Transplantation of autologous mesenchymal stem cells halts fatty atrophy of detached rotator cuff muscle after tendon repair: Molecular, micro- and macroscopic results from an ovine model

Supplemental methods

Tendon release

All operative procedures were carried out on the right shoulder of the respective anesthetized sheep, the left shoulder serving as a control essentially as described 8. In brief, the ISP tendon was released by an osteotomy of the greater tuberosity (20 x 10 x 10 mm) using an oscillating saw. The tendon stump with its attached bone chip was grasped with 2 figure-of-8 stitches using Fiberwire USP No. 2 sutures (Arthrex, Inc, Naples, Florida) through a 1.8-mm drill-hole centered in the bone chip. Subsequently, the tendon and the bone chip, were wrapped in a silicon tube (Silicone Penrose drain tube, 12 mm diameter; Fortune Medical Instrument, Taipei, Taiwan) to prevent spontaneous reattachment by scar tissue and the wound was closed. Immediately after surgery radiological measurements were effected on both shoulders.

Repair

Radiological measurements were repeated immediately prior to rotator cuff repair: The ISP tendon-bonechip complex was exposed and released from adhesions to the surrounding structures, and the silicon tube was removed. Rotator cuff repair was then performed by reattaching the bonechip to its original site, or as near as possible with attachment of the remaining sutures to a 3.5-mm self-tapping cortical bone screw with a washer.

Recovery and Sacrifice
During the first three weeks of the recovery period after repair animals were prevented from full weight bearing by attaching a ball to the sheep's claws and by using a loose suspension belt. Before sacrifice, radiological measurements were conducted on both shoulders. A biopsy (20-40 mg) was collected from the lateral aspects of the mid portion of the intact contralateral ISP muscle, frozen in nitrogen-cooled isopentane, and the entire ISP muscle excised. Then the sheep was repositioned and biopsies were collected from the different regions of the lateral aspect of the repaired ISP muscle (Fig. 1B). At the end of the experiment the animals were euthanized. Excised repaired and contralateral ISP muscles were fixed for 72 hours at room temperature in 1.5 liters of 4% buffered formalin and samples (~0.3 cm x 1 cm x 1 cm in size) were collected from the demarcated regions of the repaired muscle (Fig. 1B) and a corresponding region in the contralateral control.

**MSC preparation**

Starting 6 weeks before microtissue implantation, MSCs were extracted, characterized, and then fluorescently-labelled and seeded according to an established procedure 4,5. In brief, 20 mL of bone marrow was drawn from the pelvic medulla into tubes loaded with 0.5 ml Heparin (5000 international units/mL). The suspension was filtered in Hank’s Balanced Saline Solution (GE Healthcare), the mononuclear cell fraction recovered by centrifugation and seeded at a density of $2 \times 10^5$ cells/cm$^2$ in 10% FCS-DMEM (low glucose + glutamine 2mM, 1% penicillin-streptomycin; Gibco). Non-adherent cells were removed and the attached cells grown to 80% confluence, washed with Phosphate-buffered saline (PBS; Sigma) and detached with 500 µL of 0.25% trypsin-EDTA 1x (Gibco) before being plated in complete medium at a density of $5 \times 10^5$ cells in a T75 flask (supplemental Fig. 1) as described 4,5.
Six days prior to implantation, the cells were labeled with Q-Dots (QTracker Cell Labeling Kit, Invitrogen, Life Technologies), seeded as $5 \times 10^3$ cells 25 µL medium per well in 60-well Terasaki plates and grown upside down at 37°C and 5% CO$_2$ in an incubator as described $^9$. An aliquot of the cell suspension was classified by flow cytometry based on epitopes for phenotypic markers (CD29 and Stro-4, CD166, CD44, CD31, CD34, IgG1 and IgG2), and their osteogenic, chondrogenic and/or adipogenic differentiation potential examined by the cultivation in specific medium conditions as described (supplemental Fig. 1 and 2) $^4,5$.

