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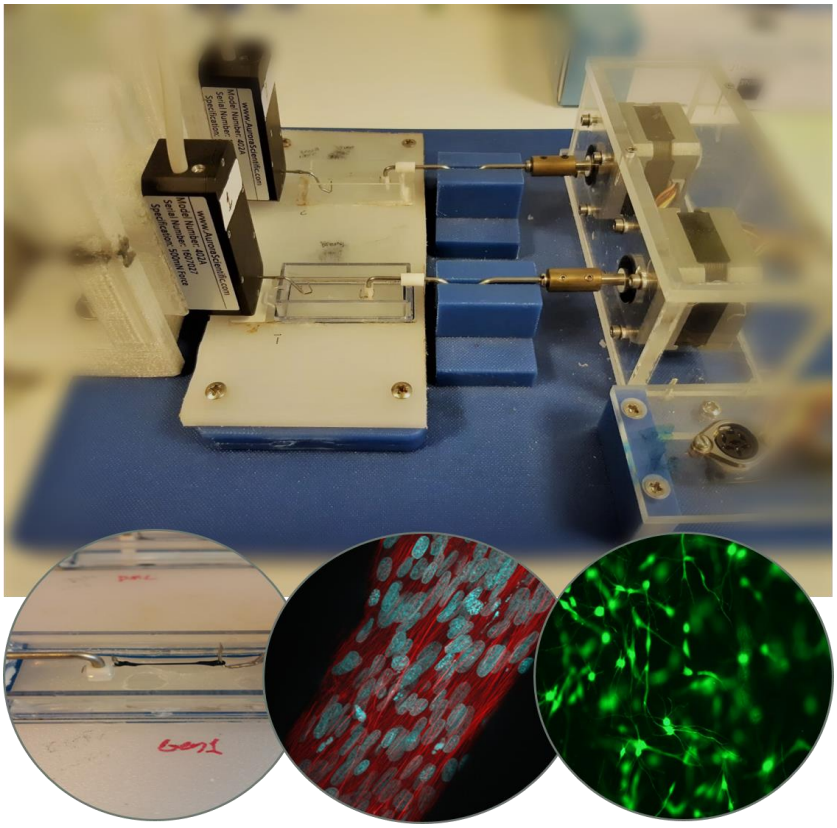

PhD Thesis

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Cell-matrix interaction in tendon constructs

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Preface and Acknowledgements

The last four years of my life I had the chance to work on my PhD at the University of Copenhagen, where I was honoured to work close to well-known and respected professionals in my field. I had the opportunity to publish my research and participate in various national and international conferences, presenting my results and meeting some of the best professors in the tendon research field. Coming from another country, I feel the need to highlight that all members of the lab (supervisors, colleagues, technicians and secretaries), offered me a supportive environment, making me feel welcomed and integrated to my new environment from my very first day at work.

I owe special gratitude to my supervisors that assisted me in overcoming whatever plausible and/or unexpected obstacle came up during my PhD journey and offered me generously with all the help that I needed. Regular meetings and thorough discussions helped my progress on both the experimental and the analytical part of my thesis.

I would like to express my deepest gratitude though to my supervisor and tutor, Peter Magnusson for all the guidance and patience that he showed throughout these years. His door was always open for me, encouraging me to reflect and express all my concerns in order to find a constructive solution, even at times when he had a rather high workload. Likewise, I need to thank my co-supervisor Michael Kjaer for offering me, and the whole lab a leader figure and exceptional academic role model to look up to.

Furthermore, Rene Svensson was always in the lab and always there for me. We shared the same office, and he generously offered me his experience and unique perspective during all the struggles that a course of PhD experiments includes. Similarly, I would like to acknowledge the contribution of Monica Bayer who had made a great stock of cells and for sharing her knowledge about the tendon constructs.

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that concerned me in and out of the lab. Additionally, the newest member of the lab, Cheng helped me to manage all the tension of my last PhD years with his calm and joyful attitude.

Last but not least, I would like to thank my family and my girlfriend, Stella, for being there for me, hearing with patience and understanding all my concerns and sometimes grouchiness. I feel very proud and honoured to have a mother like you Antigoni, a father like you Panagiotis, and a sister like you Ioanna. You give me wings to fly, such as Daedalus did with Icarus in Greek Mythology; however, you are always protecting me from being burned from the sun with your wise advice.

Dansk Resume

Senevæv har relativt få celler og en lav kollagenomsætning, men dets specifikke rolle - at forbinde muskler med knogle - er afgørende for det muskuloskeletale systems funktion. Mekaniske kræfter spiller en afgørende rolle for senens funktion med celle- og matrixinteraktioner, der dynamisk sikrer at senen kan gennemgå de nødvendige tilpasninger for at opretholde sin mekaniske og strukturelle integritet.

Under dannelsen af senen syntetiser og organiserer sene-cellerne den ekstracellulære matrix, men deres bidrag til hele vævets funktion er endnu ikke velkendt. At undersøge cellulære kræfter in vivo er kompliceret, da det er vanskeligt at skelne mellem de enkelte senekomponenters bidrag. Dette kræver en mere tilbundsgående evaluering af senens funktion med fokus på cellernes adfærd. Dette er muligt med et in vitro system bestående af en cellegenereret 3D matrix, kaldet sene-"constructs".

Målet med dette Ph.D. studie var at kvantificere celle-matrix interaktioner i humane sene-constructs. Meget få studier har direkte målt mekaniske kræfter i 3D systemer. Derfor kan den kraftmonitor, der anvendes i dette studie, være et nyttigt redskab til mere detaljeret at undersøge betydningen af mekaniske kræfter for regeneration og dannelse af senevæv. Afhandlingen består af tre studier, og det første havde til formål at detektere cellegenererede kræfter i constructs efter mekanisk aflastning (manglende spænding) og efterfølgende at evaluere betydningen af ikke-kollagenholdige proteiner (glycosaminoglycaner) for kraftoverførselen i sene constructs. I det andet studie blev tværbindinger induceret eller inhiberet, for at vise hvordan matrixændringer kan påvirke cellefunktionen, og i det tredje studie var målet at vurdere, om vækstfaktorerne IGF-1 og TGF-b direkte påvirker kraftoverførslen og tværbindingsdannelse.

Summary

Tendon tissue has relatively few cells and a low collagen turnover, but its specific role to connect the muscle with bone is crucial for the successful action of the musculoskeletal system. Mechanical forces play an essential role in tendon function with cell and matrix interactions that occur in a dynamic relationship that ensures that the tendon can undergo necessary adaptations to maintain mechanical and structural integrity.

During development the tendon cells synthesize and organize the matrix, but their contribution to the whole tissue function is not well understood. To investigate cellular forces in vivo is complicated since it is hard to distinguish the individual effect of tendon components. This requires a more in-depth evaluation of tendon function with focus on cell behaviour, which is offered by an in vitro system of cell generated 3D matrix, called tendon constructs.

This PhD study aimed to quantify cell-matrix interactions within the human tendon engineered constructs. Very few studies have measured the direct forces in 3D systems. Therefore, the force monitor used in this study can be a useful tool to investigate deeper the role of mechanical forces in tissue regeneration and development. The thesis consists of three studies, and the first aimed to detected cell-generated forces of the construct during unloading (lack of tension) and subsequently to evaluate the role of non-collagenous proteins (glycosaminoglycans) in force transmission within tendon constructs. In the second study, the induction and inhibition of cross-links showed how matrix alterations could affect cell function, and in the third study, the scope was to evaluate whether the growth factors IGF-1 and TGF-b can directly affect force transmission and cross-link formation.

Introduction

Tendon composition and structure

The musculoskeletal system is responsible for the movement of the human body through fine-tuned processes that involve the production and the transmission of forces from muscle to bone via tendons. The primary function of tendon is force transmission whilst maintaining its structural integrity at the same time, and to achieve this, its unique structure plays a crucial role [1].

During development, tendon cells produce collagen molecules, which arrange in triple helical structures and assemble into fibrils through a procedure that called fibrillogenesis, which is suggested to be mediated by the cell and takes place at the extracellular matrix (ECM) [2, 3]. The cells secrete collagen molecules to the ECM where they group into fibrillar structures [4, 5]. The collagen fibril is the fundamental building block and force-transmitting unit of the ECM [6]; fibrils form fibres and further associate in fascicles, which are organised to the highest hierarchical structure, the tendon unit [7, 8]. Observations of tendons longitudinally through the light microscope showed a wavy pattern of the collagen fibres that is called crimp and its form is dynamic and adjusts during tissue deformations [9-11].

Tendons are also categorised in larger hierarchical units; the endotenon, a thin layer of connective tissue that consists of fibre bundles, blood vessels and nerves; the epitenon, which place together fascicle bundles, and the paratenon, which contains the synovial sheath [12, 13] (see Fig. 1A). The space between different layers of tendons is called the non-collagenous matrix that is believed to be the active part of the tendon, regarding protein renewal capacity [14-16]. Each layer contains proteins that are unique to the particular region beside the common ones that fill every region (e.g. collagen) [14, 15]. For example, elastin is present in the interfascicular matrix with a possible contribution to the intrafascicular sliding and recoil [17, 18].

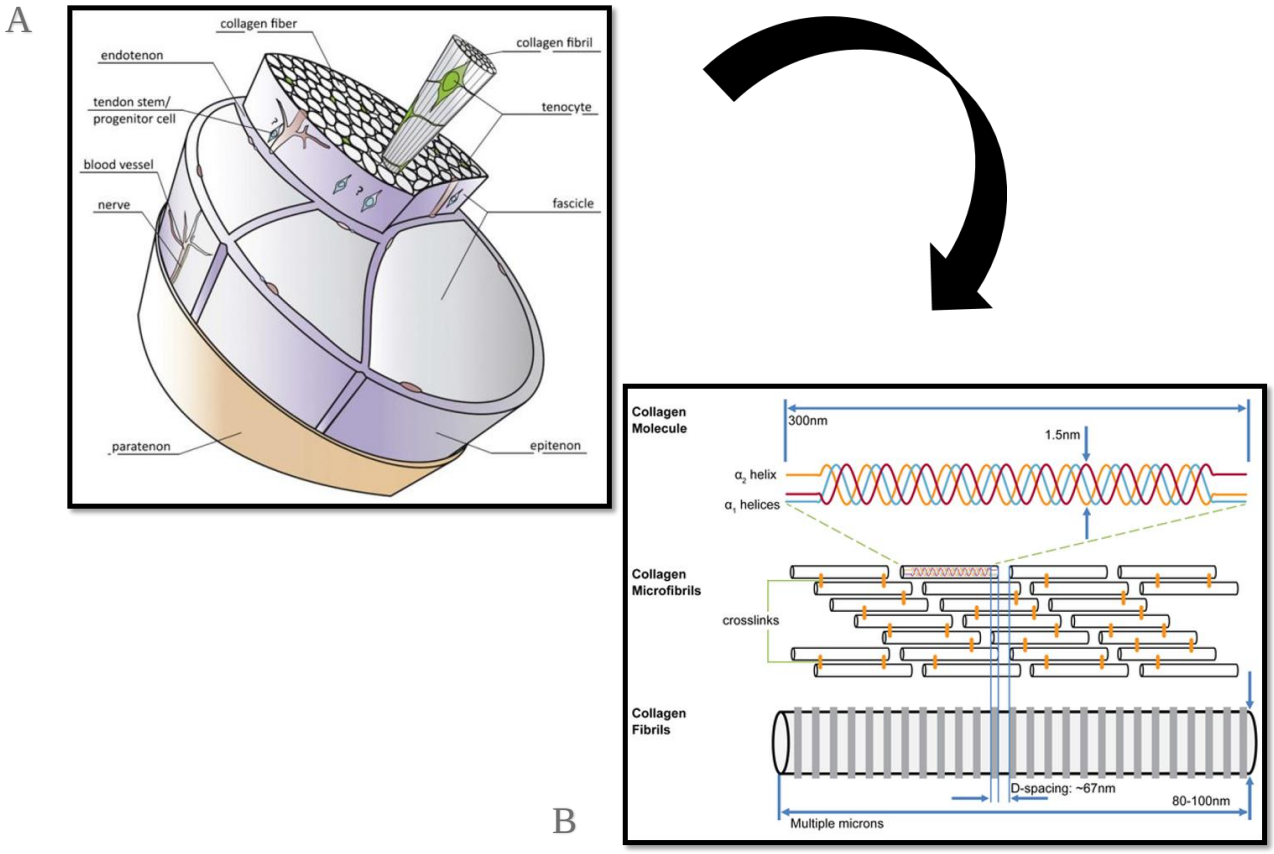

Figure 1 A) Schematic representation of tendon structure. Collagen molecules form fibrils, fibres and fascicle bundles. Larger hierarchical units compromise tendnon: the endotenon; the epitenon and the paratenon with the presence of blood vessels and nerves as well. Adapted from Docheva D et al. 2015. [19]. B) Collagen fibrils with a characteristic D-spacing and divide in microfibrils, which constitute collagen molecules. Adapted from Canelón SP et al. 2016 [20]

The viscoelastic capacity of tendons is strongly related to the hierarchical organisation of their structural properties [21]. However, the tendon regeneration and complex pathological conditions cannot be solely explained by focusing on the structure and the compositional profile should be taken into consideration. Particularly the assessment of the interplay between tendon components is necessary for better understanding tendon function.

The total dry weight of tendons consist mainly of collagens, primarily of fibrillar collagen type I (95% of the dry weigh total collagen). Other types of collagen also exist; the fibrillar collagens (collagen type II, III, V, XI, XIV and XXVII) that are part of the skeleton of different tissues [12, 22-25]; the fibril-associated collagens with interrupted helices or FACITs, which are insoluble collagen types (IX, XII and XIV), and they do not form fibrils but they contribute to the mechanical function [26-28].

Even though collagen in tendon is stable over time [29], other dynamic molecules are believed to contribute to the functional properties of the tissue [15]. Glycosaminoglycans (GAGs) and proteoglycans (PGs), which together with collagen are the most abundant elements of tendon dry weight, have also been associated with fibrillogenesis. GAG chains are linked to the PGs core via covalent bonds and are found together in the ECM. A subfamily of GAGs, the small leucine-rich PGs or SLRPs, has been shown to modulate growth factors and facilitate ECM organisation. Due to their negative charge, GAGs retain water and is critical for hydration of the tendon [30-32]. The water content is essential for tendon function as under physical load, it is transferred from the central to the periphery and affects the biomechanical behaviour of the tissue [33].

Although GAGs are also a large proportion of the tendon but their specific role in the tendon mechanical behaviour is unclear. It is also obscure whether the mechanical properties of mature tendons are regulated by lateral force transmission between adjacent fibrils, through chondroitin- and dermatan sulfate GAGs and their related PGs [34-37]. Some studies suggest the involvement of PGs (e.g. decorin and biglycan) in force transmission of tendons [38]. More specifically, decorin is believed to connect adjacent collagen fibrils but results from ex vivo experiments do not confirm this [15, 39-41] . Recently, Robinson et al. generated decorin-biglycan conditional double knock out mice and showed the lack of these SLRPs impaired mature patellar tendons mechanical properties [42]. In vitro models showed that regulation of GAGs affected binding sites essential for cell-matrix interactions [43, 44]. Ahmadzadeh et al. suggested that the diversity in the results of the various studies comes from the fact that some models refer to embryonic and some others to mature tendons [45]. The maturity of the tissue is a critical aspect for the contribution of GAGs in force transmission, as the length of the fibril changes over time (i.e. longer fibrils in mature tissues) and determines the involvement of GAGs in this process [45]. Moreover, it has been shown that GAGs do not affect the mechanics of embryonic tendons [46].

Elucidating the role of individual components in tendons *in vivo* is difficult. Consequently, in this project, we used engineered tendon constructs that allowed us to manipulate various components that are believed to be important in tendon physiology.

Cross-linking

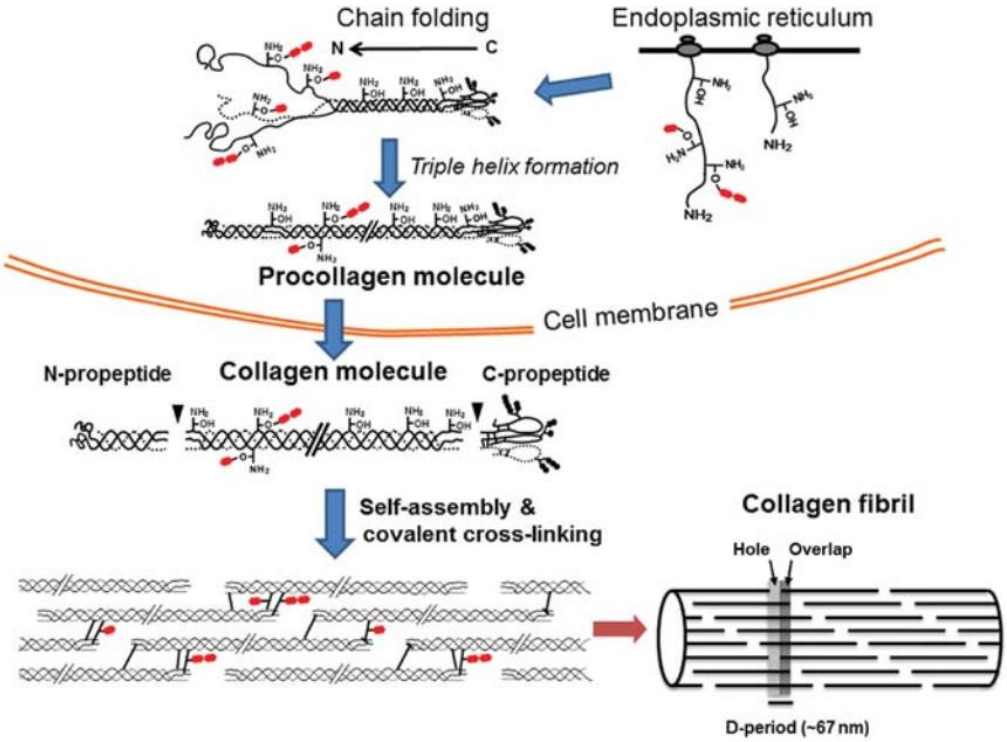

Figure 2 The collagen molecules are spontaneously self-assemble into a fibril and stabilized by covalent intra- and inter-molecular covalent cross-linking providing with stability the whole tissue. Adapted from Yamauchi 2012 [47].

Function and matrix stability of tissues are strongly related to the formation of cross-link bonds, which contribute to the mechanical integrity of collagen fibrils; inhibition of these bonds cause a dramatic reduction in the mechanical strength of the whole tissue [47-51] (Fig.2).

During development, the most abundant form is the immature enzymatic cross-links. Their formation is firmly related to lysyl oxidase (LOX) activity [52-54] and requires some days to complete [55]. It has been shown considerable effects of β-aminopropionitrile (BAPN) administration in reducing the tensile strength of connective tissues by increasing the solubility of matrix components, also of collagen [56]. Additionally, BAPN has been reported as a specific inhibitor of LOX, so it prevents the formation of LOX-related cross-links [57]. Therefore, it could be a useful tool to clarify the effects of a particular type of cross-links in tissue function.

In old tissues, another form of cross-linking is also implicated, and it is the result of modifications of the immature cross-links through glycation process. The immature cross-links become more stable through this process with the main players to be glycose and collagen molecules. These subsequent stable cross-links are called advanced glycation end-products,

AGEs [7, 58].

Cross-linking, stabilise the collagen molecules by connecting them in a way that they cannot dissociate quickly. Hence, the density of cross-links regulates tissue mechanics, such as stiffness [59]. Accumulation of collagen cross-links is related to decreased collagen turnover rate and affects dehydration of the tissues. In pathological conditions, including diabetes or naturally occurring processes, such as ageing, accumulation of cross-links drives tendons to become stiffer [60, 61]. However, it is also depend on the type of cross-links whether the induction of stiffness is beneficial for tendon function [62].

The cultivation period of engineered tissues is short compared with native tissues. For example the engineered constructs that were used in this project; they can be maintained in culture for approximately 5 weeks before they break. Therefore, supplementation of reagents to induce cross-link formation is usual to improve mechanical properties and prolong the half-life of the engineered tissues [63].

Since the natural process of cross-link formation is a combination of reactions and requires sufficient time to occur, the induction of synthetic cross-links is preferred. To achieve the accumulation of cross-links, a variety of chemical cross-linkers have been used, however, a crucial issue is the induction of high cytotoxicity [64, 65]. Recent studies suggested a natural cross-linker, genipin, to overcome these side effects. It has also been shown the ability of genipin to enhance engineered tissues mechanical properties and delay matrix degradation [66-69]. Thus, it offers a promising tool to elucidate the relation between cross-links and cellular forces.

