

Detection of slow-cycling and stem/progenitor cells in different regions of rat Achilles tendon: response to treadmill exercise

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Received: 22 November 2012 / Accepted: 29 January 2013 / Published online: 12 February 2013
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Abstract

Purpose Tendons generally exhibit poor healing capacity, probably due to slow cell regeneration potential and low vascularization. The potential to regenerate may partly be due to activation of stem/progenitor cells localized in the tendon or its vicinity. In the present study, we attempted to determine where in the rat Achilles tendon stem/progenitor cells reside and to investigate the effect of exercise on cell proliferation in the in vivo situation.

Method We used bromodeoxyuridine (BrdU) labelling to investigate proliferation and label-retaining cells (i.e. slow-cycling cells) in *non-exercised* and *exercised* rats, in combination with immunostaining of the stem cell marker nucleostemin. Rat Achilles tendons were harvested 14, 28, 56 and 105 days after BrdU administration.

Results We found the proportion of stem/progenitor cells to be twice as high in the distal tendon (DT) compared with the mid/proximal tendon (MPT) and that paratenon/endotenon regions appear to host a pool of existing stem/progenitor

cells. Exercise increased the BrdU-stained cell population after 14 days only (DT region $p = 0.032$, MPT $p = 0.065$), indicating effect mainly on more differentiated cells, since the nucleostemin-positive cells (i.e. stem/progenitor cells) remained unaffected in the intact Achilles tendon.

Conclusion Stem/progenitor cells exist in several areas of the rat Achilles tendon which implies a possible stem cell regeneration pool of different origins. The distal region has twice the amount of stem/progenitor cells compared with the mid/proximal region, indicating a potentially higher stem cell activity in this tissue. Daily moderate exercise (treadmill running) mainly improves in vivo cell proliferation in rapidly proliferating cells, whereas the stem/progenitor pool remains constant.

Keywords Achilles tendon · Stem/progenitor cells · Physical exercise · BrdU labelling · Nucleostemin · Cell proliferation

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Introduction

Tendons are not merely a rope-like structure connecting muscle and bone structures, but have their own unique tissue properties specialized for transmitting forces between muscle and bone. They consist of parallel collagen fibre bundles with specialized tendon cells positioned between the collagen fibres, responsible for construction and repair within the unique environment of the tendon. However, a tendon does not fully regain its mechanical strength, elasticity and tissue integrity after an injury.

Skin regenerates within 26–42 days depending on age, while tendon regeneration requires at least 120 days [6]. The capacity of tendons to achieve tissue homeostasis is depending on the tendon cellular content, mainly tenocytes

and the newly discovered tendon stem/progenitor cells [3]. Both tenocytes and tendon stem cells have demonstrated proliferation capacity in vitro, tendon stem cells more rapidly than tenocytes [30].

Exercise has been shown to be beneficial to the musculoskeletal system by several outcome measures such as increments of muscle mass and bone strength [10, 14]. Furthermore, tendons exposed to physical activity respond to such external stimuli in different ways: increased blood flow, collagen synthesis, growth factor expression as well as increased cross-sectional area (CSA) [4, 5, 8, 16]. However, it is not clear whether these effects of exercise are due to increased cellular activity or augmented cell proliferation. Recently, human and animal tendon cells exposed to mechanical strain during culture were seen to increase cell proliferation [25, 26] and tendon stem/progenitor cells, both increased cell proliferation and differentiation rates as a response to mechanical loading in in vitro cell culture experiments [21, 28, 29].

However, there is lack of information whether these in vitro results can be translated to an effect of physical exercise on tendon cells in vivo and whether there are differences in the response to such stimuli according to cell type.

Bromodeoxyuridine (BrdU) incorporation is widely used in studies of cell proliferation but is furthermore used as a method to identify long time label-retaining cells (i.e. slow-cycling cells). Cells which have incorporated BrdU into their DNA retain this label until BrdU has been washed out due to cell mitosis or apoptosis. Thus, slow-cycling cells retain BrdU staining for a long time and may be identified in tissues by immunohistochemistry at time points when BrdU has been washed out from most cells. In the in vivo situation, most immature adult stem cells are postulated to be predominantly quiescent [17], and the BrdU label-retaining technique has been used to detect specific stem cell niches in several tissues such as skin [22], small intestine [18], intervertebral discs [9] and knee joint [12].

Nucleostemin is a nucleolar protein first identified in neural stem cells and several cancer cell lines [24]. Nucleostemin has been shown to be expressed mainly in primitive but not differentiated cells and is down-regulated prior to terminal differentiation [24]. Lately, it has been used as a stem cell marker of tendon-derived cells since it is only expressed in the tendon stem cells and not in the terminated tenocytes [27, 30].

Our specific hypothesis was that stem cells exist in different regions within the Achilles tendon and that there are differences in the stem cell densities in the different areas of the tendon. Furthermore, since exercise has been shown to increase the in vitro tendon stem cell proliferation rate, we hypothesized that exercise is a potential inducer of tendon stem cell proliferation in the in vivo situation. Moderate exercise was performed on a rat treadmill, and

the detection of slow-cycling/stem/progenitor cells in different regions of the Achilles rat tendon was performed using BrdU in vivo label-retaining methodology and stem cell marker staining.

