Abstract

Background: Magnetic resonance imaging (MRI) is a promising tool for monitoring stem cell-based therapy. Conventionally, cells loaded with iron oxide nanoparticles appear hypointense on MR images. However, the contrast generated by iron oxide labeled cells is neither specific due to ambiguous background nor quantitative. A strategy to overcome these drawbacks is $^{19}$F MRI of cells labeled with perfluorocarbons. We show here for the first time that human neural stem cells (NSCs), a promising candidate for clinical translation of stem cell-based therapy of the brain, can be labeled with $^{19}$F as well as detected and quantified in vitro and after brain implantation.

Methodology/Principal Findings: Human NSCs were labeled with perfluoropolyether (PFPE). Labeling efficacy was assessed with $^{19}$F MR spectroscopy, influence of the label on cell phenotypes studied by immunocytochemistry. For in vitro MRI, NSCs were suspended in gelatin at varying densities. For in vivo experiments, labeled NSCs were implanted into the striatum of mice. A decrease of cell viability was observed directly after incubation with PFPE, which re-normalized after 7 days in culture of the replated cells. No label-related changes in the numbers of Ki67, nestin, GFAP, or βIII-tubulin+ cells were detected, both in vitro and on histological sections. We found that 1,000 NSCs were needed to accumulate in one image voxel to generate significant signal-to-noise ratio in vitro. A detection limit of ~10,000 cells was found in vivo. The location and density of human cells (hunu+) on histological sections correlated well with observations in the $^{19}$F MR images.

Conclusion/Significance: Our results show that NSCs can be efficiently labeled with $^{19}$F with little effects on viability or proliferation and differentiation capacity. We show for the first time that $^{19}$F MRI can be utilized for tracking human NSCs in brain implantation studies, which ultimately aim for restoring loss of function after acute and neurodegenerative disorders.

Introduction

To achieve translation of experimental stem cell-based therapy into the clinic, non-invasive imaging modalities are necessary tools. One such modality, magnetic resonance imaging (MRI), provides true three-dimensional data at high spatial resolution, enabling good detection of even small cell numbers in the living, intact individual. Commonly, contrast is achieved through in vitro labeling of cells with superparamagnetic iron oxide (SPIO) nanoparticles [1,2,3]. Although even single cells can be detected [4] with this procedure, the contrast generated by iron oxide labeled cells can easily be confounded with other sources such as bleedings or blood vessels [5]. Furthermore, since contrast is achieved indirectly through disturbances of the local magnetic field experienced by surrounding hydrogen nuclei, quantification of the number of cells in vivo is questionable [6].

A rapidly emerging field to overcome these drawbacks of ambiguity of contrast assignment and cell quantification is cell labeling with perfluorocarbon (PFC) nano-emulsions, which can be detected with $^{19}$F MRI [7,8,9]. The $^{19}$F nucleus is particularly suitable for labeling as its relative MR sensitivity is only 17% less than that of $^1$H. Furthermore, the signal intensity is directly proportional to the number of accumulated $^{19}$F, hence, allowing in vivo quantification of $^{19}$F labeled cells [10]. In addition, since the level of background $^{19}$F signal in host tissue is virtually absent [10], overlaying the $^{19}$F image on an anatomical $^1$H image allows for unambiguous, quantitative tracking of labeled cells in vivo. However, compared to labeling and tracking with metal-based contrast agents the technique is considerably less sensitive requiring a large amount of $^{19}$F to accumulate in order to generate sufficient signal-to-noise ratio (SNR).

The strategy of $^{19}$F cell labeling has already been applied to monitor cells during pathological conditions, e.g. umbilical cord blood cell localization in tumor-bearing mice [11], T-cell migration in murine models of diabetes [12], and local inflammation [13]. More recently, PFCs have proven useful in $^{19}$F MRI studies of inflammatory response to cerebral and cardiac ischemia [14] and in vivo measurements of intracellular pO$_2$ of glioblastoma cells in response to chemotherapy [15].

