

CHARACTERIZATION OF TENDON CELL CULTURES OF THE HUMAN ROTATOR CUFF

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Abstract

Rotator cuff tears are common soft tissue injuries of the musculoskeletal system that heal by formation of repair tissue and may lead to high re-rupture rates and joint dysfunction. In particular, tissue from chronic, large tendon tears is of such degenerative nature that it may be prone to re-rupture after surgical repair. Besides several biomechanical approaches, biologically based strategies such as application of growth factors may be promising for increasing cell activity and production of extracellular tendon matrix at the tendon-to-bone unit. As a precondition for subsequent experimental growth factor application, the aim of the present study was to establish and characterize a human rotator cuff tendon cell culture.

Long head biceps (LHB)- and *supraspinatus* muscle (SSP)- tendon samples from donor patients undergoing shoulder surgery were cultivated and examined at the RNA level for expression of collagen type-I, -II and -III, biglycan, decorin, tenascin-C, aggrecan, osteocalcin, tenomodulin and scleraxis (by Real-time PCR). Finally, results were compared to chondrocytes and osteoblasts as control cells.

An expression pattern was found which may reflect a human rotator cuff tenocyte-like cell culture. Both SSP and LHB tenocyte-like cells differed from chondrocyte cell cultures in terms of reduced expression of collagen type-II ($p \leq 0.05$) and decorin while higher levels of collagen type-I were seen ($p \leq 0.05$). With respect to osteoblasts, tenocyte-like cells expressed lower levels of osteocalcin ($p \leq 0.05$) as well as tenascin C, biglycan and collagen type-III. Expression of scleraxis, tenomodulin and aggrecan was similar between all cell types.

This study represents a characterization of tenocyte-like cells from the human rotator cuff as close as possible. It helps analyzing their biological properties and allows further studies to improve production of tendon matrix and osteofibroblastic integration at the tendon-bone unit following tendon repair.

Keywords: Rotator cuff, tendon healing, tenocyte, tendon-like cells, cell culture, tendon-bone.

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Introduction

Rotator cuff tendon injuries cause pain and disability. Although surgical approaches may provide good and excellent results in many patients (Severud *et al.*, 2003; Wolf *et al.*, 2004; Baysal *et al.*, 2005), the main complication remains non-healing or re-rupture of the tendon from its humeral footprint. Re-ruptures occur in up to 94% of cases and depend on several variable issues such as demographic factors, size of the initial tear, biologic characteristics (fatty infiltration, tendon tissue quality) and biomechanical aspects of the chosen surgical technique (Boileau *et al.*, 2005; Bishop *et al.*, 2006; Cole *et al.*, 2007). The natural history of re-ruptures or recurrent defects remains unknown. The therapeutic aim of rotator cuff surgery represents a secure and durable tendon-to-bone regeneration.

To improve outcome results, *biomechanical* approaches for new surgical techniques have led to increased use of strong synthetic materials in order to reattach the torn tendon stump (Mahar *et al.*, 2007; Lorbach *et al.*, 2008; Pauly *et al.*, 2010). However, the assumption of tissue damage and tendon strangulation through strong tissue adaptation to the bony insertion is possible and might even deteriorate regeneration (Gerber *et al.*, 1994; Flatow, 1996).

Biologically based strategies to stimulate cell activity and production of extracellular tendon matrix proteins have received increasing attention (Maffulli *et al.*, 2002; Hsu and Chang, 2004). Since tenocytes exhibit very low proliferative capacity (Chuen *et al.*, 2004), one possible approach for improved tendon healing is to assess their biological activity under the influence of growth factors. The latter are considered to accelerate ligament and tendon-to-bone healing (Molloy *et al.*, 2003; Hsu and Chang, 2004; Angel *et al.*, 2006) by increasing fibroblast proliferation, the synthesis of extracellular matrix (ECM) proteins, and revascularization (Lee *et al.*, 1995; Batten *et al.*, 1996; Marui *et al.*, 1997; Thomopoulos *et al.*, 2007; Chang *et al.*, 2000; Takahashi *et al.*, 2002).

Tendon cell cultures represent a precondition for studies on their biology and influence of growth factors. Hence, numerous studies have been carried out on tendon cell cultures from tissue samples of human or animal origin that were subsequently studied. However, many studies lack a specific characterization of the obtained cell cultures. Reviewing the current literature, the presence of tenocytes was assumed by phenotypic criteria or proof of collagen type-I (COL-I) (Thomopoulos *et al.*, 2005; Costa *et al.*, 2006; Anitua *et al.*, 2006; Mazzocca *et al.*, 2007) which cannot be considered tendon specific. Other studies furthermore assessed COL-III (Maffulli *et al.*,

2000; Takahasih *et al.*, 2002; Yang *et al.*, 2004; de Mos *et al.*, 2008) or proteins such as decorin and biglycan (Kardestuncer *et al.*, 2006; Yao *et al.*, 2006; Rios *et al.*, 2007; Fu *et al.*, 2008) which are non-specific tendon proteoglycans (Rees *et al.*, 2000; Yoon and Halper, 2005) and are initially increased after experimental tendon detachment (in rodents) (Yokota *et al.*, 2005).

The possibly tendon specific gene Scleraxis (Schweitzer *et al.*, 2001; Edom-Vovard *et al.*, 2002) was described for characterization of cells isolated from human or rodent tendon (Salincarnboriboon *et al.*, 2003; Schulze-Tanzil *et al.*, 2004). Only few studies looked into characterization more detailed and described the expression of several markers such as COL-I, -III, decorin, COMP, aggrecan, elastin and scleraxis (Stoll *et al.*, 2010) or COL-I, -II, scleraxis, fibronectin, proteoglycan and β 1-integrin (Schulze-Tanzil *et al.*, 2004).

In the present approach, cells were isolated from human rotator cuff tissue samples, cultivated and subsequently screened for several markers. These markers were COL-I (main component of extracellular tendon matrix (Blevins *et al.*, 1997; Kannus, 2000)), COL-III (increased in early tendon healing (Takahasih *et al.*, 2002) and experimentally in chronic tears (Yokota *et al.*, 2005)), decorin (most important proteoglycan in tendon (Rahaman and Mao, 2005; Yoon and Halper, 2005)) and biglycan (a proteoglycan present in tendon, cartilage and bone (Yoon and Halper, 2005)), aggrecan (a proteoglycan, present in cartilage as well as in compressed tendon regions (Rees *et al.*, 2000), and tenascin C (a glycoprotein, part of tendon cell adaptation to compression (Martin *et al.*, 2003)).

Cultures were furthermore screened for scleraxis (a transcription factor and possibly a tendon specific marker (Schweitzer *et al.*, 2001)), tenomodulin (a transmembrane glycoprotein, possibly another tendon-specific marker (Jelinsky *et al.*, 2010)), COL-II (cartilage specific) and osteocalcin (the major non-collagen protein in the bone matrix, bone-specific), to separate the tenocyte-like cells from other cell types of the musculoskeletal tissues.

The aim of the present study was to establish and characterize a human rotator cuff tenocyte-like cell culture in order to allow subsequent systematic studies on their biological response to growth factors.

Materials and Methods

Tendon material, donor patient demographics

Tendon samples were obtained from patients undergoing arthroscopic surgery for rotator cuff repair or open shoulder surgery such as hemiarthroplasty following humeral head fractures. Prior to biopsy, all patients gave their written informed consent. Tissue samples from the supraspinatus muscle (SSP, n=11) and the tendon of the long head of the biceps muscle (LHB, intraarticular aspect, n=7) were obtained.

