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The mechanism by which fibrous tissues adapt upon alterations in their mechanical environment remains unresolved. Here, we determine that periosteum in chick embryos resides in an identical mechanical state, irrespective of the developmental stage. This state is characterized by a residual tissue strain that corresponds to the strain in between the pliant and stiffer region of the force-strain curve. We demonstrate that periosteum is able to regain that mechanical equilibrium state \textit{in vitro}, within three days upon perturbation of that equilibrium state. This adaptation process is not dependent on protein synthesis, because the addition of cycloheximide did not affect the response. However, a functional actin filament network is required, as is illustrated by a lack of adaptation in the presence of cytochalasin D. This led us to hypothesize that cells actively reduce collagen fiber crimp after tissue shortening, i.e. that in time the number of recruited fibers is increased via cell contraction. Support for this mechanism is found by visualization of fiber crimp with multiphoton microscopy before the perturbation and at different time points during the adaptive response.

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1. Introduction

Fibrous tissues have the ability to adapt to their mechanical environment. The direction and magnitude of imposed load can lead to adapting structural and mechanical properties. This is important for proper functioning of all fibrous tissues, especially those with a load-bearing capacity such as tendons, ligaments and tissue-supporting fibrous sheets. The mechanism by which fibrous tissues adapt to alterations in their mechanical environment remains unresolved. Such knowledge would be helpful to guide repair and engineering of fibrous tissues.

Structural changes of a collagenous matrix subjected to different mechanical environments have been studied under well-controlled \textit{in vitro} conditions in the 'fibroblast-seeded collagen gel' model system (Feng et al., 2006b; Henshaw et al., 2006; Nakagawa et al., 1989; Thomopoulos et al., 2005; Wakatsuki and Elson, 2003; Huang et al., 1993). Upon embedding fibroblasts into a collagen gel, contraction will occur via interaction between collagen and embedded cells. Freely contracting collagen gels have inferior ultimate stress and material modulus and increased failure strain compared to statically loaded equivalents (Feng et al., 2006b). Correspondingly, stress-deprived tissues also have decreased material modulus \textit{in vitro} and \textit{in vivo} (Yamamoto et al., 2002; Abreu et al., 2008; Binkley and Peat, 1986; Loitz et al., 1989; Yamamoto et al., 1993; Arnoczky et al., 2007). Additionally, a disorganized distribution of cells (Henshaw et al., 2006; Nakagawa et al., 1989) and absence of collagen alignment is observed in these freely contracting constructs (Henshaw et al., 2006). Biaxially constrained gels also lack cell and collagen organization and remain mechanically isotropic (Thomopoulos et al., 2005; Henshaw et al., 2006). In contrast, uniaxially constrained gels develop high degrees of cell and fiber alignment and develop mechanical anisotropy (Wakatsuki and Elson, 2003; Thomopoulos et al., 2005; Henshaw et al., 2006; Huang et al., 1993; Nakagawa et al., 1989). This change in collagen fiber organization is driven by the embedded contractile cells (Harris et al., 1981; Grinnell and Lamke, 1984; Stopak and Harris, 1982; Sawhney and Howard, 2002; Guidry and Grinnell, 1985; Brown et al., 1998; Meshel et al., 2005) that can generate substantial contraction force (Grinnell and Lamke, 1984; Bell et al., 1979; Dodd et al., 1982; Feng et al., 2006a). Contraction is absent in cell-deprived collagen gels (Grinnell and Lamke, 1984; Guidry and Grinnell, 1985; Eastwood et al., 1996; Bell et al., 1979) and in the presence of cytochalasin B, an actin polymerization inhibitor (Bell et al., 1979; Guidry and Grinnell, 1985).

The magnitude of cell contraction appears to equilibrate around a tensional homeostatic setpoint (Brown et al., 1998). Increased contraction is observed upon a decrease in external loading, while a reduction is observed upon increased external loading (Brown et al., 1998; Tomasek et al., 1992; Petroll et al., 2004). The effect of this response is not only that cells may approach their tensional homeostatic setpoint (Brown et al., 1998), but also that tension in the tissues extracellular matrix is
altered. Hence, decreased tissue strain is restored via cell-mediated tissue contraction (Brown et al., 1998; Tomasek et al., 1992; Petroll et al., 2004).