Methods

Animals and experimental protocol

Twenty-six female Sprague-Dawley rats (Charles River, Germany), ~8–10 weeks old weighing ~200 g, were included in the study. The animals were housed three per cage and had free access to food and water, except during the BrdU administration period, described below. The animals were divided into two groups, *exercise* group ($n = 12$) and *non-exercise* group ($n = 12$). Two animals were kept as controls (not receiving BrdU and no exercise). After 1 week of acclimatizing to the desired speed and duration, the exercise group performed non-voluntary exercise on a rodent motor-driven treadmill (Exer 3/6, Columbus Instruments, Columbus, OH, USA), 5 days/week, on plain level, at a speed of 16 metre/min for 50 min. A grid at the end of the treadmill administered a low-intensity (micro voltage 0.2–0.5 mA) electrical stimulation encouraging the animals to run. Non-exercised and control rats were confined to normal cage activity. The animal experiments were approved by the local animal research ethics committee.

BrdU administration

The animals were exposed to BrdU (Sigma-Aldrich, Steinheim, Germany) diluted in the drinking water (1 mg/ml) during 14 days, which previously have been shown to be an effective and safe administration period for label-retaining cell studies [13]. Oral BrdU administration began in combination with exercise acclimatization, and rats were killed 14, 28, 56 and 105 days after the start point by an overdose of sodium pentobarbital (Apoteket Produktion & Laboratorier AB (APL), Sweden). Two animals, not exposed to BrdU, were killed at days 28 and 105 and served as negative control to the BrdU technique.

Tissue preparation, histology and immunohistochemistry

Achilles tendons from right and left side were harvested, transferred to Histofix solution (Histolab Products AB, Göteborg, Sweden) and fixed for 24 h at 4 °C. Tendon samples were then dehydrated in ethanol and embedded in paraffin. Longitudinal sections (6–7 µm) were mounted on plus-slide glasses (Histolab Products AB, Göteborg,

Sweden). For histological overview, some sections were stained with Alcian Blue, van Gieson and HTX/eosin. Immunolocalization of BrdU-positive cells was performed with an anti-BrdU antibody (DAKO, Glostrup, Denmark), and characterization of stem cells was performed with nucleostemin antibody (Neuromics, Edina, USA). Briefly, after the removal of paraffin, an antigen retrieval step was performed. For the BrdU antibody, this was carried out in 10 mM citrate buffer, pH 6.0 at $\sim 90^\circ\text{C}$ for 30 min followed by incubation in 2 M HCl for 1 h at 37°C . For nucleostemin, the sections were placed in 1XTris-EDTA buffer, pH 9.0 (Nordic Biosite, Täby, Sweden) in a water bath (60°C) over night. All sections were blocked with 2 % BSA and 0.1 % Triton-X100 diluted in phosphate saline buffer (PBS) for 30 min at RT. Anti-BrdU antibody (1/200) and nucleostemin (1/100) were added, and the sections were incubated over night at 4°C . Development with goat-anti-mouse, Alexa Fluorochrome 546 [(BrdU antibody), Invitrogen, Carlsbad, USA] and donkey-anti-goat, Alexa Fluorochrome 594 [(nucleostemin antibody), Invitrogen, Carlsbad, USA] was performed for 3 h at RT. Samples were covered with prolong gold antifade reagent with DAPI (Invitrogen, Carlsbad, USA). Positive control for the BrdU staining was small intestine samples from each time point. Negative controls for BrdU analysis were sections from rats not given BrdU. Negative antibody controls were samples where the primary antibody was excluded.

Stained cells were visualized under a Nikon fluorescence microscope (Eclipse E600), and calculations were performed using the NIS-elements software (NIKON, Tokyo, Japan).

Calculation of BrdU and nucleostemin-positive cells

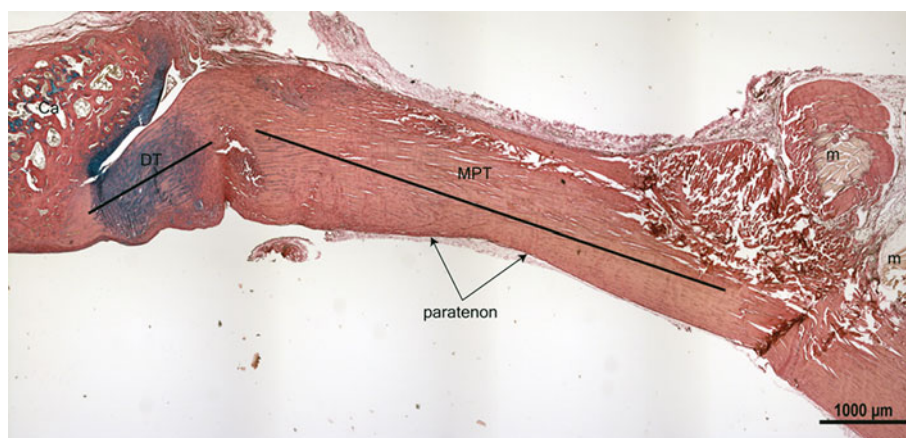
For each rat sample from both right and left Achilles tendon, counting up to a maximum of 6 tendon samples (3 rats/group) from each time point (14, 28, 56 and

