THE BLOOD-TENDON BARRIER: IDENTIFICATION AND CHARACTERISATION OF A NOVEL TISSUE BARRIER IN TENDON BLOOD VESSELS

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Abstract

Tissue barriers function as "gate keepers" between different compartments (usually blood and tissue) and are formed by specialised membrane-associated proteins, localising to the apicolateral plasma membrane domain of epithelial and endothelial cells. By sealing the paracellular space, the free diffusion of solutes and molecules across epithelia and endothelia is impeded. Thereby, tissue barriers contribute to the establishment and maintenance of a distinct internal and external environment, which is crucial during organ development and allows maintenance of an organ-specific homeostatic milieu. So far, various epithelial and endothelial tissue barriers have been described, including the blood-brain barrier, the blood-retina barrier, the blood-testis barrier, the blood-placenta barrier, and the cerebrospinal fluid (CSF)-brain barrier, which are vital for physiological function and any disturbance of these barriers can result in severe organ damage or even death. Here, we describe the identification of a novel barrier, located in the vascular bed of tendons, which we term the blood-tendon barrier (BTB). By using immunohistochemistry, transmission electron microscopy, and tracer studies we demonstrate the presence of a functional endothelial barrier within tendons restricting the passage of large blood-borne molecules into the surrounding tendon tissue. We further provide *in vitro* evidence that the BTB potentially contributes to the creation of a distinct internal tissue environment impacting upon the proliferation and differentiation of tendon-resident cells, effects which might be fundamental for the onset of tendon pathologies.

Keywords: Tendon vasculature, blood vessels, endothelial cells, barrier, tight junctions, tracer, vascular permeability, serum, tendinopathy.

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Introduction

Tendons harbour a population of multipotent stem/progenitor cells, which are heterogeneous and could be identified in both peritendon and tendon proper. These cells are described to reside both in the vascular compartment and the tendon proper (Lui, 2013; Tempfer *et al.*, 2009). The niche of these cells is not yet fully understood. The finely tuned molecular and cellular composition of this niche appears to be crucial for stem cell differentiation and maintenance (Bi *et al.*, 2007; Katenkamp *et al.*, 1976; Tempfer *et al.*, 2009; Zhang and Wang, 2013; Zhang *et al.*, 2010b). So far, besides oxygen tension and mechanical stimuli, few other niche-determining factors are known including the composition and nano-/micro-structure of the extracellular matrix (ECM) (Bi *et al.*, 2007; Rui *et al.*, 2011b; Tong *et al.*, 2012; Wang *et al.*, 2008; Zhang and Wang, 2013).

The internal milieu of the niche is severely changed during tissue degeneration, trauma and injury, often resulting in pathological alterations such as hyperproliferation, erroneous tendon cell differentiation, and calcification (Oliva *et al.*, 2012; Rui *et al.*, 2011a). Therefore, it appears reasonable to suggest that rapid restoration and stabilisation of the niche following an injury is paramount in initiating and supporting the healing process.

Healthy tendons are sparsely vascularised (Ahmed *et al.*, 1998; Hooper *et al.*, 1984; Weidman *et al.*, 1984; Zhang *et al.*, 1990), particularly sheathed tendons – such as the flexor tendon of the hand – and depend on synovial fluid diffusion to provide nutrition (Hagberg *et al.*, 1992; Jones *et al.*, 2003; Lundborg *et al.*, 1980; Manske and Lesker, 1985; Tempfer and Traweger, 2015). Between densely packed collagen fibre bundles continuous rows of tenocytes are found perfectly aligned with the collagen structure in direction of the strain (Kannus, 2000; Legerlotz *et al.*, 2014), exhibiting an exceptionally low turnover rate, which may also account for the poor regenerative capacity of tendon tissue (Heinemeier *et al.*, 2013). In contrast, chronically inflamed tendons or tendons which are recovering from injury are characterised by hypercellularity and hypervascularity, due to increased proliferation of tenocytes and vascular cells (Andersson *et al.*, 2011; Astrom and Rausing, 1995). *In vitro*, an increase in proliferation has been observed in isolated tendon cells when cultivated with serum or platelet rich plasma (Mazzocca *et al.*, 2012; Wang *et al.*, 2012).

So far, tendon vasculature has been investigated in the context of tendon development, tendinopathies and vascularisation of tendon tissue grafts (Fenwick *et al.*, 2002). Little attention has been paid to the tendon vasculature as a restrictive system impeding the passive transport of solutes and molecules across vessel walls and thereby controlling the homeostasis of the tendon-specific niche. Such influx and efflux control systems usually rely on a sophisticated network of membrane-spanning proteins (*e.g.* occludin, claudins and junctional adhesion molecules) linked to a vast array of cytoplasmic proteins, which seal the paracellular space between neighbouring cells (Bauer *et al.*, 2014; Bauer *et al.*, 2011).

The aim of this study was to investigate the structural, functional and molecular characteristics of blood vessels in human and mouse tendon tissue. By means of transmission electron microscopy (TEM), RT-PCR, immunohistochemistry, and *in vivo* tracer studies we showed that the vascular lining of tendon vessels serves as a size-selective diffusion restraint, reminiscent of the tissue barrier located in the cerebral microvasculature of vertebrates (Abbott *et al.*, 2010). Moreover, we cultivated tendon-derived cells with increasing serum concentrations, thus mimicking a barrier breakdown *in vitro*, and examined the effect on proliferation and differentiation.

We propose that this blood-tendon barrier (BTB) is imperative for the establishment of stable homeostatic conditions, thus potentially contributing to the regulation of tendon cell differentiation.

Materials and Methods

Sample collection

In total, 14 human tendon samples (biceps tendons: n = 9, 8 men, 1 woman; mean age 60.4 years, range 30 to 75, obtained from rotator cuff repairs; semitendinosus tendons: n = 3, 2 men, 1 woman; mean age 31.6 years, range 18 to 55, obtained from anterior cruciate ligament reconstruction; and Palmaris longus tendons: n = 2, 1 man, 1 woman; mean age 56 years, range 37 to 75) were obtained with patients' informed consents as approved by the Salzburg ethics committee (approval #E-Nr. 1809).

15 female C57BL/6 mice (twelve months old) were provided by Charles River Laboratories (Sulzfeld, Germany). Care of the animals and all animal experiments have been conducted according to national and international guidelines and were approved by the local ethics committee.

Cell culture

Freshly obtained biopsy specimens from human biceps (n = 7, 6 male, 1 female) and semitendinosus (n = 3, 2 male, 1 female) tendons were cut into small pieces under sterile conditions followed by a 12 h digestion step in Minimum Essential Medium (MEM), supplemented with 15 mg/mL collagenase II (Gibco, Invitrogen, Lofer, Austria) at 37 °C, 85 % humidity and 5 % CO_2. Incubated in MEM supplemented with 10 % foetal bovine serum (FBS) the resulting cell suspension gave rise to an adherent culture of tendon-derived cells (TDCs), which was passaged once before the experiments were performed.

Proliferation assay

To examine the effect of serum on cell proliferation, human TDCs were cultured in the presence of pooled human serum (Sigma, Vienna, Austria, #H6914) at concentrations of 10 %, 5 %, 1 % and without serum. Cells were counted after 3 and 5 d. For an additional experiment, we cultured human tendon cells either with 10 % serum or without serum. Cells cultured without serum were supplemented with serum on the eighth day. In parallel, serum was withdrawn from cells cultured with serum so far. Again, cell numbers were counted every second or third day until cells reached confluence after two weeks.

Differentiation experiments

To assess the effect of serum on tendon cell differentiation potential, human TDCs were cultured with and without serum supplementation for one week. After this period, either Adipolife™, Chondrolife™ or Osteolife™ (LM-0023, Lifeline Cell Technology, Frederick, MD, USA), serum-free adipogenic, chondrogenic or osteogenic media, respectively, were added to both cell types for two weeks. Subsequently, 1) adipogenic differentiation was assessed by Oil red O staining and quantitative RT-PCR (qRT-PCR) determining the expression level of peroxisome proliferator-activated receptor gamma (PPARγ), a regulator of adipocyte differentiation (Rosen and Spiegelman, 2001), 2) chondrogenic differentiation by Alcian blue, collagen II and aggrecan staining, 3) osteogenic differentiation by staining for alkaline phosphatase, qPCR on three osteogenesis markers (runt-related transcription factor 2, RUNX2; bone sialoprotein 1, BSP1; and parathyroid hormone 1 receptor, PTH1R) and visualisation of calcium deposits by Alizarin S (Sigma, Vienna, Austria) staining.

ß-Galactosidase staining

In order to exclude that lack of serum affects cell senescence, we performed a ß-galactosidase staining according to the protocol described by Dimri *et al.* (1995). To this end, cells were cultivated for 2 weeks with either Adipolife™ without serum, Adipolife™ with 10 % human serum, MEM with human serum, Osteolife™ without serum or Osteolife™ with 10 % human serum. The cells were fixed for 1 min with 3 % paraformaldehyde (PFA) at room temperature and subsequently washed 3 times with cold phosphate-buffered saline (PBS). The cells were then incubated at 37 °C for 8 h with a buffer containing 40 mM citric acid, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$ and freshly added X-Gal (1 mg/mL) at pH 6. After the cells were mounted with cover slips, they were microscopically analysed.

Alizarin S staining and quantification

In order to visualise and quantitate the amount of calcification, cells cultured in 6-well plates were fixed in 4 % PFA for 20 min at room temperature and stained with a solution of 0.1 % Alizarin S at pH 4.5 for 5 min at room temperature. After 3 washing steps in double-distilled H_2O to remove any unbound stain, the bound stain was dissolved by using 800 µL of 10 % (v/v) acetic acid under gentle agitation for 30 min. After vortexing for 30 s, the

tubes were heated to 85 °C for 10 min and then centrifuged at 15000×*g* for 10 min. 500 µL of the supernatant was removed and transferred to new 1.5 mL micro-centrifuge tubes and 200 µL of ammonium hydroxide solution 10 % (v/v) was added to achieve a pH of between 4.1-4.5. Aliquots (150 µL) of each sample were read in triplicate at an absorbance of 405 nm on a microplate reader (Tristar LB 941, Berthold Technologies, Bad Wildbad, Germany). In order to correct for varying cell numbers under different conditions, OD values were normalised to DNA content, which was determined in parallel cultures.

