % of cells positive for a given marker.