105 days) and activity group (*non-exercise* or *exercise*), calculations were performed in two different regions of the Achilles tendon as described below. The whole area of the entire tendon specimen was analysed, from the distal to the proximal region, that is, no random or other selection procedure of microscopic fields was done. Approximately 3 fields of view ($20\times$ objective) from the distal region and 8 fields of view ($20\times$ objective) from the mid/proximal portion region were analysed for each Achilles tendon sample. Some samples were excluded due to low structure integrity in the tissue. Since 4',6-diamidino-2-phenylindole (DAPI) counterstaining does not work properly (due to HCl incubation) in the BrdU staining procedure, we calculated BrdU-positive cells/ mm^2 . DAPI staining was performed on adjacent slides where the total amount of DAPI-stained cells/ mm^2 was calculated. The results of BrdU-positive cells/ mm^2 were then divided into the amount of DAPI-stained cells/ mm^2 and converted to % of total cells. Percentage of nucleostemin-positive cells were calculated from Nucl/DAPI merged pictures by dividing the number of nucleostemin-positive cells by total number of cells. All measurements were performed by a single person blinded to the samples. Additionally, 16 fields of view were analysed by an independent observer in order to evaluate inter-observer accuracy and were calculated to 88 %.

Distal tendon (DT)

The tissue at the Achilles tendon insertion site is composed of 4 zones: tendon, fibrocartilage, calcified fibrocartilage and bone [2]. These zones have different characteristics but the borders are not distinct since they blend. In this study, positive-stained cells were counted in the fibrocartilage and distal tendon zones naming it DT (Fig. 1). The part of the tendon defined as distal was characterized by the visualization of rounded cells in rows and ended where most of the cells were more elongated and separated. The median length of this area was 1.3 mm (range 0.5–2.3 mm).

Fig. 1 Section of a whole rat Achilles tendon defining the regions included in the analyses of positive cells. Distal tendon zone (DT) and mid/proximal tendon zone (MPT). Muscle (m) and calcaneus (Ca)



Mid/proximal tendon (MPT)

The tissue defined as MPT included the areas with more separated and elongated cells. This area was located directly proximal to the DT and as far as the proximity of the muscle insertion, excluding the contact region (Fig. 1). Only cells inside the tendon were calculated, excluding the cells inside paratenon. The median total tendon length calculated was 4.8 mm (range 1.8–7.2 mm).

Statistical Analysis

All nonparametric data are presented as median and interquartile range (IQR). The Kruskal–Wallis test was used to test for differences between time points in both *non-exercised* and *exercised* rats followed by Mann–Whitney *U* test (pairwise comparisons). Mann–Whitney *U* test (independent samples) was further used when analysing the effect of exercise at different time points, whereas Wilcoxon signed rank test was used when comparing % BrdU and % nucleostemin in DT and MPT regions from the same sample.

Results

Immunohistochemical staining pattern

In the BrdU staining, both strong and weaker nuclei-stained cells were calculated as positive. No positive cells were found in samples from rats not exposed to BrdU.

In the nucleostemin staining, cells with a clear punctuated staining signal inside the nucleus were considered positive.

Comparison between the distal tendon and mid/proximal tendon regions

Bromodeoxyuridine and nucleostemin-positive cells were found in tissue from both DT and MPT, in both *non-exercised* and *exercised* animals, at all time points. Significantly, higher densities of both BrdU and nucleostemin-positive cells were demonstrated in the DT area compared with the MPT area at all time points ($p < 0.05$ at all time points). The density of nucleostemin-positive cells were approximately 2 times higher in the DT region compared with MPT at all time points and regardless of activity level (*non-exercise or exercise*), whereas the amount of BrdU-positive cells was between 3 and 10 times higher in the DT area compared with MPT with the larger differences at time points 28 and 56 days.

BrdU and nucleostemin-positive cells; variation over time

Significant differences were demonstrated in the percentage of BrdU-positive cells when analysing changes over time for *non-exercised* (Fig. 2a) as well as *exercised* (Fig. 2b) tendon samples. The percentage of nucleostemin-positive cells was found to be constant over time for both *non-exercised* (Fig. 2c) and *exercised* (Fig. 2d) tendon samples.

DT region

In the *non-exercise* group, BrdU-positive cells increased from day 14 to day 28 ($p = 0.03$), whereas no differences were seen in the exercised tendons. At day 56, there was a significant drop in the percentage of BrdU-positive cells, in both non-exercised ($p = 0.03$) and exercised samples ($p = 0.03$). No significant differences were seen between day 56 and day 105 (Fig. 2a, b).

MPT region

In the *non-exercise* group, no differences were found at early time points (day 14–28), whereas there was a significant decrease in the *exercised* tendons (day 14–28, $p = 0.02$). At day 56, a decrease in BrdU-positive cells was seen in both *non-exercised* ($p = 0.009$) and *exercised* ($p = 0.002$) tendon samples followed by an increase at time point 105 days (*non-exercise*, $p = 0.002$; *exercise*, $p = 0.002$) (Fig. 2a, b).

BrdU and nucleostemin-positive cells; influences of physical activity

There was a significantly higher density of BrdU-positive cells in the *exercise* group, at day 14 when compared with the *non-exercise* group. This increased cell density was seen both in the DT and in the MPT regions, but was only significant in the DT region (*exercise vs. non-exercise*, DT region $p = 0.032$, MPT region $p = 0.065$) (Fig. 3a, b). At the other time points (day 28, 56 and 105), no differences in BrdU cell density were found between the groups. Nucleostemin density was not found to be influenced by physical activity at any time point, in any of the tendon regions (DT or MPT region) (Fig. 3c, d).

