Methods to monitor distribution and metabolic activity of mesenchymal stem cells following in vivo injection into nucleotomized porcine intervertebral discs

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Abstract Intervertebral disc (IVD) degeneration involves a series of biochemical and morphological changes leading to loss of spinal stability and flexibility. Cell therapy is promising to reconstitute IVDs with new cells, however, sustained metabolic activity seems crucial for an active contribution to regeneration. The aim of the present study was to establish methods for separate follow up of persistence and activity of autologous porcine mesenchymal stem cells (pMSC) after implantation into IVDs of Goettingen minipigs in vivo in order to conclude about the potential of such an intervention strategy. For quantitative follow up, the transfer matrix was supplemented with Al_2O_3 particles and pMSC which were retrovirally labeled with firefly luciferase (pMSC-Luc). Six mature Goettingen minipigs underwent matrix based cell transfer after partial nucleotomy of lumbar IVDs ($n = 24$). Day 0 and day 3 segments were analyzed for retained volume of Al_2O_3 particles by micro-computed-tomography (μCT) and for cell activity by luciferase enzyme assessment. Three days after injection a reduction of Al_2O_3 particles ($P = 0.028$)

to about 9% and of pMSC-Luc activity to about 7% of initial values ($P = 0.003$) was detected, which suggests loss of 90% of the implant material under in vivo conditions without evidence for reduced pMSC-Luc metabolic activity ($P = 0.887$). In conclusion, separate follow up of implant material and cell activity was possible and unravels problems with in vivo implant persistence after annular puncture rather than quick loss of cell activity. Therefore, IVD-regeneration-strategies should increasingly focus on annulus reconstruction in order to reduce implant loss due to annular failure.

Keywords Cell transfer · Intervertebral disc · μCT · Luciferase · Retrovirus · Fibrin matrix · Disc degeneration

Introduction

Intervertebral discs (IVDs) are characterized by abundant extracellular matrix and low cell density [33] coupled with absence of blood vessels, lymphatics, and nerves in all layers but the most peripheral. In many respects, this absence leads to a detrimental environment which leaves the disc prone to degeneration, without adequate sources of repair. The intervertebral disc primarily has a mechanical function in transmitting loads through the spine, thereby providing flexibility to the spinal column. The inner disc consists of the inner nucleus pulposus, a hydrophilic-gel structure containing notochordal and chondrocyte-like cells surrounded by a large amount of extracellular matrix with proteoglycan, aggrecan, and type II collagen providing viscoelastic properties to the disc. The outer region of the disc, the annulus fibrosus, contains densely packed layers of type I collagen embedded with fibroblast-like cells providing tensile strength to the disc [23, 31, 51].

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The avascular disc is connected by two endplates to the adjacent vertebral bodies. Nutrition of the disc cells arises predominantly from the endplate vasculature [9] with capillaries terminating in loops at the boundary of the cartilaginous endplates [18]. Thus, a gradient of nutrient availability builds up from endplate to nucleus with lowest levels towards the nucleus centre [21].

Disc degeneration from age or injury is considered a major cause of low back pain. Degeneration involves a series of biochemical and morphological changes, including decreased cell viability and proteoglycan synthesis leading to decreased water binding capacity of the disc. In the endplates, capillary density seems to decrease during degeneration. This factor is suggested to further contribute to disc degeneration by limiting the local nutritional supply of the disc cells [46]. These changes result in loss of stability and flexibility, which may eventually lead to back pain [17, 19].

Lumbar discectomy is an effective therapy for neurological decompression in patients suffering from a herniated nucleus pulposus (HNP), which can be safely performed via minimal invasive procedures [24, 59]. Current discectomy procedures, however, are not directed to treat the damaged intervertebral disc (IVD) and may even further aggravate existing damage [13, 16, 26]. It is therefore not surprising that successful neurological decompression is often followed by periods of persisting low back pain, severely affecting the quality of life [6, 7, 26]. Since discectomy is still the most frequently performed spinal surgical procedure worldwide and mainly affects the employed population, the resulting socio-economical consequences are dramatic [30]. This gives investigators the impetus to search for new strategies that also deal with the damaged IVD in patients treated for HNP [33, 36, 50]. Cell therapy using adult stem cells is a potential approach for the treatment of degenerative disc disease [1, 12] because it is promising to reconstitute IVDs with new cells [42], however, sustained metabolic activity seems crucial for an active contribution to regeneration. A revitalization of the degenerated IVD by methods such as cell transplantation [11, 20, 49] growth-factor injections [3, 41] or gene therapy [38] has been proposed as promising treatment approaches. However, at present, such applications are still not clinical standard procedures in humans due to open questions about safety, feasibility, and biological potency [42].