Tendon mechanical behavior

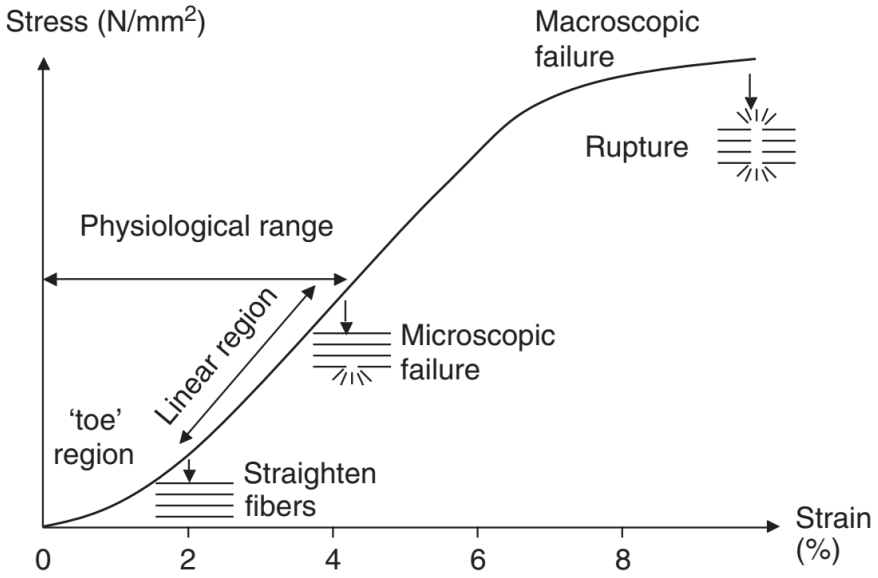

Figure 3 Stress-strain curve as adopted from Wang, J. H. 2006 [70]

Connective tissues as materials have anisotropic properties and in combination with its unique structure, resist the various types of forces that it is subjected during motion but also at rest. More specifically, tendons display a characteristic stress-strain curve with three distinct phases. The "toe region", where the fibres are aligned according to the direction of the applied forces; the "linear region", which is the result of the stretched collagen molecules helix and random micro damage start occurring, or it has occurred already; "the plastic region", which is observed at the end of the previous phase where the tissue falls apart and fail to recoil, reaching the "rupture point" [70, 71] (Fig.3). The phases as mentioned above, are a characteristic of *in vitro* testing [72]; since *in vivo* the tendon does not rupture due to simple elongation, but rather are followed by critical damages at the myotendinous junction or more rarely at the tendon-bone junction [73]. Exceptions are degenerative tendons because of pathological conditions such as tendinopathy, whereby the rupture can also occur within tendon [74, 75].

Force transmission in the tendon is a serial event of many levels and many potential regulators, however, the collagen fibrils have a crucial role at this process [4, 58, 76-78]. Therefore, studies have focused on the mechanical evaluation of the fibril unit to determine the whole tendon properties [79, 80]. More specifically, single collagen fibrils were subjected to loading patterns of various levels of strains, and the D-period spacing variations correlated to the applied deformations [80, 81]. Another study showed similar mechanical properties between fibrils but not at the inter-fibrillar properties compared with full tendon [82].

During loading, tendons present a viscoelastic behaviour as a result of their structural organisation and ECM properties (e.g. water and collagen content). Although how tendons constituents interact and participate in mechanical functions is not well understood. It has been suggested the viscoelastic properties of tendons protects them against high loads through a mechanism where fibrils or fibril bundles slide, dissipate energy between them and moderate the load.

Fibril sliding has been linked to the viscoelastic properties of the tendons and especially with the toe region phase [83]. Macroscopic alterations of tendon structure are a result of the crosstalk between macroscopic events that occur in D-period spacing or fibril sliding and adaptations of the non-collagenous matrix [15]. More studies investigating fibril mechanics are needed to provide new insights into the existing theories on whether the microscopic damage occurs within the fibril or between the fibrils (inter-fibrillar slippage) [84]. In a recent study, Svensson et al. provided significant evidence suggesting the existence of continuous collagen fibrils in mature tissues in contrast to embryonic tissues, where more likely the fibrils are discontinuous [85]. Defining fibril continuity is crucial to explain the role of fibrils in force transmission and the exact mechanism that regulates mechanical patterns.

The viscoelastic properties consist of three primary features; when the load is continuous, the stress declines over time, and this called stress-relaxation; when the stress is constant, the strain increases until reaching the failure point, and this is defined as creep; the hysteresis that represents the lost energy during cyclic loading-unloading tests on the tendon [86]. It should be noted, that strain rate can regulate the mechanical properties such as the failure stress and the stiffness, which are increased in higher strain rates [72, 87, 88].

Dynamic processes occur during mechanical evaluation protocols or cyclic loading, such as the fibre uncrimping, which happens when the crimp (wavy collagen pattern) becomes flat due to fibre deformation, and it is observed within the toe-region phase of the stress-strain curve. This pattern can be visualised by microscopy (polarised or electron microscopy etc.), and it is a mechanism of the viscoelastic properties of the tendon to prevent overloading [86, 89-91]. Another important mechanical process is the fibre re-alignment; the temporary condition, where fibres shift their distribution towards the axis of loading, and it is widely used to evaluate tendons mechanics [92, 93].

In vivo evaluation of tendon mechanics do not allow the assessment of small structures, like fibrils. Therefore, tissue engineered in combination with advanced microscopy

methods can be used to evaluate in detail the mechanical behaviour of the tendon in smaller scales.

Cells within tendon

Various cell types are present within tendon; including chondrocytes, smooth muscle, endothelial and synovial cells [1]. The prominent cell type of tendons is the fibroblast. There are different subpopulations of fibroblasts within the tendon, but there are no specific markers to distinguish them [94]. Due to the diversity in fibroblasts, the specific role of each cell type in connective tissue function is obscure.

In mature tendons, it has been observed that the cells at the core part become more elongated with a distinct spindle-like shape while in the cells at the outer part presented more rounded [95-98]. It is believed that the cells at the outer layer of tendon have stem cell properties and contribute to tendon regeneration or healing after injury. Moreover, a population of cells that have been identified in tendons possess stem cell-like characteristics, such as the differentiation capacity into various cell types [99]. Tendon-derived stem cells (TDSCs) can be distinguished from mesenchymal stem cells (MSCs) even though they express similar markers but not from other types of fibroblasts that may are present [100-107]. The most common markers for identification of embryonic tendon fibroblasts are scleraxis [108] and tenomodulin [109, 110]. However, they are also expressed in different types of fibroblasts [99, 101]. There is a need to find a fibroblast-specific marker to provide new insight into tendon function. Further, in occasions such as scar tissue formation following tendon injuries where fibroblasts deposit excess amount of collagen, it would be useful to know if a specific type of fibroblasts is responsible for the accumulation of the extra collagen so it can be targeted to prevent such conditions.

Moreover, cells within tendon are responsible for structural integrity of the tissue by depositing structural proteins (e.g. collagen) [111, 112] and in parallel by balancing the deposition of matrix components with the release of collagen degradation enzymes (lysosomal or cytoplasmic enzymes) or with the removal of the surrounding matrix through phagocytosis [113-115]. Thus, the role of the cells within tendon is critical to maintaining tissue integrity and regular function, whilst pathological conditions can be induced. In order for the cells to be functional, they require some stimuli, which can be caused by hormones or from mechanical forces. Although, in vivo, cells are shielded from many layers of a fibrillar network, and it is unknown the magnitude and the type (e.g. mechanical, shear) of forces they are subjected [116].

It is known that cells can exert forces as well, but most of the studies have been performed on 2D surfaces and in 3D artificial substrates that are lacking native tissue mechanical and structural properties [117-119]. Therefore, *in vitro* tissues that recapitulate *in vivo* conditions would be beneficial to study in detail the distribution of force within the tendon.

Further research is required to define the precise function of the cells and especially in models that resemble *in vivo* conditions. The study of mechanical forces may be a solution to reveal specific behaviours relevant to the different populations of fibroblasts in connective tissues; thus, specific markers can be identified. In this project, the so-called 'tendon constructs' (will be described later) were used as a model to demonstrate tendon tissue. Notably, the tendon constructs, have a similar structure with embryonic tendons and they present viscoelastic properties, so they recapitulate most of the tendon functions [120, 121].

Engineered tendon constructs

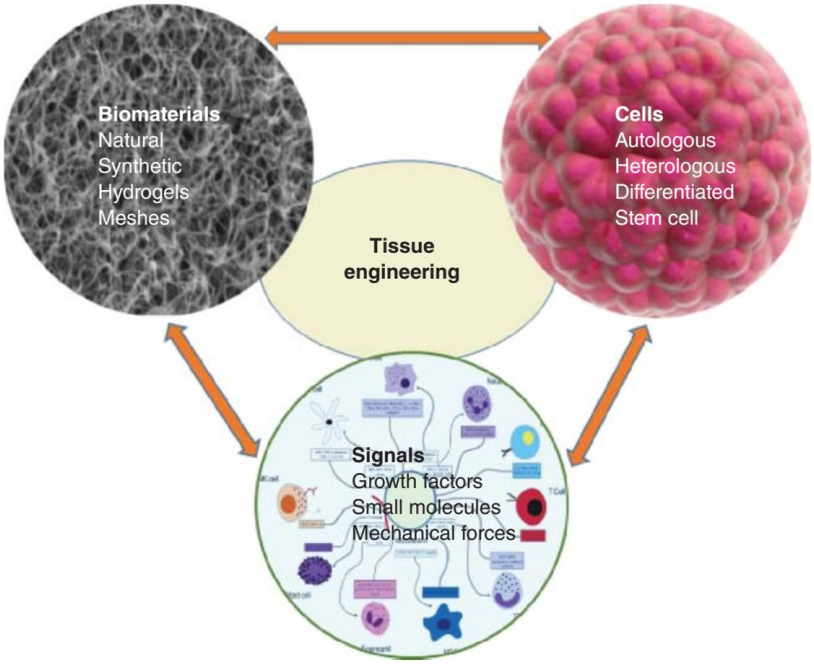

Figure 4 The triad of tissue engineering. The combination of cells, scaffolds, and signals is used to engineer functional tissues as adapted from R Mhanna, A Hasan - Tissue Engineering for Artificial Organs, 2017 [122].

The intricacy of *in vivo* conditions creates problems to distinguish the exact role of the cells and the matrix. Tissue engineering strategies offer an excellent opportunity to isolate and investigate the mechanobiology of specific tissues. However, to recapitulate native tissues *in vitro* requires a combination of the construction of the structural base of the tissue, which can be synthetic or

natural scaffold, with the appropriate type of cells and the biologically active molecules (such as growth factors)(Fig.4). The purpose of making a tissue equivalent varies as it can be for replacement of damaged tissue or for the study of specific features of tissues that are related with native conditions.

The engineered tissues need to be functional and resemble *in vivo* tissues. Hence, one of the most crucial points in tissue fabrication is the type of scaffold where the cells will be seeded. Biological scaffolds are sufficient over synthetic ones, in studies where cell-matrix interactions are assessed whereas the synthetic scaffolds are more suitable to study cell-cell interactions, since the matrix does not interfere with the cells or interactions can occur but they are far from physiological conditions [123, 124].

For engineered tendon construction, the use of collagen and fibrinogen are the most common as scaffolds [125]. Even though collagen is the main component of the tendon, it has some implications as a scaffold for engineered approaches. For example, the newly formed collagen cannot be biochemically distinguished from the collagen that has been used as a base for seeding cells. Hence, collagen scaffolds can complicate the study of collagen production or fibrilogenesis. Further, scaffold-like fibrin is believed to provide a better solution over the others since, is easily degradable by the cells, which create a natural scaffold by remodelling the ECM [126-128]. Studies have shown that fibroblasts within fibrin scaffolds are better to recapitulate the transcriptomic profile and matrix alignment of native tissues compared with collagen scaffolds, reinforcing the opinion of using fibrin among the other candidates [129, 130].

This project was focused on assessing mechanical properties of tendon and the regulation of cell contractility and matrix responses in tissue level. Kapacee et al. [120] described an engineered tendon tissue the “tendon construct” which later established by Bayer et al. [121] with human tendon cells. The tendon constructs allowed producing their collagen-rich matrix by forming a linear structure that resembles embryonic tendon structure and mechanical behaviour [121, 131, 132](Fig.5).

An essential aspect of this model is the pre-existing tension that the fibroblasts create during formation, which gives the characteristic linear shape of the construct and provides it with fibril alignment. Loss of tension by cutting the construct caused downregulation of collagen expression and the induction of inflammatory markers of COX family [133] indicating the importance of tension not only in tissue integrity but also in cell survival and protein synthesis. Tendon constructs mature over time and augment their mechanical properties (e.g. peak force, stiffness) nevertheless, five weeks after seeding, the constructs start breaking

(Herchenhan personal communication) and it could be due to the continuous rise in the tension, in combination with collagen degradation enzymes that are present in the culture medium or produced by cells. During development, the tension is important for regulating constructs morphology and when the constructs are formed, it is required to maintain their structural integrity providing a tensional homeostatic condition.

In addition, LOX related cross-links are necessary to maintain constructs stability. Treatment at an early stage with a cross-linking inhibitor (BAPN) caused a break of the constructs while supplementation with BAPN from two weeks after formation reduced dramatically the constructs mechanical features [52].

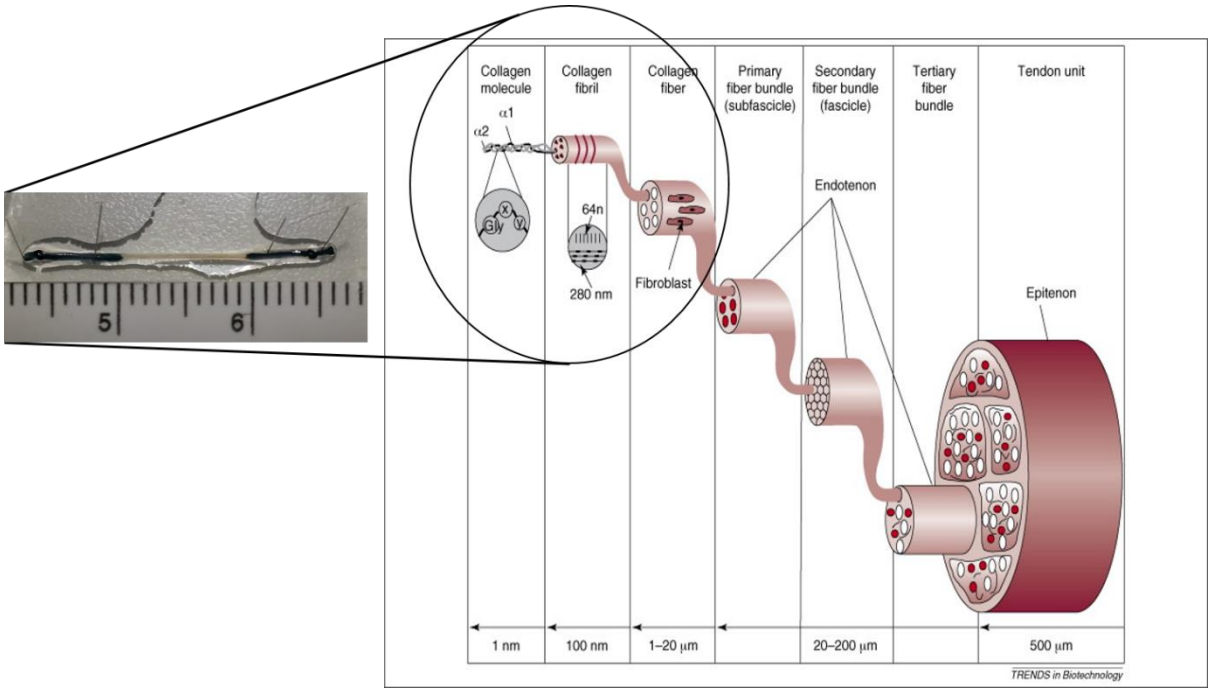

Figure 5 Comparison of tendon contract to whole tissue structure. The picture modified from Wang 2006 [70]

Overall, tendon construct provides a useful and practical model for the evaluation of understanding deeper tendon function and regeneration. This model has the advantage of incorporating most of the components that are present in a native tendon (e.g. collagen, GAGs, elastin) as the primary cell type of tendon, governs the development and formation of this tissue-like structure.

Mechanotransduction

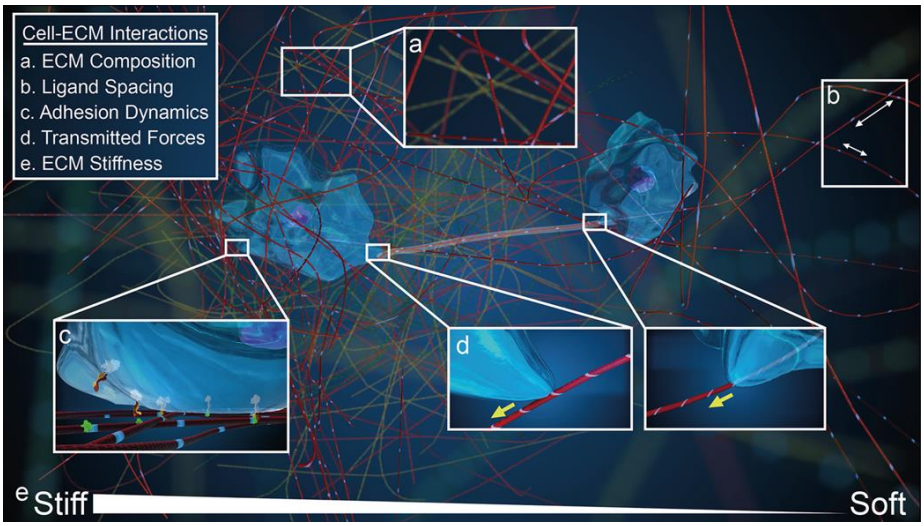

Figure 6 Cell–matrix interactions depend on various factors such as microenvironment composition, the ability of cells to bind specifically to different ECM fibres, the binding to specific ligands via integrins, the transmission of force to the ECM and to other cells and on the matrix stiffness. [134]

Cells within the human body are subjected to a diverse type of stimuli such as chemical, electrical and mechanical. The ability of the cells to probe the mechanical signals and translate them into biological cues is called mechanotransduction [135-137] (Fig.6). The biological responses can occur briefly, within milliseconds up to a few hours, followed by protein modifications or within prolonged periods that can last from days to years and can cause transcriptional alterations [138, 139]. Mechanotransduction includes a combination of events, with responses spanning from tissue to the molecular level. This process can be divided into three distinct phases; the transfer of the mechanical forces; the detection of the signals and the response by the cells (mechanoresponse) [140].

The load-bearing element of the cells is the cytoskeleton (CSK) and consists of intermediate filaments, microtubules and actin filaments. The actin cytoskeleton with several types of the motor protein myosin, are principally responsible for developing contractile forces, in muscle and non-muscle cells [141-143]. The development of contractile force by actin-myosin interactions can impose tension on cells that linked by adherent junctions [144].
Several mediators of mechanotransduction have been identified including gap junctions [145], ion and stress-activated channels [146] and MAP kinases [147]. Integrins, a cell surface receptor family, that are attached to the actin network intracellularly [142, 148, 149]; they form focal

adhesions (FAs) complexes with a variety of intracellular proteins, linking the ECM with the cytoplasm in a dynamic connection. Additionally, the FAs contribute to the transfer of the forces from inside out and vice versa as they are directly connected to actin filaments, which with myosin; they generate forces essential for cell motility and contraction [150, 151]. Actomyosin machinery links, allow the adherent cells to pull on the matrix transmitting forces to adjacent cells and the ECM; through this mechanism, the cells probe the applied force and respond respectively [152].

Alterations in ECM properties can influence many aspects of cellular function. More specifically, fibroblasts prefer to migrate to stiffer areas and exert a higher amount of forces [153, 154]. It has been identified the ability of the fibroblasts to differentiate into the more contractile type of fibroblast, the myofibroblasts [155-158], when placed in stiffer surfaces or when they subjected to different amounts of tensile strain [106, 159, 160]. The more contractile phenotype is reversible and disappears with the absence of tension [161].

