

# Human Integrin $\alpha 10\beta 1$ -Selected Mesenchymal Stem Cells Home To Cartilage Defects In The Rabbit Knee And Assume A Chondrocyte-Like Phenotype

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## Research Article

**Keywords:** intra-articular injection, xenogeneic, mesenchymal stem cell (MSC), osteoarthritis (OA), magnetic resonance imaging (MRI), superparamagnetic iron-oxide nanoparticle (SPION), integrin  $\alpha 10\beta 1$ , homing.

**Posted Date:** March 15th, 2022

**DOI:** <https://doi.org/10.21203/rs.3.rs-1434632/v1>

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# Abstract

**Background:** Mesenchymal stem cells (MSCs) have shown promising results in stimulating cartilage repair and in the treatment of osteoarthritis (OA). However, the fate of the MSCs after intra-articular injection and their role in cartilage regeneration is not clear. To address these questions, this study investigated 1) homing of labeled human adipose tissue derived integrin  $\alpha 10\beta 1$ -selected MSCs (integrin  $\alpha 10$ -MSCs), to a cartilage defect in a rabbit model and 2) the ability of the integrin  $\alpha 10$ -MSCs to differentiate to chondrocytes and to produce cartilage matrix molecules *in vivo*.

**Design:** Integrin  $\alpha 10$ -MSCs were labeled with superparamagnetic iron oxide nanoparticles (SPIONs) co-conjugated with Rhodamine-B to allow visualization by both MRI and fluorescence microscopy. A cartilage defect was created in the articular cartilage of the intertrochlear groove of the femur of rabbits. Seven days post-surgery, labeled integrin  $\alpha 10$ -MSCs or vehicle were injected into the joint. Migration and distribution of the SPION-labeled integrin  $\alpha 10$ -MSCs was evaluated by high field 9.4T MRI up to 10 days after injection. Tissue sections from the repair tissue in the defects were examined by fluorescence microscopy.

**Results:** *In vitro* characterization of the labeled integrin  $\alpha 10$ -MSCs demonstrated maintained viability, proliferation rate and tri-lineage differentiation capacity compared to unlabeled MSCs. *In vivo* MRI analysis detected the labeled integrin  $\alpha 10$ -MSCs in the cartilage defects at all time points from 12 hours after injection until day 10 with a peak concentration between day 1-4 after injection. The labeled MSCs were also detected lining the synovial membrane at the early time points. Fluorescence analysis confirmed the presence of the labeled integrin  $\alpha 10$ -MSCs in all layers of the cartilage repair tissue and showed co-localization between the MSCs and the specific cartilage molecules aggrecan and collagen type II indicating *in vivo* differentiation of the MSCs to chondrocyte-like cells. No adverse effects of the  $\alpha 10$ -MSC treatment were detected during the study period.

**Conclusion:** Our results demonstrated migration and homing of human integrin  $\alpha 10\beta 1$ -selected MSCs to cartilage defects in the rabbit knee after intra-articular administration indicating that the MSCs have a direct role in the cartilage regeneration.

## Introduction

Osteoarthritis (OA) is a progressive, degenerative joint disease characterized by inflammation, destruction of the articular hyaline cartilage and changes of the underlying bone(1, 2). In the process, the normally very tight homeostasis changes and the chondrocytes adopt a catabolic phenotype and contribute actively to the degradation of the articular cartilage(3, 4). Cartilage lesions are a major component of osteoarthritis and tend to form in early stages of the disease or as a result of trauma. These lesions typically fail to heal due to no or minimal ability for intrinsic repair in the hyaline cartilage(5–9). When a healing response takes place it typically results in formation of fibrocartilage with inferior mechanical properties containing fibrous collagen type I (COL1) instead of hyalin collagen type II (COL2) (6, 10). Currently, there are no disease modifying treatments available that can halt disease progression or stimulate regeneration of the cartilage. Thus, OA is still an irreversible condition(11) with great impact on quality of life for the affected patients and a great socioeconomic impact(7, 12).

Mesenchymal stem cell (MSC)-based therapy has developed into a promising treatment option for OA and cartilage lesions. The reason is the multipotent regenerative potential of MSCs including their ability to differentiate into chondrocytes, which is the cell-type that produces the cartilage matrix and that can repair damaged cartilage tissue(13–15). In addition to their chondrogenic potential, it is well-established that MSCs possess a variety of immunomodulatory abilities that are able to attenuate the inflammatory processes that drive OA(16–18).

There is evidence supporting that intra-articular administration of MSCs in patients with OA leads to significantly better clinical results compared to baseline or compared to various control or placebo treatments(19–24). This effect has been demonstrated using clinical outcomes such as VAS (visual analog scale) pain assessment, WOMAC score (Western Ontario and McMaster Universities Osteoarthritis Index), range-of-motion and walking distance capability(19, 20). Some authors have reported evidence of cartilage repair by magnetic resonance imaging (MRI) and second-look arthroscopy(21). MSCs embedded in a variety of scaffold preparations delivered through arthroscopic surgeries have been used in focal cartilage defects with good results in both preclinical animal studies(23, 24) and in clinical trials(22). However, this may not be the optimal method of delivery of cell therapy for multifocal or diffuse cartilage damage as seen in OA. Allogeneic MSCs prepared for “off-the-shelf” use for intra-articular injection have the potentiality to offer a relatively low-cost, minimally invasive, and easily manageable treatment option for OA.

