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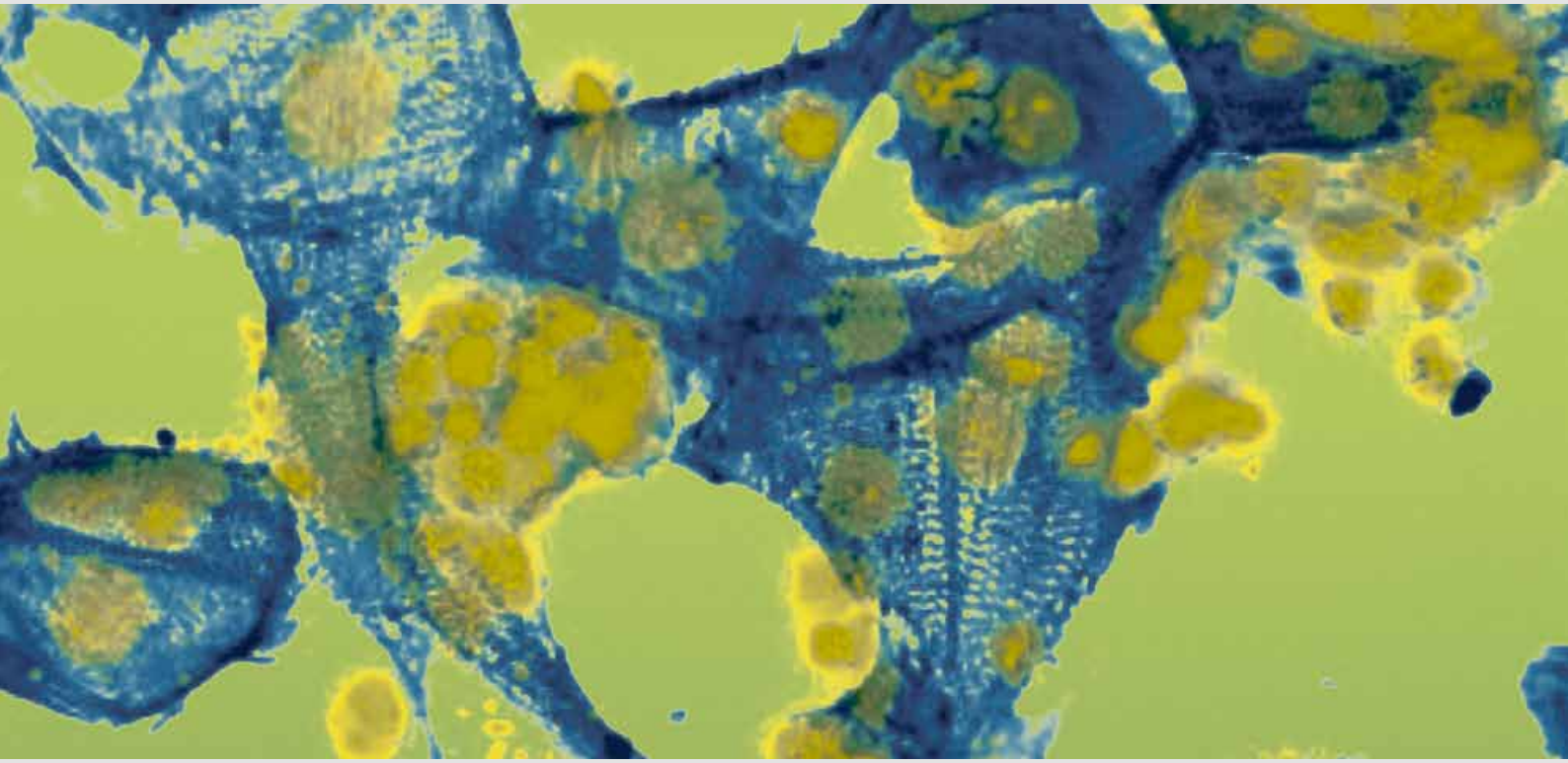
Manuel Kernbach[1], Klaus Kruttwig[2]*, Guido Piontek[3], Mathias Hoehn[2], Jürgen Schlegel[3], Andreas Bosio[1], and Dominik Eckardt[1]

[1] Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

[2] Max Planck Institute for Neurological Research, In-vivo-NMR Laboratory, Cologne, Germany

