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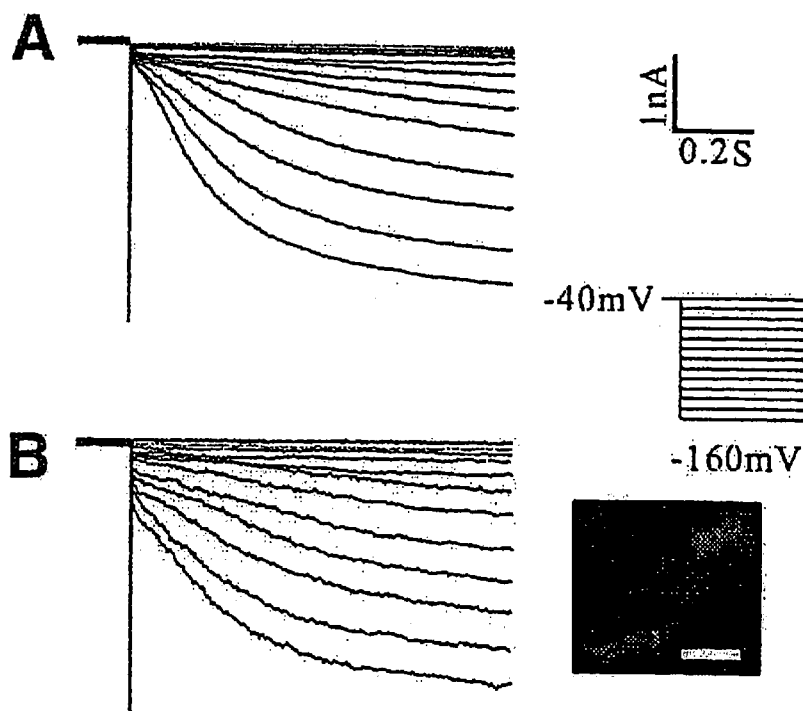
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[Continued on next page]

(54) Title: QUANTUM DOT LABELED STEM CELLS FOR USE IN CARDIAC REPAIR

FIGURE 3



(57) Abstract: The present invention provides methods and compositions relating to the labeling of target cells with nanometer scale fluorescent semiconductors referred to as quantum dots (QDs). Specifically, a delivery system is disclosed based on the use of negatively charged QDs for delivery of a tracking fluorescent signal into the cytosol of target cells via a passive endocytosis-mediated delivery process. In a specific embodiment of the invention the target cell is a stem cell, preferably a mesenchymal stem cell (MSC). Such labeled MSCs provide a means for tracking the distribution and fate of MSCs that have been administered to a subject to promote cardiac repair. The invention is based on the discovery that MSCs can be tracked in vitro for up to at least 6 weeks. Additionally, QDs delivered in vivo can be tracked for up to at least 8 weeks, thereby permitting for the first time, the complete 3-D reconstruction of the locations of all MSCs following administration into a host.



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QUANTUM DOT LABELED STEM CELLS FOR USE IN CARDIAC REPAIR**Cross Reference to Related Applications**

This application claims priority to U.S. Application No. 60/919,593, filed March 23, 2007, and 60/936,874 filed June 22, 2007, which are incorporated herein by reference in their entirety.

This research was supported by USPHS-NHLBI grants HL-28958 and HL-67101. The United States Government may have rights in this invention.

1. INTRODUCTION

[001] The present invention provides methods and compositions relating to the labeling of target cells with nanometer scale fluorescent semiconductors referred to as quantum dots (QDs). Specifically, a delivery system is disclosed based on the use of negatively charged QDs for delivery of a tracking fluorescent signal into the cytosol of target cells via a passive endocytosis-mediated delivery process. In a specific embodiment of the invention the target cell is a stem cell, preferably a mesenchymal stem cell (MSC). Such labeled MSCs provide a means for tracking the distribution and fate of MSCs that have been administered to a subject to promote cardiac repair. The invention is based on the discovery that MSCs can be tracked *in vitro* for up to at least 6 weeks. Additionally, QDs delivered *in vivo* can be tracked for up to at least 8 weeks, thereby permitting for the first time, the complete 3-D reconstruction of the locations of all MSCs following administration into a host.

2. BACKGROUND OF INVENTION

2.1 QUANTUM DOTS

[002] Quantum dots (QDs) are semiconductor nanoparticles that were discovered in the early 1980's. QDs used for biological applications consist of a cadmium selenide or cadmium tellurium semiconductor core, a zinc sulfide inner shell and an outer polymer coating. The result is a water-soluble particle 13-15 nm in diameter.

[003] Similar to organic fluorophores, QDs absorb photons of light of one wavelength and emit light of a different wavelength. Traditional fluorophores use absorbed energy to transfer electrons to excited states and energy is released in the form of fluorescent light when these electrons return to their resting states. When electrons move to different energy levels in QDs, they behave analogously, generating electron holes called excitons. The quantum system of excitons makes QD fluorescence much brighter and more photostable (less prone to photobleaching) than traditional fluorophores.

[004] The energy state of an exciton dictates the wavelength of light emitted by a particular QD after excitation. QDs have a unique property known as tunability, wherein the physical size of the QD determines the wavelength of emitted light. Smaller dots emit blue fluorescent light and as the core size of the dots increases, emitted light becomes redder. Another important feature that distinguishes QDs from conventional fluorescent dyes is the large distance between the wavelength of excitation and emission light. This energy difference, known as the Stokes' shift, means that QDs can be excited by ultraviolet light at a wavelength much lower than the peak emission wavelength. Thus, QDs can be excited by any wavelength lower than its emission wavelength. Therefore, particles are excited and emitted light is collected in a very efficient manner.

[005] The first biological uses for QDs were reported in 1998. Since then, a number of novel applications for QDs have been described. For example, Dubertret, et al (Science, 2002, 298:1759-62) reported the encapsulation of QDs within micelle-forming hydrophilic polymer-grafted lipids and delivery via microinjection into single cells of *Xenopus* embryos. Major findings from this study were that only the originally injected cells and their progeny retained QDs, labeled cells showed no signs of toxicity, all embryonic cell types were able to arise from labeled cells, the QD fluorescence was detectable above high levels of auto-fluorescence and most importantly, QDs were biocompatible. However, this group also reported that intracellular QDs tend to aggregate around the nucleus over time (Science, 2002, 298:1759-62). A similar published study demonstrated the use of QDs as lineage tracers by microinjecting into single cells of zebrafish embryos. Here it was shown that the dots were nontoxic, retained their emission spectra regardless of microenvironment, could be induced to avoid perinuclear aggregation by surface modification with streptavidin, did not pass through gap junctions, and could be imaged in aldehyde-fixed tissue (Rieger et al., Dev. Dyn 2005, 234:670-81). Both of these studies showed that the presence of intracellular QDs did not affect proliferation or differentiation of cells nor did they preclude formation of a fully-grown organism.

[006] Two later published articles demonstrated that populations of cells could be labeled with QDs. Jaiswal, et al, reported a receptor-mediated and generalizable endocytotic method for introducing QDs into the intracellular space for live cell imaging (Nat Biotechnol 2003, 21:47-51). Unfortunately, the reported images revealed the problem of perinuclear aggregation. Wu, et al, highlighted the ability of QDs for use in multiplex immunostaining of fixed cells (Wu et al., 2003, Nat Biotechnol 21:41-6). Others have compared loading populations of cells via commonly used approaches

including receptor-mediated transfection with a host of proteins (Hanaki et al., Biochem Biophys Res Commun 2003, 302:496-501; Silver et al., Nano Letters 2005, 5:1445-1449; Zhang et al., J. Biomedical Materials Research Part B: Applied Biomaterials, 2005, 76B:161-168; So et al., Nat Biotechnol 2006, 24:339-43), lipid-mediated transfection and electroporation (Derfus et al., Advanced Materials 2004, 16:961). While both lipid-mediated transfection and electroporation have the added problem of causing some degree of cell death, none of the approaches reported to date have uniformly and efficiently loaded populations of cells and all have resulted in perinuclear aggregation.

2.2 STEM CELL BASED THERAPIES

[007] The past decade has seen rapid advances in the use of embryonic and adult stem cells for tissue regeneration and repair in the heart[1-3]. These cells are believed to have the potential to differentiate into mature cardiac cells or promote native repair through angiogenesis, recruitment of host stem cells or induction of myocytes into the cell cycle[2, 4-6]. Additionally, stem cells genetically engineered to express hyperpolarization-activated cyclic nucleotide-gated (HCN) genes have been utilized to create biological pacemakers. However, one drawback associated with such studies is the inability to adequately track delivered stem cells with sufficient resolution in large animals. The ability to account for exogenous stem cells after delivery to animal models is important not only for determining the overall efficacy of intended treatments, but also to rule out potentially dangerous side effects.

[008] Traditional tracking agents such as GFP or fluorescent dyes fail to illuminate delivered cells above high levels of auto-fluorescence in the heart. Secondary staining as used to detect lacZ or amplify GFP generates false positives and would also involve

painstaking efforts to identify 100% of the exogenous cells in hundreds of tissue sections. More recently, cells have been labeled with inorganic particles for detection by MRI or PET, but these imaging approaches can resolve no fewer than thousands of cells.

[009] The present invention provides a novel approach to tracking cells, administered to a subject, using intracellular quantum dots (QDs). The invention is based on the demonstration that QD labeled hMSCs can be easily identified in histologic sections to determine their location for at least 8 weeks following delivery *in vivo*. Further, this approach has been used for the first time to generate a complete three-dimensional reconstruction of an *in vivo* stem cell “node.”

3. SUMMARY OF THE INVENTION

[010] The present invention provides a delivery system for transfer of QDs into the cytosol of target cells. Specifically, negatively charged QDs are described for use in delivering a tracking fluorescent signal into the cytosol of the desired target cells. The methods of the invention are based on the surprising discovery that negatively charged QDs are efficiently delivered into the cytosol of a target cell through a passive endocytosis-mediated delivery system. A number of benefits are found to be associated with the use of the delivery system of the invention including lack of autofluorescence or perinuclear aggregation, easy of use, reliability and reproducibility, as well as a lack of cellular toxicity. Additionally, the intracellular QDs do not interfere with cellular function and the labeled cells are capable of continued proliferation without loss of detectable label. Moreover, the labeled cells fail to transfer label to adjacent cells.

[011] The delivery system of the invention comprises contacting a target cell population with negatively charged QDs for a time sufficient to permit transfer of QDs

into the cytosol of the target cell. In an embodiment of the invention the QDs emit light at wavelengths between 655 and 800. In a specific embodiment of the invention the target cell is a stem cell, preferably a MSC.

[012] The compositions of the invention comprise labeled target cells that have taken up QDs through use of the delivery system of the invention. The QD-labeled cells of the invention lack perinuclear aggregation and show a uniform diffuse cytoplasmic labeling. In a preferred embodiment of the invention the labeled cells are stem cells, preferably MSCs. Also within the scope of the invention are QD-labeled cells that have been genetically engineered to express a desired protein of interest. For example, QD-labeled cells may be engineered to express proteins capable of promoting cardiac repair.

[013] A number of recently developed therapies are based on the administration of cells, such as stem cells, for treatment of a variety of different disorders. For example, the use of stem cells to promote cardiac repair has been described. The methods and compositions of the present invention may be used, for example, for tracking MSC mediated cardiac repair in a subject, comprising administering to said subject an effective amount of Q-labeled MSCs and determining whether there is migration of the MSCs to other sites in the body. Such methods and compositions provide a means for studying the safety and efficacy of stem cell use to treat different cardiac disorders, including but not limited to myocardial dysfunction or infarction.

4. BRIEF DESCRIPTION OF THE FIGURES

[014] **Figure 1.** Quantum dots can be loaded uniformly into hMSCs by passive incubation. QD loading was achieved by receptor-mediated uptake or passive incubation with naked dots. Panels (a) and (b) show images of QD fluorescence (655-

nm, red) with phase contrast overlays. (a) Using the receptor-mediated-based Qtracker kit (Invitrogen) resulted in non-uniform cellular loading with perinuclear aggregation. (b) In contrast, passively incubating hMSCs in naked QD media results in nearly 100% loading with a pattern that extends to the cell borders. (c) The field in (b) is imaged for QD fluorescence without the phase overlay to demonstrate homogeneity and brightness. The intracellular QD cluster distribution is diffusely cytoplasmic (c,d) and largely excludes the nucleus (blue, Hoechst 33342 dye). (e) QD loading efficiency was analyzed using flow cytometry. The threshold for plain hMSCs (gray line) was set such that the intensity range encompassed at least 98% of the control cells (red arrow indicates upper bound of control range). QD-positive status was designated for all cells in the QD-hMSC sample having intensities above the range set for the control group. In four experiments, QD-positive cells (black line) were found in 96% of over 17,000 viable cells. (f) When colchicine-conditioned cells are incubated with colchicine-containing QD media, the uptake is dramatically reduced. The cells in panel (f, colchicine) and (f, inset, no colchicine) can be directly compared, as the incubation periods were identical and the cells were imaged using the same microscope and camera settings. (g-i) hMSCs continually take up QDs from the incubation media. Cells incubated in QD media for (g) 1, (h) 3, and (i) 24 hours are all imaged using the same microscope and camera settings. Images are grayscale for clarity. Because of the different levels of loading, the exposure time (380 ms) used to image all three samples is clearly too high for the 24-hour-incubated cells; most of the QD clusters in the image are overexposed. Scale bar a, b = 50 μm . Scale bar c = 100 μm . Scale bar on d, f-i = 25 μm