It is still debated whether or not MSCs are able to migrate and attach to damaged tissue such as cartilage, as results vary significantly(19, 25–27). One reason for the varying results and conflicting data in experimental and clinical studies(19, 28, 29) is that MSC populations, defined by the minimal criteria proposed by International Society for Cellular Therapy in 2006(30), exhibit substantial heterogeneity between donors and tissues of origin and even between cells in individual MSC preparations(31).

The collagen-binding integrin  $\alpha 10\beta 1$  has been shown to be a MSC marker able to identify and select potent and consistent MSC preparations(32, 33). Integrins are transmembrane receptors consisting of an  $\alpha$ - and a  $\beta$ -subunit. They facilitate cell-to-cell and cell-to-extracellular matrix adhesion and signal transduction pathways. Integrin  $\alpha 10\beta 1$  was originally identified as the major collagen type II-binding receptor on chondrocytes(34, 35). It has been shown to be a phenotypic marker of chondrogenic differentiation(32, 36, 37), and to be expressed at the onset of chondrogenesis in the developing skeleton in the embryo(38, 39). Loss of integrin  $\alpha 10\beta 1$ , through genomic deletion, leads to chondrocyte dysfunction resulting in growth retardation of the long bones and skeletal immaturity(40, 41).

MSCs selected for a high integrin  $\alpha 10\beta 1$  expression (integrin  $\alpha 10$ -MSCs) demonstrate a significantly higher chondrogenic differentiation capacity *in vitro* and increased immunomodulatory capacity through T-cell proliferation suppression and secretion of PGE<sub>2</sub>, when compared to an unselected population of MSCs(33). In addition, integrin  $\alpha 10$ -MSCs have shown improved ability to adhere to chondral and subchondral defects *in situ* compared to unselected MSCs(33). Furthermore, it was recently demonstrated that integrin  $\alpha 10$ -MSCs are able to mitigate the progression of OA in an equine experimental model of post-traumatic OA after intra-articular injection, shown through significantly less cartilage fibrillation and less bone sclerosis compared to untreated OA joints(36).

The ability of the integrin  $\alpha$ 10-MSCs to home and adhere to damaged cartilage(33) and directly participate in the regenerative process *in vivo* has not yet been demonstrated. Therefore, the aim of this study was to investigate the homing capacity of labeled integrin  $\alpha$ 10-MSCs in a cartilage defect rabbit model. Our hypothesis was that integrin  $\alpha$ 10-MSCs have the potential to home to damaged cartilage and subsequently assume a chondrocyte-like phenotype.

## Materials And Methods

### Study design

Before initiating the *in vivo* study, *in vitro* optimization of the integrin  $\alpha$ 10-MSC superparamagnetic iron-oxide nanoparticle (SPION) labeling protocol was conducted (Additional file 1). The aim was to establish a labeling protocol that would allow good MRI contrast and fluorescence imaging while preserving cell viability, proliferation, and differentiation capacity. For the *in vivo* study, a cartilage defect was created in the right knee of 12 rabbits. Labeled integrin  $\alpha$ 10-MSCs were injected into the same knee 7 days post-injury in 8 rabbits while 4 rabbits were injected with vehicle only. Six of the rabbits injected with labeled  $\alpha$ 10-MSCs were scanned using MRI for longitudinal tracking. The cartilage defects of all rabbits were subjected to histology and immunofluorescence detection of the labeled  $\alpha$ 10-MSCs and of aggrecan, COL2 and integrin  $\alpha$ 10 $\beta$ 1. MRI baseline scans of the knee (n = 6 rabbits) were obtained on day 4 after surgery, which corresponded to day - 3 day before  $\alpha$ 10-MSC injection. Additional MRI scans were conducted 0 hours (n = 3), 12 hours (n = 2), 24 hours (n = 3), 2 days (n = 2), 4 days (n = 6), 7 days (n = 7) and/or 10 days (n = 2) after integrin  $\alpha$ 10-MSC injection (Fig. 1).

### Isolation, selection, and labeling of integrin $\alpha$ 10-MSCs

Human MSCs were isolated from lipoaspirate(33, 42), expanded and selected for expression of integrin  $\alpha$ 10 $\beta$ 1(33, 36) as previously described. Only integrin  $\alpha$ 10 $\beta$ 1-selected MSCs were used in this study.

The MSCs were labeled with the commercially available SPION Molday ION conjugated with the fluorescent dye Rhodamine B (MIRB) (BioPal Inc.) specifically designed for cell labelling and visualization both by MRI and fluorescence(43–51). MSCs were incubated with MIRB at a concentration of 25  $\mu$ g/ml with a labelling time of either 6 hours (6h-MIRB) or 16 hours (16h-MIRB). Labelling efficacy as well as MSC viability, proliferation rate, trilineage differentiation, cell marker expression and cryostability was assessed after labelling and compared to unlabeled MSCs (UL-MSCs). Chondrogenic differentiation was evaluated by real time qPCR analysis of the expression of chondrocyte specific aggrecan, COL2, and integrin  $\alpha$ 10 $\beta$ 1, relative to the reference gene GAPDH. Adipogenic and osteogenic differentiation was assessed by visual detection of intracellular lipid droplets and extracellular calcium deposits, respectively. For a detailed description of methods see Additional File 1.

#### Visualization of MIRB-labeled integrin $\alpha$ 10-MSC in vitro by magnetic resonance imaging

Different concentrations ( $10^3$ - $10^5$ ) of labeled MSCs (6h-MIRB and 16h-MIRB) and UL-MSCs were suspended in 0.2ml low-melting point agarose (Sigma-Aldrich, St Louis, MO, USA) at approximately 37°C to produce phantoms for assessment of the MRI contrast of the labeled MSCs. MRI relaxation times were measured, and

visual assessment was used to assess the efficiency of labeling and imaging contrast (for MRI details and acquisition parameters see Additional File 2).