Mechanotransduction has been mostly studied in cellular and molecular level although the whole tissue can be affected. For example in the tendon, where force transmission is the primary function and it can influence the tissue composition and mechanical properties of the tendon [162, 163]. There is a dynamic relation between the cell-ECM, and it is believed to play a crucial role in maintaining tissue homeostasis .[164, 165]

Cell-matrix interactions and tensional homoeostasis

Brown et al. introduced the concept of tensional homeostasis as the tendency of fibroblasts within a 3D scaffold to re-establish the original tension upon mechanical stimulation [166]. Cells within tissues are surrounded by a fibrous matrix and exist in critical positions regarding force transmission, as their passive components are part of the tensional homeostatic process.

Single cell studies showed that fibroblasts could reach mechanical stability at a tension level different from that they had before; after shape alterations and response to changes in matrix stiffness. However, it is not clear the existence of an accurate tensional regulatory mechanism to control the homeostatic response or it is a more a generic response to mechanical stimuli [167-169]. The failure of single-cell studies to support the tensional homeostatic theory of Brown could be due to the difference between a single cell and complex of cells, as they exist in tissues and interfere with matrix proteins.

Early studies have confirmed the presence of endogenous cell-based tension, using non-muscle cells (fibroblasts) in 3D structures [166, 170-173]. However, the above studies focused

more on the detection and quantification of cellular forces within various lattices, but they lack evidence of how these forces are regulated in response to tensile strain or by matrix alterations.

Maintaining tensional homeostasis is believed to be an essential aspect of tissue integrity and normal function. *In vivo*, excessive loading of the tendon has been correlated with pathological conditions that accompanied with the development of inflammation and disorganised fibres. In contrast, the absence of mechanical stimuli can cause dysfunctional tendons indicating the importance of mechanical forces [74].

The study of tensional homoeostasis could help to clarify the mechanism behind the interplay between the cells and the matrix, at the perspective of forces. Here we used an innovative device to detect the cellular forces applied on the matrix, and it provides an excellent opportunity to study further the tensional homeostatic mechanism since it can apply tensile forces and evaluate the response of the cells.

Growth factors and tendon function

The cell surface provides specific sites that can be activated from growth factors, independently and at the same time, from mechanical and other signals through mechanotransduction [174]. Growth factors, especially insulin growth factor (IGF-1) and transforming growth factor (TGF-ß) are involved in crucial events regarding tendon function.

TGF-ß has three isoforms (TGF-ß1, -ß2 and ß3) that share 60-80% homology [175]. It has been suggested the differential role of each isoform regarding tendon healing or regeneration but in this study, we used the whole protein, and it is referred as TGF-ß. IGF-1 is produced from many tissues of the human body, including muscle and bone. After post-translational modifications can give rise to three isoforms (IGF-1a, -1b, -1c) and they all have high affinity with the IGF-1 receptor [176, 177]. Both proteins have been suggested as regulators of tendon mechanics, with the TGF-b to be well characterised as mechanoregulator while the role of IGF-1 is not well known, regarding force regulation.

IGF-1 and TGF-ß were upregulated after heavy exercise, and they contributed to the regeneration process [178-180]. Data from in vivo and in vitro studies suggested the primary effect of IGF-1 and TGF-b in tendons is the induction of collagen synthesis by triggering fibroblasts to produce more collagen [181-183]. Interestingly, combined administration of these factors improved the mechanical properties of rabbit patellar tendon after two weeks of treatment [184]. Another study showed the optimisation of the treatment concentrations improved the formation and the mechanical properties of engineered ligaments [185].

TGF-ß has a unique role in tendon function, and particularly in the force transmission process. It has been characterised to stimulate cell contraction [157] directly through the process of differentiating fibroblasts into myofibroblasts [186, 187]. Induction of myofibroblasts in in vitro models is regular due to the presence of sufficient amount of TGF-b in the serum that is supplemented in the culture medium [158, 188-190].

IGF-1 is suggested to have beneficial effects on musculoskeletal pathologies [182, 191, 192]. In contrast to TGF-b, the precise action is not known, and it has mainly been linked to increased protein synthesis in several cell types including chondrocytes [193] and fibroblasts [194, 195].

Therefore, the regulation of serum concentration that is typically used would allow the study of IGF-1 and TGF-ß actions in relation to the cell contractility and matrix properties.

METHODS

Cell isolation

Human tendon cells were isolated from gracilis and semitendinosus tendon tissue as described previously [121]. A critical aspect of this procedure was the cleaning of the tissue from visible parts of muscle and other spare tissues. Since there are not specific markers for the distinction of tendon cells (see introduction for more details), we did not use any sorting process. Here, the tendon was digested with collagenase type 2 and the fibroblasts were obtained after centrifugation of the digested tissue. An alternative is the outgrowth method, in which the tissue is cut in little pieces and the fibroblasts allowed migrating to the surface, where the tissue was placed [196]. However, the collagenase digestion has been extensively used in the past [121, 131, 197] and is the most common method to isolate tendon fibroblasts nowadays.

In this project, they were exclusively used human primary tendon fibroblasts, and a stock of cells from various donors was made. Thus, in each study, at least two different cell lines were used to prove the observations were not cell-line specific. However, even from constructs of the same cell line, there was regularly, a variation at the formation of the constructs, such as minor morphological (i.e. thickness) differences so, a higher number of replicates were required to validate the results. The variations existed mostly at the methods that do not use normalised values and the results referring to values per construct.

Tendon Construct fabrication

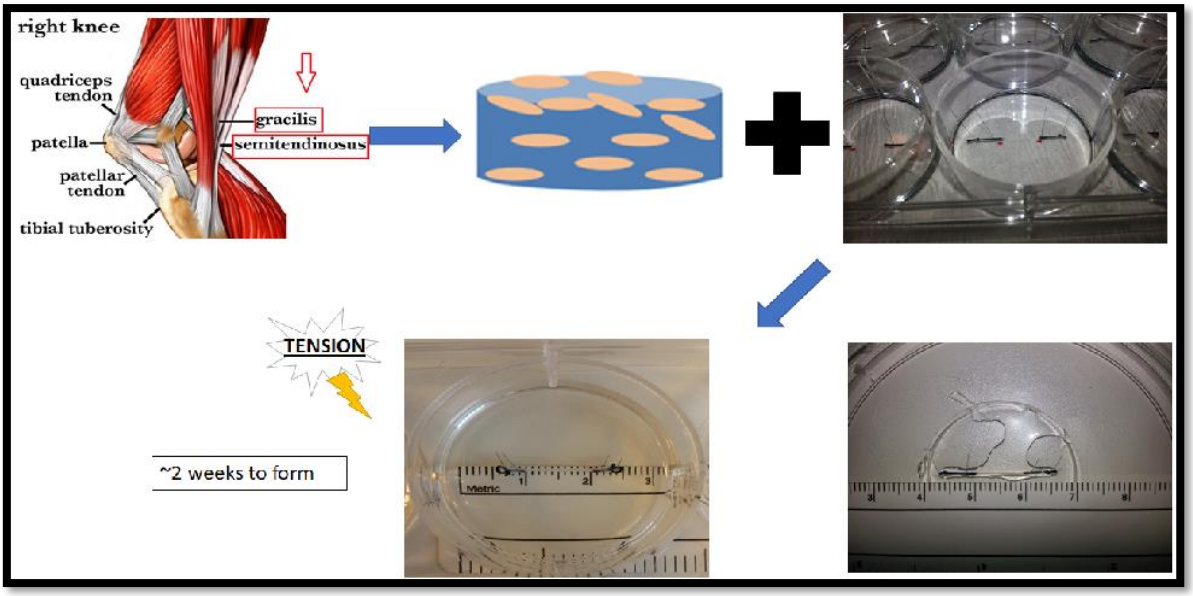

Figure 7 Schematic representation of the steps that were followed for construct fabrication.

Tendon constructs fabrication has been slightly adjusted [198] from the original protocol as described by Bayer et al. [121] (Fig.7). First, as it is shown in the pictures below, loop-shaped sutures replaced the linear shape of the sutures, so it will be easier to mount the construct in the well of the force monitor (Fig.8).

Additionally, the constructs were cultured in six-well plates, which they were pre-treated with normal medium (DMEM plus 10% FBS) to prevent the gel from attaching to the plastic surface at the edge of the wells. Thus, the manual detachment of the gel from the side (using a thin pipette tip) was not necessary and only in some cases was needed, usually at the first change of medium. With this intervention, the polymerized gels are floating although the internal tension induced by the cells, drives the morphology into a linear shape as before. Further, the time of formation has slightly shifted to an earlier time point, approximately seven days compared with the ten days without the pre-coating. In overall, the pre-coating is a useful addition to reduce the manipulation on the constructs ensuring the full integrity of the gel. Moreover, the risk of contamination is limited since there is not close and regular contact with the forming constructs.

In this project the constructs were allowed to grow in culture for 3, 4 and 5 weeks post seeding to obtain improved mechanical stability (inducing peak stress and stiffness) [132]. The newly formed constructs present poor mechanics (i.e. strength), so stronger constructs were

preferred as they expected to have more prominent changes in the results, of the various interventions (i.e. cross-link alterations) that they have been subjected.

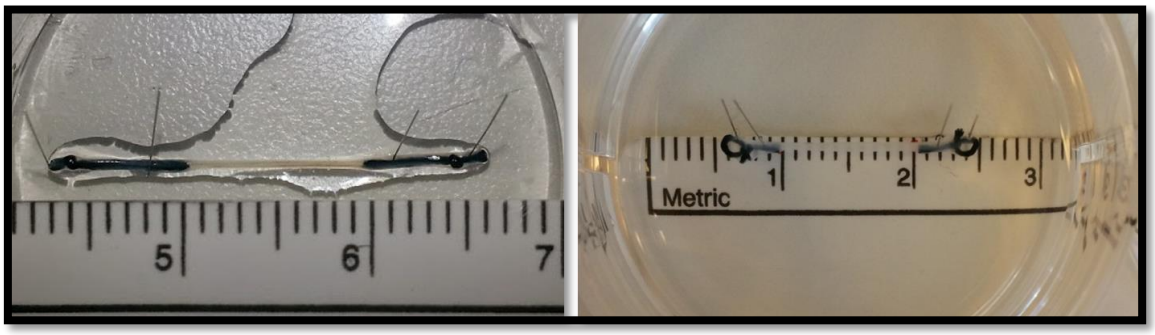

Figure 8 Photograph of the contructs without (to the left) and with (to the right) loops.

Advantages of this system consist of the rapid formation of the whole tissue within approximately 10 days and the overall culture time, which can be prolonged up to 5 weeks. Hence, tendon development or maturation can be studied in more detailed. Further, the culture conditions are similar to typical 2D cell cultures, and they can be conveniently manipulated, for example, it could be the regulation of the percentage of oxygen (inducing hypoxia) and thus, recapitulate closer *in vivo* conditions. The constructs are easily accessible to various types of treatments, by supplementing the medium with inhibitors and growth factors, or by mechanical stimulation with alteration of the length (for instance, unpinning of the one end to different positions). There are a few disadvantages of this model such as the connection of the main part of the construct with the sutures, which is usually the weakest (break) point at mechanical tests. Another important issue, is the dependence of the constructs on the serum that is supplemented to the medium (more detailed discussion following).

Digression

The tendon constructs compose a well-established model, and it was shown the importance of serum in tendon constructs development [199]. However, at some point the formation of the constructs could not be completed, and instead of gel contraction that consequently would lead to the classic linear form, the contraction process was stopped and a gel degradation was occurring. Visible holes were formed within the gel, indicating that the cells were active, so they were trying to degrade the matrix, but we could not evaluate if there were deposition of a new matrix (mainly of collagen). To test whether the above observation was an issue related to a

specific donor, we tried to make constructs with various cell lines (from different donors) but we did not observe any difference confirming that it was not a specific issue related to the donor.

The next step was to order a new batch of the reagents that were required for constructs fabrication, such as fibrinogen, aprotinin and thrombin. Thus, each reagent was tested separately, and each time constructs were made, one of the above reagents was replaced with the same but of a new batch. For example, the first time, a new batch of fibrinogen tested, but the old batch of the other reagents was maintained. Therefore, every time one reagent was tested by using a newer batch of it. However, no differences were observed, even after a new batch of all the materials was used. Additionally, the manipulation of the concentration of the reagents that were mentioned above, did not cause any change in the end product, but in some cases, such as when the concentration of aprotinin was increased, a delay in the generation of holes within the gel was observed.

An inhibitor of plasminogen, Epsilon-aminocaproic acid (EACA), that degrades the fibrin gel, was supplemented to the construct medium from the first day of seeding. As a result, after a week in culture, no visible holes were observed but neither any contraction. The above results confirming that the cells degrade the fibrin gel and produce collagen-rich matrix, as it is already known. Interestingly, two days after removal of the EACA from the medium, holes existed again.

We also tried replacing the fetal bovine serum (FBS) by horse serum, but it did not make any difference regarding gel contraction. Nevertheless, the solution was revealed when an older batch of FBS from GIBCO was used, showing that there was an issue related to the batch of the FBS we were currently using. We attempted to make constructs using different batches of FBS from GIBCO but none of them was sufficient for construct formation and the company could not provide with the specific batch that worked successfully for our model. It should be noted that GIBCO was the principle provider of FBS from the beginning of using the constructs in our lab. Later, the FBS from GIBCO was replaced with the equivalent from BIOWEST and it was sufficient for continuing the fabrication of tendon constructs.

Taken all together, issues with the serum caused a delay in the planned experiments, and we are unable to provide information about the specific component/s that prevented the constructs formation.

Force monitor (Tension Stepper)

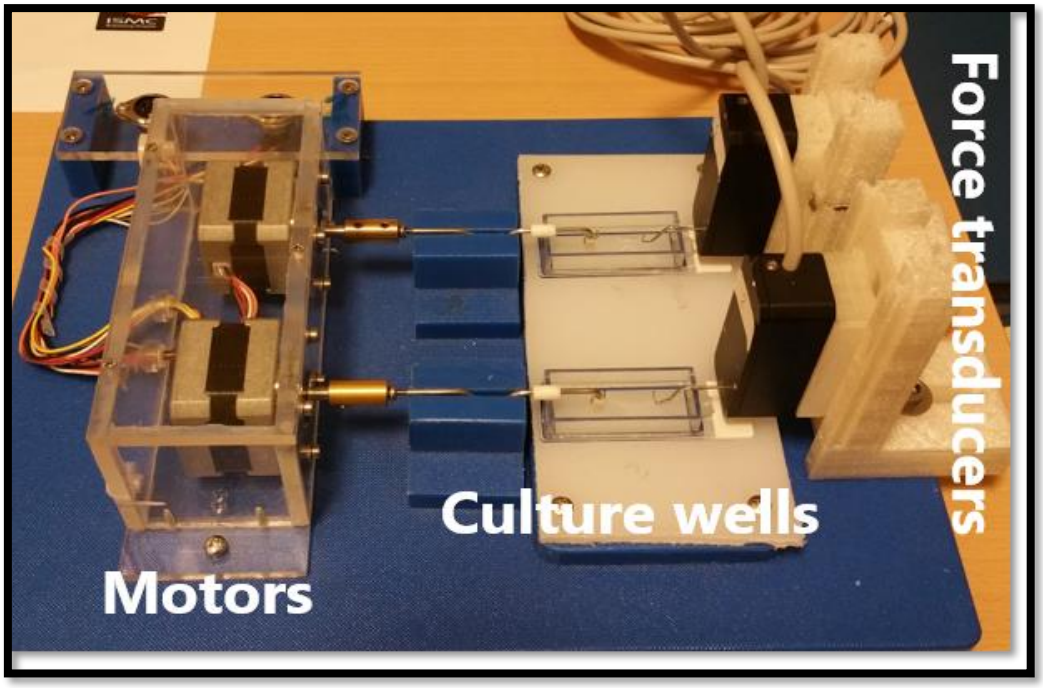

Figure 9 Photograph of the main board of the mechanical test system. The force monitor system consists of two stepper motors, culture wells and force transducers

David Holmes from Karl Kadlers lab at the University of Manchester, kindly provided the force monitor or so-called “Tension stepper”. The device has been slightly modified, by replacement of the custom force sensor with a commercial one from Aurora Scientific, due to practical issues (noise in the force signal) concerning the quality of the outcome data (Fig.9).

The force monitor can detect tension applied to the matrix of the constructs over cyclic tensile protocols. The key feature with this device is the concurrent monitoring and modulation of cell-generated and matrix-related forces upon application of external forces. It should be taken into account, the importance of this system providing quantitative data regarding cell-matrix interactions in the tissue scale. Other research groups have used mechanical test devices that either focused on the detection of forces on larger scales [17, 41] evaluating the material properties of the tested tissues or evaluated forces on single cells [140, 200]. This device offers the opportunity to fill the missing piece to the complex puzzle of mechanotransduction, quantifying forces at the cellular level within the tissue unit.

The equipment consists of two force transducers (402A, Aurora Scientific, CA), that are sensitive enough to monitor cellular forces at a resolution of 5mN/V. The constructs

were subjected to various tensile testing by regulating the strain with two stepper motors linked to a motor controller (Astrosyn, Y129-5, PC-control ltd., UK) and the output, logged through a digital converter (Microlink 751, Windmillsoft.com, UK) which allowed data collection during the testing. Strain was applied by the stepper motors via a threaded rod with a step resolution of 2.25 µm, and the deformation rate was 56 µm/s, with force data collection at 1 Hz. The output could be visualized in real time during the testing protocols.

Force monitor setup

Constructs attachment

For the construct attachment, we decided to use hooks at the ends of the force transducers and the motor arms respectively, instead of the Y-shaped form on the original design [198] (Fig.10). We ended up with this idea trying to limit the manipulation that the constructs were subjected during mounting in the testing well. Therefore, in each test we aimed to maintain the original length of the constructs by keeping them pinned to a strip of the underlying Sylgaard, thereby stabilising them during transfer to the force monitor. This consist a drawback of this system as even a few millimetres alteration of the strain can impact the existing tension. Alternatively, the constructs could have grown directly on the force monitor but at the moment this option is not supported by our equipment for practical reasons (only two wells to work at the same time). Another solution to avoid the transfer issue, could be the fabrication of the constructs within a fixed contour, more stable than the stripe of the Sylgaard we are currently use, so they can be directly fit to the testing well with the minimum manipulation.

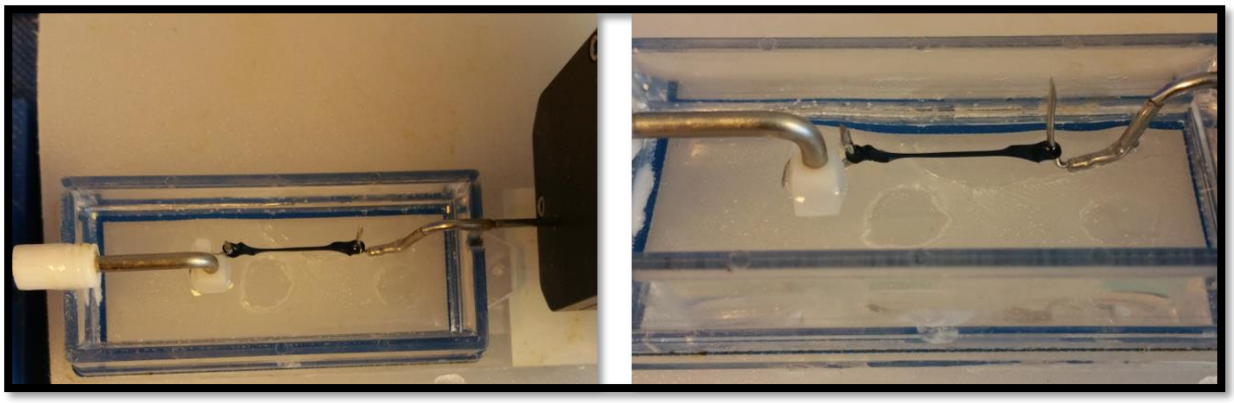

Figure 10 Pictures of genipin treated constructs represents how constructs were mounted in the wells of the force monitor

Testing conditions

The two wells require 7ml of medium each to be filled to the top. Before testing, a moist piece of tissue paper, or a plastic lid cap in later experiments, was placed on top to prevent evaporation. Replacing the medium when it was required (during the tests of some experiments) could be easily done by aspirating with a pipet directly from the well.

The deformation rate can affect tendons due to their viscoelastic properties, so if the deformation were applied fast, the stress would reach higher values compared with lower speed. Limitations of the system could not allow rate faster than (56 um/s). Furthermore, the setup of this device permits uniaxial application of stress, which fits with the direction that the constructs have formed (construct fibrils were aligned to the direction of tension).

