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PLGA-based microcarriers induce mesenchymal stem cell chondrogenesis and stimulate cartilage repair in osteoarthritis

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ABSTRACT

In the present study, we aimed at evaluating the ability of novel PLGA-P188-PLGA-based microspheres to induce the differentiation of mesenchymal stem/stromal cells (MSC) into chondrocytes. To this aim, we tested microspheres releasing TGFβ3 (PAM-T) *in vitro* and *in situ*, in a pathological osteoarthritic (OA) environment. We first evaluated the chondrogenic differentiation of human MSCs seeded onto PAM-T *in vitro* and confirmed the up-regulation of chondrogenic markers while the secretome of the cells was not changed by the 3D environment. We then injected human MSC seeded onto PAM-T in the knee joints of mice with collagenase-induced OA. After 6 weeks, histological analysis revealed that formation of a cartilage-like tissue occurred at the vicinity of PAM-T that was not observed when MSCs were seeded onto PAM. We also noticed that the endogenous articular cartilage was less degraded. The extent of cartilage protection was further analysed by confocal laser microscopy. When MSCs seeded onto PAM-T were injected early after OA induction, protection of cartilage against degradation was evidenced and this effect was associated to a higher survival of MSCs in presence of TGFβ3. This study points to the interest of using MSCs seeded onto PAM for cartilage repair and stimulation of endogenous cartilage regeneration.

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Musculoskeletal disorders are the most common cause of severe long-term pain and physical disability affecting hundreds of millions of patients worldwide [1]. The prevalence of these conditions markedly increases with age, and the burden on health and social care systems is expected to increase dramatically in the coming years. Among these diseases, osteoarthritis (OA) is characterized by loss of articular cartilage, formation of osteophytes, sub-chondral bone sclerosis and a variable degree of synovial inflammation. Other causes of cartilage damage are focal injuries arising following traumatic or instability events. The regenerative capacity of cartilage is poor and no disease modifying OA drug (DMOAD) exists. Although new therapeutic approaches are developing, most therapies do not stop or even reduce the progression of the disease and are only symptomatic [2]. Surgical techniques, such as marrow stimulation, mosaicplasty or chondrocyte implantation technique, have provided treatment options for symptomatic patients but the only option for patients in end stage disease is joint prosthesis. While this treatment is generally successful, it is not as attractive for younger patients because of prosthesis lifespan being only 10–15 years [3]. There is therefore a crucial need for the development of new treatments for this debilitating pathology. Regenerative medicine or tissue engineering approaches should reveal innovative therapeutic options.

In clinical practices, current cartilage engineering strategies utilize autologous chondrocytes combined with scaffolds [4]. However due to some drawbacks associated with chondrocyte isolation and expansion, alternative sources of cells have been sought out. Mesenchymal stem cells, also called multipotent mesenchymal stromal cells (MSCs), are an attractive source of cells

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because of their chondrogenic potential and an easy access for isolation and future expansion. They were first used with the idea to create a *de novo* cartilaginous tissue and a number of clinical studies have reported similar therapeutic effect as chondrocytes with lower cost, lower donor site morbidity and a surgical procedure less [4,5]. Scaffold-free injection of MSCs in the joints has been shown to protect against cartilage degradation in pre-clinical models, due to the trophic and anti-inflammatory properties of these cells [6,7]. Safety of such approach has been evaluated in the clinics and some efficacy has been suggested [8]. Other strategies for regenerative medicine involve scaffold-free approaches, osteochondral or particular allografts and cell-free biomaterials [4]. However, despite many efforts made in developing such strategies, the generation of a fully functional cartilage remained elusive and a technology has still to be developed to fulfill the requirements for clinical translation.

Strategies based on MSCs combined with scaffolds offer further hope for the treatment of cartilage lesions. However, efforts are still needed to overcome some problems encountered after cell transplantation such as cell survival, poor cell differentiation and integration into the host tissue. Improvement of the mechanical properties, organization of the neotissue, induction of differentiation and maintenance of chondrocyte phenotype is required to better mimic native morphogenesis. Injectable microspheres can serve as support for cell delivery and can be engineered to release bioactive factors [9]. We previously reported that PLGA-based microspheres releasing TGFβ3 could provide a suitable environment for the differentiation of MSCs into chondrocytes [10]. More recently, optimisation of TGFβ3 release thanks to a novel formulation of PLGA-P188-PLGA-based microspheres demonstrated that chondrogenic differentiation of MSCs was even more efficient [11]. In the present study, we used the most recent formulation of microspheres to evaluate *in vivo* the differentiation of MSCs into chondrocytes and their repair capacity after intra-articular implantation in the knee joints of OA mice.

1. Materials and methods

1.1. Microsphere formulation

Poly(D,L-lactide-co-glycolide)-poloxamer P188-poly(D,L-lactide-co-glycolide) polymer (PLGA-P188-PLGA) microspheres (MS) were prepared using a solid/oil/water (s/o/w) emulsion solvent evaporation-extraction process previously described [12,13]. Polymer PLGA-P188-PLGA was synthesized by IBMM-CRBA CNRS UMR 5247 (Montpellier, France) thanks to a ring-opening polymerization (ROP) of DL-lactide and glycolide using P188 [poly(ethylene oxide) $(PEO)_{80}$ - poly(propylene oxide) $(PPO)_{27}$ - poly(ethylene oxide) PEO_{80})] as an initiator, and stannous octoate [Sn(Oct)2] as catalyst (as developed in Ref. [12]). TGFβ3 loading was 1 μg of protein and 5 μg of human serum albumin (HSA)/mg of MS. The encapsulation of TGFβ3 was then performed as previously described [11]. Briefly, TGFβ3 (50 μg; Peprotech, Neuilly-sur-Seine) was precipitated with Poloxamer 188 (P188) at a TGFβ3-P188 ratio of 1:20 in 1.077 g of cold glycofurol (4 °C) diluted in 10 μL of a TRIS-HCl 0.75 M, NaCl 2 M solution (pH = 7.4). HSA nanoprecipitate with the same 1:20 protein:additive ratio was produced in a similar manner [11]. After 30 min at 4 °C, the nanoprecipitated proteins were recovered by centrifugation (10,000 g, 30 min) and the pellet was dispersed in the organic phase (670 μL of 50 mg PLGA-P188-PLGA dissolved in a 3:1 methylene chloride:acetone solution). The suspension was then emulsified in a polyvinyl alcohol aqueous solution (30 mL, 4% w/v at 1 °C) and mechanically stirred at 550 rpm for 1 min. After addition of 33 mL of deionized water and stirring for 10 min, the emulsion was added to 167 mL deionized water and stirred for 20 min to extract the organic solvent. Finally, the MS were filtered on a 0.45 μm High Volume Low Pressure (HVLP) type filter, washed and freeze-dried. Encapsulation yield, as well as release studies were carried out on these formulations as described [11]. MS without protein were prepared following the same microencapsulation process, but without protein in the organic phase.

