3D culture model of fibroblast-mediated collagen creep to identify abnormal cell behaviour

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Abstract

Native collagen gels are important biomimetic cell support scaffolds and a plastic compression (PC) process can now be used to rapidly remove fluid to any required collagen density, producing strong 3D tissue-like models. This study aimed to measure the mechanical creep properties of such scaffolds and to quantify any enhanced creep occurring in the presence of cells (cell-mediated creep). The test rig developed applies constant creep tension during culture and measures real time extension due to cell action. This was used to model extracellular matrix creep, implicated in the transversalis fascia (TF) in inguinal hernia. Experiments showed that at an applied tension equivalent to 15% break strength, cell-mediated creep over 24 hour culture periods was identified at creep rates of 0.46%/hr and 0.38%/hr for normal TF and human dermal fibroblasts respectively. However, hernia TF fibroblasts produced negligible cell-mediated creep levels under the same conditions. Raising the cell culture temperature from 4ºC to 37ºC was used to demonstrate live cell-dependence of this creep. This represents the first *in vitro* demonstration of TF cell-mediated collagen creep and to our knowledge the first demonstration of a functional, hernia-related cell abnormality.

Keywords: compressed collagen, fibroblasts, creep, tissue growth, hernia

Introduction

There is a rapidly emerging need for engineered biomimetic *in vitro* 3D models of key connective tissues such as skin, bone, blood vessel and fascia, to help understand the role changes in their properties in common human pathologies. Firstly, they can help reduce the need for animal testing of therapeutics. Secondly, they dramatically improve our understanding of the cell-matrix behaviour and bio-mechanics of native tissue which we need to replicate in structure and function for both implant production and as next generation test models for normal and pathogenic function.

For a biomimetic engineered scaffold material to fulfill its purpose successfully, there are a number of key criteria that it should meet. Ideally, this should include having a significant proportion of the structural and mechanical properties to the target native tissue. A good starting point for this is to base the model on the most common and functionally important bulk tissue support protein – native fibrillar collagen.

Collagen, and specifically type I collagen, is the major structural protein in mammalian tissues and is one of the oldest natural polymers to be used as a biomaterial (Chevallay & Harbage, 2000). The production of a collagen gel by fibrillogenesis from the soluble monomeric protein can be achieved rapidly at physiological pH and temperature (Elsdale & Bard, 1972). This process produces three-dimensional, hyper-hydrated collagen lattices capable of entrapping living cells. This is a well-established 3D biomimetic culture model of natural remodelling by cells. However, it is the nature of these gels that they trap an excess of fluid (>99%), making them weak and difficult to handle (i.e. poor non-biomimetic mechanical properties).

A technique known as ‘plastic compression’ (PC) has now been developed to rapidly remove this excess fluid increasing collagen fibril and cell density to any required point up to and beyond tissue density (Brown *et al.*, 2005). These high-density collagen-cell sheets have predictable and biomimetic structural features at the meso-scale with little or no effect on cell viability. Only after the PC process is complete do cells attach, grow and remodel the collagen matrix. Interestingly, previous studies have found that such collagen neo-tissues have a number of common structural and mechanical properties with the transversalis fascia (TF) (table 1).

TF is a thin sheet of collagen-rich connective tissue located as part of the lowermost, inner abdominal wall (fig 1). It has been postulated that this layer of tissue acts as the main barrier to herniation and that it seems to be primarily affected in the process of inguinal hernia. The focus of studies into the pathological basis of this condition has been on the TF and its apparent continuing growth/expansion under load. The process by which this connective tissue growth/expansion and ultimate failure occurs may have a similar basic mechanism to that of other tissues, such as wound and major blood vessel herniation where clinical consequences can be more dangerous (Kureshi *et al.*, 2008). The extent to which this resembles normal pre-adolescent tissue growth has been examined (Kureshi *et al.*, 2008).

Connective tissues such as fascia have an extracellular matrix consisting of a network of collagen fibres. Whilst their basket weave fibril organization allows substantial deformation in the X-Y plane, they also carry multi-directional tensile loads. It is the reorganization of these fibres under load that allows the macroscopic deformation of these tissues, and this helps explain their non-linear stress-strain curves (Kirkendall &

Garrett, 1997). These tissues display the classical visco-elastic behaviour of many connective tissues (Cornwall, 1984). Stress relaxation can be demonstrated by the application of a fixed tension on the tissue. This results in a time-dependent decrease in stress under a sustained strain, which occurs as the material geometry accommodates to the applied load over a significant time-base. Material creep can also be demonstrated when a static (sub break-stress) load is applied for prolonged periods, resulting in a continuing, slow extension over time (Purslow *et al.*, 1998).

Creep properties can be identified and measured in many materials (biological and non-biological) to different extents. However, in biological systems the ability of resident cells to remodel the matrix material during the creep period adds a unique dimension to creep in non-living materials. It is well known that connective tissue cells (typically fibroblasts) have the ability to extend or grow (or even reduce) their extracellular matrix (ECM) under certain tensile loads and mechanical cues. This remodelling adaptation results in mechanically-induced growth which is seen in abdominal skin in pregnancy, obesity-slimming cycles, over surgical tissue expanders and in transversalis fascia (TF) expansion in inguinal herniation (Brown, 2006; Wilhelmi *et al.*, 1998; Beauchene *et al.*, 1989; Kureshi *et al.*, 2008; Marenzana *et al*., 2006). Studies by Beauchene and colleagues (1989) demonstrated a significant increase in the hydroxyproline content of skin in rats, 16 days after insertion of a peritoneal tissue expander, consistent with the deposition of collagen expected during normal growth.

We have previously proposed the hypothesis that TF extension in herniation occurs due to growth/remodelling (rather than injury/repair). This is suggested to operate

by a process of cell-mediated creep in which local cues stimulate cells to remodel and extend (grow) the ECM in response to the background creep load which we know as tissue tension, leading to a gradual geometric extension of the tissue. TF fibroblasts within hernia tissues under mechanical load may then represent a natural model for this proposed cell-mediated creep process. However, this is a new concept for explaining the TF extension in herniation, with no current experimental model. The key problem for such a model is that all connective tissue materials will demonstrate some level of conventionally, cell-independent material creep. Any model or demonstration of TF extension must be able to measure specifically the cell-mediated element. This is made more difficult as increasing cell number in the biological material/ECM will inevitably also change its non-cell dependent material creep properties. As a result, simple changes in cell-mediated creep cannot simply be related to cell number, while simple cell lysis will release components such as proteases that will also affect the physical properties of the ECM.