In vivo studies

## Animals and ethical statement

Twelve young female New Zealand White rabbits (2-2.5kg) were used in this study. The animals were allowed to acclimatize for at least two weeks before entering the study. All animal experiments were approved by the Danish Animal Experiment Inspectorate (approval no. 2019-15-0201-00063) and by the local Ethical Committee of the Department of Veterinary Clinical Sciences, University of Copenhagen (project no. 2019-014). Throughout the study, animal welfare was evaluated daily through assessment of surgery site swelling, body weight, appetite and faecal output, gait symmetry and spontaneous movement. Pain was assessed subjectively on a daily basis using the Rabbit Grimace Scale(52).

## Creation of chondral defect

The chondral defect was created as previously described (53–58). In short, rabbits were anesthetized and placed in supine position, the right knee was clipped and aseptically prepared for surgery. Arthrotomy was performed by a medial parapatellar incision through the skin and joint capsule with the leg fully extended. The patella was displaced laterally, and the leg was flexed to expose the trochlear groove of the femur (Fig. 2). A chondral defect was made by a handheld drill bit with a diameter of 2.5mm and with a stop-device ensuring that all defects were 1.1 +/- 0.1mm deep at the deepest point (Fig. 2). The cone-shape of the drill ensured that the defect was mainly chondral and that the subchondral bone was only reached at the deepest point. Hence, the subchondral bone layer was not breached for this study (anaesthesia and medication protocol is available in Additional file 3).

## Intra-articular injection of MIRB labeled integrin $\alpha$ 10-MSCs

Seven days after surgical creation of the chondral defect, the rabbits were sedated, placed in supine position and the knee was aseptically prepared before injection of labeled MSCs or vehicle (CryoStor, BioLife Solutions) only. A 0.3 ml dose containing  $1.3 \times 10^6$  cryo-preserved MIRB labeled  $\alpha$ 10-MSCs were thawed in 37°C water bath and immediately injected into the cranial femoropatellar compartment using a 25G needle(59). In one rabbit (#3) the intra-articular injection failed and the MSCs were detected peri-articularly by the subsequent MRI scans. Rabbit #3 was therefore excluded from the study.

## In vivo magnetic resonance imaging

MRI scans were performed on a 9.4T Bruker horizontal bore scanner (BioSpec 94/30 USR, Bruker BioSpin), equipped with a B-GA20S gradient coil. Rabbits were anesthetized and placed in a 154mm-inner-diameter Rabbit Body Polarized volume coil (T11733V3, Bruker). A receive-only four-channel phase array surface coil (T10324V3, Bruker) was placed on top of the knee joint. For this, the right hind limb was fully extended, and the patella served as an anatomic marker. Bruker ParaVision 6.0.1 software was used for image acquisition (for acquisition parameters see Additional File 2). The time points of the scans are shown in Fig. 1.

## Magnetic resonance image analysis

Images were analyzed by subjective assessment and by objective automatic registration of changes in signal intensity (SI) in the chondral defect-area and in the surrounding joint tissue. For subjective analysis, all images were assessed by an experienced expert on veterinary diagnostic imaging (SA, DVM., Dipl. ACVR). Visual analysis was performed using Osirix software (Pixmeo). Regions analyzed subjectively included the cartilage defect, cartilage surrounding the defect, infrapatellar fat pad, and synovial membrane. The negative signal created by the MIRB labeling was subjectively graded (0 = none to 3 = marked) for each of the above regions.

For objective analysis, T2\*-weighted (T2\*W) 3-D FLASH images were analyzed as follows: DICOM images were converted to NIfTI-format using MRICROGL (<https://www.nitrc.org/projects/mricrogl>) and signal bias was corrected with 3D-Slicer (<https://www.slicer.org>). All images of the same rabbit at different timepoints were registered using ITK-SNAP software (<http://www.itksnap.org/pmwiki/pmwiki.php>) for complete 3D-alignment of the femoral bone containing the chondral defect. A region of interest (ROI) was selected in and around the chondral defect plus in a region of air immediately adjacent to the knee representing the noise. The regions were chosen based on the baseline acquisitions to eliminate observer bias. Average SI in the ROI was measured using ITK-SNAP. The noise in each image was also measured and subtracted from averaged SI in each ROI. SI drop from baseline was calculated.

## Fluorescence microscopy and immunofluorescence analysis

After euthanasia, the distal femur of both legs was fixed in formalin, demineralized in 10–20% ethylenediaminetetraacetic acid (EDTA) for 4–6 weeks until soft enough for sectioning, dehydrated and embedded in paraffin. Paraffin-embedded tissue sections (4  $\mu$ m) from the defect area and surrounding undamaged cartilage, were acetone fixed and immunostained.

Direct visualization of labeled integrin  $\alpha$ 10-MSCs in the tissue sections were conducted using a fluorescence microscope detecting the Rhodamine B signal from the MIRB labeled  $\alpha$ 10-MSCs. Immunofluorescence staining was performed using specific antibodies to aggrecan (clone 969D 4D11 2A9, Invitrogen) and COL2 (clone 5B2.5, ThermoFisher Scientific) and integrin  $\alpha$ 10 (mAb alpha10, Xintela AB) followed by fluorescence conjugated secondary antibody, donkey anti-mouse conjugated with AlexaFluor647 (Jackson ImmunoResearch). DAPI was used to stain cell nuclei. Staining, detection and co-localization was visually analyzed and recorded using a fluorescence microscope. A semi-quantitative assessment of the amount of MIRB labeled  $\alpha$ 10-MSCs (0–3) was performed for each rabbit.

