INFLUENCE OF AGE ON THE CELL BIOLOGICAL CHARACTERISTICS AND THE STIMULATION POTENTIAL OF MALE HUMAN TENOCYTE-LIKE CELLS

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Abstract

The incidence of rotator cuff tears and recurrent defects positively correlate with patient age. However, this observation has never been analysed at the cellular level. The present study aims to better understand this correlation by investigating cellular characteristics of rotator cuff tenocytes of different age groups. Additionally, previous studies reported on stimulating effects of Bone Morphogenetic Protein (BMP) -2 and BMP-7 on tenocytes. Thus, the second aim was to investigate, whether the stimulation potential of tenocytes demonstrates age-related differences.

Tenocyte-like cells from supraspinatus tendons of young and aged male patients were analysed for the following cell biological characteristics: cell density, cell growth, marker expression, collagen-I protein synthesis, stem cell phenotype, potential for multipotent differentiation and self-renewal. To analyse the stimulation potential, cells were treated with BMP-2 and BMP-7 in 2D-/3D-cultures. Measured parameters included cell activity, marker expression and collagen-I protein synthesis.

An effect of age was seen for cell growth and stem cell potential but not on extracellular matrix level. Cells from both groups responded to BMP-7 by increasing cell activity, collagen-I expression and protein synthesis. BMP-2 led to smaller increases in these parameters when compared to BMP-7. In general, 3D-cultivation improved the stimulation compared to 2D-culture.

The cell biological characteristics of tenocyte-like cells, considered important for successful restoration of the tendon-bone unit, were inferior in elderly donors. This may help explain higher rates of recurrent defects seen in elderly patients. Regarding the stimulation potential, on a cellular level young and aged patients may benefit from biological augmentation with BMPs.

Keywords: Tenocyte-like cells, age; rotator cuff; BMP-2; BMP-7; 2D-culture; 3D-culture.

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Introduction

Tears of the rotator cuff, and in particular the supraspinatus (SSP) tendon, are highly prevalent orthopaedic morbidities in the adult populations. Patients frequently suffer from pain and disability and in many cases require surgical reconstruction. However, despite biomechanical improvements in reconstruction techniques (Pauly *et al.*, 2010a; Pauly *et al.*, 2011a), clinical and radiographic mid-term results are often unsatisfactory (Burks *et al.*, 2009; Grasso *et al.*, 2009; Aydin *et al.*, 2010), and may indicate insufficient regeneration of the tendon-bone interface at the humeral insertion. In animal studies it was demonstrated that the complex process of tendon-bone healing results in the formation of a biomechanically inferior scar tissue, rather than the regeneration of the native tendon-bone tissue (Gerber *et al.*, 1999; Rodeo, 2007). Accordingly, non-healing and recurrent defects are the most frequent complications following surgical reconstructions (Boileau *et al.*, 2005; Deutsch *et al.*, 2008; Frank *et al.*, 2008; Burks *et al.*, 2009).

Several clinical and radiographic follow-up studies of large patient cohorts have investigated factors associated with these postoperative complications. These included demographic factors, such as patient age (Milgrom *et al.*, 1995; Boileau *et al.*, 2005; Yamaguchi *et al.*, 2006; Sorensen *et al.*, 2007) and gender (Grasso *et al.*, 2009; Chung *et al.*, 2011;), as well as radiographic characteristics, such as fatty infiltration of the respective muscle (Goutallier *et al.*, 2003; Gladstone *et al.*, 2007; Shen *et al.*, 2008; Cho *et al.*, 2011). However, as these were epidemiological studies, they do not provide causal or biological explanations for the reason of impaired healing. It is possible that cellular or even molecular characteristics could give rise to these correlations. These include for example weaker tenocyte cell growth or cell activity, lower cell density in the tendon, lower stem cell potential of the tenocytes or a decreased synthesis of extracellular matrix (ECM) proteins like collagen-I (Col-I).

In order to improve the quality of tendon tissue after surgical reconstruction, biologically based strategies have gained increasing interest over the last several years. Important to these strategies is the regenerative capacity of the tenocytes (e.g. cell activity, proliferation, ECM synthesis), and it has been suggested that the exogenous application of growth factors could improve tendon healing (Maffulli *et al.*, 2002; Hsu and Chang, 2004). Several experimental approaches *in vivo* (Rodeo *et al.*, 1999; Martinek *et al.*, 2002; Mihelic *et al.*, 2004; Ma *et al.*,

Table 1. Real-Time PCR primers.

Target gene (Accession number)	Product size [bp]	Annealing temperature [°C]	Sequence [5' – 3']
GAPDH (NM_002046)	115	64.2; 66.0	Forward: CCACTCCTCCACCTTTGACG Reverse: CATGAGGTCCACCACCCTGT
Col-I (NM_000088)	197	64.2	Forward: TGACCTCAAGATGTGCCACT Reverse: ACCAGACATGCCTCTTGTCC
Col-II (NM_001844)	162	66.0	Forward: CGCACCTGCAGAGACCTGAA Reverse: TCTTCTTGGGAACGTTTGCTGG
Col-III (NM_000090)	199	64.2	Forward: GCTGGCATCAAAGGACATCG Reverse: TGTTACCTCGAGGCCCTGGT
Osteocalcin (NM_199173)	209	64.2	Forward: CCCAGGCGCTACCTGTATCAA Reverse: CTGGAGAGGAGCAGAACTGG
Decorin (NM_001920)	205	64.2	Forward: CGCCTCATCTGAGGGAGCTT Reverse: TACTGGACCGGGTTGCTGAA
TGF-β1 (NM_000660)	116	64.2	Forward: AAGGACCTCGGCTGGAAGTG Reverse: AGGGCCAGGACCTTGCTGTA
TGF-β2 (NM_001135599)	185	64.2	Forward: CAACAGCACCAGGGACTTGC Reverse: AGCACAAGCTGCCCACTGAG
TGF-β3 (NM_003239)	136	64.2	Forward: CTGCTGGAGGAGATGCATGG Reverse: GGCAGACAGCCAGTTCGTTG

2007; Rodeo *et al.*, 2007), and *in vitro* (Salingcarnboriboon *et al.*, 2003; Tsai *et al.*, 2003; Thomopoulos *et al.,* 2005; Yamada *et al.*, 2008; Yeh *et al.*, 2008, Pauly *et al.*, 2011b) have demonstrated that Bone Morphogenetic Protein (BMP)-2 and -7 can stimulate tendon cells, and assist in the restoration of a solid tendon-bone complex. However, possible age-related differences in tissue susceptibility to growth factor exposure are unknown. Aged patients, who possibly have a reduced cell biological potential, may particularly benefit from growth factor augmentation during rotator cuff repair.

The primary aim of the present study was to analyse cell biological characteristics of tenocyte-like cells from male human donors in order to investigate possible reasons for increased incidence of recurrent defects with age. The second focus was to investigate, whether there are age-related differences in the stimulation potential of tenocyte-like cells treated with BMP-2 or BMP-7.

Materials and Methods

Tendon material, donor demographics, clinical data
SSP tendon samples were obtained from patients undergoing arthroscopic surgery for rotator cuff repair or open shoulder surgery, such as hemiarthroplasty after humeral head fractures. All biopsies were obtained according to a standardised protocol and were grasped 3 to 5 mm from the torn proximal tendon edge. Prior to biopsy, all patients gave their written informed consent.

Studies suggested that beyond the age of 60 to 65 years, the incidence of recurrent defects strongly increased (Milgrom *et al.*, 1995; Gerber *et al.*, 1999; Boileau *et al.*, 2005; Tashjian *et al.*, 2010). Accordingly, the age cut-off was defined at 65 years. All donors younger than 65 years were categorised "young" and 65 years or more "aged" in the current study.

In this study, the male donors differed with regard to age. Young donors had a mean age of 45.3 years (range:

42-50 years) and aged donors of 71.3 years (range: 66-75 years). Among all included donor patients, fatty infiltration of the SSP muscle was graded 0-I according to Goutallier (Goutallier *et al.*, 1994) on MRI scans (Fuchs *et al.*, 1999). Tendon retraction was graded 0-2 according to Patte (Patte, 1990) and tear size staged I-III according to Bateman (Bayne, 1984), depending on preoperative MRI assessment and intraoperative diagnostics. The radiological characteristics were evenly distributed between the two groups.

Cell density
Tenocyte-like cells were isolated from the SSP tendon biopsies as described previously (Pauly *et al.*, 2011b). Prior to digestion with 0.3 % collagenase type CLS II, the SSP biopsies were weighted under sterile conditions. Digested tendon material was then plated onto a culture flask with a ratio of tendon material (mg) / growth area (cm²) from 0.2 to 0.3 in order to allow all cells to grow under similar culture conditions. The cells were cultured with normal growth medium (DMEM/Ham's F12 with 10 % foetal calf serum (FCS) and 1 % penicillin/streptomycin, all Biochrom, Berlin, Germany) at 37 °C, with 95 % humidity and 5 % carbon dioxide, with a change of medium every 2 to 3 d. After one week of culture, cell activity was analysed with an Alamar Blue assay (Biochrom) according to the manufacturer's instructions. The cell count in the culture flask was calculated by a standard curve (defined cell number). To calculate the approximate cell density, the cell count was normalised to the weight of the tendon biopsy.