In a xenogenic rat coccygeal disc model, Wei et al. [55] reported no significant initial reduction in the amount of celltracker-orange (CTO+) labeled human CD34+ cells over 42 days in vivo. However, their study also shows a reduction trend of CTO+/CD34+ cells from day 1 and mean 120 cells/visual field to about mean 30 cells/visual field at day 10, which means a reduction to 25% of initially

implanted cells, which reconstitutes to about 60% over time. Using a rabbit model, several other authors have reported that autologous bone marrow-derived mesenchymal stem cells (MSCs) transplanted into degenerated discs proliferated, based on either fourfold increase of GFP positive cells over 46 weeks [48] or evidence of LacZ [52] positive cells 24 weeks after surgery and differentiated into disc-like cells, leading to restoration of the degenerative changes [47, 48, 60]. Using a large xenogenic animal model, one recently published study showed MSC survival according to detection of human-nuclear-antigen (HNA) and transcriptional activity of xenogenic cells in minipigs after 6 months [27]. This study did, however, not address quantitative aspects of implant retention or anabolic activity of transferred cells.

Independent of whether implanted cells physically contribute to repair tissue or secrete soluble factors triggering the repair by local IVD cells, a prerequisite for such cell based strategies is that the implanted cells remain in place and show an active metabolism for some time. Although xenogenic studies showed that human mRNA signals and co-localization of matrix proteins with implanted cells were retained at 6 weeks [55] or 6 months [27] after implantation, neither study addressed the question of implantation efficiency, implant retainment, or quantity of remaining anabolic active cells. Thus, we can only speculate, how much of the initially transferred cells contributed to the reported results. This demonstrates a need for the quantitative follow up of persistence and metabolic activity of implanted cells. To study these aspects, the selection of the firefly luciferase as a transgene for cell labeling is an attractive tool. Prior studies have already shown that the level of luciferase enzyme activity correlates well with cell anabolism [11] while its activity is rapidly lost after cell death because of its short half-life of about 4 h [8]. In addition, cell-transfer strategies benefitted from the use of a hydrogel for cell transplantation to avoid initial cell leakage and allow for the supplementation of the injectable matrix with nutrients to enhance the anabolic activity of the injected cells [11]. The use of adult stem cells seems attractive for the treatment of degenerative disc disease [12], since it has already been reported that autologous bone marrow-derived mesenchymal stem cells (MSC) transplanted into discs of rabbits with degenerated discs proliferated [48] and differentiated into disc-like cells with restoration of the degenerative changes [47, 48, 60].

To evaluate such a cell-transfer strategy quantitatively, a reproducible large animal model with appropriate anatomical properties comparable to the human situation is required before successfully introducing such methods in humans. Many different small [39, 48, 60] and large animal models [27, 37, 44] have been described and reviewed in great detail [2, 32], though no animal is identical to the

human situation and each model has individual advantages and disadvantages, which should be carefully considered. The use of the minipig model is attractive because porcine spines are close to human anatomy, IVDs are almost as large as human discs [15, 35, 44, 45, 54] and it allows to test if the transplanted cells can survive in this relatively large biologic structure without vascular supply.

The aim of the present study was to establish a strategy to both quantitatively follow up autologous porcine MSC (pMSC) implanted into IVDs of the Goettingen minipig in vivo and to conclude about their persistence and activity. Both in vivo implant retention in the disc and metabolic activity of the transferred autologous pMSC was analyzed under natural mechanical loading of treated IVD segments and quantified by μ CT imaging and biochemical analysis of luciferase transgene expression in explants.

Materials and methods

Cell isolation and cultivation

Porcine MSC (pMSC) were isolated from fresh bone marrow samples as described previously [10, 25]. Briefly, cells were washed with PBS, seeded in culture flasks and maintained at 37°C in a humidified atmosphere and 6% CO₂. pMSC culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) high glucose with 40% MCDB201 medium (Sigma-Aldrich, Deisenhofen, Germany), 2% fetal calf serum (FCS), 2×10^{-8} M dexamethasone, 10^{-7} M ascorbic acid-2-phosphate, 5 μ g/ml insulin, 5 μ g/ml transferrin, 5 ng/ml selenous acid, 100 units/ml penicillin, 100 μ g/ml streptomycin, 10 ng/ml recombinant human epidermal growth factor (EGF) (Strathmann Biotech, Hamburg, Germany) and 10 ng/ml recombinant human platelet derived growth factor BB (PDGF-BB) (Strathmann Biotech, Hamburg, Germany). After 24–48 h, pMSC cultures were washed with phosphate-buffered saline (PBS) to remove nonadherent material. During expansion, medium was replaced twice a week. Cells were used after 2–6 passages.

Retroviral gene transfer into mesenchymal stem cells

Recombinant retrovirus encoding the firefly luciferase gene (Rv-Luc) was constructed similarly as described before [5] and was used to follow up mesenchymal stem cells after injection into intervertebral discs. For optimization of the retroviral gene transfer, cells were transduced with recombinant retrovirus encoding the enhanced green fluorescent protein (Rv-eGFP) at different cell densities and using varying polybrene and retroviral concentrations. The efficiency of transduction and the long-term transgene

expression stability was evaluated by flow-cytometry at indicated time points. For retroviral transduction, cells were seeded and incubated with 30 μ l/cm² medium containing the appropriate retrovirus and polybrene concentrations for 2 h at 37°C on the following day. Fresh medium was supplemented afterwards and cells were grown to confluence.

