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Tracking of adipose tissue-derived progenitor cells using two magnetic nanoparticle types

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ABSTRACT

Magnetic resonance imaging (MRI) is to be considered as an emerging detection technique for cell tracking experiments to evaluate the fate of transplanted progenitor cells and develop successful cell therapies for tissue engineering. Adipose tissue engineering using adipose tissue-derived progenitor cells has been advocated for the cure of soft tissue defects or for persistent soft tissue augmentation. Adipose tissue-derived progenitor cells were differentiated into the adipogenic lineage and labeled with two different types of magnetic iron oxide nanoparticles in varying concentrations which resulted in a concentration-dependent reduction of gene expression of adipogenic differentiation markers, adiponectin and fatty acid-binding protein 4 (FABP4), whereas the metabolic activity was not altered. As a result, only low nanoparticle concentrations for labeling were used for *in vivo* experiments. Cells were seeded onto collagen scaffolds and subcutaneously implanted into severe combined immunodeficient (SCID) mice. At 24 h as well as 28 days after implantation, MRI analyses were performed visualizing nanoparticle-labeled cells using T2-weighted sequences. The quantification of absolute volume of the scaffolds revealed a decrease of volume over time in all experimental groups. The distribution of nanoparticle-labeled cells within the scaffolds varied likewise over time.

1. Introduction

For soft tissue augmentation as well as treatment of soft tissue defects, adipose tissue engineering using adipose tissue-derived progenitor cells has been advocated, *e.g.* seeded onto suited scaffolds or injected using different kinds of carriers like collagen microparticles or hyaluronic acid gels [1,2]. The mode of cell application as well as the efficacy of cell transplantation and the survival of transplanted cells *in vivo* represent key parameters which determine the success of tissue engineering strategies. The tracking of transplanted cells might help to monitor their fate and develop successful cell therapies.

Magnetic resonance imaging (MRI) is an excellent approach to visualize soft tissue, especially in tissues containing fat. In addition, MRI signal is affected by iron. It is known that high iron concentrations result in an increased signal decay over time [3,4]. The fact that iron destroys the signal intensity can be used to visualize iron within tissues. Using T2-weighted images, it should be possible to visualize both fat (hyperintensity=bright signal) and iron (hypointensity=dark signal). Therefore, MRI could be an useful tool for cell tracking of progenitor cells labeled with magnetic iron oxide nanoparticles in newly engineered adipose tissue.

In this study, nanoparticle-labeled adipose tissue-derived stem cells (ASC), as multipotent progenitor cells within the adipose tissue, were seeded onto collagen scaffolds, subcutaneously implanted into severe combined immunodeficient (SCID) mice, and analyzed 24 h and 28 days after implantation using a high field 7.1 T animal MR system.

Before starting the *in vivo* experiments, ASC were labeled with magnetic iron oxide nanoparticles in different concentrations and differentiated into the adipogenic lineage to assess the effects of nanoparticle-labeling on adipogenesis as well as to exclude any cytotoxic effects. Regarding adipogenesis, peroxisome proliferator-activated receptor γ (PPARγ) is known as the master regulator activating PPARγ targets like fatty acid-binding protein 4 (FABP4) and adiponectin which are expressed during terminal differentiation into mature adipocytes [5]. Furthermore, the deposition of lipid droplets is a main characteristic of mature adipocytes.

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This study provides first insights showing the successful cell tracking of nanoparticle-labeled adipose tissue derived progenitor cells in a SCID mouse model using MRI.

2. Materials and methods

Nanoparticles, bionized nanoferrite (BNF) starch and nanomag®-D-spio, were obtained from micromod Partikeltechnologie GmbH (Rostock-Warnemünde, Germany). Both nanoparticle types were 100 nm in size and coated with poly-D-lysine to ensure particle internalization. Crystal size, intercrystal structure, and polymer coating of both nanoparticle types were reported earlier [6].