Engineering of an *in vitro* model of cell-mediated creep would represent a major aid for understanding biological (living) material properties, and especially (for the first time) mechanically-induced growth in normal and pathological systems. In turn, such a model would provide great prognostic and drug development potential. The hernia/normal TF can be viewed as a potential natural model of mechanically mediated collagen matrix-cell expansion (Kureshi *et al.*, 2008; Brown & Phillips, 2007). The aim here was to identify and if possible quantify mechano-induced cell-mediated -creep using TF fibroblasts within defined 3D biomimetic collagen 'tissues' described earlier. This assumes that cells capable of contributing toward/enhancing creep (by growth and

remodelling) *in vivo* will retain this ability in 3D culture (as they retain other mechano-responsive behaviour (Marenzana *et al.*, 2006)). The definable, reproducible nature of the PC collagen construct has been used here to allow us to characterize this effect. In effect, the aim was to develop a stable, precise model of 3D cell-mediated creep to characterize the putative growth and remodelling process for the first time.

The two objectives used here to test this hypothesis and establish the model were to quantify (i) the mechanical creep properties of acellular collagen constructs and (ii) the isolated (cell-mediated) creep, under suitable standardized tensile load conditions.

Materials & Methods

Tissue harvesting and cell culture

Normal TF (NTF) was retrieved from organ transplant donors with informed family consent. Hernia TF (HTF) was obtained with consent from patients undergoing inguinal hernia repair operations. Full ethical approval was granted for this study. Human dermal fibroblasts (HDF's) were sourced from explants of normal skin cultures obtained directly from operating theatre. A minimum of 3 tissue samples was obtained from 3 different individuals for isolation of each cell type. TF fibroblasts were grown by explant culture in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomyocin (100U/ml & 100µg/ml). The cells were used between passages 6-14 at a final density of 2.32 X 10^5 cells/ml (post-plastic compression). The aponeurosis was sourced from the vastus lateralis (quadricep) muscle

of the sheep. Acellular and cellular collagen gels were prepared by addition of 4ml of sterile rat tail type I collagen (2.16 mg mL-1 protein in 0.6% acetic acid: First Link UK Ltd, West Midlands, UK) to 0.5 ml of 10x concentration of Minimum Essential Medium (10x MEM). This was neutralized drop-wise with 5M and 1M sodium hydroxide (NaOH). For cellular gels, 2 X 10^4 TF fibroblasts (per gel) were suspended in 0.5ml DMEM and mixed with the collagen solution immediately after neutralization. The gel was cast into a rectangular (33 x 22 x 10 mm^3) mold and allowed to set for 30 minutes at 37ºC, in a 5% CO_2 incubator. Following incubation, gels (with a thickness of 6.9 mm) were removed from the molds and subjected to plastic compression, as described by Brown *et al.* (2005). All compressions were carried out at room temperature under sterile conditions. A 165 μm thick stainless-steel mesh (mesh size ~300μm) and a layer of nylon mesh (~50 μm mesh size) were placed on three (240 mm diameter) circles of Whatman (Grade 1) filter paper. The collagen gel was placed on the nylon mesh, covered with a second nylon mesh, and loaded with a 120 g weight (stress equivalent to approximately 1.6 kN m^{-2}) for 5 minutes at room temperature, resulting in a flat collagen sheet with a thickness measuring 94 ± 13μm (n=6). Thickness measurements were made using a travelling microscope.

Development of Model: Fixing cell density and amount of load applied

The model was developed in stages to define the different variables and understand its limitations and reproducibility. Plastic compressed collagen sheets were selected as the scaffold for this model. Characterization of the material creep properties

of acellular constructs was an important prerequisite to identifying the separate contribution of cell-mediated creep in cellular constructs.

To characterize the creep properties of acellular PC collagen sheets, a range of loads reducing from 70% to 15% of the construct break stress (BS) were applied to the gels. A baseline stress at which the creep extension was negligible was identified at the lower end of this range. Creep testing of acellular constructs with an applied loading of 15%BS (table 2) produced minimal (not detectable) material creep. Therefore, the loading parameter was fixed at 15%BS. This provided a sound base-line above which any creep would likely to be a result of cell activity in cellular constructs.

Cell density was another main parameter that needed to be fixed, since it was predicted that the presence of cells would weaken the construct (Saddiq *et al.*, 2005) as they act as foci of relative weakness. This suggested that the creep rate of cellular constructs would be related to cell density. Therefore, it was essential to identify a cell density that would not significantly reduce the material strength of the construct with 15%BS load (i.e. leading to breakage over the 24 hour experimental period) but that would be capable of remodelling the matrix over a reasonable time course.

Constructs with cell densities in the range of 2 million to 20,000 cells per construct (i.e. 5ml gel before plastic compression, range of 2.32×10^7 cells/ml to 2.32×10^5 cells/ml) were tested for their creep properties with an applied load corresponding to 15% BS. A reduction of cell density to 2.32×10^5 cells/ml (post-plastic compression) enabled a measurable amount of creep to occur over 24 hours. Therefore the cell density was fixed at this level and used in the next stage of investigation to identify cell-mediated creep. A load was applied to cellular constructs with this cell density using the creep

device to determine their break strength and accurately calculate the amount of load corresponding to 15%BS of cellular constructs.

Creep Tests

The collagen sheets were cut into 'dog-bone' shaped strips measuring 5mm width and 10mm length using a scalpel blade. Metal mesh grips were attached to each end of the strip to ensure secure anchorage of the specimen in the tensile creep-testing device (*in vitro* culture test rig, Fig 2). Each sample was anchored in metal clamps at the lower end of the creep device and attached to a pivoting bar at the upper end. 20 ml of DMEM supplemented with 100µg/ml of ascorbate was added to the glass chamber to keep the specimen immersed in media. The entire creep test device was held in an incubator at 37ºC (4ºC for basal rate studies) and 5% CO_2 under culture conditions for the duration of the experiment. After allowing the samples to reach test temperature, a pre-determined static uni-axial load was applied to a single PC collagen sheet with real-time extension measured by a linear variable displacement transducer (LVDT) sensor (DFg5, +/-5mm stroke, RS Components Ltd, UK) connected to a computer via an ADC-11 data logger (Pico Technology Ltd, UK). The captured data was analysed using Pico software (Pico Technology Ltd, UK). Percentage creep strain was calculated as the elongation normalized to the undeformed length i.e. initial length of specimen. The strain data was plotted as a function of time to produce creep curves and enable calculations of creep rates.