For in vivo experiments, cells were transduced with Rv-Luc at a multiplicity of infection (MOI) 5 and 8 μ g/ml polybrene. Five days after primary transfection, the process was repeated to enhance transduction efficiency. During expansion of pMSC-Luc, medium was replaced twice a week and after harvest pMSC-Luc were stored in liquid nitrogen until autologous injection into porcine IVDs in vivo.

Multilineage potential—induction of differentiation

Multilineage potential of pMSC was investigated according to Steck et al. [53]. For osteogenic induction, MSC growth medium (Poietics; BioWhittaker, Taufkirchen, Germany) supplemented with 0.1 μ M dexamethasone, 0.05 mM ascorbic acid-2-phosphate, and 10 mM β -glycerophosphate was used. Adipogenic medium was composed of DMEM-HG containing 10% FCS, 0.01 mg/ml insulin, 1 μ M dexamethasone, 0.2 mM indomethacin, 0.5 mM 3-isobutyl-1-methyl xanthine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. For chondrogenic differentiation, pellets consisting of 0.4×10^6 expanded control or Rv-Luc transduced cells, respectively, were formed by centrifugation in 1.5 ml tubes in chondrogenic medium (DMEM high glucose with 0.1 μ M dexamethasone, 0.17 mM ascorbic acid-2-phosphate, 5 μ g/ml insulin, 5 μ g/ml transferrin, 5 ng/ml selenous acid, 1 mM sodium pyruvate, 0.35 mM proline, 1.25 mg/ml BSA, 100 units/ml penicillin, 100 μ g/ml streptomycin) supplemented with 10 ng/ml recombinant human TGF- β 1 (Pepro Tech GmbH) and recombinant human BMP-6 (R&D Systems) and cultivated in parallel for 8 weeks. Medium was changed every other day.

Histological and immunohistochemical analysis

For immunohistochemical staining, differentiated pMSC spheroids were fixed in PBS containing 4% paraformaldehyde for 2 h at 20°C, dehydrated in alcohol, washed in acetone and infiltrated with paraffin. Paraffin sections (3–4 μ m) were dried, deparaffinized using SEM-200 (Vogel, Giessen, Germany) and rehydrated in alcohol.

For histological staining sections were placed (3–4 μ m) on silane-coated slides and stained, using standard histochemical procedures. Acidic proteoglycan-rich extracellular matrix (GAG) indicating cartilage was visualized by

staining with 1% Alcian Blue (Chroma, Köngen, Germany) for 30 min. Counterstaining was performed for 3–5 min with nuclear fast red (Roche Diagnostics, Mannheim, Germany) before permanently mounting the sections with Eukit (HICO-MIC, Hirtz&Co, Cologne, Germany).

For immunostaining, sections were pretreated with 2 mg/ml of hyaluronidase (Merck, Darmstadt, Germany) for 15 min at 37°C and subsequently with 1 mg/ml of pronase (Roche Diagnostics, Mannheim, Germany) for 30 min at 37°C. Nonspecific background was blocked using PBS containing 5% BSA for 30 min. Sections were incubated overnight at 4°C with a monoclonal mouse anti type II collagen (ICN Biomedicals, Aurora, USA) in PBS containing 1% BSA. Following washing with Tris buffered saline, reactivity was detected using biotinylated goat antimouse secondary antibody (1:200) (Dianova, Hamburg, Germany), streptavidin-alkaline phosphatase (Dako, Glostrup, Denmark) for 30 min at 20°C and fast red (Sigma-Aldrich, Deisenhofen, Germany) for 20 min at 20°C. All sections were permanently mounted with Aquatex (Merck, Darmstadt, Germany) and examined by light microscopy.

Preparation of injectable cell fibrin gel

Polymerization time and structure of a two-component fibrin gel were adapted by varying the thrombin concentration. 1,000,000 cells were suspended in 100 µl FCS and kept at 21°C. Cells were then mixed with 100 µl of the fibrinogen solution (35–55 mg/ml, Tissucol Duo S, Baxter, Unterschleissheim, Germany) and 100 mg of Al₂O₃ powder (DEGUSSIT VM, particle size 10 µm, FRIATEC, Mannheim, Germany). To generate the gel, 200 µl of cell-fibrinogen-Al₂O₃ solution was carefully mixed with 100 µl thrombin/PBS solution (10 IU Thrombin/ml, Tissucol Duo S, Baxter, Unterschleissheim, Germany) immediately before injection. For both in vitro and in vivo segments, 150 µl of the gel was injected immediately after partial nucleotomy of the disc.