PLGA-P188-PLGA MS were coated with fibronectin (FN) and poly-D-Lysine (PDL) to allow cell adhesion. The coating solution consisted in 6 μg/mL of FN and 9 μg/mL of PDL in Dulbecco's Phosphate-Buffered Saline (DPBS), corresponding to a 60:40 ratio of FN:PDL. Coated MS with or without TGFβ3 were prepared and respectively called PAM-T and PAM.

1.2. Mesenchymal stem cell isolation and expansion

Primary human MSCs were isolated from bone marrow of OA patients undergoing hip replacement surgery, as previously described [14]. Patients gave written informed consent and sample recovery was approved by the General Direction for Research and Innovation and the Ethics Committee from the French Ministry of Higher Education and Research (registration number: AC-2010-1105). Cell suspensions were plated at 2000 cells/cm^2 in α-MEM supplemented with 10% fetal calf serum (FCS), 1 ng/mL basic fibroblast growth factor (FGF2) (R&D Systems), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. For expansion, MSCs were subcultured at a density of 1000 cells/cm^2. MSCs were positive for CD73, CD90, CD105 and negative for CD11b, CD14, CD34, CD45 and used at the third or fourth passage.

1.3. Chondrogenesis

MSCs (2.5×10^5 cells) were added to 0.5 mg of PAM or PAM-T, allowed to adhere to the MS for 4 h and then pelleted by centrifugation in 15 mL conical tubes. MSCs with PAM or PAM-T were cultured in 2 mL of incomplete chondrogenic medium, consisting in DMEM supplemented with 0.1 mM dexamethasone, 0.17 mM ascorbic acid, 35 mM proline and 1% insulin-transferrin-selenic acid (ITS) supplement. In control conditions, chondrogenesis was induced by culture of MSCs in micropellets in 15 mL conical tubes, either in incomplete or complete chondrogenic medium. Complete chondrogenic medium consisted in incomplete chondrogenic medium supplemented with 10 ng/mL of TGFβ3 [10]. Every 2–3 days, the chondrogenic media (either incomplete or complete) were replaced by fresh medium. At day 7, 14 and 21, a total of six supernatants were collected and frozen until use. At day 21, micropellets and MSC-PAM or MSC-PAM-T were washed in PBS and immediately processed or stored at −80 °C.

1.4. RT-qPCR

Total RNA was extracted from cells in micropellets or PAM aggregates using the RNeasy kit (Qiagen, Courtaboeuf, France) after mechanical dissociation using a dispenser tool (Ultra-Turrax, IKA, Königswinter, Germany). RNA (0.5 μg) was reverse-transcribed using the M-MLV enzyme (Fisher scientific, Illkirch, France). PCR reaction was carried out on 25 ng of cDNA samples using 5 μmol/L of each primer (Table 1) and 5 μL of SybrGreen PCR Master Mix (Roche Diagnostics, Meylan, France). The following conditions were used: 95 °C for 5 min; 40 cycles at 95 °C for 15 s; 64 °C for 10 s and 72 °C for 20 s in a LightCycler 480 system (Roche Diagnostics) and analyzed with the dedicated software. All values were normalized to the RPS9 housekeeping gene and expressed as fold change using the formula $2^{-\Delta\Delta Ct}$.

Table 1
Primer sequences.

Gene	Forward sequence	Reverse sequence
Col2a1 variant B	CAGACGCTGGTGCTGCT	TCCTGGTTGCCGGACAT
Aggregan	TCGAGGACAGCGAGGCC	TCGAGGGTGTAGCGTGTAGAGA
Link	TTCCACAAGCACAAACTTTA CACAT	GTGAAACTGAGTTTTGTATAACCTCTCAGT
Collagen X	TGCTGCCACAAATACCCTTT	GTGGACCAGGAGTACCTTGC
Alkaline phosphatase	CCACGTCTTCACATTTGGTG	GCAGTGAAGGGCTTCTTGTC
osteocalcin	GGCGCTACCTGTATCAATGG	TCAGCCAACTCGTCACAGTC
FABP4	ATGGGATGGAAAATCAACCA	GTGGAAGTGACGCCTTTCAT
LPL	GTCCGTGGCTACCTGTCATT	TGGATCGAGGCCAGTAATTC
RPS9	ATGAAGGACGGGATGTTCAC	GATTACATCCTGGGCCTGAA

1.5. Protein quantification

Supernatants from micropellets, MSC-PAM or MSC-PAM-T were assayed by ELISA. Human Hepatocyte Growth Factor (HGF), TGFβ1, interleukin (IL)6, IL8, Tissue Inhibitor of Metalloproteinases (TIMP)-2 and Matrix Metalloproteinase (MMP)-1 were quantified by specific sandwich ELISA according to the manufacturer's recommendations (R&D Systems).

1.6. Animal experimentation

Collagenase-induced osteoarthritis (CIOA) model was induced in immunodeficient SCID/Bg mice obtained from Charles River (L'Arbresle, France). Animal experimentation was conducted in agreement with the Languedoc-Roussillon Regional Ethics Committee on Animal Experimentation (approval CEEA-LR-10041). All surgery was performed under isoflurane gas anesthesia. Knee joints were injected with 1 unit of type VII collagenase from *Clostridium histolyticum* (Sigma–Aldrich, L'Isle d'Abeau, France) in 5 μL of saline on days 0 and 2, as previously described [15]. On day 10 or 30, MSCs (2.5×10^5 cells) were added to 0.5 mg PAM or PAM-T in 1 mL of adhesion medium (α-MEM supplemented with 3% FCS, 1 ng/mL FGF2, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin) in an ultra-low cluster plate (Costar, Corning, Avon, France) to allow cell adhesion on MS. After 4 h, MSC-PAM or MSC-PAM-T complexes were carefully washed with DMEM-F12 to eliminate serum and resuspended in a solution of Carboxymethylcellulose-Tween 80-Mannitol (CMC; final concentration 0.125%, 0.125% and 0.5%, respectively). Mice received either CMC as control or MSCs pre-incubated with PAM or PAM-T (7 μL total) using a 10 μL Hamilton syringe (NH-BIO, Massy, France). Animals were sacrificed at day 42 for histological evaluation.

For the biodistribution study, healthy SCID/Bg mice received either MSCs or MSCs pre-incubated with PAM or PAM-T into both knee joints. Euthanasia was performed after 10 or 30 days and 14 organs (lung, heart, kidney, spleen, *Tibialis anterior* muscle, brain, inguinal fat pad, BM, stomach, intestine, liver, testis or ovaries, blood, knee joint) were collected and rapidly frozen at −80 °C.