[015] Figure 2. QDs retain their brightness and cytoplasmic distribution for up to 6 weeks *in vitro* and are not transferred to unloaded cells. QD-hMSCs were fixed onto

slides and stained with Hoechst 33342 dye. The cells were imaged for QD fluorescence at (a) 2 days, (b) 16 days and (c) 44 days after loading. Only the Hoechst (blue) channels of these images have been post-processed to enhance contrast; QD channels (red) are displayed as imaged. As cells divide, they split their cytoplasmic contents to each daughter cell, diluting the ultimate concentration of QDs in progeny over time. Therefore, exposure times of excitation light need to be increased to optimally capture QD images of cells that have been through multiple divisions. Microscope and camera settings are the same for images (a) and (b), but in (c), cells are imaged for 655-nm (red) emission using triple the exposure time. The QDs in (a) are overexposed at this setting, with some dots clearly saturating the image. (d) QD-loaded and plain hMSCs proliferate similarly, as measured by a mitochondrial dehydrogenase assay (N=12). (e) . Green (GFP transfected) and red (QD-loaded) hMSCs in direct apposition were imaged live to look for QD transfer to neighboring cells (colors have been enhanced for contrast). In a separate experiment, QD-hMSCs were mechanically lysed and then added to cultured canine myocytes for 24 hours. (f) A myocyte (right) sits near a live QD-hMSC that survived the lysis and attached to the coverslip. Floating QD clusters from the lysed cells are apparent in the media but were not internalized by the myocytes. Live cell images in (e) and (f) were acquired on an Olympus inverted fluorescence microscope using a GFP and a Texas Red filter, which does not optimally image the 655-nm QD signal. Scale bar a-c = 25 μ m. Scale bar e, f = 50 μ m

[016] Figure 3. The presence of intracellular QDs does not affect ability of cells to overexpress genes after transfection. QD-hMSCs and plain hMSCs were each transfected with the HCN2-pIRES-EGFP plasmid via electroporation. Two days after transfection, both groups of cells expressed similar levels of GFP and cells expressing GFP from both groups were patch clamped to record the HCN2-induced currents. The

currents provided were from a holding potential of -40mV and included steps between -40mV and -160mV in -10mV increments. Similar levels of HCN2-induced current were recorded from (a) unloaded and (b) QD-loaded hMSCs. Additionally, the electroporation process did not alter the cytoplasmic distribution of QDs (b, inset). Imaging for these experiments was carried out on the Olympus microscope using the Texas Red filter to visualize QDs in live cells; this filter set does not optimally visualize QD loading. Scale bar on b inset = 50 μ m

[017] Figure 4. QD-hMSCs can be delivered to the canine heart on an ECM scaffold and identified 8 weeks later. QD-hMSCs were delivered to the canine ventricle via implantation of an ECM patch. Eight weeks later, tissue was explanted and fixed. Panel (a) shows fixed tissue from one animal with a blue line circumscribing the region analyzed (and imaged transmurally in panel c) and a black dotted ellipse approximating the patch borders. Straight dark black lines in the image are dissecting pins that were used to secure the tissue while photographing. The region outlined in blue was (a) frozen and sectioned transmurally at 10- μ m and (b) imaged for QD fluorescence (655nm) and phase contrast. (c) The plane of section is transmural from epicardium (top) to endocardium (bottom); a green circle highlights the region where QDs were found (imaged in b). Some tissue sections were stained with Hoechst 33342 to visualize nuclei. Panel (b, inset) shows QD-hMSCs amidst endogenous tissue (asterisks denote endogenous nuclei). Scale bar on a, c = 20 mm. Scale bar on b = 50 μ m, inset = 10 μ m

[018] Figure 5. QDs can be used to identify single hMSCs after injection into the rat heart and further used to reconstruct the 3-D distribution of all delivered cells. Rat hearts were injected with QD-hMSCs. Fixed, frozen sections were cut transversely

(plane shown in b, inset) at 10- μ m and mounted onto glass slides. Sections were imaged for QD fluorescence emission (655-nm) with phase overlay to visualize tissue borders. QD-hMSCs can be visualized at (a) low power, and (a, inset) high power (Hoechst 33342 dye used to stain nuclei blue). In (a, inset), endogenous nuclei can be seen adjacent to the delivered cells in the mid-myocardium (arrows). Serial low power images were registered with respect to one another and (b) binary masks were generated, where white pixels depict the QD-positive zones in the images. The vertical line in (b, inset) represents the z axis, which has a zero value at the apex of the heart. The binary masks for all of the QD-positive sections of the heart were compiled and used to generate the 3-D reconstruction of delivered cells in the tissue. QD-hMSCs remaining in the tissue adhesive on the epicardial surface (and not within the cardiac syncytium) were excluded from the reconstruction. (c) QD-hMSC reconstruction in an animal that was terminated 1 hour after injection. (d) Reconstruction from an animal euthanized 1 day after injection with orientation noted in inset. Our reconstructions in (c) and (d) do not account for all of the approximately 100,000 hMSCs delivered through the needle. Some of these cells undoubtedly leaked out of the needle track, while others may not have survived the injection protocol. The views of both reconstructions (c) and (d) are oriented for optimal static visualization (and also have different scales and are situated at different positions along the z-axis depending on the distance of the injection site from the apex of the heart); (e) One day after injection into the heart, the pattern of QD-hMSCs is well-organized and appears to mimic the endogenous myocardial orientation (dotted white line highlights myofibril alignment). Complete representations of the spatial localization of QD-hMSCs in the heart permits further quantitative analyses. (f) One parameter that can be computed is the distance of individual cells from the centroid of the total cell mass. The plots show the percentage

of cells at a distance less than or equal to x for both the 1-hour and 1-day rats. At both time points, most of the cells are within 1.5mm of the centroid. Scale bar on a = 500 μ m, inset = 20 μ m. Scale bar on b, inset = 1 cm. Scale bar on e = 500 μ m

[019] Figure 6. QDs do not interfere with differentiation capacity of hMSCs *in vitro* or *in vivo*. QD-hMSCs or unloaded hMSCs were induced to differentiate *in vitro* along adipogenic and osteogenic lineages. After the adipogenic induction period both (a) unloaded hMSCs and (b) QD-hMSCs displayed characteristic adipocyte morphology, with prominent lipid vacuoles. The percent of differentiated versus undifferentiated cells was similar between these two groups. (c) At high power, adipocytes from the QD-hMSC group are seen with QDs (red fluorescence) interspersed between lipid vacuoles. After the osteogenic induction period both (a) unloaded hMSCs and (b) QD-hMSCs that were initially spindle shaped adapted a more cobblestone-like morphology typical of osteocytes and tended to cluster on the dish. (f) At high power a cobblestone-shaped osteocyte from the QD-hMSC group retains the QD label (red) after the differentiation process. QD-hMSCs were delivered *in vivo* to the canine ventricle on an ECM patch. (g) After 8 weeks, some of these QD-positive cells (red) express the endothelial marker PECAM-1 (green), suggesting differentiation of these cells along an endothelial lineage. A high-power view of QD-positive cell with co-localized PECAM-1 expression is shown in (g, inset). Scale bars on a,b,d,e = 200 μ m Scale bars on c,f = 50 μ m Scale bar on g = 20 μ m, inset = 5 μ m

[020] Figure 7. QDs can be visualized using μ CT. QD-hMSCs were (a) loaded and imaged, and then formed into a pellet overnight. (b) The QD-hMSC pellet and a pellet formed from unloaded hMSCs were each embedded in a separate siloxane mold. Both phantom molds were scanned using μ CT and images were reconstructed. In (c) a

2-D image of one section through the QD-hMSC pellet is shown. (d) Average densities for pellets formed from QD-loaded (N=16) and unloaded (N=12) hMSCs were calculated and normalized to the average density of the unloaded pellet. QD-hMSCs were roughly 27% denser than unloaded hMSCs. (e) The 3-D reconstruction of the scanned region of QD-hMSC pellet is shown. Scale bar on a = 200 μ m. Scale bar on b = 1 cm. Scale bar on c = 1 mm

5. DETAILED DESCRIPTION OF THE INVENTION

[021] The present invention provides methods and compositions relating to the labeling of target cells with nanometer scale fluorescent semiconductors referred to as quantum dots (QDs). The methods and compositions of the invention provide a means for assessing the safety and efficacy of therapies based on the administration of cells, for example stem cells, into a subject in need of treatment.

5.1. QUANTUM DOT (QD) LABELING OF CELLS

[022] The present invention provides a method for transfer of QDs into the cytosol of a cell comprising contacting target cells with negatively charged QDs. The method of the invention results in delivery of a tracking fluorescent signal into the cytosol of said target cell via a passive endocytosis delivery process. The delivery system of the present invention can be used with virtually any type of biological cell population, including, mammalian cells. The specific cell type used will typically vary depending upon the type of cell tracking that is sought to be monitored. For example, mammalian cells and specifically, human cells or animal cells containing QDs are typically preferred for determining the safety and efficacy of potential human therapies. Additionally, endothelial progenitor cells may be labeled with QDs to track, for

example, early migration and incorporation of endothelial stem cells into blood vessels. QD-labeled hematopoietic stem cells may be used to track development of said labeled cells into the different functional cell types of the blood. While it is understood that the delivery system of the present invention may be used to deliver QDs into a variety of different cell types, for simplicity, the invention is described in detail below for use with stem cells. However, the methods of the invention may be applied equally as well for use with other cell types.

[023] In an embodiment of the invention, the target cells to which QDs are to be delivered are mammalian cells, including but not limited to, mammalian stem cells. In a preferred embodiment of the invention, the stem cells are mesenchymal stem cells (MSCs). In another embodiment of the invention, the stem cells are human stem cells, or human MSCs (hMSCs).

[024] As used herein, “stem cell” refers to any cell having the potential to differentiate into one or more different cell types. Such cells include, but are not limited to, stem cells derived from a variety of different sources including, for example, bone marrow, embryonic blastocysts or yolk sac, spleen, blood, including peripheral blood and umbilical cord blood, adipose tissue and other tissues and organs. Such stem cells include, but are not limited to, hematopoietic stem cells, endothelial progenitor cells or embryonic stem cells. In a preferred embodiment of the invention, mammalian MSCs are utilized in the practice of the invention. In a preferred embodiment of the invention the utilized MSCs are derived from a human.

[025] Stem cells may be obtained from a variety of different donor sources. In a preferred embodiment, autologous stem cells are obtained from the subject who is to receive the transplanted stem cells to avoid immunological rejection of foreign tissue.

In yet another preferred embodiment of the invention, allogenic stem cells may be obtained from donors who are genetically related to the recipient and share the same transplantation antigens on the surface of their stem cells. Alternatively, stem cells may be derived from antigenically matched (identified through a national registry) donors. In instances where antigenically matched stem cells cannot be located, non-matched cells may be used, however, it may be necessary to administer immunosuppressive agents to prevent recipient rejection of the donor stem cells.

[026] Procedures for harvest and isolation of such stem cells are well known to those of skill in the art and do not differ from those used in conventional stem cell transplantation. Adult stem cells may be derived from bone marrow, peripheral blood, adipose tissue and other adult tissues and organs. For derivation of embryonic stem cells, stem cells can be extracted from the embryonic inner cell mass during the blastocyst stage. Fetal stem cells may be derived from the liver, spleen, brain or heart of fetuses, 4-12 weeks gestation, following elective abortions, terminated ectopic pregnancies or spontaneous miscarriages.

[027] In a non-limiting embodiment of the invention, antibodies that bind to cell surface markers selectively expressed on the surface of stem cells may be used to identify or enrich for populations of stem cells using a variety of methods. Such markers include, for example, CD34, SSEA3, SSEA4, anti-TRA1-60, anti-TRA1-81 or c-kit.

[028] In an embodiment of the invention, MSCs may be derived from bone marrow aspirates. For example, 10 ml of marrow aspirate is collected into a syringe containing 6000 units of heparin to prevent clotting, washed twice in phosphate buffer solution (PBS), added to 20 ml of control medium (DMEM containing 10% FBS), and then

centrifuged to pellet the cells and remove the fat. The cell pellet is then resuspended in control medium and fractionated at 1100 g for 30 min on a density gradient generated by centrifugation of a 70% percoll solution at 13000 g for 20 minutes. The mesenchymal stem cell-enriched, low density fraction is collected, rinsed with control medium and plated at a density of 10^7 nucleated cells per 60 mm² dish. Alternatively, MSCs (PoieticsTM hMSGs) to be used in the practice of the invention can be purchased from Clonetics/Bio Whittaker (Walkersville, M.D.).

[029] In a specific embodiment of the invention, MSCs are grown on polystyrene tissue culture dishes and maintained at 37°C in humidified 5% CO₂ in Mesenchymal Stem Cell Growth Media supplemented with L-glutamine, penicillin and serum (MSCGM BulletKit, Cambrex). Cells are re-plated for passaging once every two weeks. The MSCs are then cultured in control medium at 37° C. in a humidified atmosphere containing 5% CO₂.

[030] In yet another embodiment of the invention, late passage MSCs, which are substantially unable to differentiate, may be labeled with QDs using the delivery system of the present invention. As used herein, “late passage MSCs” are those cells that have been passaged at least nine times. Additionally, the QD-labeled MSCs of the invention express CD29, CD44, CD54 and HLA class I surface markers while failing to express CD14, CD45, CD34 and HLA class II surface markers.

[031] In yet another embodiment of the invention, cardiogenic stem cells may be labeled with QDs. MSCs may be load with QDs and then partially differentiated into cardiogenic cells by, for example, the hanging drop method.

[032] In an embodiment of the invention, the cells to be labeled with QDs may be genetically engineered to express one or more genes encoding physiologically active

proteins of interest. Such proteins include, for example, those proteins capable of promoting cardiac repair. Such engineered cells are described in detailed below. The cells may be genetically engineered using techniques well known in the art to express proteins that further enhance the ability of such cells to promote cardiac repair. Such techniques include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. (See, for example, the techniques described in Sambrook J et al. 2000. Molecular Cloning: A Laboratory Manual (Third Edition), and Ausubel et al (1996) Current Protocols in Molecular Biology John Wiley and Sons Inc., USA). Any of the methods available in the art for gene delivery into a host cell can be used according to the present invention to deliver genes into the target cell population. Such methods include electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. For general reviews of the methods of gene delivery see Strauss, M. and Barranger, J. A., 1997, Concepts in Gene Therapy, by Walter de Gruyter & Co., Berlin; Goldspiel et al., 1993, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 33:573-596; Mulligan, 1993, Science 260:926-932; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; 1993, TIBTECH 11(5):155-215.