Cell growth
The tenocyte-like cells in passage 2 were seeded with 2.5×10^3 vital cells per well in a 48-well plate, and in triplicates. At day 1, 4, 7 and 14 after seeding, an Alamar Blue assay was performed to analyse cell activity. Between day 7 and day 14 of culture two times a half change of medium was performed. The cell count was calculated with a standard curve, and the cell count of day 4, 7 and 14 was

Table 2. Antibodies used for FACS analysis

Name	Label	Clone	Company	Dilution
CD29	Phycoerythrin (PE)	T52/16	Biolegend, Uithoorn, Netherlands	1:100
CD44	PE-Cy7	IM7	Biolegend	1:1000
CD73	Allophycocyanin (APC)	AD2	Biolegend	1:400
CD90	Peridinin Chlorophyll Protein (PerCP)-Cy5.5	5E10	Biolegend	1:400
CD105	Fluorescein Isothiocyanat (FITC)	43A3	Biolegend	1:100
CD11b	Pacific blue	ICRF44	BD Biosciences	1:1000
CD14	Pacific blue	MOP9	BD Biosciences	1:1000
CD19	Pacific blue	HIB19	BD Biosciences	1:1000
CD34	Pacific blue	581	Biolegend	1:1000
CD45	Pacific blue	HI30	BD Biosciences	1:1000
Life/Dead	Aqua Fluorescent reactive dye		Invitrogen, Darmstadt, Germany	1:1000

referred to cell count of day 1 by subtraction to assess the cell growth of the tenocyte-like cells over time.

Marker expression

Methods for cell characterisation were based on results from a previous study (Pauly *et al.*, 2010b), and included analysis of Col-I, -II, -III, and osteocalcin expression. Additionally, expression of decorin, transforming growth factor (TGF)-β1, -β2, and -β3 was analysed. RNA was isolated from the tenocyte-like cells at passage 2 with the NucleoSpin RNA II Kit (Machary Nagel, Dueren, Germany) according to the manufacturer's instructions. RNA concentrations were measured with the Nanodrop ND-1000 Spectrophotometer (PeqLab Biotechnologie, Erlangen, Germany). Subsequently, 100 ng of RNA was transcribed into cDNA with the qScript cDNA Supermix (Quanta BioSciences, Gaithersburg, MD, USA) following the manufacturer's instructions. An Epgradient Mastercycler (Eppendorf, Hamburg, Germany) was used for cDNA synthesis.

All primer sequences were designed using Primer 3 software (Freeware; http://frodo.wi.mit.edu/primer3), and were produced by Tib Molbiol, Berlin, Germany (Table 1). The Real-Time PCR was performed with the Realplex Mastercycler System (Eppendorf). The cDNA was diluted 1:20 and 5 μL was pipetted into each well as PCR template. The mastermix was prepared with the following components for each well: 12.5 μL Sybr Green Supermix (Quanta BioSciences), 1 μL primermix (10 μM, forward and reverse Primer 1:1), and 6.5 μL RNase/DNase-free water. 20 μL of the mastermix was added to each well. The following Real-Time PCR protocol was used for the amplification: a denaturation program (95 °C for 3 min), an amplification program repeated for 40 cycles (95 °C for 15 s, 64.2 °C/66 °C for 45 s, 72 °C for 30 s), a 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 Real-Time PCR results were analysed with the Realplex Software (Eppendorf). Relative expression levels were normalised to GAPDH, and calculated using the $2^{-\Delta Ct}$ method.

Col-I protein synthesis

Levels of Col-I protein were measured from the cell culture supernatant taken at day 4, 7 and 14 of cell growth analysis by using MicroVue C1CP EIA Kit (TecoMedical, Buende,

Germany) according to manufacturer's instructions. Levels were normalised to total protein content of the supernatant (Coomassie Plus™ Protein Assay, Thermo Fisher Scientific, Bonn, Germany).

Stem cell phenotype

For analysing the stem cell phenotype, fluorescent activated cell sorting (FACS) was performed. Confluent cells from a 75 cm² bottle (about 2.5-5 x 10⁵ vital cells) were harvested in passage 1. An established stem cell panel, including all antibodies, was obtained from the Core Unit of the Berlin-Brandenburg Center for Regenerative Therapies (BCRT). Only the Life/Dead staining was modified, as a different antibody was used. Half of the cells were stained with a Life/Dead reagent, CD29, CD44, CD73, CD90, CD105, and a negative mix consisting of CD11b, CD14, CD19, CD34 and CD45 for 25 min at 4 °C. All antibodies are listed in Table 2. The other half of the cells served as controls. After staining, the cells were washed with FACS buffer (0.1 % bovine serum albumin (BSA) in phosphate buffered saline (PBS)), fixed with 1 % paraformaldehyde (PFA), and measured with the BD FACS Canto II System (BD Biosciences, Heidelberg, Germany), and FACS Diva software. The data analysis was performed using the FlowJo 8.8.6 software. All viable cells were analysed for the expression of antigens.

Multipotent differentiation

The multipotent differentiation of the tenocyte-like cells was tested towards osteogenic, adipogenic, and chondrogenic phenotypes. For osteogenic and adipogenic differentiation, cells at passage 2 were seeded into 24-well plates (7.5x10³ vital cells / well), cultured until confluence and then incubated in a modified version of differentiation medium previously described (Pittenger *et al.*, 1999; de Mos *et al.*, 2007). The cells were treated for 3 weeks with osteogenic induction medium (500 μM L-ascorbic acid, 10 mM β-glycerophosphate and 100 nM dexamathasone in normal growth medium), adipogenic induction medium (1 μM dexamethasone, 1 μM insulin, 0.5 mM isobutyl-methylxanthine (IBMX) and 60 μM indomethacine in normal growth medium), or with normal growth medium (control). Medium was changed twice a week. All cell culture supplements for differentiation medium were obtained from Sigma-Aldrich (Taufkirchen, Germany).

To quantify the osteogenic differentiation, the tenocyte-like cells were stained with 0.5 % Alizarin Red S (Sigma-Aldrich) in 0.5 M HCl for 10 min. The staining was solubilised in a solution of 200 µL 5 % SDS (Roth, Karlsruhe, Germany) and 0.5 M HCl for 5 min, and then measured at 405 nm against the blank (5 % SDS in 0.5 M HCl) (Bi *et al.*, 2007). To validate the osteogenic differentiation, cells were stained for Alkaline Phosphatase (ALP) exemplarily. After formalin fixation cells were incubated in ALP staining solution (0.06 % Fast Blue Bb Salt (Waldeck, Muenster, Germany), 0.01 % Naphtol-AS-MX-Phosphate (Sigma-Aldrich), 0.5 % dimethylformamide (Sigma-Aldrich), 2 mM magnesium chloride (Merck, Darmstadt, Germany), and 0.1 M Tris-base (Sigma-Aldrich) in dH_2O, pH 8.5) for 30 min at 37 °C.

To stain the lipid-vacuoles in the adipogenic differentiated cells, a stain of 0.3 % Oil Red O (Sigma-Aldrich) in 99 % isopropanol was used for 10 min. After clearing the background with 60 % isopropanol, the staining was solubilised with 250 µL 100 % isopropanol for 10 min, and measured at 490 nm against the blank (100 % isopropanol) (Bi *et al.*, 2007). The osteogenic and adipogenic differentiation was normalised to the staining of undifferentiated cells.

For chondrogenic differentiation only *n* = 1 differentiation was performed for each young and aged donor tenocyte-like cell cultures. 2.5×10^5 vital cells were pelleted into a 15 mL falcon tube. One tube was incubated with chondrogenic induction medium (100 nM dexamethasone (Sigma-Aldrich), 175 nM L-ascorbic acid (Sigma-Aldrich), 40 µg/mL proline (Sigma-Aldrich), 100 µg/mL pyruvate (Roth), 6.25 µg/mL insulin-transferrin-sodium selenite supplement (ITS, Sigma-Adrich), 1.25 mg/mL Bovine Serum Albumine (BSA; Sigma-Aldrich), 5.35 mg/mL linolenacide (Sigma-Aldrich), 10 ng/mL TGF-β1 (R&D Systems) in normal growth medium), and one tube with normal growth medium (control), with a change of medium twice a week. After 3 weeks in culture, the cell pellet was fixed with 4 % PFA, and subsequently paraffin embedded. 4 µm slices were taken and stained with 1 % Alcian Blue solution (Sigma-Aldrich) for 30 min. Counterstaining was performed with 0.1 % Kernechtrot (Waldeck Division Chroma, Muenster, Germany) in 5 % aluminium sulphate for 5 min.

Potential for self-renewal
For testing the potential for self-renewal, a Colony Forming Unit (CFU) assay was performed. 1000 vital cells were seeded into a 10 cm Petri dish (in triplicates), and cultivated for 11 d with normal growth medium, with medium change every 2-3 d. For visualisation, the colonies were stained with 1 % methylene blue in borate buffer /1 % azur in dH_2O (1:1, Sigma-Aldrich) for 10 min. After washing, cells were dried and pictures were taken. An image analysing system with an adaptive threshold was used to quantify the number and size normalised to the number of the CFUs (ImageJ 1.44i, Wayne Rasband, National Institute of Health, Bethesda, MD, USA). All colonies between 1 mm² and 10 mm² were counted.