Besides direct effects of cell tension, preferential cleavage of unstrained fibers (Huang and Yannas, 1977; Nabeshima et al., 1996; Ruberti and Hallab, 2005; Ellsmere et al., 1999) and tissue compaction via the presence of residual strain (Bertram et al., 1998; Popowics et al., 2002) also contribute in restoring tissue tension. On the other hand, a stretch-induced increase in tissue tension is diminished in time due to the viscous properties of collagen fibers, reorientation of these fibers in the matrix (Puxkandl et al., 2002; Sawhney and Howard, 2002) and mechanical disruption or degradation of overstretched fibers (Huang and Yannas, 1977; Ellsmere et al., 1999). To meet the demands upon increased tissue load, additional collagen is synthesized (Kim et al., 2002; Parsons et al., 1999; Yang et al., 2004; Wang et al., 2003; Curwin et al., 1988) and the diameter of existing fibrils is increased (Michna, 1984; Michna and Hartmann, 1989).

These responses of fibrous tissue to imposed load suggest that fibrous tissues adapt towards homeostasis, i.e. a particular mechanical equilibrium. In search of such tissue equilibrium, and to propose a mechanism for adaptation towards this equilibrium, a step-by-step approach was adopted. (1) Determine whether growing fibrous tissues indeed attain a specific mechanical state in vivo, irrespective of its developmental stage ('native adaptation'). (2) Evaluate in vitro if this specific mechanical state is restored upon perturbation, and therefore represents a preferred equilibrium ('stretch-dependent adaptation'). (3) Assess the time-scale at which this equilibrium is restored ('transient adaptation'). (4) Determine whether this adaptation process is based on passive tissue behavior, or that protein synthesis and/or cell contractile properties contribute ('blocking cell-mediated adaptation'). (5) Propose a mechanism for fibrous tissue adaptation upon tissue shortening, by visualizing the structure of the collagen network during this process ('visualization of collagen adaptation'). By following this approach, we evaluated the hypothesis that fibrous tissues adapt towards homeostasis, characterized by a particular mechanical state, and to propose a mechanism for the adaptive behavior upon geometric changes.

2. Materials and method

2.1. Tissue preparation

Fertilized eggs of White Leghorn chickens ('t Anker, Ochten, The Netherlands) were placed in a polyhatch incubator (Brisnea, Stanford, UK). After a 15-, 16-, 17- or 18-day period of incubation, i.e. Hamburger and Hamilton stage 41–44 (Hamburger and Hamilton, 1992), chick embryos were removed from the eggs and euthanized by decapitation. Tibiotarsi were carefully dissected, without damaging the periosteum. A longitudinal incision through the periosteum over the entire length of the diaphysis was made adjacent to the fibula. The fibula was removed by cutting the proximal and distal ends with scissors. This leaves the periosteum, still encapsulating the bone, with a longitudinal gap from the proximal to distal metaphysis. The longitudinal incision was used to guide two sutures (5.0 Vicryl, Ethicon, Johnson & Johnson Medical, Amersfoort, The Netherlands) between the bone and the periosteum. The needles connected to the sutures were removed and the sutures were guided through hypodermic needles without cutting bevel (Terumo Europe, Leuven, Belgium). Subsequently, the tibiotarsus was fixed in an ElectroForce LM1 TestBench (Bose, Framingham, MA, USA) and grips were displaced until the 2 N load cell (Sensotec, Honeywell, Apeldoorn, The Netherlands) indicated a stressless situation, i.e. the tibiotarsus as well as the periosteum were fixed in the TestBench exactly at its in vivo length. Accordingly, periosteum length was unaltered with respect to its in vivo length. This length of the tibiotarsus in the TestBench was adjusted to each individual bone size, which generally increases with developmental stage (periosteum length averaged 17.5, 20.8 and 23.2 mm for embryonic day 15, 16 and 17, respectively). At this in vivo length, the inserted suture wires were moved towards the proximal and distal insertion of the periosteum to the cartilage. Subsequently, suture wires were pulled through the needles without cutting bevel to cut the proximal and distal metaphyseal cartilage. Mineralized bone tissue could then be extracted with the periosteum held at in vivo length (method is illustrated in Fig. 1, and previously described Foolen et al., 2009).