## Statistical analyses

Normality of data was assessed with a Shapiro-Wilk test, a histogram, and a qq-plot. Homogeneity of variance was assessed with Levene's test. A paired t-test between objective SI measurement at baseline and on day 4, and a Spearman correlation between SI drop at the time of euthanasia, as well as a semi-quantitative assessment of the number of MIRB labeled  $\alpha$ 10-MSCs seen on fluorescence microscopy were performed using a statistical software package R (R, version 3.6.1, The R Foundation for Statistical Computing). Graphs were created using GraphPad Prism 8.3.0.

## Results

# Optimization of the MIRB-labeling of integrin $\alpha$ 10-MSCs

Intracellular uptake of the MIRB label in the MSCs was confirmed by fluorescence microscopy (Fig. 3A). The labeling frequency of the MSCs was 100% after both 6 hours and 16 hours MIRB-labeling time as demonstrated by flow cytometry, and the median fluorescence intensity (MFI) of Rhodamine B was 11,613 and 20,545 in 6h-MIRB and 16h-MIRB, respectively. After 3 days of proliferation in culture post-labeling, the MFI had decreased to 1,322 (6h-MIRB) and 2,530 (16h-MIRB). At this time point the frequency of labeled MSCs was 27.86% (6h-MIRB) and 59.57% (16h-MIRB). After additional 2 days of proliferation in culture, the MFI of the 6h-MIRB was decreased to 841 with a labeling frequency of 8.4% (Supplementary Fig. 1, Additional File 1).

MSC viability immediately after labeling was high in both 6h-MIRB (99.69%) and 16h-MIRB (97.38%) assessed by 7-AAD (7-Amino-Actinomycin D, Biolegend) staining and flow cytometry analysis. However, total cell count of labeled integrin  $\alpha$ 10-MSCs was lower than the cell count of the UL-MSCs count, showing a 30% reduction in cell count in 6h-MIRB and 58% reduction in 16h-MIRB (Supplementary Fig. 1, Additional File 1).

Further, labeled integrin  $\alpha$ 10-MSCs showed reduced proliferation rate compared to UL-MSCs. After 3 days of proliferation in culture post-labeling, the number of cell doublings was 2.66 for 6h-MIRB and 2.11 for 16h-MIRB, compared to 2.81 cell doublings for UL-MSCs. After additional 2 days in culture, the 6h-MIRB showed 3.26 cell doublings compared to 3.5 cell doublings for UL-MSCs (Supplementary Fig. 1, Additional File 1).

## MRI detection of MIRB-labeled integrin $\alpha$ 10-MSCs in vitro

In agarose phantoms, all cell-concentrations of 6h-MIRB could be visually detected (lowest concentration of cells = 1000 cells/0.2ml) (Fig. 3B); in agarose phantoms containing the same number of 16h-MIRB, the visualization of the MSCs was better compared to 6h-MIRB. At a concentration of  $1 \times 10^5$  cells, remarkable T2\* shortening was detected with 6h-MIRB compared to UL-MSCs (16,118ms vs. 27,057ms. (Supplementary Fig. 2, Additional File 2).

Taken together, these results confirmed that 6h-MIRB had retained acceptable quality and that they could be detected by MRI. Thus, 6h-MIRB were used in the following *in vivo* experiment.

## In vitro characterization of MIRB-labeled integrin- $\alpha$ 10-MSC

Both UL-MSCs and 6h-MIRB showed a > 99% frequency of cells expressing the stem cell surface markers CD73, CD90 and CD105 (Supplementary Fig. 3, Additional File 1). The frequency of MSCs positive for integrin  $\alpha$ 10 was higher in the 6h-MIRB (81.3%) than the UL-MSCs (58.8%).

The chondrogenic differentiation assay showed that compared to the non-induced control  $\alpha$ 10-MSCs chondrogenesis was induced in both UL and the 6h-MIRB  $\alpha$ 10-MSCs, assessed by gene expression of both COL2, aggrecan and integrin  $\alpha$ 10, and that the expression levels seemed to be higher in the 6h-MIRB compared to UL (Fig. 4A).

Osteogenic differentiation in both the UL and the 6h-MIRB was confirmed by the staining of extracellular calcium deposits with Alizarin Red, while adipogenic potential was confirmed by the observation of

cytoplasmic lipid droplets stained with Oil Red O in both the UL and the 6h-MIRB (Fig. 4B). After one freeze-thaw cycle the viability of the 6h-MIRB was 97.0% and the MFI was 8,654 compared to 11,613 before freezing.

### **In vivo integrin $\alpha$ 10-MSC tracking**

The rabbits did not show any lameness or change in spontaneous movements after surgery and did not display any signs of pain during the study. Further, lameness or swelling was not observed after the intra-articular injection of the MIRB-labeled integrin  $\alpha$ 10-MSCs. At the time of euthanasia all cartilage defects were partially healed in both treated and untreated rabbits (Supplementary Fig. 4, Additional File 3).

## **MRI show homing of MIRB labeled integrin $\alpha$ 10-MSC to the osteochondral defect**

Subjective visual analysis revealed that the 6h-MIRB labeled MSCs were visible as hypointense “black dots” on the MR images. Immediately after injection they were distributed as single cells or small cell clusters in the synovial fluid, particularly in the anterior part of the knee (Fig. 5), and after 12 hours the MSCs were found lining the synovial membrane and few MSCs were found in the synovial fluid (Fig. 5). At this timepoint the MSCs were also detected in the posterior joint compartments. At 12 hours after injection, labeled integrin MSCs were found in and around the cartilage defect (Fig. 6). The amount of labeled MSCs in the cartilage defects increased up to day 4 and began to decline thereafter. In the synovial membrane, labeled MSCs seemed to decline after 2–4 days, and hypointensity in the synovial membrane was low at day 10 (Table 1). There were no MSCs attaching to healthy cartilage. Some MSCs were detected in the infrapatellar fat pad up to day 2.



