



■ **SHOULDER & ELBOW**

# Tendon-derived stem cells from the long head of the biceps tendon

INFLAMMATION DOES NOT AFFECT THE REGENERATIVE POTENTIAL

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**Objectives**

The long head of the biceps (LHB) is often resected in shoulder surgery and could therefore serve as a cell source for tissue engineering approaches in the shoulder. However, whether it represents a suitable cell source for regenerative approaches, both in the inflamed and non-inflamed states, remains unclear. In the present study, inflamed and native human LHBs were comparatively characterized for features of regeneration.

**Methods**

In total, 22 resected LHB tendons were classified into inflamed samples (n = 11) and non-inflamed samples (n = 11). Proliferation potential and specific marker gene expression of primary LHB-derived cell cultures were analyzed. Multipotentiality, including osteogenic, adipogenic, chondrogenic, and tenogenic differentiation potential of both groups were compared under respective lineage-specific culture conditions.

**Results**

Inflammation does not seem to affect the proliferation rate of the isolated tendon-derived stem cells (TDSCs) and the tenogenic marker gene expression. Cells from both groups showed an equivalent osteogenic, adipogenic, chondrogenic and tenogenic differentiation potential in histology and real-time polymerase chain reaction (RT-PCR) analysis.

**Conclusion**

These results suggest that the LHB tendon might be a suitable cell source for regenerative approaches, both in inflamed and non-inflamed states. The LHB with and without tendinitis has been characterized as a novel source of TDSCs, which might facilitate treatment of degeneration and induction of regeneration in shoulder surgery.

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**Keywords:** Biceps tendon, Tendon-derived stem cell, Mesenchymal stem cell, Tissue engineering, Shoulder

**Article focus**

- The long head of the biceps (LHB) tendon in the inflamed and non-inflamed state as a stem cell source for regenerative approaches.

**Key messages**

- The LHB tendon might be a suitable cell source for regenerative approaches, both in inflamed and non-inflamed states.
- Inflammation does not seem to affect the proliferation rate of the isolated tendon-derived stem cells (TDSCs).
- Cells from both groups showed an equivalent osteogenic, adipogenic, chondrogenic, and tenogenic differentiation potential in

histology and real-time-polymerase chain reaction (RT-PCR) analysis.

**Strengths and limitations**

- Differences between TDSCs isolated from inflamed and non-inflamed LHB tendons were studied.
- The patient population was not matched regarding sex and age, whereas it has been demonstrated that the sex of the donors, as well as age, can affect stem cell proliferation.

**Introduction**

The long head of the biceps (LHB) tendon originates from the superior labrum and the

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supraglenoid tubercle of the shoulder.<sup>1</sup> While crossing the rotator cuff (RC) interval, the LHB tendon is stabilized in the bicipital groove by the capsuloligamentous biceps pulley complex.<sup>2</sup> Before the transition to the upright gait, the LBH served as an important anterior stabilizer of the shoulder,<sup>3</sup> however, today its role in shoulder biomechanics is controversial. Some shoulder surgeons even call it the “appendix” of the shoulder joint, so frequently is it affected by inflammation. The LHB tendon may be further affected by degenerative tendinopathy, dislocation, or partial or complete tears, and intra-articular resection of the tendon is frequently performed and has consistently provided appropriate pain relief under maintained biceps muscle function.<sup>4</sup>

Interestingly, the LHB has recently been identified as a tissue source for multipotent cells characterized as tendon-derived stem cells (TDSCs).<sup>5</sup> The LHB might, therefore, be an attractive cell source to augment regenerative approaches within the shoulder.<sup>5</sup> Despite significant recent progress in the development of RC repair techniques, failure rates after RC repair still remain highly variable mainly due to biological variability of the torn RC tendon and muscle with associated fatty atrophy.<sup>6-8</sup> Histological studies have shown that tendon does not heal by rebuilding a normal fibrocartilage enthesis but by forming scar tissue with a high content of type III collagen.<sup>9,10</sup> Therefore, TDSCs might be capable of promoting repair in the tendon bone junction and may influence the tendon structure after RC repair.<sup>11</sup>

Due to the current lack of information in the literature about whether inflammation impairs the regenerative capacities of LHB tendons, in this work we comparatively analyzed TDSCs isolated from resected intra-articular portions of the LHB with and without inflammation, and analyzed the respective cultures in the context of regeneration on a tissue, cellular, and molecular level.

## Materials and Methods

**Clinical classification in inflamed and non-inflamed tendons.** Classification in inflamed and non-inflamed tendons was realized during surgery upon clinical judgement. Tendons with clear macroscopic signs of inflammation were assigned to the ‘tendinitis’ group, whereas native tendons were assigned to the ‘no tendinitis’ group. Classification was performed by two experienced shoulder surgeons (CK, PP). If the two surgeons disagreed, the samples were not included, thus, there were no borderline tendons. Macroscopic and microscopic images were taken from all samples, and representative photographs are shown in Figure 1.

**Tissue harvest.** After informed consent, LHB tendon samples were obtained from patients undergoing open or arthroscopic surgery for RC repair, LHB tenodesis, or shoulder arthroplasty. The study was approved by the local ethics committee (approval number 82/08).

Biceps tendon samples after tenotomy were gathered from 22 shoulders in total (mean donor age 59.6 years (44 to 80), 13 male patients, nine female patients) of whom 11 showed clear macroscopic signs of tendinitis. The donors’ demographics and reason for surgery are shown in Table I. Any insertion sites (bony/capsular) were removed and tendons were rinsed with saline. For each outcome measure, cells and samples from three representative donors with tendinitis and three donors without tendinitis were used. For each test, three replicates for each donor and test group were used.

**Isolation of primary cells.** The tendons were minced into small pieces and incubated with collagenase (0.175 U/ml; Sigma–Aldrich, Munich, Germany) in Dulbecco’s Modified Eagle Medium (DMEM)/Ham’s F12 (1:1; Life Technologies GmbH, Thermo Fisher Scientific, Waltham, Massachusetts) for two hours at 37°C.<sup>12</sup> After the digested tendon material was washed and the centrifugation was completed, the released cells could be collected and plated in 75 cm<sup>2</sup> tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany) at a density of 3×10<sup>6</sup> cells per flask. The cells were cultured in DMEM/Ham’s F12 (1:1), supplemented with 10% foetal calf serum (FCS) (Biochrom AG, Berlin, Germany), and 1% penicillin/streptomycin, in an incubator in a humidified atmosphere (37°C, 5% CO<sub>2</sub>) with a change of medium every two to three days. As soon as cultured cells reached 90% confluence, they were harvested for RNA isolation or seeded in well plates. The cells were passaged twice and second-passage cells were used for each donor and for each outcome measure.