2.2. Mechanical characterization

From in vivo length (Fig. 1a) tissues were subjected to different procedures (described later in Section 2.3), each aiming at exposing the adaptation response. The final test for all samples was a mechanical test to failure to obtain a standardized evaluation of mechanical properties and allow comparison of every test group. The standardized force–stretch curve was obtained by initially shortening the periosteum to 0.75 times its in vivo length, in order to relieve the tissue of any residual force. Subsequently, the tissue was strained to failure at 0.15%/s. Tissue stiffness was defined by the tangent in the linear region on the force–stretch curve (Fig. 2a), and hence not corrected for cross-sectional area. Assessment of the cross-sectional area was not possible without disturbing the adaptation process during culture, while severe tissue disruption upon straining to failure prohibited accurate measurements afterwards. Mechanical adaptation was characterized by the 'transition stretch', which is the stretch at the transition point between the low and high stiffness of the force–stretch curve (heel region). Transition stretch was defined as the stretch where the tangent to the force–stretch curve equaled 1/8th the slope of the linear stiffness region (Fig. 2a). The rationale for this definition is explained in Fig. 2b. The uniquely determine the transition stretch point, zero-phase digital filtering with a window size of 5 measurement points was used to eliminate noise in the force–stretch curves, after which the curves were differentiated. After determining the mechanical properties, periosteum was resected from all remaining tissue and stored at −30°C until further analysis, which consisted of DNA, GAG and collagen content, and HP crosslink density.

2.3. Study designs

(1) Native adaptation:
From in vivo length, i.e. immediately after extraction of mineralized tissue, periosteum of embryonic day 15 (e15) to e18 tibiotarsi was mechanically characterized (n=12 per age).

Fig. 1. Progressive steps in the mechanical test protocol. Digital images (top row) and corresponding illustrations (bottom row). (a) After making a single longitudinal cut with a scalpel alongside the fibula, which was subsequently removed, suture wires were guided in between bone and the periosteum. (b) At in vivo length, suture wires were moved proximal and distal. (c) Suture wire has cut the proximal metaphyseal cartilage. (d) Bone tissue was removed after cutting through the distal metaphyseal cartilage, with the periosteum held at in vivo length. Scale bar represents 10 mm. Fig. 1 is adopted from Foolen et al. (2009).
(2) Stretch-dependent adaptation:

From in vivo length, periosteum of e15 tibiotarsi was stretched to 0.85, 0.90, 0.95, 1.00 or 1.05 times in vivo length \( (n=4 \text{ for all stretch groups}) \) and subsequently cultured for 3 days while stretch was maintained in the ElectroForce LM1 TestBench. Experiments were performed in complete medium containing DMEM (Gibco, Invitrogen, Breda, The Netherlands), 10% FBS and 1% penicillin/streptomycin (Lonza, Walkersville, USA). The medium was kept at 37.5 °C during culturing, by circulating heated water through submerged tubing, and controlling the temperature of this circulating water based on the medium temperature, monitored close to the sample by a thermocouple. After culturing, mechanical properties were determined.

(3) Transient adaptation:

From in vivo length, periosteum of e15 tibiotarsi was stretched to 0.95 times in vivo length and evaluated immediately \( (n=4 \text{ for each group}) \), after 1, 2, 3 and 4 days of culturing \( (n=4 \text{ for each group}) \). Culture conditions were exactly as described in study design (2): ‘stretch-dependent adaptation’. It should be noted that data from the 0.95 stretch group cultured for 3 days, and native e15 (representative for 0 h in culture at 0.95 stretch) were adopted from study design (2): ‘stretch-dependent adaptation’. An overview of all in vivo experiments (study designs 2–4) is depicted in Table 1.