Force monitor testing protocol

As it has been previously described [198] (Study I), the platform of the force monitor was placed in an incubator (37 °C and 5 % CO2) and the tests were performed at basic cell culture conditions. To validate the presence of tension, the constructs were first relaxed by 0.225 mm. If the constructs were under tension, which was seen by a drop in force, the length was returned to the original position, and the test was started. In the opposite case, where the constructs lack some tension, we assumed that they became slightly slack during removal transfer from the culture plates, and they were stretched between 0.225 and 0.675 mm to regain tension level compensating the loss in tension. The new length was set as the baseline length, and afterwards, the system was allowed to stabilise for one hour. Despite the re-tension taking about 1 hour to reach a plateau, we chose to test at shorter duration to avoid possible changes in cell behaviour functions such as cell death or ECM remodelling that could affect force measurements.

For study I, the tendon constructs were subjected to three unloading / reloading sets of cycles with each cycle consisting of 0.675 mm (6.75% strain) of unloading (reducing length), 300 s of rest period followed by 0.675 mm of reloading (returning to the initial length) and another 300 s of rest. Hence, after treatment with blebbistatin or chondroitinase ABC, another set of three cycles followed whereas for study II and III the constructs performed only the first set. The amount of strain was chosen to correspond approximately to the toe-region phase and to avoid micro-damage of the constructs during the mechanical tests.

Force monitor testing calculations

Four points from each cycle were assessed to calculate the force values. More specifically the points that were determined are A) before the start of unloading. B) when re-tension starts. C) Before the start of reloading. D) Instantly after reloading (Fig.11). After three cycles the average values were calculated to define re-tension: (C - B) / (A - B), that corresponds to the normalised value of re-tension relative to the unload. Stress relaxation was defined as (D - A) / (D - C), that also corresponds to the normalised amount of relaxation. Values of re-tension and relaxation are presented as a percentage, and the absolute difference between the pre and post data represents the effect of the treatment. Additionally, in study III, absolute values of re-tension (C-B) and relaxation (D-A) are also presented (Fig.11).

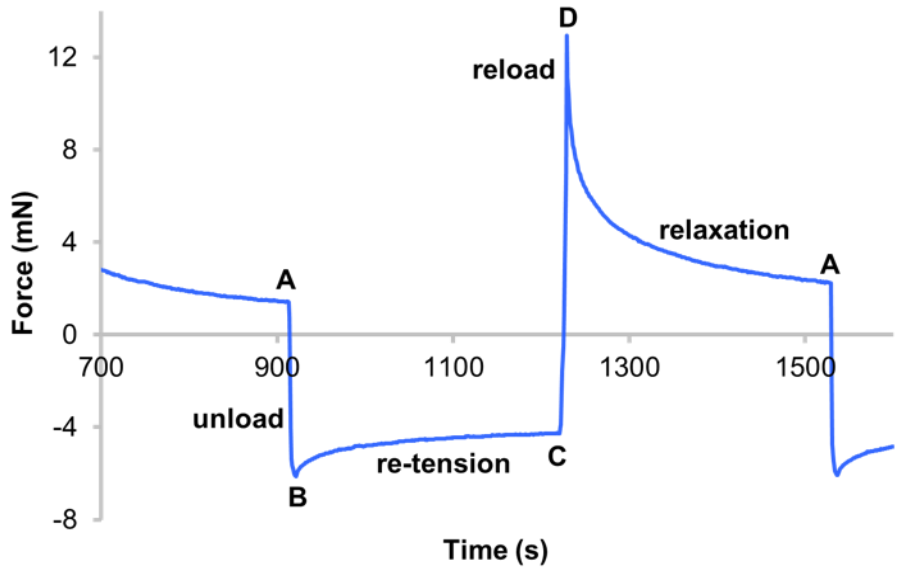

Figure 11 Example of force vs. time data for one cycle measurement. At point 'A' the construct is unloaded to point 'B' where the force is allowed to re-tension for 300s. At point 'C' the construct is stretched back to its initial position (point 'D') with the length remaining constant for 300s, relaxing back to point 'A' where another cycle starts.

Constructs treatment

The construct model is easily accessible for various treatments by direct supplementation of reagents to the culture medium [52, 133, 199]. In the study I, control samples that had normal medium replaced with fresh normal medium, were mechanically tested in the same manner to the controls for the effect of time (untreated controls). Even though the duration of the protocol was short, we thought that maybe changes concerning collagen synthesis or cell death could affect

the results. However, the untreated controls confirmed that changes that were not relevant to the treatments did not occur.

Blebbistatin

To confirm that the re-tension phase is an active cell response, we were interested in knowing that it results from the active contraction of cells and not from some passive component of the cytoskeleton. Myosin is related to cell contraction and in that context, blebbistatin, which is a specific and cell permeable non-muscle myosin II inhibitor and cell permeable, was chosen [201]. The main advantage of this reagent is the rapid, selective and reversible action. Alternative inhibitors, such as Y-27632 (Rho-associated inhibitors) and cytochalasin D (inhibits actin polymerisation) that they have been tested by others on chick embryonic constructs could also be considered [202]. The duration of the treatment was 30 min, and it was performed between the set of cycles (3 before and three after treatment). More specifically, the tendon constructs underwent three cyclic stretches in normal medium (DMEM). Immediately after, the normal medium was replaced with treatment media (DMEM plus reagent) followed by an incubation period of 30 min and three subsequent cycles.

Alternatives methods to confirm that re-tension was cell-generated, were tried but unsuccessfully. For example, killing the cells by freeze-thaw the constructs or treating them with methanol. The above methods were unable to prove that the structural integrity of the constructs after the treatment remained intact. Thus, we could not show if the results were also affected by compositional changes, such as collagen degradation.

Chondroitinase ABC

Chondroitinase ABC (CH ABC) (0.07U/ml) was used to digest glycosaminoglycans (GAGs) (Lee 04). The CH ABC treated constructs were tested similarly with the blebbistatin treated constructs.

Genipin

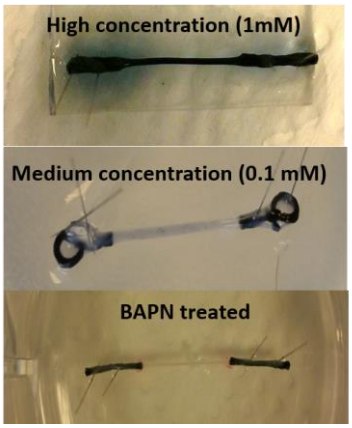

Figure 12 Representative images of tendon constructs treated with genipin and BAPN

Genipin (GN) was preferred among other cross-link reagents (e.g. glutaraldehyde) for the limited cytotoxic effects that accompany this natural compound. Further, genipin, through a chemical reaction induces the formation of cross-links between collagen, gelatin and chitosan molecules [66, 203, 204].

Genipin (GN) was prepared in DMSO at a stock solution (25 mg/ml), aliquoted and stored at -20˚C. Each time, a fresh solution of normal medium and GN were made with serial dilutions and in concentrations: 1, 0.1, 0.05 and 0.01 mM. Controls were treated with DMSO (1ul/ml) corresponding to the highest GN concentration (1mM) (Fig.12). Change of medium was performed every other day, and the supplementation of GN was from week 3 to week 4 after seeding. Genipin obtained from Sigma, and it is stated in the datasheet that is insoluble in water.

Growth factors

The measurements with this equipment correspond to the mechanics of the matrix properties of the constructs (more details in results/discussion).

The mechanical tests were performed with a micromechanical rig (200 N tensile stage, DEBEN UK). The system comprised of a petri dish so the tests can be performed in liquid and with a dissecting microscope to capture images for sample dimensions assessment. The constructs were stretched to failure, and the slope of the stress-strain curve was used for the calculation of peak modulus and stiffness.

In study III, the FBS concentration was reduced from 10 to 0.5 % as serum contains various growth factors and could interfere with or mask the potential response of the treatment we performed.

Hence, from week 3 to week 4 the 0.5 % serum was supplemented either with TGF-ß (2ng/ml) or IGF-1 (250 ng/ml). Therefore, study III consisted of three groups the TGF-ß or IGF-I treated constructs and the controls of 0.5 %. The medium with or without the growth factors was replaced every other day, and the day of mechanical testing the medium was replaced a few hours before the treatment of all constructs with fresh medium.

Mechanical testing (DEBEN)

The measurements with this equipment correspond to the mechanics of the matrix properties of the constructs (more details in results/discussion).

The mechanical tests were performed with a micromechanical rig (200 N tensile stage, DEBEN UK). The system comprised of a petri dish so the tests can be performed in liquid and with a dissecting microscope to capture images for sample dimensions assessment. The constructs were stretched to failure, and the slope of the stress-strain curve was used for the calculation of peak modulus and stiffness.

Evaluation of cell viability

In addition to the methods reported in the manuscripts, a number of other measurements were performed during the project. These methods will be described in the following section.

TUNEL assay

Two distinct types, necrosis and apoptosis characterise cell death and can be distinguished by morphological and molecular profiles of damaged cells. Routine tissue turnover includes the physiological procedure of programmed cell death (apoptosis) that occurs to maintain tissue homeostasis [205, 206]. Apoptotic cells present a discrete phenotype containing cytoplasmic and nuclear alterations which are associated with DNA fragmentation [207].

In study II, since the genipin supplementation was provided for a week, we also aimed to evaluate the potential cell cytotoxic effects at the last day of treatment so we could have a picture of the apoptotic cells within the constructs equivalent to the ones, which were mechanically tested.

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) is used to detect cell apoptosis by staining DNA fragments in nuclei that are observed at the late stage of apoptosis [208]. TUNEL staining was performed using an In Situ Cell Death Detection Kit (Roche, Indianapolis, IN) according to manufacturer's instructions. Briefly, tendon constructs were embedded (TissueTek, Sakura Finetek) and snap frozen in isopentane while pined. Sections of 5 µm were made and fixed in 4% formaldehyde. Slides were permeabilised in a freshly made solution of 0.1 % Triton X-100 and 0.1 % sodium citrate. Also, the slides were washed twice in PBS solution and left to dry at room temperature. Thus, TUNEL solution was added and the slides incubated covered to protect from light at 37˚C, in a humidified atmosphere for one hour. Afterwards, the samples were washed three times with PBS, and embedded with an antifade mounting medium without DAPI (ProLong Gold, Invitrogen). Slides were analysed under a fluorescent microscope.

XTT assay

XTT is a widely used assay for quantification of cell viability and cytotoxicity, evaluating the viable cells by the cleavage of tetrazolium salts (XTT) that were added to the culture medium. Viable cells process and cleave the XTT into formazan through a mitochondrial process. The amount of formazan in the medium that is a product of the respiratory mitochondrial process and thus, it is defined as a marker of active and intact cells (Fig.13)[209]

This assay was used in study II in 2D cells culture in 2D plates, to assess the acute effect after 24 hours of genipin treatment.

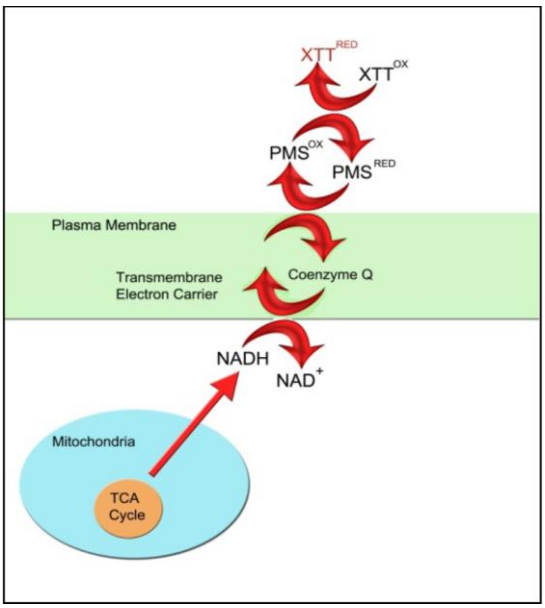

Figure 13 The colorimetric reduction of XTT by cellular enzymes, as adapted from ATCC Instruction manual.

DNA content assay

DNA content was determined in the mechanically tested constructs by DNA Hoechst quantification as slightly modified from Hoemann et al. [210] and was expressed as µg per construct (n=18). Briefly, the samples (GN001, GN005, GN01, GN1 and DMSO controls), were digested in papain solution overnight in 60˚C. Then 200ul per sample of the digested tissue, were transferred to a 96 well plate, and the fluorescence was first measured on the pure samples. After 50ul of Hoechst were added to each well of the 96 well plate, and the fluorescence was measured again. The difference of the two measurements was taken as the value of DNA specific fluorescence and was quantified via a standard curve made from known amounts of salmon DNA.

LDH assay

Lactate dehydrogenase (LDH) is an enzyme present in the cytosol of various cell types. When the plasma membrane is damaged, LDH is released in the culture media. The amount of formazan is directly proportional to the amount of LDH that is released and is an indicator of cytotoxicity.

In study II, before the mechanical testing, 150 ul of medium per condition were sampled, and the LDH assay was applied according to manufacturer's instructions [211]. Samples were taken at 8 and 24 h after replacing the medium (with and without treatment) and absorbance measured directly.

Results and Discussion

Study I

In the first study, the primary target was to show whether the re-tension phase was cell-related and subsequently to investigate a potential role of GAGs in force transmission at tendon constructs. To achieve this, we used blebbistatin to prevent the cells from contracting (as explained in the methods). Overall, we showed a reduction of the re-tension phase after the administration of the cell contraction inhibitor (blebbistatin), confirming our hypothesis, that re-tension phase is mostly generated by cells. Regardless of construct maturation (3,4 or 5 weeks) the effect of the treatment was similar across all weeks (Fig.14).In contrast, we did not observe an effect after GAGs removal by enzyme (CH ABC) digestion, indicating that GAGs do not contribute to force transmission in this model.

The quantification of cell-generated forces within collagen lattices or GAG-based hydrogels has been described in other studies with a variety of cell types [166, 170-173]. However, this study is the first to our knowledge that combines primary human tendon fibroblasts, within a natural matrix, which is created by the seeded cells. The tendon constructs have the advantage of incorporating a variety of proteins that can be found in native tissues, such as collagen, proteoglycans and elastin. Therefore, the evaluation of their potential impact on force transmission could reveal useful information regarding tendon function. Further, how the cells facilitate the tension in a more natural environment may clarify the role of mechanical forces in pathological conditions of connective tissues. For example, the formation of scar tissue following tendon injuries creates areas with a stiffer environment and evaluating the cellular forces might reveal information about the progression or prevention of matrix accumulation.

The morphology of tendon constructs depends on the endogenous tension which is a continuous process of the cells that pull on the matrix repeatedly to maintain the internal tension [133]. Although the re-tension after blebbistatin supplementation dropped ~37 % in overall, a higher reduction (maybe close to zero) would be expected as the effect of this reagent start occurring instantly, but there are some possible explanations for the non-complete loss of tension. It has been suggested that fibroblasts within the collagen lattices create a certain level of tension but after matrix cross-links, stabilise this tension, and it becomes independent of the cells. Hence, the overall tension would be part of the cells and another part of the matrix itself. However, during the cyclic loading, when the constructs are relaxed, the matrix viscoelasticity is

capable of providing only a small part of the re-tension as the contractility of the cells is the main reason of force generation. Marenzana et al., used another cell contraction inhibitor, cytochalasin D, and even the initial loss of re-tension occurred instantly; then it started to flatten with a further reduction to follow within a few hours [212]. In this study, blebbistatin was applied 30 minutes before the start of the dynamic protocol so may be the instant effect occurred during this time, and afterwards was less prominent.

In contrast to prior work, the re-tensioning was quantified over a relatively short period (300 s), whereafter the loading was repeated to confirm that the cell-generated response was a repeatable physiological phenomenon. It is more likely that the treatment time influences the ability of the blebbistatin to 'reach' all of the cells of the tendon construct and a more extended testing protocol would have yielded a near to total depletion of cell contraction, as it was observed in chick embryonic tendon constructs [202]. However, the short treatment time was chosen to avoid unrelated changes that could occur over time, such as effects on specific cell types as it has been shown with the absence of tension myofibroblasts turn to fibroblasts or the induction of signaling pathways that are not related to cellular forces.

Further, it is possible that the lower concentration of blebbistatin (17uM) in the present study was not sufficient to cause a depletion of cell contraction, compared with prior work (25-100 uM) [201, 213], although they did not quantify the level of tension. So it is not clear if the highest concentration would have helped our case. It was observed a distinct trend of the curve, during the re-tension phase of the blebbistatin treated constructs as they evaluated in the force monitor. The curve of the controls followed a constant rise over time in contrast to the blebbistatin treated equivalents that the curve start flattened after the initial rise (Fig.15) and this indicates a more extended treatment would reveal a more significant difference in re-tension between the untreated and treated groups.

The effect of blebbistatin did not differ between the weeks; however, the re-tension reduced statistically significantly from week 3 to 5. Herchenhan et al. showed that maturation of the constructs caused an increase of their mechanical properties and accumulation of collagen cross-linking also occurred [52]. Therefore, this could be the reason for decreased cell contraction. It is also suggested from others [214-216] that stiffer substrate cause decreased cell contraction and pursued us to obtain the second study (following). We also assessed the cell number between the weeks, and we did not find any difference, so the number of cells did not affect the re-tension.

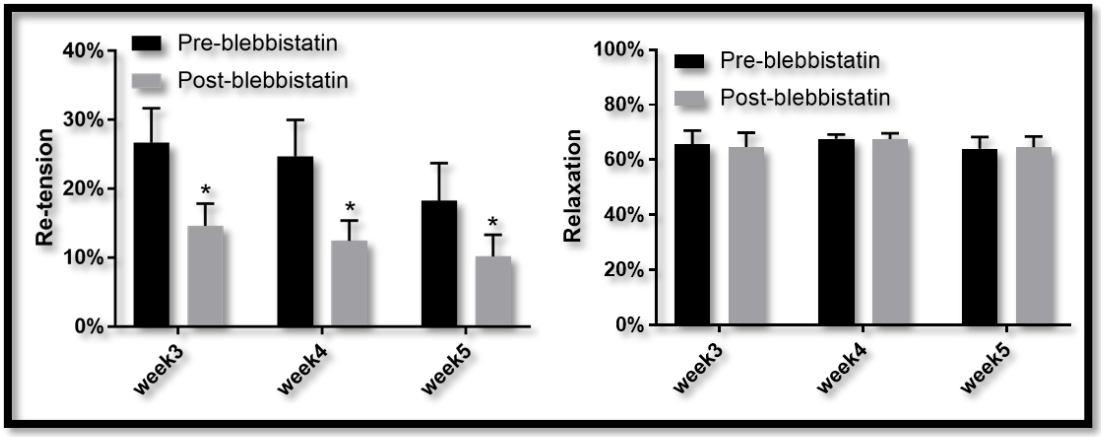

Figure 14 Blebbistatin treatment for cell contraction inhibition. A) The re-tension dropped significantly after blebbistatin treatment ($p<0.005$). B) The relaxation phase was unaffected by cell contraction inhibition. (n=24 from 5 cell lines: week 3, n=8, week 4, n=7, week 5, n=9).

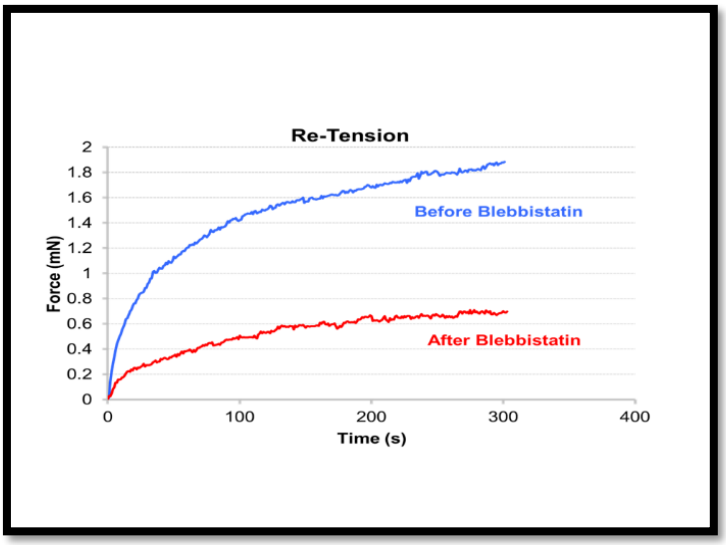

Figure 15 Zoom in at a representative force-time plot of the re-tension phase before (blue) and after (red) blebbistatin treatment.