**MSC implantation**

On the day of repair surgery, and for each animal, 720 microtissues were resuspended in DMEM, distributed in four 1-mL syringes, and stored at 37°C until being used. The resulting portions of 180 microtissues (0.9 Mio cells per 0.4 mL) were injected through 20 mm-long 27G needles at a depth of 2 cm in each quadrant of a 2 cm x 2 cm-sized area in the lateral portion of ISP muscle being demarcated with radiodense surgical suture. Another 2 cm x 2 cm-sized region being located more proximally, and injected accordingly with DMEM alone, was used as media control. A third region, which was only demarcated was used as a non-injection control. Before injection, electropulsing was performed at the lateral and medial border of the 3 demarcated regions to enhance the permeability of the extracellular matrix $^{10}$. 3 trains of 80 pulses of 100-microseconds duration at 100 mA, with 992 milliseconds interrupt were applied through 4-cm needle electrodes with a GET42EV generator (E.I.P. Electronique et Informatique du Pilat, Jonzieux, France) as described $^3$.

**Radiological assessment of structural muscle changes**

CT and MRI were performed as established $^8$ with the anesthetized animal being positioned in
way to allow the recording of transverse sections of the sheep's shoulders perpendicular to the
glenoid cavity. CT was performed with a Somatom ART (Siemens Medical Solutions, Erlangen,
Germany) recording was performed for 2 mm thick sections. Musculotendinous retraction was
documented by measuring the distance of the bonechip to its original insertion site. The density
of the muscle tissue was measured on a transverse cross-sectional area of the complete muscle as
Hounsfield units (HU). MRI was performed with a 3-Tesla system using a dedicated receive-only
extremity coil (Philips Ingenia 3T with dStream body coil Solution, Philips AG; Zurich,
Switzerland). Pulse sequences included: T1W TSE transverse; PDW aTSE transverse; T1W TSE
coronal; T2W SPIR coronar; smDIXON transverse in phase; smDIXON transverse out phase;
smDIXON transverse water only; smDIXON transverse fat only. On T1 weighted transverse images
the cross-sectional area of both ISP muscles including the central tendon (excluding the
bonechip) was marked on every slice and the muscle volume was calculated. On transverse
DIXON images the fat fraction of both ISP muscles was determined from the average of measures
from a cross section and three areas in the central portion of the muscle. Additionally, the water
and fat fraction were assessed in voxels corresponding to the three targeted regions as identified
by the radiodense sutures. For each region the fat fraction was calculated from signal intensities
derived from fat-only images (smDIXON transverse fat only (DIXON-FAT)) and water only images
(smDIXON transverse water only (DIXON-WATER)). A single observer performed readout of all
MRI and CT scans using the DICOM viewer OsiriX v.5.6 32-bit (Pixmeo) without knowledge of
how the data were assigned to the experimental groups.

*Histological analysis*

Formalin-fixed samples were processed to quantify the area percentages covered by muscle fiber
types, fat and extracellular ground substance, and MSCs based on the microscopic evaluation of
immunochemically stained structures and Q-Dot fluorescence essentially as shown. In addition, the overall morphology was described through the use of hematoxylin & eosin (H&E) and Van Gieson (VG) staining of deparaffinized sections.

In brief, 3-5 µm-thick paraffin-embedded sections were deparaffinised and fiber types visualized with serial incubation with primary mouse antibody against slow type myosin heavy chain (clone NOQ7.5.4D, Sigma-Aldrich, St. Louis, Missouri, USA), mouse antibody against fast type myosin heavy chain (clone MY32, SigmaAldrich, St. Louis, Missouri, USA), and secondary dextranpolymer-coupled antibody against mouse immunoglobulin and alkaline phosphatase, with PBS washes in between using a commercial kit (EnVision G/2 Doublestain System for rabbit/mouse (DAB+/Permanent Red), Code K5361, DAKO, Baar, Switzerland). Reactions were visualized with permanent Red chromogen, the nuclei counter-stained with hematoxylin and the sections embedded in Aquatex (MERCK AG, Darmstadt, Germany). Fat and extracellular ground substance was identified morphologically as round cells without apparent staining and the weakly stained interstitial tissue.