Overall morphological characterization

Closer morphological analysis demonstrated significant differences in the morphology of the nucleostemin, and BrdU-positive cells located in the DT region compared with tissue in the MPT region.

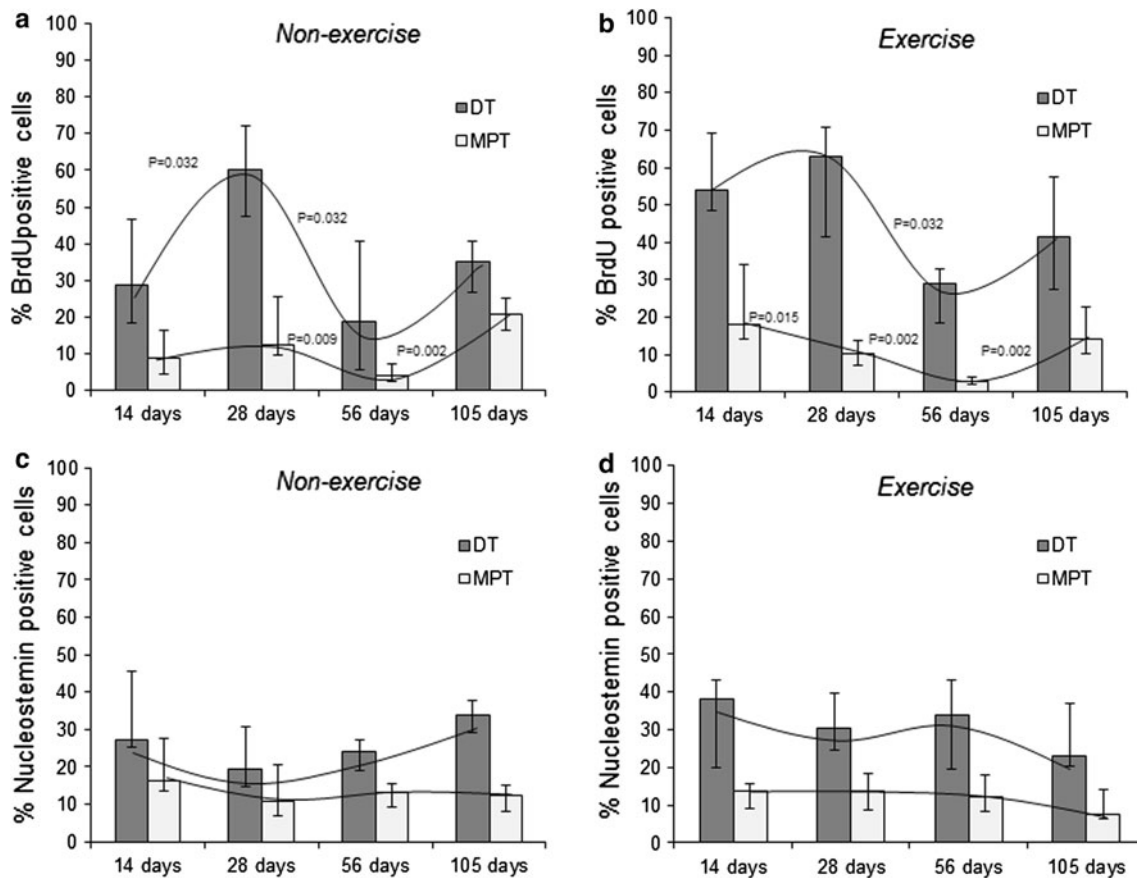


Fig. 2 Time course changes in the percentage of BrdU (a, b) and nucleostemin (c, d) positive cells from the DT and MPT regions in *non-exercised* rats (a, c) and *exercised* rats (b, d). Values are expressed as median with IQR as error bars. The *p* values in the

figure represent comparisons between adjacent time points, and only significant differences are reported. Significant higher densities of BrdU and nucleostemin-positive cells were also demonstrated in the DT area compared with the MPT area at all time points ($p < 0.05$)

DT region

The BrdU and nucleostemin-positive cells in DT had mainly rounded or cobble-stone shape with a typical pattern located in column like rows (chondrocyte-like) and in close contact with each other (Fig. 4a, b). The cells in DT with the most intensely BrdU staining were situated close to the bone, either in the fibrocartilage at the attachment site or in the calcaneal fibrocartilage site.

MPT region

The positive cells in the MPT region mainly exhibited slender elongated shapes (fibroblast-like) (Fig. 5a, b). At day 56, low numbers of intensely stained cells were seen located between the parallel chains of collagen fascicles in regions of loose connective tissue, for example, endotenon.

The loose connective tissue surrounding the tendon, the paratenon, always exhibited many intensely stained cells (myofibroblast- or endothelial-like) residing inside the paratenon or in its close proximity (Fig. 6a, b). Blood

vessels inside the paratenon or endotenon were often surrounded by many pericyte-like small intensely stained BrdU cells (Fig. 7).

The pattern of BrdU staining cells of small intestine (control tissue) was markedly different from tendon tissue, starting with a high amount of BrdU-stained cells at day 14, clearly declining already at day 28, leaving only a few or no stained cells at days 56 and 105 (picture not shown).