Application of growth factors
For testing the response of tenocyte-like cells to growth factors, cells were treated with 0 (control), 200, or 1000 ng/mL rhBMP-2 (Wyeth, New York, USA), or rhBMP-7 (R&D Systems, Wiesbaden, Germany) in both 2D- and 3D-cultures (Optimaix collagen scaffolds, Matricel, Herzogenrath, Germany). The scaffolds consist of highly oriented porcine collagen type I, in which cells can optimally attach, spread and produce extracellular matrix. Cells were seeded into 48-well plates with about 3×10^3 vital cells / well or about 2×10^4 / scaffold (size 85 mm³: 6 mm diameter x 3 mm height) in normal growth medium. For 3D-culture cells were seeded with a drop-on method directly to the scaffolds and cultured in static conditions. After cells reached about 50 % confluence in the 2D-culture (about 4 d) the medium was changed for both culturing conditions to medium without FCS to adjust the cell cycle. The next day (day 0), and at day 3 and 5, an Alamar Blue assay was performed with 500 µL /well according to the manufacturer, and then growth factor conditioned medium (200 ng/mL, or 1000 ng/mL BMP-2, or BMP-7 in DMEM/ HAM's F12 1:1 supplemented with 5 % FCS and 1 % Penicillin/Streptomycin) was applied to the cells. On day 7, a final Alamar Blue assay was performed, and RNA was isolated from the cells in the 2D- and 3D-cultures with the NucleoSpin RNA II Kit (Macherey Nagel, Düren, Germany) according to manufacturer, in order to analyse the gene expression by Real-Time PCR (see marker expression). Relative expression levels were normalised to GAPDH, and to the untreated control, and calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The Col-I protein synthesis was analysed by ELISA from the cell culture supernatant of day 7 (see Col-I protein synthesis), and normalised to the total protein content (Coomassie Plus™ Protein Assay).

Visualisation of tenocytes in collagen scaffolds
After one week of stimulation, collagen scaffolds were fixed with 4 % PFA for 30 min at 37 °C and with 25 mM ammonium chloride for 20 min at 37 °C. Afterwards, scaffolds were embedded in 5 % gelatine/5 % sucrose solution for 2 h at 37 °C and then for 4 h at 4 °C. Scaffolds were cryoembedded with TissueTec (Sakura Finetek, Alphen aan de Rijn, Netherlands) and 25 µm cryosections were taken. For the staining, cryosections were fixed with formalin and permeabilised with 0.025 % Triton-X-100 in Tris Buffer Solution (TBS) for 10 min. Afterwards, sections were incubated with Phalloidin Alexa Fluor 488 (Invitrogen, 1:500 in TBS) for 1 h at room temperature, then washed, incubate with Dapi (Invitrogen, 1:1000 in dH2O) for 5 min and covered with Fluoromount-G (Southern Biotec, Birmingham, AL, USA).

Statistics
Cells from *n* = 6 donors per each group were used for the experiments. All experiments were performed in triplicates. Accordingly, statistics were performed for *n* = 18 values for each group. For Real-Time PCR analysis, RNA of triplicates was pooled together (*n* = 6). Statistical analysis
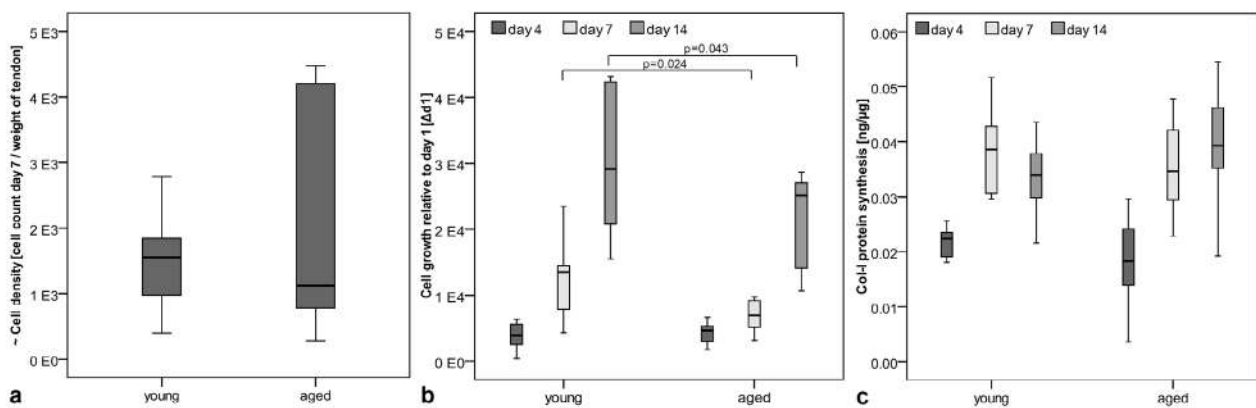
Fig. 1. Comparison of tenocyte-like cells of young and aged donors. (**a**) Cell density given as cell count of day 7 per weight of tendon biopsy showed no age-related differences. (**b**) Cell growth measured over 14 days and relative to day 1. Cells of aged donors had significantly decreased cell growth at days 7 and 14. (**c**) Col-I protein synthesis relative to total protein content was not significantly different between groups at the different time points.

was performed using SPSS 18 (SPSS, Chicago, IL, USA). The Kruskal-Wallis test was used to analyse significant differences between all groups followed by a Mann-Whitney U test to compare the young and aged group, or the different concentration groups with the untreated control. All values in the text are given as median with 25-75 percentiles. The level of significance was set at $p \leq 0.05$ for cell biological characteristics. For the stimulation potential two levels of significance were used, which are 1: $p \leq 0.05$ and 2: $p \leq 0.001$. The Bonferroni-Holm correction was performed to adjust the α-value.

Results

Cell biological characteristics

The approximate cell density revealed no significant differences between young and aged donors (Fig. 1a). The cell growth was significantly lower in the aged donor group at day 7 and 14 (Fig. 1b). The Col-I protein synthesis increased with time of culture. But in the young donor group a slightly decrease was observed from day 7 to 14. However, no significant age-related differences between the groups were observed (Fig. 1c).

The characterisation of tenocyte-like cells by Real-Time PCR showed that cells of both young and aged donors

expressed Col-I and Col-III, weakly expressed Osteocalcin (Table 3), and did not express Col-II (data not shown). The expression of Col-I, Col-III, Osteocalcin, Decorin, TGF-β1 and TGF-β3 did not show significant differences between the cells of the different donor groups. The mean TGF-β2 expression was halved in the tenocyte-like cells of young donors compared to aged donors. However, no significant changes ($p = 0.054$) could be observed (Table 3).

The tenocyte-like cells of both donor groups were found to have a stem cell phenotype. They were positive for the CD29, CD44, CD73, CD90 and CD105 antigens but negative for CD11b, CD14, CD19, CD34 and CD45 antigens. With respect to surface markers, no significant differences between cells from young and aged donors were observed (Table 4 and Fig. 2).

The cellular potential for self-renewal (number of colonies and colony size) was significantly higher in cells of young donors (Fig. 3a). 13 % (10-14 %) of tenocyte-like cells of young donors and 7 % (3-12 %) of cells of aged donors formed adherent cell colonies.

A portion of tenocyte-like cells from both groups had the capacity to differentiate into adipogenic, osteogenic and chondrogenic phenotypes (Fig. 4). Compared to the aged donors, the cells of young donors showed a significantly enhanced osteogenic differentiation potential.

Table 3. Relative gene expression of tenocyte-like cells of young and aged donors.

	Relative gene expression normalised to GAPDH [2$^{-\Delta Ct}$] given as medians with 25-75 percentiles						
	Col-I	**Col-III**	**Osteocalcin[10^{-4}]**	**Decorin**	**TGF-β1**	**TGF-β2[10^{-4}]**	**TGF-β3[10^{-3}]**
Young	2.70 (2.43-3.20)	0.45 (0.37-0.61)	8.97 (5.27-12.53)	0.14 (0.13-0.30)	0.05 (0.04-0.07)	1.27 (0.09-2.37)	1.42 (1.14-1.99)
Aged	2.65 (1.98-3.04)	0.45 (0.34-0.61)	6.65 (4.26-9.47)	0.47 (0.13-0.32)	0.05 (0.05-0.06)	2.70 (2.00-5.00)	0.96 (0.65-1.74)

