Aus dem **Julius Wolff Institut**
der **Medizinischen Fakultät Charité-Universitätsmedizin Berlin**

Dissertation

Einfluss des Alters, des Geschlechtes und der fettigen Infiltration auf die zellbiologischen Eigenschaften und das Stimulationspotential humaner Tenozyten der Rotatorenmanschette

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Abstrakt (deutsch)

Die Heilung von Rotatorenmanschetten-Rupturen stellt ein großes klinisches Problem dar, wobei häufig Komplikationen auftreten, wie die Nichtheilung des Gewebes und Rerupturen. Verschiedene Risikofaktoren können die Heilung zudem negativ beeinflussen, wie ein höheres Alter, das weibliche Geschlecht oder eine höhere fettige Infiltration des beteiligten Muskels. Die Stimulation mit Wachstumsfaktoren, z.B. Bone Morphogenetic Protein-2 (BMP-2) und BMP-7, könnte die Heilung der Rotatorenmanschette zukünftig verbessern. BMP-2 und BMP-7 sind für die Knochenheilung klinisch zugelassen und könnte neben den Osteoblasten auch Tenozyten stimulieren und die Heilung somit von zwei Seiten positiv beeinflussen.
Ziel der Arbeit war es, Tenozyten aus Supraspinatussehnen verschiedener Spendergruppen zu charakterisieren, um mögliche zellbiologische Eigenschaften zu identifizieren, die für eine schlechtere Heilung verantwortlich sein könnten. Weiterhin sollte untersucht werden, ob die Zellen von einer Stimulation mit BMP-2 oder -7 profitieren können.
Es wurden Tenozyten von 36 Spendern aus 6 Gruppen charakterisiert. Die Gruppen unterschieden sich hinsichtlich Alter (jünger und älter als 65 Jahre), Geschlecht und fettiger Infiltration (Goutalliergrad 0-1, 2, 3-4). Es wurde die Zelldichte, das Zellwachstum, das Genexpressionsprofil, die Kollagen Synthese sowie das Stammzellpotential der Tenozyten untersucht. Zusätzlich wurden die Zellen mit 200 und 1000 ng/ml BMP-2 oder BMP-7 in einer 2D-Kultur und in einer 3D-Kultur stimuliert. Untersucht wurde der Effekt auf die Zellaktivität, die Genexpression sowie die Kollagen Typ I Synthese.
Tenozyten aller Spendergruppen zeigten ein Tenozyten-spezifisches Expressionsprofil und Stammzellpotential. Tenozyten älterer Spender und von Spendern mit hoher fettiger Infiltration hatten ein signifikant geringeres Zellwachstum und Koloniebildungspotential. Tenozyten älterer weiblicher Patienten zeigten zudem eine signifikant verringerte Kollagen Typ I Synthese. Durch die Stimulation mit BMP-2 oder BMP-7 konnte vor allem die Zellaktivität sowie die Expression und Synthese von Kollagen Typ I und III in den Tenozyten signifikant gesteigert werden. BMP-7 hatte eine stärkere stimulierende Wirkung auf die Zellen als BMP-2. In der 3D-Scaffold-Kultur war die Wirkung der BMPs deutlich verstärkt gegenüber der 2D-Kultur. Zellen älterer weiblicher Spender und von Spendern mit hoher fettiger Infiltration zeigten ein verringertes Stimultionspotential.
In der vorliegenden Arbeit wurden zellbiologische Parameter identifiziert, die in Tenozyten von Patienten aus Risikogruppen verschlechtert waren. Dies könnte eine Ursache für ein schlechteres Heilungspotential bei diesen Patientengruppen sein und somit einen möglichen Ansatzpunkt für eine verbesserte Therapie darstellen. Durch die Anwendung von BMP-2 und BMP-7 konnten wichtige Eigenschaften der Tenozyten aller Patientengruppen stimuliert werden. Die Unterstützung der Heilung nach Rotatorenmanschetten-Rekonstruktionen mit BMP-2 oder BMP-7 könnte aus zellbiologischer Sicht als zukünftige Therapieoption in Frage kommen.

Abstract (english)

Healing of rotator cuff tears remains challenging. Complications, such as non-healing or re-tearing of the tendon tissue, occur often and depend on diverse risk factors such as an older age, the female sex, or a higher fatty infiltration of the corresponding muscle. Biological stimulation using growth factors, for example bone morphogenetic protein 2 (BMP-2) and BMP-7, may help to improve tendon-bone healing. BMP-2 and BMP-7 are approved for the treatment of bone defects and could stimulate tenocytes as well as osteoblasts and may therefore positively influence the healing at the rotator cuff via both cell types.

The aim of the project was to characterize tenocytes from supraspinatus tendons of different donors groups, to identify possible characteristics, which may be responsible for an inferior healing potential in these patients. Furthermore, the aim was to investigate if tenocytes can benefit from the stimulation with BMP-2 or BMP-7.

Tenocytes from 36 donors, divided into 6 groups, were characterized. The groups differed regarding age (</> 65 years), sex and muscle fatty infiltration (Goutallier grade 0-1, 2, 3-4). The cell density, cell growth, gene expression profile, collagen synthesis, and stem cell potential were analyzed. Furthermore, cells were stimulated with 200 and 1000 ng/ml BMP-2 or BMP-7 in 2D- and 3D-culture. The effect of the growth factors was determined regarding cell activity, gene expression, as well as collagen type I synthesis.

Tenocytes of all donor groups showed a specific expression profile and stem cell potential. Cells of older donors and donors with a higher grade of muscle fatty infiltration had a significantly decreased cell growth and colony forming ability. In addition, tenocytes of older females showed a significantly reduced collagen type I synthesis. By stimulating tenocytes with BMP-2 or BMP-7, the cell activity was increased, as well as the expression and synthesis of collagen type I and III. BMP-7 had a stronger stimulating effect on the cells than BMP-2. The established 3D scaffold culture of the cells highly increased the effect of the BMPs. In tenocytes of older female donors and donors with high fatty infiltration, a reduced stimulation potential was observed.

In the present study, cellular biological parameters could be identified, which were inferior in tenocytes of patients from risk groups. This may be a reason for a weaker healing potential in these donor groups and could represent a possible starting point for an improved therapy of rotator cuff healing. By stimulation of tenocytes with BMP-2 and BMP-7, important cellular biological characteristics were improved in all donors groups. From a cellular perspective, the augmentation of the healing after rotator cuff reconstructions with BMP-2 or BMP-7 may be a possible treatment option for the future.

Einleitung

Rupturen von Sehnen der Rotatorenmanschette (RM) gehören zu den häufigsten Verletzungen bei Erwachsenen und stellen aufgrund einer oftmals unzureichenden Heilung nach der chirurgischen Rekonstruktion der Sehnen ein großes klinisches Problem dar.[3-4] Die Entstehung eines biomechanisch minderwertigen Narbengewebes, anstatt einer natürlichen Insertionsstelle zwischen der Sehne und dem Humeruskopf, wird für die schlechte Heilung verantwortlich gemacht.[5-6] Das Auftreten von Rerupturen oder auch die gänzliche Nichtheilung des Gewebes sind oft die Folge.[7-8] Dabei können viele verschiedene Parameter die Sehnen-Knochenheilung der RM entscheidend beeinflussen (Abbildung 1). Vor allem das Alter stellt einen entscheidenden Risikofaktor dar, wobei die Versagensrate nach der Rekonstruktion mit dem Alter stark ansteigt.[3,7,9] Ein Alter von 60-65 Jahre scheint dabei die kritische Grenze zwischen einer Heilung und einer Nichtheilung darzustellen.[6-7,9] Ein Einfluss des Geschlechtes auf die Heilung von Rupturen der RM konnte nur in wenigen Studien gezeigt werden, in denen Frauen eine schlechtere Heilung zeigten als Männer.[4,10] Ein höherer degenerativer Status einer Sehnenruptur, der anhand der fettigen Infiltration des beteiligten Muskels (Grad 0-4 nach Goutallier) beurteilt werden kann, stellt einen weiteren entscheidenden Risikofaktor dar.[11-13] Dabei ist die Sehnenintegration ab einem Goutalliergrad von 2 vermehrt mit Komplikationen verbunden ist.[11-12] Der molekularbiologische oder zellbiologische Hintergrund für den Zusammenhang zwischen klinischen Faktoren und der Heilung der RM-Sehnen ist bisher weitgehend unbekannt. Bekannt ist jedoch, dass die Tenozyten eine wichtige Rolle bei der Sehnenheilung spielen, da sie die notwendige extrazelluläre Matrix (EZM) für die Reparatur des Sehnengewebes synthetisieren und für das Remodeling verantwortlich sind. Es liegt somit nahe, den Grund für eine schlechtere Sehnenheilung bei bestimmten Patientengruppen auf zellulärer Ebene zu suchen. Es kann vermutet werden, dass eine verringerte metabolische Aktivität der Zellen oder eine geringere EZM Synthese die Heilung negativ beeinflusst. Das Vorhandensein von Sehnenstammzellen wurde in mehreren Studien nachgewiesen[14-16] und ist möglicherweise ebenfalls ein entscheidender Faktor bei der Sehnenheilung. Eine umfangreiche Analyse von Einflussparametern auf zellulärer Ebene ist wichtig, um Ansatzpunkte für eine mögliche Therapieoption darzulegen, die die Sehnenheilung zukünftig verbessern könnten.

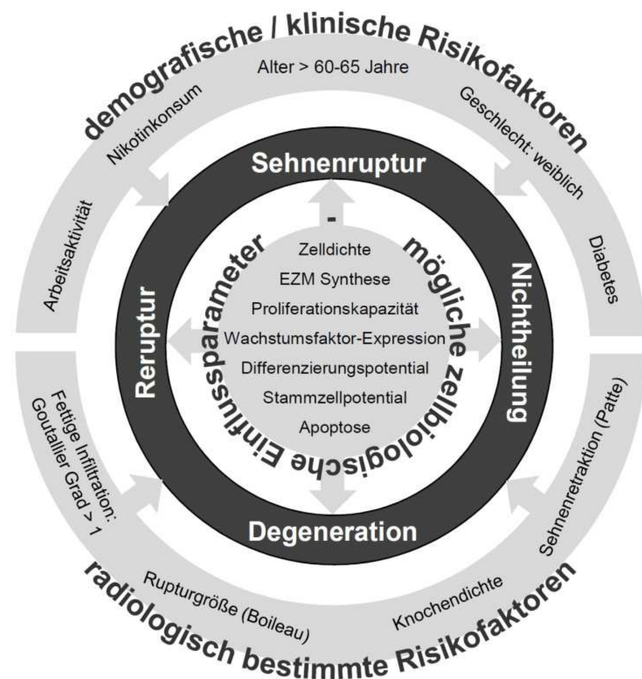

Abbildung 1: Risikofaktoren beeinflussen die Heilung nach RM-Rekonstruktionen. Zellbiologische Einflussparameter sind bisher unbekannt. Aus: Wildemann und Klatte 2011[1]

In verschiedenen biomechanischen Studien wurde der Fokus auf die Entwicklung verbesserter operativer Verfahren zur Fixierung der RM-Sehnen an den Humeruskopf durch neue Nahttechniken gelegt. Trotz verbesserter biomechanischer Eigenschaften beispielsweise von einer Doppelreihen-Rekonstruktion gegenüber einer Einfachreihen-Rekonstruktion (Abbildung 2)[17-18] konnte die Heilung der Sehnen-Knocheneinheit in der klinischen Anwendung nicht entscheidend verbessert werden.[2,19]

Der Einsatz von biologischen Verfahren wie zum Beispiel die Anwendung von Wachstumsfaktoren wäre denkbar, um die Heilung nach der Rekonstruktion zu unterstützen. Die Wachstumsfaktoren Bone Morphogenetic Protein-2 (BMP-2) und BMP-7 sind bereits für die Stimulation der Knochenheilung klinisch zugelassen.[20] Aber auch bezüglich der Sehnen-Knochenheilung konnten bereits positive Effekte beobachtet werden. Vor allem die biomechanische Belastbarkeit der Sehnen-Knocheneinheit nach vorderem Kreuzbandersatz[21-24] oder RM-Rekonstruktionen[25] konnte im Tiermodel verbessert werden. Auch in Zellkulturstudien zeigte sich ein stimulierender Effekt von BMP-2 und BMP-7 auf wichtige zellbiologische Eigenschaften, wie die Zellproliferation oder die Expression und Synthese von EZM.[26-28] Inwiefern Tenozyten bestimmter Patientengruppen von einer BMP-2 oder BMP-7 Stimulation besonders profitieren könnten, ist bisher noch nicht untersucht worden.

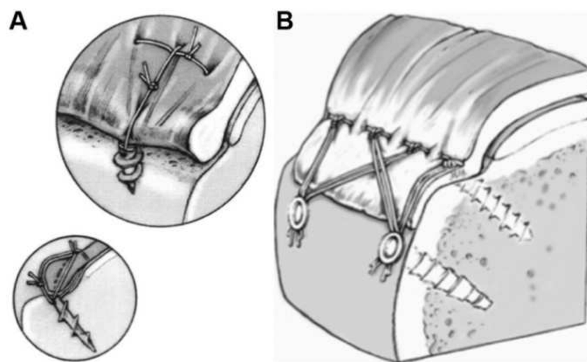

Abbildung 2: Schematische Zeichnung der Rekonstruktion der RM am Sehnen-Knochenübergang (A) Einfachreihen-Rekonstruktion (B) Doppelreihen-Rekonstruktion. Aus: Gerhardt et al. 2013[2]

Zielsetzung

Das Ziel der vorliegenden Promotionsarbeit war es, Tenozyten von RM-Sehnen ausführlich zu charakterisieren, um zellbiologische und molekularbiologische Parameter zu identifizieren, die für eine schlechtere Heilung bei bestimmten Patientengruppen verantwortlich sein könnten. Es sollte weiterhin gezeigt werden, ob nachteilige zellbiologische Eigenschaften der Tenozyten durch die Stimulation mit den Wachstumsfaktoren BMP-2 und BMP-7 verbessert werden können. Diese Erkenntnisse sollen dazu beitragen, zukünftig eine neue Therapie zur Verbesserung der Heilung nach RM-Rekonstruktionen zu entwickeln, die individueller an bestimmte Patientengruppen angepasst ist.

Methoden

Sehnenmaterial

Supraspinatus (SSP) Sehnenbiopsien wurden von Patienten gewonnen, bei denen ein arthroskopischer oder offener Eingriff zur Rekonstruktion der RM am Campus Mitte und Campus Virchow durchgeführt wurde. Alle Biopsien wurden nach einem standardisierten Protokoll von den klinischen Kollegen 3-5 mm vom proximalen Rupturbereich entnommen. Die Patienten gaben zuvor ihr schriftliches Einverständnis zur Verwendung von Material, welches ansonsten verworfen werden würde (Ethiknummer: EA1/060/09). Es wurden von jedem Patienten anonymisiert klinische Daten aufgenommen, die für die spätere Überprüfung eines Zusammenhanges von Patienteneigenschaften und zellbiologischen Charakteristika notwendig waren. Als Hauptparameter wurden die Patienten nach dem Alter, Geschlecht und der fettigen Infiltration des Muskels eingruppiert.

Zellisolierung und Kultivierung

Das Sehnenmaterial wurde nach dem Erhalt mit einem Skalpell zerkleinert und anschließend wurden die Tenozyten durch einen Verdau mit Kollagenase Typ CLS II (Biochrom AG) aus dem Gewebe isoliert. Das verdaute Material wurde in Zellkulturflaschen überführt und in DMEM/HAM's F12 Medium mit 10 % FCS und 1 % Penicillin/Streptomycin (alles Biochrom AG) unter Standardbedingungen (37 °C, 5 % CO_2) kultiviert. Ein Mediumwechsel erfolgte alle 2-3 Tage.

Zellcharakterisierung

Um den Einfluss des Alters, Geschlechtes und der fettigen Infiltration des Muskels auf Tenozyten beurteilen zu können, wurden je 6 Tenozytenkulturen aus der Gruppe der jungen und alten Männer und Frauen (je Goutallier 0-1) sowie der jungen Männer mit höheren Verfettungsgraden (Goutallier 2 und 3-4) ausführlich charakterisiert. Die Altersgrenze zwischen jungen und alten Spendern wurde auf 65 Jahre festgelegt.

Zelldichte:

Vor der Isolierung der Tenozyten wurde das Sehnenmaterial unter sterilen Bedingungen abgewogen. Nach dem Kollagenaseverdau wurde das Material mit einem Verhältnis Sehnenbiopsie (mg) zu Wachstumsfläche (cm^2) von 0,2 bis 0,3 ausgesät, um den Zellen ein vergleichbares Wachstum zu gewährleisten. Nach einer Woche wurde mit einem Alamar Blue Test (Biozol) die Zellzahl in den Zellkulturflaschen ermittelt. Der Alamar Blue Test basiert auf einer Redox-Reaktion, wobei die mitochondriale Aktivität von Zellen ermittelt wird und über eine Standardkurve mit definierten Zellzahlen die Zellzahl in der Kultur berechnet werden kann. Die ermittelte Zellzahl nach 7 Tagen wurde dann durch das Gewicht der Biopsie dividiert, um eine ungefähre Zelldichte zu erhalten.

Zellwachstum:

In Passage 2 wurden die Tenozyten in einer 48-Well-Platte in Dreifachbestimmungen ausgesät und über 14 Tage inkubiert. Mit dem Alamar Blue Test wurde die Zellzahl an den Tagen 1, 4, 7 und 14 nach Aussaat ermittelt. Zur Berechnung des Zellwachstums wurde die Zellzahl von Tag 1 von den Zellzahlen der anderen Zeitpunkte subtrahiert.

Genexpression:
Mittels Real-Time Polymerase Ketten Reaktion (RT-PCR) wurde die Expression der Gene Kollagen Typ I, II, III, Decorin, Scleraxis, Tenomodulin, Mohawk, Transforming Growth Faktor-β (TGF-β) 1, 2, 3 sowie das Haushaltsgen Glyceraldehyd-3-Phosphat-Dehydrogenase (GAPDH) untersucht.
Die RNA wurde mit dem NucleoSpin RNA II Kit (Macherey-Nagel) von Tenozyten in Passage 2 isoliert. Nach der Quantifizierung der RNA wurden 100 ng RNA mit dem qScript cDNA Supermix (Quanta BioSciences) in komplementäre DNA (cDNA) umgeschrieben. Anschließend erfolgte die RT-PCR mit 1,25 ng cDNA als Matrize. Hinzu kamen 12,5 µl Sybr Green Supermix (Quanta BioSciences), 1 µl Primermix (10 µM) und 6,5 µl RNase/DNase-freies Wasser. Das folgende PCR Programm diente zur Amplifizierung der RNA: Initiale Denaturierung 95 °C, 40 Amplifikationszyklen (95 °C 15 s, Annealing Temperatur 45 s, 72 °C 30 s). Die RT-PCR wurde mit einer Schmelzkurve zur Identifizierung von Primerdimeren abgeschlossen. Die relative Genexpression wurde auf das Haushaltsgen GAPDH bezogen und mit der $2^{-\Delta Ct}$ Methode berechnet.

Kollagen Typ I Synthese:
Um die Proteinsynthese des wichtigsten Kollagens in der Sehne zu untersuchen, wurde das MicroVue C1CP EIA Kit verwendet. Dieser ELISA basiert auf der Analyse des bei der Kollagen Typ I Synthese abgespaltenen Prokollagens. Dadurch kann proportional auf die Menge an synthetisiertem Kollagen Typ I geschlossen werden. Zur Analyse wurden die Zellkulturüberstände von Tag 14 aus der Zellwachstumsanalyse herangezogen. Zur Normalisierung der Daten wurde die Gesamtproteinmenge mit dem Bradfordassay (Thermo Fisher Scientific) untersucht.