Table 1

**Visual grading of integrin  $\alpha 10$ -MSC distribution on magnetic resonance images** Distribution of labeled mesenchymal stem cells selected for a high expression of integrin  $\alpha 10\beta 1$  (integrin  $\alpha 10$ -MSCs) after intra-articular injection in the knee of 6 rabbits with a surgically created cartilage defect. The distribution is determined by visual grading of magnetic resonance images. Distribution and cell concentration was graded subjectively as 0 = none; + = mild; ++ = moderate; +++ = marked; grey box = rabbit not scanned at that timepoint. *Rabbit #3 was removed from the study because of unintentional peri-articular injection of labeled  $\alpha 10$ -MSCs.*

		0 hours	12 hours	24 hours	2 days	4 days	7 days	10 days
Rabbit 1	Cartilage Defect		+++			++	+	
	Surrounding Cartilage		0			0	0	
	Synovial Membrane		+++			+	+	
	Infrapatellar Fat Pad		+			0	0	
Rabbit 2	Cartilage Defect			+		+		0
	Surrounding Cartilage			0		0		0
	Synovial Membrane			++		+		+
	Infrapatellar Fat Pad			0		0		0
Rabbit 4	Cartilage Defect	0			+	+		0
	Surrounding Cartilage	0			0	0		0
	Synovial Membrane	++			++	+		0
	Infrapatellar Fat Pad	0			0	0		0
Rabbit 5	Cartilage Defect	+		+		+		
	Surrounding Cartilage	+		0		0		
	Synovial Membrane	++		+		+		
	Infrapatellar Fat Pad	+		0		0		
Rabbit 6	Cartilage Defect		++		++	+	+	
	Surrounding Cartilage		0		0	0	0	
	Synovial Membrane		++		+	+	+	
	Infrapatellar Fat Pad		+		+	0	0	

The objective SI measurements confirmed the visual analysis results, demonstrating a drop in SI in the cartilage defect from 12 hours after injection, indicating accumulation of MIRB-labeled MSCs in the defect with a peak on day 2 to 4 ( $p = 0.036$  relative to baseline) after injection. On day 4 the SI was reduced up to 80% compared to baseline, and on day 10 SI had returned to baseline (Fig. 7).

## MIRB-labeled integrin $\alpha 10$ -MSCs co-localize with aggrecan and collagen type II in the regenerated cartilage tissue

Fluorescence microscopy of sections from the cartilage defect showed MIRB labeled integrin  $\alpha 10$ -MSCs in all layers of the cartilage repair tissue in all treated rabbits (Fig. 8C), while no labeled MSCs were detected in normal undamaged cartilage (Fig. 8A). No MIRB signal was detected in the untreated rabbits (Fig. 8B). The number of labeled MSCs varied between the treated rabbits (Supplementary Fig. 5, Additional File 3), however, the degree of MIRB labeled MSCs corresponded with the degree of MIRB signal on MRI images ( $r = 0.94$ ;  $p = 0.0167$ ) at time of euthanasia (Supplementary Table 1, Additional File 3).

Immunofluorescence analysis of the cartilage specific matrix components aggrecan and COL2 showed co-localization with MIRB labeled MSCs in the treated rabbits (Fig. 8D-E) suggesting differentiation of the MSCs to chondrocyte-like cells. Immunodetection of integrin  $\alpha 10$  showed that both the MIRB labeled MSCs and the resident chondrocytes expressed integrin  $\alpha 10$  (Fig. 8F).

## Discussion

This study demonstrated cartilage homing of intra-articular administered human MSCs in a cartilage defect animal model using *in vivo* MRI and post-mortem fluorescence microscopy. In the repair tissue of the defects, the injected integrin  $\alpha 10$ -MSCs were found to be co-localized with aggrecan and COL2, indicating an ability of the integrin  $\alpha 10$ -MSCs to differentiate to chondrocyte-like cells and to produce cartilage matrix molecules.

To be able to investigate homing and differentiation of the integrin  $\alpha 10$ -MSCs, we optimized the SPION labeling protocol to achieve viable and potent MSCs with maintained trilineage differentiation capacity. Our results showing chondrogenic differentiation capacity of integrin  $\alpha 10$ -MSCs after labeling is in contrast to findings in previous studies(60–62), where only adipogenic and osteogenic potential but not chondrogenic potential was retained after SPION labeling. In agreement with other studies, we found that labeling time had great impact on MSC quality(45). We used the commercially available SPION labeling agent MIRB, which is conjugated with the fluorophore Rhodamine B for visualization by both MRI and fluorescence microscopy. MIRB is a very stable SPION shown to be detectable by MRI for 18 weeks or more in an OA model in sheep(47). With a MIRB concentration of 25  $\mu\text{g}/\text{ml}$  and labeling time of 6 hours, impact on viability and proliferation was negligible and MRI contrast good, while 16-hour labeling resulted in poor MSC viability and was deemed unsuitable despite the high MRI contrast demonstrated *in vitro*. Our protocol also resulted in maintained MCS phenotype, as ascertained by expression of the cell surface markers CD73, CD90 and CD105(30, 63) and integrin  $\alpha 10\beta 1$ .