[033] In an embodiment of the invention, the target cells to be labeled with QDs may further comprise an exogenous molecule including, but are not limited to, oligonucleotides, polypeptides, or small molecules, and wherein said QD-labeled cell is capable of delivering said exogenous molecule to an adjacent cell, or cells at a greater distance, via the gap junctions of the adjacent cells. Delivery of the exogenous molecule to adjacent cell, or cells at a greater distance, via the gap junctions of the adjacent cells may be used to promote cardiac repair.

[034] QDs to be used in the practice of the invention may be composed of various semiconductor materials such as, for example, CdS, CdSe, CdTe, CdTe/ZnS or CdSe/ZnS. In a preferred embodiment of the invention, the QDs for use in the practice of the invention are those having a net negative charge. Such negatively charged QDs may be formed through conjugation of negatively charged groups onto the surface of the QD. In a specific embodiment of the invention, the negative charge of the QDs comes from carboxyl groups on the surface of a polymer surface.

[035] Further, for use in the present invention QDs are preferably those that emit light at wavelengths of between 525-800. In a preferred embodiment of the invention, the QD is one that emits light at a wavelength of 655.

[036] In an embodiment of the invention, various biological or chemical moieties may be conjugated to the surface of QDs as a means for delivery of said moiety into the cytosol of the target cell. For example, streptavidin, which binds to biotin with extremely high affinity, may be conjugated to negatively charged QDs. Through conjugation of streptavidin to QDs, a system is provided whereby streptavidin-conjugated QDs can be coupled to biotin-conjugated magnetic nanoparticles (superparamagnetic iron oxide, SPIO) through the streptavidin/biotin high-affinity reaction. Loading of MSCs using such hybrid QD-SPIO particles permits detection of said cells *in vitro* via the emitted QD fluorescence or by staining the cells with Prussian Blue to detect iron oxide. Additionally, such loaded cells can be delivered to animals and tracked non-invasively *in vivo* using MRI.

[037] The delivery system of the present invention comprises contacting a target cell population with negatively charged QDs for a time sufficient to permit transfer of the QDs into the cytosol of the target cell. In an embodiment of the invention, the target

cells are cultured, using routine tissue culture methods well known to those of skill in the art, to less than 100% confluence, preferable between 80-85 % confluence. Cells are then washed with a buffer, such as a phosphate-buffered saline (PBS) and a solution of QDs is added to the target cells. In an embodiment of the invention, the QD solution comprises a mixture of the tissue culture media in which the cells are cultured and QDs. In a preferred embodiment of the invention the media comprises fetal calf serum or calf serum. The solution of QDs contains QDs at a concentration of 8-8.5nM.

[038] Cells are incubated with the QDs for a time sufficient to permit efficient transfer of the QDs into the cytosol of the target cells. Transfer of QDs into the target cells can be monitored using, for example, fluorescent microscopy or flow cytometry. In an embodiment of the invention, the QDs are incubated for about 6-48 hours. Transfer of QDs into the target cells can be monitored using, for example, fluorescent microscopy or flow cytometry.

[039] In a specific embodiment of the invention, MSCs cells are grown to approximately 85% confluence on polystyrene tissue culture dishes. An 8.2nM solution of 655 ITK Carboxyl QDs is prepared in complete MSCGM and vortexed for 60 seconds. Cells are washed once in phosphate-buffered saline (PBS) and incubated in the QD solution for up to 24 hours at 37°C.

[040] The present invention provides labeled target cells that have taken up QDs through use of the delivery system of the invention. The QD labeled cells of the invention lack perinuclear aggregation and show a uniform diffuse cytoplasmic labeling. In a preferred embodiment of the invention the labeled cells are stem cells, preferably MSCs. Also within the scope of the invention are QD labeled cells that have

been genetically engineered to express a desired protein of interest. For example, QD labeled cells may be engineered to express proteins capable of promoting cardiac repair.

[041] The present invention further relates to pharmaceutical compositions comprising cells labeled with QDs and a pharmaceutically acceptable carrier.

Pharmaceutically acceptable carriers are well known to those skilled in the art and include, but are not limited to, 0.01-0.1M and preferably 0.05M phosphate buffer, phosphate-buffered saline (PBS), or 0.9% saline. Such carriers also include aqueous or non-aqueous solutions, suspensions, and emulsions. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, saline and buffered media.

Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Preservatives and other additives, such as, for example, antimicrobials, antioxidants and chelating agents may also be included with all the above carriers.

[042] In a specific embodiment of the invention, the carrier is an extracellular matrix protein derived from an acellularized porcine urinary bladder. The cells are seeded on this patch material (approximately 10 μ m in thickness) and then implanted to induce repair of a full thickness surgically induced defect in the ventricular wall.

[043] Another aspect of the present invention relates to kits for labeling target cells with QDs utilizing the methods of the present invention. Kits of the present invention comprise negatively charged quantum dots. Kits of the present invention may further comprise additional reagents, buffers and/or apparatus for use in labeling target cells with QDs via the method of the present invention as well as instructions for use of the kit to label cells.

5.1 USE OF QD-LABELED MESENCHYMAL CELLS FOR STIMULATION OF CARDIAC REPAIR

[044] The present invention relates to methods and compositions for tracking the fate and distribution of QD-labeled MSCs that have been administered as a means for stimulating the proliferation of cardiomyocytes for enhancement of cardiac repair. The invention is based on the discovery that upon contact with stem cells, terminally differentiated cardiomyocytes can be stimulated to enter the cell cycle. Additionally, stem cells may be QD-labeled which will eventually terminally differentiate into mature myocytes and thereby contribute to cardiac repair. The methods and compositions of the invention may be used in the treatment of cardiac disorders including, but not limited to, myocardial dysfunction or infarction.

[045] MSCs are capable of inducing native cardiomyocytes to enter the cell cycle. Accordingly, the present invention encompasses methods for tracking the distribution and fate of QD-labeled MSCs that are utilized for regenerating myocardium in a mammal comprising (i) administering QD-labeled MSCs to the myocardium in a quantity sufficient to induce native cardiomyocytes to enter the cell cycle; and (ii) determining the fate and distribution of said administered QD-labeled cells. Specifically, the invention relates to the use of QD-labeled MSCs to promote an increase in the number of cells in the myocardium through increased proliferation of native cardiac progenitor cells resident in the myocardium; stimulation of myocyte proliferation; and/or stimulation of differentiation of host cardiac progenitor cells into cardiac cells, for example. Such an increase in cell number results predominantly from stimulation of the native myocardium cells by factors produced by the administered QD-labeled MSCs.

[046] Prior to administration of the QD-labeled MSCs, the cells may be genetically engineered using techniques well known in the art to express proteins that further enhance the ability of such cells to enhance cardiomyocyte proliferation. In a non-limiting embodiment of the invention, the QD-labeled MSCs are engineered to express the Wnt-5A protein which enhances cardiomyocyte proliferation. Such techniques include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. (See, for example, the techniques described in Sambrook J et al. 2000. Molecular Cloning: A Laboratory Manual (Third Edition), and Ausubel et al (1996) Current Protocols in Molecular Biology John Wiley and Sons Inc., USA). Any of the methods available in the art for gene delivery into a host cell can be used according to the present invention to deliver genes into the QD-labeled MSCs. Such methods include electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. For general reviews of the methods of gene delivery see Strauss, M. and Barranger, J. A., 1997, Concepts in Gene Therapy, by Walter de Gruyter & Co., Berlin; Goldspiel et al., 1993, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 33:573-596; Mulligan, 1993, Science 260:926-932; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; 1993, TIBTECH 11(5):155-215.

[047] The present invention further provides pharmaceutical compositions comprising QD-labeled MSCs and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known to those skilled in the art and include, but are not limited to, 0.01-0.1M and preferably 0.05M phosphate buffer, phosphate-buffered saline (PBS), or 0.9% saline. Such carriers also include aqueous or non-aqueous solutions, suspensions, and emulsions. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, saline and buffered media.

Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Preservatives and other additives, such as, for example, antimicrobials, antioxidants and chelating agents may also be included with all the above carriers.

[048] QD-labeled MSCs can also be incorporated or embedded within scaffolds which are recipient-compatible and which degrade into products which are not harmful to the recipient. These scaffolds provide support and protection for QD-labeled MSCs that are to be transplanted into the recipient subjects. Natural and/or synthetic biodegradable scaffolds are examples of such scaffolds. Accordingly, the present invention provides methods for assessing the fate and distribution of QD-labeled cells useful for promoting cardiac repair, wherein QD-labeled MSCs are incorporated within scaffolds, prior to transplantation into a subject in need of cardiac repair.

[049] A variety of different scaffolds may be used successfully in the practice of the invention. Such scaffolds are typically administered to the subject in need of treatment as a transplanted patch. Preferred scaffolds include, but are not limited to biological, degradable scaffolds. Natural biodegradable scaffolds include collagen, fibronectin, and laminin scaffolds. Suitable synthetic material for a cell transplantation scaffold must be biocompatible to preclude migration and immunological complications, and should be able to support extensive cell growth and differentiated cell function. It must also be resorbable, allowing for a completely natural tissue replacement. The scaffold should be configurable into a variety of shapes and should have sufficient strength to prevent collapse upon implantation. Recent studies indicate that the biodegradable polyester polymers made of polyglycolic acid fulfill all of these criteria, as described by Vacanti, et al. *J. Ped. Surg.* 23:3-9 (1988); Cima, et al. *Biotechnol. Bioeng.* 38:145 (1991);

Vacanti, et al. *Plast. Reconstr. Surg.* 88:753-9 (1991). Other synthetic biodegradable support scaffolds include synthetic polymers such as polyanhydrides, polyorthoesters, and polylactic acid.

[050] In an embodiment of the invention, the scaffold is derived from porcine urinary bladder. Alternatively, in a preferred embodiment of the invention the scaffold is derived from bovine pericardium. In a specific embodiment of the invention, Veritas® which is derived from bovine pericardium may be utilized.

[051] Attachment of the QD-labeled cells to the scaffold polymer may be enhanced by coating the polymers with compounds such as basement membrane components, agar, agarose, gelatin, gum arabic, collagens types I, II, III, IV and V, fibronectin, laminin, glycosaminoglycans, mixtures thereof, and other materials known to those skilled in the art of cell culture. Additionally, such scaffolds may be supplemented with additional components capable of stimulating cardiomyocyte proliferation. Additionally, angiogenic and other bioactive compounds can be incorporated directly into the support scaffold so that they are slowly released as the support scaffold degrades *in vivo*. Factors, including nutrients, growth factors, inducers of proliferation or de-differentiation (i.e., causing differentiated cells to lose characteristics of differentiation and acquire characteristics such as proliferation and more general function), products of secretion, immunomodulators, inhibitors of inflammation, regression factors, biologically active compounds which enhance or allow ingrowth of nerve fibers, hyaluronic acid, and drugs, which are known to those skilled in the art and commercially available with instructions as to what constitutes an effective amount, from suppliers such as Collaborative Research and Sigma Chemical Co. Similarly, polymers containing peptides such as the attachment peptide RGD (Arg-Gly-Asp) can

be synthesized for use in forming scaffolds (see e.g. U.S. Pat. Nos. 4,988,621, 4,792,525, 5,965,997, 4,879,237 and 4,789,734).

[052] In another example, the QD-labeled MSCs cells may be transplanted in a gel scaffold (such as Gelfoam from Upjohn Company) which polymerizes to form a substrate in which the QD-labeled MSCs can grow. A variety of encapsulation technologies have been developed (e.g. Lacy et al., Science 254:1782-84 (1991); Sullivan et al., Science 252:718-712 (1991); WO 91/10470; WO 91/10425; U.S. Pat. No. 5,837,234; U.S. Pat. No. 5,011,472; U.S. Pat. No. 4,892,538). During open surgical procedures, involving direct physical access to the damaged tissue and/or organ, all of the described forms of stem cell delivery preparations are available options. These cells can be repeatedly transplanted at intervals until a desired therapeutic effect is achieved.

[053] In another embodiment of the invention, QD-labeled MCCs may be used to assess the safety and efficacy of using MSCs as reagents for delivery of small molecules into a target cell. Said delivery method comprises introducing the small molecule into a donor QD-labeled MSC, and contacting the target cell with the donor cell under conditions permitting the donor cell to form a gap junction with the target cell, whereby the small molecule is delivered into the target cell from the donor QD-labeled MSC. The transfer of the small molecule from a QD-labeled MSC to a target cell is via diffusion through gap junctions. The loading of specific small molecules into QD-labeled MSCs can be accomplished by electroporation or by perfusion of QD-labeled MSCs with media containing membrane permeable ester forms. Methods for delivery of small molecules into a target cell, via gap junctions, are disclosed in PCT/US04/042504, which is incorporated by reference herein in its entirety.

[054] QD-labeled MSCs form gap junction channels with other cells by containing one or more of the following connexins: Cx43, Cx45, Cx40, Cx32 and Cx26. Negatively charged small molecules with minor diameters of about 1.0 nm are all able to transit the aforementioned gap junction channels (homotypic Cx43, Cx40, Cx45, heterotypic Cx43-Cx40 and mixed or heteromeric Cx43-Cx40 and Cx32 and Cx26). The type of gap junctions and total number of channels determine the rate of transit of a specific solute between the QD-labeled MSC and target cell.