Stammzellpotential:
Damit Zellen als Stammzellen definiert werden können, müssen drei Kriterien erfüllt sein. Erstens müssen die Zellen fähig sein Kolonien aus einer einzelnen Zelle zu bilden. Weiterhin müssen mindestens 95 % der Zellen Stammzellmarker (CD29, 44, 73, 90, 105) auf ihrer Oberfläche exprimieren, während maximal 2 % der Zellen Negativmarker (CD11b, 14, 19, 34, 45) exprimieren dürfen. Als Letztes müssen die Zellen zu multipotenter Differenzierung fähig sein.[29]
Koloniebildungpotential:
Zur Untersuchung des Koloniebildungspotentials wurde der Colony Forming Unit Test (CFU-Test) durchgeführt. 1000 vitale Zellen wurden in einer 10 cm Petrischale in Dreifachbestimmungen ausgesät und über 11 Tage kultiviert. Anschließend wurden die Kolonien mit Methylenblau/Azur gefärbt. Die Anzahl der Kolonien sowie die Koloniegröße wurden mit einem Bildanalyseprogramm (ImageJ) mit adaptierbarem Treshhold ausgewertet, wobei Kolonien zwischen 1 und 10 mm^2 gezählt wurden.
Stammzellphänotyp:
Die Analyse von Oberflächenmarkern auf den Tenozyten wurde im Durchflusszytometer untersucht. Ein Drittel der Zellen wurde mit einem Leben/Tod Reagenz sowie Antikörpern gegen die oben genannten Antigene gefärbt. Die restlichen Zellen dienten als ungefärbte Kontrolle oder wurden mit entsprechenden IgG-Kontrollen gefärbt, um unspezifische Bindungen auszuschließen. Nach der Fixierung der Zellen mit Paraformaldehyd erfolgte die Messung der Färbung mit dem BD Canto II System (BD Biosciences).
Multipotente Differenzierung:
Um die multipotente Differenzierung der Tenozyten zu zeigen, wurden diese mit osteogenem, adipogenem und chondrogenem Medium stimuliert.[14-15] Für die osteogene und adipogene

Differenzierung wurden die Zellen in einer 24-Well-Platte in einer Dreifachbestimmung ausgesät und bis zur Konfluenz kultiviert. Für die chondrogene Differenzierung wurden 2,5-3 x 10^5 vitale Zellen in 15 ml Falkon Reaktionsgefäßen pelletiert. Die chondrogene Differenzierung konnte für jede Untersuchungsgruppe nur exemplarisch durchgeführt werden, da für diesen Versuch sehr viele Zellen benötigt werden. Die Stimulation der Zellen erfolgte über 3 Wochen mit 2-maligem Mediumwechsel pro Woche. Zum Nachweis der Differenzierung wurden die osteogen differenzierten Zellen mit Alizarin Red S, die adipogen differenzierten Zellen mit Oil Red O und die chondrogen Zellpellets mit Alcian Blau gefärbt. Durch das Auflösen der Farbreaktionen bei der osteogenen und adipogenen Differenzierung konnten diese quantifiziert werden. Zellen, die mit normalem Kultivierungsmedium kultiviert wurden, dienten als Negativkontrollen. Zur Validierung der osteogenen Differenzierung wurde exemplarisch eine Alkalische Phosphatase (ALP) Färbung durchgeführt.
Alle Differenzierungen wurden durch die Analyse der Expression spezifischer Differenzierungsmarker validiert. Für die osteogene Differenzierung wurde die Expression von Alkalische Phosphatase Tissue-nonspecific Isozyme (ALPL) und Runt-related Transcription Factor 2 (Runx2) untersucht, für die adipogene Differenzierung die Expression von Peroxisome Proliferator-activated Receptor Gamma (PPARγ), Lipoprotein Lipase (LPL) und Fatty Acid Binding Protein 4 (FABP4) und für die chondrogene Differenzierung diente Kollagen Typ II, Aggrecan und Cartilage Oligomeric Matrix Protein (COMP). Als Haushaltsgen diente das Ribosomal Protein L13 zur Normierung der Genexpression. Die RNA der osteogen und adipogen differenzierten Tenozyten wurden mit dem NucleoSpin RNA II Kit direkt in den Wells isoliert. Die RNA der Dreifachbestimmungen wurde gepoolt. Zur Isolierung der RNA aus den chondrogenen Zellpellets wurden 2 Pellets gepoolt und mit peqGOLD TriFast (Peqlab) und einem Precellys System mit Keramik Kügelchen unterschiedlicher Größe (Peqlab) durch schnelle Auf- und Ab-Bewegung bei 5000 rpm homogenisiert. Durch Zugabe von Chloroform wurde die RNA in die wässrige Phase überführt. Diese wurde 1 zu 1 mit 75 % Ethanol versetzt und anschließend mit dem NucleoSpin RNA II Kit aufgereinigt. Die cDNA Synthese und die RT-PCR wurden durchgeführt wie im Abschnitt Genexpression beschrieben.

Wachstumsfaktorstimulation

Test der relativen Aktivität von BMP-2 und BMP-7:
Um die relative Aktivität von BMP-2 und BMP-7 zu überprüfen wurden C2C12-Zellen, eine murine Vorläufer-Zelllinie, welche unter BMP-Stimulation in Osteoblasten differenzieren, mit 200 und 1000 ng/ml BMP-2 (Wyeth) und BMP-7 (R&D Systems GmbH) behandelt. Nach 3 Tagen wurde die Zellaktivität mit dem Alamar Blue Assay ermittelt und die ALP Aktivität bestimmt.

BMP-2 und BMP-7 Stimulation:
Je 6 Tenozytenkulturen pro Gruppe wurden zur Untersuchung des Einflusses des Alters, Geschlechtes und der fettigen Infiltration mit je 200 und 1000 ng/ml BMP-2 und BMP-7 stimuliert. Das Versuchsschema ist in Abbildung 3 dargestellt. Die Tenozyten wurden in einer 2D-Kultur in 48-Well Platten und einer 3D-Kultur in makroporösen porcinen Kollagen Typ I Scaffolds (Matricell) stimuliert. 3 Tage nach der Aussaat der Zellen wurden sie 24 Stunden in Hungermedium inkubiert, um den Zellzyklus anzugleichen. Dann erfolgte die BMP-Stimulation für 7 Tage, wobei die Wachstumsfaktoren zu 3 Zeitpunkten an Tag 0, 3 und 5 appliziert wurden. An den Tagen 0, 3, 5 und 7

der Stimulation wurde die Zellaktivität bestimmt und zum Ende des Versuches die RNA isoliert sowie die Kollagen Typ I Synthese in den Zellkulturüberständen bestimmt.

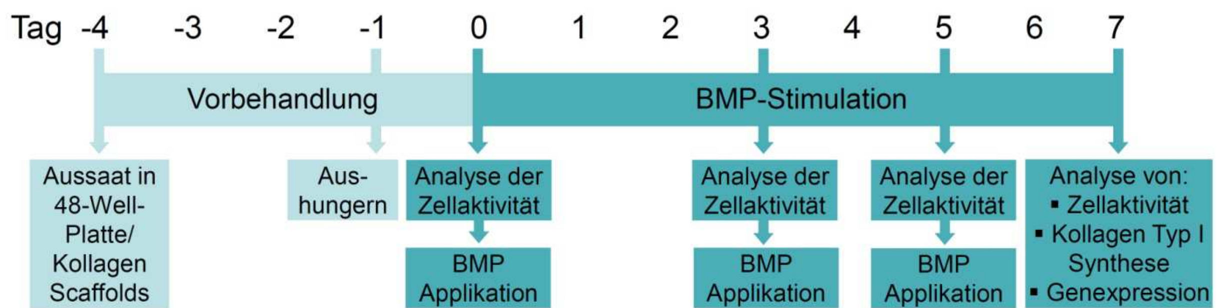

Abbildung 3: Versuchsschema zur BMP-Stimulation

Statistik

Es wurden insgesamt 6 Patientengruppen untersucht, wobei in jeder Gruppe 6 Spender einbezogen wurden. Die statistische Auswertung wurde mit dem SPSS Programm Version 18-20 durchgeführt und erfolgte für die in Dreifachbestimmungen durchgeführten Versuche für 18 Werte pro Gruppe. Für die RNA Analysen wurden die Zellen aus den Dreifachbestimmungen gepoolt (6 Werte pro Gruppe). Der Kruskal-Wallis Test wurde für die Analyse von signifikanten Unterschieden zwischen allen Untersuchungsgruppen oder Stimulationsgruppen angewendet und der Mann-Whitney-U Test zum Vergleich von einzelnen Gruppen oder den BMP-stimulierten Zellen mit der Kontrolle. Alle Werte in den Box Plots und im Text repräsentieren Mediane mit 25 % und 75 % Perzentilen. Das Signifikanzniveau wurde auf $p \leq 0{,}05$ festgelegt und mittels Bonferroni-Holm Korrektur angepasst. Für die Daten der Wachstumsfaktorstimulation wurde ein zusätzliches Signifikanzniveau für p-Werte kleiner als 0,001 festgelegt.

Ergebnisse

Tenozytenisolierung und klinische Parameter:

Es wurden 97 Tenozytenkulturen gewonnen, die nach den 3 Hauptkriterien in 12 Gruppen eingeteilt wurden (Tabelle 1). Bei 10 weiteren Isolierungen konnten keine Zellen aus dem Sehnengewebe gewonnen werden. Aus den blau hinterlegten Gruppen wurden bisher von jeweils 6 Tenozytenkulturen die zellbiologischen Charakteristika untersucht und die Zellen mit BMP-2 und BMP-7 stimuliert. Die jeweiligen Spendercharakteristika sind in Tabelle 2 zusammengefasst und es ist beschrieben, in welchen Publikationen die Daten veröffentlicht wurden.

Tabelle 1: Übersicht der gewonnenen Tenozytenkulturen pro Gruppe:

Geschlecht	Alter	Grad der Muskelverfettung (Goutallier)		
		0-I	II	III-IV
Weiblich	**< 65 Jahre**	11	6	3
	> 65 Jahre	7	6	4
Männlich	**< 65 Jahre**	20	9	8
	> 65 Jahre	7	9	7

Tabelle 2: Klinische Eigenschaften der Tenozytenspender:

Publikation	Primärkriterien [Mittelwert (Bereich)]			Sekundärkriterien [Mittelwert (Bereich)]		
	Geschlecht	Alter	Verfettungs-grad[30]	Nikotin Ja/Nein	Ruptur-größe[31]	Sehnen-retraktion[32]
1	Männlich*	45,3 (42-50)	0,5 (0-1)	4/2	2,2 (1-3)	0,7 (0-1)
	Männlich	71,3 (66-75)	0,8 (0-1)	1/5	2,0 (1-3)	1,0 (0-2)
2	Weiblich	55,7 (52-60)	0,5 (0-1)	1/5	1,8 (1-2)	1,0 (0-2)
	Weiblich	68,2 (65-74)	0,8 (0-1)	1/5	1,8 (1-3)	1,2 (0-2)
3	Männlich	59,7 (56-62)	1,9 (1,5-2)	1/5	2,3 (2-3)	1,8 (1-2)
	Männlich	55,7 (46-62)	3,2 (3-4)	2/4	3,2 (2-4)	2,7 (2-3)

*) Die Daten der jungen Männergruppe mit niedrigem Verfettungsgrad wurde ebenfalls in der 3. Publikation zum Einfluss der fettigen Infiltration veröffentlicht.

Analyse zellbiologischer Charakteristika:

Die Anzahl der Zellen, die aus den Sehnenbiopsien isoliert wurde, variierte relativ stark innerhalb der Gruppen. Die Zelldichte (Zellzahl normalisiert zum Gewicht der Biopsie) ergab keine signifikanten Unterschiede zwischen den Untersuchungsgruppen.

Tenozyten älterer männlicher und weiblicher Spender sowie Spender mit hohem Grad der fettigen Muskelinfiltration zeigten ein verringertes Zellwachstum über 14 Tage gegenüber den jeweiligen jüngeren Spendern oder Spendern mit geringerem Muskelverfettungsgrad. Der Effekt des Alters war bei den Männern stärker ausgeprägt als bei den Frauen (Abbildung 4).

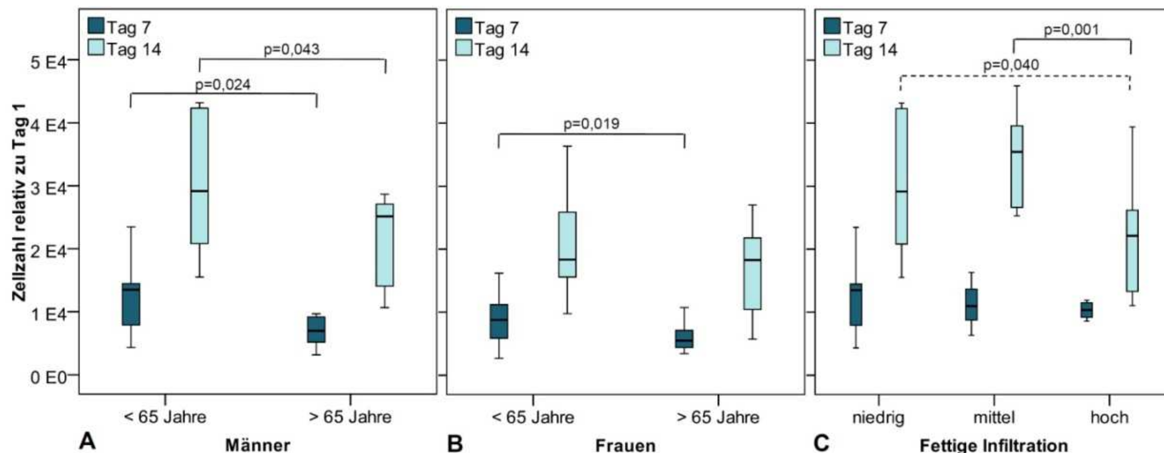

Abbildung 4: Zellwachstum der Tenozyten männlicher (A; Publikation 1) und weiblicher Spender (B; Publikation 2) unter und über 65 Jahre, sowie Spendern mit unterschiedlicher fettiger Infiltration des SSP Muskels (C; Publikation 3). Die gestrichelte Linie zeigt Unterschiede, die nach der Bonferroni-Holm Korrektur nicht mehr signifikant waren.

Die Zellen aller Spendergruppen hatten ein Tenozyten-spezifisches Expressionsmuster. Sie exprimierten große Mengen Kollagen Typ I und geringere Mengen Kollagen Typ III und Decorin. Die Sehnenmarker Scleraxis und Mohawk Homeobox konnten ebenfalls in den Zellen nachgewiesen werden. Der Sehnenmarker Tenomodulin wurde in den Zellen nur geringfügig exprimiert. Als Negativmarker konnte keine Kollagen Typ II Expression und nur eine geringe Osteocalcin Expression nachgewiesen werden. Das Alter, Geschlecht und die fettige Infiltration hatten keinen Einfluss auf die Genexpression in den Tenozyten. Lediglich die Zellen von Patienten mit niedriger fettiger Infiltration hatten eine erhöhte Kollagen Typ I, III und Osteocalcin Expression gegenüber den Tenozyten von Spendern mit höherer fettiger Infiltration. Weiterhin zeigten Tenozyten von Spendern mit hoher fettiger Infiltration eine signifikant geringere Expression des Sehnenmarkers Mohawk gegenüber den Zellen mit niedrigerem Verfettungsgrad. Die Unterschiede waren nach Bonferroni-Holm Korrektur jedoch nicht signifikant (Publikation 3).
Die Analyse der Kollagen Typ I Synthese auf Proteinebene ergab ein verringertes Synthesepotential in Tenozyten von weiblichen Spendern über 65 Jahre gegenüber den jüngeren weiblichen und den männlichen Spendern (Publikation 2).
Ein Teil der Tenozyten aller Spendergruppen zeigten ein Stammzellpotential. Sie exprimierten Stammzellmarker (CD29, 44, 73, 90 und 105) auf ihrer Zelloberfläche, jedoch keine Negativmarker (CD11b, 14, 19, 34 und 45)(Abbildung 5, exemplarische Daten aus Publikation 2). Sie waren fähig, Kolonien zu bilden und multipotent zu differenzieren. Bei dem Vergleich der Patientengruppen wurde ein verringertes Koloniebildungspotential (Kolonieanzahl und -größe) in den Tenozyten älterer und weiblicher Spender, sowie in Zellen von Patienten mit hoher fettiger Infiltration des SSP Muskels nachgewiesen (Abbildung 6). Die adipogene, chondrogene und osteogene Differenzierung wurde durch Zelltyp-spezifische Färbungen detektiert und konnte durch Expressionsanalysen von Differenzierungsmarkern bestätigt werden (Abbildung 7). Durch die Quantifizierung der Farbreaktion konnte eine signifikant erhöhte osteogene Differenzierung bei jüngeren männlichen Patienten gegenüber den älteren Patienten gemessen werden. Die Zellen von männlichen Patienten mit mittelgradiger fettiger Infiltration zeigten eine signifikant gesteigerte adipogene und osteogene Differenzierung.

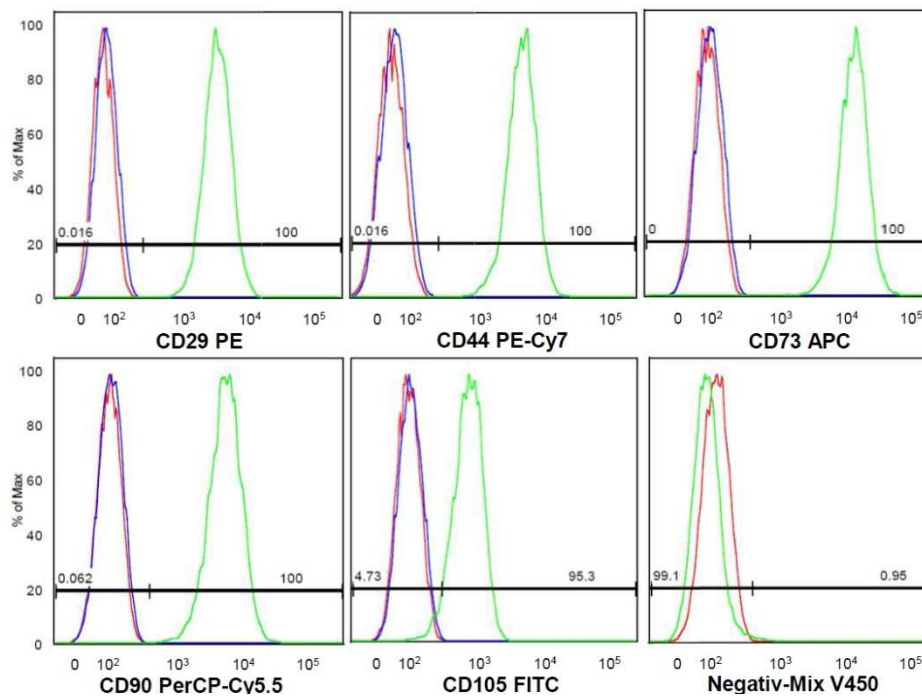

Abbildung 5: Exemplarische Ergebnisse zum Stammzellphänotyp von Tenozyten von weiblichen Spendern unter 65 Jahre: Über 95 % der Tenozyten exprimierten die Stammzellmarker CD29, 44, 73, 90 und 105 auf ihrer Zelloberfläche. Weniger als 1 % der Zellen exprimierten die Negativmarker CD11b, 14, 19, 34 und 45. Grüne Histogramme: gefärbte Zellen, rote Histogramme: ungefärbte Kontrollzellen, Blaue Histogramme: IgG Kontrollen

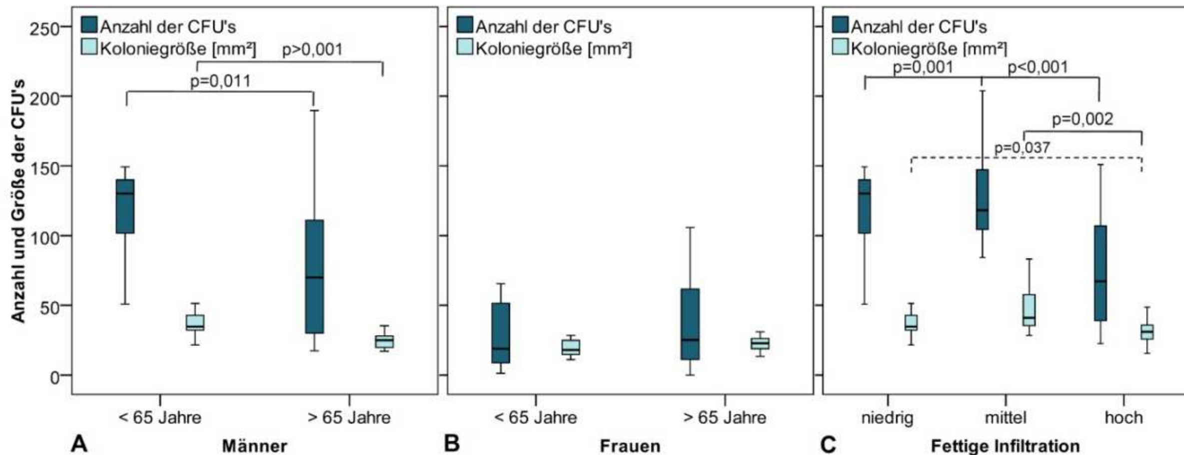

Abbildung 6: Kolonieanzahl und -größe der Tenozyten männlicher (A; Publikation 1) und weiblicher Spender (B; Publikation 2) jünger und älter als 65 Jahre, sowie Spendern mit unterschiedlicher fettiger Infiltration (C; Publikation 3) ausgehend von 1000 Zellen. Die gestrichelte Linie zeigt Unterschiede, die nach der Bonferroni-Holm Korrektur nicht mehr signifikant waren.