In experimental models of human neurodegenerative diseases cell therapies have shown that neuronal replacement and partial repair of damaged brain circuitry is possible [16]. For a successful
clinical translation, only cells of human origin will be needed, and one source of human cells are neural stem cells (NSCs) [17]. NSCs derived from the human fetal striatum have been expanded with both maintained normal karyotype and high capacity to generate different neuronal phenotypes for a long time in vitro [18,19]. Upon implantation, these cells survived in the stroke-damaged rat striatum, migrated towards the injury, and differentiated into mature neurons without tumor formation [18]. Since these cells represent a relevant cell source for clinical translation, the purpose of the present study was to establish a platform to visualize NSCs in vivo after intracerebral implantation with 19F MRI. Monitoring the spatio-temporal dynamics of NSCs grafted into the brain requires the ability to detect even low cell numbers with high spatial resolution, since pathology-related migration processes of interest may take place on a small scale, the graft size may be diluted due to these processes or be initially relatively small. A first report has already indicated that it is feasible to detect fluorine labeled, immortalized, murine neural progenitor cells in the healthy mouse brain with 19F MRI [20]. The current study is the first to show that these results can be extended to the tracking of human NSCs. Novelties of our experiments compared to the previous studies on 19F MRI of stem cells [11,20] include i) the use of a clinically relevant source of neural stem cells and detection with in vivo 19F MRI in a proof-of-concept, ii) a conservative estimate of cell detection limits for preclinical 19F MRI studies of neural stem cell implantation, and iii) a careful assessment of potential adverse effects of the 19F marker on cell viability and function both in vitro and in vivo over a period of at least one week. We show that under optimized preclinical conditions low numbers of cells can be detected in vivo at good resolution and within acceptable scan time. Our results suggest that 19F MRI may prove useful in monitoring implanted cells in models of neurodegeneration, thus allowing the optimization of preclinical protocols of graft induced brain repair.

Materials and Methods

Culturing of human neural stem cells

NSCs were obtained from the ganglionic eminences of an 8-week old aborted human fetus from Malmo/Land University Hospitals, according to the guidelines approved by the Lund/Malmo Ethical Committee. The full characterization of these NSCs, derived from the human fetal striatum, has previously been described in detail [19]. Briefly, after microdissection and dissociation of the striatal tissue, cells were maintained at 37°C in a humidified atmosphere with 5% CO2. The neurosphere expansion medium (DMEM/F-12, Gibco, Grand Island, USA; L-glutamine, 2.92 g/100 mL; HEPES, 23.8 mg/100 mL; NaHCO3, 7.5%; glucose, 0.6%; and heparin, 2%, all from Sigma–Aldrich, Hamburg, Germany) contained N-2 supplement (1%, Gibco), human leukemia inhibitory factor (10 ng/mL, Sigma–Aldrich), epidermal growth factor, 20 ng/mL and fibroblast growth factor, 10 ng/mL (both from Peprotech, Hamburg, Germany).

Labeling cells with 19F marker and determination of intracellular 19F content

A few hours prior to labeling, the small neurospheres were transferred to a Poly(2-hydroxyethyl methacrylate) (Poly-HEMA, Sigma-Aldrich) coated culture flask, in order to avoid strong attachment of the cells as a consequence of the label. We incubated cells with 5 μL, 15 μL, 50 μL, 100 μL, and 130 μL of a perfluoropolyether (PFPE) nano-emulsion (CELSENSE 1000, Celsense, Pittsburg, USA, 120 mg PFPE/ml, experimentally determined concentration of magnetically equivalent 19F c = 5.11 M/mL culture medium to determine the optimal concentration in terms of fluorine uptake. In the end the emulsion was added at the empirically determined optimal (see Results) concentration of 100 μL/mL medium 3 days after the last passage directly to the NSC culture medium and cells were incubated for 36 hours. Subsequently, the cell suspension was spun down at 184 x g for 5 min, the pellet was washed twice and finally resuspended in either fresh medium or potassium phosphate-buffered saline (KPBS) or Hank’s Balanced Salt Solution (HBSS), depending on the application.

In order to determine the amount of intracellular 19F, 10 μL of a solution containing 10 mg KF/mL H2O (i.e. 1.041014 19F spins) was added as an internal fluorine reference to the sample, which was then scanned with magnetic resonance spectroscopy (MRS, see below). The amount of fluorine was determined by comparing the integrated area of the PFPE main peak and the KF reference peak in the 19F MR spectra.

Determination of in vitro cell detectability

To determine the limits of in vitro detectability with MRI, the 19F labeled, small neurospheres were spun down, washed in KPBS, fixed with 4% paraformaldehyde (PFA), washed again and resuspended in KPBS. The fixed, labeled neurospheres were either transferred to small tubes or injected at different cell concentrations into phantoms made of 2% gelatin in small, custom-made cups 22 mm in diameter for subsequent scans with MRI.

Assessment of label influence on cell viability, proliferation and differentiation

Cell viability was determined in culture by trypan blue exclusion method before, shortly after, and at 7 days after labeling, and compared to unlabeled control cultures. Experiments were performed in triplicates. For determination of the cell phenotypes within the proliferating culture, 19F labeled neurospheres were plated on PLL coated 8-well chamber slides, allowed to attach for 5 h, then fixed and processed for immunocytochemistry. For determination of cell differentiation capacity, growth factors were removed and 1% fetal bovine serum (Gibco) was added. Labeled cells were differentiated for 9 days, then fixed and subsequently stained. Medium was changed every third day during experiments.