1.7. Confocal laser scanning microscopy

Fixed hind paws were dissected to carefully remove smooth tissues and expose articular cartilage of the tibia. Tibiae were placed in a chamber system (Labtek, Dutscher, Issy-les-Moulineaux, France) filled with PBS at room temperature. The tibial plateaux were scanned through their depth in XYZ-mode, using a confocal laser scanning microscope (TCS SP5-II, Leica Microsystems, Nanterre, France) with a voxel size of 6 μm, a 5× dry objective and a UV-laser light source ($\lambda = 405$ nm). Image stacks were then processed to evaluate articular cartilage degradations. Morphometric parameters (cartilage volume, cartilage thickness and specific cartilage surface) were quantified for the entire articular cartilage located in both lateral and medial plateau of each tibia (Avizo software, FEI Visualization Sciences Group, Lyon, France).

1.8. Histology

Knee joints were fixed in 4% formaldehyde, embedded in paraffin and cut in sections of 7 μm. Sections were stained with Safranin O-Fast green for analysis of cartilage damage. Cartilage damage was scored using the modified Pritzker OARSI score as described [15,16]. The OA score evaluated the grade of cartilage destruction and the extend of damaged cartilage and corresponded to the median value of 3 scores corresponding to 3 knee sections separated by a 140 μm thickness.

1.9. Fluorescence in situ hybridization

Paraffin sections of mouse knees were placed in Citrate Buffer pH6 at 100 °C for 20 min and then treated by pepsin for 15 min (Kreatech, Amsterdam, The Netherlands). Sections were then hybridized with human and mouse COT1 DNA as previously reported [17]. Sections were finally washed, stained with DAPI and mounted following FISH Digestion Kit protocol (Kreatech).

1.10. DNA extraction and real time PCR

DNA extraction from the different tissues or organs was performed as described [17]. Real time PCR (qPCR) was performed on 25 ng DNA using the DNA Master SYBR Green I kit (Roche Diagnostics, Meylan, France) and 0.05 μM primers for *alu* or 0.5 μM for murine Actin. According to the quantity of DNA isolated from the organ, we extrapolated the number of MSCs per organ and then normalized to the weight of each organ. The results were expressed as the percentage of human MSCs per organ.

1.11. Statistical analysis

Statistical analysis was performed with GraphPad Software (San Diego, CA). Comparison between groups used the Shapiro–Wilk normality test. When groups passed normality test, an unpaired t test was performed; otherwise, a Mann–Whitney test was used. The tests were considered as significant when p values were less than 0.05.

2. Results

2.1. Efficient chondrogenic differentiation of hMSCs in presence of TGFβ3-releasing PAMs

We previously reported that PLGA-P188-PLGA-based PAMs were more efficient to induce the differentiation of hMSCs into

chondrocytes than PLGA PAMs [11]. Here, we wanted to compare the chondrogenic differentiation of hMSCs when seeded on this novel formulation of PAMs releasing TGFβ3 (PAM-T) to that of hMSCs in standard pellet cultures where TGFβ3 was exogenously added the two or three other days. Exogenous addition of TGFβ3 in pellet cultures allowed the differentiation of hMSCs into chondrocytes as shown by induction or increase of expression level for collagen type II variant B, Aggrecan, COMP and collagen type X (Fig. 1A). Similarly, continuous release of TGFβ3 by PAM-T induced a high expression of all these markers of chondrocytes, while in absence of TGFβ3, PAMs did not induce differentiation of hMSCs. With the exception of COMP, the expression level of chondrocyte markers was slightly lower in PAM-T conditions than that obtained in pellet cultures.

We also determined whether TGFβ3 release by PAM-T influenced the differentiation of hMSCs towards osteogenesis. In presence of TGFβ3, the expression of osteocalcin was lower than that quantified in hMSCs cultured in pellet or seeded on PAMs without TGFβ3 but the expression was significantly lower with PAM-T (Fig. 1B). Expression of alkaline phosphatase was not detected in absence of TGFβ3 or with PAM-T releasing TGFβ3, while it was expressed in the pellet cultures. Expression of alkaline phosphatase in pellet cultured with TGFβ3 is likely associated with the hypertrophic chondrocyte phenotype as determined by collagen type X expression (Fig. 1A). Finally when evaluating the differentiation of hMSCs towards adipocytes, we found out that both LPL and FABP4 expression levels were decreased in presence of TGFβ3, when released by PAM-T or added exogenously (Fig. 1C). Altogether, when compared to the reference pellet culture, continuous release of TGFβ3 by PAM-T allowed efficient differentiation of hMSCs into chondrocytes while osteogenesis was significantly reduced.