To quantify material creep (cell-independent), comparative experiments of PC collagen gels seeded with TF fibroblasts under identical conditions were carried out, but at 4ºC. This assumes that at this low temperature there is minimal cell activity thus representing the effective material creep only. A critical part of the experimental design was an excellent vehicle for isolation of cell-mediated creep as it allowed identification of the effect on the same specimen (with warming). This eliminated previous problems of non-equivalence and even permitted analysis of dynamics as the temperature changed. Therefore, any creep that occurred when the temperature was increased to 37ºC could be attributed to cell activity alone, allowing quantitative measurement of cell-mediated creep.

Previous studies have shown that lowering the culture temperature to 4ºC has little effect on collagen tissue stability (Afoke et al., 1998). Therefore, it is assumed that hyper-hydrated collagen gels that were compressed to 10-12% collagen content (w/w) would behave in a similar manner. In addition, due to the temperature dependence of collagen fibrillogenesis during gel formation, lowering the temperature would if anything, expect to reduce gel stability and so increase, not reduce creep. In contrast, a temperature reduction of this magnitude is known to dramatically reduce cell metabolism and enzyme activity that are responsible for matrix remodelling.

Results

PC collagen constructs have a number of structural and mechanical properties comparable with those of native TF. The mean fibril diameter of NTF was 44.3 ± 1.7 nm compared with 30 nm for PC collagen reported previously (Cheema *et al.,* 2007) (Table 1). This contrasts dramatically with other collagen sheet tissues such as the vastus lateralis aponeurosis from sheep quadriceps. Although found in sheets, aponeurosis more closely resembles tendon in ultrastucture with a multi-modal distribution of fibril diameters and a much greater break stress than TF (2.7 fold greater than that of TF). TEM images in figure 3 illustrate the differences in fibril diameter distribution of TF and aponeurosis. The characteristic stress-strain curves for aponeurosis and TF are shown in figure 4 and illustrate the contrast in the mechanical parameters between these two tissues.

Creep-strain versus time plots for PC model constructs showed the characteristic 3 phases of creep (Fig 5). Mean creep-strain rates were calculated for a range of static loads corresponding to an increasing proportion of the ultimate break stress (10-70% BS). At 0.2MPa (70%BS) the creep strain rate was 141% per hour and this decreased with decreasing stress level until at 0.07MPa (20%), the creep strain rate was 2.4% per hour; a 60 fold reduction (Table 2). The material creep (i.e. cell-free) became undetectable with an applied stress of 0.04MPa (15%BS or less) and the gel remained intact over the 24 hour experimental period. This provided a baseline response and was used in further tests of cell-mediated creep (i.e. in cell-seeded constructs). In this case, any additional creep (above the 15%BS baseline) was attributable to cell-mediated, rather than material

collagen creep. It was essential however, to relate the creep load to ultimate break stress of the actual material type under test as the absolute break stress of cellular PC constructs was lower than acellular materials and inversely dependent on the cell density. The ultimate break stress value for cell-seeded gels was determined at mean 0.16 MPa compared to 0.28 ± 0.09 MPa for acellular gels (n=6). This meant that the 15%BS creep loading for cellular constructs was equivalent to 0.024 MPa (which corresponds to 3% of the native tissue break strength, previously reported as 0.8 MPa for normal TF tissue (Kureshi *et al.*, 2008)).

In order to demonstrate cell-mediated (as opposed to inherent collagen only material creep) it was important to establish a physiological control in which cell-mediated creep could be blocked or largely inhibited even in the presence of intact cells (i.e. in mechanically identical gels). For this demonstration, identical cellular PC constructs were set-up under the same creep (15%BS) conditions at both 4ºC (minimal cell-mediated remodelling) and 37ºC (optimal cell-mediated remodelling). Test constructs with a (post-plastic compression) cell density of 2.32 X 10^5 cells/ml NTF fibroblasts at 37ºC; 15%BS exhibited a 6-fold increase in creep-strain rate/hour, relative to gels maintained at 4ºC over 24 hours (Fig 7). This corresponded to a mean creep strain rate of 0.46 ± 0.19% per hour at 37ºC compared to 0.08 ± 0.02% per hour of gels at 4ºC ($p< 0.05$). It was possible to demonstrate this temperature sensitivity of the cell-based creep on the same PC collagen construct, sequentially by warming up a 4ºC construct (fig 6).

Figure 7 summarizes the mean creep responses for 3 cell types (NTF, HTF and human dermal fibroblasts) under identical conditions at either 37ºC or 4ºC. Human

dermal fibroblast (HDF) seeded constructs at 4ºC had a minimal mean creep strain rate as did normal and hernia TF fibroblasts (i.e. barely detectable: 0.008 ± 0.003% per hour). At 37ºC, HDF seeded construct mean creep strain rate was 0.38 ± 0.17% per hour (figure 7), almost identical to that for NTF fibroblasts at 37ºC and 12.5 fold greater than that of HTF fibroblasts at 37ºC. These results indicate that cell-mediated creep is a normal function of fibroblasts (two distinct sources). Importantly, the cell response was similar in type and magnitude for both NTF and HDF fibroblasts (table 3). This indicates that the complete lack of such cell-mediated creep behaviour in hernia fibroblasts may represent a clear cellular change in mechano-response in these cells. The survival of this difference of cell-mediated expansion through 14 passages in 2D culture is noteable.

Hernia cell-seeded constructs (HTF fibroblasts) exhibited a mean creep strain rate of 0.03 ± 0.02% per hour at 37ºC. This rate was 15 fold less than that seen in NTF fibroblasts under the same conditions (Fig 7) and this reduction was statistically significant ($p=0.0176$). Hernia cell-seeded constructs at 4ºC demonstrated an even lower mean creep strain rate, close to minimum measurable (0.0021 ± 0.0004% per hour) consistent with the conclusion that hernia cell-mediated creep was negligible in this system.