[3] Technical University, Division of Neuropathology, Institute of Pathology, Munich, Germany

* current address: University of California, San Francisco, Division of Hematology/Oncology, San Francisco, CA, USA



Intracellular labeling of multiple cell types for MRI-based *in vivo* cell tracking

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Introduction

Cell transplantation is an area of intense investigation, especially with respect to stem cell therapy of degenerative diseases, such as Morbus Parkinson or heart failure. Monitoring the location and migration of grafted (stem) cells is essential for understanding their interaction within the host and their therapeutic effects. Therefore, cell tracking is of great importance for basic research, preclinical evaluation, and monitoring of early clinical trials applying cell transplantation. Magnetic resonance imaging (MRI) is the most frequently used technique for *in vivo* cell tracking due to high resolution of soft tissues, such as brain, muscle, and heart. Detectability of transplanted cells by MRI depends on their contrast characteristics. In order to produce a strong contrast against the surrounding tissue, intracellular labeling with iron oxide particles prior to transplantation has been described. We have established an effective two-component method for *ex vivo* intracellular labeling of cells with superparamagnetic iron oxide (SPIO) particles. Contrast particles and loading reagent were optimized for proper labeling of cell lines, primary cells, and stem cells. Labeling did not affect viability or proliferation. Similar results were obtained in complex *in vitro* differentiation assays comparing labeled and unlabeled human hematopoietic stem cells (hHSCs), human mesenchymal stem cells (hMSCs), or mouse embryonic stem cells (mESCs), indicating biocompatibility of intracellular labeling. MRI analyses also demonstrated an *in vitro* detection limit of less than 200 cells and good contrast characteristics of intracellularly labeled neural progenitors after intracerebral transplantation into the mouse cortex.

Materials and methods

Ex vivo labeling of various cell types with SPIO contrast particles

Cells derived from tissues or cultures were plated on cell culture dishes. Loading reagent (lipofection reagent, optimized for introducing SPIO particles into cells) and contrast particles (FeraTrack™ MRI Contrast Agent Kit, Miltenyi Biotec) were combined and pre-incubated prior to the addition to the culture dish. For details see the data sheet available at www.viscover.com. Uptake of SPIO particles into cells was visualized either by light microscopy through staining with Prussian blue or by immunofluorescence microscopy through staining of dextran.

Differentiation of hMSCs, mESCs, and CD133$^+$ hHSCs

Adipocyte and osteoblast formation from MSCs was monitored by oil red and alkaline phosphatase staining respectively. Formation of dopaminergic neurons from mESCs was evaluated by immunofluorescence microscopy using a fluorochrome-conjugated anti-tyrosine hydroxylase (TH) antibody. The differentiation potential of hHSCs was monitored by using HSC-CFU assays.

In vitro MRI of intracellular SPIO-labeled neuronal progenitors

Neuronal progenitors were isolated from suspensions of mouse brain cells using Anti-PSA-NCAM MicroBeads. The PSA-NCAM$^+$ cells, magnetically labeled with MicroBeads on the surface, were divided into two fractions. One fraction was additionally labeled intracellularly with SPIO particles, the other fraction remained labeled extracellularly with MicroBeads. Control cells were labeled with neither MicroBeads nor SPIO particles. Defined cell numbers from both cell fractions and the control were measured with a T2*-weighted MRI sequence at 11.7 T in an agarose phantom.

Transplantation of labeled cells into experimental animals for *in vivo* MRI

Approximately 200,000 cells labeled extracellularly with MicroBeads or intracellularly with SPIO particles were grafted into the cortex of a three-week-old

CD1 mouse and measured with a T1-weighted image sequence at 7 T with TE: 5 ms, TR: 70 ms, flip angle 30°.