(4) Blocking cell-mediated adaptation:

From in vivo length, periosteum of e15 tibiotarsi was stretched to 0.95 or 1.05 times in vivo length \( (n=4 \text{ for all stretch groups}) \) and subsequently cultured for 3 days in the presence of cytochalasin D \( (10 \mu \text{g/ml, Sigma, St. Louis, MO, USA}) \) and/or cyclohexamide \( (25 \mu \text{g/ml, Sigma}) \), added to complete medium. Cytochalasins inhibit matrix contraction by disrupting the actin filament network of the fibroblasts (Bell et al., 1979; Guidry and Grinnell, 1985, 1987). Cycloheximide blocks protein synthesis by cells (Bell et al., 1979; Guidry and Grinnell, 1985, 1987). For comparison, data from the 0.95 and 1.05 stretch groups, cultured for 3 days in complete medium, were adopted from study design (2): ‘stretch-dependent adaptation’. An overview of all in vitro experiments (study designs 2–4) is depicted in Table 1.

(5) Visualization of collagen adaptation:

Upon dissection, e15 tibiotarsi were mounted in a custom-built device that could be mounted on a microscope stage. Extraction of the mineralized bone was performed as described previously. Thereafter, periosteum was stretched to 0.90 times the initial length. The central diaphyseal periosteum was scanned before as well as 1 and 72 h after applying the stretch. During culturing, with or without supplemented cycloheximide and cytochalasin D, the custom-built device was placed in an incubator \( (37 \, ^\circ \text{C, 5% CO}_2) \). Two hours prior to imaging, the medium was supplemented with \( 3 \, \mu \text{M Cell Tracker Blue (Molecular Probes, Invitrogen, Breda, The Netherlands), 10 \, \mu \text{M propidium iodide (Molecular Probes) and 25 \, \mu \text{M CNA35 probe (Krahn et al., 2006) to visualize living cells, dead nuclei and collagen, respectively. The CNA35 protein is known to have a high affinity for collagen I relative to other collagen types and shows very little cross-reactivity with noncollagenous extracellular matrix proteins. Conjugation of this protein to a fluorescent dye yields the formation of a highly specific probe for collagen imaging (Krahn et al., 2006). For scanning a multiphoton microscope (Zeiss LSM 510 META NLO, Darmstadt, Germany) in Two-Photon-LSM (TPLSM) mode was used. The excitation source was a Coherent Chameleon Ultra Ti:Sapphire laser, tuned and mode-locked at 763 nm. This wavelength resulted in the highest intensity profile for the collagen probe. Laser light was focused on the tissue with a Plan-Apochromat 40 × 0.8 numerical aperture water objective, connected to a Zeiss Axiosvert 200 M. The pinhole of the photo-multiplier was fully opened. Photo-multipliers accepted wavelength regions of 435–485, 500–550 and 600–640 nm for Cell Tracker Blue, CNA35 and propidium iodide, respectively. All single images were obtained from Z-stacks, taken through the periosteum. No additional image processing was performed.