Discussion

The aim of this study was to test the hypothesis that application of tensile load to 3D cell-matrix model constructs would cause cells to contribute towards creep and remodel that matrix (especially the load-bearing collagen) in such a way that it is extended (i.e. tension driven cell growth). This would be a quantitative measure of the cell-mediated creep process of the ECM (putative tissue growth) through remodelling and mechanically-initiated biological extension (Brown, 2006; Kureshi *et al.*, 2008). Such a cell based (growth related) tissue extension process can theoretically continue indefinitely as long as cells survive and produce more ECM to maintain material strength and thickness (i.e. to increase matrix volume/mass). Unlike non-living material creep, this cell driven extension would involve a remodelling phase as cells deposit further matrix to compensate for material thinning. (Note: by definition, 'material creep' would work mainly within constant volume rules whereas cell-mediated creep (i.e. tissue growth) would involve increase in total material volume and mass).

This study has established quantitative evidence to support the existence of this predicted cell-mediated creep in a simple *in vitro* controlled 3D model. Its findings have direct translation to connective tissue herniation, particularly as it was found that fibroblasts from hernia TF had a dramatically different ability to mediate creep than those from normal TF.

It is important to establish the differences between the two mechanical parameters measured in this study; material creep and cell-mediated creep. Material creep (demonstrated here with acellular constructs) occurs purely as a result of the mechanical properties of the nano-fibrous collagen material. Any additional creep of the material

which is a result of cell metabolic activity (remodelling with addition of new collagen under tensile load) is termed cell-mediated creep (Kureshi *et al.*, 2008). Here, the model used aimed to separate and distinguish between these two types of creep to (a) definitively identify the *in vitro* phenomenon and (b) to quantify the contribution and dynamics of cell-mediated creep. This would allow us to test for cell differences or pathogenesis to improve understanding of the controlling factors and even to develop new therapeutic approaches. In this study, the inguinal hernia model and TF tissue represents a good natural model of this process, which is predicted to occur in many normal (growth) and pathological sites. Such a process would be predicted to operate in normal physiology in childhood soft tissue growth, obesity/slimming cycles and pregnancy, in fracture callus distraction and clinically in hernia and tissue expanders where extracellular matrix grows under load (Brown & Phillips, 2007).

The method used here allowed us to quantitatively demonstrate cell-mediated creep. For normal TF fibroblasts, the rate of cell-mediated creep was considerable – giving a mean creep strain rate of almost 0.5% per hour under very modest loads of 15% of ultimate break stress and at low cell density. In practical terms, this means that a 1 cm strip of collagen tissue would increase in length by 1.2 mm each day. 12% creep strain extension/day is comparable to that reported for experimental fracture callus distraction (Kojimoto *et al.*, 1988).

Key to the success of this study was the ability to demonstrate the difference made by living cells in the collagen without changing the cell density or lysing the cells. Both of these would be likely to alter creep by other mechanisms. The 6-fold increase in creep strain rate of NTF fibroblast gels at 37ºC relative to the same gels at 4ºC is clear

evidence that this was a cell (metabolic)-mediated creep of the ECM by TF fibroblasts under load. Importantly, this effect can now be measured separately from material creep of the collagen matrix itself. The presence of temperature dependent cell-mediated creep in more widely used human dermal fibroblasts (HDF's) suggests that this type and rate of extension may be typical of normal fibroblast physiology. However, the negligible levels of cell-mediated creep found in HTF fibroblasts at 37ºC, under the same conditions (i.e. more like acellular normal TF fibroblast constructs) at 4ºC indicates a possible functional cell defect. Although it is presently too early to extrapolate this limited effect to full blown pathological mechanisms, this suggests a deficiency in matrix material properties and/or the cell-matrix remodelling process. It also represents, to the authors' knowledge, the first direct evidence for a functional defect (i.e. mechanical) in hernia cell behaviour. Further studies demonstrating matrix deposition will be critical to confirm the processes of active cell-mediated remodeling and growth. It is to some extent counter intuitive that HTF fibroblast-mediated creep was less, rather than more than normal, but such functional inversions are not uncommon in pathobiology when single components of the complex interdependent processes are dissected out from the whole.

It is also important to note that this striking difference in creep strain rate between normal and hernia TF fibroblasts seems to reflect a stable phenotypic difference in cell behaviour. The cells used in the model were cultured (expanded) for up to 14 passages outside the body on plastic, before seeding back into the collagen model. This means long-term removal from their native ECM and other micro-environmental cues. The changes identified here then, would seem to be particularly significant and deep-seated as a functionally relevant cell defect.

It is possible to speculate that reduced cell-mediated creep and remodelling in hernia fibroblasts may over long periods, lead to poor adaptation of the local TF ECM (i.e. age related reduction in ECM remodelling). Remodelling is a normal adaptive requirement for all connective tissues to accommodate for changes in tissue loading. In the case of TF this is almost inevitable as body mass, abdominal shape and muscle uses change between early and mid-life decades. In turn, failure of normal tissue adaptation may lead to increasing frequency of collagen matrix micro-scale rupture and repair, and so matrix expansion. This hypothesis would be consistent with the increased ratio of the wound repair associated type III collagen reported in hernia TF (Klinge *et al.*, 1999).

In addition, we have suggested previously (Brown, 2006 and Brown & Phillips, 2007) that fibroblast remodelling is more dependent on force vector than force magnitude. It is also possible that hernia fibroblasts have a higher sensitivity to the vector of force applied. Indeed, an important feature of the present model is the isotropic arrangement of collagen fibrils in the X-Y plane of the PC collagen sheet (though micro-lamellae are parallel in the Z plane, Brown *et al.*, 2005). As a result, uniaxial tensile loads applied here will generate micro-scale loads on the resident cells in a multi-vector fashion. This is likely to be different to the local force vectors on cells of native TF with its woven fibril arrangement and anisotropic mechanical features (Kureshi *et al.*, 2008). However, previous studies on fibroblast responses to mechanical loads suggest that such complex cell scale force vectors will result in maximal stimulation of cell responses (Mudera *et al.*, 2000). Cells strained in this way, out-of-plane of their load-bearing ECM, experience minimal stress-shielding from surrounding collagen fibrils with random orientation (Tomasek *et al.*, 2002; Brown, 2006; Mudera *et al.*, 2000).

In conclusion, this study has for the first time identified and measured *in vitro* (3D), the putative cell-mediated ECM creep. This effect was predicted based on the growth and pathological extension behaviors' of living tissues under certain tensile loads. A 3D experimental model has been developed, based on biomimetic dense collagen gels under low-level creep loads. Fibroblasts obtained from transversalis fascia of inguinal hernia tissues were used in this instance as TF herniation represents a natural model of adult cell-mediated creep, comparable in rate to that seen in clinical distraction of fracture callus (i.e. in the region of millimetres per day) (Kojimoto *et al.*, 1988). Interestingly, this rate was negligible in hernia TF cells, though the pathological significance of this is presently uncertain.