Results and discussion

Development of the two-component system for intracellular magnetic labeling

For optimization of the labeling system, particle size, iron and lipid concentration, and kinetics of particle uptake we used mouse NIH-3T3 cells. In the absence of loading reagent, virtually no uptake of contrast particles into the cells was observed (fig. 1A). In contrast, addition of a lipid-based loading reagent enabled significant uptake of contrast particles (fig. 1B). Particles larger than 100 nm formed aggregates impairing efficient particle uptake (fig. 1C, D). Titration of contrast particles (fig. 1E) and loading reagent (fig. 1F) revealed best results with regard to intracellular iron concentrations at a combination of ≥100 µg total iron and 20–30 µg total lipid per labeling reaction with 1×10^6 NIH-3T3 cells. Maximum iron load was achieved at a labeling time of 3 h (fig. 1G). Next, we used proliferating NIH-3T3 and mESCs to estimate the number of contrast particle–containing cells at days 1, 3, 5, and 7 after labeling (fig. 1H). Label retention time was prolonged in fibroblast-like NIH-3T3 cells compared to mESCs. Differences in contrast particle uptake, which depends on the cytoplasm-to-nucleus ratio, as well as variations of the proliferation rate among the two cell types might well explain the differences in label retention times. Nevertheless, similar proliferation rates were measured for labeled and unlabeled cells (data not shown).

Stem cell properties are maintained after intracellular labeling with contrast particles

To evaluate whether the newly developed labeling tool is applicable to cell types other than NIH-3T3 fibroblasts, human and mouse stem cells were analyzed by anti–dextran immunofluorescence for their contrast particle load after labeling (fig. 2A–C). Labeling efficiency again correlated with the cytoplasm-to-nucleus ratio. The largest amounts of iron oxide particles were found in fibroblast-like hMSCs (fig. 2A), lower amounts were detected in human CD133⁺ HSCs (fig. 2B) and mESCs (fig. 2C).