Oil red O staining and quantification

To determine the extent of adipogenic differentiation, cells cultured in 6-well plates were fixed in 4 % PFA for 20 min at room temperature and stained with Oil red O working solution for 15 min at room temperature. After 3 washing steps, bound Oil red O was dissolved in 400 µL isopropanol and absorbance was measured at a wavelength of 540 nm (Tristar LB 941, Berthold Technologies). In order to correct for varying cell numbers under different conditions, OD values were normalised to DNA content, which was determined in parallel cultures.

DNA quantification

Cells were lysed in 5 mM Tris-HCl (pH 8.0) by 3 freeze/thaw cycles, followed by sonication for 20 min. DNA was measured in the supernatant of the solutions using a fluorescent Quant-iT™ PicoGreen® dsDNA reagent assay kit (Invitrogen, Carlsbad, CA,USA, # P7589) according to the manufacturer's instructions.

Tracer injection

To assess the tightness of tendon blood vessels, mice (C57BL/6) were injected with 0.10-0.15 mg/g of 10 kD biotin-dextran (BDA) *per* animal (Molecular Probes; Carlsbad, CA, USA, #D-1956) or a 287 D biotinylated Neurobiotin™ tracer (Vector Laboratories, Peterborough, UK, #SP-1120) into the tail vein. The tracer was allowed to circulate for 15 min before the animals were euthanised by an overdose of sodium thiopental (Abcur, Helsingborg, Sweden) (0.4 g/kg animal) through an IP injection. Achilles tendons were removed, cut into 1 mm^3 cubes, immersion-fixed in 4 % PFA at 4 °C overnight, thoroughly washed in PBS and further processed for paraffin embedding. Heart and brain tissue as controls were processed in parallel.

Immunohistochemistry

To identify the presence of tight junction proteins in tendon blood vessels, human tendon biopsy samples (Palmaris longus, biceps tendon) were fixed in 4 % PFA in PBS at 4 °C overnight. Achilles tendons from C57BL/6 mice were perfusion-fixed with 4 % PFA in PBS and immersion-fixed in the same fixative at 4 °C overnight. Tissues were processed for paraffin embedding, sectioned (6 µm), and stained with antibodies specific for zonula occludens (ZO-1; Invitrogen, Lofer, Austria #61-7300), occludin (Invitrogen, #71-1500), claudin 3 (Abcam, Cambridge, UK, ab15102), and claudin 5 (Abcam, ab53765). Incubation with primary antibodies was performed at 4 °C overnight. Primary antibody was omitted for negative controls. After treatment with Power Vision polyHRP-anti-rabbit IgG (ImmunoLogic, Duiven, Netherlands), sections were incubated with 3,3'-diaminobenzidine (DAB) tetrahydrochloride, Sigma Aldrich, Vienna, Austria), and counterstained with Novocastra™ Haematoxylin dye (Leica Biosystems, Newcastle, UK) and mounted in Eukitt (Sigma Aldrich, Vienna, Austria).

For double-immunolabelling, mouse Achilles tendons were fixed in 4 % PFA, washed with PBS-sucrose and cryosectioned on a Leica CM 1950 microtome. Sections (15 µm) were stained with biotinylated Bandeiraea simplicifolia lectin (BSL, obtained from Sigma Aldrich, Vienna, Austria) visualising blood vessels, and antibodies specific for ZO-1, occludin, claudin 3, and claudin 5. Primary antibodies were visualised with secondary antibodies labelled with Alexa Fluor 568 (donkey anti rabbit Alexa Fluor 568, Life Technologies, Vienna, Austria), BSL was visualised with Streptavidin, Alexa Fluor 488 conjugate (Life Technologies, Vienna, Austria) and analysed on a LSM710 (Zeiss, Munich, Germany).

Tracer-injected, paraffin-embedded Achilles tendons were sectioned (6 µm) and mounted on Superfrost ultra plus glass slides (Thermo Scientific, Vienna, Austria). Following deparaffinisation and dehydration, sections were incubated in blocking reagent (Roth, Graz, Austria) for 30 min, incubated with a streptavidin-HRP labelled antibody (Dako, Glostrup, Denmark, #90397) for 1 h, and developed with DAB after several washing steps in PBS.

Immunocytochemistry

In order to better characterise the cells used for the *in vitro* experiments, human TDCs were fixed with 4 % paraformaldehyde, washed with PBS and incubated with antibodies directed against tenomodulin (Santa Cruz, Heidelberg, Germany, sc-98875), pro-collagen1a1 (Abcam, Cambridge, UK, ab64409), CD44, CD29, CD90, CD105, and CD45 (Abcam, Cambridge, UK, ab93758) at 4 °C for 48 h. Secondary antibodies labelled with Alexa Fluor 568 and cy5 (donkey anti rabbit Alexa Fluor 568 and donkey anti rat cy5, Life Technologies, Vienna, Austria) were used to visualise primary antibodies.

Laser-capture microdissection and RT-PCR

To specifically analyse tendon blood vessels, laser-capture microdissection was performed on paraffin-embedded tissue sections of mouse Achilles and human biceps tendons. Parts from the vascular bed and the dense collagenous region of the tendon were dissected using a PALM MicroBeam laser capture microscope from Zeiss and captured tissue was placed in an RNase-free PCR cap. Extracted total RNA was directly transcribed into cDNA using the High Capacity RNA-to-cDNA Master Mix (Life Technologies). PCR was performed using the smallest available intron-spanning TaqMan Gene Expression Assay (with amplicons <100 bp, Life Technologies, Vienna, Austria) for occludin (assay ID: Hs00170162_m1; Mm00500912_m1), claudin 1 (Hs00221623_m1; Mm01342184_m1), claudin 3 (assay ID: Hs00265816_s1; Mm00515499_s1), claudin 4 (assay ID: Mm00515514_s1), claudin 5 (assay ID: Hs01561351_m1; Mm00727012_s1), and claudin 12 (assay ID: Hs00273258_s1; Mm01316509_

m1). Amplification efficiency of the house-keeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT, assay ID: Hs01003267_m1; Mm01545399_ml) was used as an internal control. PCR products were separated on an agarose gel and stained with ethidium bromide.

Electron microscopy

For analysis of tendon blood vessels on the ultrastructural level, 3 mouse Achilles tendons were perfusion-fixed with 2 % glutaraldehyde in PBS and immersion-fixed in 2 % glutaraldehyde in 100 mM phosphate buffer (PB) (pH 7.0) at 4 °C overnight. After several washing steps in phosphate buffer specimens were post-fixed in 1 % glutaraldehyde and 1 % osmium tetroxide in 50 mM PB (pH 6.2) at 4 °C. Following extensive rinsing in distilled water and incubation in 1 % uranyl acetate, tissues were dehydrated in a graded ethanol series and embedded using the Ultra Bed Low Viscosity Epoxy Kit (Electron Microscopy Sciences, Hatfield, PA, USA) according to the manufacturer's instructions.

Semithin sections were cut and stained with Azur II to determine the region of interest. Ultrathin sections (~ 80 nm) were cut on an ultramicrotome Reichert Ultracut S (Optische Werke C. Reichert, Vienna, Austria), mounted on Formvar-coated copper slit grids and contrast stained with uranyl acetate/lead citrate in an ultrostainer (Leica/ Reichert, Vienna, Austria). All ultrathin sections from the proximal, the middle and the distal part of the tendons were examined in a transmission electron microscope LEO EM 910 (LEO, Elektronenmikroskopie Ltd, Oberkochen, Germany) operating at 80 kV.

Quantitative RT-PCR

To assess the expression levels of genes associated with differentiation into the adipogenic and osteogenic lineages, we performed quantitative RT-PCR. To this end, total RNA from human tendon cultures was isolated using TRI Reagent (Sigma-Aldrich, Vienna, Austria) and reverse-transcribed with iScript Reverse Transcription Supermix for RT-qPCR (BioRad, Munich, Germany, # 170-8841). Products were amplified using the Taq Man Gene Expression Master Mix (Thermo Fisher Scientific, Applied Biosystems, Vienna, Austria, # 4369016) and the smallest available PrimeTime qPCR assays from IDT (Integrated DNA Technologies, Coralville, IA, USA) for PPARγ (ID assay: Hs.PT.58.25464465), RUNX2 (ID assay: Hs.PT.56a.19568141), BSP1 (ID assay: Hs.PT.58.20755025), and PTH1R (ID assay: Hs.PT.58.3437800) using a CFX96 Touch™ Real-Time PCR Detection System (BioRad, Munich, Germany). PCR was performed applying the following temperature regimen: 2 min 50 °C uracil DNA glycosylase (UDG) incubation; 10 min 95 °C enzyme activation; 15 s 95 °C, 1 min, 60°C for 40 cycles. For normalisation the house-keeping genes hypoxanthine-guanine phosphoribosyltransferase (HPRT1, Hs.PT.39a22214821), ribosomal protein, large, P0 (RPLP0, Hs.PT.56.40434846), and TATA box-binding protein (TBP, Hs.PT.39a.22214825), were used as internal controls. Cq values were analysed using qBasePlus v. 2.4 (Biogazelle NV, Zwijnaarde, Belgium) and normalised relative quantities were calculated by normalising the data to the expression of three previously validated endogenous control genes as described by Vandesompele *et al.* (2002). The normalised quantities were then determined for the candidate genes scaled against the expression values determined for the undifferentiated control (MEM+HS) to generate fold changes in expression. Measurements from three independent experiments (n = 3) are shown (technical replicate for each sample) and expressed as mean ± standard error of the mean (SEM).