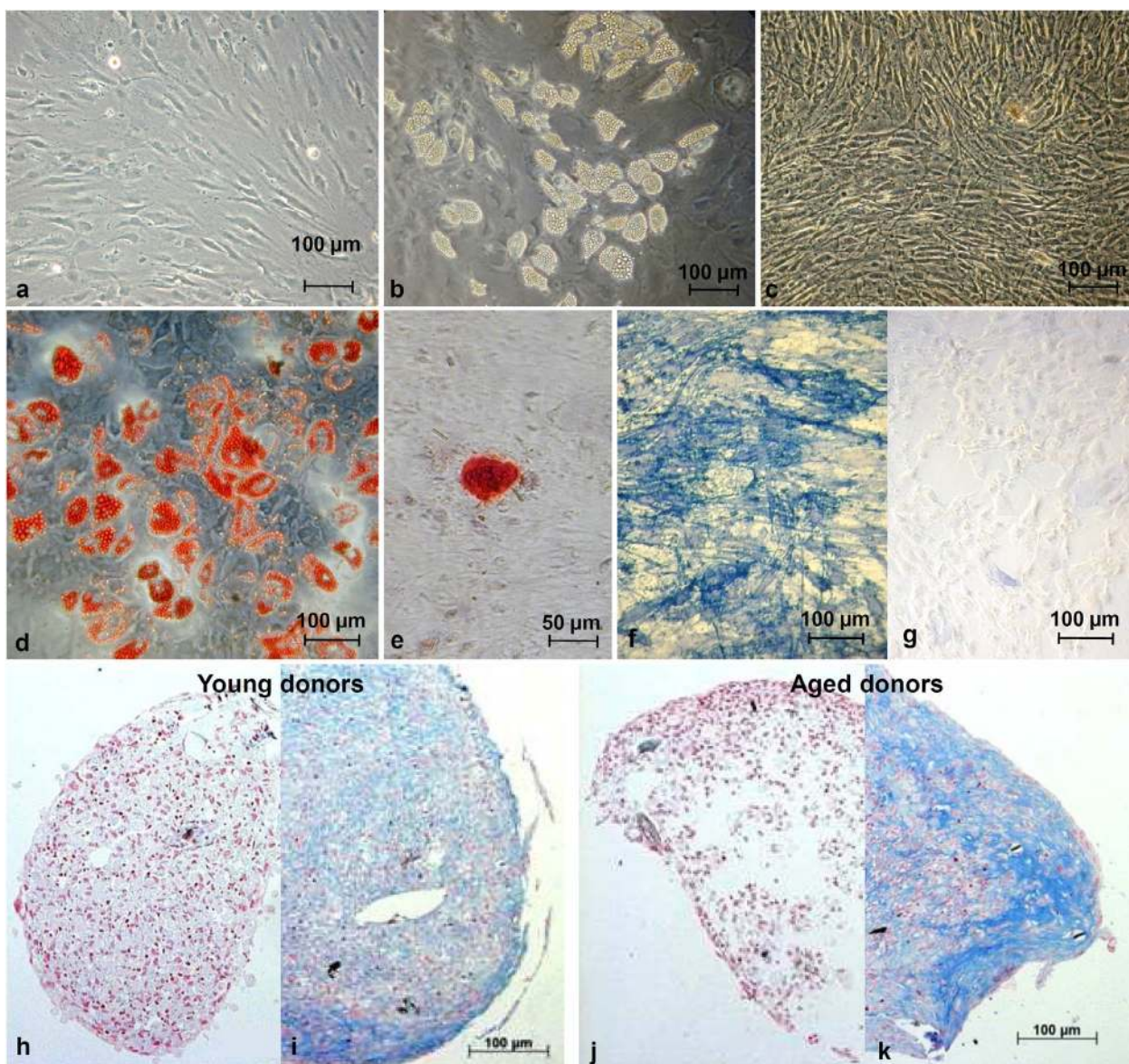**Table 4**. Stem cell phenotype of tenocyte-like cells.

	Surface marker [%] given as medians with 25-75 percentiles					
	CD29+	**CD44+**	**CD73+**	**CD90+**	**CD105+**	**Negative mix+**
Young	99.96 (99.96-99.99)	99.99 (99.92-99.99)	100.00 (99.99-100.00)	99.95 (99.87-99.96)	99.13 (97.01-99.60)	0.19 (0.03-1.81)
Aged	99.92 (99.42-100.00)	99.97 (99.90-100.00)	99.99 (99.98-100.00)	99.91 (99.39-99.97)	96.05 (94.47-98.57)	0.11 (0.03-0.25)
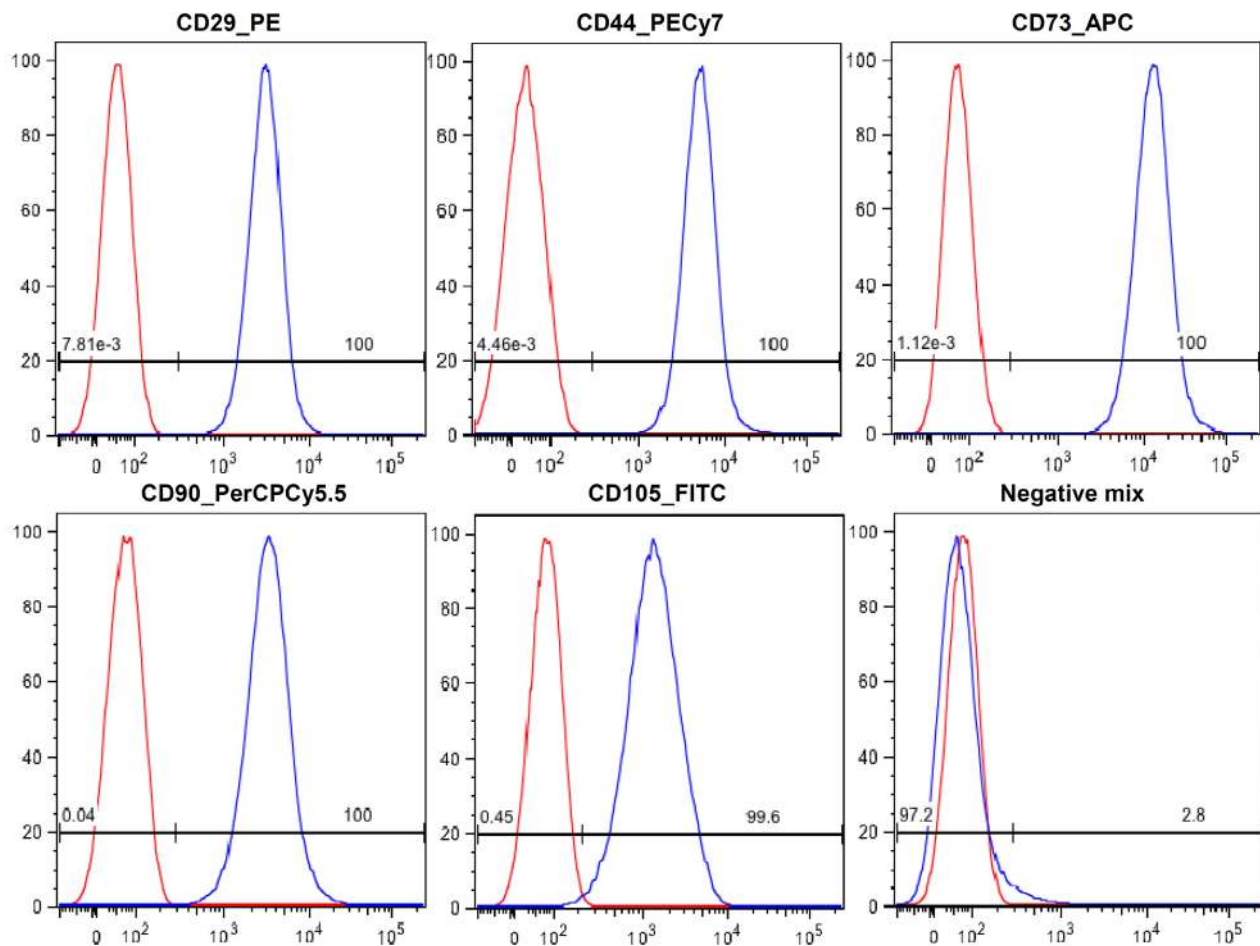
Fig. 2. Exemplary results of FACS analysis of young donor tenocyte-like cells. Graphs show the negative control cells (red curve) compared to stained cells (blue curve). Cells were positive for CD29, CD44, CD73, CD90, CD105 and negative for CD11b, CD14, CD19, CD34, CD45 (Negative mix).
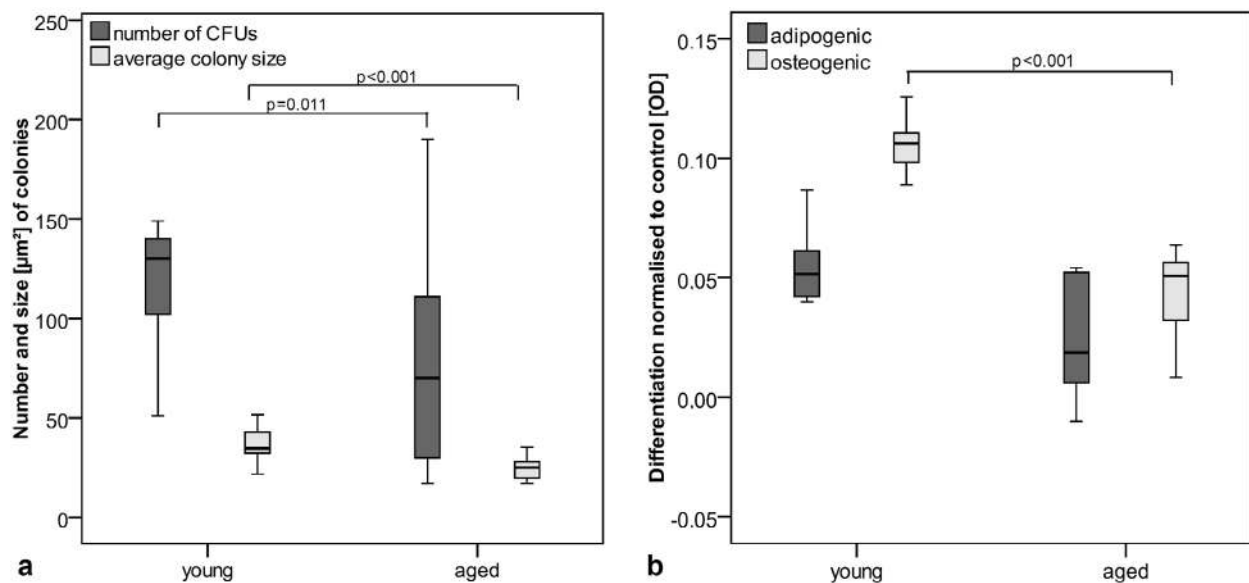
Fig. 3. Comparison of potency for self-renewal and differentiation potential of tenocyte-like cells of young and aged donors. (**a**) Number of CFUs as well as the average colony size was significantly decreased in the aged donors' cells. (**b**) Adipogenic and osteogenic differentiation was given as OD normalised to undifferentiated control cells. Adipogenic differentiation was not affected by the age of the donors, but osteogenic differentiation was significantly increased in tenocyte-like cells of young donors.