Animal experiments

All experiments were conducted according to the guidelines laid out in the German Animal Welfare Act and approved by the local authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen) under permission number 9.93.2.10.51.07.048 (dated 22 May 2007). Four adult male CD 1 mice (bodyweight 37–42 g, Janvier, Le Genest Saint Isle, France) were used for the cell implantation and 19F MRI investigations. All experiments were performed under anesthesia. Animals were housed in cages under a 12 h light/12 h darkness cycle with access to food and water ad libitum.

Implantation procedures

Human NSC cultures (passage 14) were labeled with 19F marker as stated above. At the day of implantation, 19F labeled neurospheres (diameter=100 μm) were centrifuged and resuspended in HBSS (Gibco). The neurosphere suspension had a concentration of approximately 100,000 viable cells/μL and was kept on ice during the whole procedure. Mice were anesthetized with 1–2% isoflurane in a 70/30 nitrous oxide/oxygen mixture.
and placed in a stereotaxic frame. A feedback controlled system maintained the body temperature at 37 °C (medres, Cologne, Germany). The non-labeled and 19F labeled neurosphere suspensions were injected using a Hamilton syringe into two deposits per hemisphere into the striatum (1.5 µL i.e. 150,000 cells per deposit) in two animals. Two further animals were injected bilaterally with two deposits of 19F labeled cells on the left and one deposit on the right hemisphere. The following coordinates were used: AP, +1.0, ML, ±2.0 mm from bregma, and DV, −3.0 and −2.0 mm from the brain surface. After each deposit, the needle was kept in place for 5 min before slow withdrawal. The wound was closed with suture and animals were allowed to recover in their cages. To suppress an immune reaction, animals received a subcutaneous injection of 20 mg/kg Cyclosporine A (Sigma-Aldrich) every second day starting 2 days before implantation.

MRI acquisition

MRI and MRS were carried out on a Biospec 11.7 T/16 cm dedicated animal scanner system (Bruker BioSpin, Ettlingen, Germany) equipped with actively shielded gradient coils (BGA9S, 750 mT m−1, Bruker BioSpin). For radiofrequency transmission and reception, we used custom-built, inductively coupled, single-loop surface coils of 9 mm diameter for in vitro MRS, 25 mm diameter for in vivo MRS, and 20 mm diameter for in vivo MRI, all tunable from 470 MHz for the 19F resonance frequency up to 500 MHz for 1H imaging.

In vitro 19F MRS: For the in vitro quantification of 19F content with MRS, we used a spin-density weighted pulse-acquire spectroscopic sequence with short acquisition delay of 0.05 µs, and with long repetition time (TR) of 20 s to assure full relaxation recovery (90° rectangular hard pulse, duration/bandwidth (BW) = 0.01 ms/128 kHz, 163.8 ms acquisition window, spectral points/BW = 8192/50 kHz). We chose the number of averages (NA) dependent on the strength of PFPE signal, with typically NA = 30, leading to an acquisition time (TA) = 10 min. All measured 19F concentrations are presented with respect to the main PFPE frequency (~93.2 ppm), i.e. the chemical shift signal originating from the end group of the PFPE molecule was not taken into account.

Determination of relaxation times: To optimize sequence parameters, the relaxation times were measured with the 9 mm coil on a tube containing either the pure PFPE marker or the labeled cells. The temperature was kept at 37° throughout the experiment with a feedback controlled water system (medres). T1 was measured with a pulse-acquire saturation-recovery sequence (20 experiments, TR = 90.3 ms up to 20 s, pulse identical to in vitro, 66.7 ms acquisition window, spectral points/BW = 1000/15 kHz), T2 with a multi spin echo sequence (TR = 4 s, echo spacing 20 ms, field of view (FOV) = 16 × 16 × 2.5 mm3, 32 × 32 matrix, 15 echoes, NA = 4 × PFPE/100 (cells), TA = 4 min/3:33 h). The spectral peak area or the signal in the multi-echo images S were then fitted with the software Origin (OriginLab Corporation, Northampton, USA) to the function $S = S_0 (1 - \exp(-TR/T_1))$ for $T_1$ or $S = S_0 (1 - TE/T_2)$ for $T_2$, with the equilibrium signal $S_0$.

In vivo 19F MRS: MRS was performed to determine the exact PFPE frequency in vivo using a pulse-acquire sequence identical to the in vitro experiments but with shorter TR (30° pulse, TR = 200 ms, NA = 3,000, TA = 10 min).