[055] Small molecules which are capable of being transferred include, but are not limited to, hydrophilic second messengers, drugs and their metabolites, and inorganic ions. The small molecules may also be oligonucleotides. Such oligonucleotides may be RNA that can traverse the gap junction. The oligonucleotide may be DNA. The oligonucleotide may be an antisense oligonucleotide or a cDNA that produces an antisense oligonucleotide that can traverse the gap junction. The oligonucleotide may be a siRNA oligonucleotide or a cDNA that produces a siRNA oligonucleotide that can traverse the gap junction. The oligonucleotide may be a DNA or RNA that produces a peptide that can traverse the gap junction.

[056] The oligonucleotides for use in the practice of the invention, *i.e.*, antisense, ribozyme and triple helix forming oligonucleotides, may be synthesized by standard methods known in the art, *e.g.*, by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). Alternatively, recombinant expression vectors may be constructed to direct the expression of the oligonucleotides of the invention. Such vectors can be constructed by recombinant DNA technology methods standard in the art. In a specific embodiment, vectors such

as viral vectors may be designed for gene therapy applications where the goal is *in vivo* expression of inhibitory oligonucleotides in targeted cells.

[057] According to the present invention, a method of delivering a small molecule into a target cell is provided, comprising introducing a small molecule into a donor QD-labeled MSC, and contacting the target cell with the donor QD-labeled MSC under conditions permitting the donor QD-labeled MSC to form a gap junction with the target cell, whereby the small molecule is delivered into the target cell from the donor QD-labeled MSC. As provided by the present invention, the distribution and fate of the QD-labeled cells, utilized to deliver small molecules into a target cell, can be determined following administration into a subject.

[058] According to the present invention, a method of delivering a small molecule into a syncytial target cell is provided, comprising introducing a small molecule into a donor QD-labeled MSC, and contacting the syncytial target cell with the donor QD-labeled MSC under conditions permitting the donor QD-labeled MSC to form a gap junction with the syncytial target cell, whereby the small molecule is delivered into the syncytial target cell from the donor QD-labeled MSC. As provided by the present invention, the distribution and fate of the QD-labeled cells, utilized to deliver small molecules into a syncytial target cell, can be determined following administration into a subject.

5.3. USES AND ADMINISTRATION OF THE COMPOSITIONS OF THE INVENTION

[059] The present invention provides methods and compositions which may be used to assess the safety and efficacy of treatments of various diseases associated with cardiac disorders. Specifically, through the use of QD-labeled MSCs, the fate and distribution of MSCs administered to promote cardiac repair can be tracked.

[060] The term "cardiac disorder" as used herein refers to diseases that result from any impairment in the heart's pumping function. This includes, for example, impairments in contractility, impairments in ability to relax (sometimes referred to as diastolic dysfunction), abnormal or improper functioning of the heart's valves, diseases of the heart muscle (sometimes referred to as cardiomyopathy), diseases such as angina and myocardial ischemia and infarction characterized by inadequate blood supply to the heart muscle, infiltrative diseases such as amyloidosis and hemochromatosis, global or regional hypertrophy (such as may occur in some kinds of cardiomyopathy or systemic hypertension), and abnormal communications between chambers of the heart (for example, atrial septal defect). For further discussion, see Braunwald, Heart Disease: a Textbook of Cardiovascular Medicine, 5th edition, W B Saunders Company, Philadelphia Pa. (1997) (hereinafter Braunwald). The term "cardiomyopathy" refers to any disease or dysfunction of the myocardium (heart muscle) in which the heart is abnormally enlarged, thickened and/or stiffened. As a result, the heart muscle's ability to pump blood is usually weakened. The disease or disorder can be, for example, inflammatory, metabolic, toxic, infiltrative, fibroplastic, hematological, genetic, or unknown in origin. There are two general types of cardiomyopathies: ischemic (resulting from a lack of oxygen) and nonischemic. Other diseases include congenital heart disease which is a heart-related problem that is present since birth and often as the heart is forming even before birth or diseases that result from myocardial injury which involves damage to the muscle or the myocardium in the wall of the heart as a result of disease or trauma. Myocardial injury can be attributed to many things such as, but not limited to, cardiomyopathy, myocardial infarction, or congenital heart disease. Specific cardiac disorders to be treated also include congestive heart failure, ventricular or atrial

septal defect, congenital heart defect or ventricular aneurysm. The cardiac disorder may be pediatric in origin. The cardiac disorder may require ventricular reconstruction.

[061] The present invention provides methods and compositions for tracking the fate and distribution of QD-labeled stem cells utilized for stimulating cardiomyocyte proliferation. The method comprises (i) administering an effective amount of QD-labeled stem cells to the heart; and (ii) determining the fate and distribution of said QD-labeled stem cells following administration. Accordingly, the present invention provides a method for treating a subject afflicted with a cardiac disorder comprising administering QD-labeled MSCs to said subject. The stem cells may be administered and/or transplanted to a subject suffering from a cardiac disease in any fashion known to those of skill in the art. Additionally, the stem cells to be transplanted may be genetically engineered to express molecules capable of stimulating cardiomyocyte proliferation such as, for example, Wnt-5A.

[062] Various delivery systems are known and can be used to administer a compound capable of regulating cardiomyocyte proliferation. Such compositions may be formulated in any conventional manner using one or more physiologically acceptable carriers optionally comprising excipients and auxiliaries. Proper formulation is dependent upon the route of administration chosen.

[063] The methods of the invention, comprise administration of QD-labeled MSCs in a pharmaceutically acceptable carrier, for treatment of cardiac disorders.

“Administering” shall mean delivering in a manner which is effected or performed using any of the various methods and delivery systems known to those skilled in the art. Administering can be performed, for example, pericardially, intracardially, subepicardially, transendocardially, via implant, via catheter, intracoronarily,

intravenously, intramuscularly, subcutaneously, parenterally, topically, orally, transmucosally, transdermally, intradermally, intraperitoneally, intrathecally, intralymphatically, intralesionally, epidurally, or by *in vivo* electroporation.

Administering can also be performed, for example, once, a plurality of times, and/or over one or more extended periods.

[064] The term “pharmaceutically acceptable” means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term “carrier” refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in “Remington’s Pharmaceutical sciences” by E.W. Martin. Such compositions will contain a therapeutically effective amount of the therapeutic compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

[065] The appropriate concentration of the composition of the invention which will be effective in the treatment of a particular cardiac disorder or condition will depend on the nature of the disorder or condition, and can be determined by one of skill in the art using standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses maybe extrapolated from dose response curves derived from *in vitro* or animal model test systems. Additionally, the administration of the compound could be combined with other known efficacious drugs if the *in vitro* and *in vivo* studies indicate a synergistic or additive therapeutic effect when administered in combination.

[066] The present invention provides methods and compositions for tracking the fate and/or distribution of QD-labeled MSCs administered to a subject for treatment of a particular cardiac disorder or condition. Following administration of said cells using the methods outlined above, their distribution and fate may be determined using a variety of different methods well known to those of skill in the art. In a preferred embodiment of the invention tissue samples are removed from the treated subject to determine the spatial distribution of the QD-labeled cells. Removal of such samples may be performed, for example, surgically, at different time intervals following administration. In an embodiment of the invention the sample is removed from between 1 day to 2 months.

[067] In an embodiment of the invention, tissue samples are removed from the treated subject and analyzed to determine the distribution and fate of the QD-labeled

stem cells using routine histological methods. For microscopy, histologic sections are preferably immobilized on a solid support. Any solid support can be used, with exemplary solid supports including microscope slides, wall surfaces of reaction wells, test tubes, and cuvettes, and beads. The solid support can be formed of any material known to be suitable to those skilled in this art, including glass, polystyrene, polyethylene, polypropylene, and cross-linked polysaccharides. Preferably, the sample is fixed to a glass microscope slide. The sample can be fixed to the solid support by any suitable procedure, such as air-drying or chemical or heat treatment that does not interfere with subsequent observation of the sample. It is preferred that the slide be immobilized in such a manner that it can be observed by light microscopy.

[068] The prepared sample slides can be analyzed using known fluorescent techniques, such as fluorescent microscopy. For example, the sample can be viewed using a photomicroscope equipped with an ultraviolet (UV) source such as a mercury or xenon lamp and appropriate filters, and the images photographed using conventional techniques. The cells are illuminated with a UV light source, which is the source of excitation, and must be capable of producing specific wavelengths that can be used to excite the QD-labeled cells of the invention.

[069] In a preferred embodiment of the invention, custom filters may be used to preferentially excite the QDs at a wavelength of emitted light. This is possible because QDs have a large Stokes shift, i.e., distance between wavelength of excitation and wavelength of emission, whereas this is not possible with traditional fluorophores because of the closeness in peaks of excitation and emission and the overlap in these spectra. The custom filters are designed to collect a very narrow beam of emitted light at the peak of the spectrum, so any light coming from auto-fluorescence is excluded. QD-

MSCs can also be detected using flow cytometry and labeled cells can be sorted using fluorescence activated cell sorting (FACS).

[070] In a specific embodiment of the invention, the spatial locations of QD labeled cells can be identified and from a series of binary maps visualized in 3-D. For reconstructing the 3-D distribution of injected QD-hMSCs, tissue is processed as serial transverse 10- μ m-thick sections and imaged for both QD (655-nm) fluorescence and phase contrast on the Axiovert deconvolution microscope with the 2.5X objective. Using Axiovision software, fluorescence and phase images for each section are merged to generate jpg images. The remaining image processing is executed in Matlab. The lines of code are attached as Table I. The remaining image processing is executed in Matlab. Phase contrast features echoed in each serial section are identified and the coordinates are used to spatially register the images with respect to one another. These registered RGB jpgs are converted to HSV format and the saturation and value channels are used to create new intensity images bearing only the QD-positive regions. The images are then thresholded to generate binary maps, where white pixels represent all of the QD-positive zones. The binary maps for all of the serial sections are combined into a 3-D matrix, and the total area (or volume) of white pixels is computed. High-resolution images (63X) are obtained and areas of single cells are determined. Thus, the number of cells in the reconstruction is calculated by dividing the total 3-D area by the average area of QDs per cell in a section. The centroid is determined for each individual polygon in the volume matrix, and then weighted to the polygon volume to find the centroid of the total cell mass. Next, the distance is calculated between each individual cluster and the centroid of the cell mass to characterize the distribution of QD-hMSCs in the tissue. The distribution is visualized in 3-D by extracting isosurface

data from the volume matrix and composing patch graphics objects for each of the continuous polygons in the matrix.

[071] In yet another embodiment of the invention, QD-labeled cells may be detected *in vivo* using Computer Tomography (CT) Scanning.

[072] Additionally, the progress of the recipient receiving the treatment may be determined using assays that are designed to test cardiac function. Such assays include, but are not limited to ejection fraction and diastolic volume (e.g., echocardiography), PET scan, CT scan, angiography, 6-minute walk test, exercise tolerance and NYHA classification.

6. EXAMPLE: BIOLOGICAL FEATURES OF QD LABELED MESENCHYMAL STEM CELLS

[073] The experiments described below include *in vitro* validation of hMSC loading with QDs, *in vivo* delivery of QD-hMSCs to rat and canine hearts, and development of custom computer algorithms to reconstruct the 3-D distribution of hMSCs *in vivo*.

6.1 MATERIAL AND METHODS

[074] **Computer Algorithms.** Custom algorithms were designed and executed in Matlab 6.5 and 7.0 (MathWorks, Natick, MA). Images were obtained on the Zeiss Axiovert microscope using the 2.X, 10X, or 63X objective and 655-nm custom filter set described above, and then converted to jpg using Axiovision software (ver 4.3, Carl Zeiss Vision, Germany). For QD cluster analysis, QD fluorescence intensity images (63X) were obtained from cells at various time points after loading. Images were thresholded based on Otsu's method, to minimize the intraclass variance between black and white pixels. The individual binary image clusters were identified and their major axis lengths were determined in order to calculate cluster diameters. Corresponding

nuclear images of Hoechst 33342 staining were used to compute the number of cells in the image fields. Finally, the number of QD clusters per cell was calculated for each time point.

[075] A routine was developed for reconstructing the 3-D distribution of QD-hMSCs injected into rat hearts. Tissue was processed as described above and 222 (rat terminated at 1 hour) or 126 (rat terminated at 1 day) serial transverse 10- μ m-thick sections were imaged for both QD (655-nm) fluorescence and phase contrast on the Axiovert deconvolution microscope with the 2.5X objective. Using Axiovision software, fluorescence and phase images for each section were merged to generate jpg images. The remaining image processing was executed in Matlab. Phase contrast features echoed in each serial section were identified and the coordinates were used to spatially register the images with respect to one another. These registered RGB jpgs were converted to HSV format and the saturation and value channels were used to create new intensity images bearing only the QD-positive regions. These were then thresholded to generate binary maps, where white pixels represent all of the QD-positive zones. All of the QD-positive cells that were not contained within the cardiac syncytium (cells remaining in the adhesive on the epicardial surface) were eliminated from the reconstructions. The binary maps for all of the serial sections were combined into a 3-D matrix, and the total area (or volume) of white pixels was computed. High-resolution images (63X) were obtained and areas of single cells were determined similar to the method described above. Thus, the number of cells in the reconstruction was calculated by dividing the total 3-D area by the average area of QDs per cell in a section. The centroid was determined for each individual polygon in the volume matrix, and then weighted to the polygon volume to find the centroid of the total cell mass. Next, the distance was calculated between each individual cluster and the

centroid of the cell mass to characterize the distribution of QD-hMSCs in the tissue. The distribution was visualized in 3-D by extracting isosurface data from the volume matrix and composing patch graphics objects for each of the continuous polygons in the matrix.

