Differentiation of multiple stem cells types labeled with MPIOs down multiple lineages is identical to unlabeled cells

C. Williams[1], D. Granot[2], T. Leahu[1], E. B. Lavik[3], and E. M. Shapiro[1,2]
[1]Department of Biomedical Engineering, Yale University, New Haven, CT, United States, [2]Department of Diagnostic Radiology, Yale University School of Medicine, New Haven, CT, United States, [3]Center for Translational Neuroscience, Case Western Reserve University, Cleveland, Ohio, United States

INTRODUCTION: Magnetic cell labeling using iron oxide nanoparticles, for the purpose of MRI based cell tracking, has proved successful for over fifteen years. Due to their high iron content and ease of use for cell labeling, micron sized iron oxide particles (MPIOs) have become increasingly employed as a cell tracking contrast agent (1). A great number of MRI cell tracking experiments involve the investigation of stem cell migration following transplantation or delivery to a host organism. Thus, it is critical that the contrast agent not cause cell death nor interfere with cellular processes. While it has been well demonstrated that MPIOs are non-toxic to cells (2), investigations into their effects on complex cellular functions are only now emerging. In this work, we investigated the effect of magnetic cell labeling with various sized MPIOs on differentiation of two different stem cell types, mesenchymal stem cells (MSCs) and neural progenitor cells (NPCs), down multiple cell lineages.

MATERIALS AND METHODS: MPIOs were iron oxide particles encapsulated within green fluorescent, inert polystyrene/divinyl benzene matrices (Bangs Labs).

NPCs: Green fluorescent protein NPCs were isolated and maintained in serum-free DMEM containing N2, B27, and 20ng/mL EGF. NPCs were plated overnight with 35, 1.63µm diameter MPIOs per cell. Free MPIOs were removed by vacuum filtration across an 8 µm transwell insert. For differentiation studies, NPC spheres were plated on poly-(D-lysine)/laminin coverslips in NPC media containing 10% fetal bovine serum to induce differentiation down neural, astrocytic and glial lineages. Differentiation status was assessed at 3h and 7 days. Cells were stained with primary antibodies against either nestin, glial fibrilary acidic protein, neurofilament 200 or beta-3 tubulin followed by Alexa Fluor 647 secondary antibodies. Images of neurospheres ($n \geq 4$) were acquired using a Zeiss Axiovert 200M microscope. Area of fluorescence for each stain was determined using a threshold mask in Image J. This area was normalized to the area of GFP fluorescence for each sphere.

MSCs: Upon reaching 85% confluence second passage rat MSCs were labeled with various sized MPIOs by overnight incubation, followed by extensive washing. Particle sizes were 1.63, 1.04, 0.51 and 0.19 microns. Cells were then differentiated down osteogenic (bone) and adipogenic (fat) lineages. Differentiation medium was added and changed every 3 days for 2 weeks. Standard osteogenesis and adipogenesis differentiation medium were used (3). Osteogenesis and adipogenesis assays were performed by staining with Alzarin Red and Oil Red, respectively, each following formalin fixation.

RESULTS: *Neural progenitor cells:* Cell labeling with 1.63µm diameter MPIOs resulted in 63.4 ± 2% of NPCs labeled as particles were not able to completely penetrate neurospheres. There were no gross morphological differences or differences in migration of cells from neurospheres between control plates and plates containing MPIOs. Internalization of MPIO's by NPCs was verified using confocal microscopy. **Differentiation** – Non-specific differentiation with serum containing media led to the differentiation of NPCs into neurons and glia (Fig 1, left, center). This differentiation profile was not altered by the addition of MPIOs (Fig 1, right).

Mesenchymal stem cells: Cell labeling with various sized MPIOs resulted in >90% of MSCs labeled with particles (Fig 2, left). Independent experiments also established that viability following initial labeling was also >90%. **Adipogenesis** – Both unlabeled and MPIO labeled MSCs differentiated into adipocytes and developed fat globules. MPIOs remained largely intracellular during differentiation (Fig 2, center). **Osteogenesis** - There was no visible distinction between the control plates and the plates containing MPIO of all four diameters. In all cases, MSCs formed extra cellular matrix characteristic for bone tissue (Fig 3, right). Due to the density of the newly formed extracellular matrix, it was however, difficult to determine whether particles remained inside cells.