Western blot

To determine the expression levels of extracellular matrix proteins, lysates from human tendon cells were prepared as described previously (Traweger *et al.*, 2008). Protein contents were quantified by using a BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using a 7.5 % or 10 % gel. After blotting, the polyvinylidene difluoride (PVDF) membrane was incubated in 5 % non-fat milk powder in Tris-buffered saline-Tween (TBS-T). Immunodetection was performed using primary antibodies recognising pro-collagen1a1 of Col1a1 (Santa Cruz, Heidelberg, Germany, sc-8782), MMP2 (Abcam, Cambridge, UK, ab37150), MMP9 (Cedarlane, Ontario, Canada), β-actin (Santa Cruz, sc-47778) and secondary HRP-labelled goat anti-rabbit and goat anti-mouse antibodies, respectively (BioRad, Munich, Germany). Bands were visualised using the SuperSignal West Pico Chemiluminescent Substrate from Pierce (Thermo Scientific, Vienna, Austria). All experiments were repeated at least three times. Band intensities were measured densitometrically and normalised to ß-actin expression using the Image Lab Software 5.1 from BioRad.

Zymography

Matrix metalloproteinase activities were assessed by gelatine zymography in tendon cell cultures. To this end, human TDCs were incubated with increasing serum (0 %, 1 %, 5 % and 10 %) concentrations. After 4 d, serum-containing medium was removed, cells were washed twice with PBS and serum-free medium was added to the cell cultures. After 10 min the supernatant was taken and gelatine zymography was performed as described previously (Krizbai *et al.*, 2000). Briefly, cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 % Triton X-100, and 0.1 % SDS. Equal amounts of protein were loaded on 7.5 % polyacrylamide gels containing gelatine (1 mg/mL). Following electrophoresis, the gels were given a 30 min wash with ddH_2O containing 2.5 % Triton X-100, and then incubated with a substrate buffer (50 mM Tris-HCl (pH 8.0) containing 5 mM $CaCl_2$) overnight at 37 °C. The gels were then stained with Coomassie Blue R-250 for 30 min followed by several washing steps. Band intensities were measured densitometrically using the Image Lab Software 5.1 from Biorad.

Statistical analysis

Statistical analyses were performed using Graph Pad Prism software (version 5.04). Densitometric data are presented as means with standard deviations. One-way analysis of

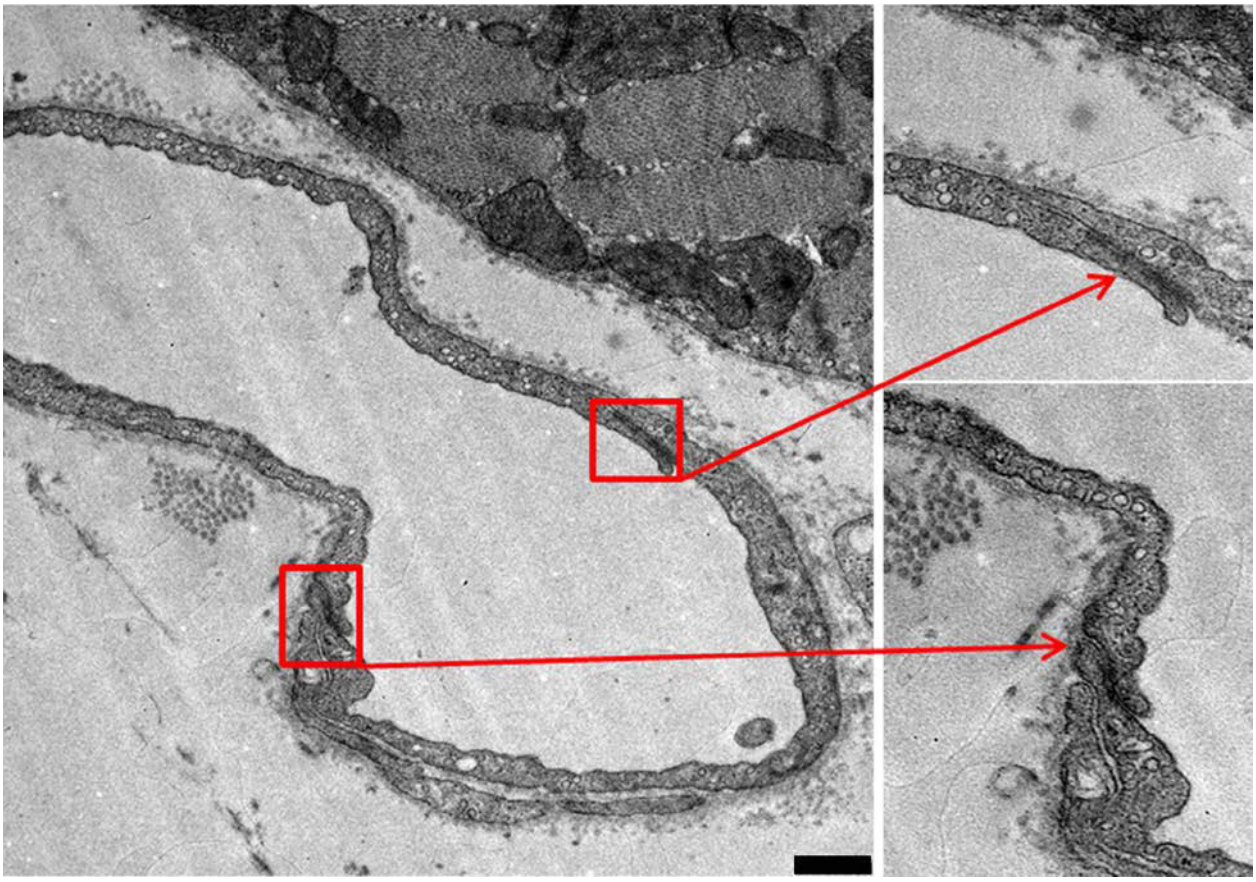

Fig. 1. Representative TEM micrograph of mouse Achilles tendon blood vessel. The endothelium displays a continuous, non-fenestrated endothelium with TJ structures (red arrows). Scale bar: 500 nm.

variation (ANOVA) using the nonparametric Kruskal-Wallis test were used to test for differences between the groups. qPCR data were analysed using the Mann-Whitney test. $p < 0.05$ was considered statistically significant.

Results

In order to characterise a putative BTB in human and mouse tendons which regulates the passage of serum components from the circulation into the tendon tissue, we first set out to demonstrate the presence of structural components known to constitute other tissue barriers. By means of transmission electron microscopy we investigated the ultrastructure of tendon vessels and found that vessels of both the proximal as well as the distal tendon region displayed a continuous, non-fenestrated endothelium with a distinct basal lamina. At higher magnification we identified "kissing points" indicative for tight-junctional complexes (Fig.1).

To demonstrate the presence of tight junctions at the tendon endothelium we performed a qualitative RT-PCR to determine the presence of mRNAs encoding occludin, claudins 1, 3, 4, 5, 7, 11, 12 and 19, as well as Tricellulin using RNA extracted from laser-microdissected human biceps and mouse Achilles tendon blood vessels (Fig.2).

With immunohistochemical staining we tested for several tight junction (TJ)-proteins known to be present at cerebral and retinal endothelial cell junctions. Immunoreactivity to at least zonula occludens-1 (ZO-1), occludin, claudin 3 and claudin 5 was detected in endothelial cells lining the blood vessels of human palmaris longus (Fig. 3**a-d**), human biceps tendon (data not shown) and mouse Achilles tendons (Fig. 3**e-h**).

Double immunolabelling using *Bandeiraea simplicifolia* lectin and antibodies directed against TJ proteins ZO-1, occludin, claudin 3 and claudin 5 on mouse Achilles cryosections showed that the TJ-proteins, occludin, claudin 3 and claudin 5 were exclusively localised to the innermost vessel layer, that is the endothelium (Fig. 3**e-p**).

Tracer studies

Since the mere presence of tight junction proteins does not necessarily reflect functional tightness of a tissue barrier, we conducted tracer studies by using both a biotinylated 10 kD dextran and a small 287 D neurobiotin tracer. Inspection of the streptavidin-HRP-labelled tissues revealed that the 10 kD tracer remained within the vessel walls of tendon and brain tissue, whereas substantial leakage into the heart muscle was evident (Fig. 4**a-f**). In contrast, the small tracer was only retained by brain vessels, but passed the endothelium in both the tendon and heart (Fig. 4**g-l**).

Tendon cell culture

Proliferation assay

To better characterise tendon cell cultures obtained by collagenase II digestion, we stained for markers

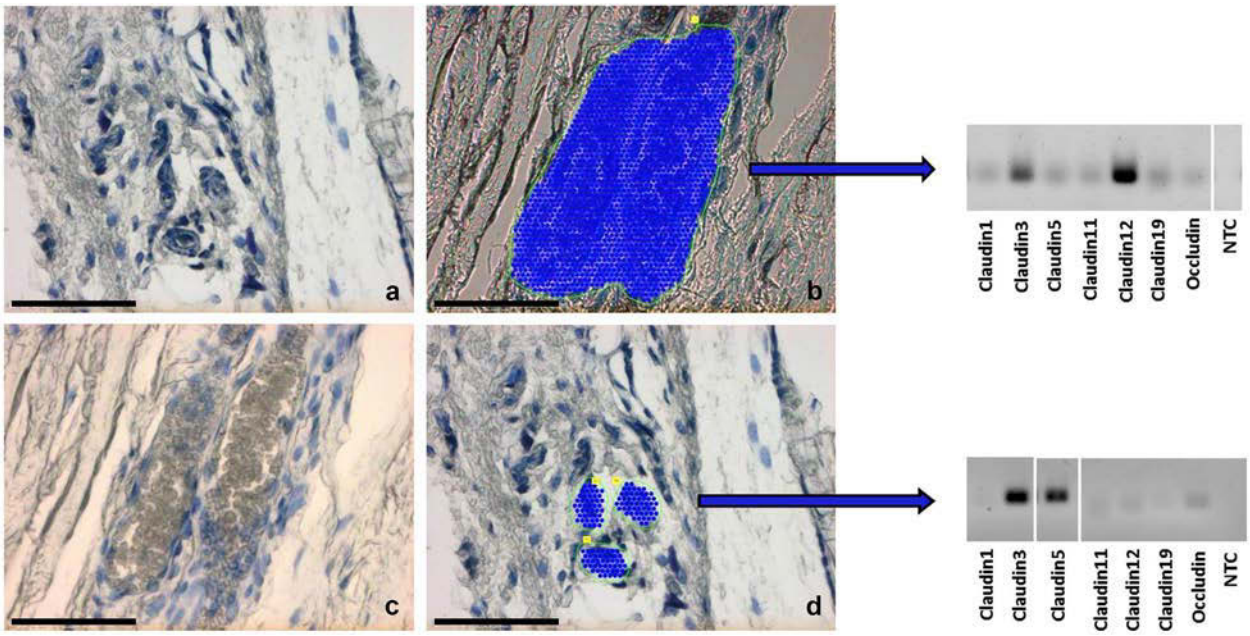

Fig. 2. RT-PCR analysis of RNA isolated from human biceps (**a**, **b**) and mouse Achilles tendon (**c**, **d**) vessels lasermicrodissected from 6 µm thick paraffin sections. mRNAs encoding TJ-associated proteins were analysed. Scale bars: 75 µm.