**Cell proliferation assay.** Cell proliferation was assessed as described previously by measuring adenosine 5’ triphosphate (ATP), using the sensitive luminometric CellTiter-Glo Luminescent Cell Viability Assay (Promega GmbH, Mannheim, Germany), according to the manufacturer’s instructions.<sup>13</sup> A total of three inflamed and three non-inflamed LHB samples were included.

**RNA isolation, RT-PCR, and qRT-PCR.** Total RNA was harvested from cells at passage two after reaching confluence in the culture flasks. Samples were isolated and real-time polymerase chain reaction (RT-PCR), as well as quantitative real-time polymerase chain reaction (qRT-PCR), were performed as described previously.<sup>13,14</sup> Elongation factor 1 $\alpha$  (*EF1A*) served as the housekeeping gene; target-specific sense and antisense primers used for RT-PCR analysis are listed in Table II, which also provides a summary of the primer details. Sequence-specific primers for qRT-PCR were obtained from biomers.net GmbH (Ulm, Germany) or Qiagen GmbH (Hilden, Germany) and are listed in Table III, which also shows the primer-specific details. Dissociation curve analysis for qRT-PCR results was carried out to verify the absence of primer dimers and/or non-specific PCR products. The expression of the genes of interest was normalized against the ribosomal



Fig. 1a

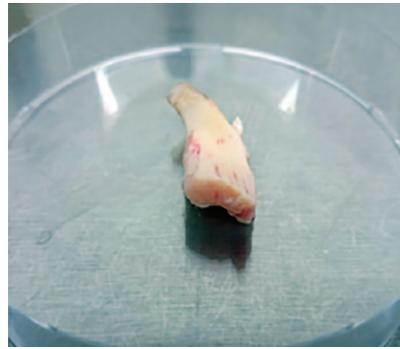


Fig. 1b

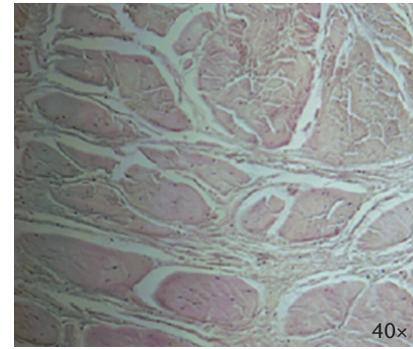


Fig. 1c

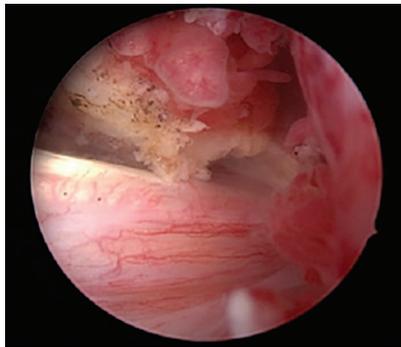


Fig. 1d



Fig. 1e

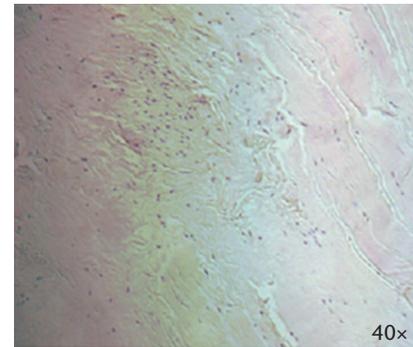


Fig. 1f

Macroscopic and microscopic view of inflamed and non-inflamed tendon samples. Long head of the biceps (LHB) tendon samples with (d, e, f) and without (a, b, c) tendinitis are shown. a) and d) Intraoperative arthroscopic view. b) and e) Tendon samples before processing in the laboratory in a 10 cm petri dish. c) and f) Microscopic appearance with haematoxylin and eosin (H&E) stain. Images are taken at 40 $\times$  as indicated.

**Table I.** Donors' demographics and reason for surgery

Feature	Tendinitis (n = 11)	No tendinitis (n = 11)
Sex, male:female, n	9:2	4:7
Mean age, yrs (range)	66 (60 to 82)	57 (46 to 67)
<b>Type of surgery, n</b>		
Arthroplasty	2	2
Rotator cuff repair	7	6
Isolated biceps surgery	2	3

protein *S27a* (*RPS27A*) housekeeping gene using the delta-delta Ct method.<sup>15</sup>

**Osteogenesis.** The LHB-derived stem cells were seeded at a density of 3000 cells/cm<sup>2</sup> into six-well polystyrene tissue culture plates (Greiner Bio-One). After reaching confluence, mineralization was induced over a period of 28 days, as described previously.<sup>14</sup> Controls were cultured in normal culture medium. Cultures were stained for matrix mineralization using Alizarin Red, as described previously.<sup>14</sup> Additionally, markers of osteogenesis were analyzed by RT-PCR, using the protocol described above. Expression of the following human messenger RNAs (mRNAs) was examined: alkaline phosphatase (*ALPL*), collagen type I alpha 2 (*COL1A2*), and runt-related transcription factor 2 (*RUNX2*). Primer sequences are provided in Table II. Expression was normalized to the housekeeping gene *EF1A*.

**Adipogenesis.** Cells were grown to confluence in six-well polystyrene tissue culture plates. Afterwards, adipogenesis was induced as described previously.<sup>13</sup> Control cultures without adipogenic supplements were also maintained. After four weeks, cultures were examined for evidence of adipogenesis staining with freshly prepared Oil red O solution, as previously reported.<sup>16</sup> In addition, RT-PCR was realized, using the protocol described above, to detect expression of the following human genes: lipoprotein lipase (*LPL*); and peroxisome proliferator-activator receptor gamma 2 (*PPARG2*). Primer sequences are provided in Table II. Expression was normalized to the housekeeping gene *EF1A*.