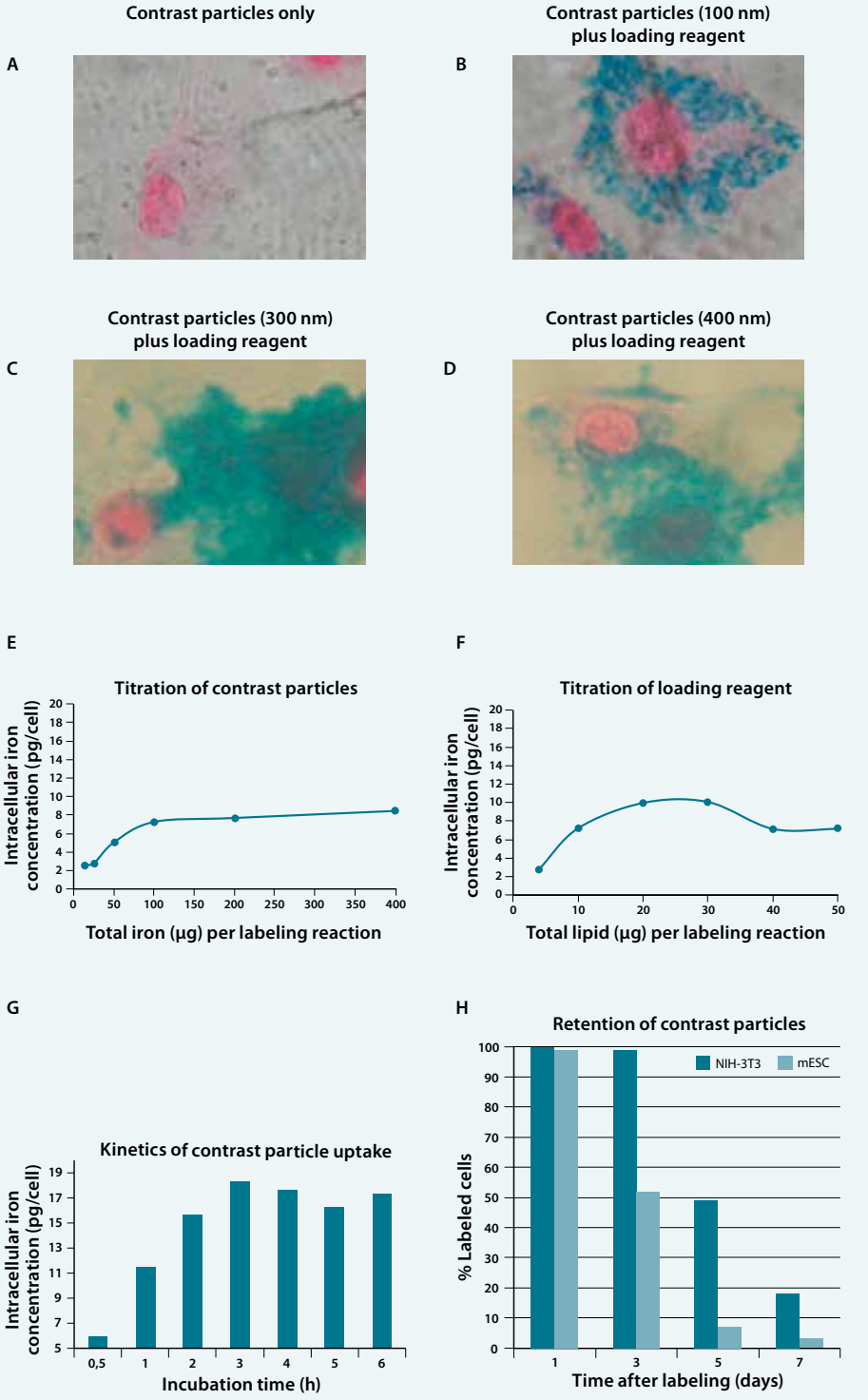

Figure 1 Development of the two-component system for intracellular magnetic labeling. Light microscopic images of cells and contrast particles (A–D). Contrast particles were visualized by Prussian blue staining, cells were counterstained with nuclear fast red. Titration curves for contrast particles (E) and loading reagent (F) as well as kinetics of contrast particle uptake (G) are shown. Retention of contrast particles by fibroblast-like NIH-3T3 cells (H, dark blue bars) was compared to mESCs (H, light blue bars).