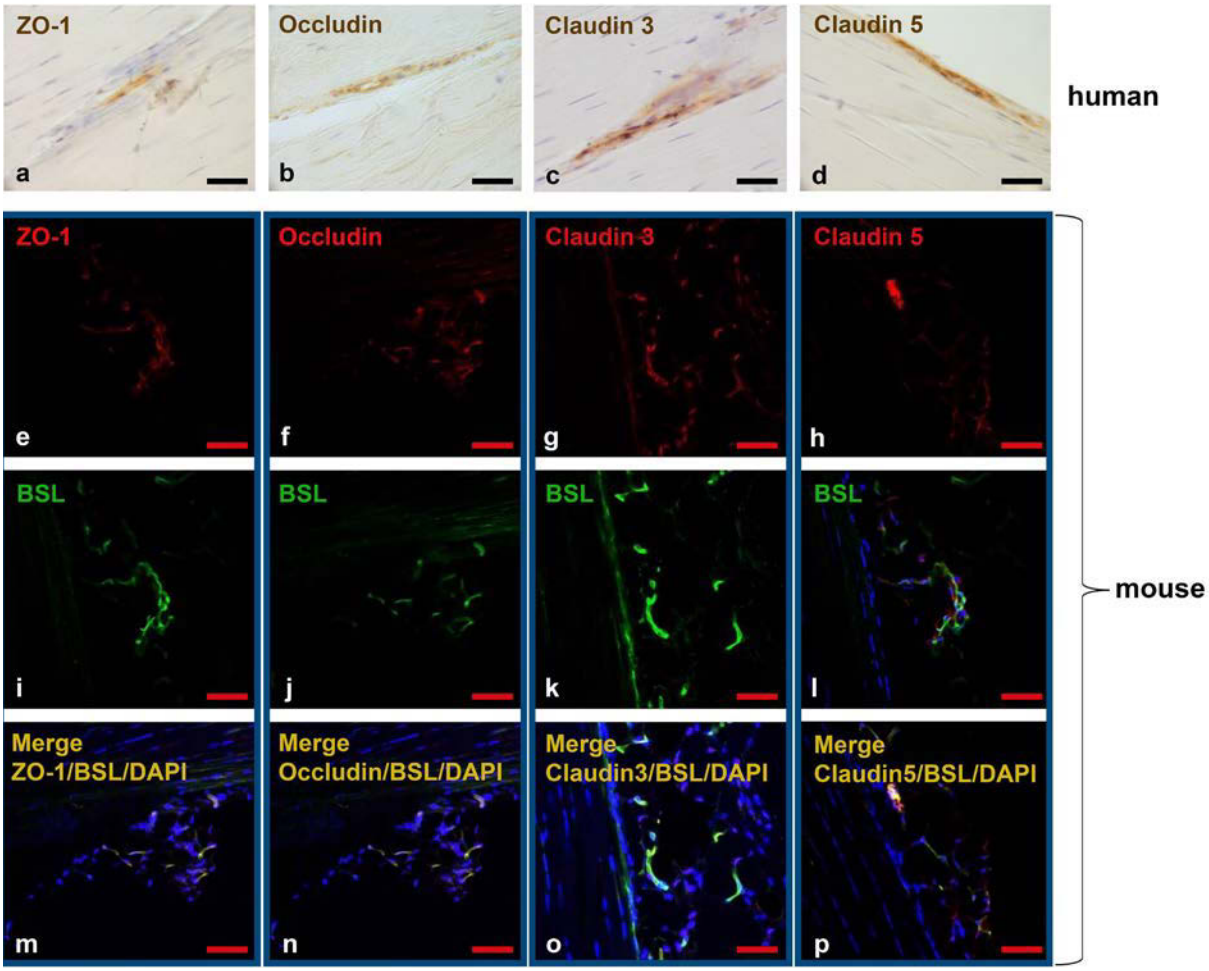

Fig. 3. Immunohistochemical staining of human palmaris longus (**a**-**d**) and mouse Achilles tendons (**e**-**p**) stained with antibodies directed against ZO-1 (**a**, **e**), occludin (**b**, **f**), claudin 3 (**c**, **g**) and claudin 5 (**d**, **h**). Blood vessels in mouse Achilles tendons are visualised by *Bandeiraea simplicifolia* lectin (BSL) (**i**-**l**), merged images show co-distribution of ZO-1, occludin, claudin 3 and claudin 5 with BSL, and nuclei are stained with DAPI (**m**-**p**). Scale bars: 50 µm.

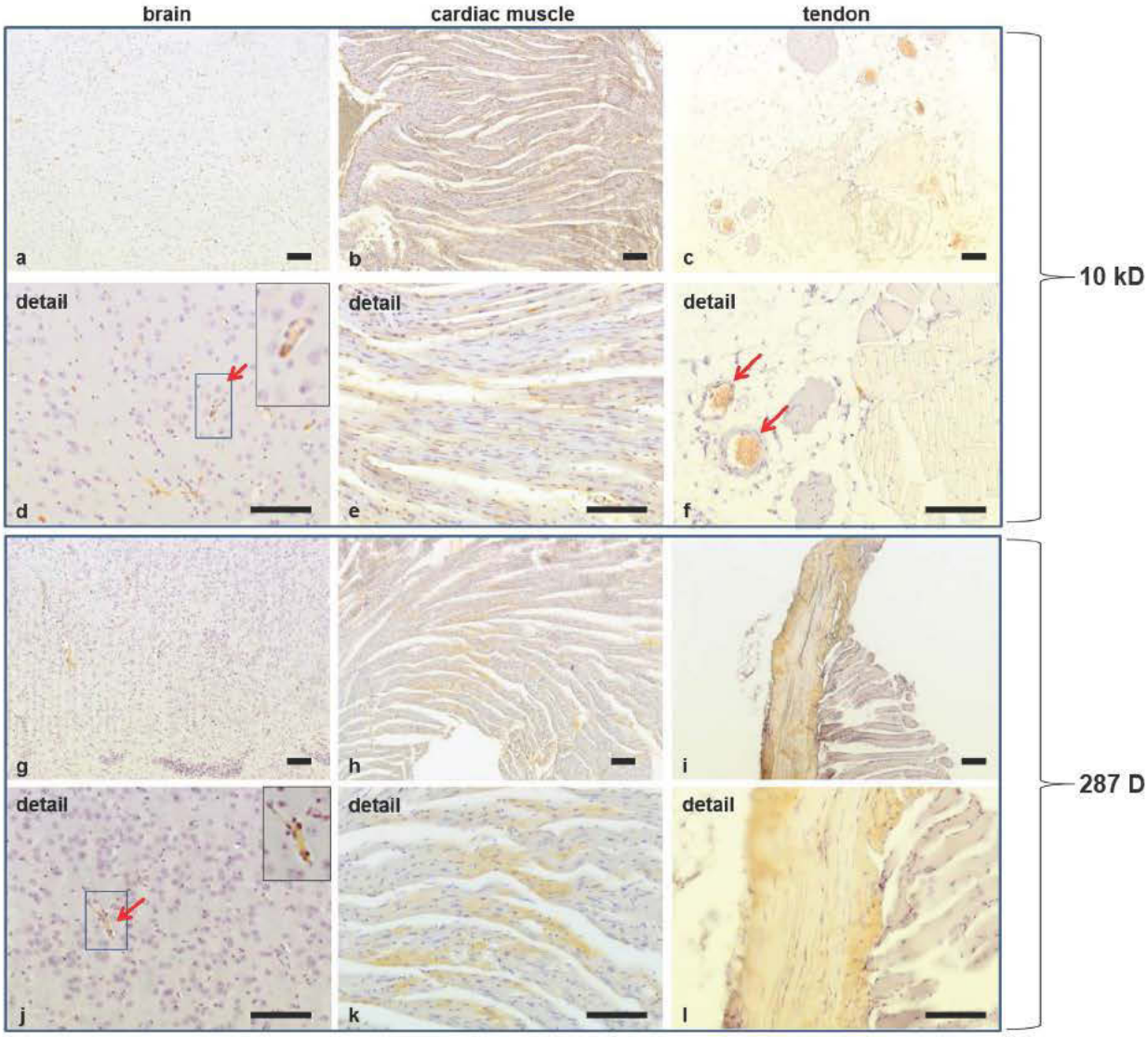

Fig. 4. Biotin immunostaining on paraffin sections of mouse brains (**a**,**d**,**g**,**j**), cardiac muscles (**b**,**e**,**h**,**k**) and Achilles tendons (**c**,**f**,**i**,**l**) of mice perfused with a 10 kD dextran tracer (**a-f**) or a 287 D tracer (**g-l**) reveal that tendon vessels are tight for the 10 kD tracer trapping it in the lumen (**c**,**f**, red arrows), whereas the 287 D tracer leaks out (**i**,**l**), causing a homogenous staining. Brain vessels are tight for both tracers (**d**,**j**, red arrows). In the cardiac muscle, both tracers leak out from the vessels (**b**,**e**,**h**,**k**). Scale bars: 100 µm.

known to be expressed by tendon cells, such as the transmembrane glycoprotein tenomodulin and the intracellular, uncleaved proform of collagen type 1, the major ECM composing protein within tendons. The tendon cells we cultured homogenously expressed tenomodulin and pro-collagen1a1, CD44, CD29, CD90 and CD105, but lacked CD45 (Fig. 5**a-i**). We cultured tendon cells in the presence of increasing serum concentrations (Fig. 6**a-f**). Tendon cell proliferation increased in a serum-dependent manner. After five days of cultivation, the number of cells incubated with 10 % human serum was almost doubled compared to cells treated with 1 % serum. Cells cultured without human serum showed only slight proliferation (Fig. 6**a**), whereas addition of serum to cultures deprived of serum until day 8 led to immediate cell proliferation, and withdrawal of serum on the day 8 let proliferation go down continuously (Fig. 6**g**).