**Chondrogenesis.** Chondrogenic differentiation was assessed by the pellet culture method as modified recently.<sup>17</sup> Pellets were incubated with chondrogenic medium as reported previously.<sup>16</sup> Aggregates were collected at three or four weeks for analysis. For detection of matrix proteoglycan, representative sections were stained with 1% (w/v) Alcian Blue (Sigma-Aldrich), as described previously.<sup>13</sup> For gene expression analyses, RNA was isolated and markers of chondrogenesis were amplified by RT-PCR, using the protocol described above. Expression of the following human mRNAs was examined: collagen type II alpha 1 (*COL2A1*); aggrecan core protein (*ACAN*); and cartilage oligomeric matrix protein (*COMP*). Primer

**Table II.** Real-time-polymerase chain reaction (RT-PCR) primer sequences and polymerase chain reaction (PCR) conditions

Gene	Oligonucleotide primer sequence	Cycles, n	Annealing temperature, °C
<b>Tenogenic marker gene</b>			
SCX	S: 5'—CCTGAACATCTGGAAATTTAATTTTAC—3' A: 5'—CGCCAAGGCACCTCCTT—3'	36	58
TNMD	S: 5'—CCATGCTGGATGAGAGAGGT—3' A: 5'—CTCGTCTCCTTGGTAGCAG—3'	35	56
<b>Osteogenic differentiation</b>			
ALPL	S: 5'—TGGAGCTTCAGAAGCTCAACACCA—3' A: 5'—ATCTCGTTGTCTGAGTACCAGTCC—3'	25	51
COL1A2	S: 5'—GGACACAATGGATTGCAAGG—3' A: 5'—TAACCACTGCTCCACTCTGG—3'	30	55
RUNX2	S: 5'—CCCCACGACAACCGCACCAT—3' A: 5'—CACTCCGGCCCCAACAATC—3'	30	64
<b>Adipogenic differentiation</b>			
LPL	S: 5'—GAGATTCTCTGTATGGCACC—3' A: 5'—CTGCAAATGAGACACTTTCTC—3'	30	51
PPARG2	S: 5'—GCTGTTATGGTGAAACTCTG—3' A: 5'—ATAAGGTGGAGATGCAGGCTC—3'	33	61
<b>Chondrogenic differentiation</b>			
COL2A1	S: 5'—TGGTGACAAAGGTGAAAAAGG—3' A: 5'—CATCAAATCCTCCAGCCATC—3'	35	51
ACAN	S: 5'—GCCTTGAGCAGTTCACCTTC—3' A: 5'—CTCTTCTACGGGGACAGCAG—3'	30	54
COMP	S: 5'—AGGATGGAGACGGACATCAG—3' A: 5'—TCTGCATCAAAGTCGTCCTG—3'	30	53
<b>Tenogenic differentiation</b>			
COL1A2	S: 5'—GGACACAATGGATTGCAAGG—3' A: 5'—TAACCACTGCTCCACTCTGG—3'	30	55
TNMD	S: 5'—CCATGCTGGATGAGAGAGGT—3' A: 5'—CTCGTCTCCTTGGTAGCAG—3'	30	56
FOS	S: 5'—CTGGCGTTGTGAAGACCATG—3' A: 5'—CTTCTCTTCAGCAGGTTGG—3'	30	55
VIM	S: 5'—TGCCCTTAAAGGAACCAATG—3' A: 5'—CTCAATGTCAAGGGCCATCT—3'	38	52
SCX	S: 5'—CCTGAACATCTGGAAATTTAATTTTAC—3' A: 5'—CGCCAAGGCACCTCCTT—3'	36	58
PTGS2	S: 5'—GCTGTCCCTTACTTCATTG—3' A: 5'—TGGCATCTTGTGATAGTGT—3'	35	55
<b>Internal control</b>			
EF1A	S: 5'—AGGTGATTATCCTGAACCATCC—3' A: 5'—AAAGGTGGATAGTCTGAGAAGC—3'	24	54

SCX, scleraxis; S, sense; A, antisense; TNMD, tenomodulin; ALPL, alkaline phosphatase; COL1A2, collagen type I alpha 2; RUNX2, runt-related transcription factor 2; LPL, lipoprotein lipase; PPARG2, proliferator-activator receptor  $\gamma$ 2; COL2A1, collagen type 2 $\alpha$ 1; ACAN, aggrecan core protein; COMP, cartilage oligomeric matrix protein; FOS, c-FOS activator protein 1; VIM, vimentin; PTGS2, prostaglandin-endoperoxide synthase 2; EF1A, elongation factor 1 $\alpha$

sequences are provided in Table II. Expression was normalized to the housekeeping gene *EF1A*.

**Tendogenesis.** For tenogenic induction, cyclic stretching was performed on cultures with  $5 \times 10^5$  cells per well, being seeded on four-well polyurethane plates and cultivated for one week in complete culture medium, supplemented with 10% FCS (Biochrom AG) and 1% penicillin/streptomycin. Cyclic stretching was performed for 30 minutes at 1 Hz and 1% extension by using a bioreactor, as described previously.<sup>18</sup> After stretching and cultivating the cells for 15 minutes, four hours, and for 24 hours, cells were harvested and total RNA was isolated as described above. Known mechanoresponsive genes such as Fos proto-oncogene, AP-1 transcription factor subunit (*FOS*), and prostaglandin-endoperoxide synthase 2 (*PTGS2*), as well as the tenogenic markers scleraxis (*SCX*), vimentin (*VIM*), and tenomodulin (*TNMD*), were analyzed by RT-PCR.

**Statistical analysis.** Statistical analysis was performed using IBM SPSS 22.0 (IBM, Armonk, New York) and p-values < 0.05 were considered significant. Determination of the statistical significance between groups was performed using Student's *t*-test, or the Mann–Whitney U test as indicated.