In vitro and in vivo 1H/19F imaging: Anatomical 1H imaging was performed with a turbo spin echo sequence (TR/echo effective time (TE eff) = 2200 ms/42.8 ms, 8 echoes per excitation, NA = 2, 10 consecutive, 1 mm thick slices, FOV = 1.92 × 1.92 cm2, 128 × 128 matrix, i.e. a resolution of 150 × 150 × 1000 µm3, TA = 1 min, BW = 50 kHz, linear phase encoding scheme) [21]. 19F images were acquired with the same sequence and matching geometry, but at lower in-plane resolution and lower BW (NA = 256, 48 × 48 matrix, i.e. a resolution of 400 × 400 × 1000 µm3, TA = 57 min, BW = 10 kHz). The transmission power needed for a 90° pulse was determined on the 1H channel on a 1 mm slice parallel to the coil through the 19F labeled cells and the same power was used after switching to the 19F frequency. Approximately the same power is needed for the 1H and the 19F 90° pulse, which we tested on a combined 1H/19F standard (diluted Trifluoroacetatic acid, data not shown).

In vivo measurement protocol: In vivo 1H and 19F imaging was carried out 2 d after implantation for those mice that received non-labeled control cells, and 2 d and 6 d after implantation for the animals that were injected with labeled cells in both hemispheres. Mice were anesthetized with an intraperitoneal injection of a ketamine (120 mg/kg) and xylazine (8 mg/kg) mixture to avoid background signal from fluorinated inhalation of anesthesia gases. Anesthesia time was prolonged by subcutaneous administration of 30–60 mg/kg ketamine at 1 h after induction. Respiration rate was monitored using a pressure sensitive pad placed under the thorax, together with DASYLab (Measurement Computing, Norton, USA) software and body temperature was maintained at 37 °C with an in-house feedback controlled system. Animals were fixed with ear bars in standard animal holders (Bruker BioSpin) and scanned with 1H MRI, 19F MRS, and 19F MRI. The total time of the imaging session did not exceed 1.5 h.

Immunocytochemistry/immunohistochemistry

After the last MRI session, animals were deeply anesthetized and perfused transcardially with saline followed by 4% PFA. Brains were post-fixed overnight and then kept in 20% sucrose solution until they sunk. Thirty micrometer thick sections were cut in the coronal plane using a freezing microtome (Leica Microsystems, Wetzlar, Germany), and kept at −20 °C in cryo-protective solution. Cells were fixed in 4% PFA for 15 min at room temperature followed by three rinses in KPBS. Before immunostaining, cells and tissue sections were pre-incubated in 5% normal serum, and 0.025% and 0.25% Triton X-100, respectively, in KPBS for 45 min at room temperature. Incubation in primary antiserum was carried out overnight at +4 °C and the following primary antibodies were used: mouse anti-b-III tubulin (1:333, Sigma-Aldrich), goat anti-doublecortin (DCX, 1:400, Santa Cruz Biotechnologies, Santa Cruz, USA), mouse anti-rat CD-68 (ED-1, 1:200, AbD Serotec MorphoSys AbD, Düsseldorf, Germany), rabbit anti-GFAP (1:500, DakoCytomation, Glostrup, Denmark), mouse anti-GFAP (1:400, Sigma-Aldrich), mouse anti-human nucleus (HuNu, 1:100, Chemicon, Temecula, USA), rabbit anti-Ki67 (1:500, Abcam, Cambridge, USA), rabbit anti-human nestin (1:500, Chemicon). Primary antibodies were detected using appropriate fluorescent Cy3 (Jackson ImmunoResearch Laboratories, West Grove, USA) or biotin-conjugated (Vector Laboratories, Burlingame, USA) secondary antibodies (1:200), which were then detected with Alexa 488-conjugated streptavidin (1:200, Molecular Probes). In order to determine the specificity of the primary antibodies and the level of background generated from the secondary antibodies, in one well per primary antibodies and the level of background generated from the secondary antibodies, in one well per primary antibodies were used: mouse anti-CD68 (1:500, AbD Serotec MorphoSys AbD, Düsseldorf, Germany), goat anti-GFAP (1:500, DakoCytomation, Glostrup, Denmark), mouse anti-GFAP (1:400, Sigma-Aldrich), mouse anti-human nucleus (HuNu, 1:100, Chemicon, Temecula, USA), rabbit anti-Ki67 (1:500, Abcam, Cambridge, USA), rabbit anti-human nestin (1:500, Chemicon). Primary antibodies were detected using appropriate fluorescent Cy3 (Jackson ImmunoResearch Laboratories, West Grove, USA) or biotin-conjugated (Vector Laboratories, Burlingame, USA) secondary antibodies (1:200), which were then detected with Alexa 488-conjugated streptavidin (1:200, Molecular Probes). In order to determine the specificity of the primary antibodies and the level of background generated from the secondary antibodies, in one well per primary antibodies were used: mouse anti-CD68 (1:500, AbD Serotec MorphoSys AbD, Düsseldorf, Germany), goat anti-GFAP (1:500, DakoCytomation, Glostrup, Denmark), mouse anti-GFAP (1:400, Sigma-Aldrich), mouse anti-human nucleus (HuNu, 1:100, Chemicon, Temecula, USA), rabbit anti-Ki67 (1:500, Abcam, Cambridge, USA), rabbit anti-human nestin (1:500, Chemicon). Primary antibodies were detected using appropriate fluorescent Cy3 (Jackson ImmunoResearch Laboratories, West Grove, USA) or biotin-conjugated (Vector Laboratories, Burlingame, USA) secondary antibodies (1:200), which were then detected with Alexa 488-conjugated streptavidin (1:200, Molecular Probes).
controlled, microscopic images acquired and double-immunoreactivities verified with a confocal laser scanning (Leica TCS SP2, Leica Microsystems) microscope equipped with a supplementary CCD camera (Leica Microsystems).