Differentiation assays

To determine whether serum also influences tendon cells apart from proliferation, we applied culture media inducing differentiation into the adipogenic, chondrogenic or osteogenic lineage, respectively. Tendon cells co-incubated with Adipolife™ and serum (with a medium change every other day) for 2 weeks displayed a high amount of lipid droplets visualised by Oil red O staining (Fig. 7**b**). Differentiation into adipocytes was confirmed by qPCR showing a significant increase in the expression levels of the adipocyte-specific gene PPARγ ($p = 0.007$), compared to tendon cells cultured with Adipolife™ alone (Fig. 7**e**).

Tendon cells incubated with Chondrolife™ exhibited collagen II expression indicative of chondrogenic differentiation (Fig. 8**a**,**b**,**g**). Interestingly, aggrecan expression was predominately present in cell cultures incubated with Chondrolife™ alone (Fig. 8**a**,**h**). In cultures

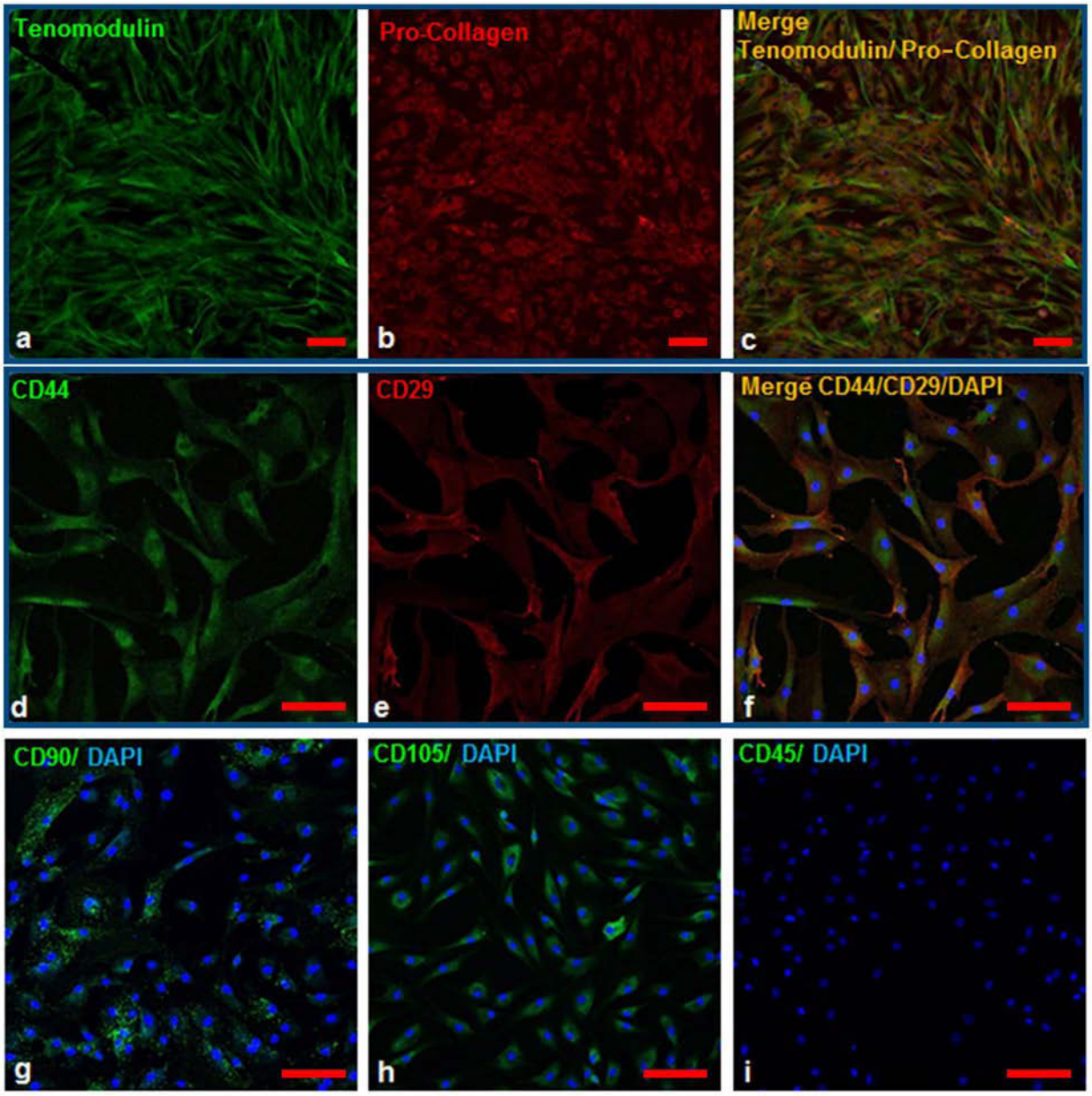

Fig. 5. Immunostaining on human biceps tendon cells show the cells coexpressing tenomodulin and pro-collagen1a1 (**a-c**), CD29 and CD44 (**d-f**). CD90 (**g**) and CD105 (**h**) are expressed, whereas CD45 (**i**) is not detectable. Scale bars: 100 µm.

incubated with serum and MEM, staining for neither collagen II nor aggrecan could be detected (Fig. 8**c**,**g**,**h**). Only cells exposed to a standard chondrogenic medium stained for Alcian blue (Fig. 8**d**,**e**), a dye staining for acidic sulphated mucosubstances and acetic mucins, whereas cells in control medium displayed a greenish colour (Fig. 8**f**).

Osteogenic differentiation was assessed by Alizarin S and alkaline phosphatase staining. The serum-free osteogenic medium Osteolife™ failed to induce mineral deposition and alkaline phosphatase activity in tendon cells (Fig. 9**a**,**e**,**h**), unless supplemented with 10 % human serum (Fig. 9**b**,**f**,**i**). In serum-free Osteolife™ treated cultures, only few nodules with calcium deposits were detectable by Alizarin S staining (Fig. 9**a**). Prominent alkaline phosphatase activity could be detected in tendon cell cultures supplemented with serum irrespective of the addition of Osteolife™ (Fig. 9**f**,**g**,**i**,**j**). Semiquantitative analysis of bound Alizarin S shows that addition of serum causes a significant ($p < 0.01$) 5.2 fold increase of mineralisation compared to cultures in unsupplemented Osteolife™ (Fig. 9**d**). Since alkaline phosphatase is not exclusively produced by osteoblasts, we performed qPCR for the osteogenesis markers Runx2, BSP1 and PTH1R (Fig. 9**k**,**l**,**m**). Although an increase in expression was evident for all genes, the difference to the control group was statistically not significant.

ß-Galactosidase staining

The number of senescent cells visualised by ß-galactosidase staining showed no difference between treatment groups (data not shown). Under all culture conditions, the number of positive cells was < 0.1 %.

Apart from its influence on proliferation and differentiation, serum also affected the expression of the