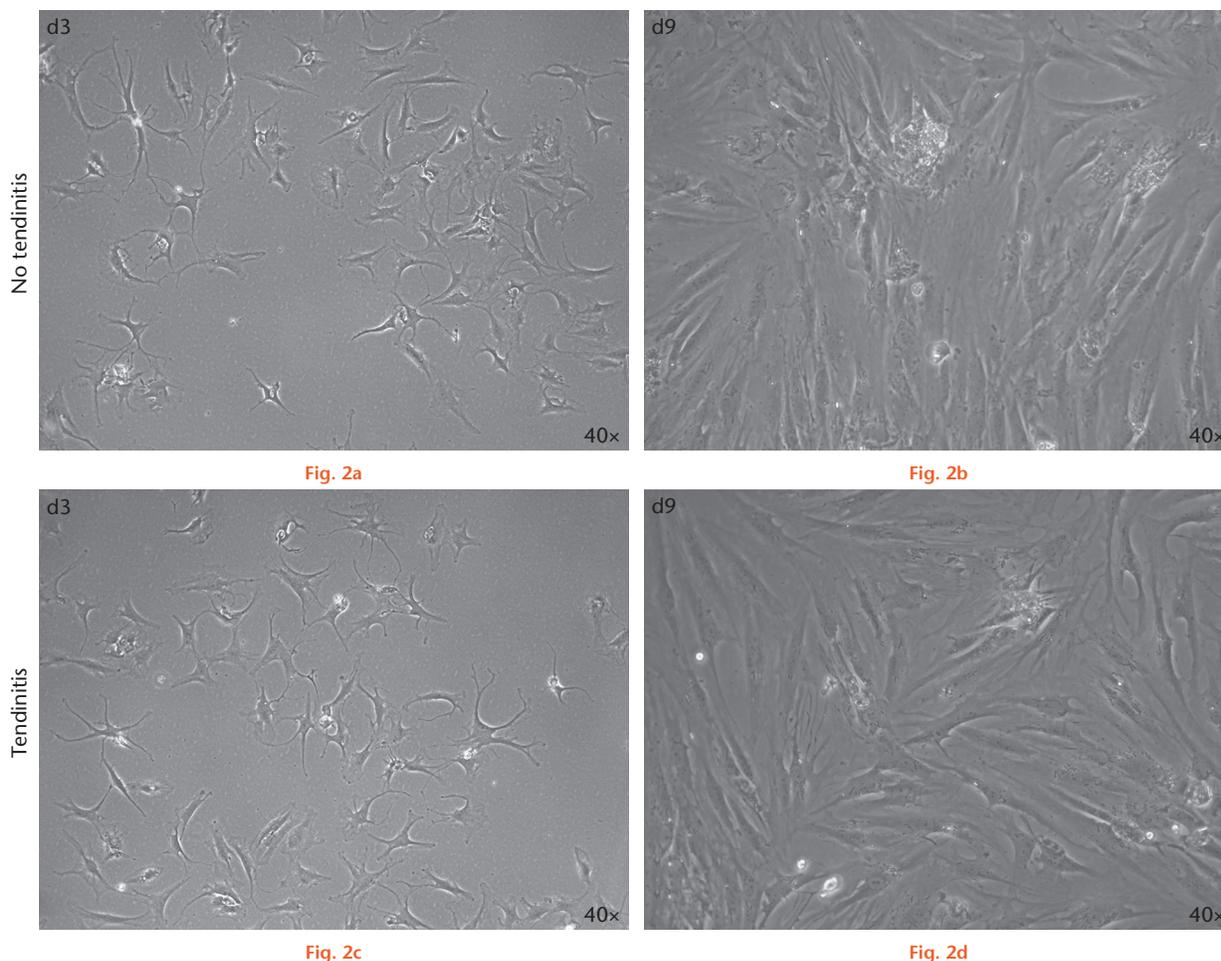
## Results

There were important differences regarding the sex and age of donors. There were nine male donors (82%) in the tendinitis group and seven female donors (64%) in the no-tendinitis group. The mean donor age in the tendinitis group was 66 years (60 to 82), and 57 years in the no-tendinitis group (46 to 67). The mean cell yield per tendon sample was  $6.8 \times 10^6$  ( $3 \times 10^6$  to  $15 \times 10^6$ ), however there was no statistical difference between the two groups.

**Table III.** Quantitative real-time-polymerase chain reaction (qRT-PCR) primer sequences and polymerase chain reaction (PCR) conditions

Gene	Primer	Sequence	Length, bp	Annealing temperature, °C	Efficiency
<b>Tenogenic marker gene</b>					
<i>SCX</i>	Forward	CCTGAACATCTGGGAAATTTTAC	111	60	1.68
<i>SCX</i>	Reverse	CGCCAAGGCACCTCCTT			
<b>Housekeeping gene</b>					
<i>RPS27A</i>	Forward	TCGTGGTGGTGCTAAGAAAA	141	60	2.01
<i>RPS27A</i>	Reverse	TCTCGACGAAGGCGACTAAT			

bp, base pairs; *SCX*, scleraxis; *RPS27A*, ribosomal protein S27a



Microscopic appearance of human long head of the biceps (LHB) tendon-derived cells with and without inflammation in culture. Human LHB tendon-derived cell cultures without tendinitis at a) day 3 (d3) and b) day 9 (d9) of culture, and c) and d) with tendinitis at the same timepoints. Images are taken as 40 $\times$  as indicated.

### Cell morphology

Cells isolated from both inflamed and non-inflamed human LHB had a spindle-shaped, fibroblast-like morphology (Fig. 2) and formed colonies upon adherent culture typical for mesenchymal cell types.<sup>19</sup>

**Cell proliferation.** Cells isolated from inflamed LHB samples proliferated well when compared with native non-inflamed LHB tendon-derived cells, and no significant differences were observed between groups (Fig. 3). Nevertheless, the mean proliferation rate was slightly decreased in the tendinitis group. Notably, there was a

high interindividual variability without clear-cut differences between groups.

**Qualitative and qRT-PCR analysis.** In RT-PCR analysis (Fig. 4a), similar expression of tenogenic marker genes, such as *SCX* and *TNMD* could be observed in the two groups. These findings were confirmed by qRT-PCR analysis (Fig. 4b), showing no significant differences regarding *SCX* expression between the two groups.