The optimized labeling protocol allowed us to inject labeled MSCs with high viability and potency, to investigate to ability of the MSCs to home and integrate in a cartilage defect. The labeling allowed us to track the cells over time after intra-articular injection and investigate distribution to different tissues in the joint.

Our study is the first to show homing of SPION labeled MSCs to a focal cartilage defect using MRI. Other studies have used MRI-based cell-tracking of labeled MSCs embedded in scaffolds and placed directly onto the cartilage defect during surgery to evaluate MSC retention(64–66). After intra-articular injection, labeled MSCs have been visualized with MRI dispersed diffusely in the joint(43, 47, 67, 68), but never previously detected in an osteochondral defect(27, 44, 53). This could be related to the sensitivity of the detection methods employed or to the performance the MSCs. Concerns over low homing ability have been raised by other authors, as this could theoretically impair therapeutic outcome of MSC therapy(25, 26, 69). We found that MSCs selected for the collagen-binding integrin  $\alpha 10\beta 1$  homed to the experimental cartilage defect in sufficient numbers to be detected with high-field MRI. This is in agreement with our previous *in situ* studies, showing improved ability of integrin  $\alpha 10$ -MSCs to home to cartilage defects in osteochondral explants along with improved capacity to differentiate to chondrocytes *in vitro*, compared to unselected MSCs(33).

Using MRI, we were able to clearly visualize the MIRB labeled integrin  $\alpha 10$ -MSCs in the joint space immediately after intra-articular injection and demonstrate their migration and homing to the cartilage defect. We detected iron-derived hypointense signal, representing MIRB labeled MSCs, in the cartilage defect from 12 hours after injection with a peak concentration on day 1 to 4 after injection followed by a decline. This decline in hypointense iron-signal may be due to a dilution of the MIRB label because of proliferation(70) or because the xenogeneic human MSCs disappeared from the defect with time due to apoptosis or an immunologic rejection. Apoptotic MSCs are known not to retain their SPION label(64).

Although we used xenogeneic human MSCs in an immunocompetent rabbit model, the integrin  $\alpha 10$ -MSCs were still present 10 days after the intra-articular injection and had engrafted in all layers of the cartilage repair tissue. The reason for this might be that cartilage is thought to be somewhat immune-privileged(71) and that MSCs(17, 72) are known to have immunomodulatory properties. In addition, the MSC used in this study have been selected for the marker integrin  $\alpha 10\beta 1$  and thus consist of a homogenous MSC preparation. We have previously shown that integrin  $\alpha 10$ -MSCs have increased PGE<sub>2</sub> secretion and T-cell proliferation suppression compared to unselected MSCs(33). PGE<sub>2</sub> mediated regulation of T-cells and macrophages and is one of the suggested mechanisms involved in the immunomodulatory effect of MSCs(17, 72–74). It has also been reported that human mesenchymal progenitor cells have been found in the cartilage repair tissue of rabbits up to 10 weeks after intra-articular injection(75) in a rabbit experimental OA model which further support our finding that human MSCs can integrate in the rabbit cartilage.

We have recently reported that intra-articular injection of equine integrin  $\alpha 10$ -MSCs mitigate cartilage degradation and bone sclerosis after articular cartilage injury in an experimental equine post-traumatic OA model(36). Although homing of the injected integrin  $\alpha 10$ -MSCs was not investigated in this study, it is likely a contributing factor for the therapeutic effect seen on cartilage and bone.

In the present study, the presence of integrin  $\alpha 10$ -MSCs in the repair tissue of the cartilage defects was confirmed by fluorescence microscopy of tissue sections collected at the time of euthanasia. The engrafted red MIRB labeled integrin  $\alpha 10$ -MSCs showed co-localization with the cartilage matrix molecules COL2 and aggrecan, indicating that the integrin  $\alpha 10$ -MSCs have the capacity to differentiate to chondrocyte-like cells *in vivo* and potentially could contribute to the regeneration of damaged cartilage. The MIRB labeled integrin  $\alpha 10$ -MSCs also co-localized with integrin  $\alpha 10$  staining in the cartilage. This may indicate a retained phenotype after

*in vivo* injection, which is encouraging, as MSCs are known to sometimes undergo phenotypic changes over time(76) or as a result of inflammatory stimulation(77, 78). However, since the integrated MSCs appear to differentiate to chondrocyte-like cells, the expression of integrin  $\alpha 10\beta 1$  is likely an additional indicator of chondrogenic differentiation, as integrin  $\alpha 10$  also is a marker of chondrocytes(32, 36, 37).

In contrast to humans, rabbits have been reported to have good potential for spontaneous endogenous healing of cartilage(79). Therefore, and due to the low number of animals in this study, cartilage defect healing was not graded in this study. For future studies, it would be interesting to study integrin  $\alpha 10$ -MSCs in an experimental model with cartilage regeneration capacity more similar to humans, such as the horse(80).

## Conclusion

Integrin  $\alpha 10$ -MSCs were successfully MIRB-labeled while maintaining MSC phenotype, viability and chondrogenic differentiation capacity *in vitro*. Using MRI, homing of intra-articularly injected labeled integrin  $\alpha 10$ -MSCs to cartilage defects in a rabbit model was demonstrated. Fluorescence analysis of the cartilage repair tissue in the defects confirmed engrafting of integrin  $\alpha 10$ -MSCs and showed co-localization with the cartilage matrix molecules aggrecan and COL2. These results indicate that integrin  $\alpha 10$ -MSCs have the capacity to differentiate into chondrocyte-like cells and produce cartilage matrix *in vivo*. This points to a promising therapeutic potential of integrin  $\alpha 10$ -MSCs in the treatment of cartilage injuries and OA related cartilage erosions.