Fig. 4. Multipotent Differentiation (**a-c**) Phenotype of tenocyte-like cells after 3 weeks in (**a**) normal growth medium (control), (**b**) adipogenic differentiation medium, (**c**) osteogenic differentiation medium. (**d**) Adipogenic differentiated cells after Oil Red O staining with red stained lipid vacuoles, (**e**) Calcium accumulation after Alizarin Red S staining. (**f**) Blue ALP staining of osteogenic differentiated cells and (**g**) undifferentiated control cells. (**h-k**) Alcian Blue staining of chondrogenic differentiated tenocyte-like cell pellets of young (**h-i**) and aged (**j-k**) donors showed a positive staining compared to the undifferentiated control cells (**h** and **j**).

The adipogenic differentiation potential of the cells was not significantly affected by age (Fig. 3b).

In terms of chondrogenic differentiation, no quantification could be performed. Only $n = 1$ tenocyte-like cell culture for each group was used for the differentiation experiment. The Alcian Blue staining of the cell pellets revealed that cells of young and aged donors could be differentiated into a chondrogenic phenotype (Fig. 4 h-k).

Stimulation potential
Cell activity
The cell activity of young donors was significantly decreased after BMP-2 treatment at day 5 and 7, and only at day 7 in aged donors in the 2D-culture. For BMP-7 treatment, a significant dose-dependent increase of the cell activity was found for nearly all days and concentrations

in the tenocyte-like cells of young and aged donors in 2D-culture. In cells of aged donors already the 3-day time point showed a significant increase (Fig. 5a and b).

The suppressing effect of BMP-2 within 2D-culture reversed in the low concentration group at day 7, and was compensated in the 3D-culture at all concentrations in tenocyte-like cells of young and aged donors. BMP-7 led to significantly increased cell activity in the high concentration in both groups and additionally to an increase at low BMP-7 concentration at day 5 in the young donors group (Fig. 5c and d).

Direct comparison of young and aged donor cells revealed a significant increase of cell activity in the aged donor group following BMP-7 application (low dosed, day 3, and 5, 2D-culture; and day 3 in 3D-culture).
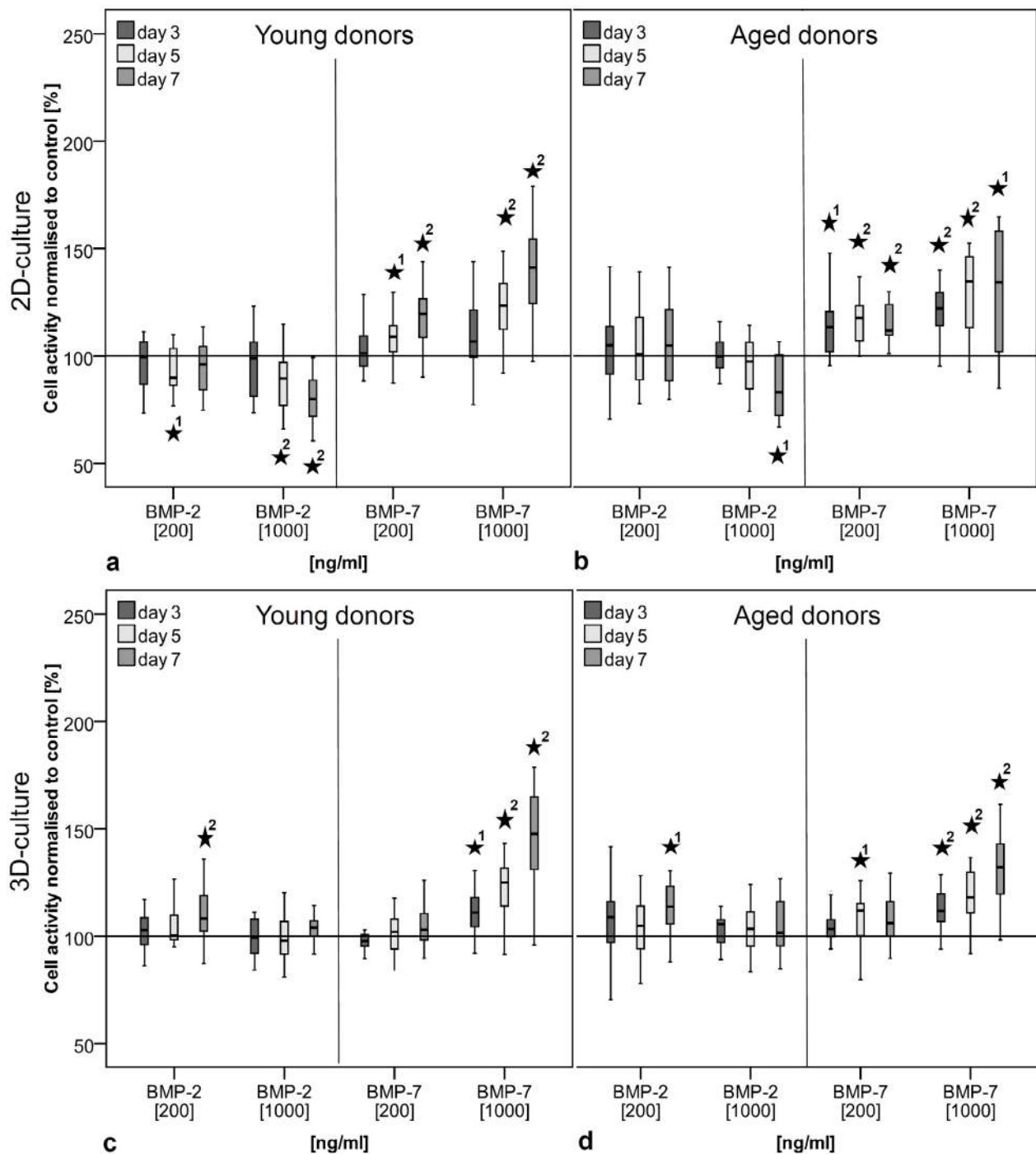
Fig. 5. Cell activity was measured by Alamar Blue assay and given as percentage of untreated control. Application of BMP-2 in 2D-culture of cells from young (**a**) and aged (**b**) donors significantly decreased activity compared to untreated control at low concentration at day 5, and at high concentration at days 5 and 7 (young donors), and at high concentration at day 7 (aged donors). BMP-7 significantly increased cell activity at nearly all concentrations and days in cells of young (**a**) and aged (**b**) donors. BMP-2 application in 3D-culture resulted in increased cell activity at day 7 at the low concentration in the cells of young (**c**) and aged (**d**) donors. Application of BMP-7 enhanced cell activity only at the high concentration. All significant differences from the untreated control are marked with an asterisk (*). The numbers give details for the *p*-value: 1: $p \leq 0.05$; 2: $p \leq 0.001$.

Relative gene expression

The Col-I expression of tenocyte-like cells in 2D-culture was increased by BMP-7 in both dosages in young and in the high dosage in the aged donor group. BMP-2 increased the Col-I expression only in the low concentration in the aged donor group. In 3D-culture, both growth factors significantly increased Col-I expression at nearly all concentrations, except for the low BMP-7 concentration in the aged donor group. Generally, BMP-7 treatment resulted in stronger effects compared to BMP-2, and the 3D-culture led to an enhancement of the results (Fig. 6).