Patient demographics and clinical data

SSP tenocyte-like cells (n=11 samples: 8 male, 3 female, mean age 63.7 yrs, range 32-85yrs):

- Tendon retraction according to Patte: grade 0-3 (Patte, 1990)
- Fatty infiltration of the SSP muscle: grade I-III according to Goutallier (Goutallier *et al.*, 1994)
- Tear size according to Bateman: stage I-IV (Bayne and Bateman, 1984).

LHB tenocyte-like cells (n=7 samples: 5 male, 2 female, mean age 59.3yrs, range 23-79 yrs).

Exclusion criteria for LHB-samples were macroscopic evidence of chronic inflammation/tendinitis. LHB-samples were obtained during hemiarthroplasty or tenotomy/tenodesis following a failed SLAP-repair. 2 LHB-biopsies were obtained during repair/-debridement of concomitant (massive) rotator cuff and pulley-defects.

All biopsies were obtained 3-5 mm from the torn proximal tendon edge.

Tendon cell cultures:

Cells were isolated from tissue samples by washing several times with phosphate buffered saline (PBS) +1% penicillin/streptomycin, dissection into small pieces and incubation with 0.3% collagenase type CLS II in PBS with Ca/Mg for 2 hours at 37°C. The digested tendon material was then placed in a tissue culture flask and cultured in DMEM / HAM's F12 (1:1) supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin.

Cells were incubated at 37°C, 95% humidity and 5% carbon dioxide with a change of medium every 2-3 days. As soon as cultured cells reached 80-90% confluence, they were treated with 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) and subcultured at a density of $1-1.5 \times 10^4$ cells/ml, maximum until passage 3 (about 8 weeks).

Chondrocyte and osteoblast cultures

Primary human chondrocytes (n=3) and osteoblasts (n=3) served as control for marker expression and immunocytochemical staining. All patients gave their written informed consent prior to cell culture.

Primary human chondrocyte cultures were kindly provided by Dr. Ringe, Department of Tissue Engineering, Berlin-Brandenburg Centre for Regenerative Therapies. Chondrocytes were cultured in DMEM / HAM's F12 (1:1) supplemented with 10% FCS and 1% penicillin/streptomycin under the same conditions as described above. Chondrocytes were used for experiments at early passages (up to passage 2) to avoid dedifferentiation.

Primary human osteoblasts were isolated as described previously (Robey and Termine, 1985). The osteoblasts were cultured in MEM EARLE / HAM's F12 supplemented with 10% FCS, 1% penicillin/streptomycin, 0.05 mM β -glycerophosphate and 0.05 mM L-ascorbate-2-Phosphate under the same conditions as described for tenocytes. Osteoblasts were used up to passage 4.

All cell culture media and supplements were obtained from Biochrom AG, Berlin, Germany.

Real-time Polymerase Chain Reaction (RT-PCR)

RNA from about $1-2 \times 10^5$ cells of the tenocyte-like cells, chondrocytes and osteoblasts was extracted with the

Table 1. Primer sequences of target genes for RT-PCR.

Target Gene	Product size	Annealing Temperature	Sequence
GAPDH (TIB Molbiol)	111 bp	59.9°C-66°C	Forward: 5' CCATGAGAAGTATGACAACAGCC 3' Reverse: 5' CCTTCCACGATACCAAAGTTG 3'
Aggrecan (Neumann <i>et al.</i> , 2007)	146 bp	64.2°C	Forward: 5' CCAGTGCACAGAGGGGTTT 3' Reverse: 5' TCCGAGGGTGCCGTGAG 3'
Biglycan (Rees <i>et al.</i> , 2000)	165 bp	64.2°C	Forward: 5' AGGCCCTCGTCCTGGTGAACA 3' Reverse: 5' GGATGCGGTTGTCGTGGATGC 3'
Decorin (Primer 3)	205 bp	64.2°C	Forward: 5' CGCCTCATCTGAGGGAGCTT 3' Reverse: 5' TACTGGACCGGGTTGCTGAA 3'
COL-I (Primer 3)	197 bp	64.2°C	Forward: 5' TGACCTCAAGATGTGCCACT 3' Reverse: 5' ACCAGACATGCCTCTTGCC-3'
COL-II (Primer 3)	162 bp	66°C	Forward: 5' CGCACCTGCAGAGACCTGAA 3' Reverse: 5' TCTTCTTGGGAACGTTTGCTGG 3'
COL-III (Primer 3)	199 bp	64.2°C	Forward: 5' GCTGGCATCAAAGGACATCG 3' Reverse: 5' TGTTACCTCGAGGCCCTGGT 3'
Osteocalcin (Primer 3)	209 bp	64.2°C	Forward: 5' CCCAGGCGCTACCTGTATCAA 3' Reverse: 5' CTGGAGAGGAGCAGAACTGG 3'
Tenascin C (Primer 3)	223 bp	64.2°C	Forward: 5' TTCCTGGAGCTGACTGTGG 3' Reverse: 5' TAGGGCAGCTCATGTCACTG 3'
Tenomodulin (Itaya <i>et al.</i> , 2009)	170 bp	59.9°C	Forward: 5' TTGAAGACCCACGAAGTAGA 3' Reverse: 5' ATGACATGGAGCACACTTTC 3'
Scleraxis	85 bp	59.9°C	Quiagen (QuantiTect Primer Assay Kit SCXB, commercial product - no sequence available)

RNeasy Mini Kit (Quiagen, Hilden, Germany) following the manufacturer's manual and then reverse transcribed into cDNA. RNA concentration was measured with the Nanodrop ND-1000 Spectrophotometer (PeqLab, Erlangen, Germany). A thermocycler (Eppendorf, Hamburg, Germany) was used for cDNA synthesis.

100 ng of RNA, 1 µL 10 mM Deoxynucleotide-triphosphate (dNTP)-mix (Invitrogen, Darmstadt, Germany) and 1 µL Randomprimer (Invitrogen) were filled up to 10 µL with *aqua ad iniectionis* (Braun, Germany) and incubated for 5 minutes at 65°C. After incubation on ice 9.5 µL reaction-mix consisting of 4 µL M-MLV RT 5x Reactionbuffer (Promega, Mannheim, Germany), 1 µL RNase-inhibitor (40 U/µL, Promega) und 4.5 µL *aqua ad iniectionis* were added and incubated for 2 minutes at 42°C. 1 µL M-MVL RT(H-) (200 U/µL, Promega) was added and incubated for 50 minutes at 42°C, then samples were cooled down and 1 µL RNase H (1.5 U/µL, Promega) was added and incubated for 20 minutes at 37°C.

The following primers were used for RT-PCR: GAPDH (housekeeping gene, serving as reference), aggrecan, biglycan, COL-I, -II, -III, decorin, osteocalcin, scleraxis, tenascin C, tenomodulin. Most primer sequences were designed using the Primer 3 software, some sequences were adapted from other publications (Table 1). All primers, except Scleraxis, were produced by Tib Molbiol, Berlin, Germany.

The RT-PCR was performed with the iCycler iQ5 RT-PCR System and the Bio-Rad iQ5 software (Bio-Rad Laboratories, Munich, Germany). The cDNA was diluted 1:10 and 2 µL were pipetted at each well as PCR Template. The mastermix was prepared with the following components for one well: 12.5 µL iQSupermix (Bio-Rad),

1 µL primermix (10 µM, forward and reverse Primer 1:1), 1 µL SYBR Green (Invitrogen) and 8.5 µL *aqua ad iniectionis*. 23 µL of the mastermix were added to each well. The following RT-PCR protocol was used for the amplification: denaturation program (95°C for 3 minutes) amplification program was repeated for 40 cycles (94°C for 30 s, 59.9°C/64.2°C/66°C for 30 s, 72°C for 30 s), melting curve program (55-95°C with a temperature change of 0.5°C holding for 30 s) and finally a cooling step at 15°C. The RT-PCR results were analysed with the Bio-Rad iQ5 Software by calculating the ratio of target gene *versus* GAPDH gene.