## Abbreviations

integrin  $\alpha 10$ -MSC: Mesenchymal stem cell selected for a high expression of integrin  $\alpha 10\beta 1$ ;

COL2: Collagen type II;

DAPI: 4',6-diamidino-2-phenylindole

MFI: median fluorescence intensity;

MIRB: Molday ION conjugated to Rhodamine B

MRI: Magnetic resonance imaging;

MSC: Mesenchymal stem cell;

OA: Osteoarthritis;

SI: Signal intensity;

SPION: Superparamagnetic iron-oxide nanoparticles;

UL-MSCs: unlabeled mesenchymal stem cells;

6h-MIRB: mesenchymal stem cells labeled with Molday Ion co-conjugated with Rhodamine B for 6 hours;

7-AAD: 7-Amino-Actinomycon D;

16h-MIRB: mesenchymal stem cells labeled with Molday Ion co-conjugated with Rhodamine B for 16 hours.

## Declarations

### **Ethics approval and consent to participate:**

All animal experiments were approved by the Danish Animal Experiment Inspectorate (approval no. 2019-15-0201-00063) and by the local Ethical Committee of the Department of Veterinary Clinical Sciences, University of Copenhagen (project no. 2019-014).

### **Consent for publication:**

Not applicable

### **Availability of data and materials:**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

### **Competing interests**

E.L.A. is the CEO and CSO of Xintela AB, holds stock in the company, is an inventor of a patent related to this study. K.U. was an employee of and holds stock in Xintela AB.

### **Funding:**

This study was funded by Xintela AB, Torben og Alice Frimodt Foundation, Direktør Emil C. Hertz og Hustru Inger Hertz' Foundation, and Clara Hansens Mindelegat (Memorial Foundation).

### **Authors' contributions**

CA: contributed to the experimental design and was the assisting in all parts of conducting the study, in charge of animal care and welfare and primary writer of the manuscript.

KU: contributed to the experimental design, is the lead of all in vitro elements of the experiment.

YM: contributed to the experimental design, optimization of MRI scanning protocol and objective scoring of MRI images.

SA: Subjective reading of MRI images and contributed to MRI scanning protocol.

SJ: First idea and contributed to the experimental design, is a part of all animal surgeries, and a major contributor in writing the manuscript.

LCB: Large contributor to study design.

ELÅ: Large contributor to the experimental design and a major contributor in writing the manuscript.

CL: First idea and major contributor to the experimental design, is the lead of all animal surgeries, and a major contributor in writing the manuscript.

All authors read and approved the final manuscript

## Acknowledgements

Bo Holmqvist (ImaGene-iT) for fluorescence microscopy and immunofluorescence, Kavitha Siva (Xintela) for help with all *in vitro* experiments, especially flowcytometry.

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## Figures

### Figure 1

**Study design.** A cartilage defect was created surgically (☒) in the right knee of twelve rabbits, 7 days prior to intra-articular injection of MSCs. Labeled integrin  $\alpha$ 10-MSCs (▲, rabbit 1-8) or DMSO freezing medium (▼, rabbit 9-12) was injected into the right knee at time 0. Baseline MRI scans (●) of the injected knee of rabbits 1-6 were performed on day -3 (4 days after surgery), while rabbits 7-12 did not undergo MRI scanning, but had their knees harvested for histology. Additional MRI scans were conducted immediately after injection of MSCs (0 hours) and 12 hours, 24 hours, 2 days, 4 days, 7 days and/or 10 days after MSCs injection (2-6 rabbits scanned at each time point). Osteochondral tissue samples of the defect area were collected for histology and immunofluorescence analysis after euthanasia (☒) from both treated and control rabbits. <sup>§</sup>*Rabbit 3 was removed from the study because of unintentional peri-articular injection of labeled  $\alpha$ 10-MSCs.*

### Figure 2

**Cartilage defect in the rabbit knee. A)** The defect was made with a handheld drill that ensured a defect size of 2.5mm in diameter and 1.1 +/-0.1 mm in depth. **B)** Arthrotomy was performed through a medial parapatellar incision and lateral displacement of the patella. A chondral defect was made in the trochlear groove of the femur of the rabbits. **C)** Magnified image of the surgically created chondral defect (arrow). **D)** Safranin O fast green-stained histology slice of a partly healed cartilage defect (rabbit #8, day 10 after integrin  $\alpha$ 10-MSC-

injection). Cartilage is red and bone is blue. **E)** Partially healed defect (arrow) (rabbit #1, day 7 after integrin  $\alpha$ 10-  
MSC-injection).

### Figure 3

**Visualization of labeled integrin  $\alpha$ 10-MSCs. A)** Fluorescence microscopy image of six individual integrin  $\alpha$ 10-MSCs labeled with Molday Ion conjugated with Rhodamine B (MIRB) (25  $\mu$ g/ml for 6 hours). **B)** MRI (T2\*W) of phantoms containing different concentrations of MIRB labeled integrin  $\alpha$ 10-MSCs. The MSCs were labeled with MIRB for 6 hours (6h-MIRB) or 16 hours (16h-MIRB) or left unlabeled (UL) and agarose phantoms were created by embedding integrin  $\alpha$ 10-MSCs in 0.2 mL agar in micro-Eppendorf tubes. Tubes were MRI scanned in transverse and sagittal view with a T2\*W FLASH sequence. The individual labeled cells can be seen as black dots, whereas unlabeled cells cannot be detected. 10k = 10,000 cells; 25k = 25,000 cells; 50k = 50,000 cells; 75k = 75,000 integrin  $\alpha$ 10-MSCs.