**Osteogenic and adipogenic differentiation.** Monolayer cultures of inflamed and non-inflamed LHB tendon cells responded equivalently to osteogenic medium in terms

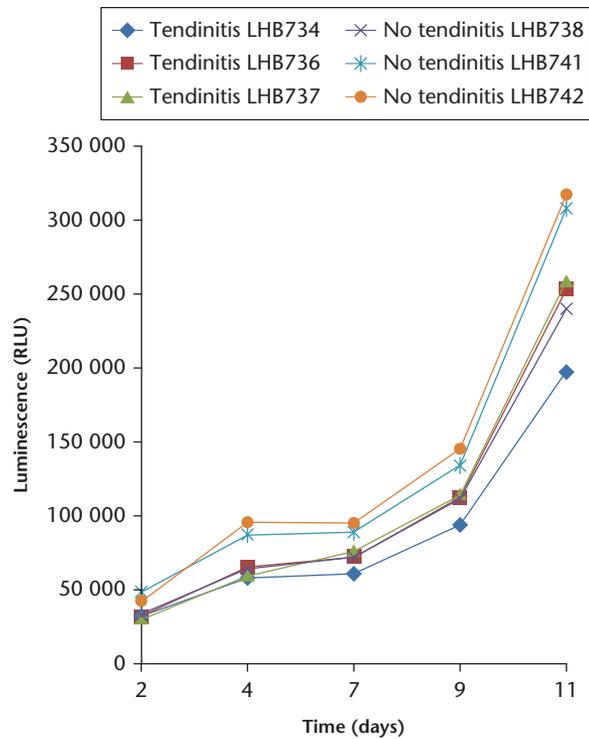


Fig. 3a

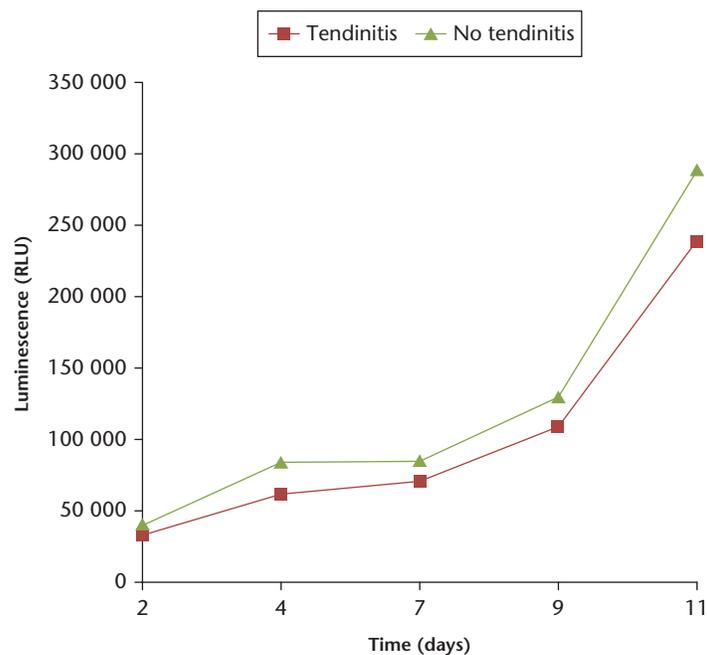
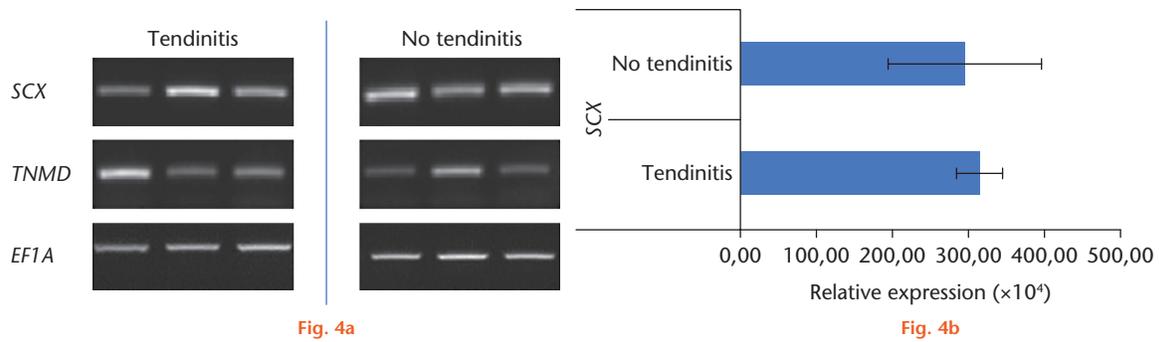