Adipose tissue-derived stem cells (ASC) were isolated from samples of human adipose tissue. The study was approved by the Ethics Committee of the Rostock University Medical Center and an informed consent was signed by every patient before surgery. The adipose tissue was processed as described previously [2]. Briefly, adipose tissue was minced and afterwards digested with collagenase (Serva Electrophoresis GmbH, Heidelberg, Germany) at 37 °C. The suspension was filtrated using a 100 μm cell strainer (BD Biosciences, San Jose, CA, USA) and centrifuged. Cells were plated in tissue culture flasks and cultured using culture medium consisting of equal volumes of Iscove's modified Dulbecco's medium and Ham's F12 Nutrient Mix (both from Life Technologies GmbH, Darmstadt, Germany) supplemented with 10% newborn calf serum (PAA Laboratories, Pasching, Germany), 10 ng/ml human fibroblast growth factor-basic (EMD Millipore Corporation, Billerica, MA, USA) and 1% penicillin-streptomycin (Life Technologies GmbH) at 37 °C in a humified atmosphere with 5% CO_2. The differentiation potential of isolated cells was reported earlier [7].

For *adipogenic differentiation*, cells were seeded at a density of 3×10^4 cells/cm² and labeled one day before starting the adipogenic differentiation with nanoparticles as follows: BNF starch and nanomag®-D-spio at concentration 10, 25 and 50 μg Fe/ml and 25, 50 and 100 μg Fe/ml, respectively. Cells were incubated for 21 days with culture medium containing 0.5 mM 3-isobutyl-1-methylxanthine, 10 μM insulin, 1 μM dexamethasone, 8 mg/l biotin and 2.5 mg/l DL-panthothenic acid hemicalcium salt (all from Sigma-Aldrich Chemie GmbH, Munich, Germany). Adipogenic differentiation was determined by staining of lipid vacuols using 1 mg/ml Bodipy® 493/503 (Life Technologies GmbH). Cells were counterstained with 2 μg/ml cell-permeant nucleic acid stain bisBenzimide H 33342 trihydrochloride (Hoechst 33342; Sigma-Aldrich Chemie GmbH). *Cell viability* was assessed by LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells (Life Technologies GmbH) according to the manufacturer's instructions. Microscopic examinations were performed using the inverted microscope Axio Observer.Z1 (Carl Zeiss Microscopy GmbH, Jena, Germany).

For testing the *metabolic activity* of adipogenic differentiated cells, the activity of mitochondrial dehydrogenases was determined using the Cell Proliferation Kit II (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) according to the manufacturer's instructions. To normalize the results to cell number, cell's DNA was stained with crystal violet. The absorbance of crystal violet solution was measured at wavelength of 600 nm using a microplate reader (Anthos Mikrosysteme GmbH, Krefeld, Germany). *Statistical analyses* were performed using IBM SPSS Statistics version 20.0.0 (IBM Corp., Armonk, NY, USA). Experiments were repeated four times using ASC of individual donors to ensure reproducibility. To test normal data distribution, the Kolmogorov-Smirnov test was used. According to data distribution, the Mann–Whitney U-test was performed and significant statistical differences were set at $p < 0.05$. All graphs were created using SigmaPlot 12.5 software (Systat Software, Inc., San Jose, CA, USA). Graphs display box-and-whisker diagrams.

For analyzing the *gene expression of adiponectin and FABP4*, RNA was isolated from nanoparticle-labeled cells cultivated for 21 days under adipogenic differentiation conditions using the NucleoSpin® RNA Kit (Macherey-Nagel GmbH, Düren, Germany). CDNA was transcribed from 1 μg of total RNA using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. Target-specific primers (5′→3′) for adiponectin (fw: GGGGAAGGAGAGCGTAATGG; rev: GAACAGGGATGAGTTCGGCA), FABP4 (fw: GCTTTGCCACCAGGAAAGTG; rev: GCGAACTTCAGTCCAGGTCA), and GAPDH (fw: CAAGGTCATCCATGACAACTTTG; rev: GTCCACCACCCTGTTGCTGTAG) were designed using Primer-BLAST [8]. To amplify target sequences, DreamTaq PCR Master Mix (Thermo Fisher Scientific Inc.), 10 μM Primer fw/rev, and 1.8 μl cDNA were used.