MRI data processing and statistical analysis
All MR images were processed with the program ImageJ (http://rsbweb.nih.gov/ij/).

For SNR analysis of the 19F signal, the measured signal and noise in the raw 19F images were corrected voxel-wise for non-Gaussian noise distribution due to the low SNR [22]. Only voxels with a corrected SNR above 3.5 were considered significant and included in the analysis.

For display purposes of the merged 1H/19F images, the fluorine SNR datasets were resized to a 128×128 matrix with bilinear interpolation, grey-levels were converted to transparency levels of red, and overlaid on the 1H image.

To estimate the minimum number of cells (N_{min}) per voxel to generate a detectable SNR of 3.5, we assumed a linear correlation between number of cells and SNR. Knowing the summed SNR of all voxels (SNR_{total}) with significant 19F signal and the overall number of cells (N_{total}) within this volume the detection limit was then calculated with the simple relationship N_{min} = N_{total} / 3.5 / SNR_{total}, similar to a method described previously [11].

Differences between in vitro labeled and non-labeled cells were evaluated with Student’s unpaired t-test. Data are expressed as mean±SEM and differences considered significant at p<0.05.

Results
Labeling of NSCs
NSCs readily took up the PFPE emulsion without the aid of transfection agents. The intracellular 19F content was maximized for 100 μL of the 19F marker substance per mL medium as confirmed with MRS (Fig. 1, A–C). With this protocol labeled cells contained on average 3.70±0.78×10^{12} (n = 3 biological replicates) 19F spins.

In order to analyze if the cells keep the fluorine marker over time, we took out 1/3 of the cells (i.e. 1/3 of the internalized 19F) directly after the labeling procedure and determined the amount of intracellular 19F with MRS. The remaining 2/3 of the cells were replated for another 7 days. The incorporated 19F was then again measured and 2.10±0.54 times (n = 4 technical replicates) the amount of the first sample was found, which is in good agreement with the expected value of 2 in case there was no significant loss of label.

The measured relaxation times were T_1/T_2 = (280±20) ms/(153±4) ms for the free PFPE marker and T_1/T_2 = (380±4) ms/(68±3) ms for the labeled cells.

Detectability and quantification in vitro
MRI of a dilution series of labeled NSCs (20,000, 10,000, 5,000 labeled and 65,000 non-labeled cells for control) suspended in gelatin clearly detected spots with 20,000 down to 10,000 cells, while there was no background 19F signal from non-labeled cells.

Figure 1. Optimization of labeling and in vitro detection limits. A–C. 19F MRS of NSCs incubated with 15 μL (A), 50 μL (B), and 100 μL (C) PFPE emulsion per mL medium together with a KF solution as an internal standard, KF frequency set to −120.9 ppm, main PFPE peak at −93.2 ppm, signal intensity in arbitrary units. The cell signal was maximized at a concentration of 100 μL/mL. D–F. MRI of 20,000, 10,000, and 5,000 labeled, and 65,000 control cells plated in gelatin, D shows the merged 1H and 19F, E the 19F image only. The 20,000 and 10,000 cells spots were clearly detectable, whereas a significant signal was not detected for the 5,000 cells, probably due to cells spreading over many voxels, thus not exceeding the critical detection limit. From the summed SNR of the two spots with 19F signal we estimate that approximately 1,000 cells need to accumulate in one image voxel to overcome the detection limit, as indicated by the crossing of the linear fit in F with the dashed line illustrating an SNR of 3.5. For the fit we assumed 0 SNR for 0 cells. Error bars in F are over n = 3 technical replicates. Note: The surface coil was adjusted parallel to the paper plane.