[076] Cell culture. Human mesenchymal stem cells (hMSCs) were obtained from Clonetics/BioWhittaker (Walkersville, MD) and passages p3-p7 were used for all *in vitro* and *in vivo* experiments. Cells were grown on polystyrene tissue culture dishes and maintained at 37°C in humidified 5% CO₂ in Mesenchymal Stem Cell Growth Media supplemented with L-glutamine, penicillin and serum (MSCGM BulletKit, Cambrex). Cells were re-plated for passaging once every two weeks. For isolation of canine cardiac myocytes, adult mongrel dogs were intravenously injected with 80mg/kg body weight sodium pentobarbital according to an approved protocol. Hearts were then removed and placed in a cold, high-potassium Tyrode solution [16]. Myocytes were isolated using a modified Langendorff system with digestion via Worthington type II collagenase [17], cultured onto laminin-coated glass coverslips and maintained in Dulbecco's Modified Eagle Medium (DMEM) with 1% penicillin/streptomycin.

[077] Quantum dot loading. Three approaches were used for loading hMSCs with QDs. First, a nucleofection protocol was followed to electroporate approximately 5×10^5 hMSCs in 8.2 nM QDs (Qdot 655 ITK Carboxyl Quantum Dots, Invitrogen Cat. No. Q21321MP) solution (supplemented Human MSC Nucleofector Solution, Amaxa Biosystems, Cat. No. VPE-1001). After electroporation, cells were re-plated in complete MSCGM media onto polystyrene tissue culture dishes. Second, a commercially available kit was used to load the cells with QDs via a carrier protein (Qtracker 655 Cell Labeling Kit, Invitrogen Cat. No. Q25021MP). Briefly, 10nM of

labeling solution was prepared according to kit directions, and approximately 0.2mL was added to a 100-mm polystyrene tissue culture dish containing roughly 5×10^5 cells. The cells were incubated at 37°C for 45-60 minutes, after which time they were washed twice with complete MSCGM. The third (and optimal) loading technique will be referred to as passive loading. Cells were grown to 85% confluence on polystyrene tissue culture dishes. An 8.2nM solution of 655 ITK Carboxyl QDs was prepared in complete MSCGM and vortexed for 60 seconds. Cells were washed once in phosphate-buffered saline (PBS) and incubated in the QD solution for up to 24 hours at 37°C.

[078] Quantum dot validation and mechanistic experiments. After the incubation period, cells were washed twice in PBS and fresh MSCGM was replaced. Loading efficiency was analyzed visually from a set of images of QD-hMSCs as well as by flow cytometry. In the first method, 181 cells from four QD fluorescence and phase contrast overlay images were studied and identified as either QD-positive or negative. Loading determination via flow cytometry was assessed using the following protocol: hMSCs were loaded with QDs for 24 hours as described above. After the loading period, cells were washed twice in PBS, trypsinized and resuspended in PBS with 5% FBS. Cells were then stained with 7-amino-actinomycin D (to determine viability) and subsequently analyzed using a LSR II true multiparameter flow cytometer analyzer (BD Biosciences with custom 655-nm filter). Four sets of QD-hMSCs (and unloaded hMSCs for control), each containing a minimum of 17,000 cells, were analyzed. The intensity range for control cells was set such as to include at least 98% of the viable cells. The same technique was used to scan the QD-hMSCs and determinations of QD-positive status were based on viable cells in the intensity range above that set for control.

[079] A number of additional *in vitro* experiments were performed. To determine the degree of loading after repeated cell divisions, cells were passaged three times at 1:4 for a minimum of 5 divisions over 44 days. For one set of experiments intended to determine the mechanism of loading, hMSCs were passively exposed to QD incubation medium for 7 hours at either 4°C or 37°C. In another approach, cells were passively exposed to QD incubation medium for 12 hours either in MSCGM or 125µM colchicine (Sigma, Prod No C9754) in MSCGM. To determine if canine cardiac myocytes (cCMs) would take up QDs, cultured myocytes were incubated for up to 24 hours in DMEM to which the lysate from approximately 10⁴ QD-hMSCs was added.

[080] **Proliferation assay.** A population of hMSCs was evenly split for passaging and both dishes were grown to ~85% confluence. One dish was passively loaded with QDs as described above while the other received a media exchange. The following day (after 24 hours of loading), both dishes were washed and re-plated at equal concentrations into 12 wells each of a 96-well dish and cells were allowed to grow for 3 days. The mitochondrial dehydrogenase assay (MDA, KKBiomed) was carried out according to instructions provided by the company. The absorbance of each of the samples was measured at 595-nm using a Polarstar OPTIMA microplate reader (BMG Technologies).

[081] **Differentiation experiments.** Induction was performed using adipogenic, chondrogenic and osteogenic kits available through Cambrex (Adipogenic Differentiation Medium, PT-3004; Chondrogenic Differentiation Medium, PT-3003; Osteogenic Differentiation Medium, PT-3002). All experiments were performed in triplicate on both QD-hMSCs and hMSCs. For adipogenesis, labeled and unlabeled cells were plated at 2x10⁴ cells/cm² tissue culture surface area and fed every 2-3 days

with MSCGM until cultures reached 100% confluence (5-13 days). Cells were fed on the following regime for a total of 3 cycles: 3 days with supplemented Adipogenic Induction Medium followed by 1-3 days with Adipogenic Maintenance Medium. Control hMSCs were fed with Adipogenic Maintenance Medium at all times. After the 3 cycles, all cells were cultured for another week in Adipogenic Maintenance Medium. Cells were analyzed using light microscopy for characteristic lipid vacuole formation. Matlab algorithms were designed to determine percent of images occupied by adipocytes. For osteogenesis, cells were plated at 3×10^3 cells/cm² tissue culture surface area and cultured overnight in MSCGM. Cells were then fed with Osteogenesis Induction Medium with replacement media every 3-4 days for 2-3 weeks. Non-induced control cells were fed with MSCGM on the same schedule. Cells were analyzed using light microscopy for characteristic cobblestone appearance.

[082] Gene transfections. For some experiments, hMSCs were transfected with pIRES-EGFP (4 μ g, Figure 3), HCN2-pIRES-EGFP (4 μ g, Figure 3), or Wnt5A (4 μ g, pUSEamp, Upstate Cell Signaling Solutions, Figure 4) plasmids using the Amaxa biosystems nucleofection technique[10].

[083] Patch clamping. Whole cell patch clamping was executed as previously described [10]. Patch electrode resistance was 4 to 6 M Ω . The pipette solution was filled with (in mM) K-aspartate 120, Mg-ATP 3, EGTA 10, and HEPES 5 (pH adjusted to 7.2 with KOH). The external solution contained (in mM) NaCl 137.7, KCl 5.4, NaOH 2.3, CaCl₂ 1.8, MgCl₂ 1, Glucose 10, and HEPES 5 (pH adjusted to 7.4 with NaOH). Recordings were made at room temperature.

[084] Visualization of QD-hMSCs. *In vitro* experiments were performed in polystyrene tissue culture dishes. For typical visualization, cells were re-plated onto

CC2-coated glass chamber slides (Lab-Tek). Several hours after re-plating, slides were rinsed in PBS and then fixed in 4% paraformaldehyde (PFA) for 15 minutes. Slides were rinsed again in PBS for 5 minutes, and then incubated in 1 μ M Hoechst 33342 nuclear dye (Cambrex) for 20 minutes. They were then washed in PBS for 5 minutes and placed in dH₂O for 20-30 seconds. The slides were rinsed successively in 30%, 70%, 95% and 100% ethanol each for 30 seconds and then placed in 100% toluene for 30 seconds. Finally, slides were mounted in CytosealTM60 (Electron Microscopy Systems) containing 1% triocetylphosphine (TOP, Sigma), with coverslips allowed to set overnight. Images were acquired on an inverted Zeiss Axiovert deconvolution microscope with AxioCam MRm CCD camera using a filter customized for 655-nm QD emission (Omega Optical, XF3305, excitation at approximately 420-nm); the DAPI filter set was used to visualize Hoechst 33342-stained nuclei. For some images, z-stacks were obtained at multiple focal planes and subsequently deconvolved using AxioVision (ver 4.3, Carl Zeiss Vision, Germany). These stacks were then reassembled (using the same software) into single 2-D images based on fluorescent pixels deemed most in plane at each section. All additional image processing was carried out using custom Matlab algorithms (Matlab 6.5 and 7.0, MathWorks, Natick, MA) or in ImageJ (ver 1.32j, NIH). For some experiments, imaging was performed on live cells using an Olympus inverted fluorescence microscope (Olympus IX51, DP70 camera) with GFP and Texas Red (for QD imaging) filter sets.

[085] *In vivo* Studies. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 85-23, revised 1985).

[086] Patch preparation. hMSCs were loaded with QDs as described above and subsequently transfected with the Wnt5A plasmid. A 15x30x0.1mm acellular ECM patch (porcine urinary bladder matrix, ACell, Jessup, MD) was rinsed twice in PBS for 10 minutes each. The patch was then soaked in MSCGM for 15 minutes, after which time the media was removed. QD-Wnt5A-hMSCs were trypsinized, resuspended in MSCGM and seeded directly onto the ECM. The patch was returned to 37°C for approximately 12 hours prior to implantation.

[087] Canine patch implants. Patches were implanted as described previously [18]. Briefly, a thoracotomy was used to expose the heart. A vascular clamp was then used to isolate a region of the right ventricular free wall. A full thickness defect was surgically induced and an hMSC-seeded scaffold was used to replace it. The chest was closed and the animal was allowed to recover. Animals were sustained under veterinary care and humanely terminated by an approved protocol at 8 weeks with pentobarbital.

[088] Rat heart injections. QD-hMSCs were prepared as described above. 24 hours after QD incubation, cells were washed twice in PBS, trypsinized and re-suspended for a final cell concentration of approximately 10^5 cells/10 μ L in DMEM at 4°C. The cell solution was stored on ice until injection. Rats (5-months-old, Charles River) were anesthetized with ketamine/xylazine intraperitoneally, intubated and maintained on inhaled isofluorane (1.5-2%) for the duration of the experiment. A left thoracotomy was performed at the 4th or 5th intercostal space. A 5-0 prolene suture was used to place a superficial stitch in the epicardium as a fiducial marker. 10 μ L of cell solution or cell lysate was injected into the free left ventricular wall apical to the suture and then a small drop of surgical grade tissue adhesive (Nexaband, JA Webster) was applied over the injection site. The thorax was closed and rats were returned to

their cages for either 1 hour or 1 day for whole cell injections, or either 1 hour or 1 week for the lysed QD-hMSC injections. Euthanasia was performed either in a CO² chamber or by administering pentobarbital (100 mg/kg body weight injected intraperitoneally) and subsequent cardiectomy.

[089] Preparation of tissue samples. Immediately after explantation, tissue samples were rinsed in isotonic saline and then fixed in 4% PFA for 24 hours. After fixation, tissue was cryopreserved in an isotonic 30% sucrose solution for at least 24 hours. Gross photographs were obtained of tissue samples with sutures in situ to identify the cell delivery zone (either patch borders or injection site). After suture removal, tissue was embedded in freezing matrix (Jung tissue embedding matrix, Leica) and stored at –20°C. 10-µm tissue sections were cut on a cryotome, transferred to Suprafrost glass slides and stored at –20°C. Slides were either imaged without mounting, or stained with Hoechst 33342 dye and mounted as described above.

[090] Immunohistochemistry. Staining for CD31 was performed as follows: 10-µm histologic sections were hydrated with PBS, and permeablized with 0.5% Tween-20 in 1X TBS followed by 0.25 % Triton X-100 in TBS. Samples were blocked with normal horse serum (Vector, Burlingame, CA) and then incubated for 3 hours in FITC-conjugated anti-human CD31 (Diacclone, Stamford, CT). Control sections were incubated in PBS instead of the antibody. Sections were washed in PBS, incubated with 1 µM Hoescht33342 for 20 minutes (Invitrogen, Carlsbad, CA), then washed in PBS before mounting with Vectashield (Vector, Burlingame, CA).

[091] Statistics. All data are listed as mean ± standard deviation. Data sets were compared by a Student's t-test with p<0.05 considered significant.

6.2. RESULTS

[092] Loading of hMSCs is optimized by passive incubation with negatively charged QDs and is blocked by inhibitors of endocytosis. Optimal use of QDs for tracking hMSCs requires nearly 100% cell survival after loading and that loaded cells behave similarly to unloaded cells. Potential approaches to loading populations of cells include electroporation[19], lipid vehicles[19-21] and passive (receptor-mediated or unmediated) incubation[22-27]. Loading using QDs with either positively or negatively-charged surface conjugations was examined using these methods . Electroporation was least effective, loading only a small fraction of the hMSC population and causing appreciable cell death. Receptor-mediated uptake was more effective (Figure 1A) but still non-uniform. Unfortunately, both methods resulted in marked aggregation of the QDs in the perinuclear spaces of the hMSCs over time. Passive incubation with naked QDs (655-nm peak emission wavelength) having carboxylic acid derivitizations on their polymer surfaces (net negatively charged) was most effective (Figure 1B, 1C). The pattern of loading was diffusely cytoplasmic (Figure 1D). Virtually all hMSCs were loaded (>98% of 181 cells analyzed visually in four images and >96% of >17,000 cells per set analyzed by flow cytometry, N=4 sets, Figure 1E). When incubations were attempted using smaller-core-sized QDs (525-nm peak emission wavelength) with either positively or negatively-charged surface conjugations, similar levels of loading where not achieved. The mechanism of QD loading was investigated by employing two protocols that reduce endocytosis: exposure to low temperature for 7 hours (4°C) and application of colchicine (125 µM, an inhibitor of microtubule aggregation[28]) for 24 hours prior to and during incubation with QDs (Figure 1F). In each case there was a dramatic reduction in QD uptake. Finally, the time course of QD loading was investigated by passively incubating cells with QDs for 1, 3, 7, 12 and 24 hours. Intracellular QDs were barely detectable at 1 hour of

incubation (Figure 1G), easily detectable after 3 hours (Figure 1H) and quite bright at 24 hours (Figure 1I), prompting us to choose an incubation range of 12-24 hours for most experiments. A summary of conditions used to optimize loading is shown in Table 1.