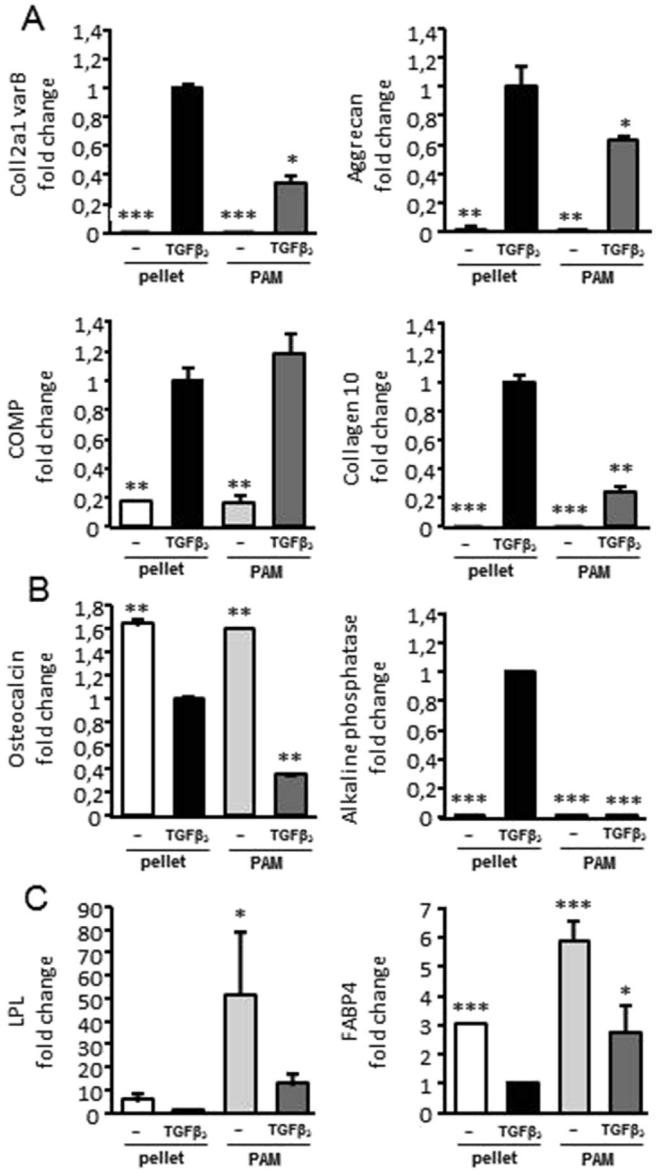

Fig. 1. *In vitro* differentiation potential of hMSCs incubated with PAMs. hMSCs were cultured in chondroinductive conditions either, in micropellet (pellet) supplemented or not with TGFβ3 or, with PAM releasing or not TGFβ3 for 21 days. (A) Expression of chondrocyte markers: collagen 2α1 variant B, aggrecan, cartilage oligomeric protein (COMP) and collagen X determined by RT-qPCR. (B) Expression of osteoblast markers: osteocalcin, alkaline phosphatase. (C) Expression of adipocyte markers: lipoprotein lipase (LPL), fatty acid binding protein 4 (FABP4). Gene expression levels were expressed as fold change compared to positive control (pellet with TGFβ3). n = 3; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

2.2. Stability of the secretory profiles of hMSCs cultured on 3D structures (pellet or PAM)

Together with their differentiation potential, the paracrine properties of hMSCs are related to their regenerative capacity. We therefore investigated whether the secretory profiles of hMSCs were altered in pellet cultures or after adhesion on PAM-T during the differentiation process. Secretion levels of factors with anti-inflammatory (IL6, HGF), anti-fibrotic (TGFβ, HGF), angiogenic (IL8) or remodeling function (MMP1, TIMP2) were quantified in culture supernatants. Independently of TGFβ3, expression kinetics of TIMP2 and MMP1 were similar in pellet or PAM conditions as shown by a high secretion of both factors at day 7 compared to day 1, followed by a huge decrease to reach undetectable levels by day 14 (Fig. 2). Expression of IL6 was higher in presence of TGFβ3 at day 1 independently of the matrix (PAM-T or pellet) and decreased thereafter to be undetectable by day 14. TGFβ3 significantly reduced the secretion of HGF by hMSCs cultured on PAM-T or in pellets while secretion of HGF remained rather stable during the 3 weeks whatever was the matrix. Finally, production of TGFβ1 was not affected by the matrix and/or TGFβ3 addition. All these results demonstrated that culture of hMSCs in pellets or on PAM did not affect their secretory profile.

2.3. In situ differentiation of hMSCs in the osteoarthritic joint environment

We then investigated the capacity of hMSCs to differentiate into chondrocytes in the joint environment, after intra-articular administration in the mouse knee. To be closer to the pathological conditions, we decided to evaluate hMSCs differentiation in the collagenase-induced OA model. We relied on the use of immunodeficient SCID/Bg mice to avoid immune rejection of hMSCs by lymphoid immune cells but allowing OA onset by inflammatory innate monocytes/macrophages. In this model, collagenase injection induced OA symptoms as visualized by degradation of articular cartilage at the surface of tibial plateaux and femoral condyles, as well as calcification of lateral ligament, as compared to saline-injected mice (Fig. 3A, upper panels). Injection of hMSCs pre-incubated with PAM or PAM-T was performed at day 28 after OA induction and mice were sacrificed 2 weeks later. PAMs were easily detectable as round, inert, blank structures surrounded by cells and were mainly localized in the synovial recessi or at the surface of menisci (Fig. 3A, middle panels). At higher magnification, the tissue surrounding PAMs appeared as a fibrotic tissue, with no sign of