2.4. Biochemical assays

2.4.1. DNA and GAG content

After lyophilization, samples were digested in papain solution (100 mM phosphate buffer, 5 mM l-cystein, 5 mM EDTA and 125–140 μg papain per ml) overnight at 60 °C. After digestion, the samples were centrifuged and the supernatant was used for both DNA and GAG assays. For measuring DNA quantity, the Hoechst dye method (Cesarone et al., 1979) was used. The supernatant was diluted in TE-buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and DNA was labeled using the Hoechst dye (Fluka, Sigma, USA) working solution \( (10 \, \text{mM Tris, 1 mM EDTA, 2 M NaCl, pH 7.4 and 2.5 μg Hoechst dye per ml}) \). After incubation in a dark environment for 10 min on a plate shaker at room temperature, fluorescence was measured using a plate reader (excitation 360 nm, emission 460 nm, Bio-Tek, Winoski, USA) and DNA quantity was determined from a standard curve prepared from calf thymus DNA (Sigma). The GAG content was determined using a modified version of the protocol described by Farnèdale et al. (1986). In short, 40 μl of supernatant was pipetted into a flat bottom 96-well plate in duplicate. To each well, 150 μl of DMMB color reagent (46 μM dimethylmethylene blue, 40.5 mM glycine, 40.5 mM NaCl, pH 3.0) was added. Absorbance was measured at 540 and 595 nm and the difference calculated. GAG amount was determined from a standard curve prepared from chondroitin sulphate from shark cartilage (Sigma).
For native adaptation: \( n = 16, 14, 14 \) and 12 for embryonic ages 15, 16, 17 and 18, respectively. For all \( \textit{in vitro} \) experiments: \( n = 4 \) for all groups.

2.4.2. Collagen content and HP crosslink density

Lyophilized tissue samples were hydrolyzed in 6 M hydrochloric acid (Merck, Germany) and used for amino acid and crosslink analyses. Hydroxyproline residues were measured on the acid hydrolysates using reverse-phase high-performance liquid chromatography after derivatization with 9-fluorenylmethyl chloroformate (Fluka) (Bank et al., 1996). The same hydrolysates were used to measure the number of the mature HP crosslinks, using high-performance liquid chromatography as described previously (Bank et al., 1997; Robins et al., 1996).

For native adaptation: \( n = 16, 14, 14 \) and 12 for embryonic ages 15, 16, 17 and 18, respectively. For all \( \textit{in vitro} \) experiments: \( n = 4 \) for all groups.

2.5. Statistics

One-Way ANOVA was used to determine the effect of the selected independent variable (embryonic age, applied stretch, culture time or addition of blocking agent) and its interaction with a dependent variable (transition stretch, stiffness, amount of GAG, DNA and collagen and HP crosslink density). The \( p \)-value was corrected with the Bonferroni criterion. Linear regression analysis was used to determine the relationship between embryonic age, applied stretch or culture time with transition stretch, tissue stiffness and biochemical composition (GAG, DNA, collagen and HP crosslinks). Paired sample comparison (paired \( t \)-test) was performed to determine significant differences in applied stretch and measured transition stretch at each time point in samples stretched to 0.95 times their initial length evaluated after 0, 1, 2, 3 or 4 days. \( p \)-values \(< 0.05 \) were considered statistically significant.

3. Results

(1) Native adaptation:

At all embryonic ages (e15–e18), the \( \textit{in vivo} \) length of the periosteum (corresponding to 1.00 stretch, Fig. 3a) maintained the tissue at its stiffness transition point, quantified by assessing the corresponding stretch value (Fig. 3b). Hence, although periosteum can grow up to 25%/day between the embryonic ages studied (Foolen et al., 2008), the \( \textit{in vivo} \) periosteum length always adapted to maintain the tissue at a similar transition stretch, indicating adaptation towards a preferred mechanical equilibrium. Tissue stiffness increased significantly with embryonic age (\( p < 0.000, R^2 = 0.83 \), Fig. 3c), which can in part be attributed to an increase in circumference of the periosteum with age (Foolen et al., 2008). In relationship to embryonic age, DNA content decreased (\( p < 0.000, R^2 = 0.31 \)), while both collagen content and HP crosslink density increased (\( p = 0.002, R^2 = 0.17 \) and \( p < 0.000, R^2 = 0.71 \), respectively, Fig. 3d). GAG content showed no relationship with embryonic age (\( p = 0.42 \), Fig. 3d).