Discussion

In the present study, we demonstrated the existence of BrdU label-retaining cells (i.e. slow-cycling cells) and nucleostemin-positive cells (i.e. stem/progenitor cells) in both the DT and the MPT and surrounding paratenon regions of the Achilles tendon in both non-exercising and exercising rats. Higher proportions of stem/progenitor cells were detected in the DT compared with MPT of the rat Achilles tendon. Moreover, treadmill running was found to increase BrdU-positive cells only at the early time point

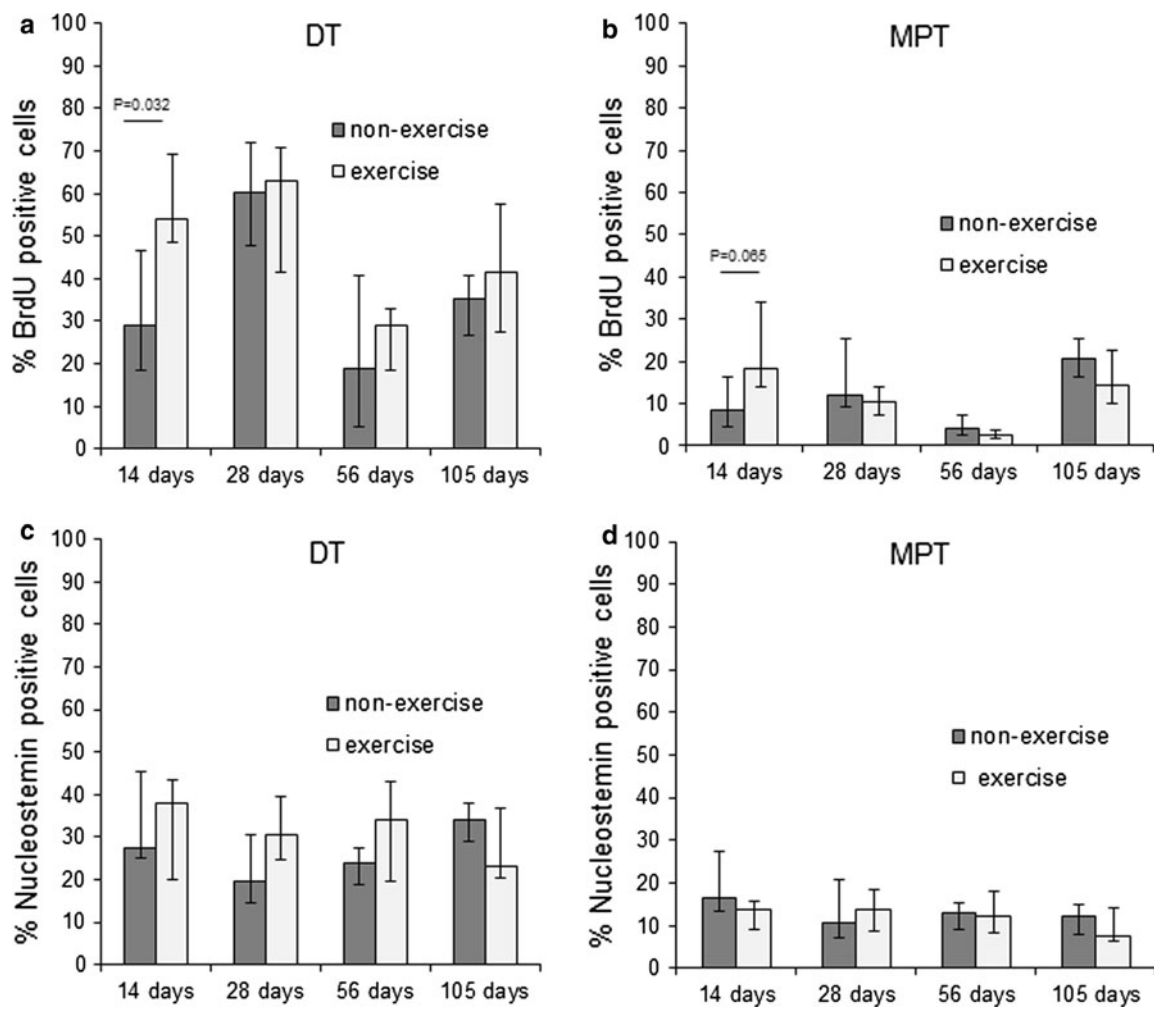


Fig. 3 Influence of physical activity on the percentage of BrdU (a, b) and nucleostemin (c, d) positive cells in DT (a, c) and MPT (b, d) regions of *non-exercised* and *exercised* rats. Values are expressed as median with IQR as *error bars*