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Spots with 5,000 cells could not be detected by in vitro MRI. From the SNR of the two cell clusters with significant signal (SNR_{total} = 112 ± 14, n = 3 technical replicates) we estimate that the minimum number of cells, which need to accumulate in one image voxel in order to generate significant signal in vitro, is approximately N_{min} ≈ 1,000 cells (Fig. 1, D–F). This finding was supported by 19F MRI of a dilution series of the 19F marker (supplementary Fig. S1). Although the 5,000 cell spot is clearly above the estimated detectable number of cells, it was most likely not discovered because cells were spread over too many image voxels.

**Effect of the 19F marker in vitro**

The labeling procedure had only very minor effects on the cells’ viability and no significant effects on their proliferation (Fig. 2, A–H) and differentiation (Fig. 2, I–P) capacity. Furthermore, carefully analyzing both the proportion and morphology of nestin+, GFAP+ and β-III tubulin+ cells, we did not detect any significant changes compared to the non-labeled control cells. Also, no differences in the numbers of Ki67+ cells were found either during proliferation (37.7 ± 3.2%; directly after the labeling) or after 7 days of differentiation (27.2 ± 1.0%) in vitro compared to non-labeled control cells (44.1 ± 2.4% for non-differentiated and 29.2 ± 1.7% for differentiated cells, n = 3 technical replicates) (Fig. 2, Q).

The percentage of living cells was equal compared to controls before labeling (95.2 ± 4.2% and 94.7 ± 5.0%) but significantly decreased directly after incubation with the 19F marker (83.1 ± 3.7% compared to 92.9 ± 6.6% for unlabeled cells, p < 0.05). However, this value normalized again after replating and subsequent culturing of the cells for 7 days (87.0 ± 5.6% for the labeled cells and 88.9 ± 3.2% for controls, n = 3 biological replicates, Fig. 2, R).

**19F signal detectability in vivo**

In order to assess whether 19F MRI is a useful tool for the longitudinal tracking of implanted cells in the brain, we injected PFPE labeled NSCs and non-labeled control cells into the striatum of mice and scanned the animals a few days later. We detected significant 19F signal from the grafts with labeled cells in all animals, while there was no signal from implanted non-labeled control cells (Fig. 3, A). Presence of Hoechst+/hum+ cells allowed definite assignment of NSC aggregations on histological sections (Fig. 3, B, D, and F). These cell spots corresponded well, both in intensity and location, to regions exhibiting significant 19F signal. In the two mice with labeled cells grafted on both hemispheres, the 19F signal persisted at least 6 days after implantation (Fig. 3, C and E). Quantitative SNR analysis of these animals revealed that total 19F SNR decreased by < 20% from day 2 to day 6.
To estimate the *in vivo* detection limit $N_{\text{min}}$ at 2 days after surgery, the overall $^{19}$F SNR in all animals was determined ($\text{SNR}_{\text{total}} = 612.5$) and used together with the known number of transplanted cells ($N_{\text{total}} = 1.5$ million) to calculate the minimum number of detectable cells at an SNR = 3.5 ($N_{\text{min}} = 9,000$).

### Effect of the $^{19}$F marker in vivo

At the end of the $^{19}$F MRI *in vivo* measurements, the animals were sacrificed and the brains were processed for immunohistochemistry to study implanted cell phenotypes. Qualitative analysis of brain sections stained for nestin, GFAP and DCX showed that...
the vast majority of the implanted NSCs were nestin+ and GFAP+ cells (Fig. 4 A–D). We also detected a portion of the implanted cells that had already differentiated into DCX+ neuroblasts (Fig. 4 E and F). Overall, these images revealed that the fraction of cells of each cell phenotype studied was similar for 19F labeled and non-labeled cell grafts.

Discussion

We show that efficient labeling of NSCs with a PFC-based marker is possible without impairment of their naive cell behavior and thus, in consequence, their therapeutic potential. We have successfully demonstrated detectability of these human neural stem cells with high sensitivity after implantation into mouse brain and the usefulness of this imaging approach for monitoring the cell dynamics longitudinally because the graft was followed with 19F MRI for a week, being most likely detectable even much longer.