Fig. 3b

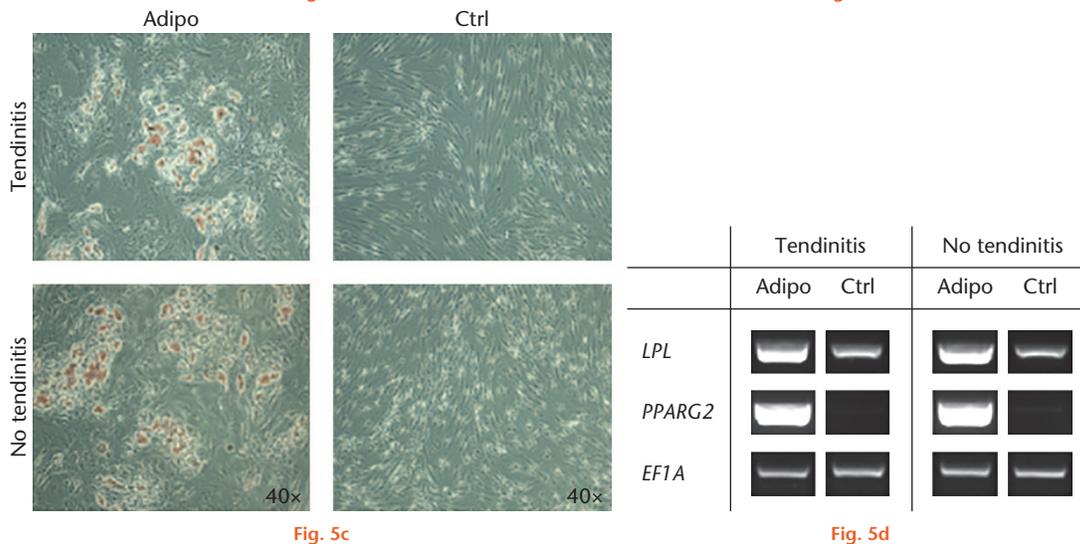
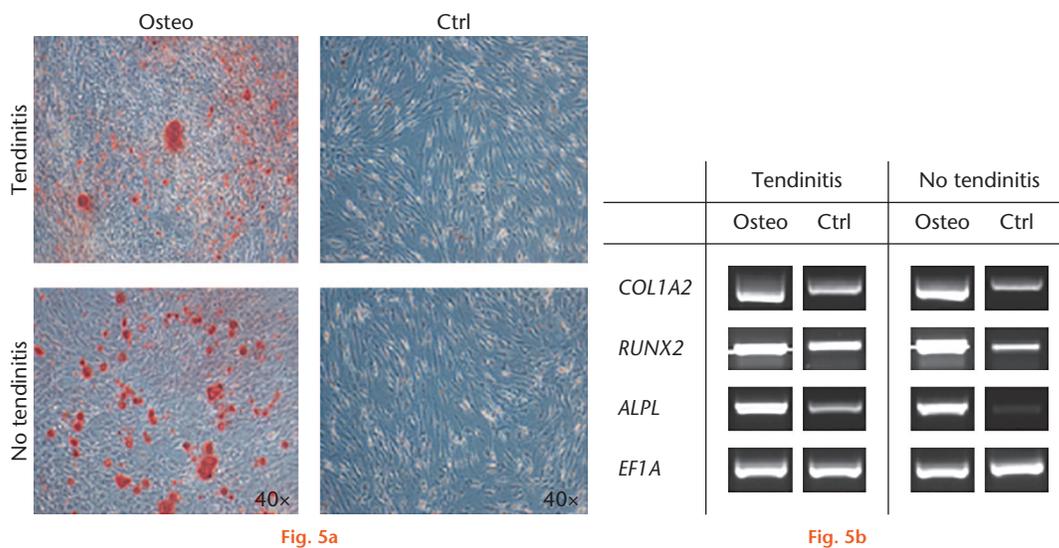
Cell proliferation behaviour of long head of the biceps (LHB) cells isolated from samples with and without tendinitis. a) Illustration of the values and sds of inflamed (LHB734/736/737) and non-inflamed (LHB738/741/742) tissue samples. b) Illustration of the means with sd. Proliferation of both cell types increased over time, but differences were not statistically significant. A total of six donors were included, and measurements were made in triplicates for each donor and timepoint.

of staining for mineralization with Alizarin Red (Fig. 5a), resulting in increased areas of positive staining (Fig. 5a) in the respective osteogenic cultures of the two cell types compared with controls. This corresponds to the induction of transcripts encoding for *ALPL* and *RUNX2*, as well as the increased expression of *COL1A2*, in all osteogenic

cultures compared with controls (Fig. 5b). Monolayer cultures of both groups showed a similar response to adipogenic conditions, as assessed by staining with Oil red O (Fig. 5c). Equivalently, adipogenic culture conditions induced an overexpression of *LPL* and *PPARG2* compared with controls within all groups (Fig. 5d).



a) Real-time-polymerase chain reaction (RT-PCR) and b) quantitative real-time-polymerase chain reaction (qRT-PCR) analyses of inflamed and non-inflamed tendon samples. Expression patterns of scleraxis (*SCX*) and tenomodulin (*TNMD*) in long head of the biceps (LHB) samples with and without tendinitis. For RT-PCR, elongation factor 1 $\alpha$  (*EF1A*), and for qRT-PCR, ribosomal protein S27a (*RPS27A*) served as housekeeping genes for normalization of the expression values. A total of six donors were included.



Osteogenic and adipogenic differentiation of cells isolated from inflamed and non-inflamed long head of the biceps (LHB) samples at four weeks. a) Cultures treated with osteogenic supplements produced a mineralized extracellular matrix as shown by intense staining for Alizarin Red. Control cultures did not produce a mineralized matrix. b) Real-time polymerase chain reaction (RT-PCR) revealed that cultures of both groups expressed the runt-related transcription factor 2 (*RUNX2*), alkaline phosphatase (*ALPL*), and collagen type 1 alpha 2 (*COL1A2*) in response to osteogenic stimuli compared with controls. c) Cells cultivated in adipogenic medium showed formation of lipid droplets in both groups as determined by Oil red O staining. Cultivation in control medium, in contrast, did not result in droplet enrichment. d) Expression of the adipogenic marker genes lipoprotein lipase (*LPL*) and peroxisome proliferator-activated receptor gamma 2 (*PPARG2*) was increased in both groups treated with adipogenic medium, but was not detectable in cells cultivated with control medium. The housekeeping gene elongation factor 1 $\alpha$  (*EF1A*) showed equal expression levels in all groups. Six donor samples were tested, and representative images from three different donors are shown. Osteo, osteogenic; Adipo, adipogenic; Ctrl, control.