Immunochemical Scleraxis staining

The tenocyte-like cells, chondrocytes and osteoblasts were seeded into 4-well chamber slides with a concentration of 2x10⁴ vital cells/ml (1 mL/well), until they reached 80% confluence. The cells were fixed for 5 minutes at room temperature in 3.7% formaldehyde in PBS. The cells were permeabilized using 1% Triton-X-100 in PBS for 15 minutes at room temperature. After incubation with normal goat serum (Vector Labs, Loerrach, Germany), the cells were stained with primary antibodies for Scleraxis (rabbit polyclonal anti SCXA, 4 µg/mL, Abcam, Cambridge, MA, USA) overnight at 4°C. Negative control was prepared by omitting the primary antibody. The cells were washed with PBS and the biotinylated anti-rabbit IgG antibody was used and subsequently incubated with the Vectastain ABC AP Kit (Vector Labs) for 50 minutes. Cells were then incubated with chromogen buffer (pH 8.2) for 10 minutes. The Vector Red Alkaline Phosphatase Substrate Kit (Vector Labs) was used and the reaction developed under visual control. The reaction was stopped with PBS and few drops

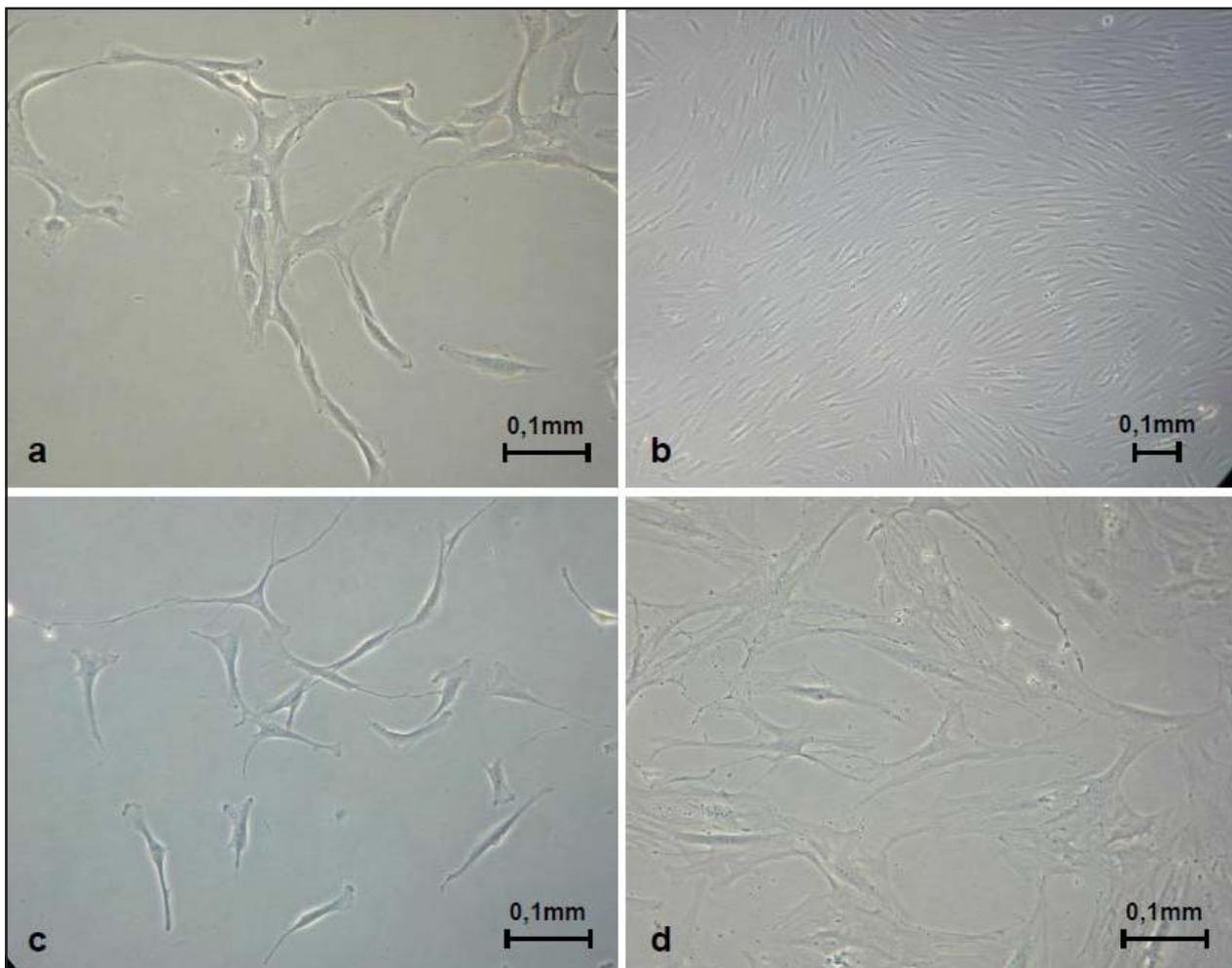


Fig. 1. Culture morphology of tenocyte-like cells from SSP tendon in monolayer culture. (a) chain formation of spindle shaped cells, Passage 0, 9 days after isolation, (b) colony formation, Passage 0, 17 days after isolation, (c) spindle shaped cells, Passage 1, 1 day after subcultivation, (d) cells with wing-like shape, Passage 1, 7 days after subcultivation. From passage 0 to 1, cells lost the appearance of colony formation and became larger.

of Kaisers Glycergelatine (Merck, Darmstadt, Germany) were added to the cells that were subsequently covered with a glass coverslip.

Statistics

Statistical analysis was performed using SPSS 14 for Windows (SPSS Inc, Chicago, IL, USA). The Kruskal Wallis test was used to analyze significant differences between all groups. The Mann-Whitney-U test was performed for non-parametric comparison of two groups. Level of significance was set up at $p \leq 0.05$. To adjust the α -Value, the Bonferroni-Holm-Correction was performed.

Results

Cell culture

Tenocyte-like cells proliferated to 80-90% confluence approximately within 14 days. The isolated cells exhibited a spindle shape and particularly built reticular colonies in cell chains (Fig. 1a, b). After subculture, the cells lost this appearance. Partially they became larger and built wing-like shapes (Fig. 1c, d).

RT-PCR

Analysis of different markers by RT-PCR demonstrated an obvious dissociation of tenocyte-like cells from chondrocytes and osteoblasts. In general, data suggest that tenocyte-like cells from either SSP or LHB origin have comparable expression patterns for all analysed markers.

COL-I and -III gene expression showed differences in tenocyte-like cells from SSP and LHB tendon compared to chondrocytes and osteoblasts.

The tenocyte-like cells isolated from the SSP and LHB tendons expressed COL-I in a comparable amount. The osteoblasts showed the highest COL-I expression compared to the other cell types. A very low COL-I expression was found for the chondrocytes, which was significantly below SSP ($p=0.016$) and LHB ($p=0.017$) tenocyte-like cells and osteoblasts ($p=0.05$) (Fig. 2a).

COL-III expression was detected in all investigated cell types and was highest in osteoblasts. However, expression was not differing significantly (Fig. 2b).