Explant preparation and aspiration of nucleus pulposus

Lumbar spine sections from domestic pigs weighing an average of 18 kg were taken by en bloc resection within 4 h after killing. IVDs with adjacent vertebral bodies were dissected using a bone-saw. The explants underwent partial nucleotomy by a special biopsy device (Bard Angiomed, Karlsruhe, Germany) consisting of a 16 G cannula with an interior needle, which was pierced through the annulus fibrosus and inserted into the nucleus pulposus in 3 different directions. The preweighted biopsy cannula and the aspirated nucleus tissue was placed into preweighted eppendorf tubes. After completion of the nucleotomy

procedures, the biopsy cannula and the tube were weighted again to assess the amount of nucleotomized tissue. 0.26 ± 0.05 g of gel-like nucleus was aspirated by repeated syringe movements. Syringe and cannula were removed and 150 µl fibrin gel supplemented with Al₂O₃ particles and pMSC-Luc was injected. One hour after polymerization of the fibrin gel, the explants were analyzed by µCT and luciferase activity was measured.

Surgical procedure, animal groups

Six mature Goettingen minipigs (2–3 years of age, weight 60–78 kg) were approved for use by the Institutional Review Board of the Animal Experimentation Committee at the Regierungspräsidium Karlsruhe, Germany. An antero-lateral retroperitoneal approach to the spine [44] was used. The L2/3 and L3/4 lumbar IVDs were surgically exposed and underwent partial nucleotomy using a special biopsy device as described for explants. 43 ± 17 mg of gel-like nucleus (~10% of total nucleus volume) was sucked inside the cannula by high intrinsic nucleus pulposus pressure and repeated syringe aspirations. The syringe and the cannula were removed and a further 16 G cannula with a lateral hole was inserted for the injection. Transplants consisted of 150 µl total volume of a fibrin gel supplemented with Al₂O₃ particles and autologous pMSC expanded and labeled by retroviral gene transfer of firefly luciferase (pMSC-Luc). After injection, the syringe was not moved for 60 s to allow polymerization of the fibrin gel. Then the fibrin clot was divided by rotating the cannula to prevent pulling it from the IVD and the cannula was removed. Treated IVDs were marked by fixation of non-resorbable suture material at the transversal extensions. Musculature and skin were closed by suture. The animals were assigned to a postoperative survival time of 3 days and were allowed free unrestricted weight bearing and activity in cages. While killing, the animals underwent surgery for the L1/2 and L4/5 untreated lumbar IVDs serving as day 0 controls and thus receiving identical partial nucleotomy and injection of pMSC-Luc/fibrin/Al₂O₃ suspension.

µCT based analysis

The total lumbar spine was resected post mortem. IVDs with adjacent vertebral bodies were dissected using a bone-saw and used for µCT-analysis. One hour after sacrifice segments were scanned using Skyscan 1076 in vivo µCT (SKYSCAN, Antwerp, Belgium) through a 0.5 aluminium prefilter with the following parameters: resolution of 17.7 µm/pixel, voltage 48 kV, source current 200 µA, exposure time of 320 ms and a rotation step of 0.6 with an averaging of 3. Scans were reconstructed and analyzed

using manufacturer's software (NRecon and CTAn, SKY-SCAN). Two-dimensional cross sections were used for gross observation of the fibrin clot and Al_2O_3 distribution in the IVD. As the fibrin clot is not radiopaque, it has a reduced X-ray opacity compared to IVD matrix and can be visualized as a negative shade. Al_2O_3 particles have a higher radio-opacity than the IVD matrix.

For quantification of the Al_2O_3 particles, a region of interest (ROI) was chosen which included the total IVD and excluded calcified vertebrae. A threshold ranging from lower gray level 65 to upper gray level of 250 was used for Al_2O_3 volumetry. Three-dimensional analyses were accomplished applying the nomenclature published as guidelines for bone histomorphometry by ASBMR [43] and therefore total Al_2O_3 volume quantification was expressed as mm^3 bone volume (BV mm^3).

Luciferase assay

Luciferase activity was measured with beetle luciferin as a substrate (luciferase assay system, Promega). Cells were rinsed in PBS and lysed with $100\text{ }\mu\text{l}$ $1 \times$ lysis buffer per 500,000 cells. Alternatively, injected nuclei pulposi were separated from the vertebral bodies with a scalpel, and pulverized in a freezer mill. Pulverized tissue was homogenized in $500\text{ }\mu\text{l}$ lysis buffer, stirred at 800 rpm in a thermomixer for 30 min at 21°C (Thermomixer Comfort, Eppendorf, Germany) and centrifuged at 12,000 rpm for 30 s to pellet debris. Ten microliters of lysate was mixed with $50\text{ }\mu\text{l}$ luciferase assay reagent (Luciferin, Mg^{2+} , ATP and coenzyme A), and the light emitted within 5 s at 25°C was detected with an ELISA reader (Wallac Victor² 1420 Multilabel Counter, Wallac, Finland). Values were expressed as relative light-emitting units (RLU).