Animal experiments were carried out using female severe combined immunodeficient (SCID) mice (CB17.Cg.*Prkdc*^{scid}*Lyst*^{bg}/Crl). The study was approved by the Local Committee on the Ethics of Animal Welfare (7221.3-1.1-039/12). For preparation of cell-seeded collagen scaffolds (MBP Biomaterial Products GmbH, Neustadt-Glewe, Germany), cells were labeled with BNF starch and nanomag®-D-spio nanoparticles at labeling concentrations of 10 μg Fe/ml and 25 μg Fe/ml, respectively, and seeded with a density of 1.5×10^6 cells per scaffold (size: $1 \times 2 \times 0.5$ cm). Scaffolds were cultured for 3 days in culture medium using a cell roller system (Integra Biosciences AG, Zizers, Switzerland). Afterwards, scaffolds were subcutaneously implanted in SCID mice. Three experimental groups were analyzed via MRI at 24 h and 28 days after implantation: (i) non-labeled cells, (ii) BNF starch- and (iii) nanomag®-D-spio-labeled cells. Within the scope of this feasibility study, only one SCID mouse per group was studied.

Magnetic resonance imaging (MRI) is an accepted approach to visualize iron in tissue, because of the increased susceptibility effects caused by iron. The signal decay caused by iron can be visualized using T2-weighted sequences resulting in signal destruction (black signal intensity) due to iron overload in tissue. For this reason, a strong T2-weighted sequence was used to visualize and to assess the distribution of ASC *in vivo* labeled with iron oxide-containing nanoparticles. Coronal T2-weighted turbo spin echo MRI was performed in a 7.1 T MR system (ClinScan, Bruker Corp., Billerica, MA, USA) and sequence was acquired using the following image parameters: TR: 1300 ms; TE: 43 ms; flip angle: 180°; matrix: 320 × 240; field of view: 41 mm; 1 averages: 1, bandwidth 130 Hz, slice thickness: 0.7 mm. Image analysis was performed using the freeware Osirix (v.5; Pixameo, Bernex, Switzerland). Manual segmentation of cell-seeded collagen scaffolds was done in each slice using the region of interest tool. After segmentation, the volume of each scaffold was automatically calculated. In addition, a subjective image analysis was performed to assess the distribution of nanoparticle-labeled cells.

3. Results and discussion

3.1. Adipogenic differentiation of nanoparticle-labeled ASC

For *in vitro* experiments, ASC were generally cultured under adipogenic differentiation conditions for 21 days. To detect any concentration-dependent effects, BNF starch and nanomag®-D-spio nanoparticles were applied at concentrations 10, 25 and 50 μg Fe/ml and 25, 50 and 100 μg Fe/ml, respectively. It could be shown by Prussian blue staining that both nanoparticle types are internalized into the cells in a dose-dependent manner (unpublished data). Because cytotoxicity can potentially be induced through nanoscale properties and exposure of superparamagnetic iron oxide nanoparticles (SPION) at high concentrations is associated

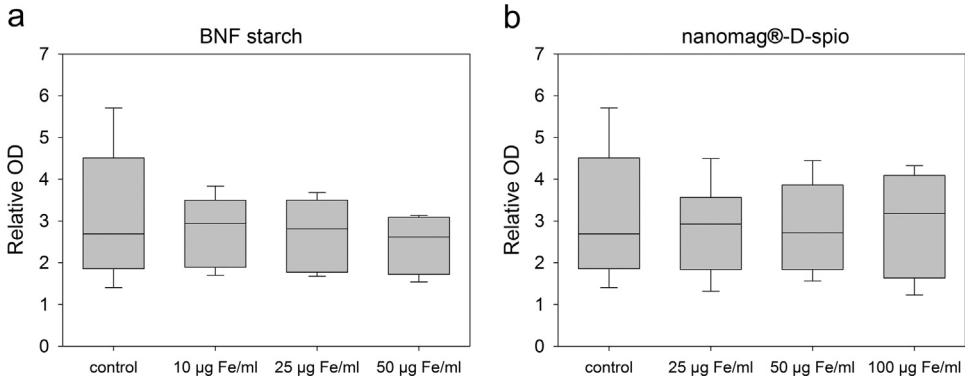

Fig. 1. Metabolic activity. Cells were treated with (a) BNF starch and (b) nanomag®-D-spio nanoparticles and differentiated into the adipogenic lineage. The activity of mitochondrial dehydrogenases was determined at day 21. No significant differences were found between nanoparticle-labeled and non-labeled cells ($n=4$; Mann–Whitney U-test).