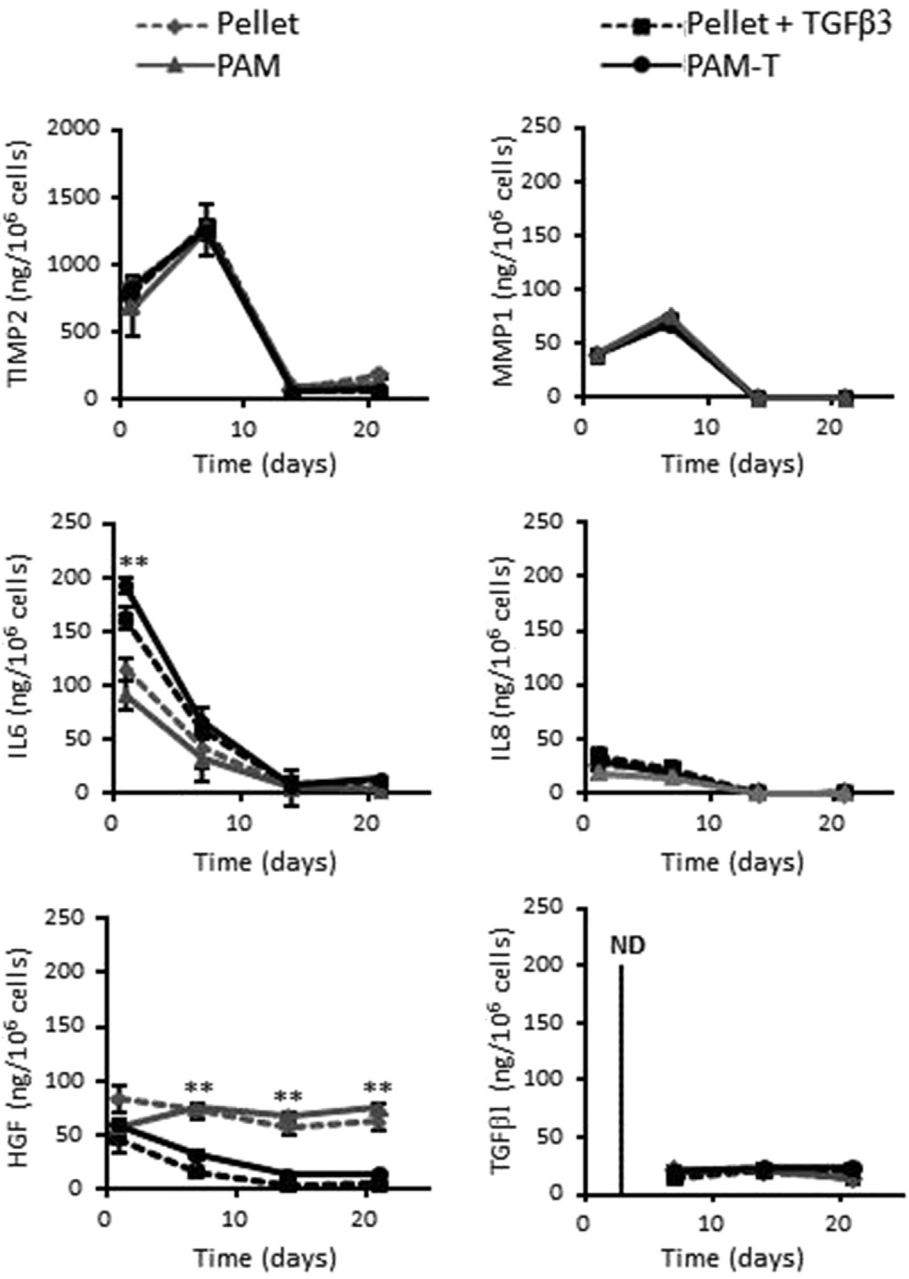

Fig. 2. Secretory profile of hMSCs cultured on PAM or PAM-T. hMSCs were cultured in chondroinductive conditions either, in micropellet (pellet) supplemented or not with TGFβ3 (pellet + TGFβ3) or, with PAM (PAM) and PAM releasing TGFβ3 (PAM-T). Cell culture supernatants were collected at different time points and secretion of TIMP2, MMP1, IL6, IL8, TGFβ1 and HGF was quantified by ELISA. n = 4; **: $p < 0.01$ (samples + TGFβ3 versus samples - TGFβ3); ND: non detected.

hMSCs differentiation (Fig. 3A, lower left panel). In contrast, the tissue surrounding PAM-T formed a tissue resembling histologically to cartilage and characterized by a dense extracellular matrix in which cells were embedded in lacunae (Fig. 3A, lower right panel). Quantification of the number of microspheres on histological sections revealed a higher number of PAM-T than PAMs, which reached almost significance (P value of 0.06) (Fig. 3B).

While evaluating the differentiation of hMSCs into chondrocytes and the formation of cartilage tissue within the knee joints, we observed lower degradation rates of cartilage at the surface of femoral condyles and tibial plateaux in mice that were injected with PAMs or PAM-T. We therefore quantified the cartilage degradation by calculating the OA scores on the knee sections and observed a very significant reduction of OA scores in mice that received hMSCs with either PAM or PAM-T (Fig. 3C). This effect was likely related to the survival of the injected hMSCs since a substantial amount of fluorescent cells were detected by FISH at day 42 indicating the human origin of the cells surrounding PAM or PAM-T (Fig. 3D). Indeed, hMSCs adherent to TGFβ3 releasing PAM-T not only could form a cartilage-like tissue after intra-articular injection in a pathological OA environment but could counteract the degradation of articular cartilage.

2.4. Chondroprotective effect of hMSCs on joint articular cartilage after implantation in osteoarthritic environment

We therefore evaluated the effect of continuous release of TGFβ3 by PAM-T on the trophic effect of hMSCs in terms of cartilage

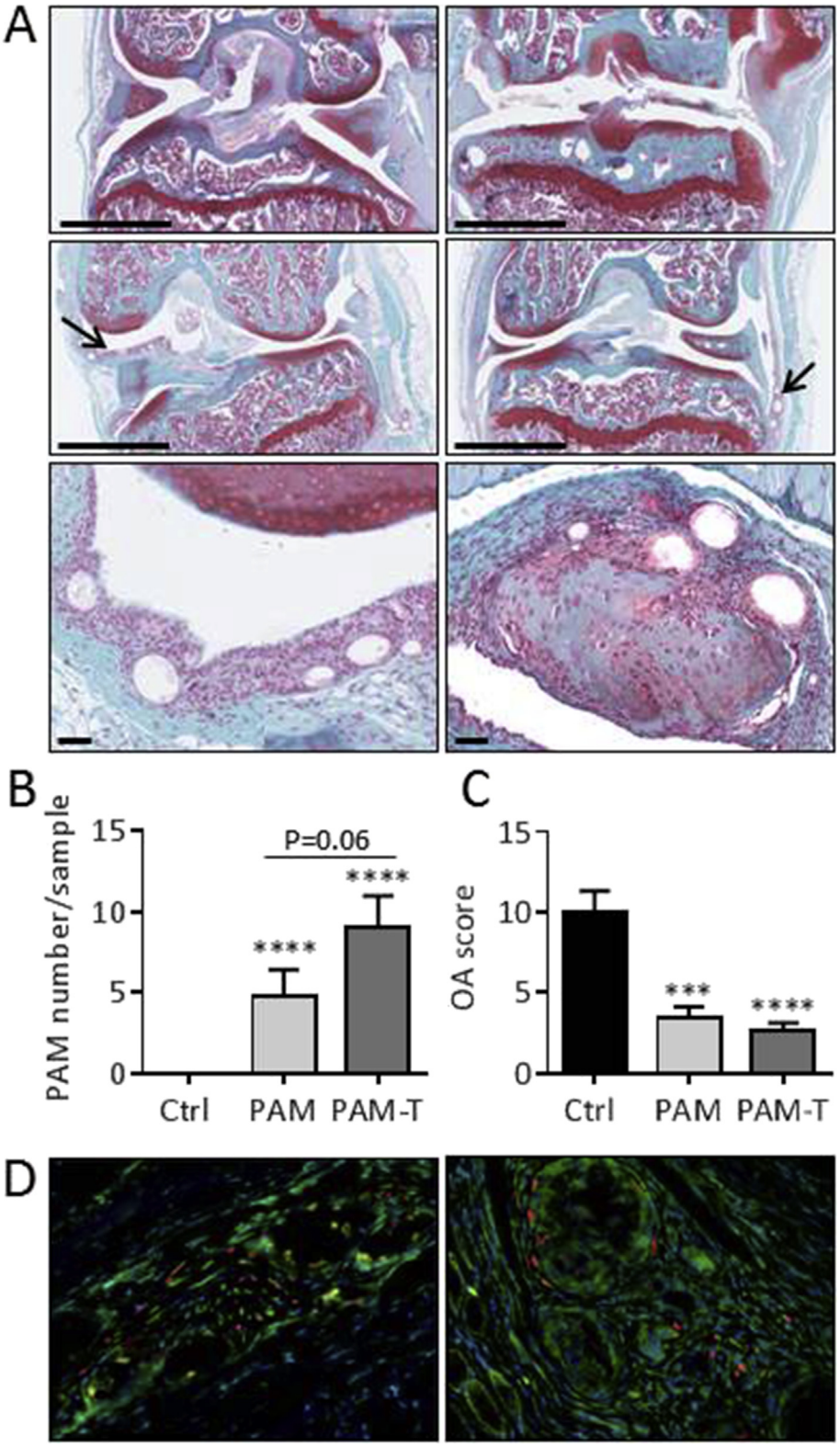

Fig. 3. *In vivo* differentiation of hMSCs associated with PAM-T in the collagenase-induced model of osteoarthritis. hMSCs were pre-incubated with PAM or PAM releasing TGFβ3 (PAM-T) for 4 h before implantation in the intra-articular space of mouse knees, 30 days after OA induction. Mice were euthanized on day 42 and knee joints processed for histology. (A) Representative pictures of Safranin O-Fast green stained sections of knee joints: healthy control (left upper panel), collagenase control (right upper panel), PAM associated hMSC group (left middle and lower panel), PAM-T associated hMSC group (right middle and lower panel). Bars indicated 1 mm (upper and middle panels) or 100 μm (lower panels). Arrows localized PAM or PAM-T in knee joints. (B) Average number of PAM or PAM-T per histological section (3 sections/mouse). (C) Average OA score evaluated on 3 sections/mouse in OA mice (Ctrl) or OA mice receiving hMSCs on PAM or PAM-T. (D) Fluorescence *in situ* hybridization of hMSC (red) and murine cells (green) in joint sections of PAM (left) and PAM-T (right) treated mice. Original magnification ×20. n = 7–10/group; ****: $p < 0.0001$. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