Proteoglycans and glycoproteins

A slight increase of decorin expression was observed for chondrocytes when compared to tenocyte-like cells and

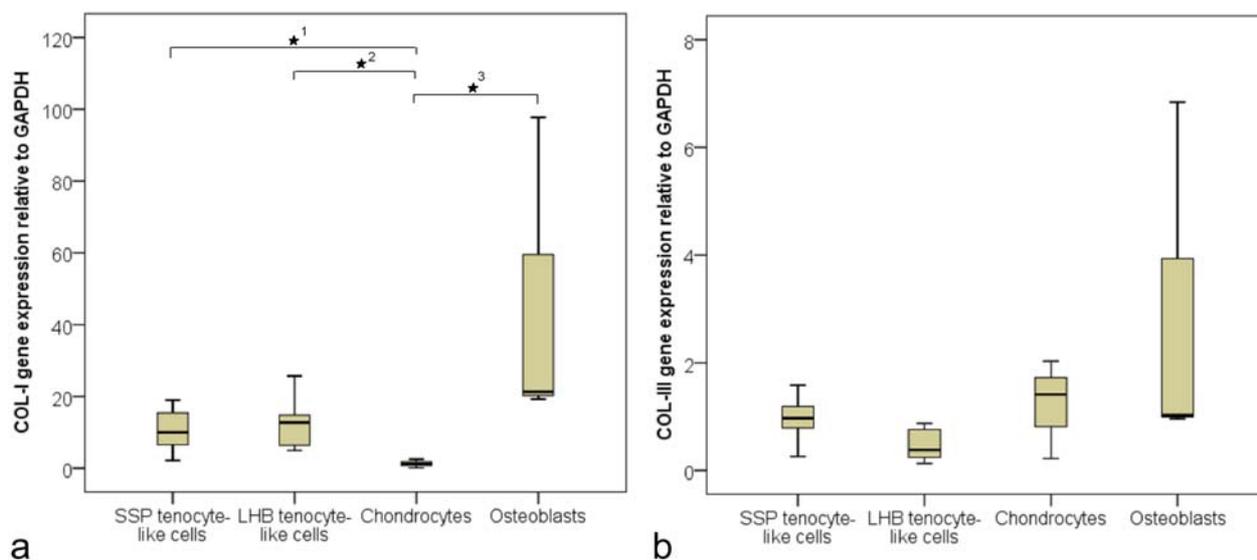


Fig. 2. Real-time quantitative polymerase chain reaction of collagens relative to GAPDH and respective tissue origin of cell cultures of SSP tenocyte-like cells (n=11), LHB tenocyte-like cells (n=7), chondrocytes (n=3) and osteoblasts (n=3); * $p \leq 0.05$; (a) COL-I expression of reference chondrocytes differed significantly to SSP tenocyte-like cells ($p^1=0.016$), LHB tenocyte-like cells ($p^2=0.017$) and osteoblasts ($p^3=0.05$); (b) COL-III expression showed no significant differences between the cells types. Osteoblasts served as reference group for statistical analysis.

to osteoblasts. Expression of decorin was similar between tenocyte-like cells and osteoblasts (Fig. 3a).

The expression of biglycan was comparable between chondrocytes and tenocyte-like cells. A marginal increase of its expression was found for osteoblasts (Fig. 3b). The difference was significant compared to SSP tenocyte-like cells after Mann-Whitney-U-Testing ($p=0.036$) but not when adjusted by Bonferroni-Holm-Correction.

Expression of aggrecan was slightly elevated in chondrocytes compared to tenocyte-like cells from LHB and SSP tendon as well as to osteoblasts ($p > 0.05$ each, Fig. 3c).

Osteoblasts showed higher expression of tenascin C compared to tenocyte-like cells and to chondrocytes ($p > 0.05$ each, Fig. 3d).

Cartilage, bone and tendon markers

A very low level of COL-II expression was measured for tenocyte-like cells and osteoblasts. Its expression in chondrocytes was significantly higher compared to the other cell types (SSP: $p=0.01$; LHB: $p=0.014$; osteoblasts: $p=0.046$, Fig. 4a)

Regarding bone markers, osteocalcin showed significantly higher expression in osteoblasts when compared to SSP tenocyte-like cells ($p=0.009$), LHB tenocyte-like cells ($p=0.017$) and chondrocytes ($p=0.05$) (Fig. 4b).

Scleraxis expression was low in all three cell types and no significant differences were observed (Fig. 4c).

Expression of tenomodulin was very low and almost similar in all cell types without significant differences (Fig. 4d).

In summary, both SSP and LHB tenocyte-like cells differed from chondrocyte cell cultures in terms of significantly reduced expression of COL-II and lower decorin expression while significantly higher COL-I-levels were seen.

With respect to osteoblasts, tenocyte-like cells expressed significantly lower levels of osteocalcin as well as reduced tenascin C, biglycan and collagen type-III.

Immunocytochemical scleraxis staining

In order to confirm RT-PCR results for the possible tendon specific marker scleraxis, SSP tenocyte-like cells, chondrocytes and osteoblasts were immunocytochemically stained for scleraxis. The staining showed high positive findings in all cell types. No visible distinctions in intensity between the cell types could be detected (Fig. 5 a-c). For the negative control cells, no staining could be detected (Fig. 5b, data of negative osteoblast and chondrocyte control not shown).

Discussion

Following surgical rotator cuff repairs, re-tear or non-healing of the tendon is a relevant clinical problem (Boileau *et al.*, 2005; Cole *et al.*, 2007; Lafosse *et al.*, 2007). Besides biomechanical improvements (Lorbach *et al.*, 2008; Mahar *et al.*, 2007; Pauly *et al.*, 2010), recent approaches have suggested an influence of growth factors on tendon cell behaviour and their potential value in rotator cuff repair.

Numerous studies on cell cultures from human tendon tissue were carried out (Table 2). Few of these surveys focus on cells from human rotator cuff tissue (Gumina *et al.*, 2009; Takahasih *et al.*, 2002; Tempfer *et al.*, 2009). However, many of these studies lack characterizations that allow the specific assumption of tendon cells. In particular, phenotypic assessment or proof of different collagen-types (Gumina *et al.*, 2009; Li *et al.*, 2008; Maffulli *et al.*, 2000; Takahasih *et al.*, 2002; Yang *et al.*, 2004) are not precise enough as these are expressed in various fibroblast-dependent connective tissues. A similar situation is the