### Figure 4

**Trilineage differentiation of unlabeled (UL-MSCs) and 6-hour MIRB-labeled (6h-MIRB) integrin  $\alpha$ 10-MSCs. A)** Chondrogenic differentiation was induced and evaluated by qPCR analysis of collagen type II (COL2), aggrecan and integrin  $\alpha$ 10 in UL-MSCs or 6h-MIRB. Uninduced UL-MSCs and 6h-MIRB served as controls. The relative quantity of all genes was normalized to chondrogenically induced UL-MSCs. Uninduced integrin  $\alpha$ 10-MSCs (both UL-MSCs and 6h-MIRB) showed no expression of COL2 or aggrecan but did show expression of integrin  $\alpha$ 10. IntgA10 = integrin  $\alpha$ 10; MIRB = Molday Ion conjugated with Rhodamine B. **B)** Adipogenic and osteogenic differentiation was confirmed by positive staining of intracellular lipid droplets with Oil Red O and extracellular calcium deposits with Alizarin Red, respectively. There was no visual difference between UL-MSCs or 6h-MIRB.

### Figure 5

**Magnetic resonance imaging (MRI) detection of labeled integrin  $\alpha$ 10-MSCs in the synovial fluid.** Baseline MRI scans were performed before injection of MIRB labeled integrin  $\alpha$ 10-MSCs and at different timepoints after injection. Coronal views of the knee of rabbit #1 before injection (Baseline, A), immediately after (0h, B) and 12 hours (12h, C) after injection of  $1.3 \times 10^6$  MIRB labeled  $\alpha$ 10-MSCs. The MSCs are detected as black dots. At baseline the synovial fluid is clear (surrounded by white line). Immediately after injection, MSCs were distributed in the synovial fluid (arrows)(B), and after 12 hours they were found lining the synovial membrane (arrows) (C). MIRB = Molday Ion conjugated with Rhodamine B.

### Figure 6

**Magnetic resonance imaging (MRI) detection of MIRB labeled integrin  $\alpha$ 10-MSCs in a cartilage defect.** A focal cartilage defect was created surgically and MIRB labeled integrin  $\alpha$ 10-MSCs were injected 7 days later. 3D-FLASH MRI coronal (A) and 3D-FISP MRI sagittal (B) views of the knee of rabbit #1 and #6 at baseline (after surgery but before injection), 12 hours (12h), 24 h, 4 days (4d) and 7d after integrin  $\alpha$ 10-MSC injection. The chondral defect is indicated (dotted lined box) and inserts show the magnification of the cartilage defect area and labeled integrin  $\alpha$ 10-MSCs aggregating in the are detected as hypointense black material. MIRB = Molday Ion conjugated with Rhodamine B.

## Figure 7

**Magnetic resonance imaging (MRI) signal intensity (SI) in the cartilage defects after homing of labeled integrin  $\alpha$ 10-MSCs.** All images of the same rabbit were aligned. A 3D region of interest (ROI) was marked in the area of the cartilage defect (indicated in Figure 6) and SI in the ROI was automatically calculated. The graph shows the % drop in SI from baseline. An SI drop indicates accumulation of labeled  $\alpha$ 10-MSCs in the defect. The SI drop was statistically significant on day 4 ( $p = 0.036$ ) relative to baseline. *Note that rabbit #3 was removed from the study because of unintentional peri-articular injection of labeled  $\alpha$ 10-MSCs.*

## Figure 8

**Fluorescence analysis of labeled integrin  $\alpha$ 10-MSCs and cartilage specific molecules aggrecan, collagen type II (COL2) and integrin  $\alpha$ 10.** Osteochondral sections obtained after euthanasia from rabbits with a surgically created and partially healed cartilage defect (4-10 days after intraarticular injection of either MIRB-labeled integrin  $\alpha$ 10-MSCs or from untreated rabbits). MIRB labeled integrin  $\alpha$ 10-MSCs are detected in the cartilage repair tissue of treated rabbits (red, C-F). The cartilage specific molecules aggrecan (green, A-D), COL2 (green, F), and integrin  $\alpha$ 10 (green, E) are stained with specific antibodies, and cell nuclei are stained with DAPI (blue, A-F). **A)** Normal undamaged cartilage from a treated rabbit (rabbit #1) showing aggrecan (green) in chondrocytes and in the extracellular matrix. **B)** Repair tissue in an untreated cartilage defect (rabbit #10 euthanized on day 10) showing aggrecan (green). **C)** Repair tissue in a treated cartilage defect (rabbit #4 euthanized on day 10) showing MIRB labeled integrin  $\alpha$ 10-MSCs (red) and aggrecan (green). **D)** MIRB labeled integrin  $\alpha$ 10-MSCs co-localizes with aggrecan, which is seen as cells with both red and green stain (white arrows) as well as red cells surrounded by green aggrecan-containing extracellular matrix. **E)** MIRB labeled integrin  $\alpha$ 10-MSCs co-localizes with collagen type II, which is mainly seen as cells with both red and green stain (arrow). **F)** The collagen binding receptor integrin  $\alpha$ 10 (green) is expressed on both MIRB labeled  $\alpha$ 10-MSCs (red) and resident chondrocytes. Co-localization of MIRB labeled  $\alpha$ 10-MSCs and integrin  $\alpha$ 10 is seen as cells expressing both red and green (white arrows). MIRB = Molday Ion conjugated with Rhodamine B.

## Supplementary Files

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