regeneration or protection. We previously described that 3D imaging of cartilage using CLSM allowed evaluating the benefit of one therapy on the whole cartilage architecture through morphometric analysis [18]. In this experiment, we wanted to finely and quantitatively analyze the totality of articular cartilage using CLSM. hMSCs pre-incubated on PAMs or PAM-T were implanted either at day 10 or day 30 after OA induction and animals were euthanatized at day 42. CLSM analysis was performed on lateral and median tibial plateaux before 3D reconstruction of cartilage tissues. In collagenase-induced OA mice, the architecture of cartilage revealed high tissue degradation, principally on the edges of the tissue, which was less severe in mice implanted with hMSCs pre-incubated on PAMs or PAM-T (Fig. 4A). Some differences were however observed depending on the time of injection or PAM/PAM-T samples. In mice treated at day 10, morphometric analysis of the 3D reconstructions indicated higher volume, higher thickness and less surface irregularities (as measured by the area/volume ratio) in the PAM/PAM-T samples compared to control (Fig. 4B). Significantly lower surface defects of cartilage were observed when mice received hMSCs with PAM-T. In mice treated at day 30, significantly higher volume, higher thickness and lower surface degradation were recorded for mice injected with hMSCs on PAMs, while these parameters tended to improve for mice implanted with hMSCs on PAM-T (Fig. 4B). To validate these data with the "gold standard" histological scoring, samples were processed for histology and OA scoring of sections was performed. As compared to the collagenase control mice, the mean OA score was significantly lower for mice implanted with hMSCs on PAMs or PAM-T at both time points, day 10 and day 30 (Fig. 4C). The histological scoring on 3 sections/animal confirmed the morphometric analysis on the entire cartilage tissue, indicating protective effect of hMSCs in presence of PAMs or PAM-T against cartilage degradation, when injected 10 or 30 days before. On the basis of data acquired by CLSM on the full cartilage, release of TGFβ3 did not add to the therapeutic effect of hMSCs on the short term (10 days after implantation) and hMSCs on PAM-T was even less efficient than on PAM. However, the chondroprotective effect of hMSCs on the long term (30 days after implantation) was higher when TGFβ3 was released.

2.5. TGFβ3 release by PAM-T increases the survival of hMSCs after intra-articular implantation in osteoarthritic environment

We next determined whether the chondroprotective effect of hMSCs may be related to their survival, 10 or 30 days after implantation. Using qPCR for quantification of human specific *Alu* sequences, we checked for the biodistribution of hMSCs injected with or without PAMs or PAM-T, in 16 different organs. hMSCs were detected only in the injected joints in 80–100% of injected mice, on day 10 after implantation (Fig. 5A). Human *Alu* sequences were not detected in the other tested organs. On day 30 after implantation, hMSCs were identified in the joints of all treated mice when implanted with PAM-T but not in the other groups (Fig. 5B). When we evaluated the percentage of hMSCs that survived in the joints after implantation, 30–40% of the initially injected hMSCs were detected at day 10, with no significant differences between groups (Fig. 5C). After 30 days, still 20% of the initially injected hMSCs survived only when implanted in presence of PAM-T. These results suggest that the superior therapeutic effect of hMSCs associated with PAM-T on the long term was related to a better survival of hMSCs.

3. Discussion

The aim of the present study was to evaluate *in vivo* the interest of a novel formulation of microspheres releasing TGFβ3 for its ability to support chondrogenic differentiation of MSCs and induce cartilage formation in a pathological OA environment. We showed that 1) hMSCs combined with PAM-T releasing TGFβ3 were able to differentiate *in situ* into chondrocytes forming a cartilage-like neotissue in the local inflammatory OA microenvironment, 2) hMSCs-derived chondrocytes combined with PAM or PAM-T protected the endogenous murine cartilage from OA degradation and 3) TGFβ3 released by PAM-T allowed hMSCs survival for at least 30 days, which could be related to the protection of cartilage from degradation.

We previously reported that PLGA-based PAMs releasing TGFβ3 could provide an appropriate environment for hMSCs to differentiate *in vitro* into chondrocytes and form a cartilage-like neotissue when implanted in subcutaneous or intra-muscular ectopic locations [10]. We then improved PAM formulation to allow higher release of TGFβ3 and more efficient differentiation of hMSCs [11]. Here, we confirmed that the second generation of PAMs was as efficient as the standard pellet culture system for hMSC chondrogenesis, while it was more potent to inhibit osteogenesis. The hypertrophic marker collagen 10 was also reduced when using PAM-T as compared to pellet culture but whether it could be related to lower hypertrophic differentiation or slightly reduced chondrocyte differentiation was not investigated in further details. The interest of PAMs was the progressive and continuous release of TGFβ3 during the culture, in contrast to the standard pellet culture, where TGFβ3 had to be added every two days to the culture medium. Such approach for *in situ* differentiation of hMSCs is particularly relevant for cartilage engineering. It allows availability of TGFβ3 at proximity of hMSCs for inducing their differentiation while avoiding side effects of the bioactive factor such as osteophyte formation or inflammation [19]. We also showed that the secretory profile of hMSCs was not impacted by the culture on microspheres. Although we did not perform a high throughput screening of proteins secreted by hMSCs, we evaluated the expression level of key proteins involved in cartilage differentiation, remodeling, inflammation, chemoattraction and fibrosis. All tested factors were expressed at similar levels when cultured on microspheres or in pellet; differences were only observed when TGFβ3 was present, independently of the 3D culture. In presence of TGFβ3, IL6 was secreted at higher levels at day 1 while HGF was downregulated by day 7 and throughout the culture period. Increase of IL6 in very early time points in TGFβ3-induced chondrogenic differentiation of hMSCs has been reported to positively regulate chondrogenesis [20]. HGF down-regulation by the TGFβ pathway has also already been described [21]. Therefore, TGFβ3 supplied by the engineered PAM-T induced *in vitro* the chondrogenic differentiation of hMSCs without major modification of their secretory profile.