Statistical analysis

Mean $\pm$ standard deviation (SD) were calculated for all variables. The Student's *T* test was applied to analyze differences between groups. A two-tailed *P* value ≤ 0.05 was considered significant. Data analysis was performed with SPSS for Windows 11.0.1 (SPSS Inc., USA).

Results

Retroviral gene transfer of porcine mesenchymal stem cells

To optimize retroviral gene transfer efficiency, cells were transduced with Rv-eGFP at different conditions and the amount of eGFP-expressing cells was determined by flow

cytometry. Variation of the seeding density between 2,000, 4,000, and 6,000 cells per cm^2 and of the polybrene concentration between 8, 16 and $32\text{ }\mu\text{g/ml}$ showed no effect on transfer efficiency (results not shown). Transduction with a MOI of 1, 2, and 5 resulted in an increase of up to 50% of transduced cells at MOI 5, whereas no further improvement was achieved with MOI 10 (not shown). Transgene expression remained detectable with a slight gradual decline throughout the monitored culture period of 64 days in vitro (pMSC-1 and pMSC-2, Fig. 1a). The highest transduction rate of 75% was achieved after two consecutive transductions within 5 days with MOI 5 and $8\text{ }\mu\text{g/ml}$ polybrene (pMSC-3, Fig. 1a), which was applied for all further transductions. Luciferase activity was proportional to cell number and the detection limit was 100 genetically modified cells (Fig. 1b). The multilineage potential of the pMSC was confirmed as described recently [53]. Cells expressing the luciferase transgene still had the ability to undergo chondrogenic in vitro differentiation and accumulated collagen type II and proteoglycans in the presence

Fig. 1 Retroviral gene transfer into porcine mesenchymal stem cells. Autologous porcine mesenchymal stem cells were isolated and underwent retroviral gene transfer with rv-LUC encoding the firefly luciferase transgene at an M.O.I. of 5. Transgene expression after primary infection with an eGFP encoding virus was detectable by flow cytometry (FACS) as long as 64 days in vitro (a) with a gradual decline over time (pMSC-1 and pMSC-2). Secondary infection resulted in 75% genetically modified cells (pMSC-3) 5 days after primary infection as determined by FACS (a). Luciferase activity was proportional to cell number and the detection limit was 100 genetically modified cells (b). These cells showed the ability to undergo chondrogenic differentiation in the presence of 10 ng/ml TGF- β 1 and BMP-6 over 56 days. Cells accumulated collagen type II (red stain) and proteoglycans (insert, blue stain) as determined by immunohistology and alcian blue stain. Representative results of chondrogenic differentiated pMSC-Luc shown in (c) and identically treated pMSC as control shown in (d)

Fig. 2 Labeling of the implant material for follow up by μ CT in explants. Domestic pig lumbar IVD explants underwent injection of cell-matrix constructs including pMSC-Luc and Al_2O_3 particles of 10 μm size after partial nucleus pulposus aspiration. Visualization of injection area in **a** and **b** by μ CT show a representative segment immediately after minimal invasive nucleotomy. The nucleotomized area is shown in black. **c**, **d** The identical segment after injection of pMSC-Luc, fibrin and embedded Al_2O_3 particles. Arrow shows localization of Al_2O_3 particles. (EP Endplate)

of TGF-beta and BMP-6 over 56 days (Fig. 1c). No difference was evident to identically treated non-genetically modified controls (Fig. 1d).

Labeling of the implant material for imaging by μ CT in explants

Successful nucleotomy was clearly visible as an area of reduced contrast after μ CT analysis (Fig. 2a, b black). To visualize the cell-fibrin-gel construct in the porcine nucleus pulposus by μ CT, several materials have been evaluated for suitability in our study. UltravistTM, a negative contrast agent, was tested but the stain diffused in the whole nucleus pulposus. Additionally, three different Al_2O_3 particle sizes (DegussitTM 10, 60, and 180 μm) as well as Dyna-(2.8 μm) and MACS-beads (50 nm) were mixed in the fibrin matrix and their detection was followed up in porcine IVD explants prior to the in vivo study. Whereas Dyna- and MACS-beads were not detectable in the μ CT at the required instrument settings of 17.7 μm resolution, the Al_2O_3 particles produced good visualization results independent of size. However, particles of 60 and 180 μm size showed a high tendency of sedimentation once combined with the fibrinogen while the 10 μm sized Al_2O_3 particles showed an appropriate distribution and were selected for colocalization of the implanted gel in the disc (Fig. 2c, d arrow). The mean detectable volume of Al_2O_3 particles after injection was $4.79 \pm 2.03 \text{ mm}^3$. When cells were included in the fibrin matrix the mean amount of colocalized retrievable luciferase activity was $1,278 \pm 518 \text{ RLU}$ (Table 1). Intersegment variation deviated between 19% of mean aspirate weight, 41% of mean detectable RLU, and 42% of mean Al_2O_3 volume.