(2) Stretch-dependent adaptation:

The results of study (1); ‘native adaptation’ imply that a change in periosteum length triggers adaptation towards a mechanical equilibrium, which is the stiffness transition region. In support of this hypothesis, we showed that after artificially inducing a length change over a range of \(-10\%\) to \(+5\%\) in our \( \textit{in vitro} \) culture system, the transition stretch approximated the applied stretch after 3 days of culturing (Fig. 4a and b). Only at a length change around \(-15\%\), an offset became apparent (Fig. 4b). This shows that upon a change in length, periosteum is able to mechanically adapt by shifting its stiffness transition point to its current length. The shape of the curve also changed, with an increased heel region length towards lower applied stretch values (Fig. 4a). An important finding is that tissue stiffness (Fig. 4c) increased significantly with applied static stretch (\( p < 0.000, R^2 = 0.73 \)). Hereby, stiffness of the 1.05 stretch group was significantly higher after 3 culture days compared to native e15 periosteum,
but all stretch groups had significantly lower stiffness compared to native e18 periosteum (One-Way ANOVA). No relationship was found between applied stretch and DNA or GAG content (Fig. 4d, $p = 0.78$ and 0.16, respectively). Collagen content and HP crosslink density showed a negative relationship with applied stretch ($p = 0.011$, $R^2 = 0.31$ and $p = 0.015$, $R^2 = 0.29$, respectively) (Fig. 4d). DNA content of the stretched samples was not significantly different from native periosteum at any age between e15 and e18 (One-Way ANOVA). However, DNA content of stretched samples corresponded most to native e18 periosteum. HP crosslink density in all stretch groups after 3 days of culture was significantly higher compared to native e15, but not different from native e18 periosteum (One-Way ANOVA). (3) Transient adaptation:

Tissue adaptation resulted in a transient shift in transition stretch (Fig. 4a and b). No significant difference was detected between applied stretch and measured transition stretch from day 3 (Fig. 5b, paired t-test). A non-significant drop in stiffness was observed after 4 days of culture (One-Way ANOVA, Fig. 5c). DNA content showed a negative relationship ($p = 0.001$, $R^2 = 0.31$) and HP crosslink density a positive relationship ($p < 0.000$, $R^2 = 0.63$) with culture time. From the first culture day on, HP crosslink density was significantly higher compared to native e15 periosteum. Interestingly, the slopes of regression in DNA and HP crosslinks with time were the same in vivo and in vitro (Fig. 3d and Fig. 5d compared). Both GAG and collagen content showed no transient response (Fig. 5d, $p = 0.58$ and 0.17, respectively).

(4) Blocking cell-mediated adaptation:

Blocking protein synthesis by adding cycloheximide (CH) led to a shift in transition stretch, indistinguishable from controls, not treated with CH (Fig. 6a and b). However, blocking the cells’ ability to contract by adding cytochalasin D (CD) in addition to CH, abolished the shift in transition stretch (Fig. 6a and b). Tissue stiffness of samples stretched to 0.95 times in vivo length treated with CH, was significantly lower compared to samples treated with both CH and CD (Fig. 6c). DNA and GAG content decreased in the presence of CH relative to samples in complete medium, but collagen content increased (Fig. 6d, One-Way ANOVA). Note that this does not necessarily imply that this is due to increased collagen synthesis; selective loss of other matrix components such as proteoglycans also induces these results. Addition of blocking agents (CD and/or CH) did not significantly change HP crosslink density.

(5) Visualization of collagen adaptation:

The proportional relationship between applied stretch and tissue stiffness (Fig. 4c) did not result from increased collagen content or HP crosslink density (Fig. 4d), suggesting that tissue stiffness was dominated by structural changes in the collagen network. Furthermore, blocking protein synthesis alone had no effect on the shift in transition stretch, while disrupting the actin filament network with cytochalasin D prevented this shift (Fig. 6b). This suggests an adaptation

![Figure 4](image-url)
mechanism that is independent of matrix protein expression, but highly dependent on the contractile properties of the cells. Evaluation of collagen morphology confirmed that tissue adaptation upon sustained shortening, involved a reorganization of the collagen fiber network, only when the cells had a functional actin filament network (Fig. 7).