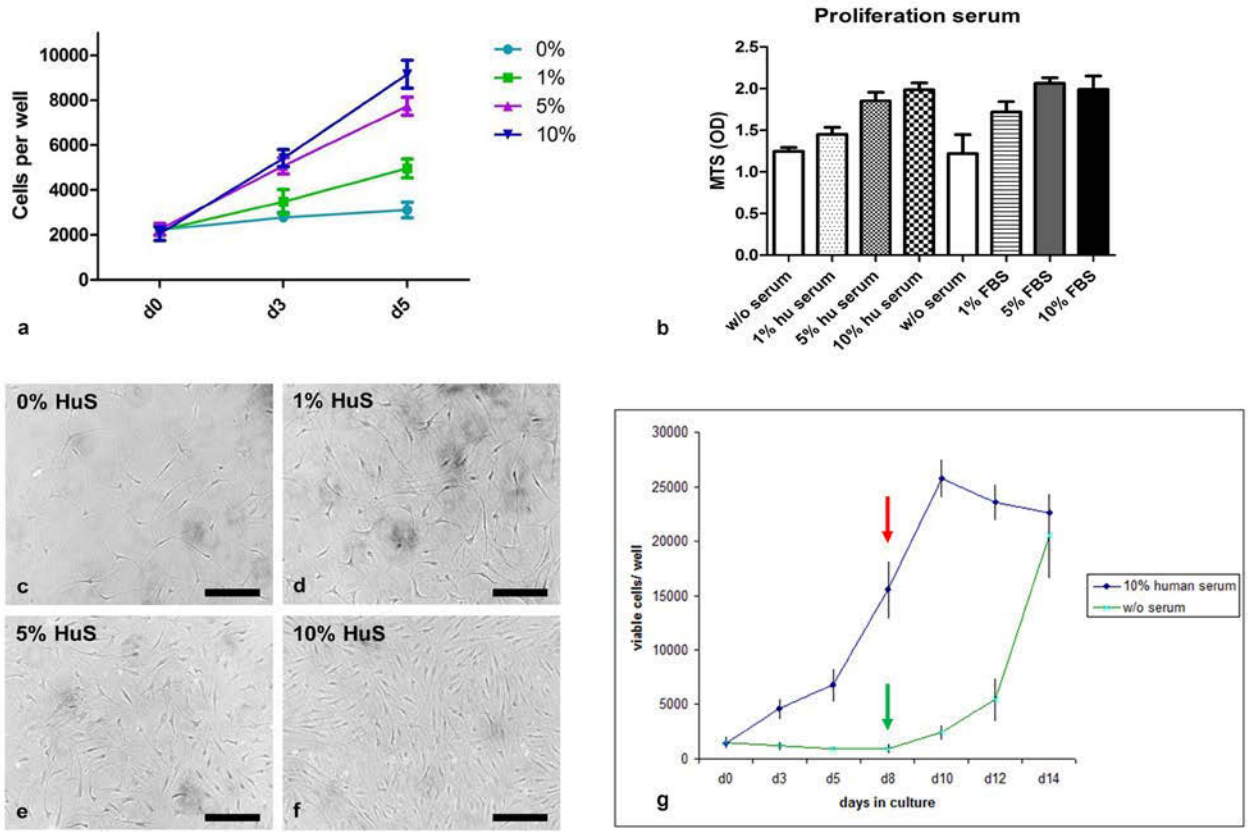

Fig. 6. Proliferation of human tendon cells (n = 3 donors) is serum-dependent, as shown by cell counting (**a**) and MTS assay after 5 d of culture (**b**). MTS assay shows no difference in cell proliferation between human and bovine serum (FBS). Representative photomicrographs of human tendon cells cultured for 5 d at different serum concentrations illustrate the effect of serum on tendon cell proliferation (**c**-**f**), Scale bar: 100 µm. Under serum-free conditions, human tendon cells do not proliferate, addition of serum after 8 d (green arrow) induces proliferation (**g,** green line). Serum withdrawal (red arrow) in cells cultured for 8 d with 10 % human serum leads to an abatement of proliferation (**g**, blue line).

matrix degrading matrix metalloproteinases MMP2 and MMP9 and of pro-collagen1a1, the precursor form of the ECM building protein collagen1. Whereas MMP2 ($p = 0.0572$) and MMP9 ($p = 0.0972$) expression increased markedly with increasing serum concentration, pro-collagen1a1 expression appeared unchanged between 1 and 10 % serum (Fig. 10).

Discussion

In this work, we focus on the structure, composition and permeability of blood vessels in healthy tendons. Moreover, we show effects of serum on tendon cells *in vitro*. Our findings provide evidence to suggest the existence of a blood-tissue barrier in tendons allowing the establishment of a stable, internal milieu important for tendon cell proliferation and tendon function.

Since tendons are sparsely vascularised tissues, ultrastructural analysis has so far mainly focussed on fibril size and composition. In our study, therefore, we investigated the ultrastructure of tendon blood vessels. On TEM micrographs we found that tendon vessels display a continuous, non-fenestrated endothelium with junctional complexes between endothelial cells, similar to vessels in the brain and retina (Garbuzova-Davis *et al.*, 2013; Masaki *et al.*, 1995; Wallow and Geldner, 1980).

We further demonstrated the presence of a series of tight junction molecules, both on the RNA as well as the protein level, in human and mouse tendon vessels constituting the prerequisite for the formation of a functional barrier. Interestingly, various core TJ-proteins expressed by tendon endothelial cells (tECs) resemble the set of TJ-proteins expressed at the blood-brain barrier (BBB) and the blood-retina barrier (BRB). At the TJs of central nervous system (CNS)-capillaries, occludin, ZO-1, claudin 3 and claudin 5 have been detected in both human and mouse (Nitta *et al.*, 2003; Schrade *et al.*, 2012; Wolburg *et al.*, 2003). In rodent retinal endothelial cells, the TJ-proteins ZO-1, occludin, claudin1/3, claudin 2, claudin 5 and JAM1 have been shown to be expressed (Klaassen *et al.*, 2009; Luo *et al.*, 2011; Xu *et al.*, 2005), and claudin 12 transcripts have been detected in bovine retinal endothelial cells (Klaassen *et al.*, 2009). Claudins 1, 3 and 5 are considered to be barrier-sealing proteins (Pfeiffer *et al.*, 2011; Zhang *et al.*, 2010a). In overexpression experiments using Madin-Darby canine kidney (MDCK) cells, Milatz *et al.* (2010) showed that claudin 3 acts as a barrier-forming tight

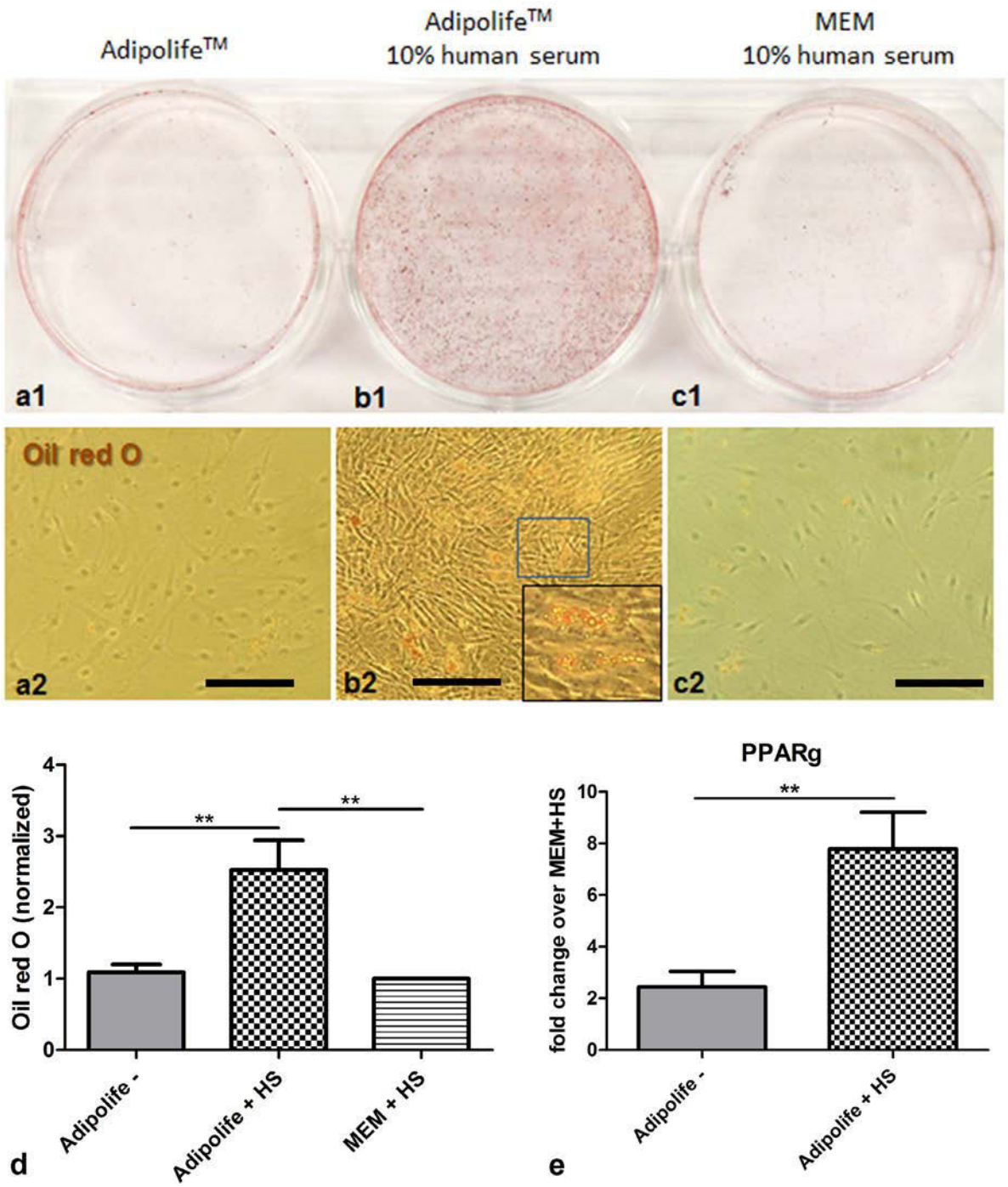

Fig. 7. Oil red O staining of human tendon cells cultured with Adipolife™ (**a1** overview, **a2** photomicrograph), Adipolife™ supplemented with 10 % human serum (**b1** overview, **b2** photomicrograph), and MEM with 10 % human serum (**c1** overview, **c2** photomicrograph) reveals that serum promotes the formation of lipid droplets. Scale bar: 100 µm. Semiquantitative analysis of bound Oil red O shows that addition of serum causes a significant (**$p < 0.01$) 2.5 fold upregulation of lipid droplets (**d**). Transcripts of PPARγ are significantly (**$p < 0.01$) upregulated in the cells cultured with Adipolife™ and human serum (**e**). Scale bars: 150 µm.

junction component that strongly reduces the paracellular permeability to ions and larger uncharged solutes but not to osmotically driven water.