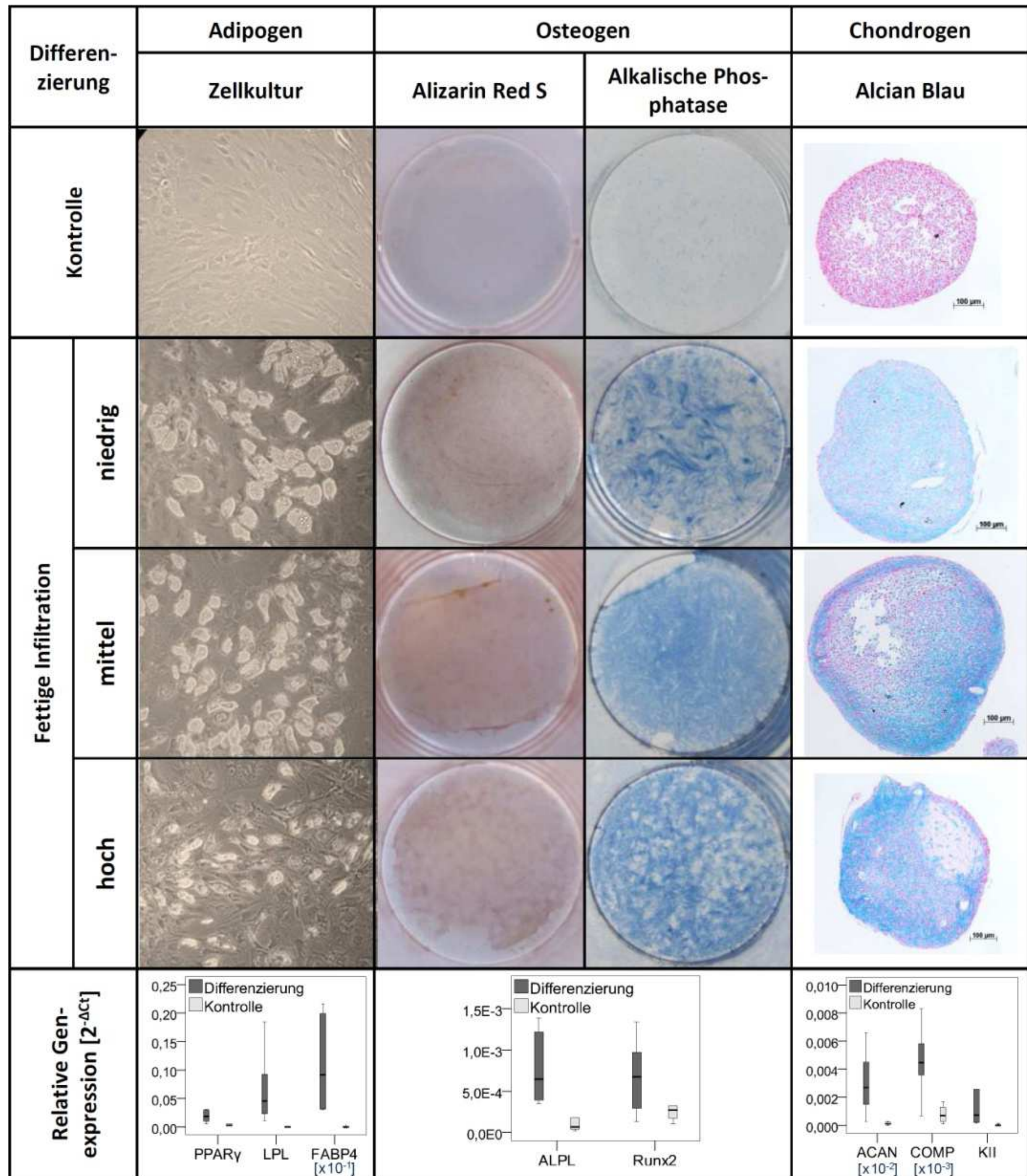

Abbildung 7: Exemplarische Ergebnisse zur multipotenten Differenzierung von Tenozyten mit niedriger, mittlerer und hoher fettiger Infiltration: Adipogen differenzierte Zellen bildeten Lipidvakuolen aus. Die Alizarin Red S Färbung nach der osteogenen Differenzierung war schwach, die ALP Färbung hingegen zeigte deutlich blaue Zellen. Die chondrogen differenzierten Zellpellets wurden durch Alcian Blau deutlich angefärbt. Die Genexpression von linienspezifischen Markern war in den differenzierten Zellen stark erhöht gegenüber den undifferenzierten Kontrollzellen. (PPARγ: Peroxisome Proliferator-activated Receptor Gamma, LPL: Lipoprotein Lipase, FABP4: Fatty Acid Binding Protein 4, ALPL: Alkalische Phosphatase Tissue-nonspecific, Runx2: Runt-related Transcription Factor 2, KII: Kollagen Typ II, ACAN: Aggrecan, COMP: Cartilage Oligomeric Matrix Protein**)**

Wachstumsfaktorstimulation:

Die relative Aktivität von BMP-2 und -7 wurde durch die Bildung von ALP in C2C12-Zellen nach BMP-Gabe untersucht. Die ALP-Aktivität relativ zur Zellaktivität wurde bei einer Konzentration von 1000 ng/ml durch beide Wachstumsfaktoren ähnlich stark stimuliert (BMP-2: Absorption bei 405 nm (A_{405})=2,7 (2,5-2,8); BMP-7: A_{405}=3,0 (2,7-3,1)). Bei einer Konzentration von 200 ng/ml konnte eine stärkere ALP induzierende Wirkung durch BMP-2 beobachtet werden (A_{405}=2,2 (2,0-2,5)), als durch die BMP-7 Gabe (A_{405}=0,6 (0,6-0,7))(Publikation 2).

Die 3D-Kultur zur BMP-2 und -7 Stimulation der Tenozyten führte zu verbesserten Ergebnissen gegenüber der konventionellen Monolayer-Kultur der Zellen. Die Expression und Synthese von EZM wie Kollagen Typ I wurde in der Scaffoldkultur deutlich verstärkt. Hohe Konzentrationen von BMP-2 (1000 ng/ml) hatten einen supprimierenden Effekt auf die Zellaktivität der Tenozyten in der 2D-Kultur. Dieser negative Effekt trat in der Scaffoldkultur nicht auf (Abbildung 8A, Publikation 1).

Tenozyten aller Spendergruppen profitierten von der Wachstumsfaktorstimulation mit BMP-2 und BMP-7. BMP-2 hatte in der 3D-Kultur nur einen geringfügig stimulierenden Einfluss auf die Zellaktivität der Tenozyten. BMP-7 führte hingegen sowohl in der 2D- als auch in der 3D-Kultur zu einer deutlich verstärkten Zellaktivität. Die Kollagen Typ I Expression und Synthese konnte durch beide Wachstumsfaktoren dosisabhängig gesteigert werden (Abbildung 9, Publikation 2). Die Kollagen Typ III Expression wurde vor allem durch BMP-7 signifikant erhöht. Nur bei Tenozyten der Frauen wurde die Kollagen Typ III Expression auch durch die hohe BMP-2 Konzentration signifikant gesteigert (Abbildung 9, Publikation 2). In den Tenozyten männlicher Spender wurde gezeigt, dass die Decorinexpression durch BMP-7 herunterreguliert wurde, während BMP-2 keinen Einfluss auf die Expression von Decorin hatte (Abbildung 8B, Publikation 1).

Bei dem Vergleich der Untersuchungsgruppen zum Effekt des Alters, Geschlechtes und der fettigen Infiltration auf das Stimulationspotential wurde deutlich, dass ältere Frauen ein signifikant verringertes Stimulationspotential aufwiesen als jüngere Frauen (Publikation 2). Auch Tenozyten von männlichen Spendern mit hoher fettiger Infiltration sprachen weniger stark auf die Wachstumsfaktorstimulation an, als Zellen von Spendern mit geringerer fettiger Infiltration. Dies äußerte sich vor allem in der Stimulation der Zellaktivität und der Kollagen Typ I Synthese (Abbildung 10, Publikation 3).

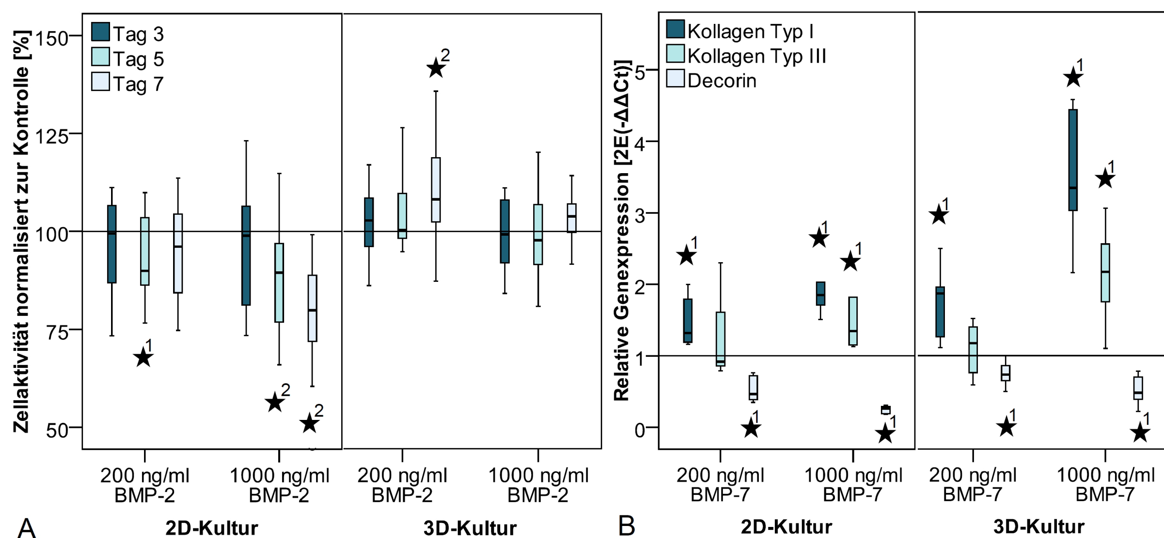

Abbildung 8: Stimulationspotential der Tenozyten zum Vergleich von 2D- und 3D-Kultur (exemplarische Daten der jungen männlichen Spender): A) Die Zellaktivität wurde durch die BMP-2 Stimulation in der 2D-Kultur gegenüber der Kontrolle (100 %) signifikant reduziert. Der reduzierende Effekt wurde in der 3D-Kultur

aufgehoben. B) Die Genexpression von Kollagen Typ I und III nach BMP-7 Gabe wurde in der 3D-Kultur stärker stimuliert als in der 2D-Kultur. Die Decorin Expression wurde verringert. Die Sterne symbolisieren signifikante Unterschiede zur unstimulierten Kontrolle. Die Zahlen geben das Signifikanzniveau an 1: p<0,05, 2: p< 0,001.

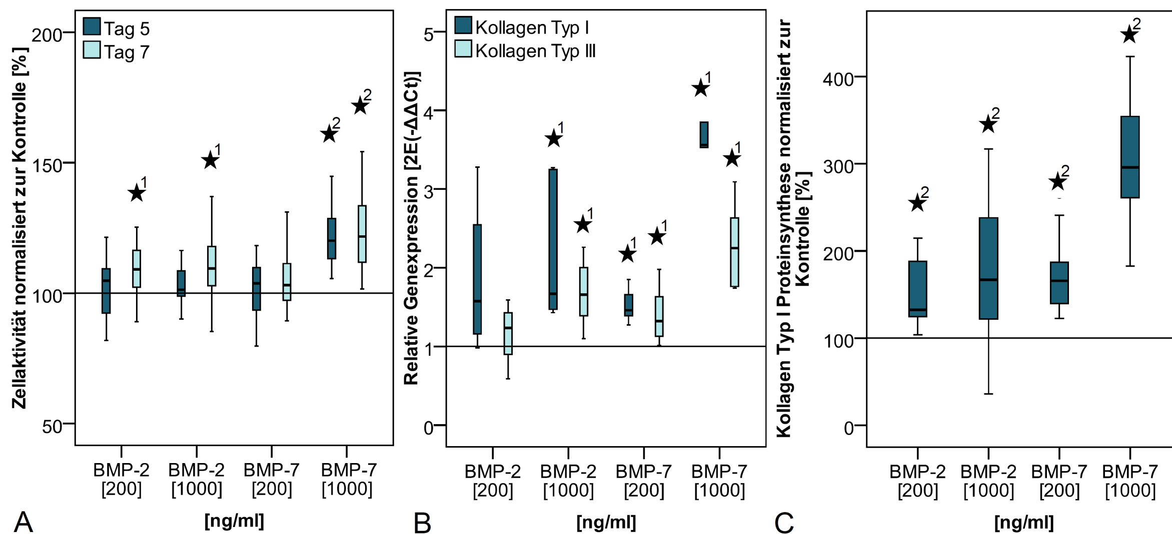

Abbildung 9: Exemplarische Ergebnisse zur BMP-Stimulation in 3D-Kultur von Tenozyten von weiblichen Spendern unter 65 Jahre: A) Die Zellaktivität wurde durch BMP-2 in beiden Konzentrationen an Tag 7 signifikant gesteigert. Durch BMP-7 wurde die Zellaktivität in der hohen Konzentration an den Tagen 5 und 7 hoch signifikant stimuliert. B) Nur die hohe Konzentration von BMP-2 führte zu einer signifikanten Steigerung der Kollagen Typ I und III Expression. BMP-7 reguliert die Expression beider Kollagene in beiden Konzentrationen hoch. C) Die Kollagen Typ I Proteinsynthese wurde durch beiden Faktoren in beiden Konzentrationen hoch signifikant gesteigert. Die Sterne symbolisieren signifikante Unterschiede zur unstimulierten Kontrolle. Die Zahlen geben das Signifikanzniveau an 1: p<0,05, 2: p< 0,001.

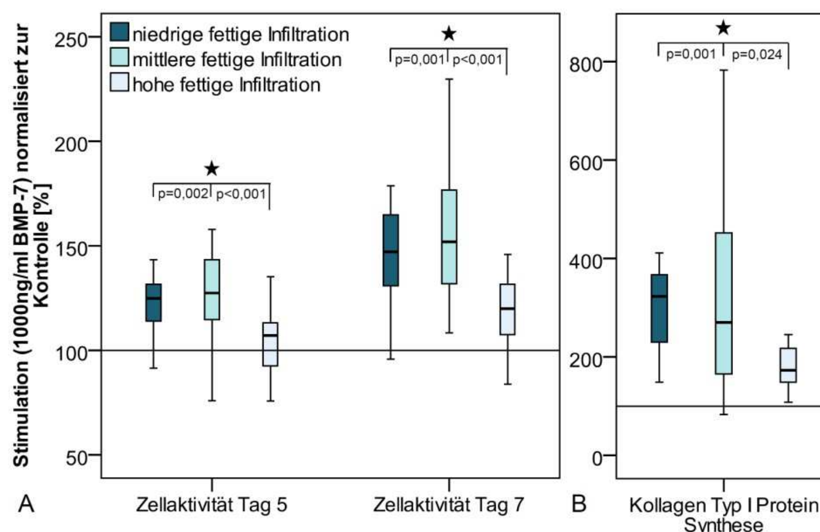

Abbildung 10: Gruppenspezifische Unterschiede im Stimulationspotential von männlichen Spendern mit niedriger, mittlerer und hoher fettiger Infiltration in 3D-Kultur. Die Zellaktivität an den Tagen 5 und 7 (A) sowie die Kollagen Typ I Proteinsynthese (B) konnte in den Zellen von Spendern mit hoher fettiger Infiltration weniger stark stimuliert werden gegenüber Zellen von Spendern mit niedrigerer fettiger Infiltration.

Diskussion

In der vorliegenden Promotionsarbeit wurde das erste Mal der Versuch unternommen, klinische Merkmale von Patienten mit den zellbiologischen Eigenschaften der entsprechenden Tenozyten aus RM-Sehnen in Zusammenhang zu bringen. Dabei wurden als Hauptkriterien das Alter, das Geschlecht und die fettige Infiltration ausgewählt, da diese in der täglichen klinischen Praxis immer wieder als Risikofaktoren diskutiert werden.

In der vorliegenden Arbeit wurden Tenozyten aus SSP-Sehnen isoliert und ausführlich charakterisiert. Da es keinen Tenozyten-spezifischen Marker gibt, wurde eine Vielzahl von Genen analysiert, die mit Sehnen assoziiert sind. Dazu wurde in einer vorhergehenden Studie die Genexpression von Tenozyten mit der von Osteoblasten und Chondrozyten verglichen.[33] Anhand dieser Studie wurden die Charakterisierungsmarker auf RNA-Ebene ausgewählt. Kollagen Typ II und Osteocalcin wurden als Marker identifiziert, die eine eindeutige Abgrenzung der Tenozyten zu den anderen Zellen des muskuloskeletalen Systems zulassen. In den Tenozyten konnte keine Kollagen Typ II Expression und nur eine geringe Osteocalcin Expression nachgewiesen werden. Kollagen Typ I und III, als wichtigste Kollagene, sowie Decorin, als wichtigstes Proteoglycan der Sehne, wurden ebenfalls untersucht und in den Tenozyten aller Spendergruppen vergleichbar exprimiert. Der Transkriptionfaktor Scleraxis wurde als Sehnen-spezifischer Marker beschrieben,[34] zeigte in der vorhergehenden Studie jedoch keine Unterschiede zwischen den Tenozyten, Osteoblasten und Chondrozyten.[33] Eine Expression von Scleraxis konnten in den Tenozyten der vorliegenden Arbeit von allen Spendergruppen nachgewiesen werden. Tenomodulin wird als Marker beschrieben, der hauptsächlich in Sehnen und Bändern zu finden ist, jedoch mit höheren Passagen und in einer 2D-Kultur verloren geht.[35-36] Dies konnte in der vorliegenden Arbeit bestätigt werden, da Tenomodulin nur geringfügig exprimiert wurde. Auf RNA Ebene konnten keine signifikanten Unterschiede zwischen den Spendergruppen aufgezeigt werden. Mögliche Unterschiede im Expressionsprofil gehen vermutlich durch die Kultivierung der Zellen verloren. Nur Zellen von Spendern mit niedriger fettiger Infiltration zeigten eine verstärkte Kollagen Typ I, III und Osteocalcin Expression gegenüber denen von Spendern mit hoher fettiger Infiltration. Weiterhin zeigte sich, dass die Expression des Sehnen-assoziierten Markers Mohawk, welcher bei der Entwicklung von Sehnen eine regulatorische Bedeutung hat,[37] in den Tenozyten von Spendern mit hoher fettiger Infiltration signifikant verringert war. Die Unterschiede waren jedoch nach Bonferroni-Holm Korrektur nicht signifikant. Eine verringerte Mohawk Expression deutet möglicherweise auf den Verlust des tenogenen Potentials der Zellen hin.

In verschiedenen Studien konnte gezeigt werden, dass Tenozyten Stammzellpotential besitzen.[14-16] Dies konnte in der vorliegenden Arbeit bestätigt werden. Ein Teil der Zellen erfüllte die minimalen Stammzellkriterien,[29] indem sie fähig waren Kolonien zu bilden, multipotent zu differenzieren und Stammzellmarker auf der Zelloberfläche zu exprimieren. Das Differenzierungspotential in die osteogene Richtung zeigte sich anhand der Alizarin Red S Färbung relativ schwach in den Tenozyten. Da nicht von einer reinen Stammzellpopulation ausgegangen werden kann, ist eine schwächere Differenzierung gegenüber mesenchymalen Stammzellen zu erwarten. Die Analyse osteogener Gene und die ALP Färbung konnte das Ergebnis untermauern.