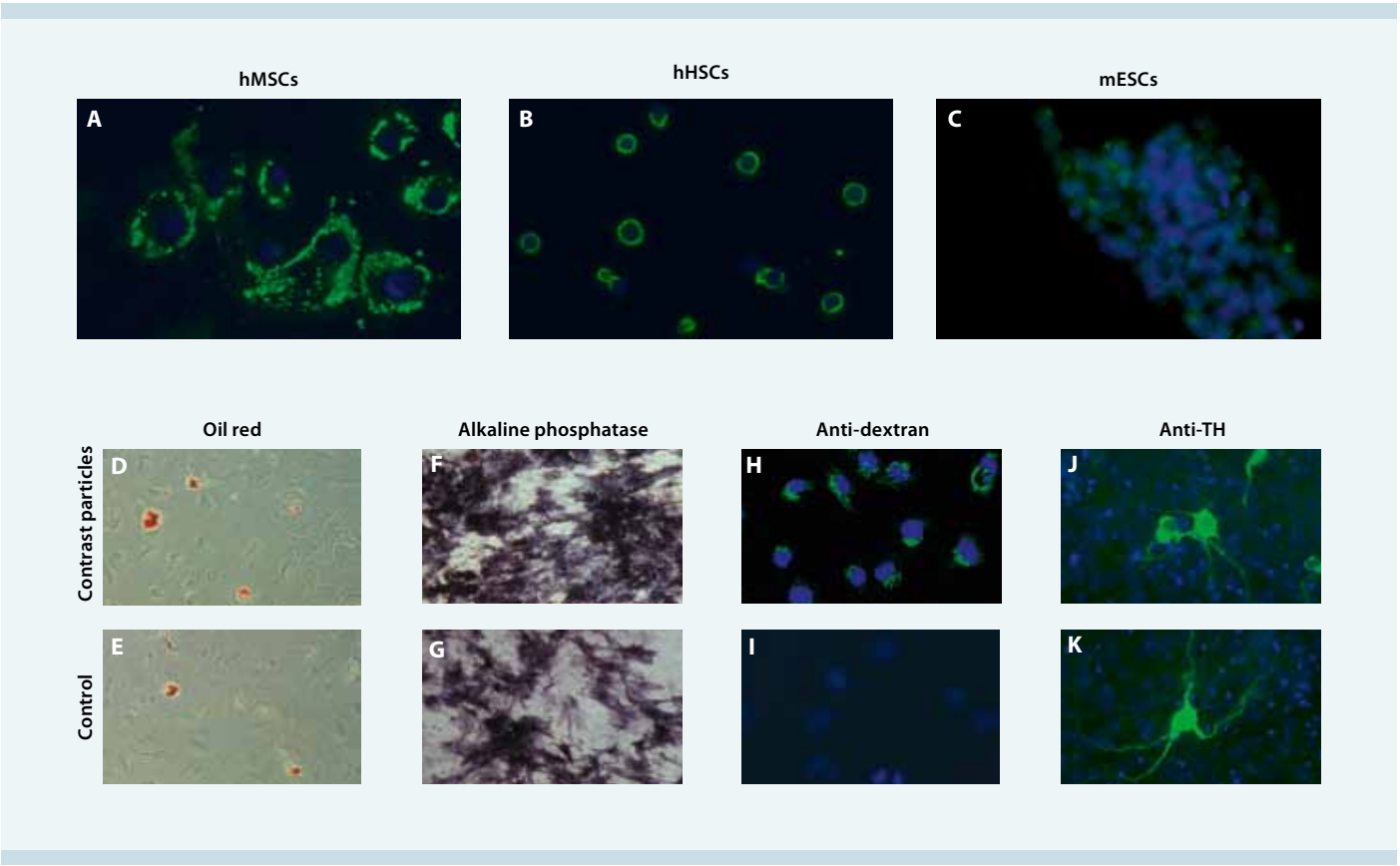

Figure 2 Stem cell properties in the absence or presence of intracellular labeling with contrast particles. Human MSCs (A) and HSCs (B), and mESCs (C) were intracellularly labeled with contrast particles, visualized in green by immunofluorescence microscopy. Unlabeled and intracellularly labeled human MSCs were compared by *in vitro* adipocyte (D, E; oil red staining) and osteoblast (F, G; alkaline phosphatase staining) differentiation. Mouse ESC-derived neural progenitors were intracellularly labeled with contrast particles at day 3 of differentiation. At day 21 of differentiation, retention of contrast particles (H, I) and generation of dopaminergic neurons (J, K) were evaluated by (anti-dextran) and anti-TH staining, respectively, both indicated in green. Cell nuclei were stained with DAPI (A–C, H–K; blue).

Next we analyzed whether intracellular magnetic labeling affects cellular characteristics. The differentiation potential of unlabeled and intracellularly labeled human MSCs was compared by *in vitro* adipocyte (fig. 2D, E) and osteoblast (fig. 2F, G) differentiation. Similar differentiation efficiencies for unlabeled and intracellularly labeled cells were found by oil red staining at day 21 (fig. 2D, E) or alkaline phosphatase staining at day 12 of differentiation (fig. 2F, G). Mouse ESC-derived neural progenitors were intracellularly labeled with contrast particles at day 3 of differentiation. At day 21 of differentiation, retention of contrast particles and generation of dopaminergic neurons were evaluated by immunofluorescence analysis. Immunofluorescent labeling clearly identified contrast particles in mESC-derived cells (fig. 2H). Formation of neuronal cells, however, was not affected by the contrast particles, as similar numbers of TH-expressing neurons were detected in control or labeled cell populations (fig. 2J, K). Additionally, intracellular magnetic labeling of enriched CD133$^+$ HSCs gave rise to similar colony morphologies and colony numbers in a CFU assay when compared with unlabeled controls (data not shown). These findings clearly show that even long-term and complex *in vitro* differentiation processes are not affected by our intracellular labeling method with contrast particles.

In vitro and *in vivo* MRI of intracellular SPIO-labeled neuronal progenitors

Next we evaluated MRI contrast enhancement properties of the SPIO particles and defined the detection limits for extracellularly and intracellularly labeled cells. To this end, we used neural PSA-NCAM$^+$ neuronal progenitors magnetically isolated from suspensions of mouse brain cells by using Anti-PSA-NCAM MicroBeads. We analyzed three cell fractions by MRI as indicated in materials and methods: i) cells labeled only extracellularly with MicroBeads (fig. 3A, B; red); ii) cells additionally labeled intracellularly with contrast particles (green), and iii) unlabeled cells (blue). Discrimination of single hypo-intensive signals was only possible with cells bearing an intracellular magnetic label. Cells labeled extracellularly with MicroBeads were only detectable, in rare cases, in cellular aggregates (fig. 3A and insert). Control cells did not show a detectable signal.