It is well established that composition and architecture of junctional complexes are critically involved in regulating vessel permeability (Bazzoni, 2006). Tendon vasculature in general and tendon vessel properties in particular have hardly been investigated so far. To our knowledge, no data on the permeability of tendon vessels exist. Our tracer studies revealed that mouse tendon vessels, in contrast to heart vessels, are tight to a 10 kD tracer, but not to a 287 D tracer, suggesting that tendon vessels are not as tight as brain capillaries which exclude both tracers. The fact that the 10 kD tracer does not permeate the blood vessel wall implies that most serum proteins are excluded from paracellular entry into the tendon tissue. However, we

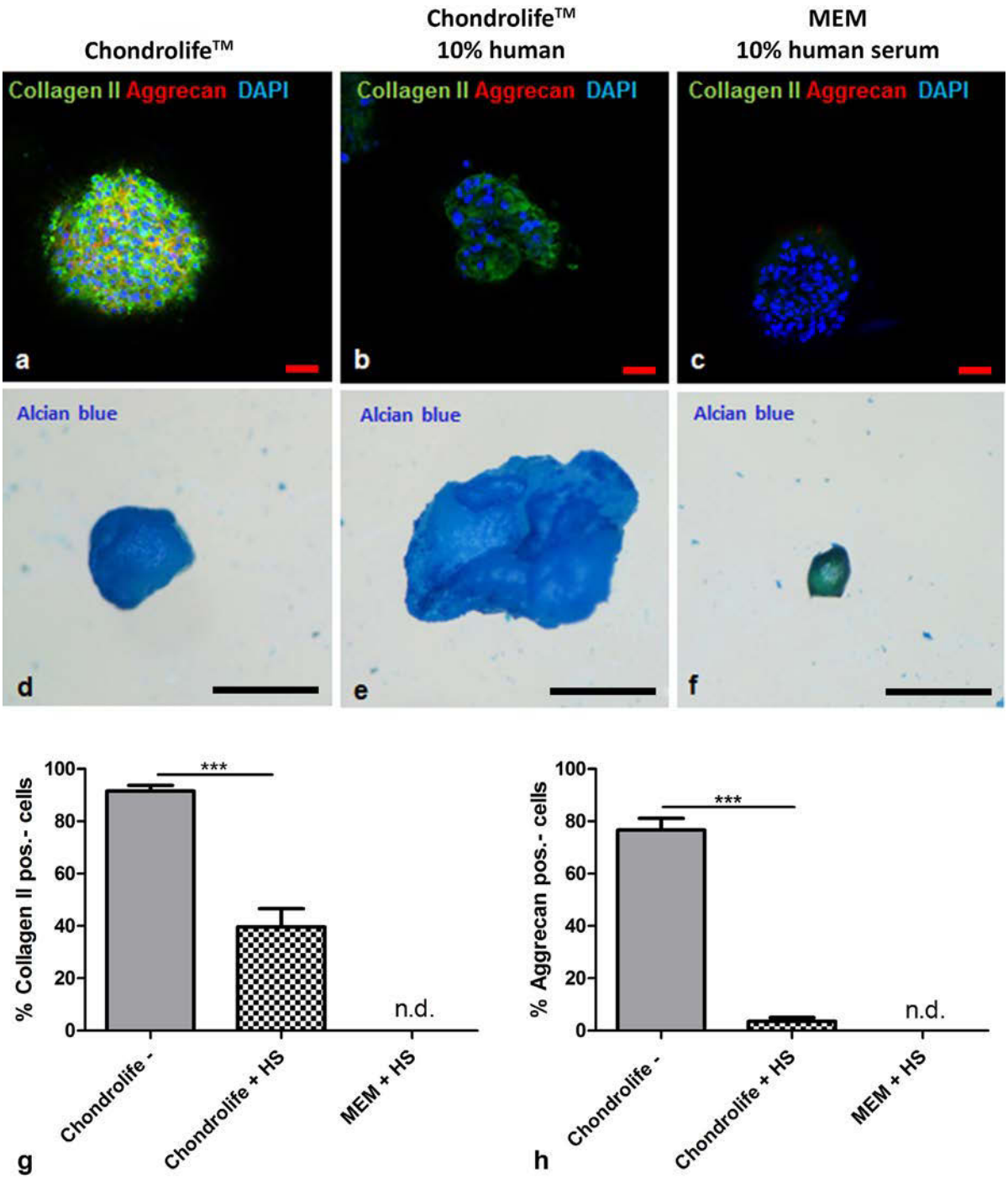

Fig. 8. Immunohistochemical staining of pellet cultures from human tendon cells (**a**-**c**) show that collagen type II (91.6 % cells positive, **g**) and aggrecan (76.7 % positive, **h**) are expressed when the pellets were cultured in unsupplemented Chondrolife™. Upon culture in Chondrolife™ supplemented with 10 % human serum, collagen type II is expressed by 39.6 % of all cells (**g**) and aggrecan by 3.6 % (**h**). Neither collagen type II nor aggrecan are detectable when cells were cultured in MEM and serum (**g**,**h**) (***$p < 0.001$). The pellets are positive for Alcian blue staining under all conditions (**d**-**f**). Scale bars: 50 µm (**a**-**c**), 1 mm (**d**-**f**).

cannot exclude an active transport *via* a transporter protein mediating transcellular passage.

Modulation of vessel permeability by altered TJ composition has clearly been shown by Nitta *et al.* (2003). By performing tracer experiments in claudin 5-deficient mice, the authors demonstrated a size-selective increase in the permeability of morphologically normal blood vessels allowing the passage of small (< 800 D), but not larger molecules.

Since the charge- and size-selective properties of the paracellular barrier are determined by the combination and interaction of the claudin family members present within a cell, alterations in the expression of these proteins have a strong influence on ion homeostasis and, thus, the microenvironment of the tissue niche (Mizutani *et al.*, 1995; Wolburg *et al.*, 2003).

In order to obtain information on the effects of serum on tendon cells, we utilised an *in vitro* approach to mimic blood

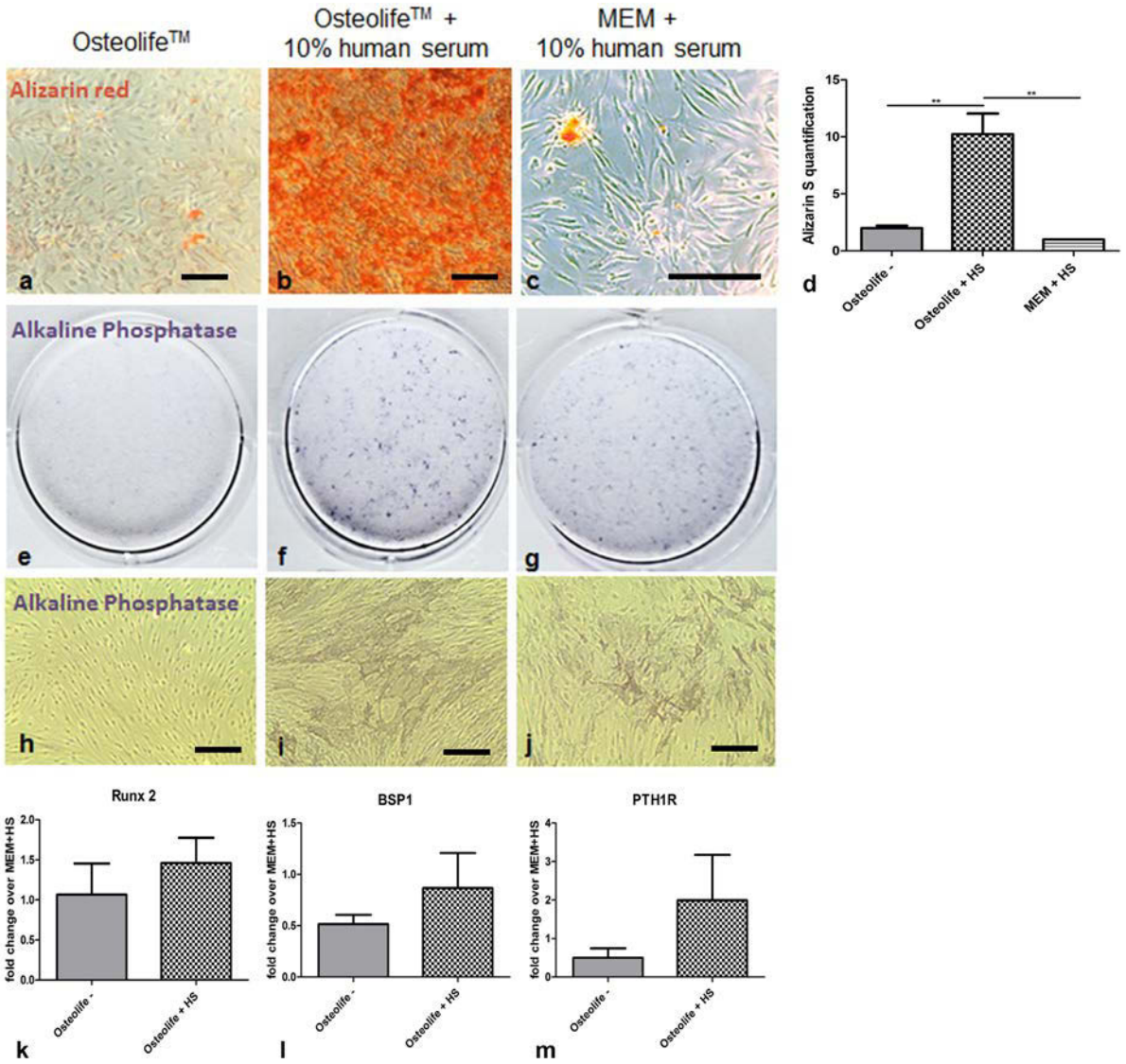

Fig. 9. Alizarin S staining of human tendon cells cultured with Osteolife™ (**a**), Osteolife™ supplemented with 10 % human serum (**b**), and MEM with 10 % human serum (**c**) reveals that serum promotes the deposition of minerals. Scale bars: 100 µm. Semiquantitative analysis of bound Alizarin S shows that addition of serum causes a significant (**$p < 0.01$) 10.3 fold increase of mineralisation (**d**). Alkaline phosphatase (AP) staining shows no AP activity in cells cultured with Osteolife™, whereas AP activity is detectable in cells cultured in Osteolife™ with serum and in cells cultured with MEM and serum (**e-g** overviews, **h-j** photomicrographs – Scale bars: 150 µm). Quantitative RT-PCR shows that Runx2 (**k**), BSP1 (**l**) and PTH1R (**m**) transcripts showed a trend of upregulation in cells cultured in Osteolife™ supplemented with 10 % human serum.

vessel leakage by adding increasing amounts of human serum to the culture medium. We found that serum seems to play a fundamental role in proliferation and differentiation of human tendon cells and their expression of matrix-forming and matrix-degrading proteins. Under adipogenic culture conditions, serum promoted the formation of cytoplasmic lipid droplets and significantly increased the expression of the ligand-activated transcription factor PPARγ, which is the master regulator of adipogenesis (Rosen and Spiegelman, 2001). In contrast, Adipolife™ culture medium without serum did not induce adipogenesis. From these results, we conclude that tendon cells need both a stimulus directing the cells towards an adipogenic differentiation programme and the presence of serum for final maturation into fat cells. While Osteolife™ without serum triggered only a small population of tendon cells to deposit calcium crystals, addition of serum substantially increased the percentage of cells containing calcium deposits. This finding indicates that serum increases the susceptibility of tendon-derived cells to osteogenic stimuli. In contrast, successful osteogenic differentiation of MSCs cultured in Osteolife™ does not require the presence of serum (Bortolotti *et al.*, 2015; Uzer *et al.*, 2013).