Das Koloniebildungspotential war zusammen mit dem Zellwachstum der Tenozyten der am stärksten regulierte Parameter zwischen den Untersuchungsgruppen. Zellen älterer und weiblicher Spender, sowie Spender mit hoher fettiger Infiltration hatten ein verringertes Koloniebildungspotential und Zellwachstum. Der Effekt des Alters äußerte sich dabei stärker zwischen den jungen und alten

Männern als bei den Frauen, da der Altersunterschied bei den weiblichen Patienten nicht so stark ausgeprägt war.
Bei den weiblichen Spendern zeigte sich ein Unterschied in der Kollagen Typ I Synthese der Zellen, wobei Tenozyten von Frauen über 65 Jahre eine signifikant verringerte Kollagen Typ I Synthese gegenüber jüngeren Frauen sowie älteren Männern aufwiesen. Eine verringerte Kollagen Typ I Synthese in Tenozyten weiblicher Spender könnte möglicherweise auf die Wirkung von Hormonen wie Estradiol zurückzuführen sein, da gezeigt werden konnte, dass Estradiol einen hemmenden Einfluss auf die Kollagensynthese *in vivo* und *in vitro* hat.[38-41]

Anhand der Stimulationsversuche mit BMP-2 und BMP-7 konnte gezeigt werden, dass wichtige zellbiologische Charakteristika, wie die Zellaktivität und die Kollagen Typ I Expression und Synthese, in den Tenozyten durch die Wachstumsfaktoren verbessert wurden. Die Etablierung einer 3D-Scaffoldkultur stellte sich dabei als wichtige Voraussetzung dar, da hierdurch die Ergebnisse der Genexpression und Kollagen Synthese deutlich verstärkt werden konnten. Die 3D-Kultur imitiert die *in vivo* Umgebung von Tenozyten besser als die Monolayer-Kultur und kann somit den Erhalt des natürlichen Phänotyps fördern und Redifferenzierungen vorbeugen.[42-44] In der ersten Publikation wurden die beiden Kultivierungsmethoden unter BMP-Stimulation verglichen. In den zwei nachfolgenden Studien wurde die BMP-Stimulation in der 2D- und 3D-Kultur durchgeführt, jedoch nur die Ergebnisse der 3D-Scaffoldkultur veröffentlich.
In Vorarbeiten wurde die optimale Konzentration der Wachstumsfaktoren BMP-2 und BMP-7 durch die Gabe der klinischen Produkte beider Faktoren (BMP-2: InductOS, Wyeth, USA; BMP-7: Osigraft, Stryker, USA) von 100 bis 2000 ng/ml getestet.[45] Von 100 bis 1000 ng/ml fand ein dosisabhängiger Anstieg der analysierten Parameter statt. Eine höhere Konzentration von 2000 ng/ml führte zu einer Verschlechterung der Ergebnisse. Für die Promotionsarbeit wurden dann die Konzentrationen 200 und 1000 ng/ml ausgewählt. In der Vorarbeit konnte ebenfalls die Expression der, für die Signalverarbeitung notwendigen, BMP und Activin Rezeptoren in den Tenozyten gezeigt werden.
In der zweiten Publikation wurde die relative Aktivität von BMP-2 und BMP-7 mit Hilfe von C2C12-Zellen getestet. Beide Wachstumsfaktoren bildeten bei einer Konzentration von 1000 ng/ml die gleiche Menge ALP, während bei einer Konzentration von 200 ng/ml eine stärkere Reaktion der C2C12-Zellen durch BMP-2 hervorgerufen wurde. BMP-7 hatte in den Stimulationsversuchen einen stärkeren Effekt auf die Tenozyten als BMP-2. Dies zusammen mit der relativen Aktivität von BMP-2 und BMP-7 deutet darauf hin, dass BMP-2 in den Tenozyten eine schwächere Wirkung hervorruft als BMP-7. Möglicherweise könnte dies durch eine geringere Anzahl an Rezeptoren auf den Zellen begründet sein, oder durch intrazelluläre Prozesse. Die Rezeptor-Bindung der BMPs ist unterschiedlich, wobei BMP-2 mit hoher Affinität an Typ I Rezeptoren und BMP-7 an Typ II Rezeptoren bindet.[46] Um den genauen Grund für die unterschiedliche Wirkung der BMPs identifizieren zu können, müsste eine detaillierte Untersuchung der Rezeptortypen, der Rezeptorbindungen und der Signalkaskaden nach BMP-Gabe erfolgen. Dies wurde in Ansätzen bereits durchgeführt, jedoch noch nicht publiziert. Smad8 wurde dabei als wichtigstes Signalmolekül in den Tenozyten identifiziert. Weiterführende Untersuchungen der BMP-2 und BMP-7 Signalkaskade sind zukünftig vorgesehen.
Die BMP-Gabe stimulierte die Zellaktivität sowie die Expression und Synthese von Kollagen Typ I und III. Diese Faktoren könnten wichtig sein für die Sehnen-Knochenheilung der RM *in vivo*. BMP-7 hatte insgesamt einen stärkeren stimulierenden Effekt auf die Tenozyten und könnte damit als Faktor für eine klinische Anwendung bevorzugt in Frage kommen. Die hemmende Wirkung von BMP-7 auf die

Decorin-Expression könnte sich hingegen negativ auf die Heilung der RM auswirken, da Decorin als Proteoglycan beschrieben wurde, welches der Narbenbildung entgegen wirkt und sich positiv auf die biomechanischen Eigenschaften von Sehnen auswirkt.[47]

Die Tenozyten aller untersuchten Patientengruppen konnten durch die BMPs stimuliert werden. Die, bei der Charakterisierung der Zellen identifizierten, nachteiligen zellbiologischen Eigenschaften der Tenozyten spiegelten sich auch in dem Stimulationspotential wieder. Tenozyten von älteren weiblichen Patienten und Patienten mit hohem Grad der fettigen Infiltration zeigten ebenfalls ein verringertes Stimulationspotential, vor allem im Hinblick auf die Zellaktivität und Kollagen Typ I Synthese (hohe fettige Infiltration). Das untersuchte Alter scheint keinen Einfluss auf die Stimulierbarkeit der Zellen zu haben. Erst die Kombination aus einem höheren Alter zusammen mit dem weiblichen Geschlecht beeinflusst die Tenozyten negativ. Es scheint, dass die Tenozyten von älteren weiblichen Spendern und Spendern mit hohem Goutalliergrad einen langsameren Zellmetabolismus haben.

Als Ursache für die gruppenspezifischen Unterschiede könnten im Hinblick auf das Geschlecht Sexualhormone vermutet werden. Es konnte in Zellkulturstudien gezeigt werden, dass Tenozyten von männlichen und weiblichen Spendern Östrogen-Rezeptoren exprimieren.[48] Es konnte weiterhin beobachtet werden, dass die Zellen durch eine direkte Estradiol-Gabe mehr Kollagen Typ III und Elastin exprimierten[49] und die Zellproliferation und Kollagen Typ I Synthese inhibiert wurde.[39] *In vivo* wurde demonstriert, dass Frauen nach aktivem Training eine verringerte Kollagen Typ I Synthese aufwiesen als Männer.[38] Selbiges konnte für Frauen festgestellt werden, die regelmäßig orale Kontrazeptiva (Estrogen und Progesteron) nahmen, gegenüber denen, die keinen Kontrazeptiva ausgesetzt waren.[40-41]

Aus klinischer Sicht wird ein Goutalliergrad zwischen 1 und 2 als Grenze zwischen heilenden und nicht heilenden RM-Sehnen beschrieben.[11-12] In der vorliegenden Arbeit lag die Grenze aus zellbiologischer Sicht zwischen einem Goutalliergrad von 2 und 3. Für den langsameren Zellmetabolismus in Spendern mit hohem Grad der fettigen Infiltration könnte die fehlende aktive Bewegung der Schultersehnen, aufgrund von stärkeren Schmerzen und damit verbunden die fehlende mechanische Stimulation vermutet werden. Eine Responsivität von Tenozyten auf eine mechanische Stimulation konnte in mehreren Studien nachgewiesen werden, wobei eine dynamische Zugbelastung zu einer vermehrten Zellproliferation und Kollagen Synthese führte.[50-51]

Eine Stimulation der Sehnenheilung allein durch BMPs bei Patienten mit hohem degenerativen Status, würde möglicherweise keinen Erfolg bringen. Es müsste zusätzlich die Wiederherstellung der natürlichen Struktur des SSP Muskels adressiert werden. Gerber et al. konnten dazu an einem Schafsmodel zeigen, dass eine kontinuierliche Elongation des Muskels zu einer Verbesserung der Architektur des Muskels führt.[52] Die BMP-Stimulation kombiniert mit der Regeneration des Muskels in einem Heilungsmodel zu untersuchen, könnte eine vielversprechende zukünftige Studie darstellen.

Abschließend kann geschlussfolgert werden, dass in Tenozyten nachteilige zellbiologische Eigenschaften identifiziert wurden, die möglicherweise für eine schlechtere Heilung bei bestimmten Patientengruppen verantwortlich sein könnten. Diese zellbiologischen Parameter konnten durch die Wachstumsfaktoren BMP-2 und BMP-7 stimuliert werden. Die vorliegende Studie könnte somit einen Beitrag leisten, die Sehnenheilung zukünftig zu verbessern, indem Ansatzpunkte für mögliche Therapieoptionen dargelegt wurden. Die Arbeit stellt einen Beitrag für die Grundlage zur Entwicklung einer mehr patientenorientierten Therapie dar.

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Eidesstattliche Versicherung

„Ich, Franka Klatte-Schulz, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Einfluss des Alters, des Geschlechtes und der fettigen Infiltration auf die zellbiologischen Eigenschaften und das Stimulationspotential humaner Tenozyten der Rotatorenmanschette“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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Meine Anteile an den ausgewählten Publikationen entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o.) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

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Datum Unterschrift

Anteilserklärung an den erfolgten Publikationen

Franka Klatte-Schulz hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1:

Franka Klatte-Schulz*, Stephan Pauly*, Markus Scheibel, Stefan Greiner, Christian Gerhardt, Gerhard Schmidmaier, Britt Wildemann
„Influence of Age on the cell biological Characteristics and the Stimulation Potential of Male Human Tenocyte-like cells"
European Cells and Materials 2012 Jul 12;24:74-89

*) geteilte Erstautorenschaft

Anteil: 70%
Beitrag im Einzelnen: Beteiligung an der Versuchsidee, Versuchsplanung, Zellisolierung, Etablierung der 3D-Kultur, Etablierung und Durchführung der Versuche zur Charakterisierung und Wachstumsfaktorstimulation, Auswertung der *in vitro* Ergebnisse, statistische Auswertung der Daten, Erstellung des Manuskripts

Publikation 2:

Franka Klatte-Schulz, Stephan Pauly, Markus Scheibel, Stefan Greiner, Christian Gerhardt, Jelka Hartwig, Gerhard Schmidmaier, Britt Wildemann
„Characteristics and Stimulation Potential with BMP-2 and BMP-7 of Tenocyte-like Cells Isolated from the Rotator Cuff of Female Donors"
PLoS One 2013 Jun 25;8(6)

Anteil: 70%
Beitrag im Einzelnen: Versuchsplanung, Zellisolierung, Etablierung der Versuche, Aktivitäts-Untersuchung von BMP2 und BMP-7, bei der Durchführung der Versuche zur Charakterisierung und Wachstumsfaktorstimulation wurde ich von der Bachelor-Studentin Jelka Hartwig unterstützt, Auswertung der Ergebnisse, statistische Auswertung der Daten, Erstellung des Manuskripts

Publikation 3:

Franka Klatte-Schulz, Christian Gerhardt, Markus Scheibel, Britt Wildemann, Stephan Pauly
„Relationship between Muscle Fatty Infiltration and the Biological Characteristics and Stimulation Potential of Tenocytes from Rotator Cuff Tears"
Journal of Orthopaedic Research 2013, Sep 10

Anteil: 80%
Beitrag im Einzelnen: Versuchsplanung, Zellisolierung, Etablierung der Versuche, Durchführung der Versuche zur Analyse zellbiologischer Charakteristika und zum Stimulationspotential, Auswertung der Ergebnisse, statistische Auswertung der Daten, Erstellung des Manuskripts

<u>Publikation 4:</u>

Britt Wildemann, <u>Franka Klatte</u>
Review Artikel „Biological Aspects of Rotator Cuff Healing“
Muscles, Ligaments and Tendons Journal 2011, 1:160-167

<u>Anteil: 60%</u>
Beitrag im Einzelnen: Literaturrecherche, Erstellen der Graphiken, Beteiligung an der Erstellung des Manuskripts

Unterschrift des Doktoranden/der Doktorandin

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Ausgewählte Publikationen

Publikation 1:

„Influence of Age on the cell biological Characteristics and the Stimulation Potential of Male Human Tenocyte-like cells"

Franka Klatte-Schulz*, Stephan Pauly*, Markus Scheibel, Stefan Greiner, Christian Gerhardt, Gerhard Schmidmaier, Britt Wildemann

*) Geteilte Erstautorenschaft

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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.

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

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Abstract

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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., 2*005; 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 $2.5x10^3$ 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 10^5 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 (7.5x10^3 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. $2.5x10^5$ 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 $3x10^3$ vital cells / well or about $2x10^4$ / 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

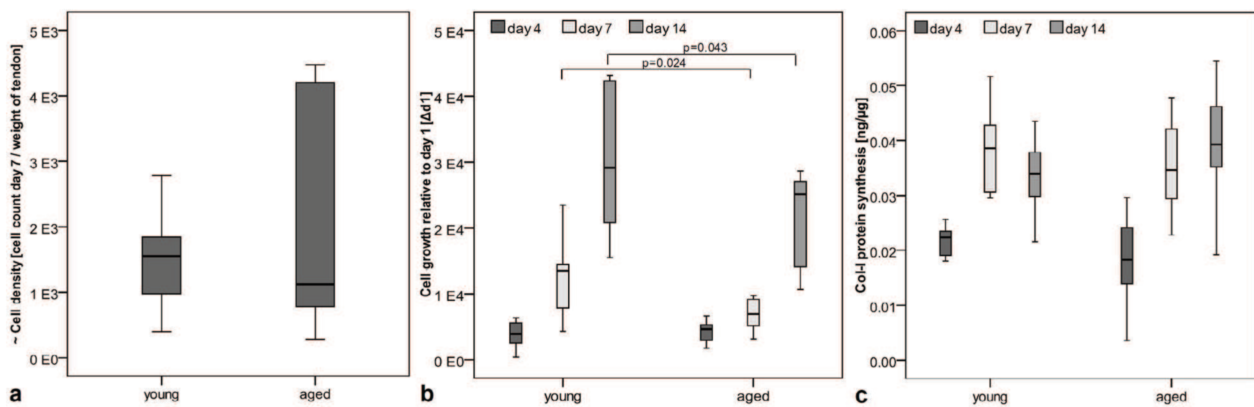

Fig. 1. Comparison of tenocyte-like cells of young and aged donors. (**a**) Cell density given as cell count of day 7 per weight of tendon biopsy showed no age-related differences. (**b**) Cell growth measured over 14 days and relative to day 1. Cells of aged donors had significantly decreased cell growth 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)

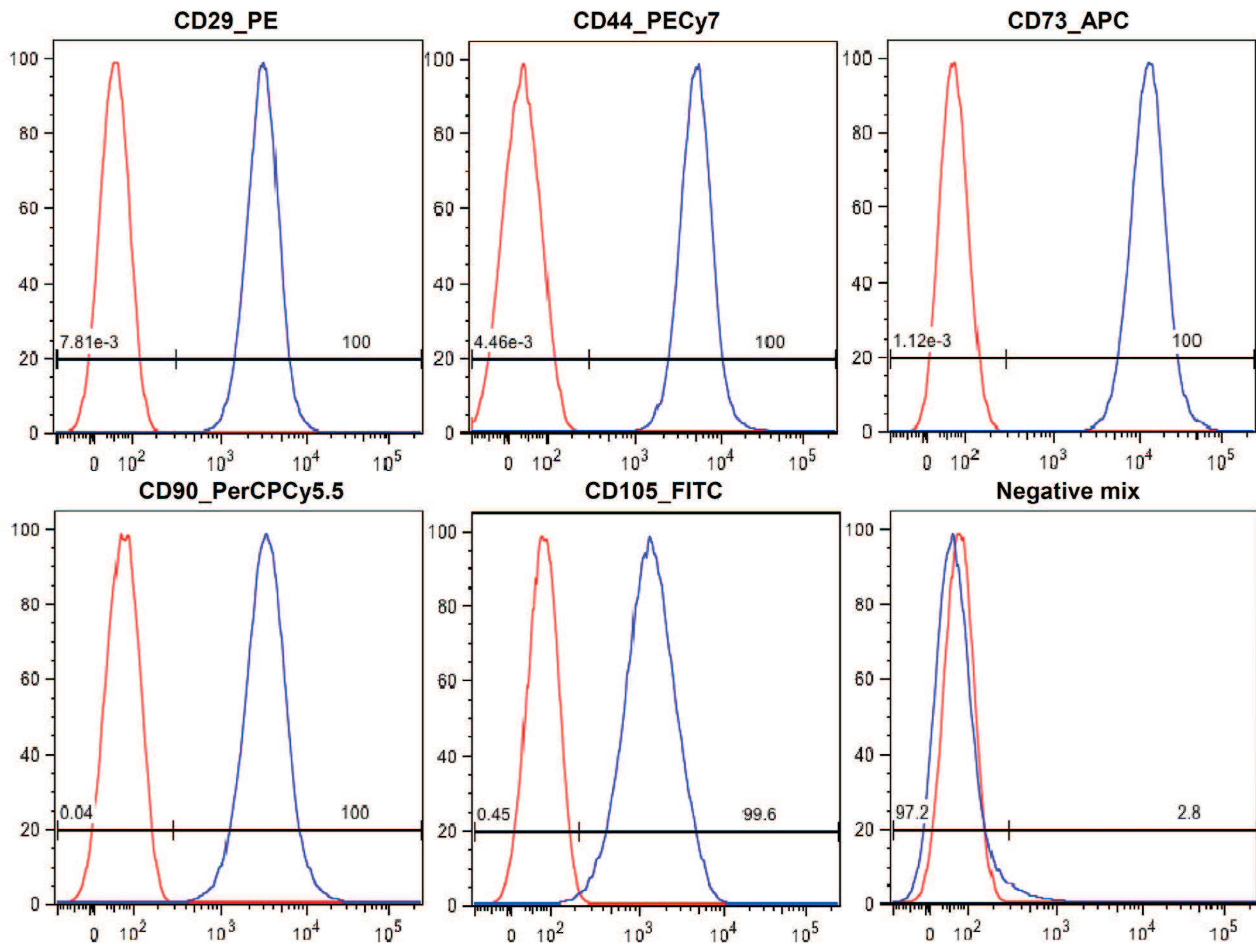

Fig. 2. Exemplary results of FACS analysis of young donor tenocyte-like cells. Graphs show the negative control cells (red curve) compared to stained cells (blue curve). Cells were positive for CD29, CD44, CD73, CD90, CD105 and negative for CD11b, CD14, CD19, CD34, CD45 (Negative mix).

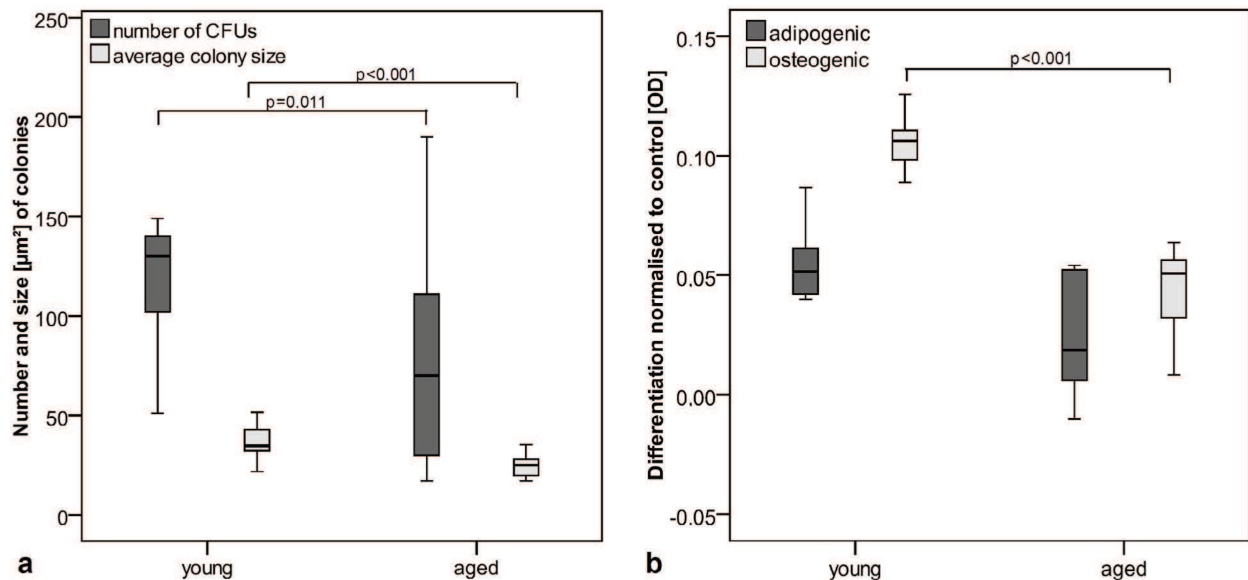

Fig. 3. Comparison of potency for self-renewal and differentiation potential of tenocyte-like cells of young and aged donors. (**a**) Number of CFUs as well as the average colony size was significantly decreased in the aged donors' cells. (**b**) Adipogenic and osteogenic differentiation was given as 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.