To evaluate *in vivo* the differentiation of hMSCs implanted with the second generation of PAMs, we chose the SCID/Bg immunodeficient murine model to avoid an adaptative host immune response and destruction of human cells after implantation. However, the mouse model is too small to generate reproducible partial-thickness cartilage defects, which could mimic focal lesions observed in patients. Ectopic implantation, either subcutaneously or intra-muscularly, is a good model to evaluate *in situ* the differentiation of hMSCs but it does not reproduce the local joint environment [10]. We therefore utilized an inducible model of OA to generate OA-like lesions in mouse knee joints and determine whether hMSCs implanted with PAM or PAM-T could differentiate in a pathological joint environment. Using the collagenase-induced OA model, we observed *in situ* differentiation of hMSCs but only when TGFβ3 was released by PAM-T. We never or rarely observed PAM and/or hMSCs at the surface of the articular cartilage, which was not surprising since cells likely tended to be rejected to the edges of the joint with animal motion. Formation of a cartilage-like

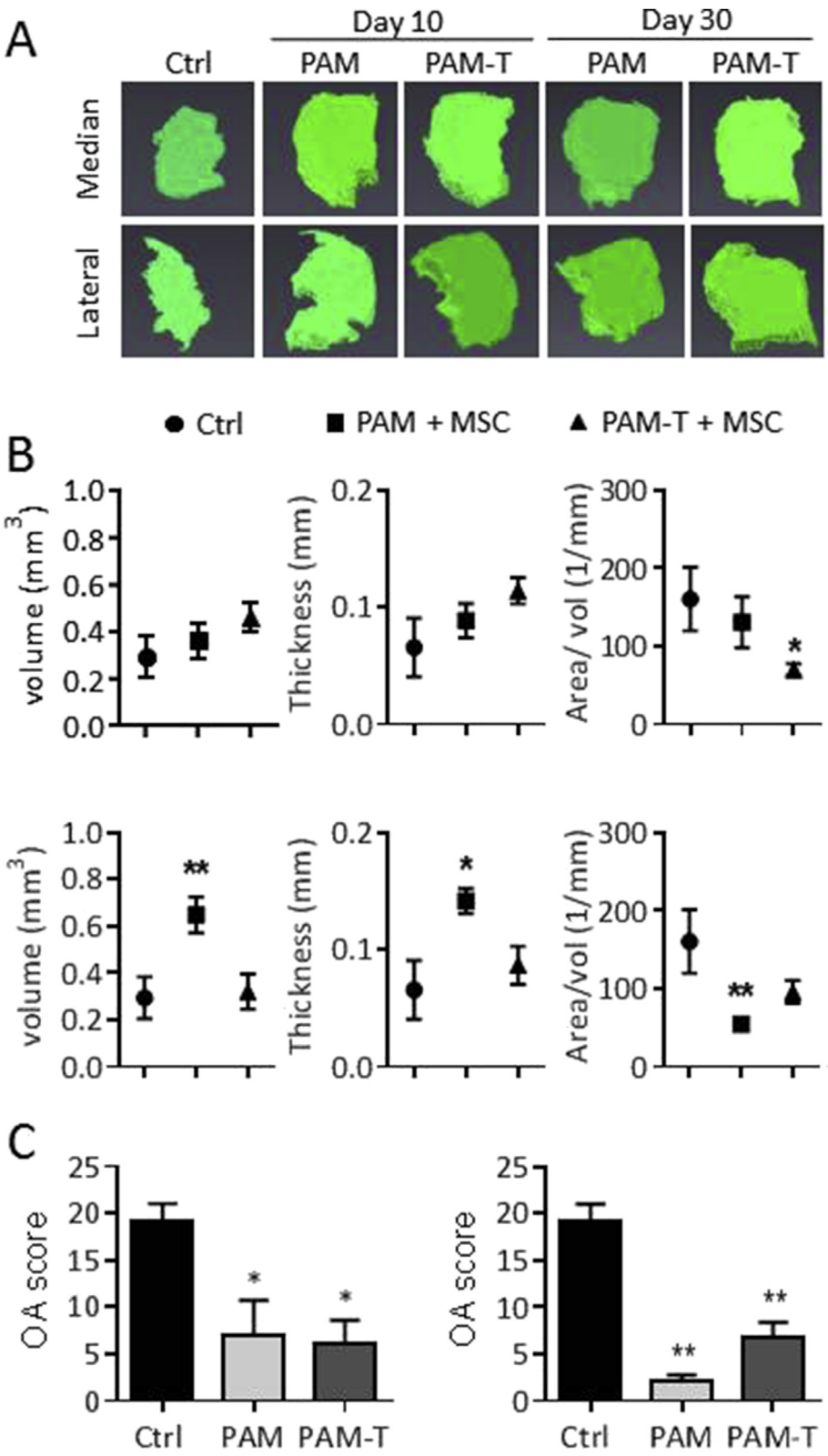

Fig. 4. hMSCs associated with PAM or PAM-T exert a chondroprotective effect on endogenous articular cartilage. hMSCs were pre-incubated with PAM or PAM releasing TGFβ3 (PAM-T) for 4 h before implantation in the intra-articular space of mouse knees, 10 or 30 days after OA induction. Mice were euthanized on day 42 and knee joints processed for histology. (A) Representative 3D pictures of median and lateral tibial plateaux of collagenase-induced OA control mice (Ctrl) or groups of mice implanted with hMSCs on PAM or PAM-T. (B) Morphometric analyses of reconstructed 3D pictures for the three groups implanted on day 10 (upper panels) or on day 30 (lower panels). (C) Average OA score evaluated on 3 sections/mouse in OA Ctrl mice or OA mice receiving hMSCs on PAM or PAM-T on day 10 (left panel) or on day 30 (right panel). n = 8/group; *: $p < 0.05$; **: $p < 0.01$.

neotissue was detected lining the synovium in the joint recessi, near the patella or the menisci. This finding indicated that the pathological milieu of the joint, rich in inflammatory cytokines and degrading enzymes, did not inhibit the differentiation of hMSCs.