Native periosteum contains straight collagen fibers, oriented in the longitudinal direction (Foolen et al., 2008). After mounting periosteum in the custom-built stretch device and observing the tissue with multiphoton microscopy, this collagen morphology was confirmed (Fig. 7a and e). Immediately after shortening the tissue to 0.90 times the initial length, all collagen fibers were crimped (Fig. 7b and f). After 72 h of culturing at 0.90 times the initial length, the fibers were predominantly straight, although a portion of them still displayed a crimped morphology (Fig. 7c and g). After 72 h of culturing in the presence of CH and CD, all collagen fibers remained crimped (Fig. 7d and h). Both with and without CH and CD, cells remained viable over the culture period.

4. Discussion

We hypothesized that adaptation directs fibrous cellular tissue towards mechanical equilibrium. We set out to test this hypothesis by characterizing this mechanical equilibrium. For this, force–stretch curves of growing native periosteum were evaluated. In vivo, the periosteum is constantly functioning in the heel region of the force–stretch curve. Therefore, we postulated that native adaptation would drive periosteum towards a state, in which its stiffness is in the transition from pliant to much stiffer. Subsequently, we studied stretch-dependent and transient adaptation in vitro. In agreement with our hypothesis, upon induced alterations in tissue length, periosteum adapted by transiently restoring this preferred mechanical state. Restoration was not dependent on the cells' ability to synthesize proteins, but highly dependent on their contractile properties. The underlying mechanism for the adaptive response was exposed by visualization of the collagen morphology upon shortening the tissue to 0.90 times the initial length. The resulting collagen fiber crimp was gradually reduced during three days of culturing. This process was cell-mediated and required a functional actin filament network; reduction of collagen crimp was absent in periosteum cultured in the presence of cycloheximide and cytochalasin D. Interestingly, the structural organization of the collagen network, and not the collagen content or HP crosslink density, determined the stiffness of the tissue. Therefore, we conclude that changes in the force–stretch relationship upon shortening are caused by collagen crimp, while the adaptive response is due to a gradual straightening of the collagen fibers by active cell contraction via the actin filament network.

The consideration to use chick periosteum as a model system originated from the ability to subject the tissue to stretch regimes starting exactly from its very well-defined in vivo length. Also, the collagen network was known to be well-aligned (Foolen et al., 2008). Another advantage is that embryonic chick tibiotarsi have a high incremental growth rate (Church and Johnson, 1964) and therefore adapt at a very fast rate. Periosteum can grow up to 25%/day (Foolen et al., 2008), and still maintain low in vivo force (Foolen et al., 2009). Nevertheless, periosteum has a potential for osteogenesis (Augustin et al., 2007) and chondrogenesis (O’Driscoll et al., 1994; Ravetto et al., 2009; Emans et al., 2005).
However, differentiation was negligible in our experiments, because GAG content did not change and mineralization was not observed. Therefore, extrapolation of the adaptation mechanism to other fibrous tissues remains valid.

Mechanical adaptation was assessed by determining the transition point between low and high stiffness on the force–stretch curve. For appropriate comparison, this transition point needed to be uniquely defined. We did that as follows. The transition point in unstretched samples (i.e. stretched to 1.00 times in vivo length, Fig. 4a and b) after 3 days of culture was considered the reference situation. In the force–stretch curve of these samples, the transition point between low and high stiffness was then defined as the point where stretch equaled 1.00. At this point, the tangent to the force–stretch curve appeared to equal 1/8th of the slope in the linear stiffness region. Subsequently, the transition point in the force–stretch curves for each individual sample was determined as the point where the tangent to that force–stretch curve equaled 1/8th of the slope in its linear stiffness region. It should be noted that the choice for the condition that exactly defines the transition point is arbitrary. However, an evaluation of our data showed that the results and conclusions are not sensitive to this definition (Fig. 2b).

Tissue stiffness was determined by straining the tissues to failure at 0.1%/s relative to their in vivo length. For visco-elastic tissue, stiffness increases with strain rate. Pilot studies showed that at the low strain rate of 0.1%/s, strain-rate-dependent effects are minimal. Yet, in fact, the applied strain-rate during failure testing was higher for samples that were adapted to a shorter length during culture. Correction of strain-rate to the newly acquired length would have enhanced the proportional relationship between applied stretch and stiffness, and would therefore have strengthened the relationship that was already determined.