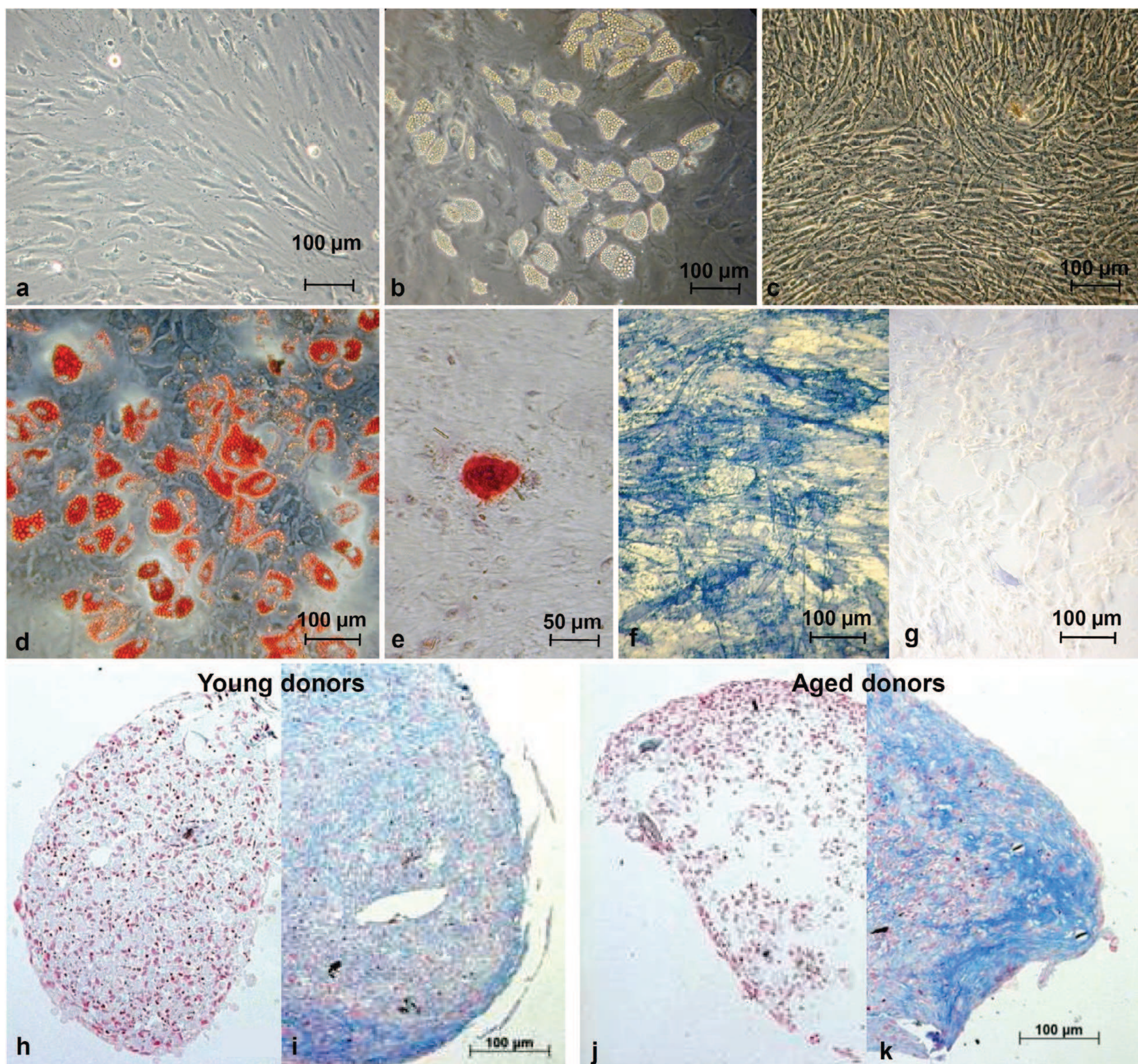

Fig. 4. Multipotent Differentiation (**a-c**) Phenotype of tenocyte-like cells after 3 weeks in (**a**) normal growth medium (control), (**b**) adipogenic differentiation medium, (**c**) osteogenic differentiation medium. (**d**) Adipogenic differentiated cells after Oil Red O staining with red stained 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).

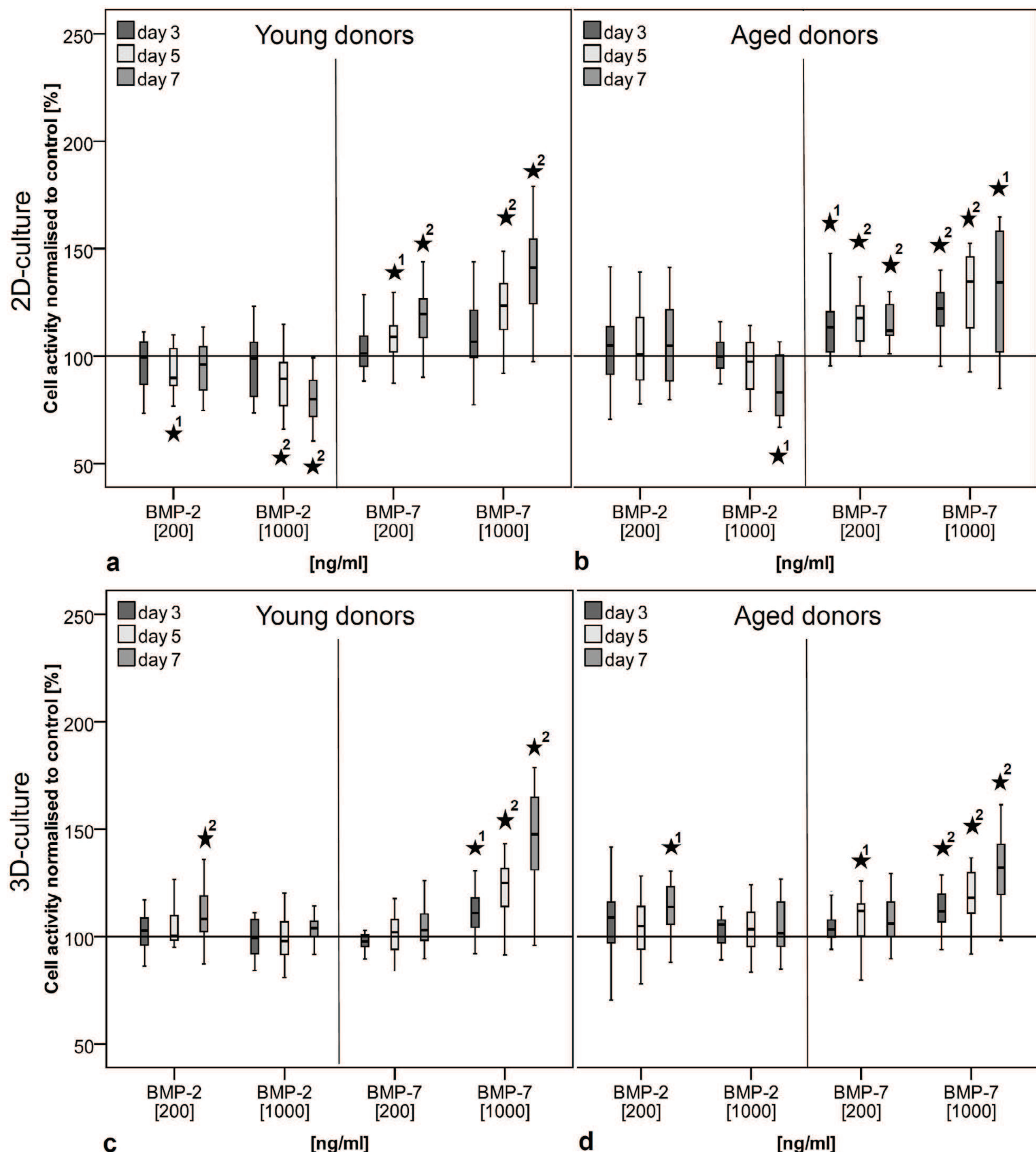

Fig. 5. Cell activity was measured by Alamar Blue assay and given as percentage of untreated control. Application of BMP-2 in 2D-culture of cells from young (**a**) and aged (**b**) donors significantly decreased activity compared to untreated control at low concentration at day 5, and at high concentration 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

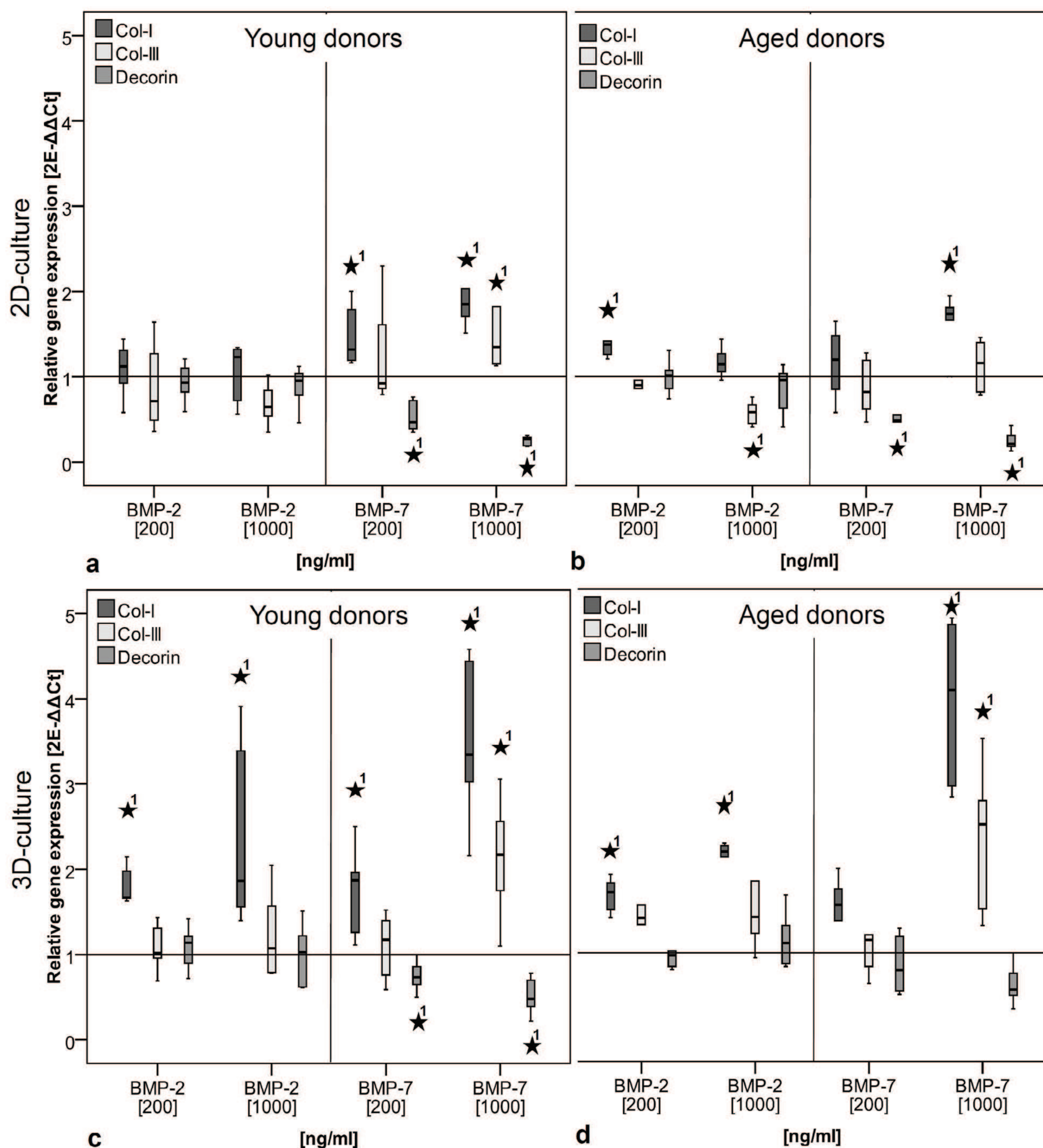

Fig. 6. Effect of growth factors on Col-I (dark gray bars), Col-III (light gray bars), and Decorin (grey bars) expression of tenocyte-like cells. (**a**) Marker expression of cells of young donors in 2D-culture was not significantly affected when treated with BMP-2. BMP-7 significantly increased Col-I 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: $p \leq 0.05$; 2: $p \leq 0.001$.

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).

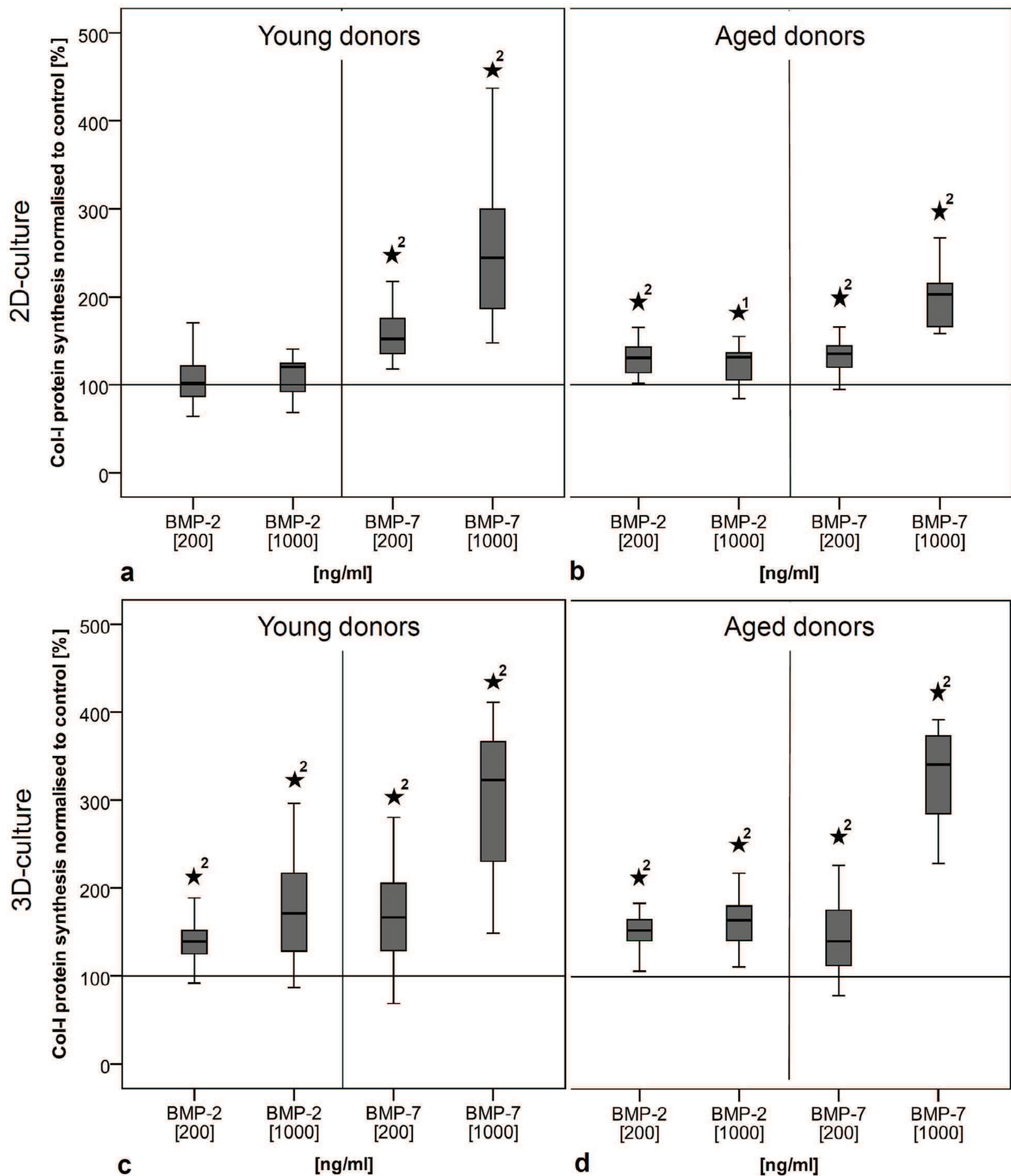

Fig. 7. Col-I protein synthesis in cell culture supernatant of day 7 after growth factor application. Col-I synthesis was calculated relative to total protein and given as percentage to untreated control. (**a**) Application of BMP-7 but not BMP-2 in 2D-culture of cells of young donors increased the 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

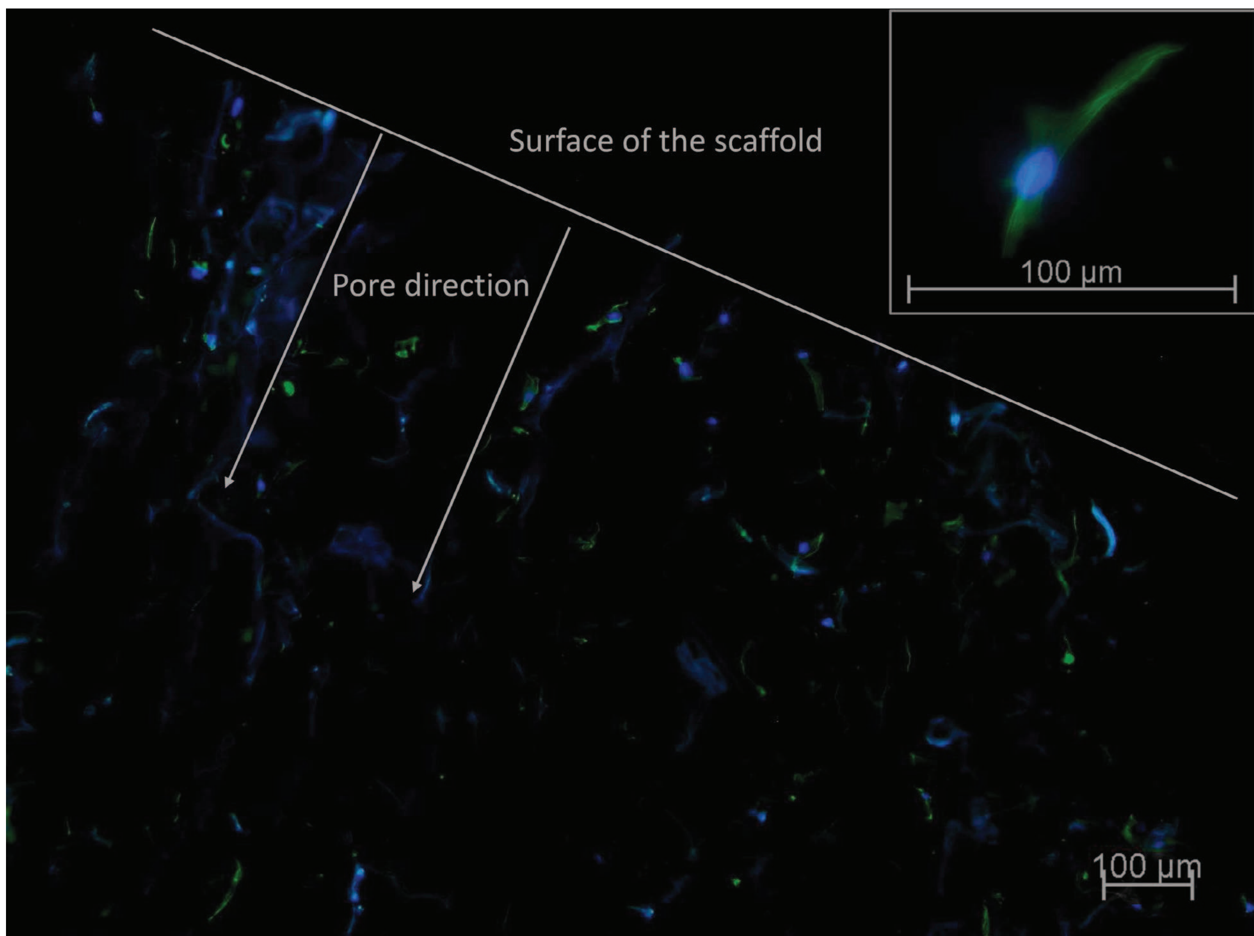

Fig. 8. Exemplary overview of tenocyte-like cells in Optimaix collagen scaffold after 7 days of stimulation with 1000 ng/mL BMP-7. Green staining represents Alexa Fluor Phalloidin stained actin filaments of tenocyte-like cells. Blue dots within the actin filaments are Dapi stained cell nuclei. The other 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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Publikation 2:

„Characteristics and Stimulation Potential with BMP-2 and BMP-7 of Tenocyte-like Cells Isolated from the Rotator Cuff of Female Donors“

Franka Klatte-Schulz, Stephan Pauly, Markus Scheibel, Stefan Greiner, Christian Gerhardt, Jelka Hartwig, Gerhard Schmidmaier, Britt Wildemann

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Abstract

Tendon bone healing of the rotator cuff is often associated with non-healing or recurrent defects, which seems to be influenced by the patient's age and sex. The present study aims to examine cellular biological characteristics of tenocyte-like cells that may contribute to this impaired rotator cuff healing. Moreover, a therapeutic approach using growth factors could possibly stimulate tendon bone healing. Therefore, our second aim was to identify patient groups who would particularly benefit from growth factor stimulation. Tenocyte-like cells isolated from supraspinatus tendons of female donors younger and older than 65 years of age were characterized with respect to different cellular biological parameters, such as cell density, cell count, marker expression, collagen-I protein synthesis, and stem cell potential. Furthermore, cells of the donor groups were stimulated with BMP-2 and BMP-7 (200 and 1000 ng/ml) in 3D-culture and analyzed for cell count, marker expression and collagen-I protein synthesis. Female donors older than 65 years of age showed significantly decreased cell count and collagen-I protein synthesis compared to cells from donors younger than 65 years. Cellular biological parameters including cell count, collagen-I and –III expression, and collagen-I protein synthesis of cells from both donor groups were stimulated with BMP-2 and BMP-7. The cells from donors older than 65 years revealed a decreased stimulation potential for cell count compared to the younger group. Cells from female donors older than 65 years of age showed inferior cellular biological characteristics. This may be one reason for a weaker healing potential observed in older female patients and should be taken into consideration for tendon bone healing of the rotator cuff.