Three regions of interest in an artifact-free region of the sections were identified at a preset distance using a isosceles triangle with side lengths of 4.6mm and 6.6 mm, and digitally recorded at a 10x magnification on a microscope with a DFC 320 camera (Leica DMR Microsystems GmbH, Wetzlar, Germany) and being operated with Image Access Standard software (Imagic Bildverarbeitung A G, Glattbrugg, Switzerland). Captured images were processed in Photoshop Elements 12 (Adobe Systems Inc., San Jose, USA) to quantify the stained slow type and fast type muscle fibers, the histologically identified fat compartment and extracellular ground substance of the connective tissue structures were falsely colored. Subsequently pixel values of the stained structures were determined with an automated procedure through use of Image J software (v1.48v National Institutes of Health, USA) and the relative area of each type of structure was
calculated from the measurements in the three regions of interest. The MCSA of muscle fibers was
determined from the quotient between the surface area of the respective muscle fiber type and
the number of muscle fibers per region of interest.

Fluorescent signal was excited at 380nm, filtered (center wavelength of 647 nm, bandwith of 70
nm) and the paraffin section recorded at 655 nm with a 10x-objective (Plan Apochromat NA 0.45
air) on an automated upright slide scanning microscope (Axio Scan.Z1, Zeiss, Germany).
Fluorescence-positive microscopic fields were subsequently separately digitally recorded at a 40-
fold magnification and used to determine the number and individual area of fluorescence-positive
signals (counts) in the respective fields from the red color channel with a standard procedure
using Image J software (v1.48, National Institutes of Health, USA). Lastly the number of
fluorescent nano-particle positive microscopic fields per scanned section was determined by
manual counting and was used to calculate the total area being covered by Q-tracker in the
studied section.

H&E staining and VG staining were performed according to standard procedures, visualizing
the cytosplasm (pink) and nuclei (blue); or connective tissue/collagen fibers (red), muscle tissue
(yellow), and nuclei (brown-black).

Biochemical analysis
Homogenates were prepared from paraffin embedded blocks with a modification of a published
protocol 1. In brief, a tissue volume corresponding to 10 mm³ was collected by pooling 5
micrometer-thick sections. The sample was rehydrated and incubated in a series of Ethanol
bathes at decreasing concentration under vigorous vortexing, rotating, and centrifugation at
12’000 g. The pellet was recovered in 350 µL of extraction buffer (20 mM Tris, 2% SDS, 200 mM
beta-mercapto-ethanol, pH 8.8), incubated for 35 minutes at 100°C with 15 passages through a
20G 1” needle (BD Microlance). Following a 2-hours incubation at 80°C at 550 rpm in a thermomixer (Vaudaux-Eppendorf, Basel, Switzerland) and a centrifugation step (15 min at 17200 g) at 4°C, extracted proteins were recovered in the supernatant and transferred to the new 1.5 mL tube (Huberlab, Aesch, Switzerland).

10 microgram protein in Lämmli buffer were separated using a precast 4-15% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis system (Mini Protean TGX Precast Gel, Bio-Rad) and subsequent immunoblotted to detect the abundance myogenin, tenascin-C, and PPARG respective to sarcomeric α-actin with enhanced chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce) using the PXi multi-application gel imaging system (Syngene) as described 2. The following primary antibodies and dilutions were used: Myogenin, 1:200 of MyoG F5D (sc12732, Santa Cruz); tenascin-C, 1:500 of monoclonal mouse antibody B28.13 (gift of Prof. Ruth Chiquet-Ehrismann, Friedrich Miescher Institute); PPARG, 1:1000 of antibody LS-C178333 (LSBio); sarcomeric α-actin, 1:5000 of 5C5 antibody (A2172, Sigma). Goat α-mouse HRP conjugate (A9917, Sigma) was used to detect immunoreactivity. Samples from the media- and MSC-injected region, or from non-injected regions from the T and T-MSC group, respectively, of a repaired muscle as well as an internal reference sample, were loaded on the same polyacrylamide gel. Immunoreactive signals were standardized to the α-actin values for the respective protein extract, standardized to the internal reference and pooled between experiments. For each analyzed biopsy from an MSC-injected region a single section was inspected to validate the presence of Q-Dots with an IX50 microscope fluorescent microscope (Olympus Schweiz AG) under the settings described above.