The expression of Col-III was significantly increased in tenocytes of both donor groups by BMP-7 in the high concentration with both culturing conditions. A decreased
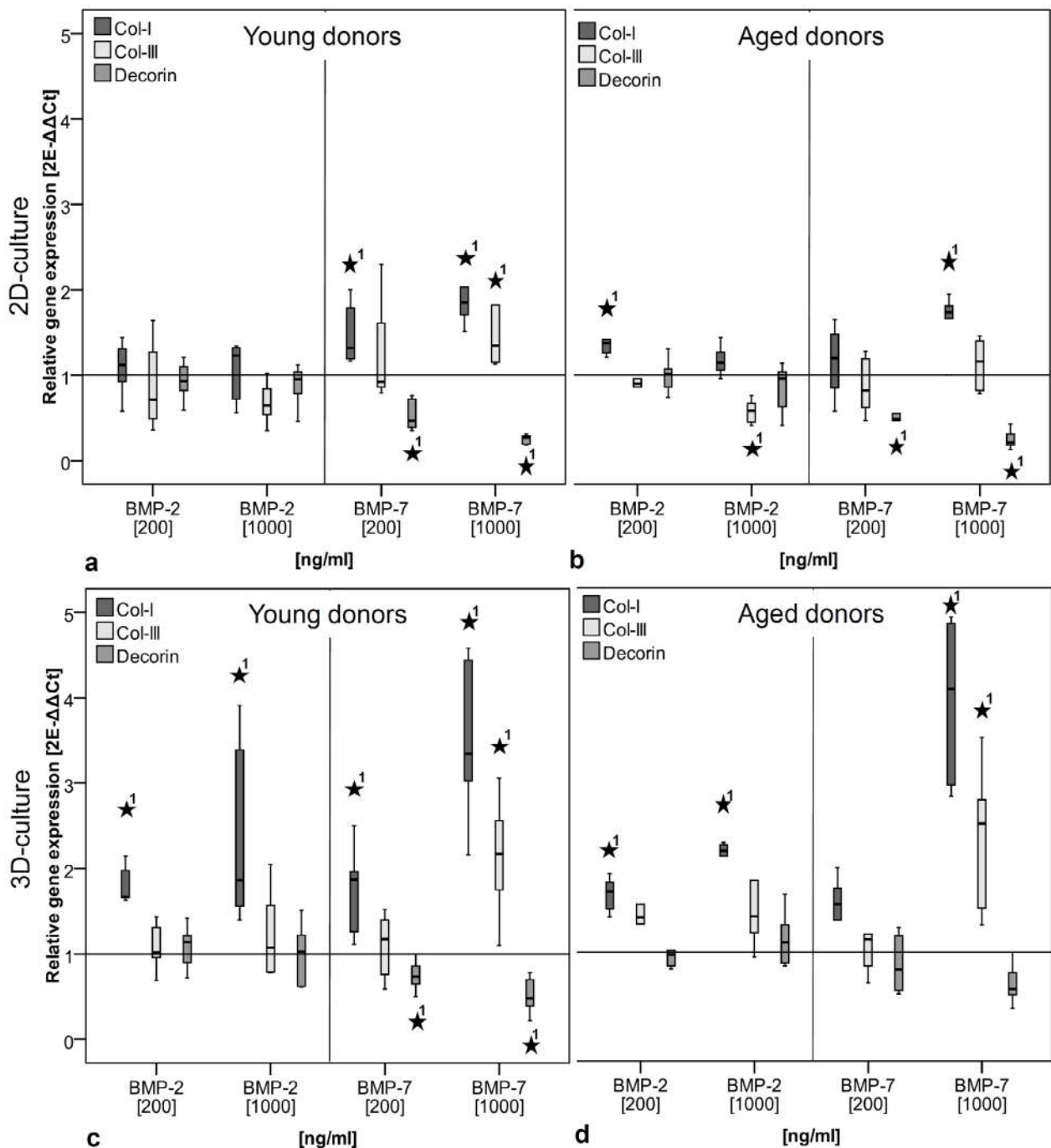
Fig. 6. Effect of growth factors on Col-I (dark gray bars), Col-III (light gray bars), and Decorin (grey bars) expression of tenocyte-like cells. (**a**) Marker expression of cells of young donors in 2D-culture was not significantly affected when treated with BMP-2. BMP-7 significantly increased Col-I expression in both and Col-III expression in high concentration, and decreased the Decorin expression. (**b**) Cells of aged donors in 2D-culture showed increased Col-I expression in low BMP-2 and high BMP-7 concentration. High BMP-2 concentration significantly decreased Col-III expression. Decorin expression was significantly decreased after BMP-7 treatment. (**c**) In 3D-culture of cells of young donors Col-I expression was significantly increased by BMP-2 and BMP-7 in all concentrations. Col-III expression was increased by high BMP-7 concentration, and Decorin was decreased by both BMP-7 concentrations, but not by BMP-2. (**d**) Cells of aged donors in 3D-culture showed significantly increased Col-I expression after BMP-2 application in both and BMP-7 in the high concentration. BMP-7 significantly increased Col-III expression in high concentration. The asterisks (*) mark significant differences from the untreated control. The numbers give details for the *p*-value: 1: $p \le 0.05$; 2: $p \le 0.001$.

Col-III expression was found for high BMP-2 treatment of the cells of aged donors in the 2D-culture (Fig. 6).

The expression of decorin, the most important proteoglycan in the tendon, was significantly decreased

after application of BMP-7 dose-dependently, except the decrease was not significant in the aged donor group in 3D-culture. BMP-2 did not affect the decorin expression (Fig. 6).
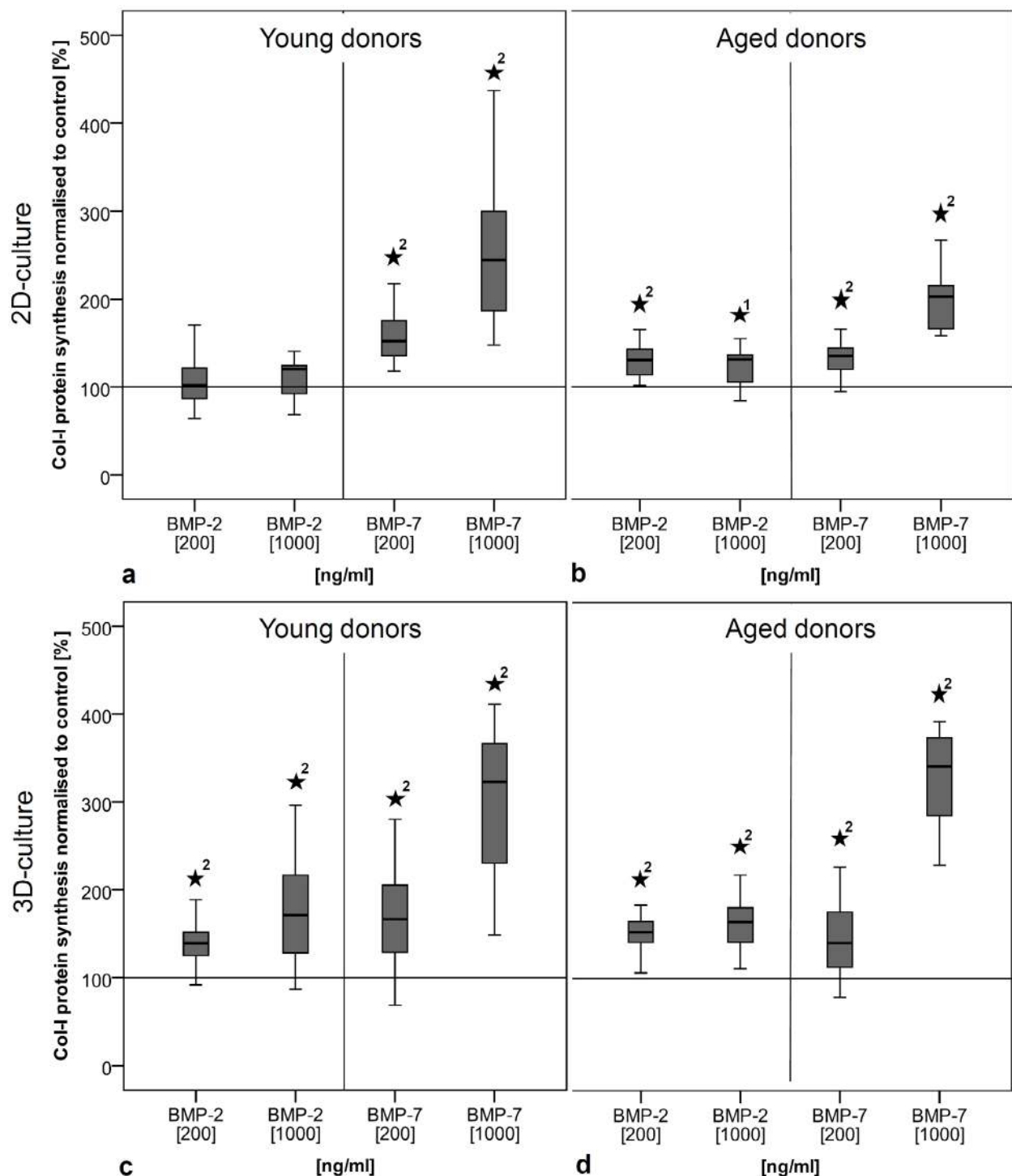
Fig. 7. Col-I protein synthesis in cell culture supernatant of day 7 after growth factor application. Col-I synthesis was calculated relative to total protein and given as percentage to untreated control. (**a**) Application of BMP-7 but not BMP-2 in 2D-culture of cells of young donors increased the Col-I protein synthesis significantly. (**b**) Col-I protein synthesis was significantly increased by both BMP-2 and BMP-7 concentrations in cells of aged donors in 2D-culture. (**c** and **d**) The BMP-2 and BMP-7 treatment in 3D-culture of tenocyte-like cells of young (**c**) and aged (**d**) donors significantly increased Col-I protein synthesis at all concentrations. The asterisks (*) mark significant differences from the untreated control. The numbers give details for the *p*-value: 1: $p \leq 0.05$; 2: $p \leq 0.001$.

The results for the expression of osteocalcin, as a marker for osteoblasts, and Col-II, as a marker for chondrocytes, did not suggest an osteogenic or chondrogenic differentiation of the tenocyte-like cells within the experimental period after BMP-2 or BMP-7 treatment (data not shown).