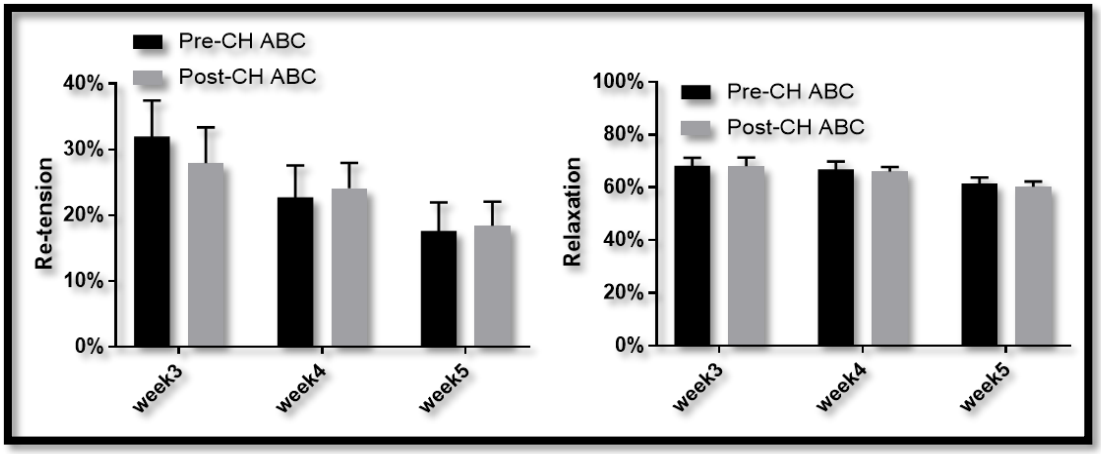

Figure 16 Chondroitinase ABC (CH ABC) treatment for glycosaminoglycan digestion. A) The re-tension did not change after CH ABC treatment. B) The relaxation also remains unaffected after the treatment. (n=23 from 5 cell lines: week 3, n=8, week 4, n=7, week 5, n=8).

While the unloading allowed evaluation of the cell-generated response, the force response during the constant elongation permitted evaluation of the extracellular component of the matrix, since inhibition of cell contraction did not affect the stress relaxation response. Although it has been shown that removing the proteoglycan decorin and glycosaminoglycans chondroitin sulphate and dermatan sulphate complex in connective tissue leaves the mechanics unaffected [41, 58] and that fibrils are likely continuous [85], we hypothesised that in constructs that are similar to embryonic tendon the fibrils would be discontinuous. So the GAGs may be used as interfibrillar bridges to connect adjacent fibrils, as a potential mechanism for force transmission. Although, we could not detect any difference indicating that such a mechanism for lateral force transmission is not present in the immature tissue.

Notably, in the present study, we showed that a 30 % reduction of GAG content did not impact at the relaxation phase of the 3-5 week constructs, implying that this is not important for the mechanics in immature tendon tissue (Fig.16). As discussed in the introduction the role of GAGs in the mechanical properties of the tissues is debetable, but we expected that the sensitivity of the force monitor would allow us to detect any potential effects. In a previous study on similar constructs, a dry weight of ~0.25 mg per construct was measured, making the ~7µg GAG content equal to ~2.8%, which is around fivefold higher than in mature tendon. We did not have access to the ultra-micro balance for accurately measuring the very small dry weight of the constructs so before digesting the constructs with papain we did not measure the dry weight. Additionally, it was observed an acute decrease of the force for approximately 30s during the

relaxation phase, and a stable curve was followed for the rest (270 s). Analysis of this short phase (30s relaxation) did not reveal any difference between the CH ABC treated groups compared with the controls (data not shown).

Robinson et al. recently developed a conditional decorin-biglycan knock out model and induced the effects after birth to study the defects of these proteins on mature mice tendons. They showed that the absence of biglycan and decorin caused poor mechanical properties in tendons, such as decreased stiffness and dynamic modulus at knock out mice compared with the controls, and the tests performed 30 days after the knock out [42]. However, it cannot be distinguished whether the effects of decorin-biglycan disruption are due to matrix regulation or a direct effect on force transmission. In contrast, to our study, we performed an acute treatment (for 30 min) as we did not aim to assess the effect of GAGs on matrix formation, but rather the direct effect on mechanotransduction.

While we showed that manipulation of the cells affected the re-tension, we did not see an effect by altering the matrix properties. Thus, at the next study targeting more the matrix rigidity, we evaluated more in-depth the stress-relaxation phase.

Study II

Matrix properties influence various cell functions such as cell proliferation and differentiation, although, little is known about cell-mediated forces. The results from the study I indicated that stiffness might affect cell re-tension and we sought to examine this further by cross-linking the matrix. The force monitor was used for the cell-matrix mechanical tests and for quantifying cell-mediated forces as produced in response to matrix stiffness. A natural cross-linker, genipin, was applied to induce cross-link formation and augment the mechanical properties of the constructs. We aimed to evaluate any potential acute and long-term cytotoxicity related to genipin since the cell number is expected to correlate with the re-tension. For the acute effect, we performed the LDH assay on samples collected 8 and 24 hours post-treatment, to assess the released LDH of the damaged cells as a marker of cytotoxicity. However, when genipin reacts, produce a blue colour that interfered with the LDH reaction; thus the measurements were not accurate. Therefore, we performed the XTT assay in fibroblasts seeded on a 2D surface, and the cell viability was evaluated 24 hours after the treatment (Table 1). All the concentrations remain unaffected by genipin treatment except for the GN1 concentration that killed the cells (98% reduction in cell viability). The 10-fold difference between GN01 and GN1 concentrations of genipin, maybe explains the fact that we did not observe a dose-dependent pattern at the cell-

matrix mechanical tests. Hence, the GN005 concentration was supplemented in the 2nd batch of experiments to investigate deeper the dose effect.

The long-term effect of genipin was evaluated a week after treatment to be representative of the conditions; the cells exist in the mechanically tested constructs. TUNEL assay was used to stain the apoptotic cells of control, GN01 and GN001 concentrations at constructs of 4 weeks old. The quality of the sections was not sufficient for quantification of the staining (representative images below) (Fig.17). Further, we evaluated the DNA content of all genipin-treated constructs, but it did not show any difference compared with the controls. The DNA measurement did not detect any difference between the treatments, even at the GN1 concentration where the constructs were visibly fixed. The weakness of the DNA content evaluation to distinguish the non-viable cells can be explained from the fact that DNA fragments can be detected for some time before they fully degrade [217-219].

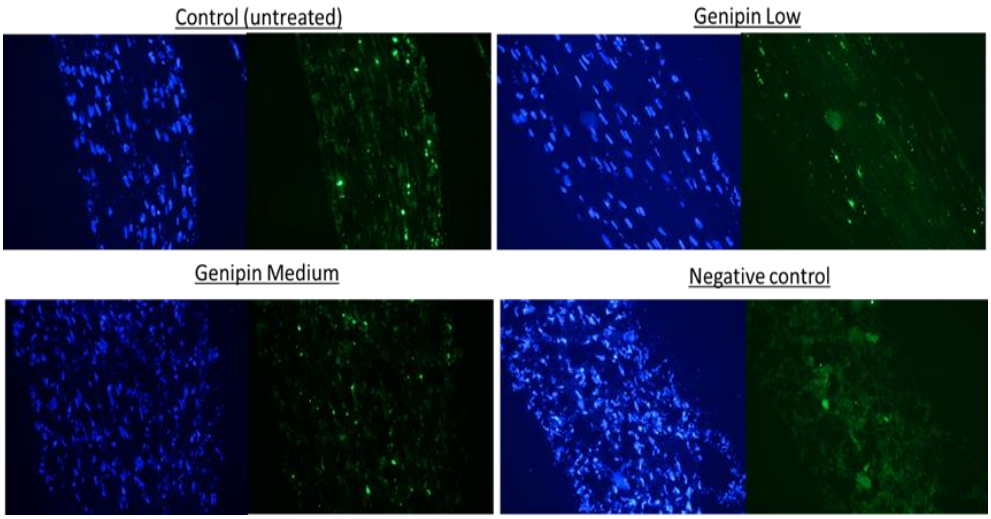

Figure 17 Representative images of cryosections stained with TUNEL and made from constructs treated with genipin, plus the DMSO control.

Alternative methods (such as construct digestion with cell counting or cryosections for nuclei counting) for the evaluation of cell number in whole constructs were used although practical challenges regarding the quality of the sections (the nuclei seemed to be fragmented and could not be distinguished from other non-specific stained parts) did not allow the collection of useful data.

Table 1 Cell-viability measured in 2D, presented as 100% of the DMSO controls. Whole constructs solubilized and DNA, Hydroxyproline and GAG content measured in ug per construct. Geometric mean [geometric SE]. Significant differences from DMSO control are marked with asterisks (*) ($p<0.05$).

	Cell viability, XTT assay (% of control)	**DNA content (ug per construct)**	**Hydroxyproline content (ug per construct)**	**GAG content (ug per construct)**
DMSO control	100%	15.8 [14.7-17.0]	10.6 [9.9-11.5]	11.5 [9.9-13.4]
Gn001 (0.01 mM)	93.5%	16.7 [14.8-18.9]	11.5 [10.4-12.6]	11.2 [9.5-13.2]
Gn005 (0.05 mM)	93.2%	12.9 [11.1-15.1]	11.0 [10.3-11.7]	9.3 [7.4-11.5]
Gn01 (0.1 mM)	94.3%	13.2 [11.4-15.2]	Not measured	8.8 [7.6-10.2]
Gn1 (1 mM)	1.2%*	14.9 [12.7-17.5]	10.8 [10.3-11.3]	7.9 [6.7-9.4]
BAPN (50 uM)	Not measured	19.6 [16.8-23.0]	10.5 [9.5-11.5]	14.0 [12.3-15.9]

As we showed in study I, inhbition of cell contraction did not affect the relaxation phase indicating the relaxation phase depends on the matrix properties. Hence, we saught the measurements from the test to failure experiments, that also provide information about matrix properties would have a similar trend. Nevetherless, genipin treatment did not reveal alterations at peak force or tensile strain while GN005, GN01 and GN1 caused a significant reduction in the re-tension (Table 2, Fig.18). Besides, a dramatic decrease occurred with BAPN treatment compared with the DMSO controls, indicating that the constructs become more elastic. However, no difference was observed in BAPN treatment at cell-matrix mechanics, relative to the controls (Fig.18). However, cell-matrix mechanical testings were performed in low strains (approximately 5%), in contrast to failure testing that corresponds to higher strains. Moreover, the magnitude of the forces that the cells can reach is approximately 5mN whereas the failure strength is approximately 150 mN [198]. Therefore, the difference in strain magnitude and the outcome force might explain the absence of correlation between the two testing methods. The toe region of the stress-strain plot corresponds to lower strain levels, and we thought that it would follow a similar pattern with the stress-relaxation phase but the comparison between the two phases did not reveal any correlation. Thus, these data indicate that the sensitivity of the force monitor allows changes to be detected that cannot be seen on a larger scale.

Regardless of the treatment, DNA, collagen and GAG content did not affect, indicating the changes in the mechanics occurred mainly by cross-linking of the current matrix, without the addition of new components (Table 1). Herchenhan et al., which showed the effect of BAPN treatment on fibrils size but in their number, caused mechanically weaker constructs [52]. This indicates the quality rather than the amount of the matrix was responsible for the mechanical defects, corroborating our data.

Table 2 Constructs mechanics, assessed by stretch to failure testing. Geometric mean [geometric SE]. Significant differences from DMSO control are marked with asterisks (*) ($p<0.05$).

	Peak stress (MPa)	**Peak modulus (MPa)**	**Strain (%)**	**Peak force (N)**	**Stiffness (N/mm)**
DMSO control	1.73 [1.63-1.83]	12.5 [11.8-13.2]	21 [21-22]	0.13 [0.12-0.15]	99 [91-107]
Gn001 (0.01 mM)	1.61 [1.46-1.77]	11.9 [10.7-13.2]	23 [22-25]	0.16 [0.14-0.19]	122 [108-137]
Gn005 (0.05 mM)	1.53 [1.42-1.65]	12.1 [11.2-12.9]	22 [21-23]	0.15 [0.14-0.18]	115 [100-132]
Gn01 (0.1 mM)	2.02 [1.80-2.26]	16.6 [14.8-18.7]	21 [20-21]	0.17 [0.15-0.20]	150 [135-166]*
Gn1 (1 mM)	2.68 [2.29-3.14]*	24.3 [21.4-27.7]*	21 [20-21]	0.21 [0.19-0.24]	194 [173-217]*
BAPN (50 uM)	0.49 [0.44-0.55]*	3.8 [3.5-4.3]*	16 [15-17]*	0.06 [0.04-0.09]*	30 [26-34]*

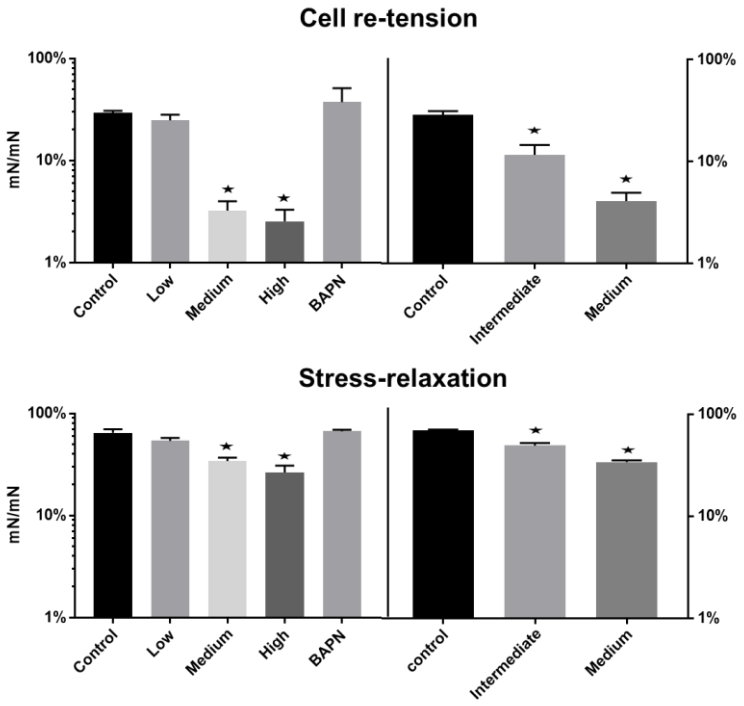

Figure 18 Cell-matrix testing of the constructs measured with the force monitor. The line separates the results from the two different batches of experiments (batch1 = left, batch2 = right). Geometric mean [geometric SE]. Significant differences from DMSO control are marked with asterisks (*) ($p<0.05$).

One point worth noting is the fact that re-tension phase is not solely cell-generated, but it refers to the outcome force that the cells exert on the matrix. So far, studies have shown disparate results with some that suggest that cell-generated forces are dependent on the matrix rigidity [214, 220] while others propose that the cellular forces are standard per cell type and autonomous from their surroundings properties [221]. Herein, the cell-matrix mechanical tests showed that the cell re-tension was lower in stiffer constructs and followed a dose-dependent manner; likewise, similar changes occurred in the stress-relaxation phase. More specifically, GN005, GN01 and GN1 concentrations showed a significant reduction in re-tension compared with the DMSO controls and the same concentrations caused a decrease in the relaxation phase (Fig.18). Interestingly, even though the stiffness of the constructs was reduced radically by BAPN treatment, we did not detect any difference at the cell-matrix tests. Hence, a decrease of the re-tension in more rigid substrates, such as in GN001 concentration, could be explained if we assume that the cells tend to balance the increased stiffness by generating higher forces, although, the overall force drops due to stress shielding and that is why the outcome force of GN001 is the same as the controls. Likewise, in less rigid constructs (BAPN treated) the cells might pull more on the matrix to compensate for the lower stiffness, but the measured force

reaches the same level as in GN001 and controls. In GN005, GN01 and GN1 concentrations it is more likely that the cellular forces reached a plateau value and the stiffer constructs showed decreased re-tension. The limitation of the cellular forces has been shown in single cell studies and can support the data mentioned above. On the other hand, the cell-generated forces could have remained the same (independently of the matrix stiffness), but the matrix itself is stiffer, so the outcome force is lower. However, this assumption cannot support the fact we did not observe any difference in cell-matrix mechanics of the BAPN treated constructs vs the controls.

The various types of scaffolds (collagen-based or GAG-sponges) might be the reason for the variation of the results across the different studies regarding force transmission and matrix compliance in the tendon. It has been shown that the type of the scaffold can affect the cell behaviour and more specifically, it has been suggested, fibrin to be the sufficient scaffold for the fabrication of engineered tendon constructs. This is supported by the fact that fibrin is degradable so the fibroblasts can remodel the matrix by creating their "own" collagenous-rich matrix [129, 130]. Thus, the newly formed ECM consists of all the components that required for tendon development whereas in synthetic scaffolds the variety of the components that exist in the system is limited to the chosen materials.

As mentioned earlier, the cells were dead at GN1 concentration although the re-tension did not drop to zero. The presence of tension after the treatment can be explained since tensional homoeostasis of this system is part of the cells and part of the matrix exclusively (explained in the introduction). As a result, the constructs exist in a pre-tensed state, and when the tension is decreased, the passive components of the cells in combination with the viscoelastic properties of the constructs could generate some level of force. Besides, the absolute forces in Gn1 concentration treated constructs were meagre compared with the controls. Accordingly, we considered separating the re-tension into two-phases in an attempt to distinguish the forces from the passive components and the active cell contraction, but it was not possible from our set of data since it seems the two possible phases are overlapping each other.

The limitation of this study to measure cellular forces of a complex of cells and not being able to distinguish single cell forces simultaneously represents the strength of this study. Similar to native tissues, cells in tendon constructs are linked to other cells and the matrix, so the whole tissue function depends on cell-cell and cell-matrix interactions. Therefore, in this project, the use of the force monitor, which can quantify the overall outcome force from tendon constructs, provides with results that closely represent natural conditions.

Taken together, after manipulating cells function by inhibiting the cell contraction in the study I and by altering matrix properties with the induction and inhibition of cross-link formation in study II, the next step was to perform treatment with growth factors. An exciting aspect of the supplementation of growth factors is that they can affect molecular pathways relevant to force transmission independently of mechanical stimuli.

Study III

Growth factors and particularly IGF-1 and TGF-ß, have been shown to have an impact on a variety of effects regarding tendons, spanning from induced matrix formation to increased cell proliferation or induced differentiation [176, 178, 222]. However, there are not many studies that they have investigated the potential effect of the proteins mentioned above in force transmission within tendons.

Tendon constructs, as mentioned earlier, demonstrate accurately embryonic tendon structure. Hence, elucidating the impact of growth factors in the constuct model will provide usefull information regarding their role in native tendons. Further, the role of TGF-ß in fibroblast differentiation to myofibroblasts and consequently the effects on cell contraction is well established. In the present study, we used this growth factor to confirm the effect on cell contractility as evaluated by cell-matrix mechanical tests and investigate whether the IGF-1 regulates force transmission in our system.

The growth factors were supplemented for a week, aiming to evaluate the long-term effect in contrast to previous studies that used shorter periods of treatment [188, 190, 223]. Then, the tendon constructs were subjected to a dynamic mechanical protocol at the force monitor as in the previous studies. It is important to be noted that in this study the results will be presented both in absolute and relative values as described in the methods. The reason behind presenting both types of data is that we expected the growth factors to affect the matrix and cell behaviour (at least with TGF-ß).

Table 3 Functional tests of the constructs measured with the force monitor. Geometric mean [geometric SE]. Significant differences showed with asterisks (*)

Absolute Values	**Re-tension (mN)**	**Relaxation (mN)**
Control (untreated)	0.28 [0.24-0.32]	5.15 [4.66-5.70]
IGF-1	0.32 [0.29-0.35]	6.30 [5.77-6.80]
TGF-b	1.15 [0.97-1.35]*	10.16 [8.88-11.63]*
Normilized Values (%)	**Re-tension (mN/mN)**	**Relaxation (mN/mN)**
Control (untreated)	7.6% [5.6-10.1%]	58.5% [53.5-63.9%]
IGF-1	8.3% [7.4-9.3%]	63.8% [61.5-66.1%]
TGF-b	11.0% [9.3-13%]	52.4% [50.9-53.9%]

The results by calculating the absolute values, showed a significant increase in the re-tension and relaxation phase of TGF-ß treated constructs, compared with the untreated controls, but no differences were shown with IGF-1 treatment (Table 3). Interestingly, calculations of the normalized values for the two phases, depleted the differences that were mentioned above, and all the groups presented similar values (Table 3).