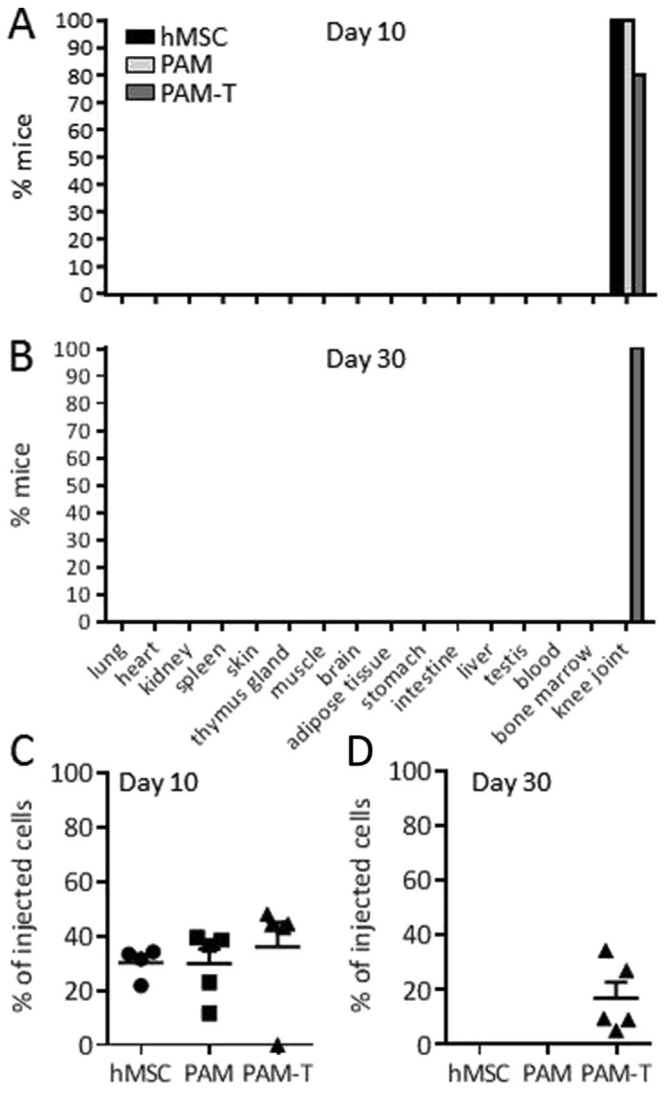

Fig. 5. hMSCs associated with PAM-T survived on the long term after intra-articular implantation. hMSCs were pre-incubated or not with PAM or PAM releasing TGFβ3 (PAM-T) for 4 h before implantation in knee joints of SCID/Bg mice. Mice were euthanized 10 or 30 days after implantation and several organs were collected and processed for DNA extraction. Human MSCs were identified by *Alu* sequences detection by qPCR and normalization to murine Actin sequences. (A) Biodistribution of hMSCs as expressed as percentage of mice in which *Alu* sequences were detected in the indicated organs, on day 10. (B) Biodistribution of hMSCs as expressed as percentage of mice in which *Alu* sequences were detected in the indicated organs, on day 30. (C) Percentage of hMSCs retained of the total number injected in the knee joints of mice, on day 10. (D) Percentage of hMSCs retained of the total number injected in the knee joints of mice, on day 30.

This is in agreement with a previous study reporting differentiation of hMSCs into chondrocytes expressing collagen II, after intra-articular injection in a xenogeneic spontaneous OA model of 7-month-old pigs [22]. However, no or few data exist on the effect of the OA milieu on the differentiation of hMSCs within a scaffold. Neocartilage formation observed in the present study included both human and murine cells, as detected by FISH analysis, suggesting that hMSCs attracted murine cells. Homing of murine cells could partly be attributed to TGFβ3 release as previously described [23] but also to hMSCs themselves, through the secretion of a variety of chemokines [24]. The ability of MSCs to form cartilage in a pathological OA environment is of importance for future clinical application. Nevertheless, further investigation in a large animal model is important to strengthen the results obtained with the murine model. Indeed, a significant improvement in cartilage repair was detected based on histology and ultrashort time echo sequences in magnetic resonance image analysis in joints treated with MSCs associated with PAM releasing TGFβ3 in an equine model (article in press).

Besides the differentiation of hMSCs into chondrocytes, their trophic effect on the endogenous murine cartilage was also noticed. A number of studies have reported the therapeutic effect of intra-articular administration of MSCs in a variety of OA models [6,25]. However, the role of chondrocytes derived from *in situ* differentiated hMSCs in presence of scaffolds on OA cartilage lesions is poorly documented. Here, we provide evidence that after implantation of PAM or PAM-T with hMSCs, hMSC-derived chondrocytes as well as undifferentiated hMSCs protected endogenous articular cartilage from degradation. For OA scoring, we compared the gold standard histological analysis to CLSM morphometric analysis that allowed analyzing the entire cartilage tissue [18,26]. We are among the first to demonstrate that morphometric data acquired by CLSM analysis provided quantitative evaluation of OA-induced cartilage changes on the medial and tibial plateaux, which was comparable to the histological analysis. The interest of the CLSM analysis was a comprehensive evaluation of the whole tissue in contrast to the analysis of 3 histological sections per knee joint, which were supposed to be representative of the whole tissue. Moreover, a quantitative evaluation of several cartilage parameters can be performed and even combined to micro-computed tomography (μCT) to quantify and visualize bone changes [27]. Chondroprotection was observed when hMSCs were implanted at early time points (day 10) but also at late time points (day 30), suggesting that hMSCs may stimulate cartilage regeneration. An unexpected higher therapeutic effect of hMSCs implanted with PAMs versus PAM-T was observed when hMSCs were implanted at day 30 after OA induction in the second experiment. Because such effect was not observed in the first experiment, we can hypothesize that it was experimentation-dependent. When hMSCs were implanted with PAM-T at day 10 after OA induction, the chondroprotective effect was significantly better than that observed with PAMs, using CLSM analysis of the entire tissue. This is likely due to the prosurvival effect of TGFβ3 on hMSCs as demonstrated by survival of human cells after 30 days in the biodistribution experiment. Indeed, it has been reported that TGFβ-induced Smad1/5/8 signaling is required for chondrocyte proliferation and survival in the early stage of chondrogenesis while during cartilage maturation, TGFβ/Smad2/3 pathway stimulates Sox9, collagen 2 and aggrecan production [28]. The TGFβ pathway has also been shown to induce the secretion of anti-apoptotic genes, such as BCL-XL and XIAP [29]. Better survival of MSCs after 30 days may therefore be partly explained by the pleiotrophic function of TGFβ pathway both on proliferation, survival and apoptotosis. Altogether, these experiments demonstrated that hMSCs when implanted with PAM or PAM-T were able to exert their trophic function on endogenous murine cartilage, leading to a chondroprotective and chondroregenerative effect in OA.

The main findings of the present study is that hMSCs implanted onto a scaffold releasing a chondroinductive factor can differentiate *in situ* and form a cartilage-like neotissue. This allows a “one-shot” treatment avoiding repeated injections of the bioactive factor. This strategy of tissue engineering is of particular interest for cartilage repair through its chondroinductive and chondroprotective effects but also for cartilage regeneration through the stimulation of endogenous tissue. This approach is therefore promising for future clinical applications in cartilage repair.

Competing interests

Authors declare no conflict of interest.

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