Col-I protein synthesis
In general, the Col-I protein synthesis was dose-dependently increased after BMP-2 and BMP-7 application in 2D- and 3D-culture in tenocyte-like cells of young and aged donors. Only the BMP-2 treated cells of young donors
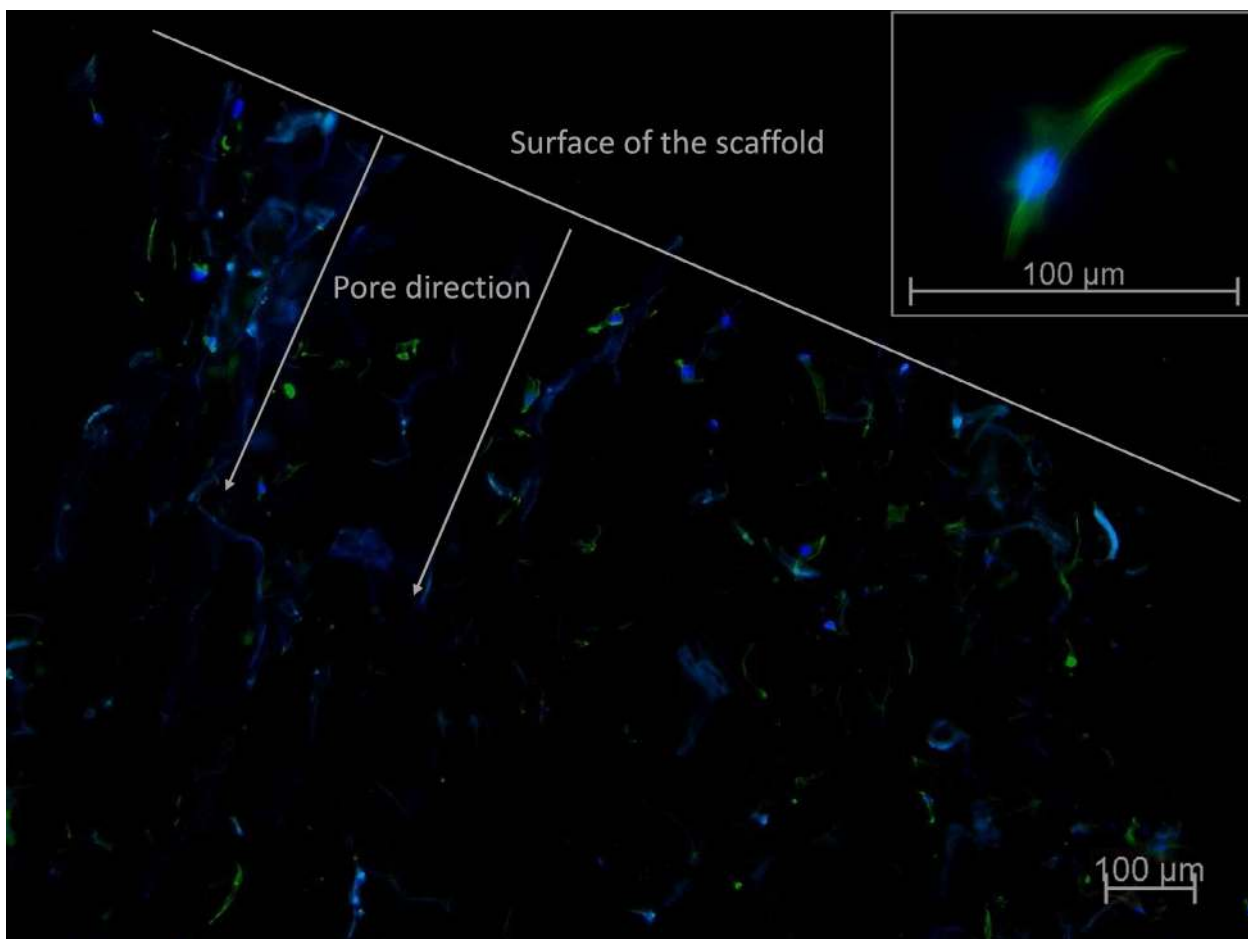
Fig. 8. Exemplary overview of tenocyte-like cells in Optimaix collagen scaffold after 7 days of stimulation with 1000 ng/mL BMP-7. Green staining represents Alexa Fluor Phalloidin stained actin filaments of tenocyte-like cells. Blue dots within the actin filaments are Dapi stained cell nuclei. The other blue parts show some autofluorescence of the scaffold.

in the 2D-culture revealed no significant differences when compared to the untreated control (Fig. 7).

In 2D-culture, the low dosed BMP-2 treatment increased Col-I protein synthesis in the aged donor group as compared to the cells of young donors. Contrary results were found for the low BMP-7 concentration group.

Discussion

In patients undergoing surgical rotator cuff repair, recurrent defects and non-healing are the primary postoperative complications. Clinical observations have indicated a correlation between increased retear rates and patient age (Boileau *et al*., 2005; Milgrom *et al*., 1995; Sorensen *et al*., 2007; Yamaguchi *et al*., 2006). To better understand this relationship, the present study has identified cell biological characteristics that might account for this correlation. Furthermore, the stimulation with growth factors such as BMP-2 and BMP-7 revealed no distinct age-dependent effects on tenocyte-like cells.

The results of the present study demonstrate that the cell biological characteristics of tenocyte-like cells differ depending on their donor characteristics. Cells from aged donors showed a decreased cell growth and stem cell

potential in terms of their potential for self-renewal and osteogenic differentiation. These findings indicate a slower cell metabolism in cells of aged donors and might be a reason for a weaker tendon-bone healing of the rotator cuff. It was reported in the literature that additionally to the age of the donors and amongst other factors, the tear size can influence the characteristics of the tenocytes of the rotator cuff (Chaudhury and Carr, 2012). Microarray analysis for example revealed that the expression of Collagen 4, 12 and 14 were upregulated in small tears compared to massive tears and the expression of Aggrecan was upregulated in massive but not small tears compared to normal controls (Chaudhury *et al*., 2011). While keeping all clinical and radiological parameters equal between the young and the aged donors group, an influence of the respective parameters on the results of the study should be excluded.

Regarding the stimulation potential of the tenocyte-like cells, no clear age-related differences could be observed. Cells from both young and aged donors could be stimulated with BMP-2 and BMP-7, but more with BMP-7. This suggests that BMP-7 might have therapeutic potential to biologically enhance tendon-bone healing of the rotator cuff.

There have been very few studies investigating tendon cell cultures with respect to their age-dependent

differences. In previous studies, foetal and adult cells from animal ligaments and tendons were compared, and it was shown that higher levels of Col-I and –III were present in foetal cells (Brink *et al.*, 2006; Stalling and Nicoll, 2008). In the present study, no age-related differences were found with respect to Col-I protein synthesis, or the expression of Col-I and -III. This indicates that age-related changes are not seen at the level of the extracellular matrix in the present study.

Decorin is the most important proteoglycan in tendons, and it has been reported to enhance tissue regeneration while decreasing scar formation (Jarvinen and Ruoslahti, 2010). The anti-scarring activities of Decorin are caused by its inhibitory effects on TGF-β. TGF-β1 is the main scar-inducing isoform, and TGF-β2 augments the actions of TGF-β1, whereas TGF-β3 antagonises scar formation (Brunner and Blakytny, 2004; Bandyopadhyay *et al.*, 2006; Ferguson *et al.*, 2009). No significant differences in the expression of Decorin, TGF-β1 and TGF-β3 were found in the two groups of tenocyte-like cells. While the expression of TGF-β2 was lower in aged donor cells, this change was not significant ($p = 0.054$). A higher expression of TGF-β2 in aged donor cells might lead to increased scar formation *in vivo*, and may therefore translate into higher rates of recurrent defects.

Several *in vitro* studies have demonstrated that tendon cell cultures express stem cell markers and are able to both self-renewal and to undergo multipotent differentiation (Bi *et al.*, 2007; de Mos *et al.*, 2007; Zhang and Wang, 2010; Steinert *et al.*, 2011). Our results are consistent with these previous findings, and additionally, it was shown that tenocyte-like cells of both young and aged donors have this stem cell potential. Cells from both groups fulfilled the minimal stem cell criteria (Dominici *et al.*, 2006). However, in cells from aged donors, the stem cell potential was decreased with respect to their potential for self-renewal and osteogenic differentiation. This may be another reason for inferior tendon-bone healing in elderly patients. To the best of our knowledge, there have been no previous tendon cell culture studies, analysing the age-related differences in stem cell potential. However, in a cell culture study with rat MSCs, no age-related changes were observed for stem cell phenotype (CD11b, CD29, CD31, CD44, CD45, CD81, CD90, CD105, CD172a), or osteogenic and adipogenic differentiation (Tokalov *et al.*, 2007). Moreover, Stenderup *et al.* have reported that the bone forming capacity of human MSCs maintained in both young (24-27 years) and aged (71-81 years) donors (Stenderup *et al.*, 2004). However, both studies lack a quantitative analysis and therefore cannot evaluate quantitative changes in the differentiation capacity of the cells, as it was done in the present study. Several *in vitro* studies using rat osteoprogenitors (Bellows *et al.*, 2003), and human bone marrow cells (Oreffo *et al.*, 1998a; Oreffo *et al.*, 1998b; D'Ippolito *et al.*, 1999; Stenderup *et al.*, 2001) have demonstrated that the capacity for self-renewal is not age-dependent. These contrasting findings may result from the use of different cell types (progenitor cells *versus* tenocyte-like cells) or different study design. Results consistent with the findings in this study were found by Oreffo *et al.*, who reported that colony size is reduced

in bone marrow cells of aged donors (Oreffo *et al.*, 1998a; Oreffo *et al.*, 1998b).