Study III, is an ongoing study and here are presented the results that obtained from the force monitor as to our perspective, they look promising to pursue further research on the direct role of growth factors relative to force transmission.

CONCLUSIONS

The primary goal of this study was to quantify cell-matrix interactions in human tendon constructs.

In the first study, it was shown cells have the ability to produce forces during the relaxation of the constructs, which serves to maintain tensional homeostasis. Maturation of the constructs reduced the cell-generated forces while the number of the cells remain unaffected, which indicates a stiffer matrix could be a critical regulator for cell contraction. Disruption of GAGs did not cause any difference on re-tension or relaxation phase.

In the second study, genipin, a natural cross-linker affected the mechanics of the tendon constructs but not their compositional profile as measured by the cell number, and GAG and collagen content. Aside from the GN1 concentration, which induced cell death, the peak force and strain remain unaffected by the genipin treatment while BAPN caused a dramatic reduction, compared with the DMSO controls. Stiffness increased by GN01 and GN1 concentrations but decreased with BAPN treatment relative to DMSO controls. GN1 also increased peak stress and peak modulus, in contrast to BAPN treatment, which caused a significant reduction. The re-tension was reduced with GN005, GN01 and GN001 concentrations but no changes occurred with supplementation of BAPN compared with DMSO controls. The same pattern was seen in the relaxation phase.

In the third study, TGF-b increased the absolute values of the re-tension and relaxation phase compared with untreated controls. However, the effect of treatment disappeared when the re-tension normalised to the amount of unloading and the relaxation to the amount of loading (normilised values as presented to the previous studies). The treatment with IGF-1 did not affect the cell-matrix mechanics compared with the untreated controls.

Perspectives

This project show that mechanical forces are important for cellular behaviour and consequently for whole tissue function. Thereby, interventions directly targeting the regulation of cell forces could be a key element in preventing pathological conditions related to connective tissues. The force monitor used in this project could be an advantageous tool to probe the role of mechanical forces by expanding the application of protocols for extended periods. The force monitor could be used as a bioreactor by subjecting the constructs to constant loading from the start of their formation and assessing the forces during development.

Cross-linking induces tissue stiffness, but it can be a double-edged sword as it can cause dysfunctional tissues although it is also important for their structural stabilization. The information that revealed in study II could be useful to understand which level of cross-links can be beneficial for tissue function. Targeting specific types of cross-links would provide great insight for the development of targeting treatments against pathologies, such as irregular matrix stiffness that follows tendon injuries.

The study of the impact of growth factors on tendon function would provide valuable information for the clinicians. IGF-1 has been used as a potential treatment for tendon injuries and pathological conditions, at animal and human models. It has been shown that IGF-1 affects fibroblast collagen synthesis and proliferation, but its contribution to mechanical properties of the cells is unknown. The fact that IGF-I might affect both cell and matrix functions, such as TGF-b, complicates the experimental setup. Thus, focusing more on the activity of IGF-1 isoforms could narrow the range of actions and simplify the studies.

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Short communication

Cellular homeostatic tension and force transmission measured in human engineered tendon

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ABSTRACT

Tendons transmit contractile muscular force to bone to produce movement, and it is believed cells can generate endogenous forces on the extracellular matrix to maintain tissue homeostasis. However, little is known about the direct mechanical measurement of cell-matrix interaction in cell-generated human tendon constructs. In this study we examined if cell-generated force could be detected and quantified in engineered human tendon constructs, and if glycosaminoglycans (GAGs) contribute to tendon force transmission. Following de-tensioning of the tendon constructs it was possible to quantify an endogenous re-tensioning. Further, it was demonstrated that the endogenous re-tensioning response was markedly blunted after interference with the cytoskeleton (inhibiting non-muscle myosin-dependent cell contraction by blebbistatin), which confirmed that re-tensioning was cell generated. When the constructs were elongated and held at a constant length a stress relaxation response was quantified, and removing 27% of the GAG content of tendon did not alter the relaxation behavior, which indicates that GAGs do not play a meaningful role in force transmission within this system.

1. Introduction

The chief function of tendon is to transmit contractile muscular force to bone to produce movement. It has been shown that placing sizeable repetitive loads on the tendon may influence numerous cell responses (Spiesz et al., 2015), tissue composition (Langberg et al., 1999) and mechanical properties of the tendon (Hansen et al., 2003), which indicates that tendon tissue is mechanoresponsive although the precise pathway is unknown (Harris et al., 1980; Wang et al., 2012). This conversion of a mechanical stimulus into an electrochemical action and intracellular biochemical response demonstrate that tendons are capable of mechanotransduction. While the tendon can impart forces on the cell, it is also possible for cell to generate endogenous forces on the extracellular matrix (ECM) (Eastwood et al., 1994; Kolodney and Wysolmerski, 1992), which allows for a fine-tuned dynamic interaction between the cell and the ECM to maintain tissue homeostasis (Freedman et al., 2015; Joshi et al., 1985).

The ability for cells to exert forces on the ECM has previously typically been quantified using a polymeric collagen lattice to show that cells can control homeostatic tension when measured over hours to days (Delvoye et al., 1991; Eastwood et al., 1994). Sponge gels with defined properties have also been used as scaffolds to evaluate cell responses (Brown et al., 1998; Delvoye et al., 1991; Kolodney and Wysolmerski, 1992). However, cell-generated scaffolds comprise a mixture of ECM components that more likely resemble that of the in vivo situation compared to the aforementioned models. Such cell-derived tendon construct have therefore been developed using both animal (Kapacee et al., 2008) and human cells (Bayer et al., 2010), with similar composition (Kapacee et al., 2008) and mechanical properties (Herchenhan et al., 2013) to embryonic tendon tissue (Kalson et al., 2010). Tension appears critical for the formation and development of these constructs, which underscores the importance of mechanotransduction (Bayer et al., 2014; Kapacee et al., 2008). However, direct mechanical measurement of cell-matrix interac-

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tion in cell-generated human tendon constructs has never been reported before.

The principle force transmitting structure of the ECM in mature tendon tissue is the fibril (Cribb and Scott, 1995; Parry et al., 1978). It has been suggested that force is transferred between adjacent fibrils via proteoglycans and their associated glycosaminoglycan (GAG) chains, including chondroitin- and dermatan-sulfate (Ryan et al., 2015; Scott and Thomlinson, 1998). However, removing this complex in tendon (Svensson et al., 2011) and ligament (Provenzano and Vanderby, 2006) does not appreciably affect the mechanical properties of the tissue. Moreover, it was recently shown that fibrils appear to be continuous in mature tendon tissue, suggesting that the importance of lateral force transmission between fibrils may be negligible (Svensson et al., 2017). However, in the early stages of developing tendon tissue the fibrils are discontinuous (Birk et al., 1995), and the relative amount of non fibrillar matrix is larger. Therefore it is possible that a mechanism for lateral force transmission is necessary, but this has never been investigated. Hence, the purpose of this study was two-fold; (1) to examine if cell generated force could be detected and quantified in engineered human tendon constructs, and (2) to assess if GAGs contribute to transmission of force in this human cell generated tendon tissue.

2. Materials and methods

2.1. Tendon construct preparation

Cells were obtained as previously described (Bayer et al., 2010) (see supplement for details). In brief, tendon fibroblasts were isolated from semitendinosus and gracilis tendon from patients that underwent reconstructive anterior cruciate ligament (ACL) surgery. All the cell lines from different donors were obtained from the same source. Informed consent was obtained from all tissue donors in accordance with ethical approval [H-3-2010-070]. Cells were isolated using collagenase type II and seeded into culture flasks (DMEM/F12, 10% FPB). Cells between passages 2 and 6 were used for experiments.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jbiomech.2018.07.032.

Tendon constructs from human tendon fibroblasts were made as previously described (Bayer et al., 2010) (see supplement for details). Briefly, each well of a six well plate was coated with Sylgard (DoW Chemicals). Two loop shaped silk sutures were pinned 10 mm apart to the coated plates and sterilized in 70% ethanol. Fibroblasts were suspended in a mix of fibrinogen, aprotinin and thrombin (all from Sigma Aldrich) to a final concentration of 0.2 million cells per well. The 3D gels were incubated in construct medium (DMEM/F12, 10% FBS, 0.2 mM L-ascorbic acid 2-phosphate, 0.05 mM L-proline), which was replaced every other day. Approximately 2 weeks after seeding the constructs were fully formed (the matrix contracted to a 10 mm long narrow linear structure between the sutures).

2.2. Mechanical evaluation

A custom made system was used to measure forces in cell derived human tendon constructs. Briefly, the system consisted of force transducers (402A, Aurora Scientific, CA), stepper motors with a motor controller (Astrosyn, Y129-5, PC-control ltd., UK), culture wells and a PC data collection system (Microlink 751, Biodata ltd., UK). Strain was applied by the stepper motors via a threaded rod with a step resolution of 2.25 μm. Deformation was applied at a rate of 56 μm/s and force data sampled at 1 Hz. Constructs were attached by their silk suture loops to the motor and force transducer via stainless steel hooks (Fig. 1A).

2.3. Mechanical testing protocol

Mechanical tests were performed in an incubator (37 °C and 5% CO_2). The Sylgaard coating underneath the construct was cut into a strip and transferred together with the pinned construct to the force monitoring system to avoid altering the original length and tension. Thereafter, the constructs were relaxed by 0.225 mm to confirm the presence of tension. If tension was present the length was returned to the original position. If there was no tension the constructs were considered to have become slightly slack during transfer and were stretched in 0.225 mm steps up to 0.675 mm to re-establish tension. This position was defined as the baseline length and subsequently the system was allowed to stabilize for 1 h. The tendon constructs were subjected to a protocol that consisted of three cycles, with each cycle consisting of 0.675 mm of unloading (reducing length), 300 s of rest period followed by 0.675 mm of reloading (returning to the initial length) and another 300 s of rest (see Fig. 1B).

2.4. Construct treatment

Tendon constructs were tested at either 3, 4 or 5 weeks after seeding (based on 5 cell lines). The tendon constructs underwent three cyclic stretches in normal medium (DMEM). Immediately after, the normal medium was replaced with treatment media (DMEM plus reagent) followed by an incubation period of 30 min and three subsequent cycles. Blebbistatin (B0560, SIGMA) (17 μM) was used for inhibiting non-muscle myosin-dependent cell contraction (n = 24: week 3, n = 8, week 4, n = 7, week 5, n = 9), and chondroitinase ABC (C3667, SIGMA) (0.07U/ml) was used to digest glycosaminoglycans (n = 23: week 3, n = 8, week 4, n = 7, week 5, n = 8). Control samples (n = 10 from 2 cell lines: week 3, n = 4, week 5, n = 6) that had normal medium replaced with fresh normal medium were also mechanically tested in the same manner to control for the effect of time (untreated controls).

2.5. Glycosaminoglycan determination

Sulfated GAG content was determined in the mechanically tested constructs using a 1,9-dimethylmethylene blue (DMMB) assay slightly modified from (Hoemann, 2004) and was expressed as μg per construct (see details in supplement).

2.6. Statistics and data reduction

The force values were determined at four different points in each cycle (see Fig. 1B): A) at the end of relaxation (300 s). B) Immediately following unloading. C) At the end of re-tension (300 s). D) Immediately following reloading. Average values of the 3 cycles were used for each sample before and after treatment. Re-tension was calculated as: (C – B)/(A – B), which corresponds to the relative amount of re-tension. Stress relaxation was calculated as: (D – A)/(D – C), which also corresponds to the relative amount of relaxation. Re-tension and relaxation are expressed as a percentage and the treatment effect is the absolute difference between the pre and post percentage values.

The effect of treatment and construct maturity on mechanical behavior was examined with 2-way ANOVA's with post hoc Sidak's multiple comparison tests (GraphPad Software, La Jolla California USA). Unpaired t-tests were used to compare GAG content and change in re-tension between chondroitinase and blebbistatin treated constructs and to compare the baseline mechanics (before treatment) between week 3 and 5. The primary comparison was

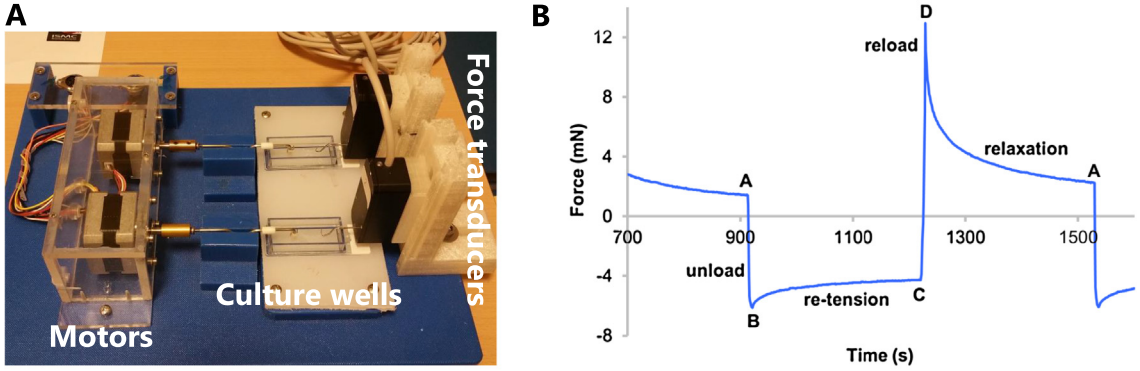

Fig. 1. Mechanical test system. (A) The force monitor system which consists of two stepper motors, culture wells and force transducers. (B) Example of force vs. time data for one cycle measurement. At point 'A' the construct is unloaded to point 'B' where the force is allowed to re-tension for 300 s. At point 'C' the construct is stretched back to its initial position (point 'D') with the length remaining constant for 300 s, relaxing back to point 'A' where another cycle starts.

between blebbistatin and chondroitinase treated samples but as an additional control, unpaired t-tests were also made against the untreated controls. Results are reported as mean ± SE.

3. Results

In untreated controls re-tension decreased over time (−3.5 ± 1.2%, n = 10, $p < 0.05$) but relaxation was unaffected (−0.8 ± 0.5%, n = 10, $p = 0.14$) indicating little effect of time. Blebbistatin treatment significantly reduced re-tension compared to pre-treatment (Fig. 2A, $p < 0.005$, main effect) but had no effect on relaxation (Fig. 2B, $p = 0.97$, main effect). Blebbistatin treatment did not affect GAG content (7.17 ± 0.35 μg, n = 29) compared to the untreated controls (6.84 ± 0.63 μg, n = 7). Chondroitinase treatment reduced the total GAG content of the tendon constructs (5.24 ± 0.22 μg, n = 27) by 27% compared to blebbistatin treated constructs ($p < 0.0001$). Chondroitinase did not affect re-tension (Fig. 3A, $p = 0.87$, main effect) or relaxation ($p = 0.74$, main effect) of the constructs (Fig. 3).

The change in re-tension with blebbistatin (-10.6 ± 1.6%, n = 24) was significantly different from the change with chondroitinase treatment (−0.8 ± 1.5%, n = 23, $p < 0.0001$) and from the change in untreated controls ($p < 0.05$). While the effect of treatment did not differ between weeks there was a baseline reduction in re-tension from week 3 (29.4 ± 3.6%, n = 16) to 5 (18.0 ± 3.4%, n = 17, $p = 0.029$). There was no significant baseline difference in relaxation from week 3 (66.9 ± 2.8%, n = 16) to 5 (62.8 ± 2.4%, n = 17, $p = 0.165$).

4. Discussion

In the present study we sought to examine if cell-generated force could be detected and quantified in engineered human tendon constructs. When the constructs were unloaded an endogenous force was generated that could be quantified (see Fig. 1A), and following inhibition of cell contractility by blebbistatin (an inhibitor of non-muscle myosin) (Cai et al., 2006; Even-Ram et al., 2007; Kalson et al., 2013), the re-tension was dramatically reduced, which indicates that the endogenous force is cell-generated. Complete loss of re-tension did not occur, which may relate to the short treatment time.

The fact that the cells have the ability to generate internal tissue tension and thereby maintain homeostasis is well known and has been studied in different models. However, to the best of our knowledge, this is the first report of cell-generated tissue tension in a 3D human tendon scaffold. The advantage of this model is that

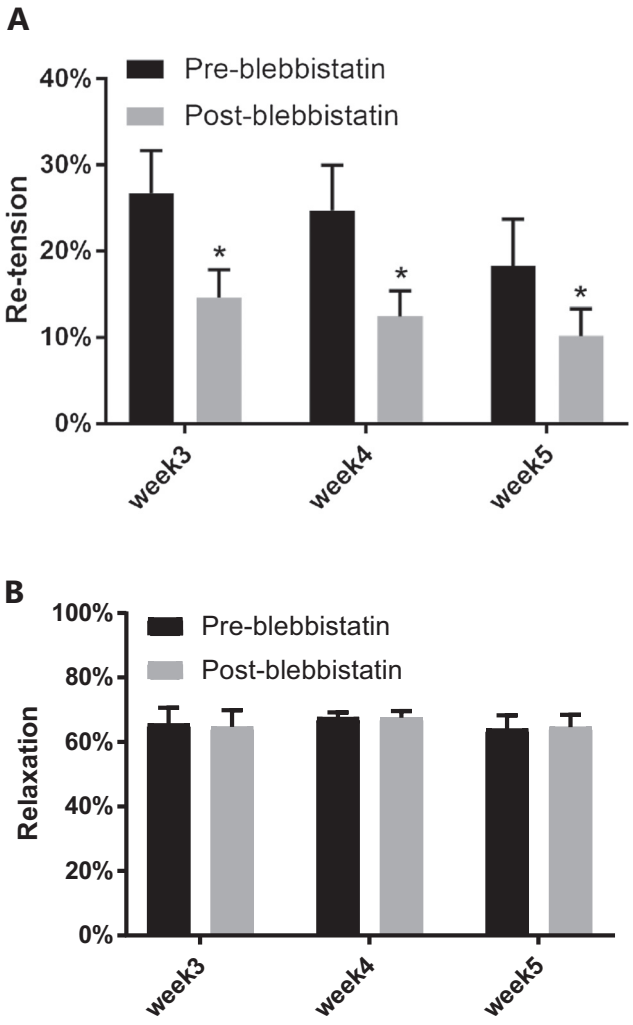

Fig. 2. Blebbistatin treatment for cell contraction inhibition. (A) The re-tension dropped significantly after blebbistatin treatment ($p < 0.005$). B) The relaxation phase was unaffected by cell contraction inhibition. (n = 24 from 5 cell lines: week 3, n = 8, week 4, n = 7, week 5, n = 9).

the cells produce and organize their own collagen matrix (Herchenhan et al., 2013; Kalson et al., 2010).

The magnitude of re-tensioning was less in week 5 compared to week 3 constructs. This could be the result of a lower cell number, but an assessment of nuclei area fraction did not reveal a difference

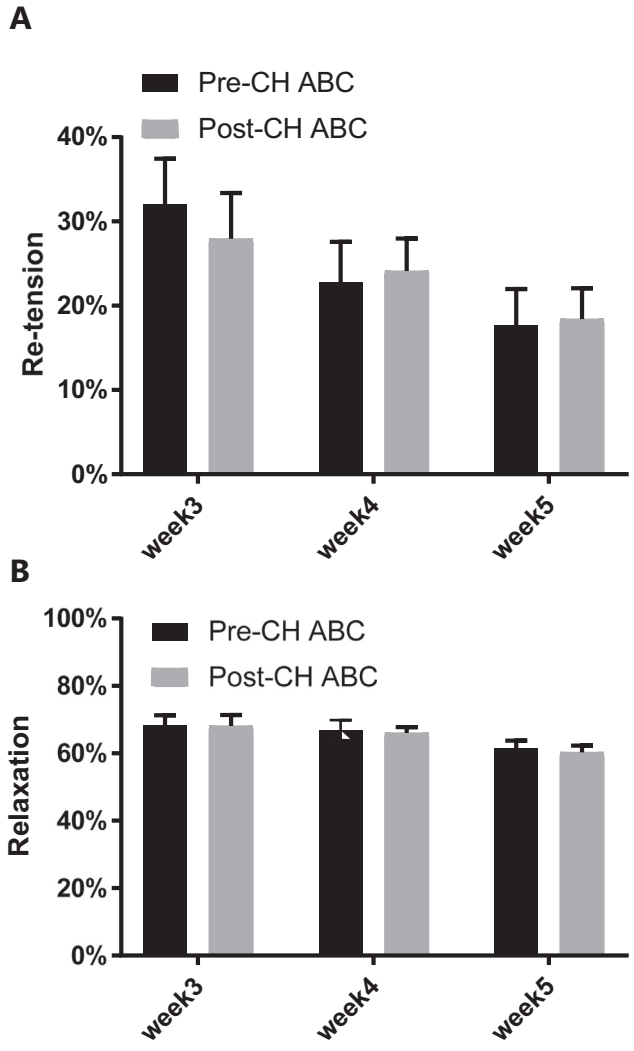

Fig. 3. Chondroitinase ABC (CH ABC) treatment for glycosaminoglycan digestion. (A) The re-tension did not change after CH ABC treatment. (B) The relaxation also remain unaffected after the treatment. (n = 23 from 5 cell lines: week 3, n = 8, week 4, n = 7, week 5, n = 8).

between the two weeks (see supplement). However, others have shown that cell number declines over time in similar constructs (Delvoye et al., 1991; Kalson et al., 2010). On the other hand, it has been shown that the stiffness of the construct increases dramatically from week 2 to 5 (Herchenhan et al., 2013), and it is possible that the increased collagen stiffness itself reduces the magnitude of the cell contraction (Karamichos et al., 2007). The relative contribution of a potential decline in cell number or augmented construct properties cannot be ascertained in the present study.