DISCUSSION: Critical to the use of magnetic particles for MRI-based cell tracking is that particles not interfere with cellular processes. This is especially the case with stem cells. It is also imperative that particles remain with cells during migration and differentiation to avoid non-specific background contrast. NPCs are nestin positive and a hallmark of differentiation is the loss of nestin staining. Indeed it was observed that for unlabeled and labeled cells, nestin expression subsided during differentiation. Additionally, GFAP and beta-3 tubulin are expressed on glial cells and neurons, respectively and both markers were expressed in equal quantities in unlabeled and labeled cells. Thus it can be determined that MPIOs are non-interfering in NPC differentiation. MSCs have three easy lineage differentiation pathways – adipocytes, osteocytes and chondrocytes. Here we demonstrated that all of the various sized MPIOs are permissive for adipocyte and osteocyte differentiation. Furthermore, it can be determined that MPIOs remain intracellular during differentiation.

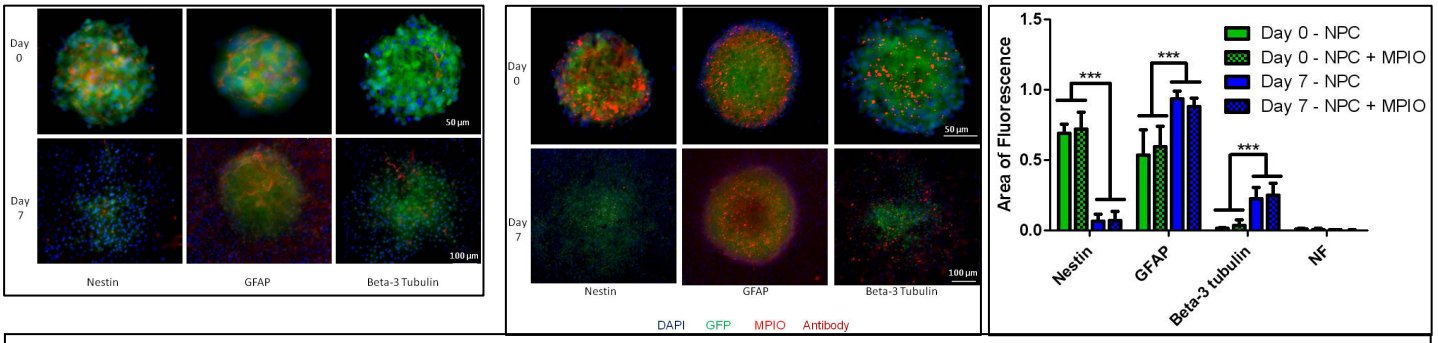

Figure 1: Differentiation of unlabeled **(Left)** and labeled **(Center)** NPCs. Blue is DAPI, staining nuceli. Green is GFP from the cells. Orange is the MPIOs. Red is the appropriate marker. **Right:** Quantification of differentiation for unlabeled and labeled cells. Differentiation was identical for unlabeled and labeled NPCs.

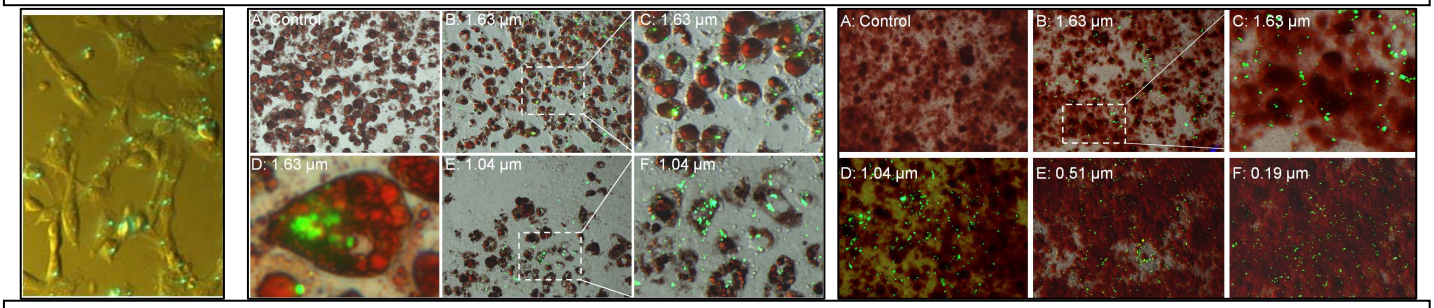

Figure 2: Left: MSCs labeled with 1.63 µm MPIOs in culture, before differentiation. **Center:** Adipogenesis. Red stain indicates fat globules. Note the intracellular particles. **Right:** Osteogenesis. Red stain indicates bone extracellular matric formation.Particles sizes are as indicated in pictures.

References: 1) Shapiro, EM, et al, *MRM* **53**, 329-338 **(2005)** 2) Shapiro, EM, et al, *MRM* **55**, 242-249, **(2006)** 3) Peister A, et al, *Blood* **103(5)**, 1662-8, **(2004)**.