Interestingly, differentiation of tendon-derived cells into the chondrogenic lineage did not seem to be serum-dependent. Aggrecan, the cartilage-specific proteoglycan core protein, appeared to be expressed in cells cultured in Chondrolife™ medium without serum supplementation. Expression of collagen II, the predominant collagen type in articular cartilage, was induced by Chondrolife™ in both

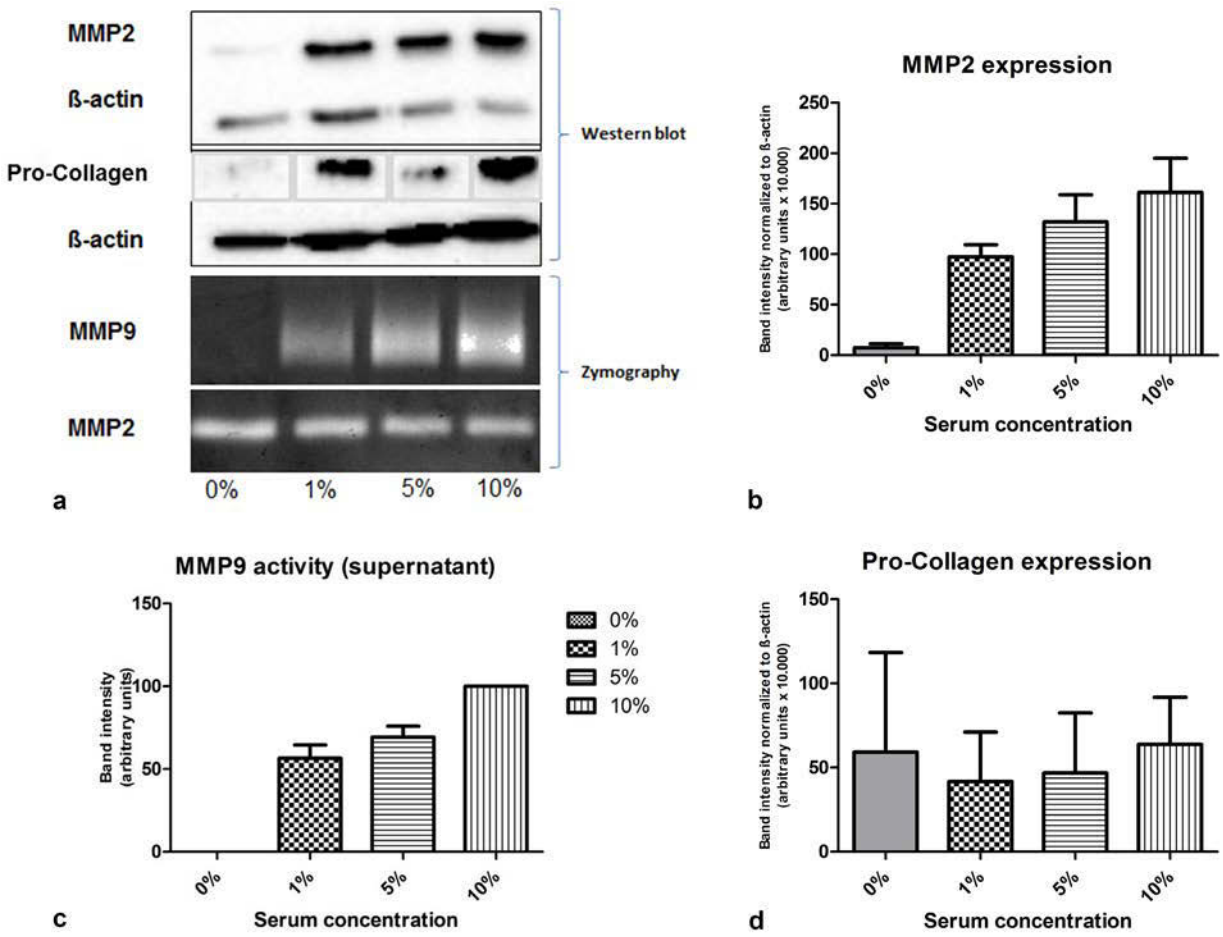

Fig. 10. Representative Western blots and zymograms of human tendon cell lysates showing MMP2, MMP9, and Pro-collagen1a1 expression of human tendon cells in dependence of different serum concentrations (0, 1, 5 and 10 %). ß-actin serves as loading control (**a**). Corresponding densitometric quantification of expression levels of MMP2 (**b**), MMP9 (**c**) and Pro-collagen1a1 (**d**) from three different experiments are normalised to ß-actin.

the absence and presence of serum. The same applied for proteoglycans evidenced by Alcian blue staining.

Future studies will have to address the potential role of the described vascular barrier in the diseased tendon. This is tempting, as there are many parallels between our *in vitro* observations and symptoms known from diseased tendons:

1. Tissue turnover and cell proliferation are very limited in healthy tendons (Heinemeier *et al.*, 2013), in diseased tendons hypercellularity and hyperproliferation are common features (Rolf *et al.*, 2001). In our work, we show that serum strongly affects tendon cell proliferation. Tendon cells hardly proliferate when cultured without serum. Upon addition of serum, cells immediately started to divide (Fig. 6**g**).
2. In tendinopathic or tenotomised tendons, calcification and ossification processes, mucoid and fatty infiltrations have been observed (Buck *et al.*, 2009; Mavrikakis *et al.*, 1989; McClure, 1983; Oliva *et al.*, 2012), the underlying mechanism for it being largely unknown. In this work, we show that serum contact is crucial for tendon-resident cells to undergo differentiation to the osteoblast and adipocyte lineage.
3. Remodelling processes, occurring in tendinopathic and ruptured tendons, correspond to an increased expression of MMP2 and MMP9 (Alfredson *et al.*, 2003; Robertson *et al.*, 2012). Our results demonstrated that serum increases the expression of the ECM degrading matrix metalloproteinases MMP2 and MMP9. Pro-collagen1a1, the major matrix constituting protein, does not show comparable serum dependence. Pro-collagen1a1 levels appear similar in tendon cell cultures supplemented with 1 or 10 % human serum. Apparently, tendon cells do not require high serum concentrations since they are capable of producing collagens also under low serum conditions (Fig. 10**d**).

To underpin a putative role of an impaired barrier in tendon disorders, the permeability status and TJ expression of blood vessels in compromised (*e.g.* diabetic) tendons will be in the focus of our future studies.

If tendon degenerative processes are a consequence of, or at least abated by, increased permeability of leaky tendon vessels, the application of drugs leading to a barrier tightening might be a feasible strategy to slow down or halt this detrimental progression. Therefore, restoration of a functional barrier and consequently the niche may stand at the basis for promising regeneration.

Conclusion

We provide evidence for a TJ-based blood-tendon barrier restricting the passage of molecules larger than 10 kD. This barrier contributes to the niche in which tendon stem cells reside and differentiate and may be a novel therapeutic target in tendon regeneration strategies.

Acknowledgements

We would like to thank Prof. Dr. Wolf-Dietrich Krautgartner and Ing. Michaela Klappacher from the Department of Organismic Biology of the University of Salzburg for technical support and for providing access to the electron microscope facility. Moreover, we acknowledge Dr. Katja Heinemeier and Dr. Peter Schjerling from the Institute of Sports Medicine of the University of Copenhagen for their collaboration and scientific input. The authors would like to acknowledge funding from the Paracelsus Medical University (project R-10/05/022-LEH), the Herrmann and Marianne Straniak Foundation (Sarnen, Switzerland) and the Lorenz Böhler Funds (Vienna, Austria). We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Discussion with Reviewers

Reviewer I: The potential involvement of the tendon-blood barrier in the development of tendinopathies is an interesting idea, which does indeed lend itself to be tested in an animal model. Particularly as to date there are no valid tendinopathy animal models out there, what would your approach be to translate/transfer that idea into an animal model?

Authors: We agree with the reviewer regarding the notion that there is no valid tendinopathy small animal model available. If there was, we would tracer inject such animals and look whether the tracer leaks out of the vessels. Naturally occurring tendinopathies in horses are due to size reasons no option for tracer perfusion. One approach to test our hypothesis could be to make the barrier leaky by mannitol injection and look whether a tendinopathy develops.

Reviewer I: How does the concept of a tendon blood barrier fit in with current tendinopathy treatment methods? On the one hand, sclerosing agents are used to destroy neovessels, whereas on the other hand, platelet rich plasma (PRP) is injected into the tendon to promote healing.

Authors: The concept of a blood-tendon barrier fits in well with the reported efficacy of sclerosing agents shown to destroy neovessels. Reduction of leaky neovessels reduces extravasation of serum into the surrounding tissue and consequently, in line with our *in vitro* observations, proliferation and erroneous differentiation of tendon cells. Interestingly, methods shown to be beneficial for treatment of tendinopathies at high levels of evidence, such as eccentric loading, reduce tendon vascularity. Injection of platelet rich plasma into the tendon is believed to promote cell proliferation and angiogenesis by delivery of various growth factors such as vascular endothelial growth factor and is therefore regarded to promote the healing process. But so far there is no convincing evidence from studies with high levels of evidence that treatment of diseased tendons with PRP is superior to control treatments. The findings presented in this paper may shed new light on potential mechanisms of PRP on tendon tissue.

Editor‘s Note: Scientific Editor in charge of the paper: Juerg Gasser.