While the unloading allowed for an evaluation of the cell-generated response, the relaxation phase represents the response of the extracellular component of the matrix. As mentioned earlier, GAGs could influence force transmission in the immature constructs. However, in the present study we show that a 27% reduction of GAG content did not impact the relaxation phase of the 3–5 week constructs, which implies that this is not a critical force transmission pathway in these tissues.

In conclusion, these data show the presence of cell-generated tension within the human tendon constructs. This observation was supported by a 38% decline in cell re-tension after introducing a cell contraction inhibitor. In addition, a 27% reduction in GAG content did not seem to affect force transmission in this system. The ability of our system to apply, detect and quantify the generated forces in real time provides new insight to the field of tendon biomechanics. The force monitor is a useful tool to investigate tissue development and regeneration by evaluating cell-matrix interactions.

5. Conflict of interest statement

All authors declare no conflicting interests.

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Supplementary material

1 Materials and Methods

1.1. Tendon construct preparation

Tendon fibroblasts were isolated from human semitendinosus and gracilis tendon from patients that underwent reconstructive anterior cruciate ligament (ACL) surgery. Informed consent was obtained from all tissue donors in accordance with ethical approval [H-3-2010-070]. Under aseptic conditions the tissue was minced into pieces of ~ 2 mm^3 and digested overnight in DMEM/F12 (Gibco, Invitrogen) supplemented with 0.1% collagenase type II (Worthington) and 20% fetal bovine serum (FBS) (Gibco, Invitrogen). Following repeated washes in culture medium (DMEM/F12, 10% FBS) the cells were seeded into flasks and cultured until the next passage. Cells between passages 2 and 6 were used for experiments.

Tendon constructs from human tendon fibroblasts were made as previously described (Bayer et al., 2010). Briefly, each well of a six well plate was coated with Sylgard (DoW Chemicals) and incubated at 55 °C for 48 h. Two loop shaped silk sutures were pinned to the coated plates with minutien insect pins (0.1 mm diameter) (Fine Science Tools GmbH), 1 cm apart. Plates were sterilized in 70 % ethanol for 45 min. Human fibroblasts were suspended in a mix of human fibrinogen (4 mg/ml)(F3879), 1U thrombin (T6884) and bovine aprotinin (A3428)(10 µg/ml) (all from Sigma Aldrich) to a final concentration of 0.2 million cells per 815 µL and rapidly spread over the complete surface of the wells. The plate was left for 30 min at 37 °C and then 3 ml of construct medium (DMEM/F12 plus 10% FBS supplemented with 0.2 mM L-ascorbic acid 2-phosphate and 0.05 mM L-proline) was added to each well. Construct medium was replaced every other day. Constructs were considered to be fully formed when the matrix had contracted to a narrow linear structure between the sutures (2 weeks after seeding).

1.2. Glycosaminoglycan determination

Sulfated GAG content was determined in the mechanically tested constructs using a 1,9 dimethylmethylene blue (DMMB) assay slightly modified from (Hoemann, 2004) and was expressed as µg per construct. Standards of chondroitin sulfate (C3788, SIGMA) in water were used. Constructs (chondroitinase and blebbistatin treated) and 10 µL of standards were digested in 100 µL of papain solution (0.125 mg/mL papain (P3125, SIGMA), 10 mM L-cysteine, 100 mM sodium phosphate, 10 mM EDTA, pH 6.5 at 60 °C for 2

hours with vortexing every 30 min. Triplicates of 10 µL digested sample were transferred into an untreated 96-well plate and 190 µL of DMMB reagent (16 µg/mL DMMB (341088, SIGMA), 40 mM glycine, 40 mM sodium chloride, 9.5 mM HCl, pH 3.0) was added to each well. The absorbance at 540 nm minus 590 nm was used for quantification. Blebbistatin treated constructs were considered to be controls.

2 Microscopy

2.1 Cell quantification

Tendon constructs (n=4) were embedded (TissueTek, Sakura Finetek) and snap frozen in isopentane while pinned, at week 3 and 5 after formation. Sections (20 µm) were cut and stained with DAPI to visualize the nuclei, and three images (425 by 320 µm) per section were taken (one for each end and one of the middle part). Nuclei appeared fragmented (Fig.S1) prohibiting consistent counting, so instead the area fraction of nuclei was determined using color thresholding in FIJI (Schindelin et al., 2012) as a measure of cell density. Area of nuclei fraction in weeks 3 and 5 was evaluated with an unpaired t-test.

There was no significant difference in the area fraction of nuclei from week 3 (38 ± 3%) to week 5 (34 ± 4%, p=0.088).

2.2 Cytoskeletal structure

Fluorescence microscopy images were obtained using laser confocal microscope (LSM 700, Axio Imager 2, Zeiss). A control and a blebbistatin treated construct were fixed while pinned with 10 % formalin for 25 min. Subsequently they were stained with Hoechst (nuclei) and phalloidin (F-actin) to visualize the cytoskeleton.

For blebbistatin treatment confocal microscope images showed a disruption in actin cytoskeleton with the presence of aggregates at the treated (Fig.S2.A) but not at the untreated controls (Fig.S2.B).

3 Supplementary Figures

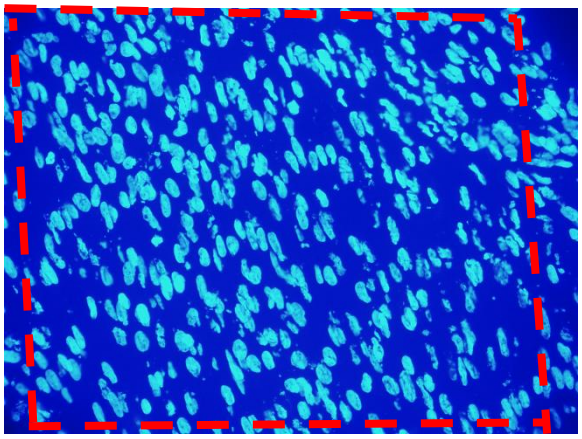

Fig.S1. Representative fluorescence image of human tendon construct stained with nuclear counterstain (DAPI). Red outlined section indicates the area analyzed for nuclei area fraction to exclude the edges of the construct.

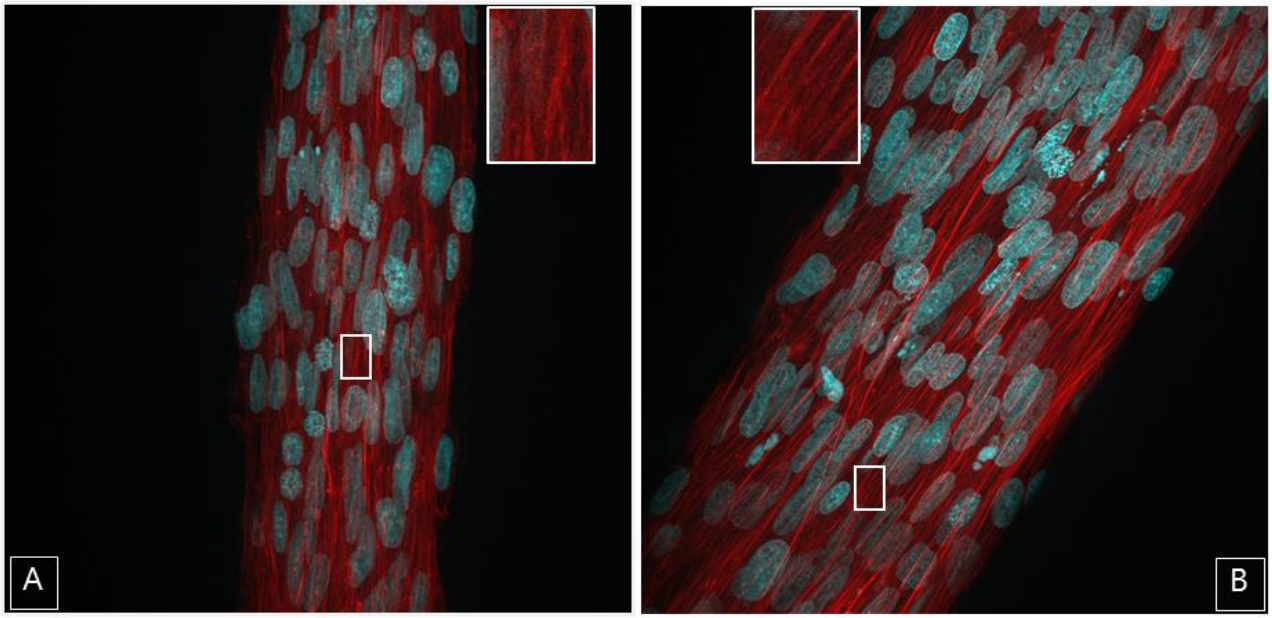

Fig.S2. Representative confocal images of human tendon constructs whole mount stained with phalloidin (F-actin, red) and counterstained with Hoechst (nuclei, blue). (A) Blebbistatin treated construct and (B) untreated control construct. Inset shows phalloidin stained actin stress fiber organization is disrupted by blebbistatin treatment.

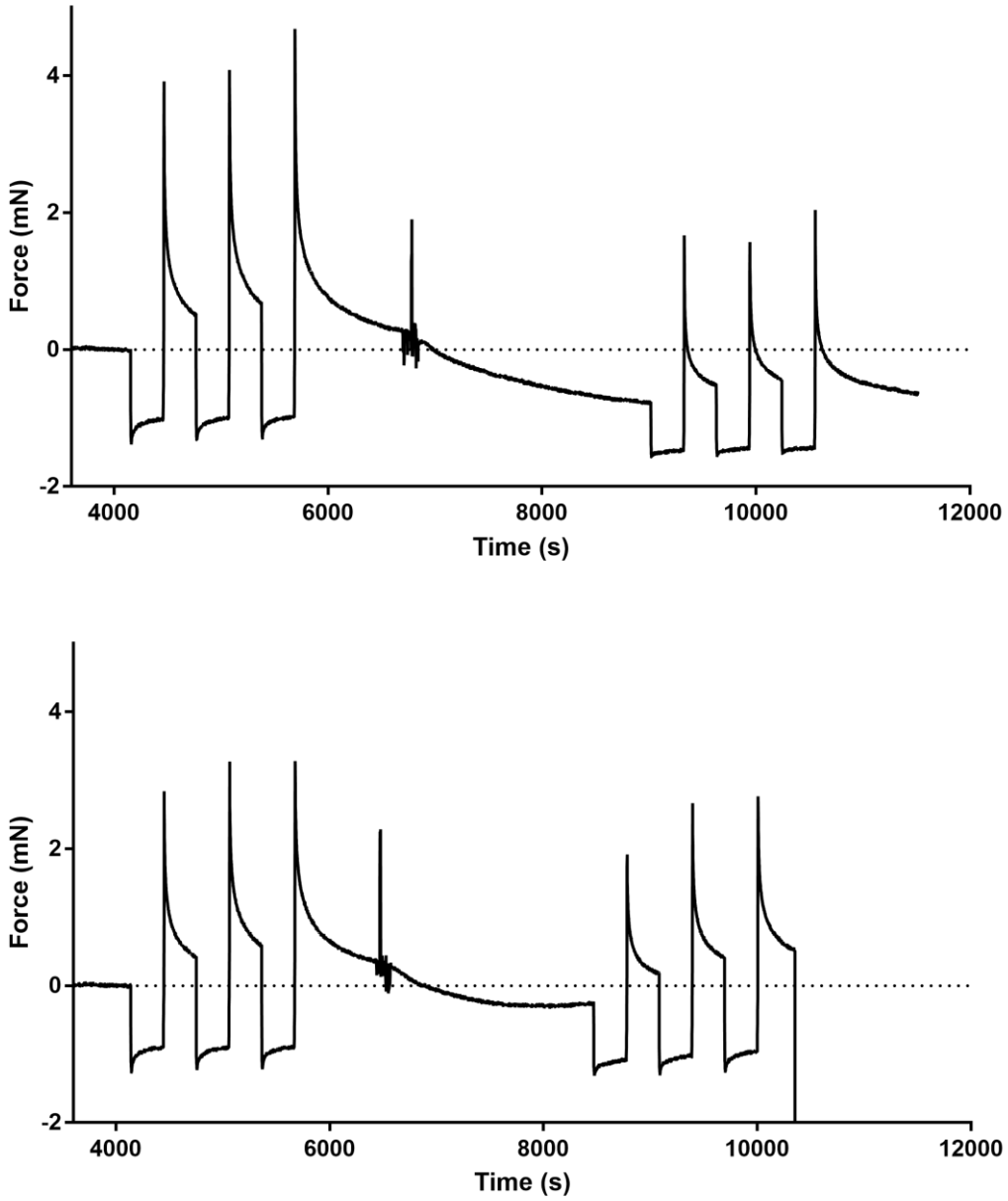

Fig. S3. Representative graphs of construct mechanical evaluation. One hour after mounting the construct to the well, a 3-cycle cyclic protocol was performed. Then the medium was replaced with the treatment medium followed by a 30 min incubation time. Finally three post-treatment cycles were performed. Top: Blebbistatin treatment. The force dropped as it can be seen in the graph indicating loss of tension due to the treatment. Bottom: CH ABC treatment.

4 Supplementary Tables

Table S1. Table of absolute forces (mN) measured in the four different phases of the mechanical test (see Fig.1 in the main article). Median [inter quartile range].

Treatment	Time	Unload (A-B)	Re-tension (B-C)	Reload (C-D)	Relaxation (D-A)
Blebbistatin	Pre (n=24)	-2.16 [-4.34;-1.75]	0.43 [0.29;0.67]	5.49 [4.05;8.19]	-3.67 [-4.70;-2.83]
Blebbistatin	Post (n=24)	-1.17 [-2.63;-0.89]	0.09 [0.07;0.29]	3.41 [2.49;6.10]	-2.36 [-3.48;-1.59]
CH ABC	Pre (n=23)	-2.41 [-3.78;-1.75]	0.41 [0.31;1.03]	6.82 [4.18;9.32]	-4.13 [-6.29;-2.56]
CH ABC	Post (n=23)	-2.02 [-3.27;-1.24]	0.32 [0.25;0.63]	4.47 [3.40;6.89]	-2.90 [-4.31;-2.10]
Control	Pre (n=10)	-4.37 [-6.92;-3.55]	1.69 [1.03;3.16]	8.93 [6.95;11.14]	-5.99 [-7.21;-4.66]
Control	Post (n=10)	-4.55 [-7.53;-3.53]	1.56 [1.05;2.64]	9.04 [7.54;12.03]	-6.06 [-6.85;-5.31]

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Manuscript II

(*In submission form*)

The influence of matrix rigidity on cell-mediated mechanical responses

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Abstract

It is well known that cells can generate endogenous forces onto the extracellular matrix, but to what extent the mechanical properties of the matrix influences the endogenous cellular forces remains is not well known. We therefore sought to quantify cell-matrix interactions and the influence of matrix rigidity by inducing cross-links using different concentrations of genipin (Gn001=0.01, Gn005=0.05, Gn01=0.1 or Gn1=1 mM) or by blocking cross-link formation using BAPN (50 uM), in human tendon constructs. The cell-matrix mechanics of tendon constructs were evaluated as cell generated tissue re-tensioning and as stress-relaxation response using a novel custom-made force monitor, which can apply and detect tensional forces. Genipin treatment had no influence upon the compositional profile of the construct (hydroxyproline, glucosaminoglycan and DNA content). Cell viability remained the same in genipin treated and controls except for the highest genipin concentration. The peak stress at mechanical resting of tendon constructs increased by 56% at Gn1 concentration whereas it decreased by 72% with BAPN treatment compared to controls. The stiffness increased by 51% and 96% at Gn01 and Gn1 concentrations, respectively, whereas it decreased by 70% in BAPN treated constructs relative to the controls. The endogenous re-tension phase was significantly decreased with rising genipin concentrations by 61% (Gn005), 89% (Gn01) and 91% (Gn1) compared to controls. These data show that human tendon fibroblasts produce lower forces in a dose-dependent manner when the cell-generated matrix is stiffened by cross-linking, which support the notion of a interaction between the cells and matrix properties and thus a role for this interplay in mechanical homeostasis of the tissue.

Introduction

The cells that reside in the extracellular matrix (ECM) of connective tissues respond to mechanical stimuli and they establish the foundation of the ECM during development [1]. The cells can also generate endogenous forces onto the ECM [2-5], and it is well established that ECM rigidity can affect cellular behaviour and functions [6, 7]. This constant fine-tuned cell-matrix homeostatic interaction ultimately determines the structural composition and mechanical properties of the ECM [2, 8, 9]. However, to what extend ECM rigidity can influence the mechanical responses of the cells by regulating the endogenous tensional homeostatic mechanism is not well understood. Studies using tissue engineered constructs have suggested that a stiffer matrix results in a reduction in cell-generated forces [10, 11], while others have shown that the forces are independent of matrix rigidity [12].

The collagen fibril is the fundamental building block and force-transmitting unit of the ECM and it consists of triple-helical collagen molecules that aggregate in a highly ordered staggered form [13, 14]. The mechanical integrity of the fibril is dependent on cross-links that increase matrix stability and regulate tissue function [15]. Immature enzymatic cross-links are the most abundant form in tissues during development, and their formation is based on lysyl oxidase (LOX) activity [15, 16]. In older tissues these cross-links can mature to more stable products in addition to the accumulation of non-enzymatic cross-links based on advanced glycation [17]. *In vivo* accumulation of collagen cross-links occur in pathological conditions like diabetes and cancer or in natural processes of maturation [18] and ageing [19], which stiffens the matrix. How this accumulation of cross-links/stiffened ECM will affects cell-generated forces remains mostly unknown. We hypothesized that a stiffening of the

matrix tissue would result in higher endogenous cell generated forces.

Different tools have been used to study cell-matrix interactions on a molecular, cellular and tissue scale in 3D environments [20, 21]. Most studies have focused on single cell forces and these may not necessarily be representative of native tissues environment, in which the exerted forces are the result of cell-cell and cell-matrix interactions. Furthermore, previous studies have often used synthetic or pre-formed collagen matrices that may not properly recapitulate the natural cell environment [22]. In this study, we used the engineered human tendon constructs as they provide a model that resemble *in vivo* embryonic tendons [23, 24] and allow for the manipulation of specific features, such as cross-linking. A variety of cross-linkers have been used although cytotoxicity is often a concern. Recent studies have suggested that a natural cross-linker, genipin, has the ability to augment the mechanical properties of engineered tissues with limited cytotoxicity [25, 26]. Hence, we sought to quantify cell-matrix interactions and the influence of matrix rigidity by inducing cross-links using genipin in tendon constructs. A recently developed custom-made force monitor that allows us to detect passive tensioning of the tissue as well as cell-driven re-tensioning was used to evaluate the cell forces exerted from the constructs in real time under culture conditions [27](Kadler PNAS).