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Characteristics and Stimulation Potential with BMP-2 and BMP-7 of Tenocyte-Like Cells Isolated from the Rotator Cuff of Female Donors

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Abstract

Tendon bone healing of the rotator cuff is often associated with non-healing or recurrent defects, which seems to be influenced by the patient's age and sex. The present study aims to examine cellular biological characteristics of tenocyte-like cells that may contribute to this impaired rotator cuff healing. Moreover, a therapeutic approach using growth factors could possibly stimulate tendon bone healing. Therefore, our second aim was to identify patient groups who would particularly benefit from growth factor stimulation. Tenocyte-like cells isolated from supraspinatus tendons of female donors younger and older than 65 years of age were characterized with respect to different cellular biological parameters, such as cell density, cell count, marker expression, collagen-I protein synthesis, and stem cell potential. Furthermore, cells of the donor groups were stimulated with BMP-2 and BMP-7 (200 and 1000 ng/ml) in 3D-culture and analyzed for cell count, marker expression and collagen-I protein synthesis. Female donors older than 65 years of age showed significantly decreased cell count and collagen-I protein synthesis compared to cells from donors younger than 65 years. Cellular biological parameters including cell count, collagen-I and –III expression, and collagen-I protein synthesis of cells from both donor groups were stimulated with BMP-2 and BMP-7. The cells from donors older than 65 years revealed a decreased stimulation potential for cell count compared to the younger group. Cells from female donors older than 65 years of age showed inferior cellular biological characteristics. This may be one reason for a weaker healing potential observed in older female patients and should be taken into consideration for tendon bone healing of the rotator cuff.

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Introduction

Non-healing and recurrent defects are the most frequent complications following surgical reconstructions of the tendon bone unit of the rotator cuff [1–4]. The outcome after rotator cuff reconstructions depends on many different biological and clinical factors. Patient's age has been shown to be highly correlated with tendon tears and recurrent defects [1,5–8], while over the age of 65 the risk of a poor clinical outcome is strongly increased [1,6]. The influence of sex on the healing outcome is controversial. Some clinical cohort studies have shown an influence of sex, with a higher failure rate after arthroscopic rotator cuff repair [9] or a higher disability of the shoulder, arm and hand (DASH) score and decreased strength in female patients [10]. However, other authors have found no association between the sex of the patients and the healing outcome [5,7,11]. It has been hypothesized that sex hormones such as estrogens may cause differences in healing capacities between men and women. Magnusson et al. reported that estrogens can influence the healing by influencing collagen synthesis in the tendon [12]. Furthermore, postoperative complications may be associated with "social" components of gender, with different personality traits, attitudes and behaviors potentially causing these differences [13,14]. Additionally, different working activities of men and women have been investigated and suggested to influence shoulder disorders [14].

Differences in healing rates of rotator cuff tears between men and women have only been shown in epidemiological studies. However, to date no relationship between the cellular characteristics of tenocytes of the rotator cuff and the sex of the patient has been demonstrated. In a previous study, we demonstrated differences between tenocytes of rotator cuffs of young (average 45.3 years) and aged (average 72.3 years) male donors [15]. Cells differed with respect to their cell count and stem cell potential, with cells of aged donors showing inferior parameters. The same experimental set up was also performed in the present study for cells of female donors and results were discussed with previous findings to investigate sex-related differences.

In daily clinical practice rotator cuff disorders are treated in the same manner for different patient cohorts. However, as different healing rates in various donor populations may be associated with differing cellular characteristics, it may be useful to reconsider the uniform treatment of rotator cuff tears. Many in vivo and in vitro studies have demonstrated that the application of growth factors, such as bone morphogenetic protein (BMP)-2 and -7, in rotator cuff surgery may be a potential treatment option for an improved tendon bone healing. It has been reported that important cellular characteristics of tenocytes, such as cell proliferation and matrix production, can be stimulated with BMP-2 and BMP-7 [16–21]. Additionally, BMP-2 and BMP-7 have been found to increase tendon bone biomechanical strength during healing in several in vivo experiments [22–26]. In the present study, the stimulation potential of tenocyte-like cells (TLCs) of the rotator cuff from women younger or older than 65 years of age was investigated to allow for the development of more patient specific therapies.

Methods

Ethic Statement

The Ethic commission of the Charité-Universitaetsmedizin Berlin, Germany, authorized the use of tendon samples under anonymous conditions, that otherwise would be discarded (Ethic number: EA1/060/09).

Tendon Material

Supraspinatus (SSP) tendon samples were taken from patients undergoing either arthroscopic surgery for rotator cuff repair or from open shoulder surgery for reasons such as hemiarthroplasty after humeral head fractures. The biopsies were obtained 3 to 5 mm from the torn proximal tendon edge according to a standardized protocol. All patients gave their written informed consent.

Several clinical studies have reported that patients over the age of 65 years have a higher risk of sustaining recurrent defects post rotator cuff surgery [1,7,11,27]. Accordingly, in the present study the age cut-off was set at 65 years. The demographic data of the female donor groups younger and older than 65 years of age are listed in table 1.

Analysis of Cell Density

TLCs were isolated from the SSP tendon samples as described previously by collagenase type CLS II digestion [15]. After 1 week of culture in normal growth medium (DMEM/Ham's F12 with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin, all Biochrom AG, Germany) with 3 medium changes per week, an Alamar Blue assay (Biozol, Germany) was performed according to the manufacturer to quantify the number of cells in the flask by a standard curve. The cell count was normalized to the weight of the tendon sample to assess an approximate cell density.

Analysis of Cell Count Over 14 Days

A total of 2.5×10^3 TLCs at passage 2 were seeded per well in a 48-well plate in triplicates and cell count was analyzed at day 1, 4, 7, and 14 with an Alamar Blue assay according to a previous study using a standard curve method [15]. The cell count of the TLCs at day 4, 7 and 14 was normalized to cell count of day 1 by subtraction.

Gene Expression Analysis

At passage 2, RNA was isolated from the cells and cDNA was synthesized as described previously [15]. Cells were characterized by analyzing gene expression of collagen-I (Col-I), Col-II, Col-III, and osteocalcin. Furthermore, tendon-related genes like scleraxis, tenomodulin, and mohawk were analyzed, as well as the gene expression of decorin, transforming growth factor (TGF)-β1, TGF-β2, and TGF-β3. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control (Primer sequences see table 2). The Real-Time PCR protocol is described under paragraph "validation of multipotent differentiation by Real-Time PCR". The relative gene expression levels were calculated with the $2^{-\Delta Ct}$ method.

Analysis of Col-I Protein Synthesis

The Col-I protein synthesis was analyzed from the cell culture supernatant of day 14 of cell count analysis. The MicroVue C1CP EIA Kit (TecoMedical, Germany) was used according to the manufacturer. The level of Col-I protein synthesis was normalized to total protein content, which was analyzed with the Coomassie Plus™ protein assay (Thermo Fisher Scientific, Germany).

Analysis of Stem Cell Phenotype

A total of $2.5–5\times10^5$ vital cells in passage 1 were stained according to an established stem cell panel from the core unit of the Berlin-Brandenburg Center for Regenerative Therapies (BCRT). The cells were stained as follows: Live/Dead reagent, antibodies against CD29, CD44, CD73, CD90, CD105 and a negative mix consisting of CD11b, CD14, CD19, CD34 and CD45 for 25 min at 4°C (for further details see table 3). These antibodies were selected according to the study by Dominici et al. [28], who reported on the minimal stem cell criteria. Unstained cells as well as isotype controls were used as controls. After fixation, cells were measured with the BD FACS Canto II system (BD Biosciences, Germany) and FACS Diva software. The data was analyzed using FlowJo 8.8.6 software. The expression of surface markers related to stem cells as well as negative markers is an important characteristic for the stem cell potential of cells.

Table 1. Demographic data of the donor groups.

Group	Mean age [years] (Range)	Mean muscle fatty infiltration (range)	Mean tendon retraction (range)	Mean tear size (range)
		MRI assessment [53]	MRI assessment, intraoperative diagnostics [54]	MRI assessment, intraoperative diagnostics [55]
Female <65 years (N = 6)	55.7 (50–60)	0.5 (0–1)	1.0 (0–2)	1.8 (1–2)
Females >65 years (N = 6)	68.2 (65–74)	0.8 (0–1)	1.2 (0–2)	2.0 (1–3)

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Table 2. Primer.

Gene	Sequence forward primer	Sequence reverse primer	Source	°C
Housekeeping genes				
GAPDH	CCACTCCTCCACCTTTGACG	CATGAGGTCCACCACCCTGT	Primer3	
RPL13	CCTGGAGGAGAAGAGGAAAG AGA	TTGAAGGACCTCTGTGTATT TGTCAA	Primer3	
Characterization markers				
Col-I	TGACCTCAAGATGTGCCACT	ACCAGACATGCCTCTTGTCC	Primer3	64
Col-III	GCTGGCATCAAAGGACATCG	TGTTACCTCGAGGCCCTGGT	Primer3	64
Scleraxis	QuantiTect Primer Assay Kit SCXB (Qiagen, Hilden, Germany)			60
TNMD	TTGAAGACCCACGAAGTAGA	ATGACATGGAGCACACTTTC	[56]	60
Mohawk	TGGTTTGCTAATGCAAGACG	CCTTCGTTCATGTGGGTTCT	Primer3	60
Decorin	CGCCTCATCTGAGGGAGCTT	TACTGGACCGGGTTGCTGAA	Primer3	64
TGF-β1	AAGGACCTCGGCTGGAAGTG	AGGGCCAGGACCTTGCTGTA	Primer3	64
TGF-β2	CAACAGCACCAGGGACTTGC	AGCACAAGCTGCCCACTGAG	Primer3	64
TGF-β3	CTGCTGGAGGAGATGCATGG	GGCAGACAGCCAGTTCGTTG	Primer3	64
Adipogenic markers				
PPARγ	TGCAGTGGGGATGTCTCATA	CAGCGGGAAGGACTTTATGT	Primer3	60
LPL	TCCGTGGCTACCTGTCATTT	ACATCCTGTCCCACCAGTTT	Primer3	60
FABP4	TCAGTGTGAATGGGGATGTG	CCACCAGTTTATCATCCTCTCG	Primer3	60
Osteogenic markers				
OC	CCCAGGCGCTACCTGTATCAA	CTGGAGAGGAGCAGAACTGG	Primer3	64
ALPL	GGAAATCTGTGGGCATTGTG	CCCTGATGTTATGCATGAGC	Primer3	60
Runx2	GCCCCCAAACAGTATCTTGA	GCCTGAAGTGAGGTTTTAGGC	Primer3	60
Chondrogenic markers				
ACAN	CCAGTGCACAGAGGGGTTTG	TCCGAGGGTGCCGTGAG	[57]	64
COMP	GCAACACGGACGAGGACAAG	CGCCATCACTGTCCTTCTGG	Primer3	64
Col-II	CGCACCTGCAGAGACCTGAA	TCTTCTTGGGAACGTTTGCTGG	Primer3	66

RPL13: ribosomal protein L13; TNMD: tenomodulin; PPARγ: peroxisome proliferator-activated receptor gamma; FABP4: fatty acid binding protein; LPL: lipoprotein lipase; OC: osteocalcin; ALPL: alkaline phosphatase tissue-nonspecific isozyme; Runx2: runt-related transcription factor 2, ACAN: aggrecan, COMP: cartilage oligomeric matrix protein.

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Multipotent Differentiation

Cells with stem cell potential need to have the ability for multipotent differentiation, which was analyzed with the following method. Cells at passage 2 were seeded into a 24-well plate and cultured until confluence. Afterwards, cells were incubated in differentiation medium: osteogenic medium (500 μM L-ascorbic acid, 10 mM β-glycerophosphate and 100 nM dexamathasone in normal growth medium), adipogenic 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). Cells were incubated in osteogenic or adipogenic induction medium for 3 weeks with a change of medium every 3–4 days. Formed calcium deposits were visualized using Alizarin Red S staining (0.5%, Sigma-Aldrich, in 0.5 M HCL, 10 min). For quantification, staining was solubilized with 5% SDS (Roth, Germany) in 0.5 M HCL for 5 min, and then measured at 405 nm against the blank (5% SDS in 0.5 M HCL). For validation of the osteogenic differentiation, TLCs were stained for alkaline phosphatase (ALP) exemplarily. Cells were fixed with formalin and incubated in ALP staining solution (0.06% Fast Blue Bb salt (Waldeck GmbH & Co KG, Germany), 0.01% naphtol-AS-MX-phosphate (Sigma-Aldrich), 0.5% dimethylformamide (Sigma-Aldrich), 2 mM magnesium chloride (Merck, Germany), 0.1 M tris-base (Sigma-Aldrich) in dH_2O, pH 8.5) for 30 min at 37°C. For adipogenic differentiation, the lipid vacuoles in the cells were stained with 0.3% Oil Red O (Sigma-Aldrich) for 10 min. The background was cleared with 60% isopropanol, and the staining was solubilized with 100% isopropanol for 10 min and measured at 490 nm against the blank (100% isopropanol). The osteogenic and adipogenic differentiation was normalized to the staining of undifferentiated cells.

For chondrogenic differentiation 2.5×10^5 vital cells were pelleted into 15 ml falcon tubes. One cell pellet was incubated with chondrogenic 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-Aldrich), 1.25 mg/ml bovine serum albumin (BSA, Sigma-Aldrich), 5.35 mg/ml linolenacide (Sigma-Aldrich), 10 ng/ml TGF-β1 (R&D Systems GmbH, Germany) in normal growth medium, and 1 pellet with normal growth medium served as a control. Medium was changed every 3–4 days for 3 weeks. After fixation (4% PFA), cell pellets were 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 GmbH & Co KG) in 5% aluminiumsulfate for 5 min. Alcian Blue staining for chondrogenic differentiation was

Table 3. Antibodies used for FACS analysis.

Antibody	Marker specification	Results	
		<65 years	>65 years
CD29 PE (BL)	Cell adhesion	+++	+++
CD44 PE/Cy7 (BL)	Hyaluronic acid receptor	+++	+++
CD73 APC (BL)	Mesenchymal, endothelial, epithelial marker	+++	+++
CD90 PerCP/Cy5.5 (BL)	Fibroblast and stromal cell marker	+++	+++
CD105 FITC (BL)	Mesenchymal cell marker	+++	+++
CD11b V450 (BD)	Leukocyte marker	−	−
CD14 V450 (BD)	Monocyte marker	−	−
CD19 V450 (BD)	B-cell marker	−	−
CD34 PB (BL)	Hematopoietic progenitor marker	−	−
CD45 V450 (BD)	Pan-leukocyte marker	−	−
PE mouse IgG1, κ isotype control (BL)		−	−
PE/Cy7 rat IgG2b, κ isotype control (BL)		−	−
APC mouse IgG1, κ isotype control (M)		−	−
PerCP/Cy5.5 mouse IgG1, κ isotype control (BD)		−	−
FITC mouse IgG1, κ isotype control (BD)		−	−
Live/Dead AF (I)			

BL: Biolegend, Uithoorn, Netherlands; BD: BD Biosciences; M: Miltenyi; I: Invitrogen.
PE: phycoerythrin; APC: allophycocyanin; PerCP: peridinin chlorophyll protein; FITC: fluorescein isothiocyanat; PB: Pacific Blue; AF: Aqua Fluorescent reactive dye.
Results: +++: >95% positive staining; −: <2% positive staining.
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exemplarily performed only for n = 1 TLC culture for each donor group.

Validation of Multipotent Differentiation by Real-Time PCR

Individual stimulation experiments were additionally performed to validate the multipotent differentiation. The osteogenic and adipogenic differentiation was performed in triplicates and the chondrogenic differentiation in duplicates for 2 cultures per group (n = 4) and lineage specific markers were analyzed by Real-Time-PCR.

RNA was isolated from the osteogenic differentiated cells after 1 and 2 weeks of culture and from the adipogenic and chondrogenic differentiated cells after 3 weeks. For osteogenic and adipogenic differentiation RNA was directly isolated from the 24-well plates with the NucleoSpin RNA II Kit (Macherey Nagel, Germany). For chondrogenic differentiation 2 cell pellets from each donor were pooled and homogenized using peqGOLD TriFast (Peqlab, Germany) with the Precellys system (25× 1.4 mm ceramic pellets and 3× 2.8 mm ceramic pellets, Peqlab) at 5000 rpm for 30 s repeated 3 times. Using chloroform, RNA was extracted to the aqueous phase, which was afterwards diluted 1:1 with 75% ethanol and transferred to the NucleoSpin RNA II columns to purify RNA according to the manufacturers manual.

100ng RNA were transcribed into cDNA with the qScript cDNA Supermix (Quanta BioSciences, USA) according the manufacturer's instructions. Real-Time PCR was performed with the Realplex Mastercycler system (Eppendorf). 1.25 ng cDNA was used as PCR template. The Sybr Green mastermix was prepared with the following components for each well: 12.5 μl Sybr Green Supermix (Quanta BioSciences), 1 μl primer mix (10 μM, forward and reverse primer 1:1), and 6.5 μl RNase/DNase-free water. 20 μl of the master mix was added to each well. Details for the primers used are listed in table 2.

After an initial denaturation step, the following Real-Time PCR amplification protocol was repeated for 40 cycles: 95°C for 15 s, annealing temperature for 45 s, and 72°C for 30 s. The protocol was finished with a melting curve program. The Real-Time PCR results were analyzed with the Realplex software (Eppendorf). Several housekeeping genes were tested, but were regulated with differentiation. The housekeeping gene ribosomal protein L13 (RPL13) showed the weakest regulation between differentiated and undifferentiated cells. Relative expression levels were normalized to RPL13 ($2^{-\Delta Ct}$ method).

Potential for Self Renewal

A total of 1000 TLCs in passage 2 were cultured for 11 days in a 10 cm petri dish with normal growth medium and a change of medium 3 times a week. The experiment was performed in triplicates. For quantification, colonies were stained with 1% methylene blue in boratbuffer/1% azure in dH_2O (1:1, Sigma-Aldrich) for 10 min. Pictures were taken and number and average size of the colonies (range: 1–10 mm^2) were analyzed using an image analyzing system with an adaptive threshold (ImageJ 1.44i, Wayne Rasband, National Institute of Health, USA). The colony forming unit (CFU) assay allows the quantitative analysis of the self renewing capacity of TLCs, which is one important criterion for stem cells.

Test for Relative Activity of BMP-2 and BMP-7

The mouse myogenic cell line C2C12 react with osteogenic differentiation after BMP-2 and BMP-7 stimulation and serve therefore as an established system for testing the relative activity of BMP-2 and BMP-7. The osteogenic differentiation was measured by an increase in ALP activity. C2C12 cells were seeded at a

density of 5×10^4 vital cells per well in a 24-well plate in triplicates. 5 hours after seeding cells were stimulated with 200 ng/ml or 1000 ng/ml rhBMP-2 (Wyeth, USA) or rhBMP-7 (R&D Systems GmbH, Germany) in DMEM supplemented with 1% FCS and 1% penicillin/streptomycin. After 3 days of incubation cell count was analyzed by Alamar Blue assay and subsequently cells were incubated with ALP substrate solution (0.13% 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich), 50 mM glycine, 100 mM tris-base, 1 mM magnesium chloride in dH_2O, pH 10.5) for 60 min and reaction was measured at 405 nm. The ALP activity after BMP-2 and BMP-7 stimulation was normalized to the cell count.