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References

Afoke A, Meagher PJ, Starley I, McGrouther DA, Bailey AJ, Brown RA (1998) Biomechanical characterization of tissues in Dupuytren's disease. J Hand Surg Br 23(3):291–296

Beauchene JG, Chambers MM, Peterson AE, Scott PG (1989) Bio- chemical, biomechanical, and physical changes in the skin in an experimental animal model of therapeutic tissue expansion. J Surg Res 47:507–514

Brown RA (2006) Cytomechanics in connective tissue repair and engi- neering. In: Chaponnier C, Desmouliere A, Gabbiani G (eds) Tissue repair, contraction and the myofibroblast. Landes Bio- science and Springer Science+Business Media, Georgetown, pp 6–24

Brown RA, Phillips JB (2007) Cell responses to biomimetic protein scaffolds used in tissue repair and engineering. Int Rev Cytol 262:75–150

Brown RA, Wiseman M, Chou CB, Cheema U, Nazhat SN (2005) Ultra- rapid engineering of biomimetic materials and tissues: fabrication of nano- and microstructures by plastic compression. Adv Funct Mater 15:1762–1770

Cheema U, Nazhat SN, Alp B, Foroughi F, Anandagoda N, Mudera V, Brown RA (2007) Fabricating tissues: analysis of farming versus engineering strategies. Biotechnol Bioprocess Eng 12:9–14

Chevallay B, Herbage D (2000) Collagen based biomaterials as 3D scaffold for cell cultures: applications for tissue engineering and gene therapy. Med Biol Eng Comput 38:211–218

Cornwall M (1984) Biomechanics of non-contractile tissue. Phys Ther 64(2):1869–1973

Elsdale T, Bard J (1972) Collagen substrata for studies on cell behavior. J Cell Biol 54(3):626–637

Kirkendall DT, Garrett WE (1997) Function and biomechanics of ten- dons. Scand J Med Sci Sports 7(2):62–66

Klinge U, Zheng H, Si ZY, Bhardwaj R, Klosterhalfen B, Schumpelick V (1999) Altered collagen synthesis in the fascia transversalis of patients with inguinal hernia. Hernia 3(4):181–187

Kojimoto H, Yasui N, Goto T, Matsuda S, Shimomura Y (1988) Bone lengthening in rabbits by callus distraction. The role of periosteum and endosteum. J Bone Joint Surg Br 70(4):543–549

Kureshi A, Vaiude P, Nazhat SN, Petrie A, Brown R (2008) Matrix mechanical

properties of transversalis fascia in inguinal herniation as a model for tissue expansion. J Biomech 41(16):3462–3468

Majima T, Yasuda K, Tsuchida T, Tanaka K, Miyakawa K, Minami A, Hayashi K (2003) Stress shielding of patellar tendon: effect on small-diameter collagen fibrils in a rabbit model. J Orthop Sci 8(6):836–841

Marenzana M, Wilson-Jones N, Mudera V, Brown RA (2006) The origins and regulation of tissue tension: identification of collagen tension-fixation process in vitro. Exp Cell Res 312:423–433

Mudera VC, Pleass R, Eastwood M, Tarnuzzer R, Schultz G, Khaw P, McGrouther DA, Brown RA (2000) Molecular responses of human dermal fibroblasts to dual cues: contact guidance and mechanical load. Cell Motil Cytoskel 45:1–9

Purslow PP, Wess TJ, Hukins DW (1998) Collagen orientation and molecular spacing during creep and stress-relaxation in soft con- nective tissues. J Exp Biol 201(Pt1):135–142

Saddiq ZA, Barbenel JC, Grant MH (2005) The mechanical strength of collagen gels containing glycosaminoglycans and populated with fibroblasts. Eur Cells Mater 10(2):64

Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA (2002) Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol 3(5):349–363

Wilhelmi BJ, Blackwell SJ, Mancoll JS, Phillips LG (1998) Creep vs. stretch: a review of the viscoelastic properties of skin. Ann Plast Surg 41(2):215–219

Figures

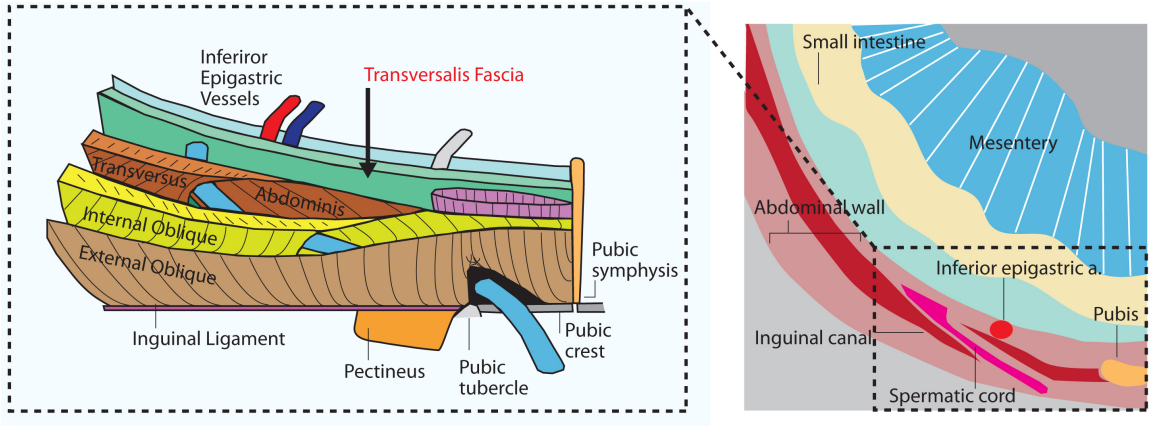


Fig 1 Illustration depicting anatomical location of Transversalis Fascia (TF). Thin sheet of collagen-rich connective tissue found on the inner layer of the abdominal wall beneath the three muscle layers