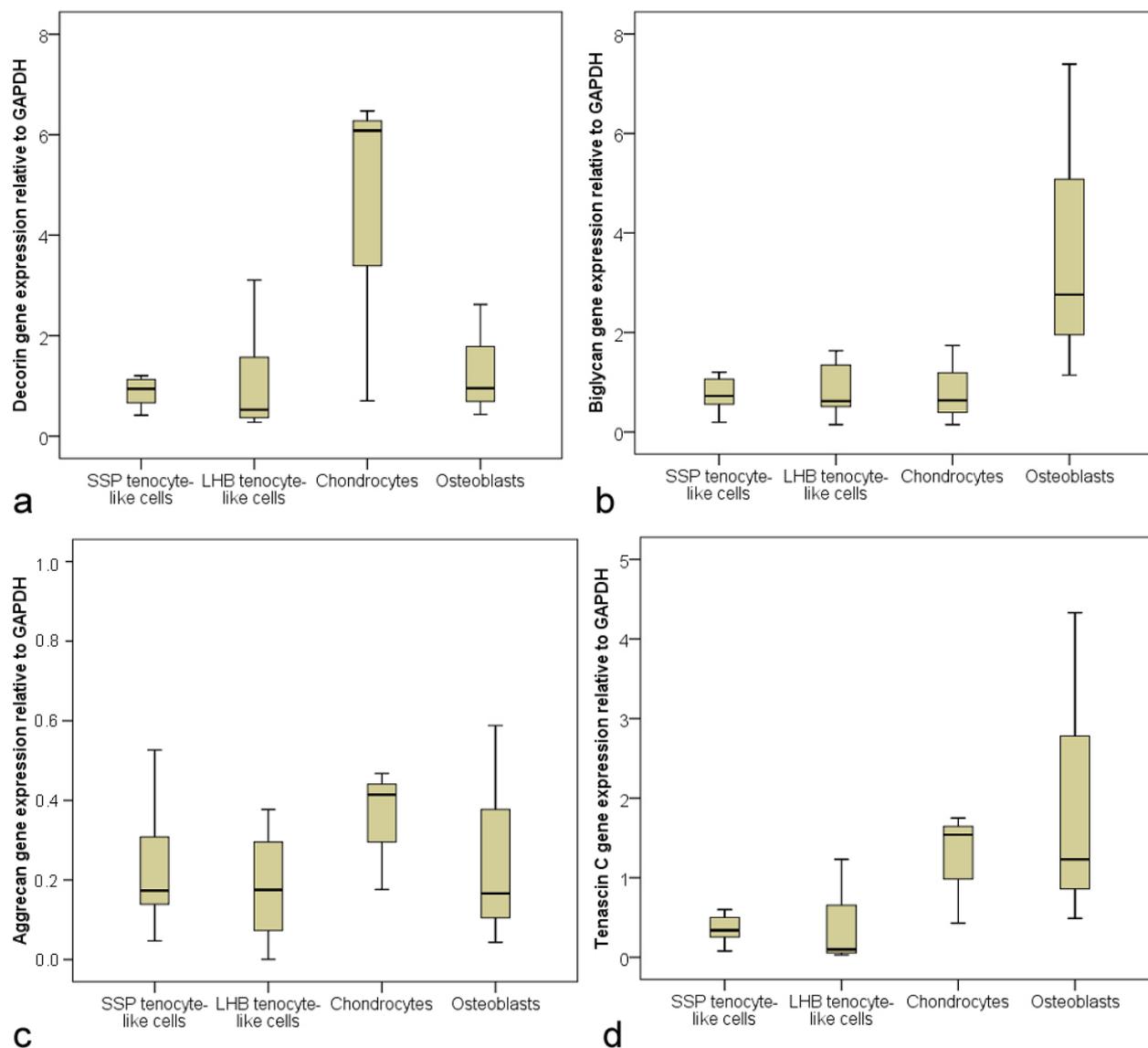


Fig. 3. Real-time quantitative polymerase chain reaction of proteoglycans and glycoproteins relative to GAPDH and respective tissue origin of cell cultures of SSP tenocyte-like cells (n=11), LHB tenocyte-like cells (n=7), chondrocytes (n=3) and osteoblasts (n=3); * $p \leq 0.05$. (a) Decorin expression of tenocyte-like cells and osteoblasts did not differ significantly from reference chondrocytes. (b) Biglycan expression of reference osteoblasts showed no significant differences compared to the other cell types. (c) Aggrecan expression showed no significant differences between the cell types. Chondrocytes served as reference group for statistical analysis. (d) Tenascin-C expression without significant differences of the cell types compared to osteoblast reference group.

examination of growth factors expressed *in vitro* (Anitua *et al.*, 2006; De Mos *et al.*, 2008), Matrix metalloproteinases (MMP) (Tempfer *et al.*, 2009) or surface clusters of differentiation (Scutt *et al.*, 2008) which are not specific for tendon tissue. Other authors assessed the presence of possibly tendon specific gene markers (Schulze-Tanzil *et al.*, 2004) or non specific proteoglycans (Kardestuncer *et al.*, 2006) while characterization of tendon cells obtained for further experiments was not described in some studies (Mazzocca *et al.*, 2007; Wang *et al.*, 2008). A characterization of the cultured cells therefore seems mandatory as a precondition for subsequent investigations of their biology.

The aim of the present study was to characterize tenocyte-like cells from the human rotator cuff as precisely

as possible using a spectrum of tendon markers on gene- and protein-base. Furthermore, tenocyte-like cells were compared to other cell types of the musculoskeletal system such as osteoblasts and chondrocytes. The majority of cells within healthy tendon tissue cells are tenocytes besides small amounts of chondrocytes (Kannus, 2000). Moderate COL-II expression is observed *in vivo* in human rotator cuff tendon (Gigante *et al.*, 2004; Martin *et al.*, 2003) and was presently confirmed for cultivated rotator cuff tendon cells.

Increased amounts of chondrocytes can be present in overused tendon (Archambault *et al.*, 2007) or chronic rotator cuff tears: *In vivo* transdifferentiation was observed in larger rotator cuff tears, especially in tendon stump areas of low fibroblast presence (Fukuda *et al.*, 1990; Matthews