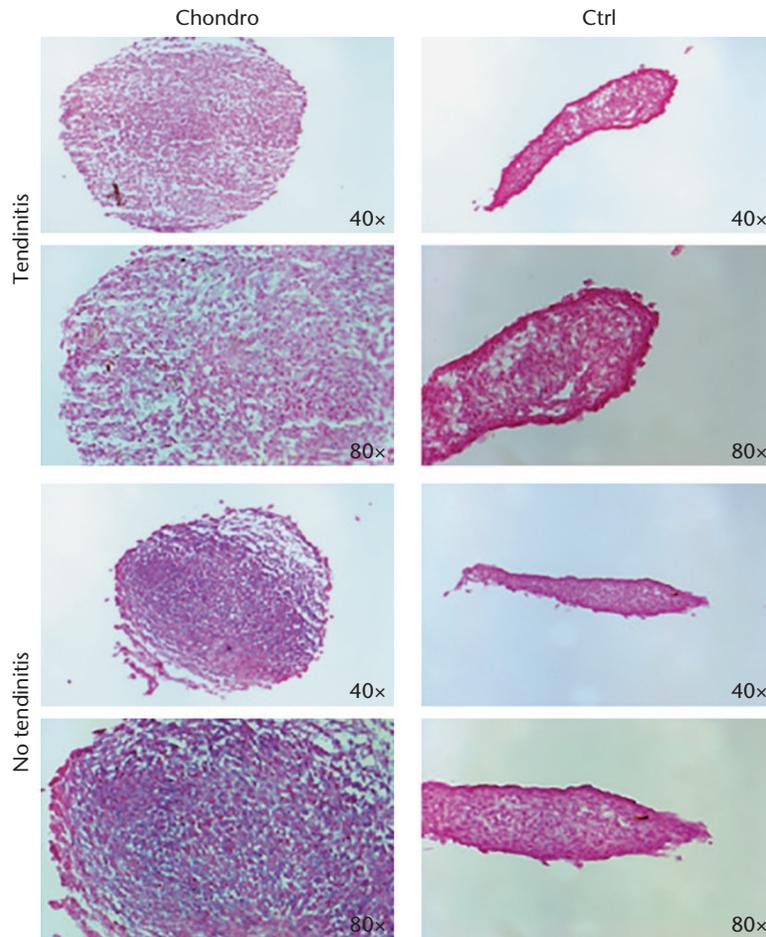


Fig. 6a

	Tendinitis		No tendinitis	
	Chondro	Ctrl	Chondro	Ctrl
ACAN				
COL2A1				
COMP				
EF1A				

Fig. 6b

Chondrogenic differentiation by cell cultures isolated from inflamed and non-inflamed tendon samples. After four weeks in chondrogenic medium, aggregates derived from both groups were stained with Alcian Blue. a) Unlike controls, aggregates treated with transforming growth factor beta 1 (*TGFB1*) populations produced an extracellular matrix rich in sulphate. b) Aggregate cultures of the two cell types collected at three weeks expressed the cartilage-specific genes encoding collagen type 2 (*COL2A1*) and aggrecan core protein (*ACAN*) in response to *TGFB1* treatment, in contrast with controls lacking *TGFB1*. Control aggregates expressed cartilage oligomeric matrix protein (*COMP*) mRNA at low levels, but expression was markedly increased in the presence of *TGFB1*. The expression of elongation factor 1 $\alpha$  (*EF1A*) was included as an internal control for RNA loading. Results are presented using representative patient populations from at least three independent experiments at four weeks. Six donor samples were tested, and representative images from three different donors are shown. Chondro, chondrogenic; Ctrl, control.

**Chondrogenic differentiation.** When placed into pellet culture, both inflamed and non-inflamed LHB-derived stem cells underwent chondrogenic differentiation in the presence, but not absence, of transforming growth factor beta 1 (*TGFB1*) as judged by histology (Fig. 6a) and

by the expression of transcripts encoding *COMP*, *ACAN*, and *COL2A1* (Fig. 6b). Pellets formed by the two different groups showed no differences in staining for proteoglycans with Alcian Blue. However, chondrogenic pellets from both cell types produced more glycosaminoglycans

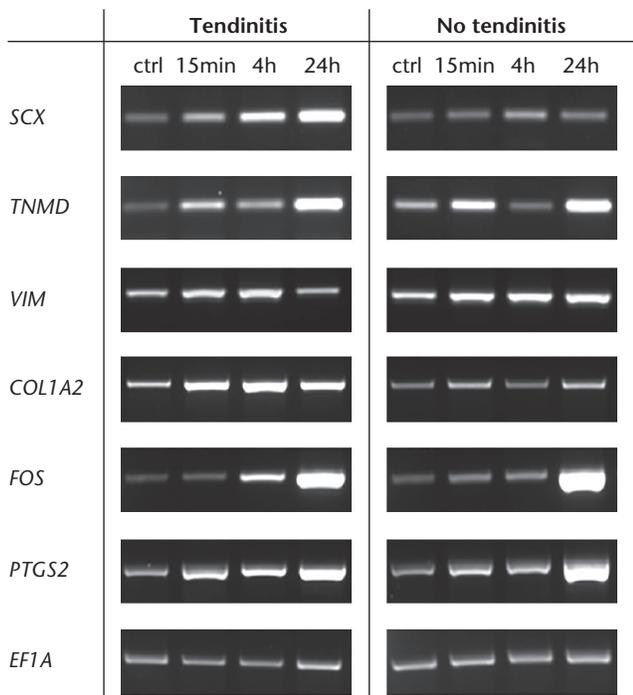


Fig. 7

Tenogenic differentiation of cells isolated from inflamed and non-inflamed tendon samples. Real-time polymerase chain reaction (RT-PCR) analysis after cyclic stretching of cells isolated from inflamed and non-inflamed long head of the biceps (LHB) samples for 15 minutes, four hours, and 24 hours. After cyclic stretching, cells of the two groups showed a time-dependent increase in the expression of both tenogenic and mechanoresponsive genes. Tendogenesis-related genes including scleraxis (*SCX*), tenomodulin (*TNMD*), vimentin (*VIM*), and collagen type 1 alpha 2 (*COL1A2*) were examined, while prostaglandin-endoperoxide synthase 2 (*PTGS2*) and Fos proto-oncogene, AP-1 transcription factor subunit (*FOS*) served for the analysis of mechanosensitivity. The expression of elongation factor 1 $\alpha$  (*EF1A*) was included as an internal control for RNA loading. A total of six donors were included.

(GAG) compared with non-chondrogenic controls, as can be seen in Figure 6a. Consistent with this, chondrogenic pellets from both cell types produced significantly more mRNA transcripts encoding *COMP* and induced an overexpression of *ACAN* as well as *COL2A1* compared with respective non-chondrogenic controls (Fig. 6b).

**Tenogenic differentiation.** Undergoing cyclic stretching for 15 minutes, four hours, and for 24 hours, cells from the two different groups showed a time-dependent overexpression of tendogenesis-related genes such as *SCX*, *VIM*, and *TNMD*, as well as mechanoresponsive mRNA transcripts such as *PTGS2* and *FOS* (Fig. 7).<sup>20,21</sup> Both the tendinitis and no tendinitis groups showed an increasing expression of the examined genes at the three different timepoints after cyclic loading.