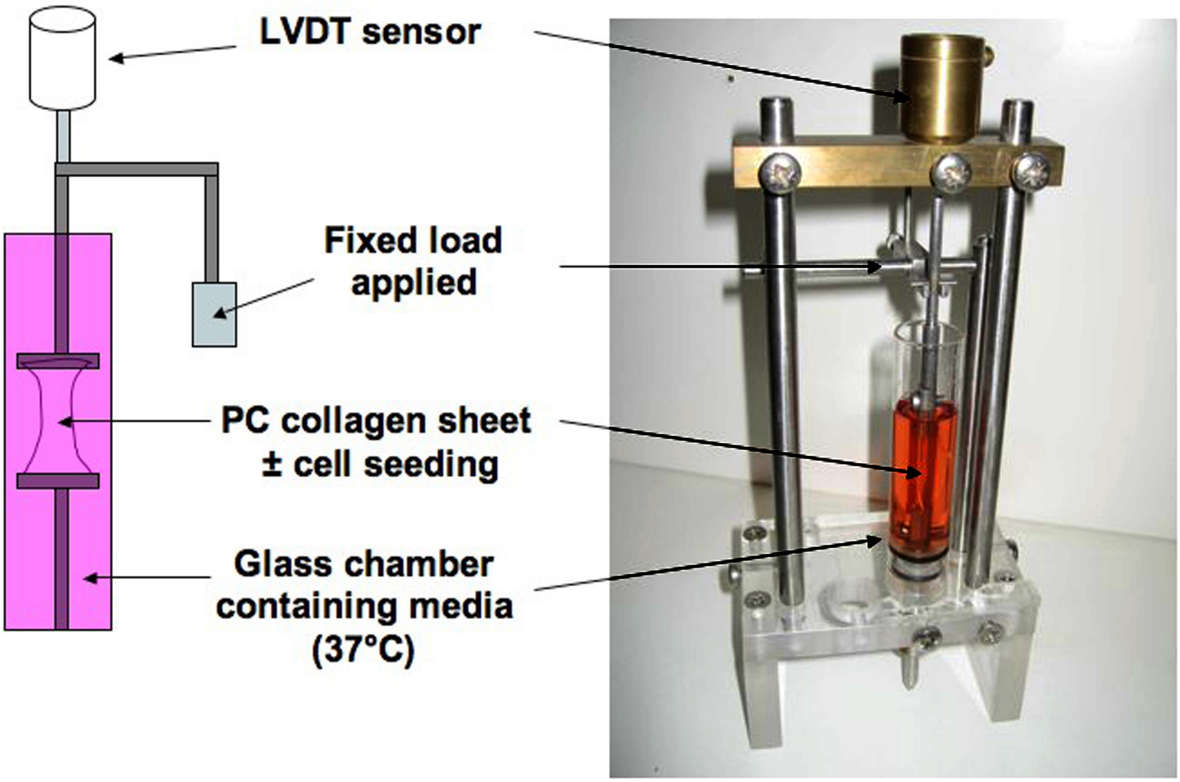


Fig 2 Diagrammatic representation of *in vitro* creep device. (LVDT = linear variable displacement transducer). The whole of this device is placed in a 37ºC CO_2 incubator for the culture period with sensor wires exiting through a wall-port. A recent version of this device now has specimen chambers for replicate runs

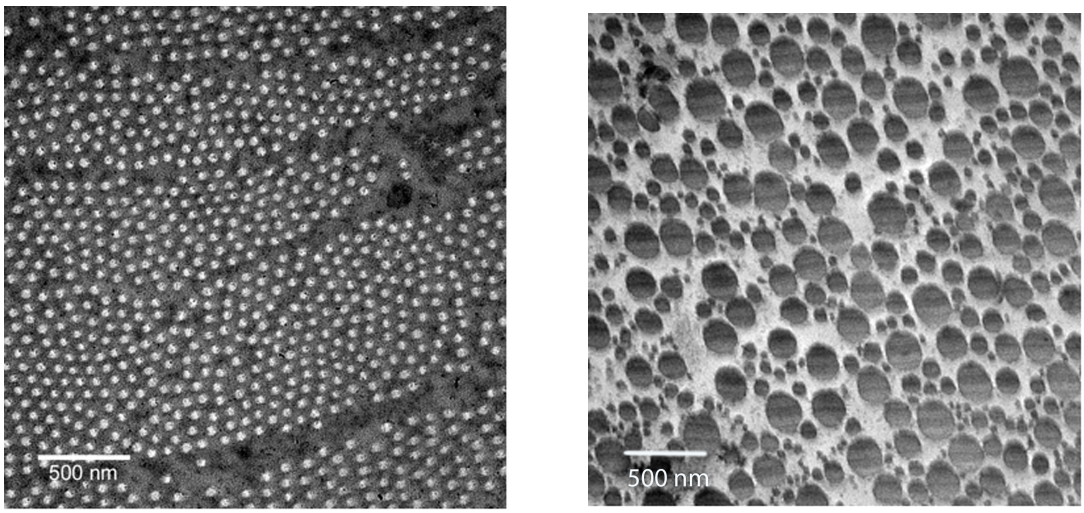


Fig 3a & 3b Transmission Electron Micrographs showing fibril cross section and diameter differences in (a) Normal Transversalis Fascia (TF) and (b) in vastus lateralis aponeurosis (sheep). There is a unimodal distribution of collagen fibrils observed in TF (fibril diameter = 44 ± 1.7) compared to a multi-modal distribution seen in aponeurosis which has a tendon-like structure (typical tendon fibril diameter ranges from 30 – 330nm in rabbit patellar tendon, Majima *et al.*, 2003)

	PC Collagen	Normal TF	Aponeurosis
Modulus (MPa)	1.3 ± 0.3	1.21 ± 0.71	n/a
Tensile Strength (MPa)	0.6 ± 0.1	0.85 ± 0.65	2.3 ± 1.4
Break Strain (%)	55.0 ± 13.8	65.5 ± 39.6	40.5 ± 10.6
Mean fibril diameter (nm)	30	44.25 ± 4.04	~30-330

Table 1 Mechanical parameters and fibril diameter comparing (hydrated) PC collagen (published data: Brown *et al.* (2005) and Cheema *et al.* (2007) normal TF tissue (weakest plane) (Kureshi *et al.*, 2008) and aponeurosis (strongest plane). Mean values ± SD are shown (n=12 for mechanical parameters; n=3 fibril diameter) for TF and Aponeurosis (n=2)