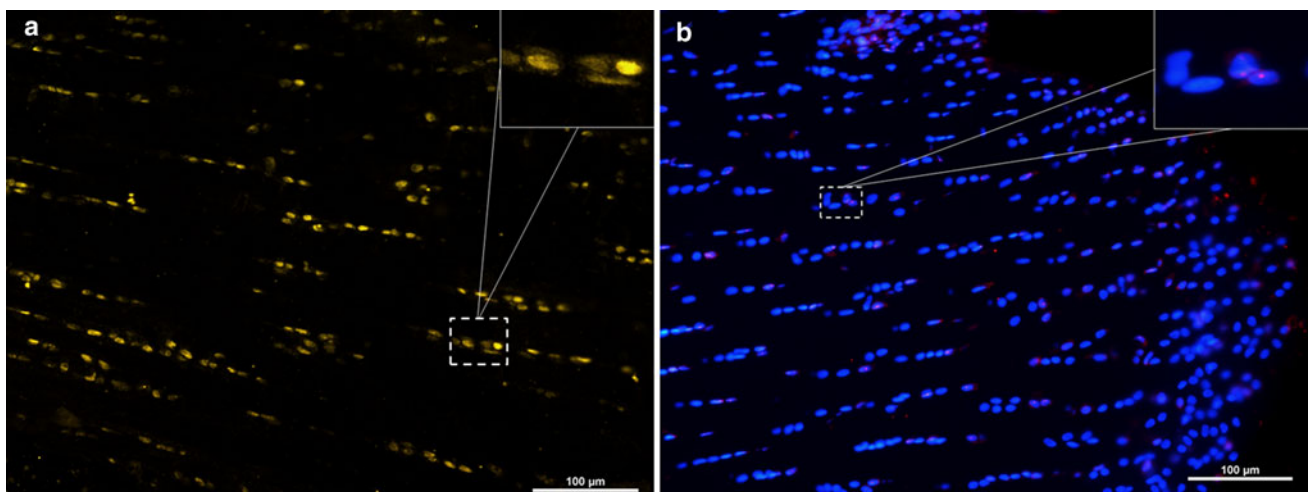


Fig. 4 Characteristics of stained cells in DT zone of rat Achilles tendon. Rounded cells in column like rows with nucleus staining of BrdU (a) and nucleostemin (b). Samples from rat Achilles tendon, *non-exercised* group, day 14

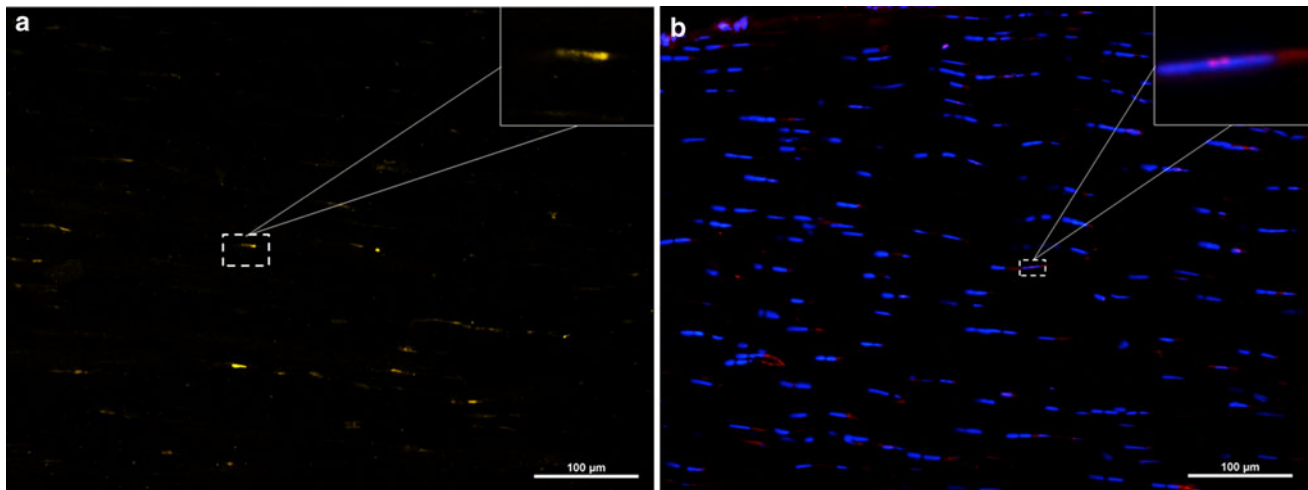


Fig. 5 More elongated cells in the MPT region stained positive for BrdU (**a**) and nucleostemin (**b**). Samples from rat Achilles tendon, *non-exercised* group, day 14