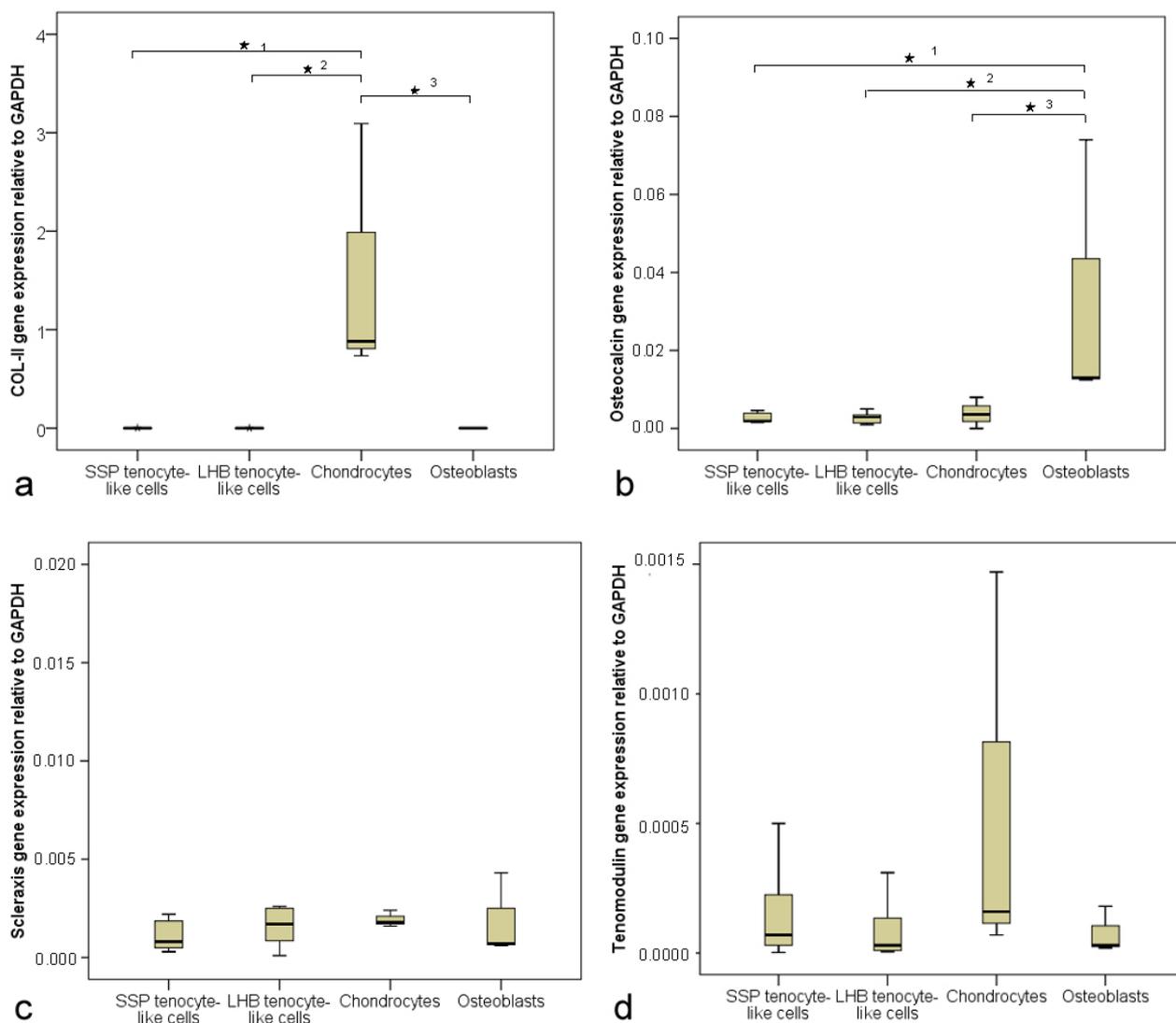


Fig. 4. Real-time quantitative polymerase chain reaction of cartilage, bone and tendon markers relative to GAPDH and respective tissue origin of cell cultures of SSP tenocyte-like cells (n=11), LHB tenocyte-like cells (n=7), chondrocytes (n=3) and osteoblasts (n=3); * $p \leq 0.05$. **(a)** COL-II expression of reference chondrocytes showed significant differences compared to SSP tenocyte-like cells ($p^1=0.01$), LHB tenocyte-like cells ($p^2=0.014$) and osteoblasts ($p^3=0.046$). **(b)** Osteocalcin expression differed significantly for SSP tenocyte-like cells ($p^1=0.009$), LHB tenocyte-like cells ($p^2=0.017$) and chondrocytes ($p^3=0.05$) compared to reference osteoblasts. **(c)** Scleraxis expression without significant differences between the cell types compared to reference tenocytes. **(d)** For tenomodulin expression, chondrocytes served as reference group. No significant differences could be detected.

et al., 2006). Other authors suggested that chondroid metaplasia may be influenced by both compression and tension forces in human rotator cuff prior to complete tear (Gigante *et al.*, 2004). Furthermore, tendon cells isolated from their natural biomechanical surrounding may transdifferentiate into fibrocartilage cell lines *in vitro* (Ehlers and Vogel, 1998; Jelinsky *et al.*, 2010; Schulze-Tanzil *et al.*, 2004).

Present tenocyte-like cells differed from chondrocytes in terms of reduced expression of COL-II while higher COL-I-levels were found. COL-I is the main component of extracellular tendon matrix (Blevins *et al.*, 1997; Kannus, 2000). Its synthesis is increased initially after rotator cuff tendon injury and then decreased with time in chronic tears (Yokota *et al.*, 2005). Tenocyte-like cells

presently expressed COL-I. Hence, COL-I cannot be considered tendon specific. Tenocyte-like cells furthermore showed significant differences from osteoblasts in terms of osteocalcin expression, a bone-specific non-collagen protein in the bone matrix.

Cultivation of small amounts of chondrocytes or osteoblasts cannot be ruled out by the present approach. However, tenocyte-like cells in total showed expression patterns significantly different from chondrocyte and osteoblast cultures with regard to characteristic markers. Besides significant differences to other musculoskeletal cell types, several other markers were investigated. Even though these cannot be considered specific, however, their respective presence in tendon was described and hence should be confirmed *in vitro*.

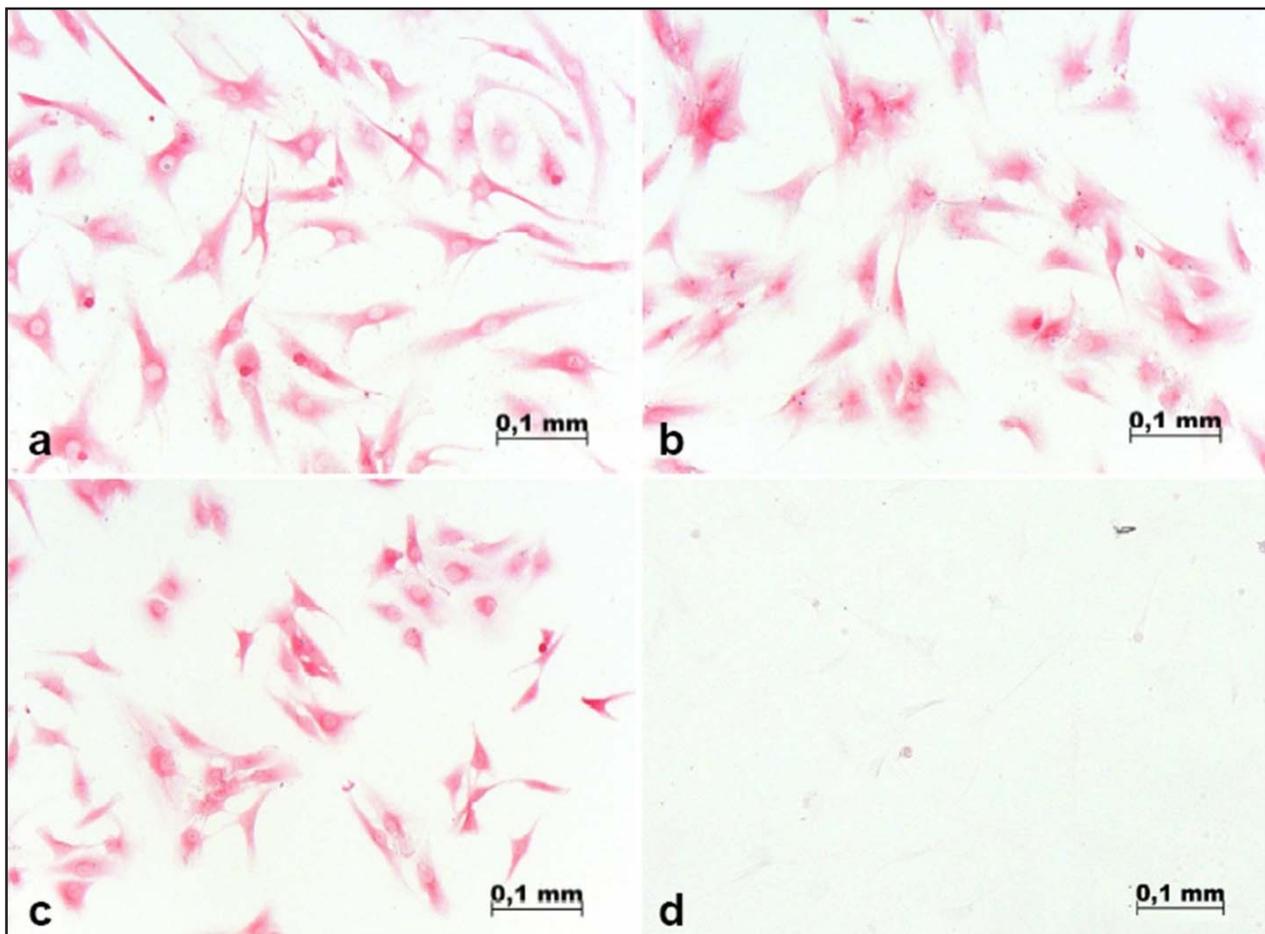


Fig. 5. Immunocytochemical staining for Scleraxis of formaldehyde-fixed (a) SSP tenocyte-like cells, (b) Chondrocytes and (c) Osteoblasts showed comparable intensities. (d) SSP Tenocyte-like cells negative control without primary antibody.