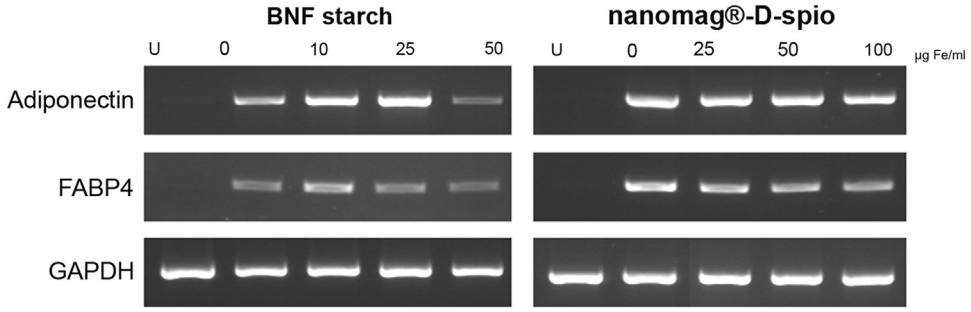

Fig. 2. Expression of adipogenic differentiation markers: adiponectin and FABP4. Cells were labeled with nanoparticles and differentiated into the adipogenic lineage for 21 days (U: undifferentiated and non-labeled control). Both markers were expressed due to adipogenic differentiation conditions and slightly down-regulated due to nanoparticle application (GAPDH: housekeeping gene).

with significant toxic effects like impaired mitochondrial function [9], the activity of mitochondrial dehydrogenases was analyzed. It was revealed that the metabolic activity of neither BNF starch-labeled cells nor nanomag®-D-spio-labeled cells was altered compared to non-labeled control cells (Fig. 1). Focussing on adipogenic differentiation, the expression of adiponectin and FABP4, as markers for terminal adipocyte differentiation, was assessed (Fig. 2). Both adiponectin and FABP4 expression was not detected in undifferentiated ASC. In ASC cultured under adipogenic differentiation conditions, nanoparticle-labeling caused a diminished expression of adiponectin and FABP4 in a concentration-dependent manner. Effects were more pronounced for BNF starch-labeled cells. Both nanoparticle types exhibit hydrodynamic diameters around 100 nm (unpublished data). In contrast, several studies found no effects of SPION labeling on adipogenesis using varying SPIONs with hydrodynamic diameters less than 70 nm [10,11].

To influence ASC as little as possible in terms of their adipogenic differentiation potential, BNF starch and nanomag®-D-spio nanoparticles were applied for *in vivo*-experiments at concentrations of 10 µg Fe/ml and 25 µg Fe/ml, respectively. Using these labeling concentrations, cell's viability (Fig. 3) as well as the deposition of lipid droplets (Fig. 4) was verified 21 days after inducing adipogenic differentiation. No cytotoxic effects were revealed during adipogenic differentiation due to nanoparticle labeling. Apparently, nanoparticle-labeled cells deposited lipid droplets in comparable levels as non-labeled cells.

3.2. *In vivo MRI of nanoparticle-labeled ASC*

For MRI visualization of nanoparticle-labeled ASC seeded onto collagen scaffolds in SCID mice, T2-weighted sequences were used to detect signal decay caused by iron (Fig. 5). Image analysis demonstrated a loss of signal intensity in scaffolds containing ASC labeled with BNF starch nanoparticles as well as nanomag®-D-spio nanoparticles. After 24 h, nanoparticle-labeled cells are homogeneously distributed. In contrast, the signal loss indicating nanoparticle-labeled ASC is located in the boundary of the scaffolds at day 28 after implantation. Absolute volumes of cell-seeded scaffolds were calculated 24 h after implantation and revealed a volume of 0.4368 cm³, 0.4873 cm³, and 0.4913 cm³ for the following groups: non-labeled cells, BNF starch- and nanomag®-D-spio-labeled cells, respectively. The absolute volume of scaffolds was decreased more than twofold at day 28 after implantation resulting in 0.132 cm³ (non-labeled cells), 0.1592 cm³ (BNF starch-labeled cells), and 0.1675 cm³ (nanomag®-D-spio-labeled cells). Apparently, all experimental groups were affected in equal measure. Volume loss of collagen scaffolds may be due to enzymatic degradation resulting in collagen resorption [12].