In a previous study, BMP-2 and BMP-7 was applied to tenocyte-like cells that had been pooled from rotator cuffs and biceps tendons, without distinguishing between young and aged patients (Pauly *et al.*, 2011b). In the present study, only SSP tendons with a MRI classification of fatty muscle infiltration of grade 0 and 1 were biopsied. Also in contrast to this previous study, other concentrations of BMP-2 and -7 were used. Additionally, cells were stimulated with BMP-2 and -7 in 3D-culture, which represents a system closer to *in vivo* conditions (Stoll *et al.*, 2010).

In general, tenocyte-like cells of young and aged donors showed a very robust response to BMP stimulation. As reported in the previous study, BMP-7 exposure exerts stronger effects than BMP-2 (Pauly *et al.*, 2011b). While other groups have found no effect of BMP-2 on tenocyte cultures (Salingcarnboriboon *et al.*, 2003; Thomopoulos *et al.*, 2007), the previous findings that BMP-2 increased Col-I expression and protein synthesis were confirmed. This was observed in previous findings regarding BMP-7, where once again, cell activity, Col-I expression and protein synthesis were strongly increased following BMP-7 application. These results are consistent with the findings of Tsai *et al.*, Yamada *et al.* and Yeh *et al.*, who have independently reported of significantly increased cell activity / proliferation and Col-I protein synthesis following BMP-7 stimulation in rat and bovine tendon cells of different origin (Tsai *et al.*, 2003; Yamada *et al.*, 2008; Yeh *et al.*, 2008).

Various *in vivo* studies have reported on improved tendon-bone healing following application of BMP-2 and BMP-7 (Rodeo *et al.*, 1999; Martinek *et al.*, 2002; Mihelic *et al.*, 2004; Higuera *et al.*, 2005; Ma *et al.*, 2007). In general, these studies have shown improved tendon-bone integration and higher biomechanical strength following ACL reconstructions or SSP tendon repair. The results from the present study suggested that such *in vivo* findings after BMP treatment may be explained by increased cell activity or Col-I expression and protein synthesis.

Decorin expression was decreased after BMP-7 treatment. Tendon healing of the rotator cuff often results in the formation of a biomechanically inferior scar tissue (Gerber *et al.*, 1999; Rodeo, 2007). Decorin reduces scar formation, and may improve the biomechanical properties of tendons (Jarvinen and Ruoslahti, 2010). Thus, a reduced decorin expression after BMP-7 application may enhance scar formation, and therefore impair regeneration at the tendon bone insertion site. Further *in vivo* studies will be necessary to better understand this process.

Currently, the effects of BMP-2 and BMP-7 were analysed in 2D- and 3D-culture, with a more pronounced effect seen in 3D-cultures. The 3D-approach is a better model of the *in vivo* milieu, and it prevents tenocytes from redifferentiating or changing of phenotypes (Schulze-Tanzil *et al.*, 2004; Yao *et al.*, 2006; Stoll *et al.*, 2010). As demonstrated, the 3D-culture seems to be a better system for analysing the response of cells to growth factors. This has also been reported in a study using human skin fibroblasts after epidermal growth factor (EGF) treatment (Colige *et al.*, 1990). However, the analysis of cell activity

in 3D-culture by Alamar Blue assay was not as sensitive as it was in 2D-culture, because the red colour mainly formed inside the 3D-scaffold, and the transfer to the surrounding medium was limited. 2D-culture remains important to visualise cell morphology within the experimental period, a capability, which is hindered when using light microscopy in 3D-cultures. Moreover, as it has been shown that cells change morphology with increasing passages (Yao *et al*., 2006), cells at the earliest passage as possible were used, with passage 2 being the maximum.

Marginal significant differences between young and aged donor cells responded to BMP were observed. Increased cell activity (with 200 ng/mL BMP-7) and Col-I protein synthesis (with 200 ng/mL BMP-2) were found in the aged donor cells compared to the cells of young donors. In contrast, the Col-I protein synthesis was decreased after incubation with 200 ng/mL BMP-7 in cells of aged donors. The differences were significant between both groups, but did not follow a consistent pattern. These results suggest that the effect of BMP-2 and BMP-7 on cells of aged donors is comparable to those of young donors.

Study limitations
Due to limitations in obtaining tissue biopsies, the present data were extracted exclusively from cells harvested at the time of rotator cuff surgery, with no cells used from pre- or post-operative stages. Prospective clinical and radiographic (MRI) follow up over a period of at least 24 months are needed (and currently being compiled) to investigate a possible correlation with baseline biologic parameters. This would provide a holistic approach to assess for age-related differences in rotator cuff regeneration potential.

Conclusions

Age-related differences were found in the cell biological characteristics of the tenocyte-like cells, which may be one reason for varying healing rates following rotator cuff repair of patients from different age groups. It may follow that a reduced cell growth and stem cell potential, observed *in vitro*, correlates with a reduced number of tenocyte-like cells *in vivo*, and therefore may contribute to an inferior SSP tendon repair in elderly patients.

However, no distinct age-related differences were found in terms of cell response to BMP-2 or BMP-7. In other words, both young and aged male patients might benefit from BMP-2 or BMP-7 treatment. At the cellular level, our findings suggest that BMP-7 seems to be most promising for the treatment of rotator cuff tears to reduce rates of non-healing or recurrent defects following surgery.

The study might contribute to a more patient specific surgical repair of the rotator cuff instead of the current uniform therapy.

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Discussion with Reviewers

Reviewer I: Please justify the 1-day sampling point rather than longitudinal analysis!

Authors: We did a longitudinal analysis of the cell growth for the cell biological characteristics and cell activity for the stimulation potential, because it is a non-toxic method for the cells and can be used at different time points within a culture. For the RNA isolation and Real-Time PCR for each time point additional tenocytes would be needed, which would have required much more primary tenocytes. The amount of cells at early passages for each patient, however, is very limited. We therefore decided to use only the cell

growth/cell activity analysis as longitudinal parameter and the others as endpoint parameters. However, we analysed the Col-I synthesis from cell culture supernatant at day 4 and 7 additionally to the 14-day time point.

Reviewer II: Why were the cells fixed with formalin before the staining? The reaction here is based on the enzymatic activity of the ALP, which might be reduced by fixation!

Authors: As stated in the literature the fixation of cells prior to ALP staining is a common procedure (Abukawa *et al.*, 2009, additional reference), and is also recommended for commercial kits from for example Sigma-Aldrich. The fixation of the cells should avoid detachment of the cells from the bottom within the incubation time.

Reviewer II: Were there signs of hypoxia in the 3D scaffold?

Authors: Unfortunately, we cannot provide a satisfactory answer to that question, since we did not analyse any hypoxic effect like apoptosis in the scaffolds. The scaffolds are relatively small, have a macroporous structure and cells were seeded at a low density. Furthermore, the cell activity of the tenocytes in the scaffolds was good over the entire culture period. In the sections we stained as examples, the macroscopic images revealed good cell morphology even in the middle of the scaffold. We therefore assume that no hypoxia takes place in the scaffolds. Additionally to that another group working with these scaffolds seeded larger scaffolds (diameter 13 mm versus 6 mm) with a much higher cell density (7.5×10^3 / μL *versus* 2.35×10^2 / μL) and morphological analysis of the cells in the scaffolds revealed an overall viable phenotype (Petersen *et al.*, 2012, additional reference).

Reviewer II: The authors showed the stem cell-like phenotype as well as the ability of cells to differentiate towards osteoblasts in response to dexamethasone. Furthermore the cells respond to BMP by upregulating Col I, indicating that the receptors are present. Nevertheless, in stimulation experiments with BMP no osteoblast differentiation was observed. Can the authors give an explanation or speculation about the mechanism involved in the suppression?

Authors: We agree that BMP-2 and BMP-7 can facilitate ectopic bone formation *in vivo* and promote and regulate osteoblastic differentiation of various cell types *in vitro*. We speculate that a 7-day BMP stimulation is a too short a time frame to see a direct effect on the osteoblastic differentiation evaluated by osteocalcin expression of the

tenocytes. Most differentiation studies for BMP-2 and -7 last about 2-3 weeks before analysis of the respective markers (Shen *et al.*, 2010; Steinert *et al.*, 2011; Luong *et al.*, 2012). We assume that the BMP stimulation at first led to an induced proliferation and matrix production (Col-I and –II) of the tenocytes and that differentiation takes place later on when cells are more than 100 % confluent. Furthermore, in the literature additional factors like L-ascorbic acid, β-glycerophosphate or dexamethasone are added to the cell cultures (Asahina *et al.*, 1996, additional reference; Bi *et al.*, 2005; Steinert *et al.*, 2011, text references), which may additionally influence the differentiation capacity. We speculate further, that since only a portion of the tenocyte-like cells in our study seem to have a stem cell potential, the potential for osteogenic differentiation after stimulation with the BMPs may be slower or weaker compared to the stem cell approaches found in the literature (Luu *et al.*, 2007; Shen *et al.*, 2010).

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