Application of Growth Factors

A total of 2×10^4 vital cells were seeded with a drop-on method into a macro porous collagen scaffold (size 85 mm^3: 6 mm diameter × 3 mm height, Optimaix, Matricel, Germany) consisting of highly oriented porcine Col-I for 3D-culture. The remaining experimental set up is listed in table 4. TLCs were treated with 200 ng/ml or 1000 ng/ml rhBMP-2 or rhBMP-7 in DMEM/HAM's F12 (1:1) supplemented with 5% FCS and 1% penicillin/streptomycin.

Using Real-Time PCR, relative gene expression levels of Col-I, -II, -III and osteocalcin were normalized to GAPDH and to the untreated control, and calculated using the $2^{-\Delta\Delta Ct}$ method [29]. The Col-I protein synthesis was analyzed by MicroVue C1CP EIA Kit (TecoMedical) and normalized to the total protein content measured with the Coomassie Plus^{TM} protein assay according to the manufacturer.

Statistics

Statistics were performed for n = 18 values (N = 6 donors per group in triplicates) for each donor group. For Real-Time PCR analysis, RNA of triplicates was pooled (n = 6). The results are always given as median with 25 and 75 percentiles. Statistical analysis was performed using SPSS 20 (IBM, USA). Significant differences between all groups for the stimulation potential were analyzed with the Kruskal-Wallis test. The Mann-Whitney U test was performed to compare the female groups younger and older than 65 years of age or the different concentration groups with the untreated control. The level of significance was set at $p<0.05$ and adjusted with the Bonferroni-Holm correction. For the analysis of stimulation potential an additional level of significance was investigated to indicate high significant values ($p\leq0.001$).

Table 4. Experimental set up for growth factor application.

Day	Experimental procedure	Analysis
−4	Seeding in 3D-culture	
−1	Medium replacement (w/o FCS)	
0	Alamar Blue assay; Growth factor treatment	Cell count
3	Alamar Blue assay; Growth factor treatment	Cell count
5	Alamar Blue assay; Growth factor treatment	Cell count
7	Alamar Blue assay; RNA isolation, Real-Time PCR MicroVue C1CP EIA	Cell count Gene expression Col-I protein synthesis

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Results

Cell Biology

No significant differences between female donors younger and older than 65 years of age were observed regarding cell density of the tendon samples (Figure 1A). Cell proliferation was reduced in the cells from donors <65 years, as seen in the lower cell count at day 7 (Figure 1B). The Col-I protein synthesis was significantly increased in TLCs of female donors younger than 65 years of age compared to the older group (Figure 1C). The TLCs of both donor groups expressed Col-I and Col-III, the most important collagens in the tendon. They expressed low amounts of osteocalcin but no Col-II. Tendon-related genes like scleraxis, and mohawk were also expressed in the cells, as well as decorin, TGF-β1, -β2, and -β3. Tenomodulin expression was not present in the TLCs. No significant differences in the expression profile were detected between both donor groups (Table 5). A portion of TLCs revealed a stem cell potential, as was evident by their expression of stem cell markers, potency for self renewal, and multipotent differentiation. Cells were overall more than 95% positive for the markers CD29, CD44, CD73, CD90 and CD105, but more than 98% negative for the markers CD11b, CD14, CD19, CD34 and CD45, without differences between the groups (Table 3, Figure 2). With respect to the potency for self renewal, no differences were found between the groups, while 1.9% (0.8–5.2%) of the cells of donors <65 years and 2.5% (1.0–6.2%) of the cells of donors >65 years were able to form adherent cell colonies (Figure 3). A portion of cells differentiated into an adipogenic, osteogenic and chondrogenic phenotype (Figure 4). However, the osteogenic Alizarin Red S staining as well as the chondrogenic Alcian Blue staining was relatively weak in both donor groups. The ALP staining was positive in cells differentiated to the osteogenic direction. Gene expression analysis of osteogenic differentiated cells revealed an increased expression of the differentiation marker alkaline phosphatase tissue-nonspecific isozyme (ALPL) after 1 and 2 weeks compared to undifferentiated cells. The expression of runt-related transcription factor 2 (Run×2) was upregulated in differentiated cells after 1 and 2 weeks compared to controls, however without significant differences ($p = 0.114$). The osteocalcin expression was not distinctly different in cells incubated in osteogenic or normal growth medium. TLCs, which were differentiated into the adipogenic direction showed an increased expression of adipogenic markers like peroxisome proliferator-activated receptor gamma (PPARγ), lipoprotein lipase (LPL) and fatty acid binding protein 4 (FABP4). In the chondrogenic differentiated TLC pellets a strong upregulation of aggrecan and cartilage oligomeric matrix protein (COMP) expression was found. Furthermore, cell pellets incubated with chondrogenic induction medium expressed Col-II, while no Col-II expression was present in pellets cultured with normal growth medium (Figure 4). The quantification of adipogenic and osteogenic differentiation by solubilizing of the stainings revealed no significant differences between the TLCs of both donor groups.

Stimulation Potential

The relative activity of BMP-2 and BMP-7, tested by ALP activity normalized to cell count in C2C12 cells, were comparable between both factors at a concentration of 1000 ng/ml (BMP-2:2.7 (2.5–2.8), BMP-7:3.0 (2.7–3.1). At a concentration of 200 ng/ml ALP inducing activity of BMP-2 was stronger compared to the same concentration of BMP-7 (BMP-2:2.2 (2.0–2.5), BMP-7:0.6 (0.6–0.7)).

The application of BMP-2 to TLCs of donors younger and older than 65 years of age in the 3D-culture slightly increased the

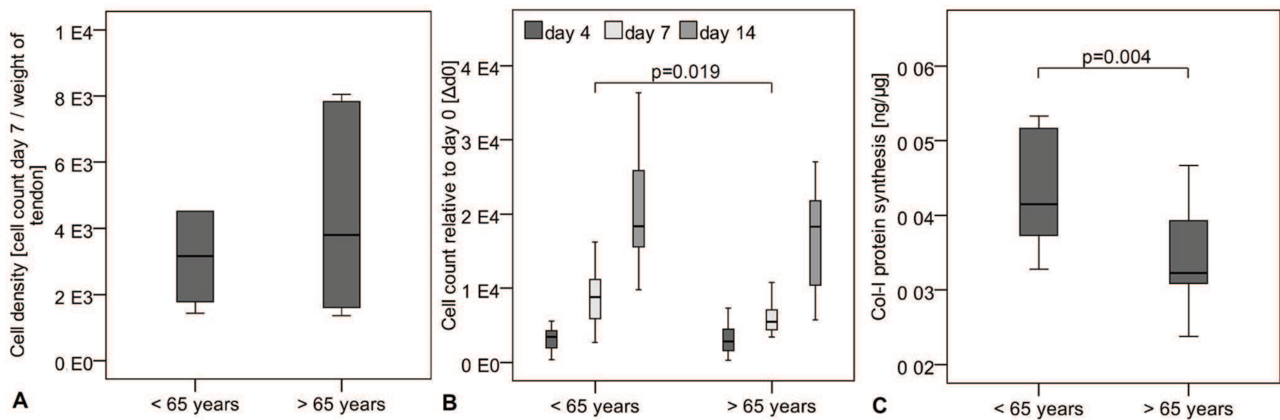

Figure 1. Cellular biological characteristics of TLCs of female donors younger and older than 65 years of age. A Cell density, measured by cell count 7 days after isolation relative to the weight of the tendon biopsy, showed no significant differences between the groups. **B** Cell count measured over 14 days and relative to day 1 after seeding was significantly decreased in TLCs of females >65 years compared to females <65 years. **C** Col-I protein synthesis relative to total protein content was significantly increased in female cells younger than 65 years of age.
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cell count at day seven in cells of females younger than 65 years of age, but not in cells of females older than 65 years. With application of BMP-7, cell count was increased at the high concentration at days 5 and 7 in both donor groups (Figure 5A). A significantly different stimulation potential between cells of female donors younger and older than 65 years of age was found, while the stimulation of cell count was weaker in the older female group compared to the younger female group at high BMP-2 and BMP-7 concentrations (Figure 5A, gray boxes). The Col-I expression and protein synthesis was increased in TLCs of both groups after application of BMP-2 and BMP-7 at both concentrations, while the treatment of the cells with BMP-7 had a stronger effect than that with BMP-2. The BMP-2 stimulation increased the Col-III expression in the TLCs at the high concentration in both donors groups (Figure 5B). BMP-7 additionally led to an increased Col-III expression in the low concentration in cells of females younger than 65 years of age (Figure 5C).

Discussion

The purpose of this study was to characterize TLCs isolated from SSP tendons of female donors of 2 different age groups to investigate differences in biological characteristics of the cell and stimulation potential with growth factors. Expanding upon our previous study investigating the effect of age on cells of male donors [15], the current study analyzed cells of female donors younger and older than 65 years of age. The results from female donors are also compared to previously published data from male donors to investigate additionally sex-associated differences. These cellular differences may be a reason for an inferior healing potential observed after rotator cuff tears in older or female patients.

Consistent with our previous study investigating age-related differences in male TLCs, the present study demonstrates that cells of older female donors have inferior cell biological characteristics compared to the younger female group. Cells of female donors older than 65 years of age had a lower cell count and Col-I protein synthesis. In comparison to our previous study, the age-related differences in the female TLCs were not as pronounced as those

Table 5. Relative gene expression of TLCs of female donors.

Relative gene expression normalized to GAPDH [$2^{-\Delta Ct}$] (median with 25–75 percentile)	Female <65 years	Female >65 years
Col-I	2.8 (2.4–3.3)	2.7 (2.2–3.3)
Col-II	–	–
Col-III	0.3 (0.2–0.8)	0.3 (0.2–0.5)
Osteocalcin [10^{-4}]	5.5 (4.1–6.6)	5.9 (5.8–8.8)
Scleraxis [10^{-3}]	2.5 (1.5–5.9)	2.3 (1.8–2.7)
Tenomodulin	–	–
Mohawk [10^{-2}]	1.5 (0.9–3.2)	0.8 (0.7–2.1)
Decorin	0.2 (0.1–0.2)	0.2 (0.1–0.2)
TGF- β1 [10^{-2}]	5.1 (3.7–5.8)	4.0 (3.3–4.9)
TGF-β2 [10^{-4}]	2.1 (1.3–4.0)	1.8 (1.0–3.5)
TGF-β3 [10^{-4}]	8.4 (4.7–11.4)	7.0 (4.7–9.3)

doi:10.1371/journal.pone.0067209.t005

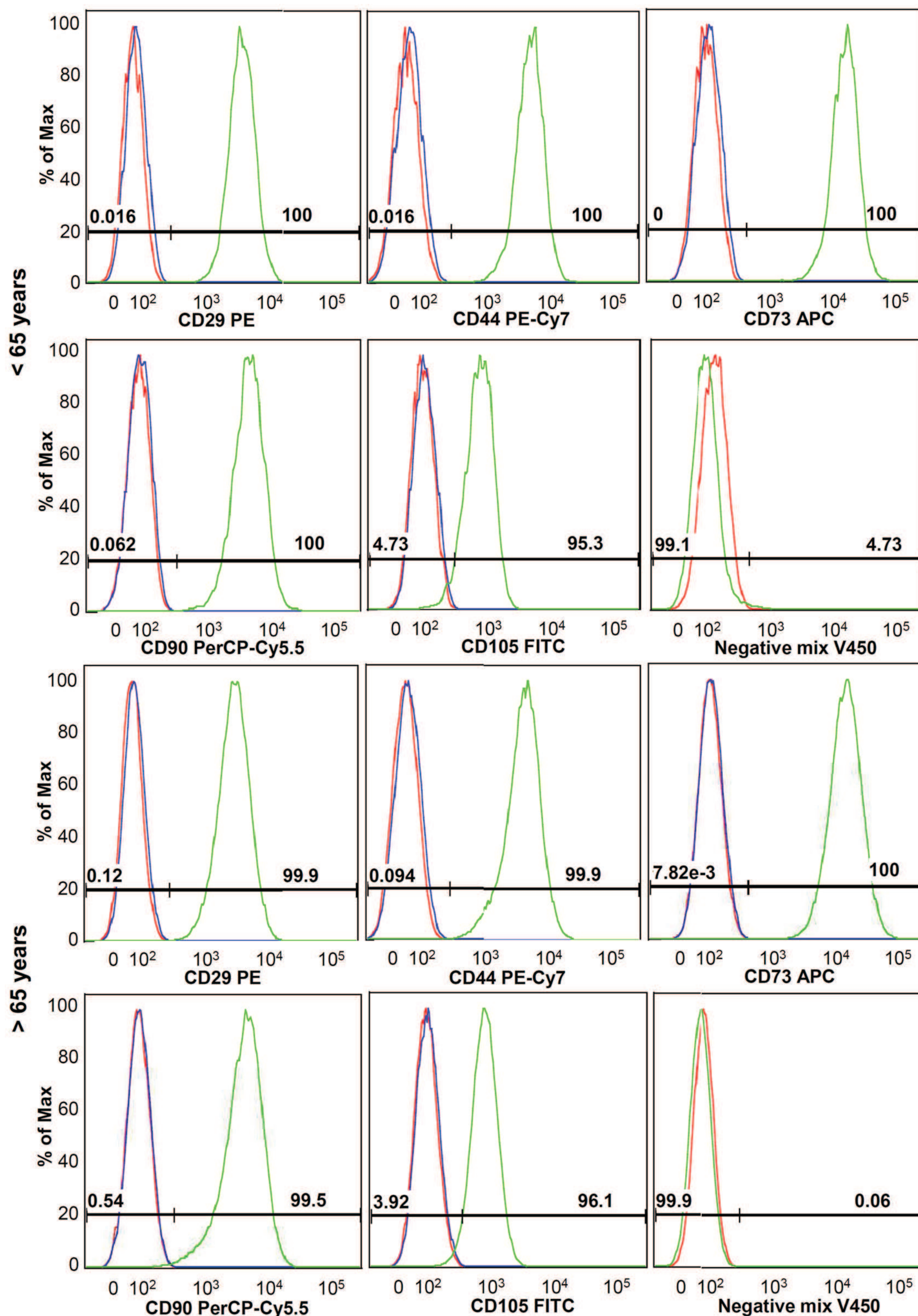

Figure 2. Representative histograms of FACS analysis of female donors </>65 years. Antibodies against stem cell markers as CD29, CD44, CD73, CD90 and CD105 revealed an overall more than 95% positive staining. Negative markers were found negative in more than 99% of cases. Green histograms represent stained cells, blue histograms represent isotype controls and red histograms show unstained cells.
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observed in males, but this may be explained by a lower mean age difference among female donors (15 years) compared to the male donors (25 years).

TLCs of female donors younger and older than 65 years of age showed a typical gene expression profile for tenocytes; cells expressed Col-I and Col-III the most important collagens in the

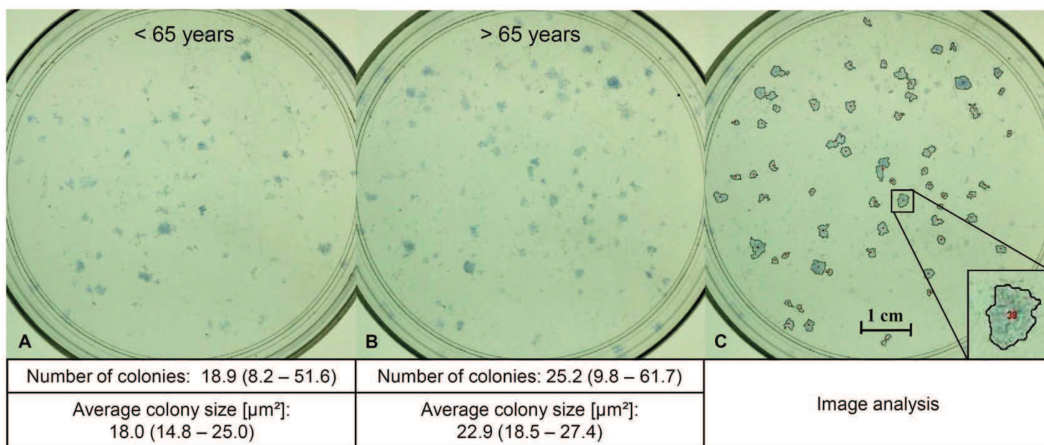

Figure 3. Representative images of CFU assay. Blue colonies formed from 1000 TLCs seeded on a 10 cm petri dish after methylen blue/azure staining from female TLCs. **A** <65 years, and **B** >65 years, **C** marked cell colonies after image analysis.
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tendon. The tendon related transcription factor scleraxis and the mohawk homeobox gene, which is a regulator in tendon development [30], were also expressed in the TLCs. Furthermore, decorin, the most important proteoglycan in the tendon [31], as well as the 3 TGF-β isoforms were expressed in the cells. Tenomodulin expression was not present in the TLCs. We reported previously that TLCs at passage 0 or 1 have a very weak tenomodulin expression [32]. This time mRNA expression from cells at passage 2 was analyzed, without measurable tenomodulin expression. Also other authors showed that tenomodulin expression is reduced in cells cultured 2 dimensionally and with higher passages [33,34]. As negative control, no Col-II and low amounts of osteocalcin were expressed.

When comparing the results of female donors to the cell biological characteristics of male donors from the previous study sex-related differences became apparent. Cells of female donors older than 65 years of age showed less cell count, Col-I protein synthesis, and potential for self renewal, compared to the TLCs of older male donors. Only a direct comparison of the older female and older male group was meaningful, due to a comparable mean age of the groups. The cell count at day 4 and 14 in older female TLCs was 2793 and 18303, respectively, lower than in older male cells, 4648 and 25140 ($p = 0.021/0.025$). Also the Col-I protein synthesis was lower with 0.027 ng Col-I per μg total protein in female cells compared to 0.039 ng Col-I per μg total protein in older male TLCs ($p = 0.012$). Additionally, only 3.6% of TLCs of female donors were able to form adherent cell colonies, while 7% of older male donors formed colonies ($p = 0.007$). A study on human muscle derived stem cells also revealed differences between male and female cells [35]. The authors found increased cell count and differentiation rates, as well as lower apoptosis rates after oxidative stress injury in human female cells. Fossett et al. reported that synovial fat pad derived mesenchymal stem cells (MSCs) of female donors tend to show an increased proliferation rate and surface marker expression compared to male cells [36]. However, this correlation was not statistically significant. Another study on human MSCs from bone marrow revealed no significant differences between male and female donors for cellular characteristics including CFU potential, single cell cloning efficiency, generation time and multipotent differentiation [37]. Most of these studies indicate that female cells may be stronger or more resilient than cells from men, which is in contrast to our findings. However, differences may be a result of different cell types used, as these studies use a variety of cells from mesenchymal precursors versus specialized cells of the musculoskeletal system.

The analysis of stem cell phenotype revealed a more than 95% expression of surface markers related to stem cells like the cell adhesion molecule CD29, the hyaluronic acid receptor CD44, the mesenchymal cell markers CD73 and CD105, and the fibroblast marker CD90. The negative markers for leukocytes CD11b and CD45, the monocyte marker CD14, the B-cell marker CD19, as well as the hematopoietic progenitor marker CD34 were not expressed on the TLCs. This result is comparable to other studies on adult cell cultures of the anterior cruciate ligament (ACL) [38,39] and hamstring tendon [40]. Steinert et al. reported that the isolation method, either by collagenase digestion or explants migration, has no influence on the expression of these respective surface markers [38].