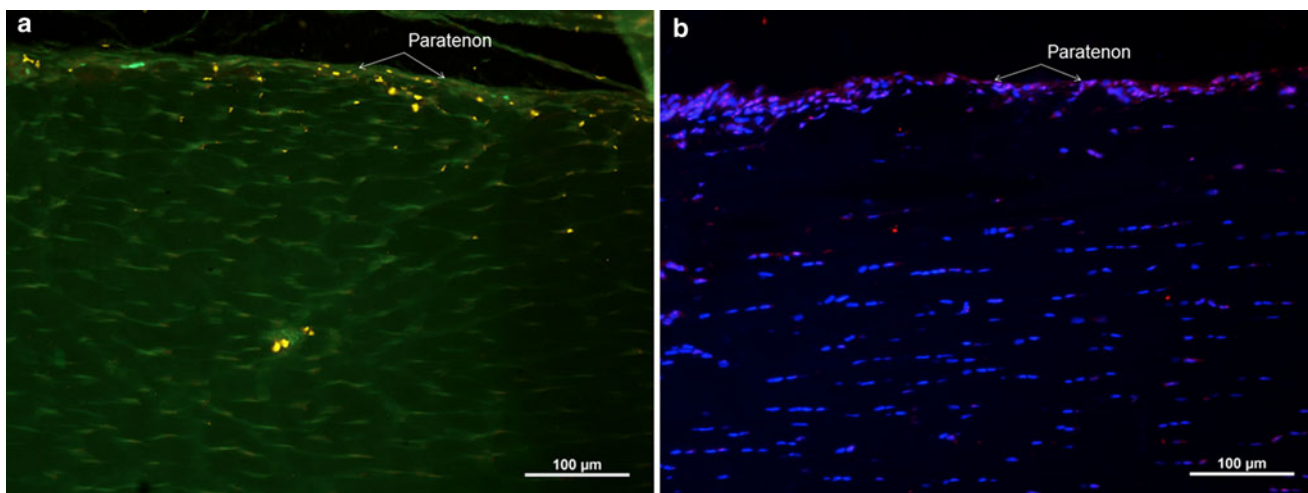


Fig. 6 Section showing many intensely stained BrdU (**a**) and nucleostemin (**b**) positive cells inside and in close contact with paratenon. Samples from rat Achilles tendon, *exercise* group, day 14. BrdU picture is merged with green auto-fluorescence

(14d), whereas nucleostemin-positive cells were not increased at any time point. These results suggest the existence of several pools of stem/progenitor cells localized in different regions of the Achilles rat tendon and that moderate exercise mainly activates *in vivo* proliferation of a more differentiated cell population, whereas the Achilles tendon pool of stem/progenitor cells remains unaffected.

In the DT region from both *non-exercised* and *exercised* tendon samples, the amount of BrdU-stained cells peaked at 28 days after BrdU administration. At this time, around 60 % of the cells stained BrdU positive indicating a high proliferating state in this region. Since the percentage of the stem/progenitor cell marker nucleostemin in the distal region remained constant during all time points, many of these proliferating cells are probably a result from division

of both more differentiated cells and/or stem cell offspring which incorporated the BrdU during the administration time. In the mid/proximal region, no such peak could be seen at the early time point, and the levels of BrdU-stained cells were comparable with the levels of nucleostemin-positive cells. The amount of BrdU-positive cells significantly declined at 56 days (both distal and mid/proximal region), which is in accordance with the study of Bi et al. [3], detecting low amounts of BrdU-stained cells in mouse patellar tendon 8 weeks post-BrdU administration. However, the increased number of BrdU-positive cells seen in the mid/proximal region at 105 days is intriguing. This phenomenon may be explained by migration of BrdU-stained cells (e.g. stem/progenitor cells) from paratenon or other external tissues or by increased mitosis in a slow-cycling cell population initially marked by BrdU.

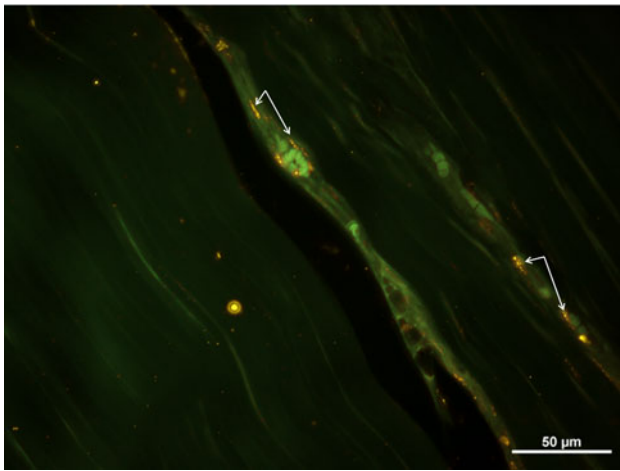


Fig. 7 Small BrdU-positive cells (pericyte-like) surrounding blood vessels inside the endotenon region of Achilles tendon. Sample from rat Achilles tendon, *non-exercise* group, day 56. BrdU picture is merged with green auto-fluorescence

This may though be a part of the normal homeostatic cell proliferation rate of the Achilles tendon where cell renewal is an ongoing process. Support for this suggestion is that collagen I has an estimated turnover of around 120 days in tendon tissue [6]. A regular renewal of the cell pool in the tendon may therefore be a necessity in order to keep tissue homeostasis.

Like Bi et al. [3], we found BrdU-retaining cells (slow-cycling cells possibly stem or progenitor cells) in the mid/proximal region after 56 days mainly unevenly scattered inside the tissue and residing between the collagen fibrils indicating a local tendon stem cell niche in this area. This pattern was also seen with the nucleostemin staining. We further found accumulation of both BrdU and nucleostemin-stained cells located in the thin layer of connective tissue surrounding the internal collagen fibre bundles (endotenon), and blood vessels were often located in such areas. This could be an indication of a more complex stem cell niche in the tendon, comprising a set of stem cells of different origin. Perivascular cells from human supraspinatus tendon have been found to exhibit both tendon and mesenchymal stem cell-like characteristics, and these cells also expressed α -SMA, indicating a specific pericyte population with capacity of tendon cell renewal and regeneration [23], further supporting a possible mixture of different stem cells in tendons.