The promising results presented in this study demonstrate that MRI is an useful technique for cell tracking *in vivo* using magnetic iron oxide nanoparticles.

Future studies comprising a statistical relevant number of animals per group as well as additional time points are necessary to consolidate the results. In addition, the impact of special MRI approaches such as T2 mapping and T2* mapping should be evaluated in future work providing promising options for iron quantification [3,4,13]. In our opinion, the possibilities of MRI are far from being exhausted. Furthermore, it should be possible to draw conclusions about the number of cells present on the scaffolds using iron quantification MRI techniques. This hypothesis will be further explored.

So far, both nanoparticle types seem to be suitable for cell tracking experiments, however, BNF starch nanoparticles may

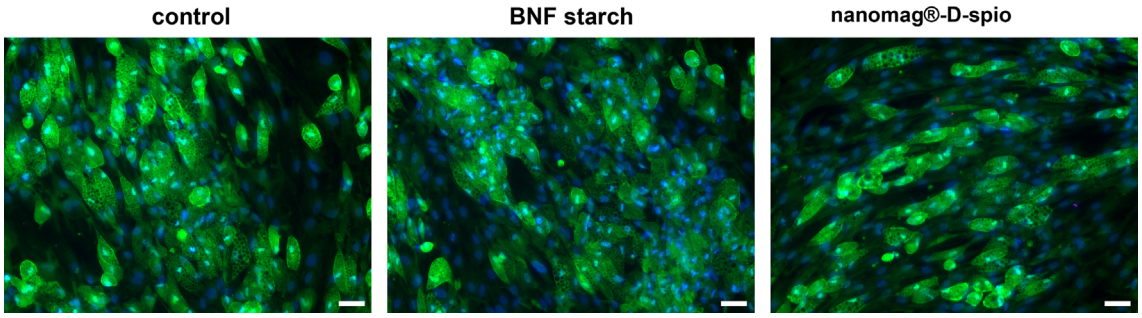

Fig. 3. Viability/cytotoxicity test. No cytotoxic effects were detected due to nanoparticle labeling. Viable cells and nuclei of apoptotic cells were stained with calcein AM (green) and ethidium homodimer (red), respectively. Cells were counterstained with Hoechst 33342 (blue) (scale bars = 50 μm).

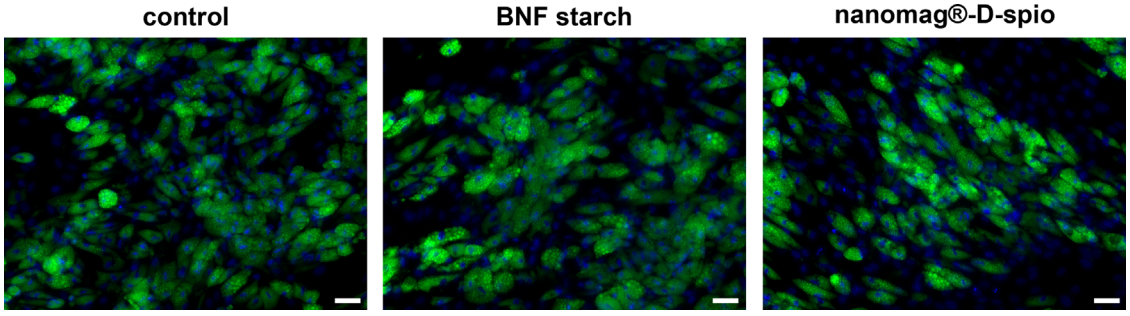

Fig. 4. Deposition of lipid droplets. Adipogenic differentiation of ASC was verified by staining of lipid droplets (green). Cells were counterstained with Hoechst 33342 (blue) (scale bars = 50 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