[093] QD-loaded hMSCs continue to proliferate and retain label for more than 6 weeks *in vitro*. To be useful for stem cell tracking, intracellular QDs must not interfere with cellular function or proliferation. QD-hMSCs were studied for up to 44 days *in vitro*. During this time period the cells divided at least five times (consistent with the proliferative behavior of unloaded hMSCs, see below) and retained sufficient label to be easily imaged (Figure 2A-C). The intracellular QD cluster sizes were stable over this period ($0.84 \pm 0.11\mu\text{m}$, $0.91 \pm 0.21\mu\text{m}$, $0.94 \pm 0.13\mu\text{m}$, average cluster diameters for 2, 16 and 44 days after loading respectively) and the distribution remained cytoplasmically diffuse. As shown in Figure 2D, a proliferation assay on QD-hMSCs and plain hMSCs revealed no difference between the two groups (0.1405 ± 0.0165 a.u. vs. 0.1186 ± 0.0230 a.u. respectively, $p > 0.05$, $N = 12$ per group).

[094] QDs do not transfer to adjacent cells. To prevent the occurrence of false positives, a tracking agent must not transfer from labeled to unlabeled stem cells. The only direct path of contact between the intracellular space of one cell and that of another is the gap junction channel. It was previously demonstrated that hMSCs express connexins 43 and 40 and form functional gap junctions when placed in close apposition[29]. An experiment was designed to investigate possible transfer of QDs from loaded to unloaded hMSCs. QD-hMSCs were co-cultured with hMSCs transfected to express green fluorescent protein (GFP-hMSCs). The co-culture was

grown to near confluence and GFP-hMSCs in close proximity to QD-hMSCs were imaged, as depicted in Figure 2E.

[095] No evidence of internalized QDs in GFP cells was observed in four experiments. This is consistent with the known diameters of QDs (~10nm) and gap junction channels (~1nm).

[096] **QDs are not taken up by adult cardiac myocytes in culture.** hMSCs have been shown to enhance cardiac regeneration in animal trials[4]. If QDs are used to track the fate of stem cells delivered to the heart, myocytes must not take the dots up from the extracellular space should these exogenous cells die in their vicinity. To simulate the *in vivo* situation of dying hMSCs, cultured cardiac myocytes were exposed to the cell lysate from mechanically disrupted QD-hMSCs for 24 hours. Figure 2F provides one example, demonstrating that the myocytes did not take up QDs. An equivalent control was performed using lysed cells *in vivo*, which is discussed below.

[097] **QD-loaded hMSCs can be transfected to overexpress genes.** Because hMSCs are an attractive vehicle for gene delivery to the heart[10], it was investigated whether the presence of intracellular QDs would affect expression of exogenous genes. QD-hMSCs were transfected with the HCN2-pIRES-EGFP plasmid. The HCN2 gene expresses a time dependent inward current, which is the basis of cardiac pacemaker activity. This plasmid was previously used with hMSCs as the cellular vehicle to create a biological pacemaker in the canine heart[10]. After 48 hours, QD-loaded cells were visualized for GFP expression and compared to control hMSCs that underwent the same transfection protocol but were not first exposed to QDs. GFP-positive cells from each group were then selected for patch clamping to record membrane currents (Figure 3a and 3b). QD-hMSCs expressed the HCN2 gene and generated a family of pacemaker

currents similar to those recorded in unloaded cells. The current amplitudes recorded at -150mV for both control and QD-hMSCs were -1459.48 ± 616.83 and -1352.68 ± 864.70 respectively ($p > 0.05$, $N = 5$ per group).

[098] Intracellular QDs do not interfere with differentiation potential of hMSCs *in vitro*. hMSCs are one of several stem cell types being studied for use in tissue repair and regeneration. We queried whether the presence of intracellular QDs would affect the ability of hMSCs to differentiate along mesodermal lineages. We cultured QD-loaded and unloaded hMSCs under conditions of adipogenesis or osteogenesis. After 23 days of adipogenic induction, both unloaded and QD-loaded hMSCs showed similar levels of differentiation (44.9% and 40.4% area occupied by adipocytes respectively for fields of view shown in Figure 5a and b). Furthermore, terminally differentiated adipocytes originating from QD-loaded hMSCs retained the QD label (Figure 5c). Both unloaded and QD-loaded hMSCs responded to the osteogenic induction similarly, with both groups of cells showing characteristic changes in morphology from spindle shaped to cobblestone shaped and tendency toward clustering by day 15 (Figure 5d and e respectively). Again, cobblestone-shaped osteocytes derived from QD-hMSCs still contained QDs at the end of the differentiation process (Figure 5f).

[099] QD-hMSCs can be implanted into canine ventricle and identified up to 8 weeks later. Both cellular and functional cardiac regeneration was previously observed after replacing a full thickness right ventricular defect in the canine heart with an acellular extracellular matrix (ECM) patch derived from porcine urinary bladder[18]. If a naked ECM patch induces regeneration it might be possible to enhance the regeneration process by delivering hMSCs on a patch. Therefore, ECM patches (~15x30x0.1 mm) seeded with QD-hMSCs were implanted, the animals were

terminated 8 weeks after implantation and a region of myocardium circumscribing the patch implant area was excised (Figure 4A and 4C). Transmural sections (10- μ m) within the patch region were imaged to identify QD-hMSCs (Figure 4B). Figure 4B illustrates that QD fluorescence can be imaged in tissue without any detectable contribution from background autofluorescence. Further, individual hMSCs can be easily imaged and continue to display a diffuse cytoplasmic pattern of QD fluorescence (Figure 4B, inset).

[0100] QDs do not affect differentiation of hMSCs *in vivo*. hMSCs have been found to spontaneously differentiate along an endothelial lineage and participate in angiogenesis in response to tissue injury[31]. We sought to determine whether the presence of QDs in these cells would affect their ability to develop an endothelial fate. Histologic sections from the 8-week QD-hMSC ECM patch explant were stained for the marker CD31 (PECAM-1) using a human-specific antibody. Many of the QD-containing regions in these sections stained positively for CD31 (Figure 5g), with several areas showing clear co-localization (Figure 5g inset), suggesting that these cells were differentiating along an endothelial lineage. As a means of control, endogenous canine endothelium from the same tissue sections were negative for the marker (Figure 5h), as were cultured hMSCs *in vitro* (Figure 5j), whereas human endothelial cells in culture stained positively (Figure 5i).

[0101] QDs are not internalized by cardiac cells *in vivo*. A set of experiments were performed to determine whether native myocardial cells internalize QDs *in vivo*. QDs can exist extracellularly if QD-hMSCs die and leak their contents. Therefore, a suspension of approximately 100,000 QD-hMSCs that were mechanically disrupted to cause cell lysis were injected into the rat ventricle and the animals were terminated at

either 1 hour (N=2) or 1 week (N=2). QDs were not observed in any cell type in these hearts. This finding is expected, as free carboxylated QDs will be removed by the reticuloendothelial system in less than one hour[31].

[0102] The number and distribution of QD-hMSCs injected into a rat heart can be reconstructed in 3-D. One million hMSCs had previously been injected into canine myocardium to create a biological pacemaker and traditional means of identifying these cells in histologic sections were used (GFP and secondary staining)[10].

Although it was possible to demonstrate the presence of some of our delivered cells, it was not possible to reconstruct their three-dimensional locations. Such added information could help to understand how these HCN2-transfected stem cells generated pacemaker activity and to assess their potential to develop unwanted arrhythmic events. With this need in mind, a series of experiments were performed in rats to enumerate delivered cells *in vivo* and reconstruct their spatial distribution in three dimensions. Approximately 100,000 QD-hMSCs were injected into the left ventricular free wall. Hearts were harvested at either 1 hour or 1 day after injection. Serial 10- μ m transverse sections were imaged for QD fluorescence (Figure 5A and 5E). Using algorithms described in Supplementary Methods, these fluorescence images were filtered and thresholded to generate binary maps of QD-positive zones from all of the tissue sections (Figure 5B). The spatial locations of QD-hMSCs were identified from the series of binary maps and visualized in 3-D (Figure 5C and 5D). **The algorithms described herein** permit enumeration of the total number of QD-hMSCs in the whole heart from these models (approximately 50,000 at 1 hour and 30,000 at 1 day). A distance parameter was also computed to characterize the distribution of cells, based on the distance between individual cells and the centroid of the total stem cell mass. Most of

the cells were clustered in close proximity (85% of cells within 1.5mm at 1 hour and 95% within 1.5mm at 24 hours, see Figure 5E).

[0103] QDs do not interfere with differentiation capacity of hMSCs *in vitro* or *in vivo*. QD-hMSCs or unloaded hMSCs were induced to differentiate *in vitro* along adipogenic and osteogenic lineages. After the adipogenic induction period both (a) unloaded hMSCs and (b) QD-hMSCs displayed characteristic adipocyte morphology, with prominent lipid vacuoles. The percent of differentiated versus undifferentiated cells was similar between these two groups. (c) At high power, adipocytes from the QD-hMSC group are seen with QDs (red fluorescence) interspersed between lipid vacuoles. After the osteogenic induction period both (a) unloaded hMSCs and (b) QD-hMSCs that were initially spindle shaped adapted a more cobblestone-like morphology typical of osteocytes and tended to cluster on the dish. (f) At high power a cobblestone-shaped osteocyte from the QD-hMSC group retains the QD label (red) after the differentiation process. QD-hMSCs were delivered *in vivo* to the canine ventricle on an ECM patch. (g) After 8 weeks, some of these QD-positive cells (red) express the endothelial marker PECAM-1 (green), suggesting differentiation of these cells along and endothelial lineage. A high-power view of QD-positive cell with co-localized PECAM-1 expression is shown in (g, inset). Scale bars on a,b,d,e = 200 μm Scale bars on c,f = 50 μm Scale bar on g = 20 μm , inset = 5 μm

[0104] Computer Tomography (CT) Scanning of QDs. QDs are semiconductor nanoparticles comprised of a CdSe core and ZnS shell. Because of the very high densities of these materials (5.816g/cm³ and 4.09g/cm³ respectively), it was investigated whether QD-hMSCs could be imaged using computed tomography (CT) scanning. In order for QD-hMSCs to be detected within a block of tissue, two criteria

must be satisfied: 1) the resolution of the CT scanner must be sensitive enough to detect single cells (mean diameter, 10 μ m) and 2) the overall physical density of a QD-loaded hMSC must be at least 10% higher than the physical density of the surrounding tissue. Currently, micro CT (μ CT) scanners are available with resolutions as low as 1 μ m.

[0105] After uptake by hMSCs, QDs exist within the cells in clusters with an average diameter of 0.75 μ m. Prior to cell division the average cell contains approximately 200 of these QD clusters, as determined by fluorescence imaging. Since an individual cluster will occupy approximately 0.22 μ m³ in the cell, the total volume of QDs in a given cell is roughly 44 μ m³. An average hMSC has a volume of approximately 500 μ m³. Therefore, based on these calculations, QDs occupy approximately 9% of the volume of the cell. This is a low-end estimate of the percent volume, with alternate calculations yielding a value as high as 25%. Assuming a cell and tissue density of 1.05g/cm³, the expected overall physical density of QD-hMSCs should range from 1.40-2.00g/cm³. These densities are well above the threshold for detection of the μ CT scanners.

7. EXAMPLE : USE OF QDs TO TRACK LABELED MSCS NON-INVASIVELY IN VIVO

[0106] The long term future of stem cell therapies will depend on the ability to track non-invasively. Both the core of the QD and the passivating shell contain metal ions (cadmium and zinc respectively). Metals are radiopaque, meaning they do not allow penetration of x-ray waves, and can therefore be imaged using x-ray technology. Traditional x-rays are too large to interfere with nano- or micro-scaled metals. However, since they are radiopaque, it may be possible to use micro computed tomography (μ CT) scanning to image QDs *in vivo*.

7.1. MATERIALS AND METHODS

[0107] ***Preparation of cells for phantom.*** QD-hMSCs were prepared as described above. After 24 hours, cells were washed and visualized to confirm QD loading (Figure 7a). Cells from the QD-hMSC group and a separate control unloaded hMSC group were each trypsinized and centrifuged at 1000g for 4 minutes in polypropylene tubes to prevent adhesion of cells to tube walls. The pellets were resuspended in MSCGM and the solution was then centrifuged at 1500g for 5 minutes. Pellets were incubated at 37°C overnight. After the incubation period and without breaking them up, pellets were gently washed in PBS and fixed in 4% paraformaldehyde for 2 hours.

[0108] ***Creation of phantom mold.*** A curable siloxane compound was prepared by mixing vinylmethylpolysiloxane (GE silicones RTV615A, s.d.1.02 g/cm³) and vinyl MQ resin (GE silicones RTV615B, s.d.0.99 g/cm³) in a 10:1 ratio and stirring for 5 minutes. The mixture was then poured into two wells of a 4-well chamber slide to cover the bottom of the well and cured at 50°C for 2 hours. The cell pellets were placed in each well and additional siloxane mixture was poured over the top to complete cover the pellets. The materials cured overnight at room temperature (Figure 7b).

[0109] ***μCT scanning.*** High resolution (9μm) μCT scanning (Scano Medical μCT 40, Basserdorf, Switzerland) was used to visualize cell pellets within the siloxane mold. Individual 2D images from the unloaded hMSC and QD-hMSC molds were visualized and densities in the cell pellet regions were measured from image intensities (12 and 16 images sampled respectively). A constrained 3D Gaussian filter was applied to reduce noise in the images (“support”= 9, “sigma”= 5). Cell pellet regions were segmented from the surrounding siloxane by thresholding (lower threshold = 124, upper threshold =1000). The 3D reconstruction was generated from the stack of binarized images.