The ability of cells to differentiate into the adipogenic, osteogenic, and chondrogenic direction is one of the minimal stem cell criteria investigated by Dominici et al. [28]. A better differentiation potential of the cells may therefore be linked to a greater number of tendon stem cells in the culture. However, the osteogenic Alizarin Red S staining, as well as the chondrogenic Alcian Blue staining was relatively weak in cells of both donor groups. A reason may be that the analyzed TLCs are a mixture of tenocytes and some tendon stem cells. A weaker staining or differentiation compared to pure MSC cultures was therefore expected. Real-Time PCR analysis of differentiation markers were additionally performed to prove differentiation towards multiple directions. In adipogenic differentiated TLCs the upregulation of lineage specific markers PPARγ, LPL and FABP4 proved adipogenic differentiation. For chondrogenic differentiated cell pellets, the weak Alcian Blue staining was strengthened by strongly increased aggrecan, Col-II and COMP expression. With upregulation of important osteogenic markers like Runx2 and ALPL differentiation towards an osteogenic direction was underlined. The expression of Runx2 was not statistically significant since only $n = 4$ differentiations were performed for validation. It would be expected to find significant differences with more TLC cultures used. Furthermore, a longer differentiation of the cells as done for validation of adipogenic and chondrogenic differentiation could have improved the Runx2 expression. The early osteogenic marker osteocalcin was not increased in differentiated cells, which

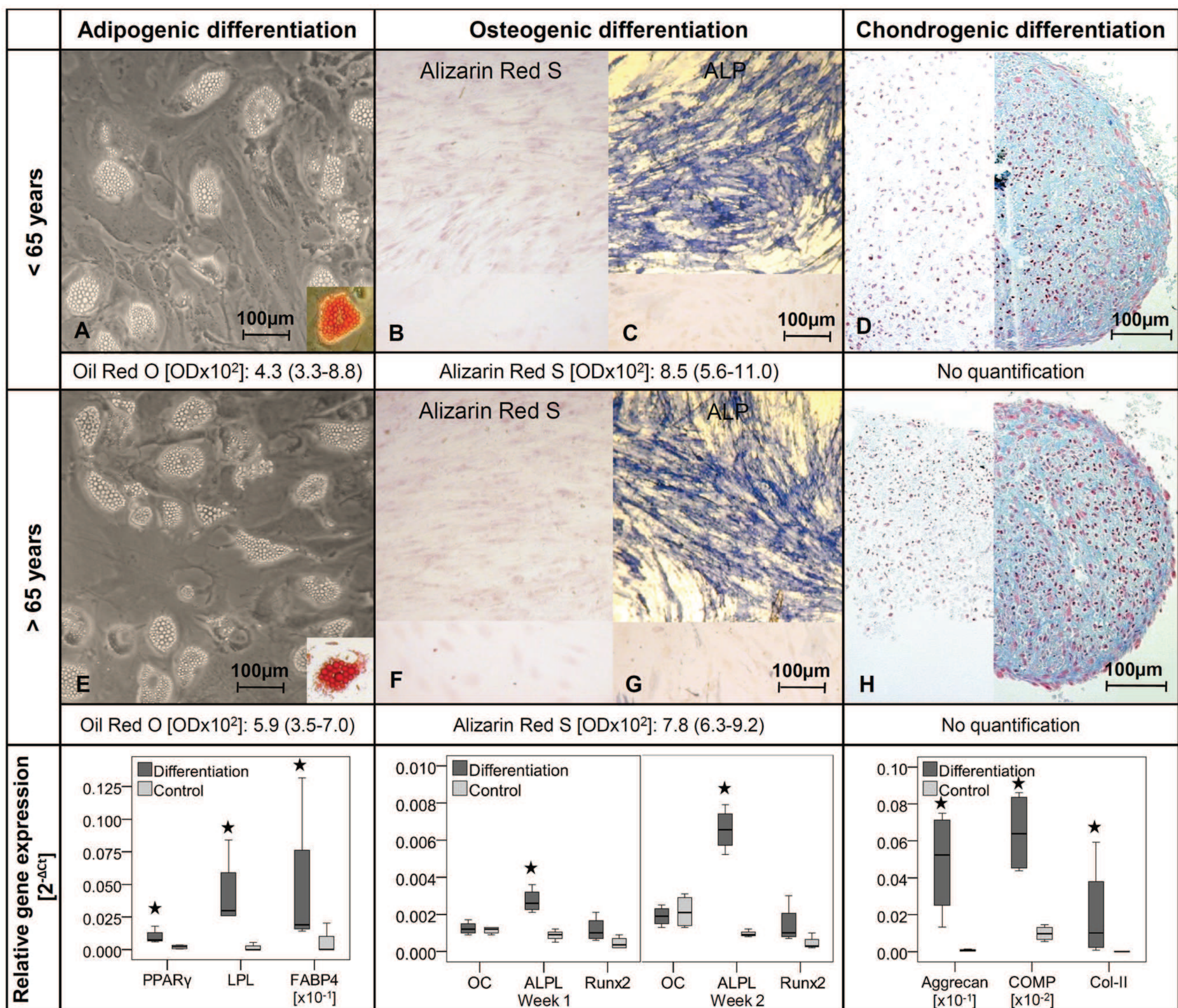

Figure 4. Multipotent differentiation potential of female TLCs. Representative images of multipotent differentiation of TLCs of female donors younger (A–D) and older (E–H) than 65 years. **A and E** Adipogenic differentiated cells showed lipid vacuoles, which were stained red after Oil Red O staining (cut-out). **B and F** Alizarin Red S staining after osteogenic differentiation was relatively weak in TLCs compared to the undifferentiated control cells (bottom). **C and G** ALP staining revealed a strong blue color in osteogenic differentiated TLCs compared to undifferentiated cells (bottom) **D and H** Blue stained chondrogenic differentiated cell pellets after Alcian Blue staining (right) versus control cell pellets (left). **Relative gene expression** of lineage specific markers was upregulated in the differentiated cells compared to undifferentiated control cells ($p = 0.029$).
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may be due to the fact that a 1 week time point was already too late. Furthermore the investigated alkaline phosphatase staining served as an additional control for a differentiation into the osteogenic direction. In the present study, the osteogenic differentiation potential was stronger in the female cells compared to the male cells from the previous study regarding the quantitative Alizarin Red S staining (OD: 0.08 versus 0.05; $p = 0.007$). A similar effect was seen in a study by Leskelä et al., who described an increasing osteogenic differentiation in bone marrow MSCs of women with age, but an unchanged differentiation rate in cells of male donors with age [41].

The cellular basis of sex-related differences is still a topic of controversy, when reviewing the current literature. The most likely factor causing differences between cells of men and women seems to be sex hormones such as estradiol. It has been reported that tenocytes of the posterior tibial tendon and flexor digitorum longus tendon of male and female donors express estrogen receptors, which are activated by estrogens such as estradiol [42]. Moreover, it has been found that estradiol had an effect on tendon fibroblasts by increasing Col-III and elastin expression [43], and inhibiting proliferation, and Col-I synthesis [44], when the hormone was directly added to the cell cultures. The influence of estrogens has also been shown in vivo, with the finding that collagen synthesis decreases in women with a higher hormone status [12,45]. Despite these findings, the menstrual cycle seems to have no impact on tendon mechanical properties [46,47], or collagen synthesis [12]. The present study was not able to prove a relationship between patients' specific hormone levels and the cell biological findings presented. Sex hormones may not play a major role beyond the in vivo situation, when they are not directly added to the cells.

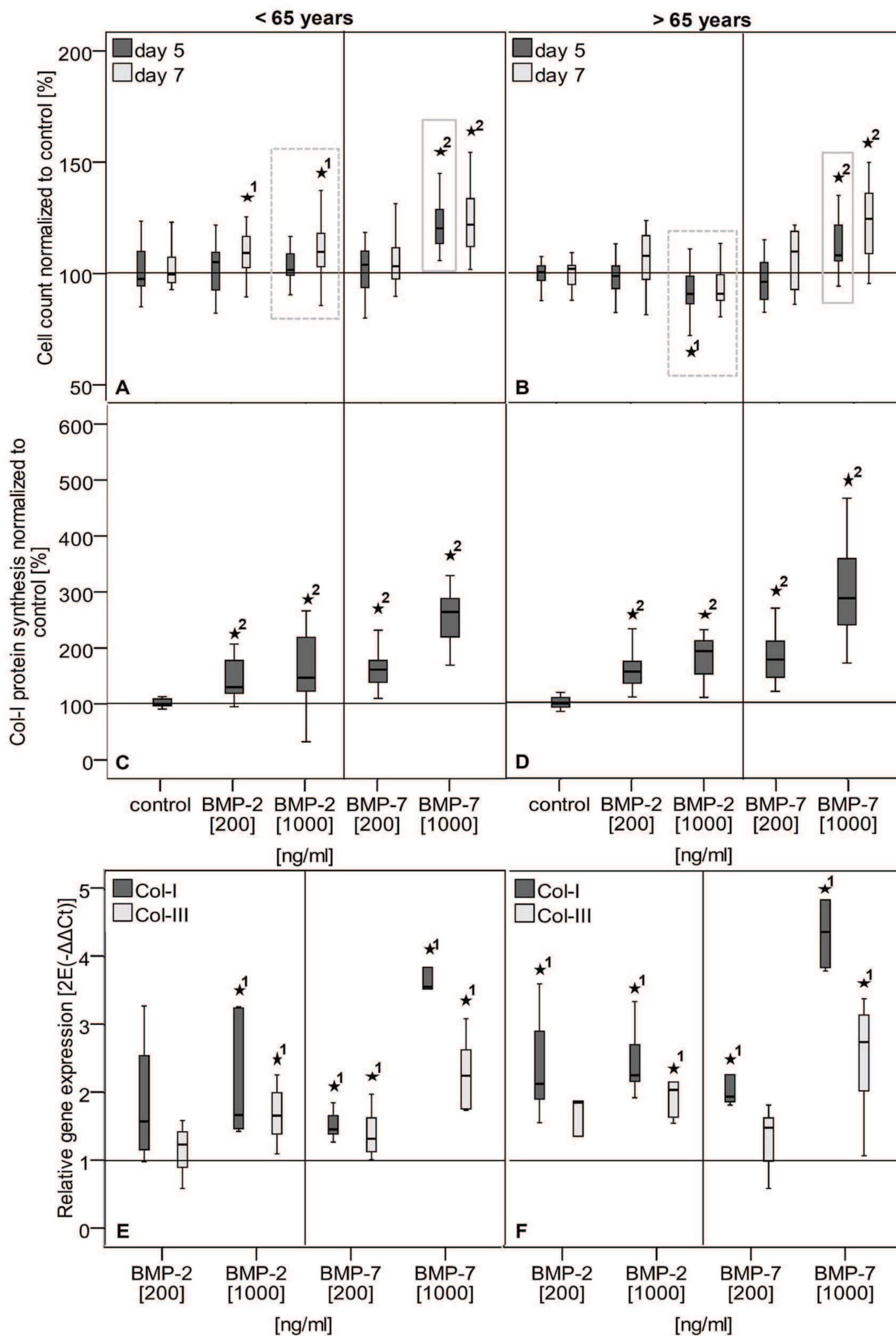

Figure 5. Stimulation potential of TLCs of female donors younger and older than 65 years of age. A–B Cell count of TLCs of female donors younger (A) and older (B) than 65 years was measured by Alamar Blue assay and given as percentage relative to untreated controls. BMP-2 application increased cell count at day 7 at the low and high concentration in cells of donors younger than 65 years. High BMP-2 concentration significantly decreased cell count at day 5 in TLCs of donors older than 65 years of age. Application of BMP-7 enhanced cell count at the high concentration at day 5 and 7 in cells of both donor groups. Gray boxes in graphs indicate significant differences between donors </>65 years, while older donors showed a decreased stimulated cell count. **C–D** Col-I protein synthesis in cell culture supernatant of day 7 after growth factor

application of TLCs of female donors younger (C) and older (D) than 65 years of age. Col-I synthesis was calculated relative to total protein and given as percentage to the untreated control. The BMP-2 and BMP-7 treatment of TLCs of both donor groups significantly increased Col-I protein synthesis at all concentrations. **E–F** Col-I and Col-III gene expression after growth factor application of TLCs of female donors younger (E) and older (F) than 65 years. Gene expression is given as $2^{-\Delta\Delta Ct}$ and was normalized to the untreated control. The Col-I and Col-III expression was significantly increased by high BMP-2 and both BMP-7 concentrations in the female group$<$65 years. In cells of females $>$65 years both factors increased the Col-I expression, but Col-III expression was only increase by high concentrations of BMP-2 and BMP-7. The asterisks (*) mark significant differences to the untreated control. The numbers give details for the p-value: 1: $p \leq 0.05$; 2: $p \leq 0.001$.
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However, it has to be kept in mind that the FCS in the medium contains an undefined amount of hormones and also phenol red, which is present in cell culture medium, representing a weakly acting estrogen mimic [48]. Since tenocytes of male and female donors were shown to express estrogen receptors [42], both components may influence TLCs in the same manner.

It has been suggested that the negative association linking female sex to inferior rotator cuff healing [9,10] may, in addition to hormone levels, be due to differences in working activities and/or general daily activities between men and women [13,14]. However, we were unable to investigate these associations as we were not provided with any additional information regarding our donors' working activities or activities of daily living.

The cell count, Col-I expression, and protein synthesis, which seem to be important factors for the tendon bone healing of the rotator cuff, were stimulated in cells of female donors younger or older than 65 years of age with application of BMP-2 and BMP-7. These results are in accordance with several studies, which have reported that BMP-2 and BMP-7 have a positive effect on tendon and ligament cell cultures [16–21]. As observed in earlier studies [15,16], BMP-7 had a stronger stimulating effect on the cells than BMP-2. The varying effect of BMP-2 and BMP-7 on the TLCs may be due to their different molecular structure and the different binding affinities of the molecules to BMP and activin receptors [49,50]. BMP-7 binds with high affinity to the activin receptors I, II and IIB, whereas BMP-2 binds with high affinities to the BMP receptors IA, IB and II [51,52]. We have previously shown that TLCs express BMP receptor IA and II, as well as activin receptor I and II, but only slightly express BMP receptor IB and activin receptor IIB [16]. However, we cannot draw any conclusions from the previous findings of receptor expression in TLCs, as they relate to the different stimulation capacities of BMP-2 and -7 seen in the present findings, since the needed receptors for both BMPs are expressed on the cells. However, when BMP-2 and BMP-7 were tested on C2C12 cells by osteoinduction (ALP activity) it was shown that both factors have the same relative activity at a concentration of 1000 ng/ml in this cell line and a higher relative activity of BMP-2 compared to BMP-7 was observed at a concentration of 200 ng/ml. This indicates that TLCs may not be able to process BMP-2 as good as BMP-7. Further studies are necessary to elucidate BMP/activin receptors and their ligands within this TLC population in more detail.

The important cellular biological characteristics can be augmented in TLCs of female donors younger and older than 65 years of age. Interestingly, however, cells of older female donors had a weaker stimulation potential with respect to cell count compared to TLCs of younger females and of older males from the previous study. The stimulation of cell count was slightly increased in the younger female and older male group [15] compared to the control. But the TLCs of females older than 65 years of age showed a slightly decrease in cell count compared to the untreated control. The stimulation of cell count was significantly decreased in the older female group compared to the other groups ($p = 0.034$–0.004), primarily at the earlier time points of days 3 and 5. We therefore speculate that the cell response to the growth factors takes longer in the cells of females older than 65 years of age, because of a slower cell metabolism, as already discussed by the biological characteristics of the cells.

In the previous study no distinct age-related differences for the stimulation potential in young and aged male donors were observed [15]. In general, it appears that the clinical parameters of older age in combination with the female sex have a negative influence on the stimulation potential of TLCs compared to other donors groups. Possibly, a higher dosage of growth factors would be needed to obtain the same effect in cells of older female donors compared to other donor groups. In vivo animal models could be used to identify the effect and the optimal dosage of BMP-2 and BMP-7 in different age and sex groups. For the potential future clinical application of BMP-2 or BMP-7 in rotator cuff repair, a higher dose for the treatment of older females should be considered.

Conclusion

The present study revealed differences in the cell biological characteristics between different patient groups. From the present findings we suggest that inferior rotator cuff healing, which is often present in donors older than 65 years of age or females, is possibly associated with inferior cellular biological characteristics, which we have observed in TLCs of older female donors. A slower cell metabolism may also have an impact on the stimulation potential of TLCs.

We conclude from the present findings that for treatment options, such as growth factor application, the patients' clinical characteristics should be considered in order to allow for more personalized therapy.

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Author Contributions

Conceived and designed the experiments: FK SP MS SG CG GS BW. Performed the experiments: FK JH. Analyzed the data: FK JH BW. Contributed reagents/materials/analysis tools: SP MS SG CG GS. Wrote the paper: FK BW. Important for discussion of clinical relevance: SP MS SG CG GS.

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Publikation 3:

„Relationship between Muscle Fatty Infiltration and the Biological Characteristics and Stimulation Potential of Tenocytes from Rotator Cuff Tears“

Franka Klatte-Schulz, Christian Gerhardt, Markus Scheibel, Britt Wildemann, Stephan Pauly

Journal of Orthopaedic Research 2013, Sep 10

Link: http://dx.doi.org/10.1002/jor.22481

Impact Faktor: 2,875

Abstract:
The healing after rotator cuff surgery is still dissatisfying, and increased muscle fatty infiltration even more impairs the healing success. To achieve sufficient healing after rotator cuff reconstructions, the use of growth factors may be one possibility. The aim of the study was to identify a possible relationship between fatty infiltration of the Supraspinatus muscle and cellular biological characteristics and stimulation potential in tenocyte-like cells (TLCs). TLCs of 3 donors groups differing in grade of muscle fatty infiltration were analyzed for their cellular characteristics and were stimulated with BMP-2 or BMP-7 in a 3D scaffold culture. The cell count and potency for self renewal were significantly decreased in TLCs from donors with high muscle fatty infiltration compared to the lower fatty infiltration groups. Cell count and Collagen-I expression as well as protein synthesis were stimulated by growth factors. Interestingly, TLCs of the high fatty infiltration group exhibited a weaker stimulation potential compared to the other groups. TLCs from donors with high muscle fatty infiltration generally revealed inferior characteristics compared to cells of lower fatty infiltration groups, which may be one reason for a weaker healing potential and may represent a possible starting point for the development of future treatment options.

Publikation 4:

Review Artikel „Biological Aspects of Rotator Cuff Healing“

Britt Wildemann, Franka Klatte

Muscles, Ligaments and Tendons Journal 2011, 1:160-167

Link: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3666482/

ohne Impact Faktor

Abstract:
Tendon tears of the rotator cuff show a high prevalence in today’s population. Patients suffer from permanent pain and disability, and surgical reconstruction may be the only possibility for abatement. The complex process of tendon-bone healing leads to mechanically inferior scar-tissue, which often results in retears or non-healing. In the current literature, factors such as patients age, sex and fatty muscle infiltration are highly correlated to the presence of rotator cuff tears and the incidence of retears. To improve the tendon tissue quality after surgical reconstructions biologically based strategies with use of growth factors arouse more and more interest in the last years. However, to optimize the treatment of rotator cuff tears the biological background of tears and retears must be investigated in more detail. This article will elucidate different aspects that have an impact on rotator cuff healing and give a brief insight in tendon/ligament cell culture and animal studies focusing on growth factor treatments.

Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Publikationsliste

Klatte-Schulz F, Gerhardt C, Scheibel M, Wildemann B, Pauly S
„Relationship between Muscle Fatty Infiltration and the Biological Characteristics and Stimulation Potential of Tenocytes from Rotator Cuff Tears“
Journal of Orthopaedic Research 2013, Sep 10
Impact Faktor: 2,875

Klatte-Schulz F, Pauly S, Scheibel M, Greiner S, Gerhardt C, Hartwig J, Schmidmaier G, Wildemann B
„Characteristics and Stimulation Potential with BMP-2 and BMP-7 of Tenocyte-like Cells Isolated from the Rotator Cuff of Female Donors“
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Impact Faktor: 3,730

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Impact Faktor: 4,558

Wildemann B, Klatte F
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Pauly S, Klatte F, Strobel C, Schmidmaier G, Greiner S, Scheibel M, Wildemann B (2011)
“BMP-2 and BMP-7 affect human rotator cuff tendon cells in vitro.”
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Pauly S, Klatte F, Strobel C, Schmidmaier G, Greiner S, Scheibel M, Wildemann B. (2010)
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Eur Cell Mater 20, 84–97.
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Kongressbeiträge

Klatte-Schulz F, Stephan Pauly, Markus Scheibel, Christian Gerhardt, Britt Wildemann
Vortrag: **„Tenozyten der Rotatorenmanschette von Spendern mit hoher fettiger Infiltration haben nachteilige zellbiologische Eigenschaften“**
20. Jahreskongress der Deutschen Vereinigung für Schulter und Ellenbogenchirurgie, Würzburg, 2013

Klatte-Schulz F, Pauly S, Scheibel M, Greiner S, Gerhardt C, Schmidmaier G, Wildemann B
Poster: **“Muscle Fatty Infiltration Influences Tenocytes of the Rotator Cuff In Vitro.”**
Transactions Vol. 37, San Antonio, USA, 2013

Klatte-Schulz F, Pauly S, Scheibel M, Greiner S, Gerhardt C, Schmidmaier G, Wildemann B
Vortrag: **„Das Geschlecht beeinflusst die zellbiologischen Eigenschaften und das Stimulationspotential humaner Tenozyten in vitro.“**
Deutscher Kongress für Orthopädie und Unfallchirurgie (DKOU), Berlin, 2012

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