While the TestBench allowed us to mount the tibiotarsus at in vivo length using feedback from the force-signal, the custom-built stretch device for the microscope did not allow force measurement. A possible effect on initial tissue length is however minimal compared to the induced strains. Also, the fiber structure after mounting in the stretch device (Fig. 7a and e) resembles in vivo structure (Foolen et al., 2008) very well, indicating a proper transfer of the in vivo situation to the experimental setup.
The CNA35 probe (Krahn et al., 2006) used to visualize periosteal collagen in the custom-built stretch device has a drawback of potentially preventing proper collagen fibril formation (Boerboom et al., 2007). We therefore chose to visualize separate samples per time point, instead of one sample at multiple times. In this setup, we chose to apply a stretch of 0.90 times the initial length. We aimed at relating tissue mechanical properties to structural organization of the collagen network. The stretch-dependent adaptation experiment revealed that tissue stiffness decreased after 3 culture days at 0.90 times in vivo length, while at 0.95 times in vivo length, such decrease was not present.

In post-hatched quails the in vivo strain state corresponded to that of the heel region of the force–stretch curve, i.e. the strain where transition in stiffness from pliant to much stiffer behavior occurs (Bertram et al., 1998). This is in agreement with the in vivo loading state of periosteum in the current study.

Stiffness of e18 periosteum was significantly higher than e15 periosteum cultured for 3 days. Part of this difference can be attributed to an increase in circumference of native periosteum, while in vitro cultured tissue does not gain width. The metaphyseal cartilage from embryonic age e12 to e17 increases its circumference on average by 20% per day (e12–e15 data adopted from Foolen and coworkers (Foolen et al., 2008); e16 and e17 data not shown).

HP crosslink density significantly increased with development in subchondral bone of newborn foals and tibial bones from male broiler breeder chickens (Rath et al., 2000). In our study, HP crosslink density also increased with developmental age, which may have contributed to increased stiffness (Balguid et al., 2007). We therefore chose to visualize separate samples per time point, instead of one sample at multiple times. In this setup, total dry weight were presented.

Based on observations from our various experiments, we propose the following adaptation mechanism for fibrous tissue upon sustained shortening. In full equilibrium, all fibers are straight. Fibers become crimped when the tissue is shortened. This weakened environment unbalances tensional homeostasis of the cells, which therefore start to contract. As a result, crimp from individual fibers is transiently removed and the transition point gradually shifts from the old to the new tissue length, until a new equilibrium is reached with minimal crimp. Complete removal of crimp from all fibers is prolonged with increased shortening. Therefore, a discrepancy between applied stretch and transition stretch is introduced at low applied stretch values and a proportional relationship between applied stretch and tissue stiffness is obtained. Another consequence is that with increased shortening, differences in crimp between individual fibers become larger, whereby the length of the heel region is increased. Namely, the amount of crimp present in the fiber and the stretch value from where a fiber starts to become recruited determines which fibers will bear the imposed load over the course of the tensile test. While protein synthesis does not contribute to this adaptation process, the cells’ ability to contract is crucial. Cells, inactivated by cytochalasin D, will not remove fiber crimp and tissue properties including the transition stretch, length of the heel region and stiffness are unaltered.

5. Conclusion

This research adds to our current insight in mechanical adaptation of fibrous tissue by showing that (1) periosteum in chick embryos resides in a particular mechanical state, irrespective of the developmental stage. This mechanical state is characterized by a residual tissue strain that corresponds to the strain in between the pliant and the stiffer region of the stress–strain curve. (2) Periosteum is able to regain that mechanical state in vitro within three days upon perturbation of that equilibrium. (3) Pursuing the particular mechanical state is cell-contraction mediated. (4) Cell contraction reduces the crimp of slack collagen fibers after tissue shortening, which increases the number of recruited fibers in time.

Conflict of interest

The authors declared that no conflict of interest exists.

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