[0110] *Statistics.* All data are listed as mean $\pm$ standard deviation. Data sets were compared by a Student's t-test with $p < 0.05$ considered significant.

7.2. RESULTS

[0111] QDs are semiconductor nanoparticles comprised of a CdSe core and ZnS shell. Because of the very high densities of these materials (5.816g/cm³ and 4.09g/cm³ respectively), it was investigated whether QD-hMSCs could be imaged using micro-computed tomography (μ CT) scanning. In order for QD-hMSCs to be detected within a block of tissue, two criteria must be satisfied: 1) the resolution of the CT scanner must be sensitive enough to detect single cells (mean diameter, 10 μ m) and 2) the overall physical density of a QD-loaded hMSC must be at least 10% higher than the physical density of the surrounding tissue. Currently, micro CT (μ CT) scanners are available with resolutions as low as 1 μ m. The scanner used herein is a μ CT scanner with 6 μ m resolution which should satisfy the first criterion listed above.

[0112] As described above, after uptake by hMSCs, QDs exist within the cells in clusters with an average diameter of 0.75 μ m. Prior to cell division the average cell contains approximately 200 of these QD clusters, as determined by fluorescence imaging. Since an individual cluster will occupy approximately 0.22 μ m³ in the cell, the total volume of QDs in a given cell is roughly 44 μ m³. An average hMSC has a volume of approximately 500 μ m³. Therefore, based on these calculations, QDs occupy approximately 9% of the volume of the cell. This is a low-end estimate of the percent volume, with alternate calculations yielding a value as high as 25%. Assuming a cell and tissue density of 1.05g/cm³, the expected overall physical density of QD-hMSCs should range from 1.14-2.00g/cm³. These densities are well above the threshold for detection of the μ CT scanner utilized.

[0113] Based on these findings, it is theoretically feasible for QD-hMSCs to be detected with μ CT in both explanted tissue samples and in living animals.

[0114] Both unloaded hMSC and QD-hMSC pellets were detectable within the siloxane mold (Figure 7c) as areas of hypointensity. Regions of interest within the pellet area from sample images in each group were selected in order to measure densities (N=12 for unloaded hMSC, N=16 for QD-hMSC). Density measurements were normalized to the average density of the unloaded hMSC pellet. The values were 1.000 ± 0.103 and 1.276 ± 0.039 for unloaded and QD-hMSC pellets respectively (Figure 7d). A 3-D reconstruction of the QD-hMSC pellet was generated from the stack of images (Figure 7e).

[0115] The development of a tracking agent that can visualize delivered cells in vivo non-invasively with high resolution is highly desirable. Existing techniques for non-invasive tracking of stem cells include loading cells with radioactive substances like truncated thymidine kinase for positron emission tomography (PET) detection or radiometals for single photon emission computed tomography (SPECT). Concerns have arisen, however, over the uptake of the label by host tissue, endogenous tissue photon attenuation and high levels of label needed for detection. Another approach is the transfection of cells with the gene for luciferase; this allows cells to be visualized using bioluminescence. Major problems with this approach include the absorption and scatter of visible light and use of non-human genetic material. More commonly used for non-invasive cell tracking are radioopaque metals like iron (super paramagnetic iron oxide, SPIOs) and gadolinium. These materials are visualized using magnetic resonance imaging (MRI). For gadolinium, difficulties arise in loading the cells with sufficient concentrations to permit T1 contrast. Since high concentrations are needed,

the dilution effects are pronounced as cells divide. SPIOs like ferridex are most frequently used, but conflicting studies exist on whether these particles interfere with chondrogenesis. If true, this would suggest they are not a “stealth” particle within the cell and could potentially interfere with other important physiologic functions. Further, should the technique be extended to clinical trials in humans, individuals with electronic pacemakers or implantable defibrillators would be excluded from the study. This would isolate a potentially needy patient population.

[0116] The present example demonstrates that passive QD loading of hMSCs yields cells that are labeled with sufficient QD clusters to theoretically permit detection via μ CT. When pelleted and embedded in a siloxane mold, the labeled cells are detectable and found to be approximately 27% denser than unlabeled cells. This result is consistent with theoretical calculations. Based on these findings, it should be possible to detect a cluster of QD-hMSCs within heart tissue using μ CT.

[0117] To confirm feasibility QD-hMSCs will be injected into heart tissue and the sample will be scanned. Once done, non-invasive scanning can be tested in living animals. To synchronize the scanning with the heart beat the use of gating algorithms may be required. Scano Medical manufactures in vivo scanners for animals (vivaCT 40) and humans (XtremeCT) that have resolutions of 16 and 1001 μ m respectively. These resolutions are acceptable (approximately single cell resolution for the animal scanner and 125-cell resolution for the human scanner) and superior to that attainable with SPIOs and MRI.

[0118] The present invention is not to be limited in scope by the specific embodiments described herein which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the claims. Throughout this application, various publications are referenced to by numbers. The disclosures of these publications in the entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to those skilled therein as of the date of the invention described and claimed herein.

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What is claimed:

1. A method for transfer of quantum dots into the cytosol of a cell comprising contacting a target cell population with negatively charged quantum dots for a time sufficient to permit transfer of the quantum dots into the cytosol of the target cell.
2. The method of claim 1 wherein the quantum dots are composed of material selected from the group consisting of CdS, CdSe, CdTe, CdTe/ZnS or CdSe/ZnS.
3. The method of claim 1 wherein the negatively charged quantum dots are formed through conjugation of negatively charged groups onto the surface of the quantum dots.
4. The method of claim 1 wherein the quantum dots emit light at wavelengths of between 525-800.
5. The method of claim 1 wherein the cell is a mesenchymal stem cell.
6. The method of claim 1 wherein the cell is a genetically engineered cell.
7. A stem cell comprising negatively charged quantum dots.
8. The cell of claim 7 wherein the quantum dots are composed of material selected from the group consisting of CdS, CdSe, CdTe, CdTe/ZnS or CdSe/ZnS.
9. The cell of claim 7 wherein the negatively charged quantum dots are formed through conjugation of negatively charged groups onto the surface of the quantum dots.
10. The cell of claim 7 wherein the quantum dots emit light at wavelengths of between 525-800.
11. The cell of claim 7 wherein the cell is a mesenchymal stem cell.
12. A pharmaceutical composition comprising cells labeled with quantum dots and a pharmaceutically acceptable carrier.
13. The pharmaceutical composition of claim 12, wherein the carrier is an extracellular matrix.

14. The pharmaceutical composition of claim 12 wherein the quantum dots are composed of material selected from the group consisting of CdS, CdSe, CdTe, CdTe/ZnS or CdSe/ZnS.

15. The pharmaceutical composition of claim 12 wherein the negatively charged quantum dots are formed through conjugation of negatively charged groups onto the surface of the quantum dots.

16. The pharmaceutical composition of claim 12 wherein the quantum dots emit light at wavelengths of between 525-800.

17. The pharmaceutical composition of claim 12 wherein the cell is a mesenchymal stem cell.

18. The pharmaceutical composition of claim 12 wherein the cell is a genetically engineered cell.

19. The pharmaceutical composition of claim 13 wherein the extracellular matrix is derived from an a cellularized porcine urinary bladder.

20. A method for tracking the distribution and/or fate of quantum dot-labeled cells that have been administered to a subject afflicted with a cardiac disorder comprising (i) administering quantum dot-labeled cells, to a region of the subject's heart and (ii) detecting the distribution and/or fate of the quantum dot-labeled cells that have been administered to said subject.

21. A method for tracking the distribution and fate of quantum dot-labeled cells that are utilized for regenerating myocardium in a mammal comprising (i) administering quantum dot-labeled cells to the myocardium in a quantity sufficient to induce native cardiomyocytes to enter the cell cycle; and (ii) determining the fate and distribution of said administered quantum dot-labeled cells.

22. The method of claim 20 or 21 wherein the quantum dots are composed of material selected from the group consisting of CdS, CdSe, CdTe, CdTe/ZnS or CdSe/ZnS.

23. The method of claim 20 or 21 wherein the negatively charged quantum dots are formed through conjugation of negatively charged groups onto the surface of the quantum dots.