Compared with the MPT region, the amount of nucleostemin and BrdU-positive cells in the DT region was higher at all time points, regardless of activity level. Since we calculated the percentage of stained cells compared with the total amount of cells, this could not be due to the difference in cell-to-matrix ratio between the regions, but rather an indication of higher metabolic and cellular

activity in the DT region compared with the MPT region. The amount of stem/progenitor cells, as indicated by the amount of nucleostemin-positive ($\sim 25\%$) and BrdU label-retaining cells ($\sim 25\%$) at day 56 in the DT region, seems to be twice as high as in the MPT region ($\sim 12\%$ nucleostemin-positive and $\sim 4\%$ BrdU-positive cells, respectively). In the DT region, approximately the same proportion of BrdU- and nucleostemin-positive cells was seen at day 56, whereas in the MPT region at the same time point, there were approximately three times higher proportions of nucleostemin-positive cells compared with BrdU-positive cells. Since BrdU is only incorporated into proliferating cells during the administration period, and stem cells divide infrequently, whereas nucleostemin stains all stem/progenitor cells, this may indicate higher mitotic stem/progenitor cell activity in the distal region compared with the mid/proximal region. The higher proportion of the stem cell marker nucleostemin in positive cells (12–13%) in the MPT region from our study may further reflect the true stem cell population in this region.

The concentration of stem/progenitor cells as indicated by nucleostemin seems to be fairly high, especially in the DT. To our knowledge, no information exists about the stem cell population size in the Achilles tendon. However, in patellar tendon from mouse puppets, Bi et al. [3] found approximately 6% BrdU label-retaining cells resisting after 14 weeks post-BrdU administration, representing tendon stem cells. This is in accordance with our study where the proportion of BrdU label-retaining cells in mid/proximal Achilles tendon at day 56 where approximately 3–4%.

BrdU as well as nucleostemin-positive cells in the paratenon were observed in high numbers, with intensive staining and in close vicinity to blood vessels at all time points. This finding could indicate that the paratenon, where blood vessels and nerves normally reside, serves as a pool of stem/progenitor cells [1, 23]. After overuse or rupture of the tendon, these progenitor cells may migrate into the tendon tissue together with ingrowing nerves and blood vessels, in order to repair injured tissue and sustain homeostatic cell renewal [1]. Ingrowing nerves release substance P, which may in fact act as an injury-inducible messenger for mobilization of progenitor cells [11]. In the acute tendon injury, the repair and regeneration process is indeed working in this way, with invasion of cells from the surrounding sheath together with the internal tenocytes trying to optimize the healing process [15].

In the present study, we found physical exercise to influence *in vivo* cell proliferation only at early time points. This early increase in cell proliferation by exercise was also seen in annulus fibrosus in intervertebral discs from the same animals [19]. Annulus fibrosus, a region consisting of connective tissue comparable with tendon tissue, had a

similar cell proliferation time curve as demonstrated in the mid/proximal region of the Achilles tendon in the present study. It appears as an additional exercise level (even at the low-intensity level used in this study) triggers cells quickly after an exercise boost. This is in accordance with a study by Skovgaard et al. [20] where treadmill running was seen to increase cell proliferation in the Achilles tendon of rats using positron emission tomography (PET) to study the uptake of 3'-(F-18)fluoro-3'-deoxythymidine (FLT).

The early exercise response seen in our study seems to be due to activation of the more differentiated cells since we could not observe any differences in the nucleostemin staining cells between *exercised* and *non-exercised* Achilles tendon samples. However, a similar moderate input of physical exercise resulted in nearly double proliferation rate of tendon stem cells isolated from mice after treadmill running [28] which suggests that exercise exerts its anabolic effect on tendons at least in part by increasing proliferation to expand the pool of tendon stem cells. Since the exercise level used in both studies was almost equal, the discrepancy between these studies may be that in the *in vivo* situation, stem cells are more protected from mechanical influences, whereas they may be quickly triggered when they are isolated from the surrounding protective tissue as in the study of Zhang et al. [28]. Another reason may be the use of different species (rat vs. mice) since we have no knowledge if physical activity effects stem/progenitor cells in the tendon differently between species.

Some limitations of this study should be noted. First, there were technical difficulties getting high-quality sections of all parts of the tendon (bone–tendon and muscle–tendon insertion) in some specimens. However, since we analysed and calculated the BrdU and nucleostemin-positive cells in all intact areas of all specimens, from the distal to the proximal region (i.e. no random selection procedure of microscopic fields was done), the number of analysed areas should be sufficient and representative.

Second, the moderate exercise level used in this study might be too low to induce differences in stem/progenitor cell densities. However, a too high exercise level might induce changes as seen in overused tendons [7] which were not the purpose of this study.

In conclusion, we have demonstrated that slow-cycling cells and stem/progenitor cells exist in several areas of the rat Achilles tendon which implies a possible stem cell regeneration pool of different origins. The distal region has twice the amount of stem/progenitor cells compared with the mid/proximal region, indicating a potentially higher stem cell activity in this tissue. Furthermore, our study indicates that daily moderate exercise (treadmill running) mainly improves *in vivo* cell proliferation in rapidly proliferating cells, whereas the stem/progenitor pool remains constant.

Acknowledgments The study was supported by grants from Sahlgrenska University Hospital, LUA/ALF Research project: 44931, Dr. Felix Neubergs Foundation (ER) and by the regional agreement on medical training and clinical research (ALF) between Stockholm County Council and Karolinska Institutet (project nr. SLL20100168).

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