Table 1 Quantification of aspirate, volume of implanted Al_2O_3 and luciferase activity of transferred cells in nucleotomized porcine explants

Segment	Aspirate (g)	BV ($\text{Al}_2\text{O}_3 \text{ mm}^3$)	RLU
A	0.22	3.02	952.0
B	0.19	1.24	662.0
C	0.32	5.69	1,464.0
D	0.32	7.35	2,278.0
E	0.25	5.74	977.0
F	0.24	5.69	1,335.0
Mean	0.26	4.79	1,278.0
SD	0.05	2.03	518.7

BV bone volume based on ASMBR guidelines, RLU relative light unit, SD standard deviation

Follow up of implant and matrix embedded porcine mesenchymal stem cells in intervertebral discs in vivo

μ CT visualized the transferred Al_2O_3 particles at day 0 immediately after transfer (Fig. 3a, b, arrow), the signal-negative nucleotomized area (Fig. 3b, d, black) and the transferred Al_2O_3 particles in significantly smaller quantity 3 days after transfer (Fig. 3c, d, arrow). Quantitative assessment of μ CT imaging (Table 2) showed significant amounts of Al_2O_3 particles detectable directly after injection at day 0 (Fig. 4a). After 3 days, the volume of the co-injected Al_2O_3 particles had significantly decreased to about 9% of initially transferred material ($P = 0.035$). In parallel, the mean activity of detectable luciferase declined significantly ($P = 0.003$) over time (Fig. 4b). Three days after matrix-based injection of genetically modified mesenchymal stem cells into the NP, 7% of initial luciferase activity was recovered. Intersegment variation in vivo

Fig. 3 Follow up of implant material in vivo. Goettingen minipig lumbar IVDs underwent injection of cell-matrix constructs including autologous pMSC-Luc and Al_2O_3 particles of 10 μm size after partial nucleus pulposus aspiration. μCT visualization of injection area in **a** and **b** show a representative segment which received cells, fibrin and embedded Al_2O_3 particles and was analyzed at day 0. **c**, **d** μCT visualization of a representative segment after co-injection of Al_2O_3 particles and three days in vivo. *Arrows* point to injected Al_2O_3 particles

Table 2 Three day follow-up of implant (Al_2O_3 volume) and pMSC enzymatic activity (luciferase RLU) after nucleotomy in vivo

Animal	Day 0 (L1/2; L4/5)			Day 3 (L2/3; L3/4)		
	BV (Al_2O_3 mm^3)	RLU	RLU/mean BV	BV (Al_2O_3 mm^3)	RLU	RLU/mean BV
1a	2.70	2,287	424.9	0.01	281.0	675.0
1b	3.51	1,167	216.8	0.00	227.0	545.3
2a	n/a	n/a	n/a	0.32	1,623.0	3,898.9
2b	2.51	14,198	2,637.7	3.49	1,741.0	4,182.3
3a	0.00	6,899	1,281.7	0.69	1,672.0	4,016.6
3b	6.78	2,037	378.4	0.00	1,505.0	3,615.4
4a	2.26	10,099	1,876.2	0.00	33.0	79.3
4b	15.67	12,578	2,336.7	0.43	45.0	108.1
5a	2.90	23,063	4,284.6	0.00	33.0	79.3
5b	3.02	14,261	2,649.4	0.02	28.0	67.3
6a	0.35	12,741	2,367.0	0.00	113.0	271.5
6b	19.52	1,498	278.3	0.04	169.0	406.0
Mean	5.38	9,166.2	1,702.9	0.42	622.5	1,495.4
SD	6.05	6,713.4	1,247.2	0.95	721.8	1,734.0
<i>P</i> value versus day 0				0.035	0.003	0.887

BV bone volume based on ASMBR guidelines, RLU relative light unit, SD standard deviation

ranged from 112% for Al_2O_3 volume at day 0 to 226% at day 3 and from 73% for detectable RLU at day 0 to 115% for detectable RLU at day 3. Since at day 3 after implantation 5/12 segments showed no detectable Al_2O_3 volume we decided to consistently normalize the detectable amount of retrievable luciferase activity to the calculated mean volume of the Al_2O_3 particles of all segments instead of the Al_2O_3 volume of the corresponding segment (Table 2). By normalization, we found that the mean cellular transgene activity was not significantly affected ($P = 0.887$) over time.