Efficacy and effects of labeling NSCs with 19F

PFC nano-emulsions are particularly suitable agents for stem cell tracking because of the excellent MR properties for imaging and the high biocompatibility [10,23]. Important for a possible clinical translation, PFCs are chemically stable, biologically inert, and related compounds have already been evaluated in preclinical and clinical trials as blood-substitutes [23,24,25].

Our labeling procedure with PFPE lead to an uptake of $3.7 \times 10^{12}$ spins/cell, similar to reports on other cell types. For experimental studies, 19F uptake could potentially be further...
increased through the use of transfection agents, however, most of these are not approved for use in the clinic [10]. We found a high $T_2/T_1$ ratio both for the free PFPE tracer and for the labeled cells. This indicates that the MR properties of the tracer are in deed optimal in terms of maximizing the SNR particularly when using turbo spin echo sequences. We note however, that the reported relaxation times may not be universal, e.g. due to slice thinning or excitation/refocusing flip angle imperfections and echo train signal variations.

Careful assessment of important immunohistochemical markers revealed that the label has only minor effects on viability, differentiation capacity, and proliferation in vitro and in vivo. In other reports the viability of PFC labeled stem cells was not different from non-labeled controls [11,20] whereas we observed a decrease in viability directly after incubation. This could either be a specific feature of the human NSCs or be due to the relatively high concentration of $^{19}$F marker in the medium compared to those other studies. We observed a decrease in viability over time, although non-significant, also in the non-labeled control cells. This suggests that the impairment in viability may rather be related to other factors such as the PolyHEMA coating of flasks.

The use of a fluorescent agent, attached to the PFPE, could provide a more direct proof that the agent is internalized, analyzing localization by fluorescence microscopy. However, most fluorophores are not suitable for clinical use and, therefore, we used an emulsion without fluorescent tag. We included thorough washing steps in all procedures in order to remove excessive $^{19}$F marker particles. Moreover, a study with a similar, fluorescent PFC emulsion showed localization of the $^{19}$F marker in the cytosol of murine neural progenitor cells and persistence of $^{19}$F MRI signal over 2 weeks [20]. Importantly, we did not detect a decrease in MRS-determined $^{19}$F content from labeled, replated cells over a period of one week. In vivo the $^{19}$F SNR only slightly decreased between day 2 and day 6 after implantation. Overall, there is strong evidence that NSCs retain the label over time and can be tracked longitudinally, even for longer periods than the time span of this study. This feature of long-term tracking of cells was not provided employing alternative fluorine markers such as Poly-L-lysine CFS, for which labeled cells lost half the $^{19}$F signal within 7 days [26,27]. However, thorough long-term evaluations of both effects of PFCs on grafted stem cells and efficacy of the labeled cells in the pathological brain e.g. stroke models are needed in the future.

**Cell quantification and detection limits**

The $^{19}$F MR signal is proportional to the amount of magnetically equivalent fluorine nuclei within a certain voxel. This allows quantification of the number of cells. If the total number of implanted cells is known, one strategy is to link this number to the sum total of SNR and to assume linear correlation between both. This way each SNR can be assigned a cell number. We employed this for the determination of detection limits by extrapolation of the number of cells generating an SNR of 3.5. However, the strategy works only under the assumption that cells are labeled homogeneously, do not lose the label, and do not proliferate as this leads to a dilution of $^{19}$F content/cell, and that partial volume effects can be neglected. The assumption of a homogeneous labeling is reasonable when fluorine content per cell is averaged over a few hundred cells. Further, we have shown containment of the $^{19}$F marker within NSCs over one week under in vitro conditions. The dilution of label through cell division is a potential threat to our strategy, since injected cells can divide after transplantation. However, the cells used here cease to proliferate shortly after implantation. The previously reported time span of 2 days for proliferation in vivo is rather short considering a doubling time of approximately 72 hours under ideal in vitro conditions [18,19,28,29].