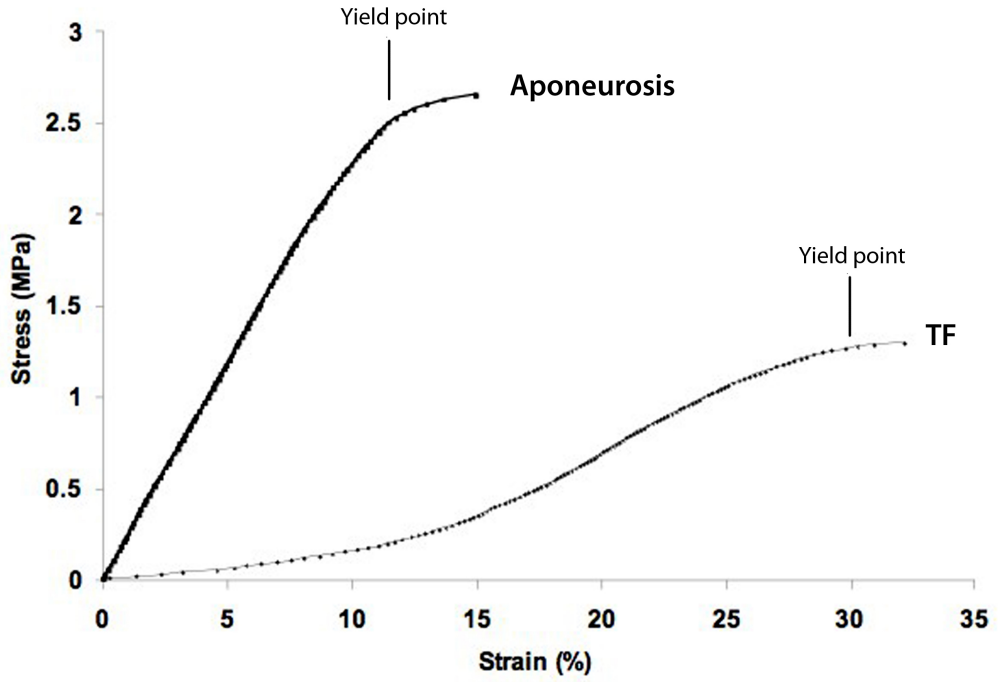
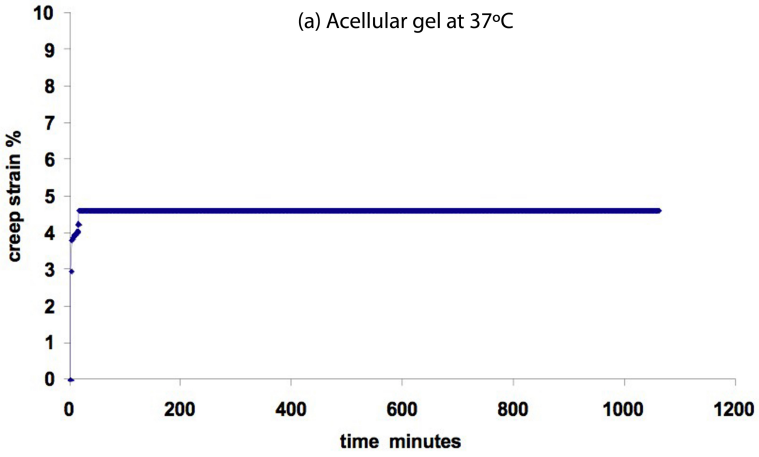
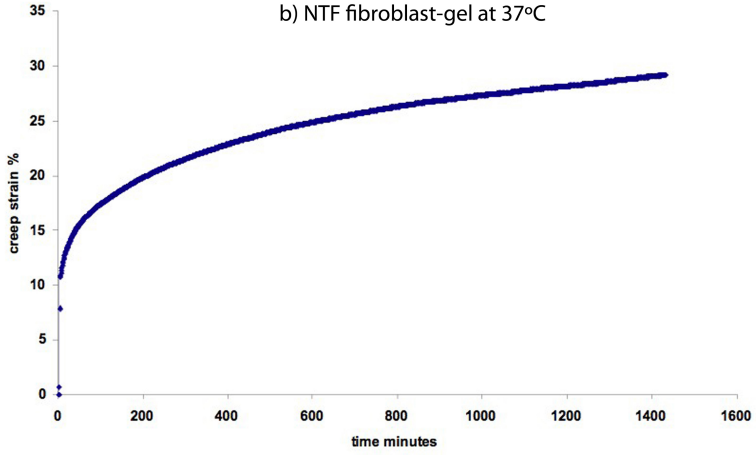


Fig 4 Representative stress-strain curve comparing TF to Aponeurosis. TF is relatively compliant for a collagenous tissue, extending by a much greater amount (>2x) than tendon-like aponeurosis, before failure and with a yield stress again >2x TF

(a) Acellular gel at 37°C



b) NTF fibroblast-gel at 37°C



(c) Aponeurosis tissue

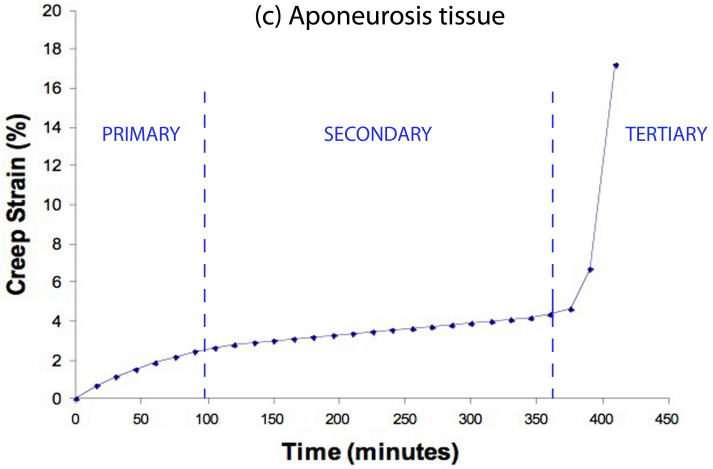


Fig 5 Representative creep strain-time graphs of constructs and tissue specimens tested using the creep model to illustrate the effects of material and cellular (bio) creep: (a) acellular gel at 37ºC with an applied load corresponding to 15% break stress, represents material creep of a collagen gel (b) NTF fibroblast-populated gel at 37ºC tested under defined conditions with cell density of 2.32 X 10^5 cells/ml and applied load of 15%BS, represents the effect of cellular creep in addition to collagen material creep (c) whole tissue specimen of aponeurosis, illustrating the three characteristic phases of a creep curve. Primary phase shows the initial non-linear extension of the tissue under load, secondary phase shows the effect of creep and the tertiary phase represents the effect of tissue failure