Discussion

Cell therapeutic approaches to the disc still suffer from unsolved problems with cell-transfer effectiveness and cell survival. Therefore, the aim of the present study was to establish a strategy to both quantitatively follow up pMSC implanted into IVDs in vivo and to conclude about their persistence and activity. In the in vivo model used in this study, injury to the AF and partial nucleotomy is carried out before the cell transfer to generate conditions which mimic the human situation, where after discectomy the

Fig. 4 In vivo transfer of porcine mesenchymal stem cells into intervertebral discs. Six mature Goettingen Minipigs (2–3 years age, weight 60–78 kg) received autologous cell-matrix injection into two segments of the lumbar spine. 3 days after killing, the animals underwent surgery again for two remaining untreated lumbar IVDs serving as day 0 controls and thus receiving identical partial nucleotomy and injection of pMSC-Luc/fibrin/Al₂O₃ suspension. **a** The amount of co-injected Al₂O₃ particles significantly decreased

after 3 days to about 9% of initially transferred material ($P = 0.035$). **b** The mean luciferase activity which was rescued from the treated segments. The amount of detectable luciferase activity declined significantly ($P = 0.003$) over time. 3 days after matrix-based injection of pMSC-Luc into the NP, 7% of initial luciferase activity was detectable. Mean $\pm$ SD is shown. * $P < 0.05$ versus day 0; ** $P < 0.005$ versus day 0

protruded nucleus pulposus is removed. Current discectomy procedures in humans, are presently not directed to regenerate the damaged intervertebral disc (IVD) and, thus, are only symptomatic [13, 16, 26].

One important novum in our study was the separate analysis of implant retention and anabolic activity of transferred cells in an in vivo model, which offers for the first time the possibility to follow up tissue engineered bioconstructs after transplantation in vivo. With the support of the established analytical strategy, we were able to answer two important questions: Is the chosen implant material retained in the implantation site under conditions of physiological mechanical loading in vivo? And is the anabolic activity of the implanted cells significantly affected by the physico-chemical characteristics of the intervertebral disc?

The volume of Al₂O₃ particles in our study decreased significantly ($P = 0.035$) within 3 days to about 9% of initial quantity. Therefore, it is tempting to attribute the loss of injected Al₂O₃ particles to implant extrusion through the fibrin sealed injection canal due to the natural physiological loading of the implant occurring during the 72 h of normal animal movements post implantation.

Although during the last years, increasing knowledge and technical advancements in the field of tissue engineering has resulted in numerous promising strategies to repair, replace, or regenerate the herniated nucleus pulposus [26, 50], none of these advancements has yet resulted in a clinically proven effective therapy. One of the major limitations is the lack of effective strategies that deal with the damaged annulus fibrosus [56]. Since optimal regeneration of the nucleus pulposus should lead to restoration of

the physiological intradiscal pressure, the surrounding annulus fibrosus is generally of too inferior quality to withstand these forces. Others recently addressed the question of an appropriate annular closure technique by using a mechanical in vitro model where all tested closure methods failed [28] and highlighted the future demand for an appropriate annulus reconstruction method with good long-term reliability. Our data support this demand for the first time with large animal in vivo results and suggest to both investigating annulus closure techniques as well as in vivo hydrogel stability separately. Investigating a biomaterial which actively crosslinks to the nucleus pulposus matrix might help to support implant retainment and might reduce extrusion likelihood for future applications. The follow up strategy presented in this work would allow investigating both new annular closure techniques and/or biomaterials for their in vivo suitability.

The second question we were interested in was to evaluate whether the anabolic activity of the implanted cells is significantly affected by the physico-chemical characteristics of the intervertebral disc. Earlier studies showed significant changes in cell metabolism of genetically modified cells once implanted in IVD explants based on transgene expression [11] in vitro. We found that 3 days after injection pMSC-Luc activity was reduced to about 7% of initial values ($P = 0.003$). Normalization of the detectable amount of metabolic cellular activity after 3 days in vivo to the mean of the detectable amount of co-localized Al₂O₃ particles suggests that the metabolic activity of transferred autologous cells inside the treated IVD segments may remain relatively stable compared to controls ($P = 0.887$), if only few cells are retained. This

corroborates studies of others who found transplanted human MSCs to be detectable at 42 days in a xenogenic rat model [55] or in a xenogenic minipig model even after 6 months [27]. Sakai et al. used gene transfer of green fluorescent protein (GFP) for cell detection, and thus a convincing cell labeling technique found, that GFP-positive MSCs were detected in the nucleus from 2 to 48 weeks post-transplantation; these cells increased proportionally over time, suggesting that some of the cells had survived and proliferated [48]. In our present study, however, conclusions about cell expansion cannot be drawn, since only few cells were retained after 3 days. Hence, we will analyze this in future studies addressing longer follow-up times and technical modifications to reduce cell loss.

Before one could envisage performing successful cell-therapeutic application in humans, it is desirable to address effectivity of such a treatment in light of questions of annular failure and/or implant retention. One possible solution would be to compensate the loss of implant material by increasing the cell numbers in the transplant to assure that a sufficiently high amount of metabolic active cells is retained inside the damaged disc over time. Calculating with an initial loss of 90% of implant material after surgery however is ineffective and would mean that it is necessary to produce at least 5×10^6 cells to reach a final augmentation of the disc with 5×10^5 cells, which corresponds to a final concentration of 5×10^6 cells/ml, a concentration that has been reported as the natural cell density in the human nucleus pulposus [34]. The other possibility is, of course, to develop new strategies for implant retention and annular closure, to assure that the injected/implanted cells remain in place and contribute to the regeneration of the diseased segment.