24. The method of claim 20 or 21 wherein the quantum dots emit light at wavelengths of between 525-800.

25. The method of claim 20 or 21 wherein the cell is a mesenchymal stem cell.

FIGURE 1

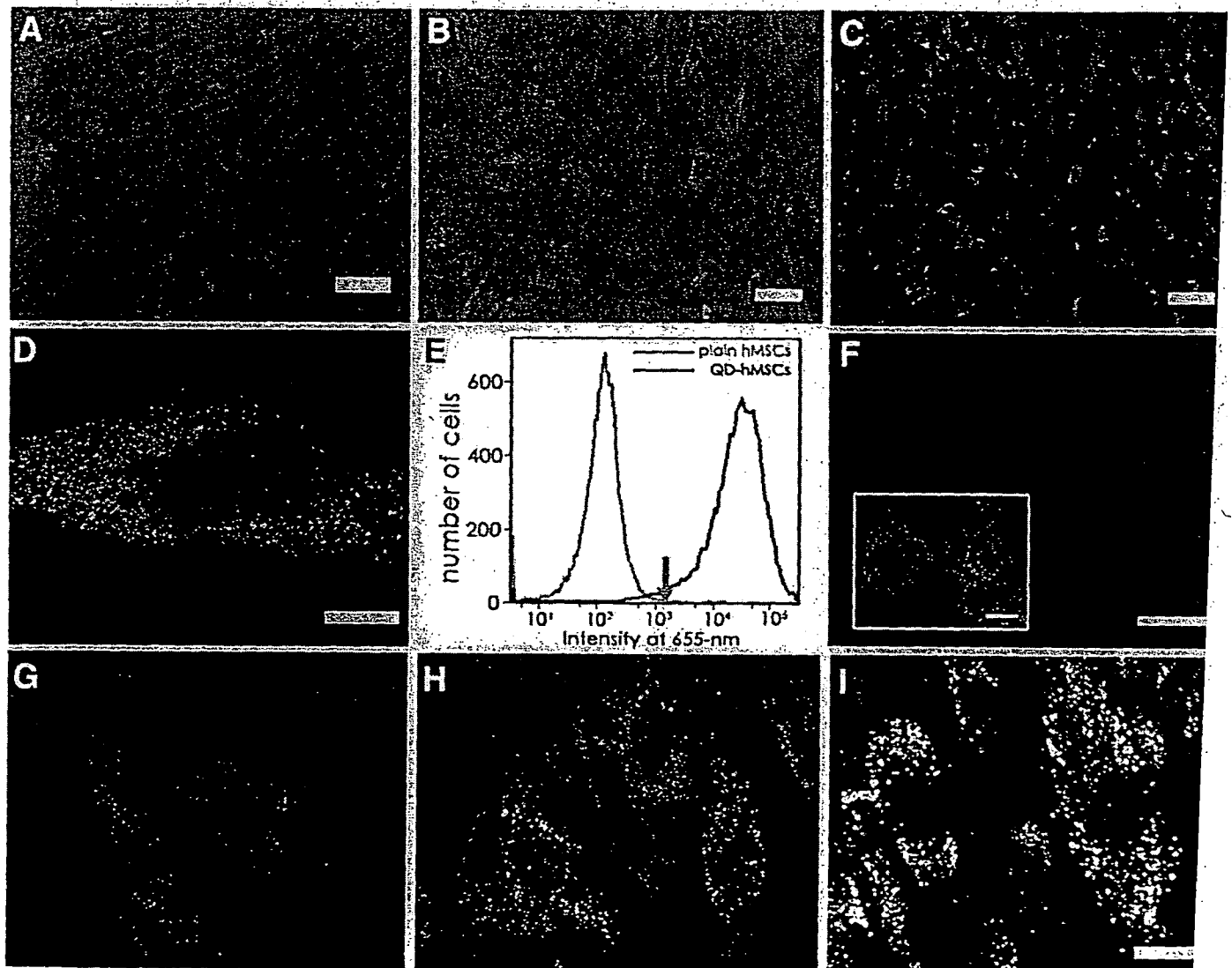


FIGURE 2

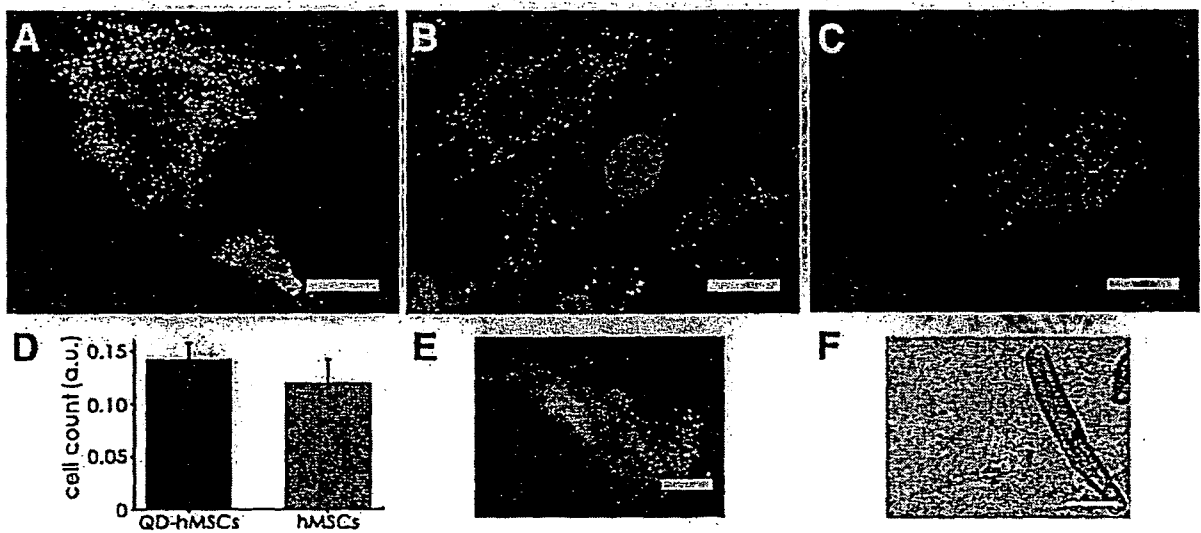


FIGURE 3

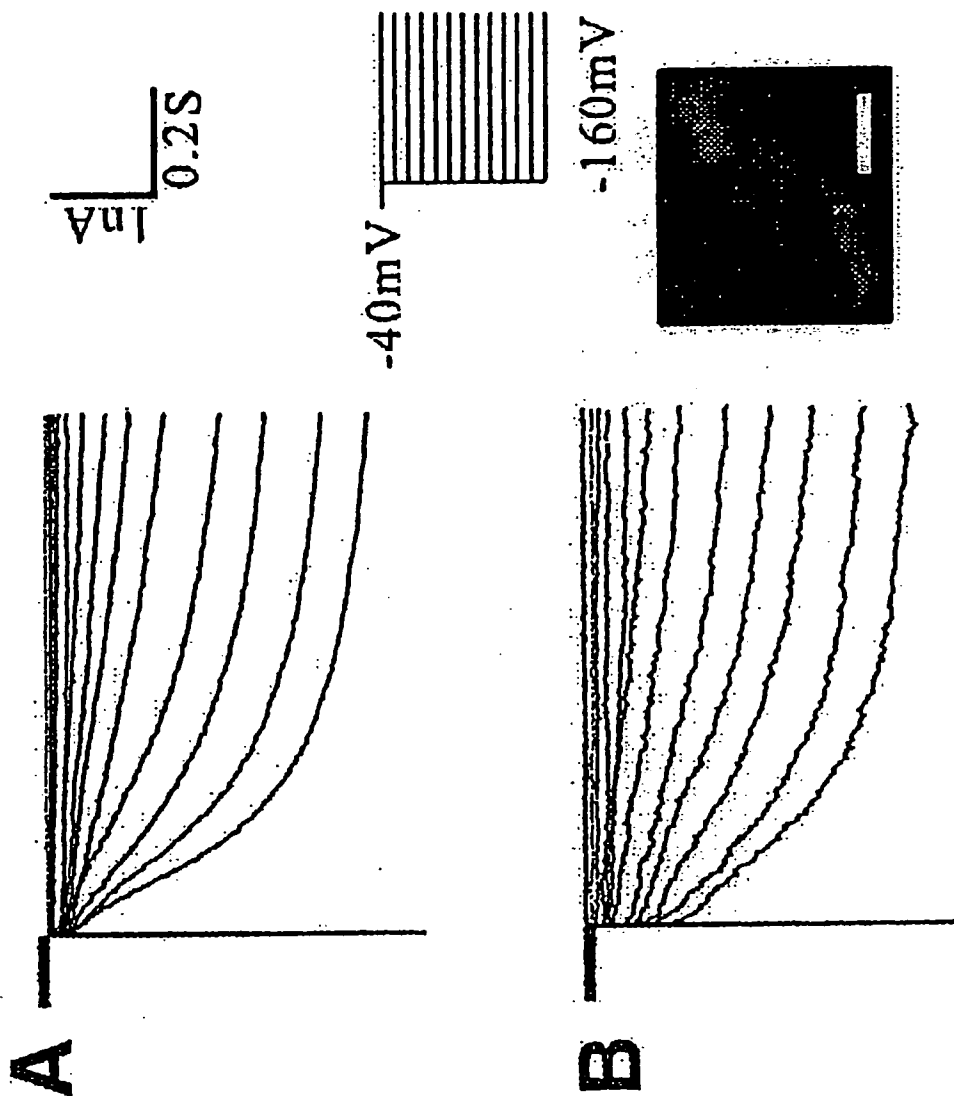


FIGURE 4

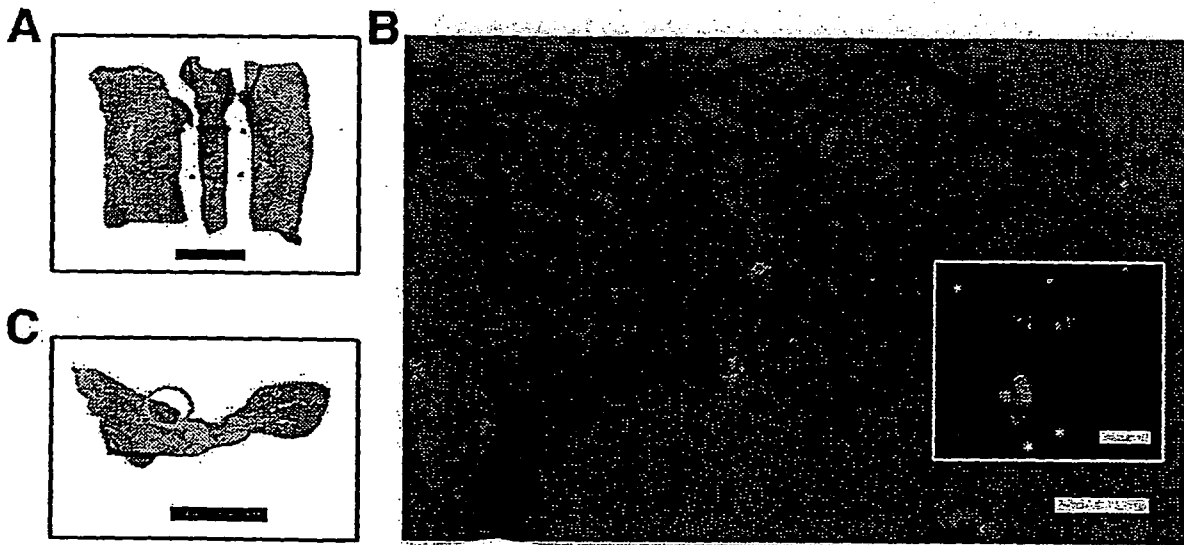


FIGURE 5

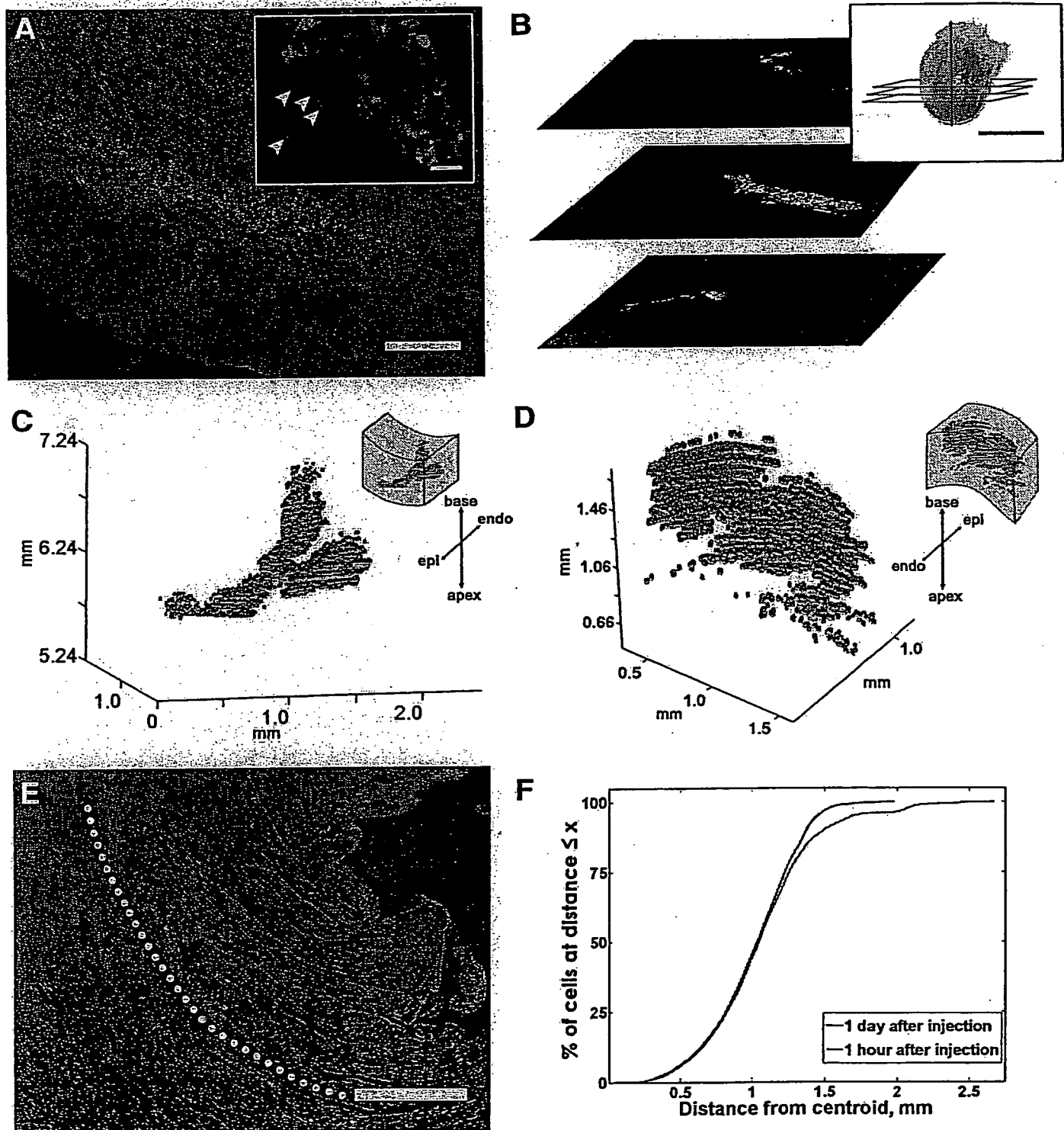


Figure 6

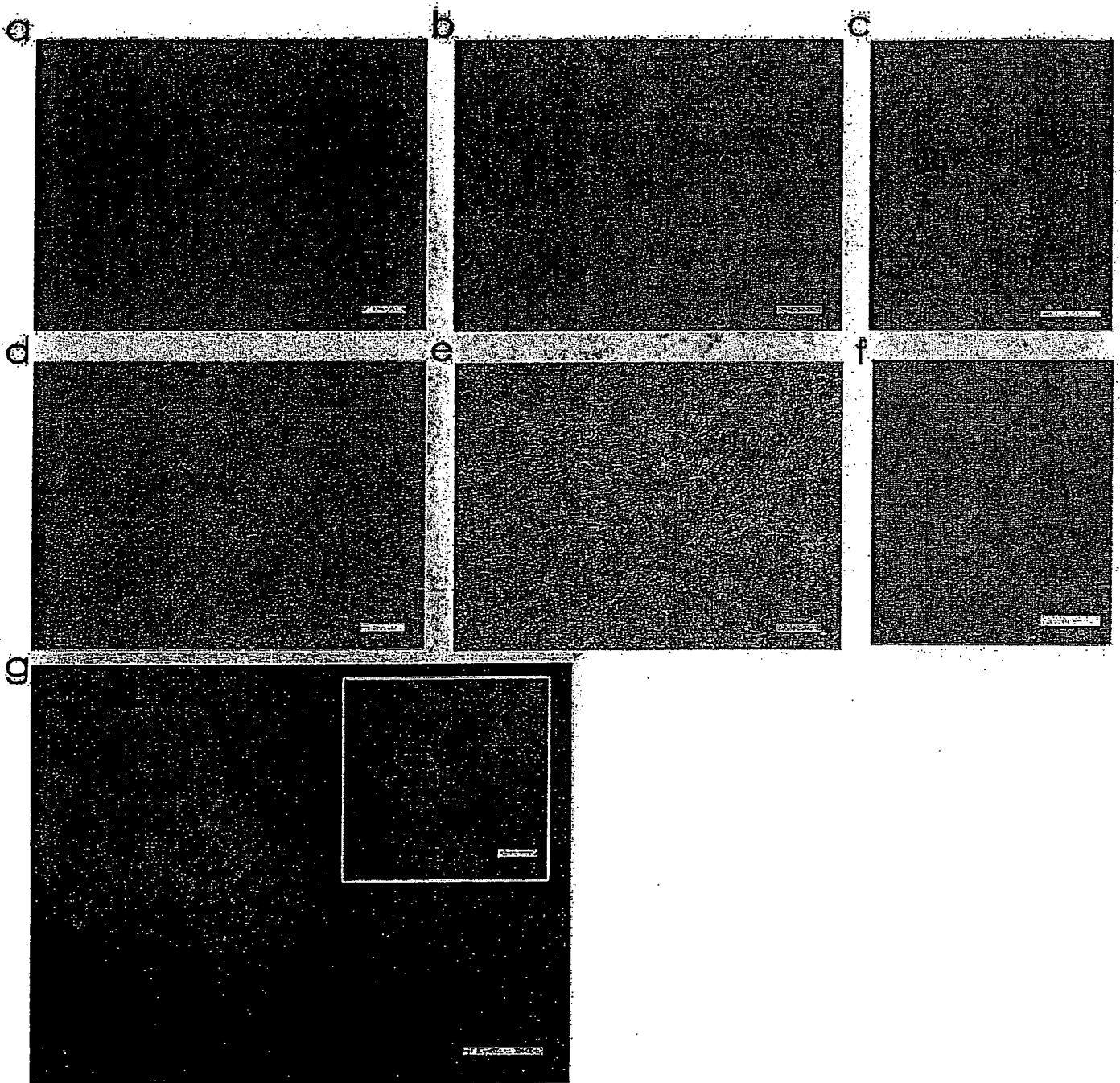


Figure 7