## Discussion

This study confirms that human LHB samples have multilineage potential in the inflamed, as well as non-inflamed state, and share several characteristics with mesenchymal stem cells (MSCs) as previously isolated from tendon, muscle, ligament, or bursa tissue.<sup>13,16,22-26</sup> Up-to-date

clinical outcomes after massive RC tears are still unsatisfying and the development of stem cell-based therapies could be an option in the future.<sup>27</sup> In general, LHB-derived stem cells (LHBSCs) may be a new, endogenous, patient-specific tendon-derived stem cell source for tissue regeneration. With the present investigation, we report that cells from the LHB can be easily isolated, cultured, expanded, and differentiated in chondrocytes, adipocytes, and osteocytes *in vitro*. Interestingly, we showed that cells isolated from inflamed and non-inflamed LHBs significantly overexpressed both tenogenic and mechanoresponsive marker genes after cyclic loading for 24 hours. This endorses the *in vivo* results by Zumstein et al<sup>28</sup> that tendon regeneration is facilitated by mechanical stimulation. Randelli et al<sup>5</sup> showed the multipotency of the LHBSCs by inducing differentiation of these cells towards osteoblasts, adipocytes, and skeletal muscle, as well as MSC-specific surface antigen expression, thus defining them as a new MSC source. However, it is not clear whether inflamed samples were used in this study. We showed that inflamed samples do not present a significant difference in cell yield, differentiation potential, or proliferation rate compared with LHBSCs harvested from native LHB tendons, which means that both groups are a suitable stem cell source. In the future, tendon stem cells could be collected directly *in situ* at the same time as shoulder surgery, without requiring additional invasive procedures for cell harvesting. Recent human *in vivo* studies that applied MSCs harvested from the iliac crest showed promising results. Hernigou et al<sup>29</sup> performed augmentation by injecting a mean 51 000 MSCs (SD 25 000) into the tendon-to-bone junction and in the bone at the site of the footprint after arthroscopic RC repair. At ten-year MRI follow-up, intact RCs were found in 39 of the 45 patients (87%) in the MSC treatment group, but in just 20 of the 45 patients (44%) in the control group.<sup>29</sup> Ellera Gomes et al<sup>30</sup> injected a mean of  $5 \times 10^6$  MSCs per tendon sample into the tendon-to-bone junction and the footprint of 14 patients undergoing open RC repair. The two-year MRI follow-up revealed intact RC muscles in 13 of the 14 patients (93%), however, the study did not include a control group.<sup>30</sup> Taking into account the mean cell yield per tendon sample in our study ( $6.8 \times 10^6$  cells per sample), the LHB seems to be a viable cell source for regenerative approaches. Nevertheless, randomized controlled trials and animal studies with histological analysis of the tendon-to-bone junction are still necessary to prove positive effects on RC healing of this procedure, and further studies are required to determine if cell-based strategies are sufficient. Another future study could be the use of matrix- or scaffold-based delivery of multipotent stem cells such as the LHBSCs for RC augmentation or even for irreparable RC tears. Chen et al<sup>31</sup> and Funakoshi et al<sup>32</sup> showed the effectiveness of tenocyte-seeded scaffolds regarding histological healing potential and biomechanical strength in rabbit RC models. To

further increase regenerative potential, combination with growth factors could be an option. However, the transfer from bench to bedside is challenging and these promising findings should be implemented in clinical trials in the future.

At present, only a few genes have been reported as general tendon markers. Recently, SCX has been reported as an important marker of tendon progenitor populations and tenocytes, promoting tendon development and differentiation.<sup>33</sup> Shukunami et al<sup>34</sup> were able to show that SCX positively regulates the expression of *TNMD*, a differentiation marker of tenocytes. Mice lacking *TNMD* display a severe decrease in tenocyte proliferation in newborn tendons and a disrupted adult collagen fibril structure.<sup>22</sup> In our study, we found no significant difference in the expression pattern of *TNMD* and SCX in the tendinitis and no tendinitis groups, therefore we conclude that the ongoing inflammatory processes do not negatively affect the tenogenic regeneration potential of cells isolated from inflamed LHBs. However, more samples are required in order to determine any subtle differences in expression between the test conditions.

Today, very little is known about the regenerative capacity of inflamed tissues in musculoskeletal pathologies. Therefore, in previous studies, we analyzed the regenerative capacity of cells derived from inflamed subacromial bursa tissue of the shoulder relative to bone marrow stromal cells, and found that both cell types revealed the main characteristics of MSCs, including their capacity to proliferate and differentiate into cartilage, fat, bone, and tendon under appropriate *in vitro* culture conditions.<sup>13</sup> Notably, the tissue sources were different in this study, and it is currently unknown whether stem cells isolated from the LHB tendon can be used effectively to augment tissue repair *in vivo*; cells might behave differently under defined *in vitro* conditions as when they are incorporated in inflamed tissue i.e. in a potential catabolic environment. Similar results were found when investigating the MSC characteristics from anterior cruciate ligament (ACL) cells, which were either isolated from the traumatically ruptured ACLs of young donors (18 to 35 years) or from degeneratively altered ACLs from older patients (45 to 67 years) with knee osteoarthritis, with almost equipotent MSC capacities between both cell types.<sup>16</sup> Although the tissue source was similar in this work (ACL), the influence of inflammation on the MSCs and presumably on their regenerative capacity, remains uncertain. As an inflammatory component is regularly present, both after trauma and in a degenerative setting as part of the respective disease processes, phenomena frequently overlap.<sup>35</sup> Interestingly, an inflammatory phase is also seen during regeneration and wound healing in almost every tissue of the body. Therefore, the role of the inflammatory response in the repair processes of mesenchymal tissue is of great interest. However, it is not yet

well characterized so the question of whether tissue inflammation during the regenerative process constitutes friend or foe is still a matter of controversy and of great debate among clinicians and basic scientists.<sup>36</sup>

A limitation of this study is the fact that the patient population was not matched regarding sex, whereas it has been demonstrated that the sex, as well as the age,<sup>37</sup> of the donors<sup>38</sup> can affect stem cell proliferation. Since only small central tissue pieces from each sample were used for histological confirmation of the treatment groups, there was not enough material to perform all of the tests on all samples, which is another limitation of this study.

In conclusion, being a source of anterior shoulder pain, the LHB tendon is often resected during shoulder surgery and could therefore be used as a possible cell source for tissue-engineering approaches. In this study, the LHB with and without tendinitis has been characterized as a suitable, novel source of multipotent tendon-derived cells. However, it remains unclear whether such cell types might be suitable in the treatment of degeneration and induction of regeneration in shoulder surgery.

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