Within the light of recently published results about the nutritional situation of the intervertebral disc [22], cell therapeutic approaches will be generally limited by the harsh nutritional situation of the disc. However, nutrient concentrations around the cells depend not only on the rate of supply but also on the rate of cellular consumption; glucose or oxygen, for example, can be exhausted in the disc center either if capillary contact area decreases or if cell densities or consumption rates are high. Thus, an increase of cell densities may lead to more nutritional stress with less cell survival. This suggests that the number of new cells has to be carefully selected to allow them to survive.

Since nutrients are only supplied to the avascular disc through the cartilaginous endplates, the inner disc nucleus is end of the line in regard to nutrient supply and disposal of waste products [18]. In degenerated discs, which are the target for therapeutic approaches, this situation is further deteriorated due to degenerated endplates [46]. In the present study, porcine discs of mature Goettingen minipigs

were used without prior experimental induction of disc degeneration, but moderate degenerative changes may generally exist in the endplates of mature Goettingen minipigs, while disc degeneration with black disc signs are evident only in some animals at this age. The degenerative endplate changes were similar to those in healthy adult humans and thus not comparable to those in severe disc degeneration [40]. Interestingly, however, severely degenerated discs are characterized by lower intradiscal pressures due to disc dehydration and less disc height with a shorter nutritional diffusion distance, which may facilitate nutrient diffusion from the endplates to the inner nucleus [22]. Whether in the end, severe degeneration will also facilitate survival of more transplanted cells can again be addressed by the methods developed in this study.

We here observed a considerable intersegment variability of implanted material and co-implanted cells in spite of standardized procedures, which questions the interpretation of *in vivo* studies which did not quantify injected material and remaining material after the end of the study. One possible reasons why variability is higher *in vivo* is certainly the limited angular accessibility of the treated segment while operating under open visual control. A possible improvement for further large animal studies might be the use of X-ray image intensifier control for precise placement of the injection needle [14].

One general limitation of our study is the use of the minipig model to gain relevant information about implantation strategies aimed to be used in humans. In humans, the weight of the upper body acts on the lumbar spine. This is not the case in quadrupeds. Therefore, loading of the lumbar spine is often assumed larger in humans than in quadrupeds. This, however, may not be correct since muscle contraction and tension of passive structures such as ligaments also adds substantially to the loads acting on the spine [58]. According to Alini et al. [2], this additional load may be higher in large quadrupeds such as calves, sheep, or pigs than in humans since stabilizing a horizontally aligned spine requires higher muscle forces and passive tension than stabilizing an almost balanced vertically aligned spine, e.g. suspension bridge versus an inverted pendulum.

Hence, although there are no directly measured forces in the literature, and therefore no consensus, loading of the lumbar spine is probably not smaller but even larger for large quadrupeds than for humans. This would also be consistent with the up to fourfold higher bone mineral density found in sheep, calf, and pig lumbar vertebral bodies compared to the human [57].

One other limitation of our study is the use of Al_2O_3 particles as a means to follow up implant retention. We have to emphasize, that Al_2O_3 has no known therapeutic benefit, nor was its use ever planned to have any biological

benefit. Its use was solely based on the technical requirement to visualize parts of the implant by μ CT. Al_2O_3 by its nature has a higher density than water and therefore its distribution inside the transfer matrix is not absolutely homogeneous. Further studies which might use magnetic resonance imaging (MRI) to visualize discs and implants, however, could follow a similar strategy to follow up implant retention by using paramagnetic beads which are incorporated by the cells [29]. One further limitation is the small animal ($n = 6$) and segment number ($n = 12$ per group) used in the in vivo study. Considerable variations were found between different animals, however, we considered the animal number sufficient to establish the presented methods. Although the normalized data suggest no evidence for a reduced pMSC-Luc metabolic activity over time ($P = 0.887$), such effects may become apparent when means for enhanced implant retention become available.

In summary, we have established a strategy which allowed separate quantitative follow up of the implant material by μ CT on the one hand and the activity of contained autologous mesenchymal stem cells by biochemical analysis on the other hand after implantation into partially nucleotomized intervertebral discs of Goettingen minipigs in vivo. Since we found that rather implant persistence in the disc than quick loss of cell marker activity were a concern in our study, we propose that intervertebral disc engineering strategies should increasingly focus on the regeneration or repair of the annulus fibrosus in order to reduce the potential of implant extrusion. This would increase the potential of nucleus pulposus engineering strategies and mechanically assist nucleus pulposus replacement therapies [4, 56].

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