COL-III was expressed by the tenocyte-like cells with a slight decrease compared to osteoblasts. As another component of ECM (Kannus, 2000), COL-III production is increased in tenocytes from ruptured and tendinopathic-compared to intact Achilles tendon (Maffulli *et al.*, 2000). In the present study, tendon material was obtained from torn rotator cuff and expression of COL-III was not compared to intact rotator cuff biopsies to potentially confirm this observation.

The expression profile of COL-III over time during tendon healing was described (Hefti and Stoll, 1995; Takahashi *et al.*, 2002; Yokota *et al.*, 2005), but present amount of COL-III expression does not allow approximation of the time point of rotator cuff tear.

Decorin and biglycan are non-specific proteoglycans in several tissue types such as tendon, cartilage and bone (Rees *et al.*, 2000; Yoon and Halper, 2005). Both were increased initially after experimental tendon detachment (chronic tear model) and then decreased, although Decorin remained elevated from normal for months after injury (Yokota *et al.*, 2005). In the present cell culture study, decorin was expressed lower in both SSP and LHB tenocyte-like cells when compared to chondrocytes. Biglycan was expressed in a comparable quantity for both chondrocytes and tenocyte-like cells while osteoblasts

expressed slightly more biglycan. Both markers were detected in the tenocyte-like cells as expected but do not exclusively allow the characterization of tenocyte-like cells.

As a proteoglycan present in fibrocartilage and tendon (Rees *et al.*, 2000; Yoon and Halper, 2005), aggrecan was expressed by both tenocyte-like cell types while slightly higher amounts were seen in chondrocytes. High levels are expressed in compressed regions of tendon and particularly in fibrocartilage, while low levels are expressed in tensional parts (Yoon and Halper, 2005). *In vivo*, both SSP and LHB tendons are subjected to both traction- and compression forces depending on the position of the humeral head. Osteoblasts expressed aggrecan as described before (Wong *et al.*, 1992).

Tenascin-C is an anti-adhesive ECM protein and present in musculoskeletal regions that are transmitting mechanical forces from one tissue component to another (Jarvinen *et al.*, 2000), such as osteotendinous junctions as well as articular cartilage (Kannus *et al.*, 1998). Isoforms of tenascin-C in degenerated tendon were suggested to potentially stimulate tenocyte proliferation (Riley *et al.*, 1996). The expression of tenascin-C is regulated by mechanical stress (Chiquet-Ehrismann and Tucker, 2004) and is elevated in degenerative SSP tendon (Riley *et al.*,

Table 2. Overview on human tendon cell markers used in the current literature

Markers/parameter used		Author/study group	Tissue origin
Before experiment	After experiment		
morphology	COL-I, -III, Transforming Growth Factor β (TGF- β 1)	(Yang <i>et al.</i> , 2004)	human patellar tendon
none	COL-I, -III, MMP-1, -3, -13, Vascular Endothelial Growth Factor (VEGF), TGF- β	(de Mos <i>et al.</i> , 2008)	human hamstring tendon (3 children)
morphology	COL-I, -II, -III	(Takahasih <i>et al.</i> , 2002)	human rotator cuff
none	COL-I, III	(Maffulli <i>et al.</i> , 2000)	human achilles tendon
COL, CD44, CD90, D7fib	COL, CD44, CD90, D7fib	(Scutt <i>et al.</i> , 2008)	human vs. rodent patellar tendon and anterior cruciate ligament (ACL)
morphology	COL-I, -II, scleraxis, fibronectin, proteoglycan, β 1-integrin	(Schulze-Tanzil <i>et al.</i> , 2004)	human finger flexor tendon (of one donor) vs. cartilage tissue
none	haematoxylin and eosin staining, polarized microscopy (collagen maturation level); mechanical properties	(Wang <i>et al.</i> , 2008)	human foetal extensor tendon
COL-I, Decorin	COL-I, Decorin	(Kardestuncer <i>et al.</i> , 2006)	human hamstring tendon
COL-I	COL-I, Hepatocyte Growth Factor (HGF), VEGF, TGF- β 1	(Anitua <i>et al.</i> , 2006)	human ACL
morphology	COL-I, Actin filament, Vinculin	(Li <i>et al.</i> , 2008)	human patellar tendon
none	None (adhesion and proliferation rate)	(Mazzocca <i>et al.</i> , 2007)	human tendon tissue vs. bone tissue
COL-I	None	(Gumina <i>et al.</i> , 2009)	human rotator cuff
none	COL-I, MMP-2, -8, -12, -13, Tissue Inhibitor of Metalloproteinase (TIMP), Sox9	(Tempfer <i>et al.</i> , 2009)	human rotator cuff
Tenomodulin, Thrombospondin 4		(Jelinsky <i>et al.</i> , 2010)	human tendon tissue
COL-I, -III, decorin, COMP, aggrecan, elastin, scleraxis, Sox9		(Stoll <i>et al.</i> , 2010)	human hamstring tendon
COL-I, -III, decorin		(Yao <i>et al.</i> , 2006)	human achilles tendon
COL-I, -III, fibronectin, MMP-12, TIMP-1		(Almarza <i>et al.</i> , 2008)	Human medial collateral ligament (MCL), ACL, patellar tendon

1996). Compared to tenocyte-like cell cultures, slightly elevated expression of tenascin C was found in chondrocyte and osteoblast controls.

Several approaches to establish effective primers for scleraxis were unsuccessful: no products were obtained or non-desired dimers were formed, respectively. The targeted Scleraxis-gene is rather short compared to other gene-sequences (with only about 2600 bases) and has just two exons which reduces the chance of designing a reliable primer. Using a commercially available primer, comparable amounts of scleraxis were obtained for tenocyte-like cells, chondrocytes and osteoblasts in both RT-PCR and in

immunocytochemical staining. These findings might suggest that scleraxis is non-specific for tendon which was supported by its detection in several other musculoskeletal tissues (Edom-Vovard *et al.*, 2002; Jelinsky *et al.*, 2010). In contrast, other authors reported on Scleraxis as a tendon-specific gene (Schweitzer *et al.*, 2001) which regulates the expression of tenomodulin (Shukunami *et al.*, 2006). Significant differences in scleraxis expression were reported between human chondrocyte and flexor tendon cells of one adult donor. However, a 3D-culture was used which potentially may explain different expression patterns (Schulze-Tanzil *et al.*, 2004).

Tenomodulin is a transmembrane glycoprotein which is non-specific, but mainly expressed in tendon and ligament (Shukunami *et al.*, 2006). Other authors claimed tenomodulin as a selective tendon gene (Jelinsky *et al.*, 2010). Its high expression compared to different other tissues, however, is fading when cells are cultured two-dimensionally (Jelinsky *et al.*, 2010). The tenocyte-like cells in the present study were cultured in monolayers, which may explain its low expression. Furthermore, moderate expression of tenomodulin was reported in cartilage and bone (Jelinsky *et al.*, 2010), which is in accordance with the present low expression in chondrocytes and osteoblasts.

Brune *et al.* analyzed tendon cells from intact *vs.* torn ligament and observed differences in expression patterns but similar behaviour when seeded for tissue engineering (Brune *et al.*, 2007). Other authors suggested fibroblast cultures from healing instead of intact tendon to be more appropriate for studying cellular tendon regeneration (Fu *et al.*, 2008). In contrast to other authors focusing on intact human rotator cuff material (Tempfer *et al.*, 2009), donor material for the present study was obtained from torn rotator cuff. This cannot be classified as healing but was submitted to structural and biomechanical changes prior to biopsy.