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Appendix Figure A1: Characterization of the microtissues of MSCs.
Microscopic image visualizing the appearance of the expanded MSCs in culture (A), and the microtissues (B). Bar, 100 micrometer. C) Spider diagram depicting the expression of the selected markers in aliquots from the numbered MSC cultures from the different sheep. Abbreviations: CD29, cluster of designation 29, integrin beta-1; CD166, cluster of designation 166, activated leukocyte cell adhesion molecule; CD44, cluster of designation 44, extracellular matrix receptor III; CD31, cluster of designation 31, platelet endothelial cell adhesion molecule; CD34, cluster of designation 34, hematopoietic progenitor cell antigen; IgG1, Immunoglobulin heavy chain type G1; IgG2, Immunoglobulin chain type G2; Stro-4: antigen of stromal / mesenchymal stem cells.
**Appendix Figure A2: Adipogenic and osteogenic differentiation of MSCs**

Micrographs exemplifying the differentiation of the isolated MSC cultures after cultivation under adipogenic (A-C) and osteogenic conditions (D-F). Aliquots from the same MSC preparation of one sheep were subjected to adipogenic or osteogenic differentiation. Subsequently the degree of differentiation was inspected microscopically, for chondrocytes based on Alcian blue staining for glycosaminoglycans (A, D), for osteocytes based on Van Kossa staining for calcium complexes (B, E), for adipocytes based on lipid staining with Red Oil (C, F). Note the intense positive staining for calcium complexes under the osteogenic conditions (E, brown) and intense lipid staining under adipogenic conditions (C, in red).
**Appendix Figure A3:** Computed tomography based composition of MSC-injected and repaired muscle.

Box Whisker plots visualizing the percentage changes in Hounsfield units (U) in the repaired infraspinatus muscle and its contralateral control, 6 weeks after repair in groups T and T-MSC. ** denotes p<0.01 for the indicated comparison. ANOVA with posthoc test of Fisher.
Appendix Figure A4: Regional differences in muscle composition in the repaired muscle in groups T and T-MSC. Box-Whisker plots of the water fraction as assessed from MRI-based DIXON sequences (A), and CT-based Hounsfield units (B) in the different muscle regions of the repaired infraspinatus muscle six weeks after repair in groups T and T-MSC. +, * and ** denote p<0.10, p<0.05 and p<0.01 for the indicated comparison. ANOVA with post-hoc test of Fisher.
**Appendix Table A1: Macroscopic consequences of tendon release.** Mean values and SD and p-values for the observed differences in retraction of the bone chip of the ISP muscle after osteotomy and body mass in the two experimental groups T (24.6 ± 4.5 months, n=6) and T-MSC (18.5 ± 1.2 months, n=6) at the different time points. 0w: immediately after osteotomy, 16w, 16 weeks after osteotomy and immediately after tendon repair; 22w, 6 weeks after repair of the released tendon. Underlines values were deemed significant. ANOVA with post hoc test of Fisher.

| group | time point | body mass [kg] | retraction mean [mm] |
|-------|------------|----------------|----------------------|
|       |            | mean ± SD      | vs. 0w               | vs. 16w | vs. T-MSC | mean ± SD | vs. 0w | vs. 16w | vs. T-MSC |
| T     | 0w         | 45.3 ± 4.8     |                      | 0.022   |           | 2.9 ± 0.4 | 0.017  |           |           |
| T     | 16w        | 55.7 ± 4.5     | <0.001               | 0.085   |           | 5.8 ± 1.0 | 0.002  | 0.662    |           |
| T     | 22w        | 59.4 ± 4.6     | <0.001               | 0.001   | 0.413     | 4.7 ± 1.3 | 0.024  | 0.011    | 0.724     |
| T-MSC | 0w         | 55.3 ± 7.7     |                      | 3.6 ± 0.5|           |           |         |          |           |
| T-MSC | 16w        | 62.0 ± 6.8     | <0.001               | 5.6 ± 0.7| <0.001    |           |         |          |           |
| T-MSC | 22w        | 62.2 ± 6.4     | 0.001                | 0.001   |           | 4.5 ± 0.7 | 0.008  | 0.363    |           |
**Appendix Table A2: Size distribution of fluorescence-positive cellular structures**