We also tested various cell numbers of the three fractions (fig. 3B). Cells labeled only extracellularly with MicroBeads did not show

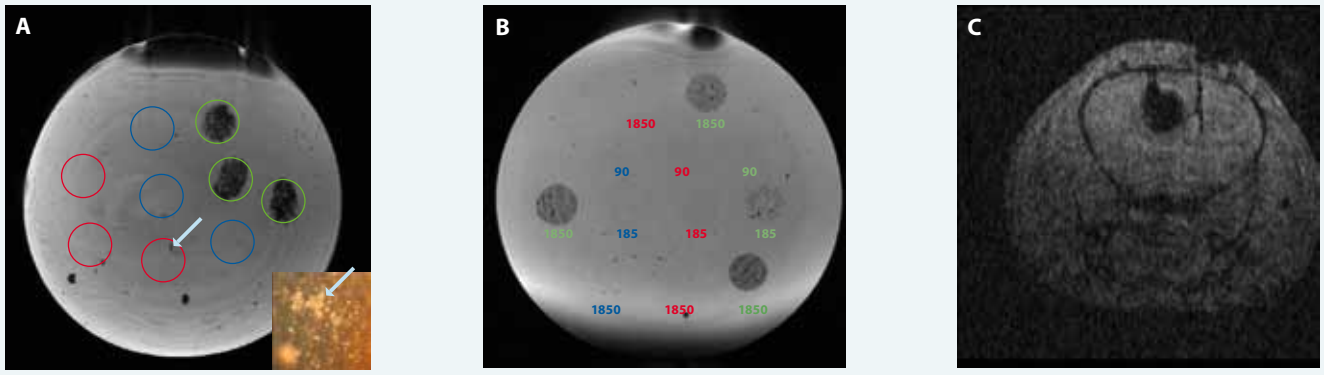

Figure 3 MRI analyses of intra- and extracellularly magnetically labeled neuronal progenitors. MRI contrast enhancement properties and detection limits of the SPIO particles were analyzed with neuronal progenitors. Primary neuronal progenitors were magnetically enriched from neonatal mouse brain after extracellular labeling with Anti-PSA-NCAM MicroBeads. Three different cell fractions were characterized by MRI analyses of agarose phantoms (A, B) and mouse brain (C). (A, B) Cells only labeled extracellularly with Anti-PSA-NCAM MicroBeads are indicated in red. Cells additionally labeled intracellularly with SPIO contrast particles are indicated in green. Cells without extra- or intracellular magnetic label are indicated in blue. (A) Discrimination of single hypo-intensive signals was only possible with cells carrying an intracellular magnetic label (8,500 cells measured). Extracellularly labeled cells were only rarely detectable in cellular aggregates (9,200 cells; insert: arrow points to cellular aggregates in bright-field microscopy). Control cells (13,500 cells). Parameters: TE: 18 ms, TR: 200 ms, flip angle: 30°. (B) Serial dilution of extracellularly (red) or intracellularly labeled (green) or unlabeled (blue) cells revealed only distinct signals for cells additionally labeled intracellularly (cell numbers are shown in the figure). Parameters: TE: 12 ms, TR: 200 ms, flip angle 30°. (C) After transplantation into the cortex of mouse brain, cells labeled only extracellularly with Anti-PSA-NCAM MicroBeads gave a faint signal (right cortex). However, a strong contrast was detected with cells additionally labeled intracellularly with contrast particles (left cortex).

a detectable signal at the tested numbers (90, 185, or 1850 cells). In contrast, distinct signals were observed in the samples containing 185 or 1850 intracellularly labeled cells (fig. 3B). Finally, extracellularly and intracellularly labeled cells were grafted into the cortex of a three-week-old CD1 mouse (fig. 3C). A strong contrast was detected for the intracellularly labeled cells in the left cortex. In contrast, cells with extracellularly bound Anti-PSA-NCAM MicroBeads were barely detectable (right cortex), although the same cell numbers (approx. 200,000) were injected.

Conclusion

We have developed a novel two-component tool allowing intracellular labeling of cells with superparamagnetic nanoparticles for subsequent MRI analysis. Highly effective intracellular labeling of cell lines and tissue-derived stem or progenitor cells does not impair cellular characteristics, such as their differentiation potential. As proven by *in vitro* and *in vivo* MRI analysis, magnetic labeling generates strong T1 and T2*-weighted contrast images allowing for efficient and reliable cell detection and tracking and could become a broadly applicable tool for pre-clinical imaging in cellular therapy.

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