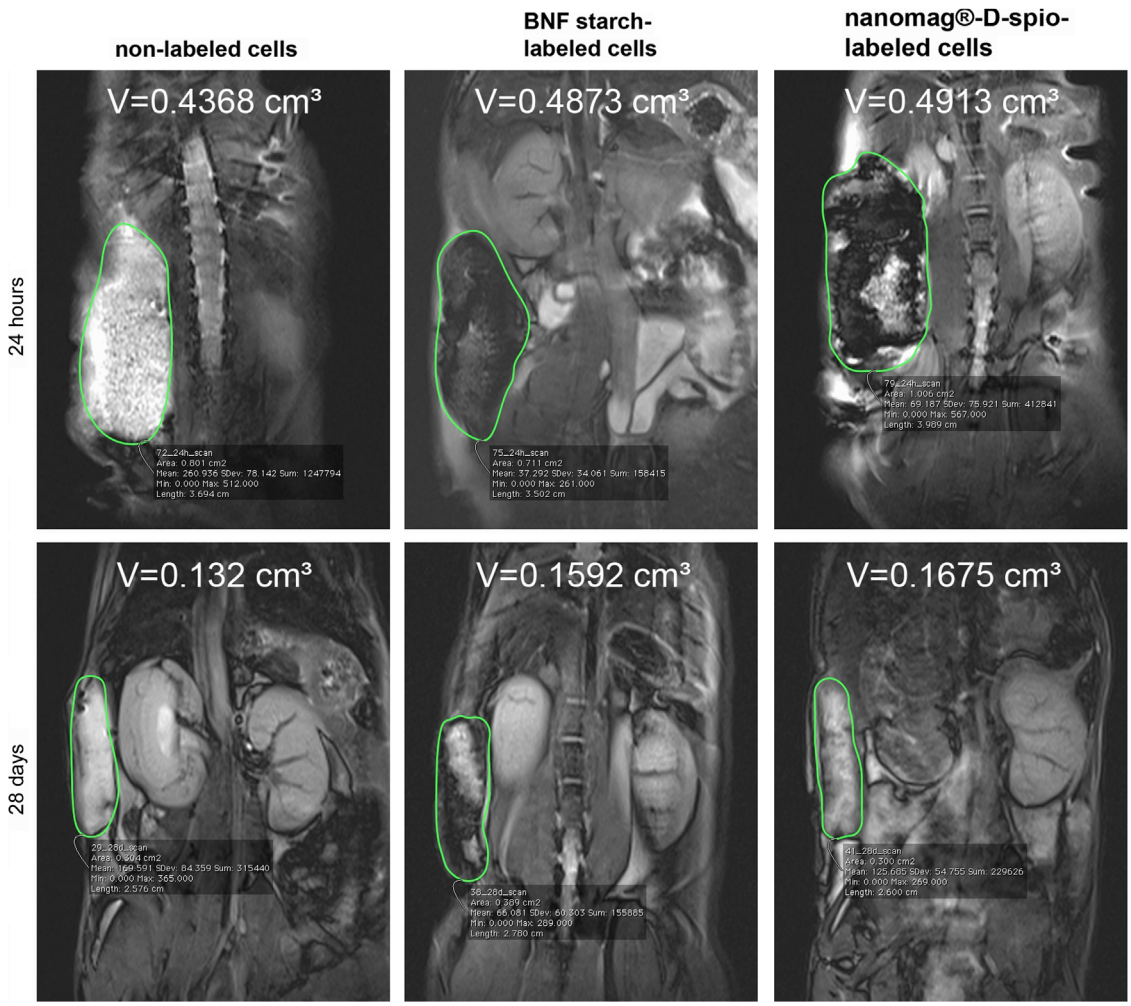

Fig. 5. *In vivo* MRI of nanoparticle-labeled ASC. T2-maps of collagen scaffolds implanted in SCID mice are shown 24 h and 28 days after implantation. Scaffold borders are marked with a green line and calculated absolute volumes of scaffolds are specified in each picture. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

have more impact on the adipogenic differentiation potential of ASC than nanomag®-D-spio nanoparticles. In general, perfect nanoparticles should exhibit both excellent MRI properties and minimal impact on cell functions. Further experiments are needed to address this question properly.

4. Conclusions

First insights are provided showing the successful visualization of BNF starch and nanomag®-D-spio-labeled ASC seeded onto collagen scaffolds for adipose tissue engineering in a SCID mice model. Initial *in vitro* experiments revealed suitable labeling concentrations for both nanoparticle types to ensure minimal influence on adipogenic differentiation of ASC. Further studies are needed to establish iron quantification *in vivo* using MRI and, moreover, be able to calculate the number of cells within the scaffolds.

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