Mean + SD of the distribution of the size of fluorescence-positive cellular structures and chance to identify fluorescence-positive cellular structures of a given diameter in 100 micrometer².

| diameter          | <10 mm | 10-20 mm | >20 mm |
|-------------------|--------|----------|--------|
| distribution [%]  | 92.6 ± 2.6% | 4.9 ± 3.2% | 2.5 ± 0.8% |
| chance [% hits per 100 um²] | 3.7 ± 1.0 % | 0.3 ± 0.2% | 0.1 ± 0.0% |
Appendix Table A3: Consequences of microtissue injection on muscle parameters. Mean ± SD of the MCSA and frequency of muscle fibers.

| group         | muscle        | treatment       | slow type          | fast type          | all types          |
|---------------|---------------|-----------------|--------------------|--------------------|--------------------|
| T-MSC         | operated      | non-injected    | 3769.5 ± 837.1     | 2776.5 ± 904.2     | 3327.0 ± 800.1     |
| T-MSC         | operated      | media-injected  | 4817.3 ± 1589.4    | 3841.8 ± 1387.4    | 4282.8 ± 1456.0    |
| T-MSC         | operated      | MSC-injected    | 3518.3 ± 907.8     | 2812.2 ± 898.5     | 3110.8 ± 899.4     |
| T             | operated      | non-injected    | 3006.3 ± 809.7     | 3372.3 ± 998.9     | 3197.8 ± 841.8     |

p-value: media- vs. non-injected 0.065 0.050 0.077
MSC- vs. media-injected 0.024 0.058 0.032
MSC- vs. non-injected 0.650 0.946 0.681
p-value: T-MSC- vs. T for non-injected 0.174 0.262 0.806

| group         | muscle        | treatment       | slow type          | fast type          | all types          |
|---------------|---------------|-----------------|--------------------|--------------------|--------------------|
| T-MSC         | contralateral | non-injected    | 4207.8 ± 659.7     | 3377.8 ± 263.9     | 3815.2 ± 629.2     |
| T             | contralateral | non-injected    | 4030.0 ± 511.8     | 3469.8 ± 524.9     | 3675.3 ± 465.8     |

p-value: T-MSC vs. T 0.748 0.861 0.790

| group         | muscle        | treatment       | slow type          | fast type          | all types          |
|---------------|---------------|-----------------|--------------------|--------------------|--------------------|
| T-MSC         | operated      | non-injected    | 100.0 ± 11.9       | 111.0 ± 21.2       | 105.5 ± 9.6        |
| T-MSC         | operated      | media-injected  | 73.2 ± 7.4         | 89.5 ± 11.6        | 81.4 ± 8.4         |
| T-MSC         | operated      | MSC-injected    | 92.2 ± 7.9         | 120.9 ± 15.3       | 106.5 ± 10.4       |
| T             | operated      | non-injected    | 96.1 ± 11.6        | 115.9 ± 22.8       | 106.0 ± 15.6       |

p-value: media- vs. non-injected 0.131 0.705 0.101
MSC- vs. media-injected 0.491 0.473 0.090
| Comparison                  | Value 1  | Value 2  | Value 3  |
|----------------------------|----------|----------|----------|
| MSC- vs. not injected      | 0.440    | 0.720    | 0.951    |
| p-value: T-MSC vs. T       | 0.240    | 0.484    | 0.979    |
| T-MSC contralateral non-injected | 100.6 ± 9.5 | 120.5 ± 18.2 | 110.5 ± 6.3 |
| T contralateral non-injected | 86.5 ± 10.0 | 146.7 ± 4.8 | 116.6 ± 7.1 |
| p-value: T-MSC vs. T       | 0.165    | 0.155    | 0.692    |