Indeed, intact tendon tissue may not reflect the biological situation in chronic rotator cuff tears as assessed during surgical reconstruction. The present analysis of torn human rotator cuff tissue was in accordance with expression profiles in rodent experiments on chronic cuff tear models (Yokota *et al.*, 2005).

Study limitations

As a limitation of the present study, no possible correlations of the investigated tenocyte-like cell culture characteristics with respective patient subgroups were investigated. A prospective study in a rather small series of cases was performed to generally analyze whether or not a human rotator cuff cell culture can be established. As a further weakness, neither 3D-cell cultures nor biomechanical stimulation of obtained cell cultures were investigated. We hypothesize that tenocyte-like cells grown under different culture conditions on scaffolds could show a variable phenotype compared to monolayer culture as shown by other publications (Theisen *et al.*, 2010; Stoll *et al.*, 2010).

Conclusions

The present study describes the successful cultivation of human rotator cuff cells. Characterization of these rotator cuff cells showed an expression pattern of different gene markers that is unique for these cell types and in parts significantly different to osteoblasts and chondrocytes.

As a conclusion of our results it seems to be important to look at the collective expression profile of the tenocyte-like cells. COL-I is the most important protein for tenocyte-like cells and should be highly expressed. COL-III, biglycan, decorin, and tenascin C should also be expressed while COL-II and osteocalcin expression should be present just in low amounts.

Since no specific gene- or protein markers for tendon are known so far, the present approach represents a description as close as possible based on currently discussed markers. This characterization is necessary for further studies on the biology and stimulation of tendon cells in order to optimize therapy outcome in rotator cuff cells.

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Discussion with Reviewers

Reviewer I: Which expression profile and level will give evidence that tenocytes were isolated and cultured. At what level do you think it is better to discharge a culture?

Authors: To answer this question it seems to be important to look at the collective expression profile of the tenocyte-like cells. COL-I is the most important protein for tenocyte-like cells and should be highly expressed. COL-III, biglycan, decorin, and tenascin C should also be expressed while COL-II and osteocalcin expression should not be present. It is difficult to define concrete values for the expression level but on the basis of the presented results cell cultures with a higher relative COL-II and osteocalcin expression to GAPDH than 0.01 should not be considered as tendon-like cell cultures.

Reviewer II: Could you justify the use of monolayer cultures with all the attendant problems of dedifferentiation – this is stated in the manuscript but reasons for using this method would be useful to know here. Please comment!

Authors: To isolate cells from the native tendon it is necessary to culture them in monolayer and no other methods are known to do this in 3D culture. For further expanding of the isolated cells, with regard to very little native material we could obtain and the small amounts of tendon-like cells in the material, a monolayer culture seems the best method to ensure growth of cells as quickly as possible. A second important fact is that normal microscopic cell evaluation is impossible in 3D culture, for example in scaffolds. The morphology of the tenocyte-like cells was also important for the characterization and to estimate the condition of the cells. It is known that the tenocyte-like cells expanded in monolayer show an unstable phenotype and a tendency to dedifferentiate, but there is no evidence that this can be avoided when cells are cultured in 3D. For example, Stoll *et al.* also demonstrated phenotypic differences of tenocyte-like cells cultured on scaffolds and in high-density culture compared to native tendon and with the growth of the culture (Stoll *et al.*, 2010; text reference).

Reviewer II: Why was immunohistochemistry carried out for scleraxis and not for the other proteins?

Authors: As already mentioned, scleraxis is discussed in the literature as one possible tendon specific marker. As only low scleraxis expression was found at the RNA-level on tenocyte-like cells we decided to investigate the expression of scleraxis also on the protein level.

Reviewer II: Your controls not adequate: you should have used serum or immunoglobulins of the species of the primary antibody, as well as a control excluding the first antibody. Also, positive controls would have been useful.

Authors: We agree with the reviewer that more controls could have been performed. We just used the first antibody control to exclude the unspecific binding of the secondary antibody. In order to test the specificity of the primary antibody by precipitation, a scleraxis protein would be necessary. Since there is no scleraxis protein available, this control could not be carried out.

Reviewer II: The rotator cuff-derived cells appear to do very little – all parameters give low values. Given that the rotator cuff cells routinely give low values compared to the other cell types, I would be concerned about the culture conditions being appropriate for the cells.

Authors: We respectfully disagree that rotator cuff cells “routinely give low values” when compared to the other cell types. Some relevant markers were expected to give low values in the tenocytes when compared to other cell types, i.e., expression of COL-II (compared to chondrocytes) or osteocalcin (compared to osteoblasts). Unfortunately, the current literature does not allow a quantitative comparison of the present results to other studies elucidating different expression profiles between human rotator cuff tendon-like cells, chondrocytes and osteoblasts. However, we do not assume that these differences necessarily represent inappropriate culture conditions. With respect to the second part of the reviewer’s suggestion, indeed there is no evidence that chosen culture conditions are inadequate for these cells. When searching the literature, different culture media were used for tenocyte-like cells. Other research groups also used DMEM (Guoguang *et al.*, 2004), HAM’s F12 (Klein *et al.*, 2002) or DMEM/HAM’s F12 (Tsai *et al.*, 2003) medium without supplements such as ascorbate. We followed the decision to culture the cells this way and did not observe any problems with cell viability or activity.

Reviewer II: I am also unconvinced by the scleraxis labels; as indicated above I think an extra control is necessary, along with some positive controls. It seems odd that all cells should stain with a very low level of expression as measured by supposedly high sensitivity qPCR methodology should label, along with cell types that are not generally associated with expression of this molecule.

Authors: Two different methods to analyse the scleraxis expression and production were performed. By quantitative RT-PCR we found low levels of scleraxis. Using this quantitative method we did not expect high levels of this transcription factor as seen for all matrix proteins in this study. In contrast to RT-PCR, immunocytochemistry is no quantitative method and can only visualize whether or not a protein is present. Therefore, we suggest that the unequal results are not caused by wrong protocols but are normal variances obtained from methods that are different in principle.

Reviewer II: There is no doubt that you have cultured cells from the rotator cuff tendon that are different from chondrocytes and osteoblasts, but it is difficult to draw conclusions as to their behaviour in culture as they seem to be doing very little. Perhaps supplementation with ascorbate, as you do for osteoblasts, might help – osteoblasts seem to be the most active cells in your experiments. It is very difficult to make comparisons between these cell types given the differences in culture medium supplements.

Authors: We agree that differences in cell culture supplements like ascorbate could influence the matrix expression. But no comparative study on tenocytes was found to prove this. Ibold *et al.* showed the influence of

different ascorbate supplementations on chondrocyte pellet cultures. 100 μM L-ascorbate-2-phosphate (we used 50 μM) resulted in decreased COL-I expression at the beginning of the culture and slightly increased COL-II and aggrecan expression (Ibold *et al.*, 2009). Another study on mesenchymal stem cells revealed that ascorbate supplementation (0-500 μM) had almost no effect on growth and proliferation until passage 3 (Choi *et al.*, 2008). Son *et al.* demonstrated that ascorbate supplementation (60 and 125 μM) did not affect the collagen expression (Son *et al.*, 2007). To rule out an effect of the different culture conditions on matrix expression, we agree that it would have been better to culture all cells with the same supplementations. But with respect to the mentioned studies we would not expect a marked difference in expression profiles. We therefore think that our comparison is reasonable.

Additional References

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