With the small graft sizes used in brain implantation studies, an image voxel is typically only partly filled by labeled cells. This becomes particularly severe with $^{19}$F MRI as one strategy to enhance sensitivity is to measure with rather low spatial resolution [10]. As a consequence, quantification in regions with cell densities below the detection limit leads to underestimated cell numbers, graft borders are only inaccurately defined on $^{19}$F MR images, and migratory processes on a small scale cannot be monitored. A post-processing scheme to ease the problem has been applied in models of T-cell homing to the pancreas or lymph node by dilation of regions of interest by one-half voxel [12,13]. This scheme could potentially be included in future MRI studies of $^{19}$F labeled NSCs depending on expected cell distribution. In order to minimize these partial volume effects in the first place, we used a relatively high in vivo resolution (160 nL voxel size in the present report compared to for example 305 nL used before in the mouse brain [20]). To our knowledge there is only one in vivo study with a higher resolution, but in mouse hind limb (15.6 nL voxel size [30]). To compensate the loss of sensitivity at the higher resolution, we decided for the use of surface radiofrequency coils with inhomogeneous field profile and $T_2$-weighted turbo spin echo pulse sequences. Thus the measured $^{19}$F signal from a certain region depended not only on the amount of fluorine contained therein but also on $^{19}$F relaxation times and the region’s distance from the coil. The use of time-consuming, purely spin-density weighted pulse sequences can improve quantification only at the cost of SNR per unit time. A volume coil with more uniform magnetic field profile may be better suited for quantification. However, the coil is often larger and less sensitive compared to a surface coil. To minimize the problem we adjusted the setup in a way that the cell spots were placed within the same distance from the coil for the in vitro experiments. For the in vivo experiments cells were spread over a slightly larger region and further away from the coil, thereby leading to loss of signal. This may partly explain the order of magnitude difference of in vitro and in vivo detection limits. In conclusion, coil design and choice of pulse sequence must be carefully adapted to the specific application in future studies employing $^{19}$F MRI for cell tracking.

Using a $^{19}$F marker with high spin density, using optimized radiofrequency coils and a very high field of 11.7 T we show detection of small numbers of cells in a preclinical setting. Our data indicates that within ~1 h acquisition time in vitro detection of ~1,000 cells/voxel is feasible corresponding to less than 10$^{16}$ fluorine spins/voxel. In vivo we estimate a detection limit in the order of ~10,000 cells/voxel. The limits are in agreement with earlier studies [11,12,14]. These numbers are usually sufficient for imaging the graft core and possibly even for larger cell clusters migrating away from injection sites in the brain.

**Potential of $^{19}$F MRI of NSCs in cell replacement therapy**

The usefulness of cell tracking with $^{19}$F MRI mainly depends on its sensitivity. Although the $^{19}$F marker and MRI methods used in this study are likely to be safe for clinical translation, detection sensitivity will be orders of magnitude lower in a clinical setting due to lower field strengths, larger radiofrequency coils, and shorter acquisition times [31]. Still, $^{19}$F MRI of NSCs may become clinically relevant in the near future as a result of further advances in MRI hardware and pulse sequence development.

However, before stem cell based therapy of neurological disorders can be used in human patients the action of these cells needs to be further investigated in animal models [16]. $^{19}$F labeling
may prove superior to labeling with magnetic iron oxide nanoparticles in such preclinical investigations particularly when the pathology induces changes in $^1$H relaxation times that make distinction between diseased tissue and labeled cells difficult.

Possible scenarios include vasogenic edema after ischemic stroke or lesions involving bleedings, in which heterogeneous background detection of magnetically labeled cells with $^1$H MRI. As $^{19}$F MRI allows quantification of cell numbers it may also help to optimize efficacy of stem cell therapy e.g. in terms of injection sites, monitoring graft size changes [29] and routes of migration.

Conclusion

In future clinical applications, NSCs are one of the most potential and attractive cell sources for stem cell based treatment of diseases affecting the central nervous system. However, for the successful translation of cell therapy additional live imaging tools such as MRI are a prerequisite. $^{19}$F MRI is a promising, quantitative, non-invasive technique to monitor NSC grafts after implantation. We have shown that labeling NSCs with a PFC marker, potentially suitable for clinical use, does not significantly impair cell function both in vitro and in vivo. Our high detection sensitivity and resolution have allowed for the first time true in vivo tracking of small groups of human NSCs. This is particularly interesting for studies of brain replacement therapy, in which the graft can initially be small and may become further diluted through lesion-induced cell migration.

Supporting Information

Figure S1 Dilution series of $^{19}$F marker. $^{19}$F MRI of six tubes containing different concentrations of the PFPE agent (1, 2, 3, 4, 5, and 6 times 31.1 mM corresponding to multiples of $3\times10^{15}$ $^{19}$F spins/voxel). The tube with $3\times10^{15}$ spins/voxel can clearly be depicted (SNR ~ 6). Assuming a labeling efficacy of 3–4$\times10^{12}$ spins/voxel this would translate to detection of less than 1,000 cells/voxel in agreement with the results obtained with our quantification strategy (Fig. 1). Pulse sequence parameters were chosen identical to the cell dilution series in Fig. 1. Note: The surface coil was oriented parallel to the paper plane.

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Author Contributions

Conceived and designed the experiments: PBS TK MH SW. Performed the experiments: PBS TK LM. Analyzed the data: PBS TK LM. Contributed reagents/materials/analysis tools: PBS TK LH. Wrote the paper: PBS TK MH.

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