% ultimate break stress	Applied stress (MPa)	Mean creep strain rate per hour (%)	± SD
70	0.20	141	97.6
50	0.14	31.8	2.97
30	0.08	11.4	0.85
20	0.06	2.4	1.02
15	0.04	Nil	Nil
10	0.03	Nil	Nil

Table 2 Mean creep strain rates per hour shown for a range of loads that correspond to a varying % of the break stress (BS) applied to cell-free PC collagen sheets. The effects of creep became undetectable at an applied load of 15% BS; therefore this load was used to investigate the effects of cell-mediated creep in cellular PC collagen sheets

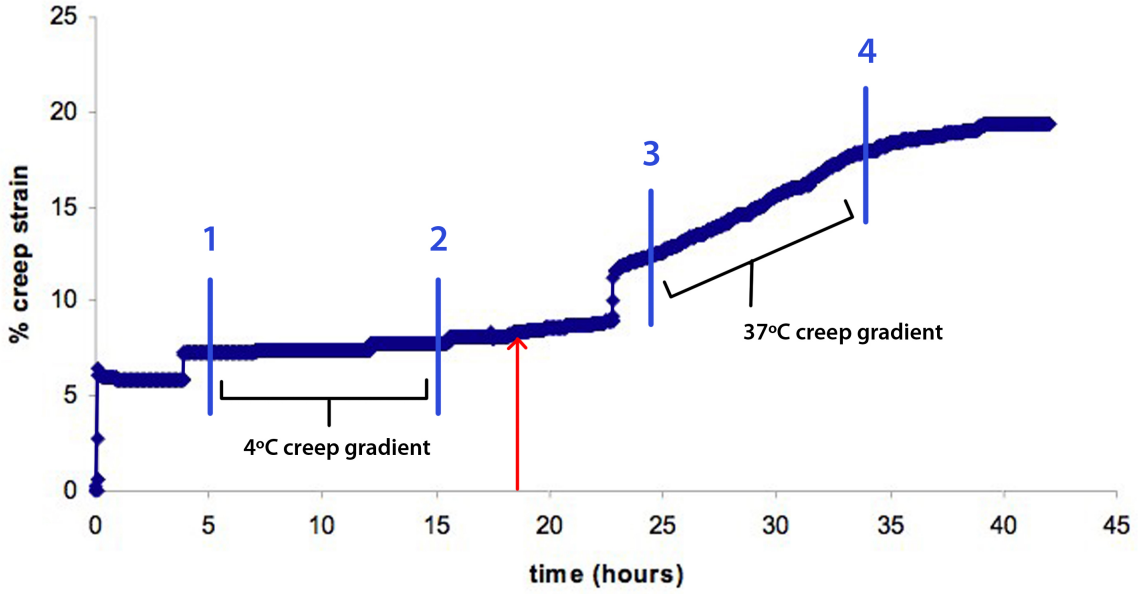


Fig 6 Effect of incubation temperature on creep rate in cell-seeded collagen sheets. Representative creep strain-time graph of normal TF fibroblasts in PC collagen sheet. The first phase (0-18hrs) was maintained at 4ºC after which the temperature was raised and maintained at 37ºC (red arrow) for a further 24hrs. Rate was calculated from the slope between points 1 and 2 for 4ºC, and between points 3 and 4 for 37ºC. (Note: the culture took approximately 6 hours to rise in temperature from 4ºC to 37ºC after the arrow)

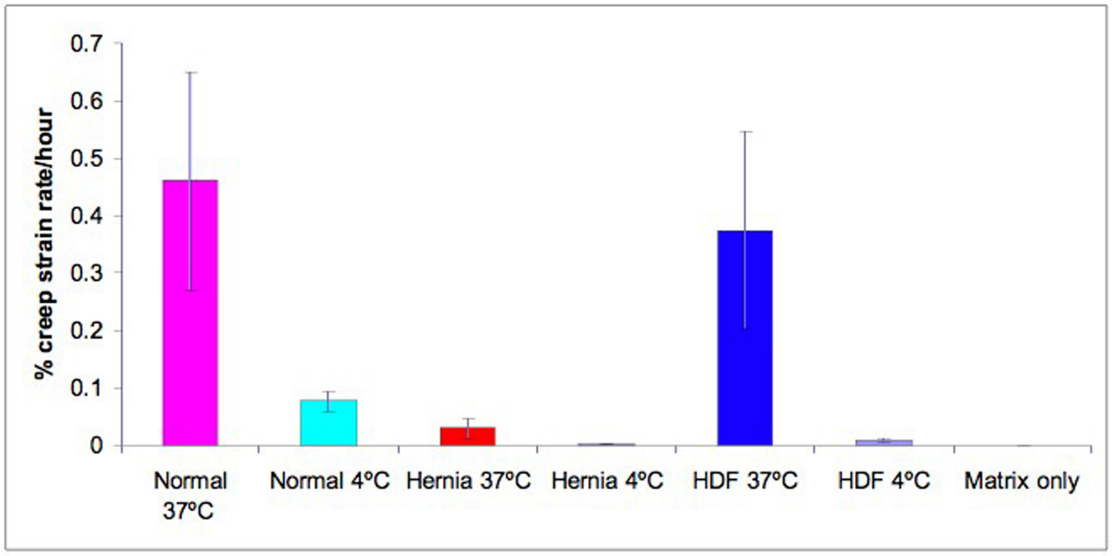


Fig 7 Histogram showing % creep strain rate/hour of normal TF vs. hernia TF vs. human dermal fibroblasts in PC collagen gel. Applied load corresponds to 15% break stress. At 37ºC the creep rate/hr for normal, hernia and HDF were 0.46 ± 0.19%, 0.03 ± 0.016% and 0.375 ± 0.17% respectively. At 4ºC the creep rate/hr for normal, hernia and HDF were 0.078 ± 0.017%, 0.0021 ± 0.0004% and 0.008 ± 0.003% respectively. The creep response of HDF was very similar to that of normal TF fibroblasts at 37ºC. This further highlights the reduced capacity for cell-mediated creep by hernia fibroblasts in this system

Mean creep-strain rate/hour at:	NTF	HTF	HDF
4ºC	0.08 ± 0.02%	0.002 ± 0%	0.01 ± 0.0%
37ºC	0.46 ± 0.19%	0.03 ± 0.02%	0.37 ± 0.17%

Table 3 Comparison of mean creep-strain rates/hour (with ± SD) of collagen gels seeded with normal TF (NTF) fibroblasts, hernia TF (HTF) fibroblasts and human dermal fibroblasts (HDF) at 4ºC and 37ºC