Methods

Tendon construct preparation

Collection of cells and construct preparation was performed as previously described [24, 27]. In brief, tendon fibroblasts were isolated from human tendons (gracilis and semitendinosus). Informed

consent was obtained from all tissue donors [ethics approval H-3-2010-070]. Cells were isolated using collagenase type 2 (Worthington) and seeded into culture flasks (DMEM/F12, 10% FBS). Cells between passages two and six were used for experiments. In each well of a silicone coated six-well plate, two loop-shaped silk sutures were pinned 10 mm apart and sterilized in 70 % ethanol. Fibroblasts were suspended in a mix of fibrinogen, aprotinin and thrombin to a final concentration of 0.2 million cells per well. The 3D gels were incubated in construct medium (DMEM/F12, 10% FBS, 0.2 mM L-ascorbic acid 2-phosphate, 0.05 mM L-proline), which was replaced every other day. Approximately two weeks after seeding the linear constructs were fully formed.

Construct treatment

In order to induce cross-link formation, we supplemented the medium with genipin that (SIGMA) was dissolved in DMSO (25mg/ml) and the stock aliquoted and stored at -20 degrees. Genipin concentration of 0.01 (Gn001), 0.05 (Gn005), 0.1 (Gn01) and 1mM (Gn1) were prepared after serial dilution of the stock in construct medium and supplemented to the constructs from week 3 after seeding. Controls treated with DMSO, corresponding to the DMSO concentration that used to dissolve the Gn1 concentration (1mM). β-aminopropionitrile (BAPN), (dissolved in ddH20) treated constructs were used as a negative control. The constructs were incubated with freshly made genipin-supplemented medium for one week, and the medium was changed every other day. Genipin treated constructs had a blue hue to them, which is normal after genipin reacts with primary amines [25]. After one week of treatment, mechanical testing was performed (tested at week four after seeding). A second batch of experiments was performed using Gn01 and Gn005 concentrations plus controls treated with DMSO, which now corresponds to the concentration of DMSO of Gn005 concentration.

The Gn005 concentration was added after evaluating the first batch of experiments as we observed a marked difference in the mechanics between Gn001 and Gn01 concentration. This was performed in order to get more detailed dose-response information.

Cell viability

Cell viability was tested in 2D culture using XTT Cell Proliferation Kit (Sigma) according to manufacturer's instructions. Fibroblasts were plated at a concentration of $40*10^3$ per well of a 24 well plate. A standard curve of 20, 40 and $60*10^3$ cells was made. The cells were incubated for 24 h with the different concentrations of genipin. Controls had a DMSO concentration corresponding to that of GN1 treated samples. The next day, fresh solutions of XTT (SIGMA X4626) in DMEM and phenazine methosulfated (PMS) in ddH_2O were prepared (Stock PMS: 100mM and XTT: 1mg/ml) and mixed at a ratio of 5µl stock PMS to 5ml XTT stock. To avoid the blue color from the genipin treatment medium was replaced by 1 ml of pure DMEM per well.. Then 250µl of the XTT/PMS mix was added to each well and allowed to react for five hours. From each well 100 µl was transferred to 96-well plate in triplicates and absorbance measured at a wavelength of 450-500 nm [15].

Glycosaminoglycan determination and DNA content

Sulfated GAG and DNA content was determined in the mechanically tested constructs using a 1,9-dimethylmethylene blue (DMMB) assay slightly modified from Hoemann [28], and it was expressed as µg per construct (n=18). The procedure has described elsewhere [27].

Collagen content by hydroxylproline assay

Following mechanical testing two samples from each cell line (n=3) per treatment were used for hydroxylproline assay to determine collagen content as previously described [18]. In brief, samples were hydrolysed in 6 M HCl at 110°C overnight. Hydroxyproline was detected by chloramine-T reduction and color reaction with 4-dimethylbenzaldehyde measured at 570nm.

Failure testing

Tensile testing to failure of the constructs was performed after one week of treatment (four weeks after cell seeding). The testing was performed in a PC-driven micromechanical rig with a sample bath containing PBS (20 N load-cell, sampling rate of 10 Hz; Deben, Suffolk, UK). A stereoscopic microscope was used for capturing images to determine the diameter and length using in ImageJ (NIH, USA). Tendon constructs were attached to hooks that were affixed to the specimen plates. Samples were stretched at 4 mm/min until failure. Force was filtered by a running average over 10 data points (equal to 1 s or ~0.5% strain) before calculating stress based on the cross-sectional area. Strain was determined from the length at the onset of force (the point where stress first exceeds 10 kPa). Tensile modulus was calculated as the peak slope determined by linear regression over a 5% strain range. The mechanical testing was performed in triplicates for 4 cell lines.

Cell-matrix mechanics

A custom made system was used to measure forces in cell-derived human tendon constructs [29]. Briefly, the system consisted of force transducers (402A, Aurora Scientific, CA), stepper motors with a motor controller (Astrosyn, Y129-5, PC-control ltd., UK), culture wells and a PC data collection system

(Microlink 751, Biodata ltd., UK). Constructs were attached by their silk suture loops to the motor and force transducer via stainless steel hooks. Deformation was applied at a rate of 56 µm/s, by the stepper motors via a threaded rod with a step resolution of 2.25 µm. Data were sampled at 1 Hz. As it has been previously described [27], the functional tests at the force monitor were performed in an incubator (37 °C and 5 % CO_2). The Sylgaard coating underneath the construct was cut into a strip to maintain the initial length and tension when mounted on the hooks. To verify the presence of tension, the constructs were relaxed by 0.225 mm. If tension was present the length was returned to the original position. If there was no tension, the constructs were considered to have become slightly slack during transfer and were stretched in 0.225 mm steps up to 0.675 mm to re-establish tension. This position was defined as the baseline length, and subsequently the system was allowed to stabilize for one hour. After stabilizing, the tendon constructs were subjected to three unloading / reloading cycles, with each cycle consisting of 0.675 mm (6.75% strain) of unloading (reducing length), 300 s of rest period followed by 0.675 mm of reloading (returning to the initial length) and another 300 s of rest. For the cell-matrix testing duplicates from 3 cell lines were used for the 1st batch and triplicates from 2 cell lines for the 2nd batch.

Statistics

The magnitude of re-tensioning was normalized to the amount of unloading and the magnitude of relaxation was normalized to the amount of loading, with both values expressed as percentages. The average of the 3 cycles was used to calculate the relative amount of re-tension and relaxation for each construct. The effects of treatment were statistically analyzed using 1-way ANOVA comparing each treatment to DMSO controls. Dunnett's multiple comparison tests were used for posttests (GraphPad

Software, La Jolla California USA). The mechanical parameters showed a log-normal residual distribution and were therefore log transformed prior to statistical analysis. Results are shown as back-transformed geometric means and geometric standard errors (shown as [lower upper] due to the asymmetric nature of the log-normal distribution).

Results

Cell-matrix interactions

The re-tension phase was significantly decreased by 61%, 86% and 91% respectively in Gn005, Gn01 and Gn1 concentration treated constructs compared with the DMSO control (Fig.1). The relaxation followed a similar pattern with reductions of 29%, 47% and 59% respectively in Gn005, Gn01 and Gn1 concentration treated constructs relative to DMSO controls (Fig.1).

Cell viability and construct composition

Cell viability remained the same in genipin treated and DMSO controls except for the Gn1 genipin concentration in which fibroblast viability was reduced by 99% ($p<0.0001$)(table 1). DNA content, hydroxyproline and GAG content were unaltered by genipin treatment (table 1).

Failure tests

The peak stress increased with Gn1 (56%) and Gn001 (17%) genipin concentrations whereas it decreased by 72% with BAPN treatment compared to the DMSO controls (table 2). The tensile strain was unaffected by genipin, but decreased by 25% with BAPN treatment compared to DMSO controls

(table 2). The peak modulus was increased by 95% at Gn1 genipin concentration and decreased by 69% in BAPN treated constructs. Genipin treatment did not affect the peak force whereas the BAPN treatment decreased by 54% relative to the DMSO controls (table 2) Finally, the stiffness also increased by 96% with Gn1 and by 51% with Gn01 concentrations of genipin whereas it decreased by 70% in BAPN treated constructs compared with the DMSO controls (table 2).

Table 1. Cell-viability measured in 2D, presented as 100% of the DMSO controls. Whole constructs solubilized and DNA, Hydroxyproline and GAG content measured in ug per construct. Geometric mean [geometric SE]. Significant differences from DMSO control are marked with asterisks (*) ($p<0.05$).

	Cell viability (% of control)	**DNA content (ug per construct)**	**Hydroxyproline content (ug per construct)**	**GAG content (ug per construct)**
DMSO control	100%	15.8 [14.7-17.0]	10.6 [9.9-11.5]	11.5 [9.9-13.4]
Gn001 (0.01 mM)	93.5%	16.7 [14.8-18.9]	11.5 [10.4-12.6]	11.2 [9.5-13.2]
Gn005 (0.05 nM)	93.2%	12.9 [11.1-15.1]	11.0 [10.3-11.7]	9.3 [7.4-11.5]
Gn01 (0.1 mM)	94.3%	13.2 [11.4-15.2]	Not measured	8.8 [7.6-10.2]
Gn1 (1 mM)	1.2%*	14.9 [12.7-17.5]	10.8 [10.3-11.3]	7.9 [6.7-9.4]
BAPN (50 uM)	Not measured	19.6 [16.8-23.0]	10.5 [9.5-11.5]	14.0 [12.3-15.9]

Table 2. Constructs mechanics, assessed by stretch to failure testing. Geometric mean [geometric SE]. Significant differences from DMSO control are marked with asterisks (*) ($p<0.05$).

	Peak stress (MPa)	**Peak modulus (MPa)**	**Strain (%)**	**Peak force (N)**	**Stiffness (N/mm)**
DMSO control	1.73 [1.63-1.83]	12.5 [11.8-13.2]	21 [21-22]	0.13 [0.12-0.15]	99 [91-107]
Gn001 (0.01 mM)	1.61 [1.46-1.77]	11.9 [10.7-13.2]	23 [22-25]	0.16 [0.14-0.19]	122 [108-137]
Gn005 (0.05 nM)	1.53 [1.42-1.65]	12.1 [11.2-12.9]	22 [21-23]	0.15 [0.14-0.18]	115 [100-132]
Gn01 (0.1 mM)	2.02 [1.80-2.26]	16.6 [14.8-18.7]	21 [20-21]	0.17 [0.15-0.20]	150 [135-166]*
Gn1 (1 mM)	2.68 [2.29-3.14]*	24.3 [21.4-27.7]*	21 [20-21]	0.21 [0.19-0.24]	194 [173-217]*
BAPN (50 uM)	0.49 [0.44-0.55]*	3.8 [3.5-4.3]*	16 [15-17]*	0.06 [0.04-0.09]*	30 [26-34]*

Discussion

In the current study, we were able to quantify cell-matrix interactions and the influence of matrix rigidity by inducing cross-links (genipin) in engineered human tendon constructs. The main findings showed that human tendon fibroblasts produced lower forces in a dose-dependent manner when cross-linking stiffened the cell-generated matrix. Similarly, the stress-relaxation response decreased in stiffer constructs. These results are in contrast to our hypothesis that cells in the tendon constructs would exert higher forces in the stiffer matrix to re-establish tensional homeostasis.

It has been suggested that the amount of cell-generated forces is dependent on the cell type but independent of the matrix rigidity [30]. Herein, we observed differences in forces generated by constructs of the same cell type with treatments affecting matrix stiffness (see Fig. 1). However, it is difficult to isolate distinct cell responses from the overall mechanical outcome when cells are embedded in matrix. Thus, the re-tension measured in the present study does not exclusively represent cell-generated forces since the force is mediated via the matrix before being transmitted to the force sensor and therefore matrix properties would influence the detected force. Therefore, stiffer matrices that require higher forces to contract (i.e. the cells are stress-shielded by the stiffer matrix), could cause a reduction of the measured re-tension despite a potentially higher force developed by the cells. The reduction in re-tension is only observed for the stiffest matrices (GN005 to GN1). One possible interpretation is that the cells compensate the increasing matrix stiffness (going from BAPN to GN001) by generating larger forces, but is only able to do so to a certain point after which the external force generated by the construct drops due to stress shielding.I This idea is supported by single cells studies showing that the cells prefer to move towards stiffer areas and are

able to produce higher forces [31-33], although at a certain level of high matrix stiffness, cellular forces decrease, indicating a plateau value for cellular contraction [33, 34]. It should be noted that others have found that the cell contraction is reduced in stiffer matrices [11, 35].

With Gn1 concentration (1 mM) the cell viability assay showed that the cells were not alive, although surprisingly some re-tension could be detected. The load-bearing element of the cells is the cytoskeleton, and its components (microtubules, actin filaments) contribute to the viscoelastic properties of the cell as well as to generation and preservation of forces [36-38]. Prediction models suggest an instant response of the cell to external deformations to maintain its shape integrity, through a procedure that takes place independently of the actin-myosin contractility. [4, 39, 40]. Thus, it is possible that the viscoelastic properties of the constructs in combination with the passive components of the cytoskeleton can explain the generation of forces in GN1 treated constructs.

Hydroxyproline content, which is a marker for collagen, was unaffected by the treatment and therefore differences at the matrix properties can likely be attributed to the conformational changes due to cross-linking (see Table 1). In contrast to the cell-generated forces of ~1-5 mN, the failure tests reached force of ~150 mN, and therefore reveal information about constructs material properties [27]. Therefore, it is likely that the observed increases in peak stress and stiffness are related to increased collagen cross-links in the absence of increased collagen content. In contrast to the effect of genipin, BAPN treatment caused a dramatic reduction in the mechanical properties of the constructs confirming the relation between cross-linking and mechanical properties (see Table 2). It should be noted the increased peak stress in Gn01 and Gn1 was not associated with an increase in peak force, which indicates that the treated constructs were thinner since the main difference between these

parameters is that the stress values are normalized to the cross-sectional area (length is the same for all the constructs). It has been shown that the number of fibrils remains the same after BAPN treatment although they display a disordered shape and reduced mechanical properties [16]. Collectively these data indicate that the changes in mechanical behaviour of the contructs are due to changes in cross-linking, which is corroborated of the effect of BAPN treatment on construct mechanics in the absence of any change in the compositional profile (hydroxyproline and GAG content).

The stress-relaxation response appears to be unaffected by cell cytoskeletal integrity [23, 27], indicating that it is primarily governed by matrix properties. Assessing cell-matrix mechanics with the current system, we observed that the stress-relaxation responses did not match entirely with the stretch to failure experiments, which was contrary to our expectations since both measure matrix properties. However, we observed a dose-dependent response in the relaxation phase as evaluated by the force monitor. A possible explanation for the lack of similarity between the two measures might be that force monitor tests are performed at very low strain to avoid potential damage of the constructs whereas the stretch to failure experiment assesses mainly the high stress/strain response. We also tried to correlate the toe region of the stress-strain curve, but the pattern was the same.

The present study has inherent limitations that should be considered. Genipin was used to cross-link the matrix of the constructs, but this can result in crosslinking of various proteins in addition to that of collagen. Consequently, it cannot be ruled out that changes in water content or ligand availability could also be affected [41]. In contrast, BAPN is more target specific since it is known to inhibit LOX related collagen cross-link formation, and it has been related to constructs mechanical

disruption [16, 42, 43]. Additionally, the cell viability assay (XTT) was performed in 2D cultures to assess the potential acute cytotoxicity of the treatment after 24 hours of treatment. The XTT assay can not be used on 3D constructs due to the interference of the blue colour that occurs in constructs with genipin. Therefore, the DNA content was measured instead to evaluate the long-term effect of the treatment. The DNA measurement did not detect any treatment effect, however, at the highest concentration; the constructs were visibly fixed. This is a limitation since DNA fragments can be detected for some time before they fully degrade . Finally, this study could not assess the mechanical response of individual cells but rather we measured the overall cell response of a in a structure, which may be more relevant to tissue level behaviour.

In conclusion, we manipulated the stiffness of human cell-generated tendon construct matrix by using a natural crosslinker, genipin. The stiffness and peak stress of the construct were increased in a genipin dose-dependent manner while the compositional profile of the constructs (hydroxyproline content, GAG and DNA content) was unaltered. In contrast, the endogenous cellular re-tensioning phase was significantly decreased, which indicates that there is an interaction between the cells and the matrix properties, which may influence the tissue homeastasis. Importantly, the quantitave measure of the overall cellular forces of a within a self-genereted matrix provides a model that closely resmebles *in vivo* conditions. Future investigations can examine whether changes in cell-tissue interaction are related to pathological conditions such as tendinopathy, or healing tendon ruptures.

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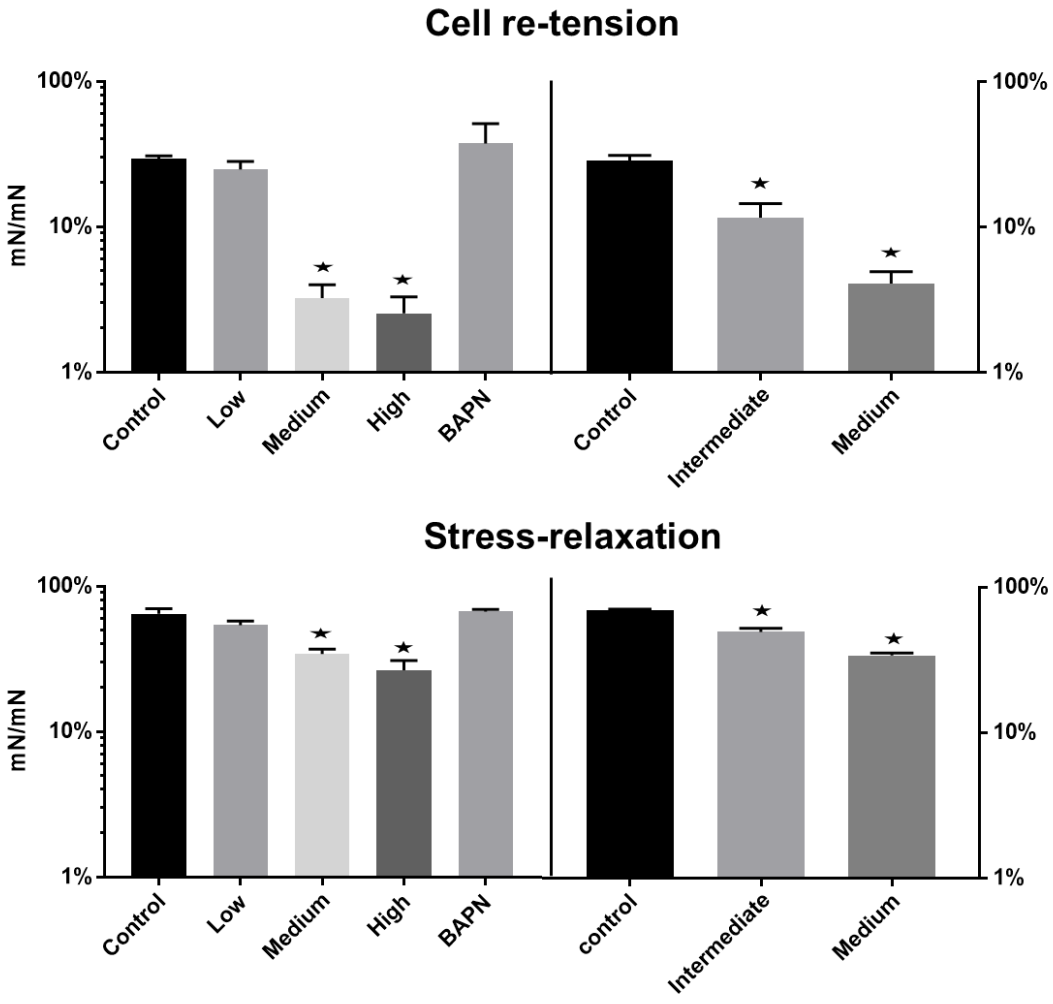
Figure captions

Fig.1. Functional tests of the constructs measured with the force monitor. The line separates the results from the two different batches of experiments (batch1 = left, batch2 = right). Geometric mean [geometric SE]. Significant differences from DMSO control are marked with asterisks (*)($p<0.05$).



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Cell-matrix interaction in tendon constructs

This declaration concerns the following article:

Cellular homeostatic tension and force transmission measured in human engineered tendon

The PhD student's contribution to the article: *(please use the scale (A,B,C) below as benchmark*)*	(A,B,C)
1. Formulation/identification of the scientific problem that from theoretical questions need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable by experiments	C
2. Planning of the experiments and methodology design, including selection of methods and method development	C
3. Involvement in the experimental work	C
4. Presentation, interpretation and discussion in a journal article format of obtained data	C

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A. refers to:	*Has contributed to the co-operation*	*0-33 %*
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C. refers to:	*Has predominantly executed the work independently*	*67-100 %*

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