

IMPROVEMENT OF TENDON REPAIR USING MUSCLE GRAFTS TRANSDUCED WITH TGF- β 1 cDNA

Martin Majewski^{1*}, Ryan M. Porter^{2,4}, Oliver B. Betz², Volker M. Betz², Harald Clahsen³, Rudolf Flückiger² and Christopher H. Evans^{2,4}

¹ Orthopädische Klinik, Universität Basel, Basel, Switzerland

² Center for Molecular Orthopaedics, Brigham and Women's Hospital, Harvard Medical School, Boston, USA

³ Anatomisches Institut, Universität Düsseldorf, Düsseldorf, Germany

⁴ Center for Advanced Orthopaedic Studies, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, USA

Abstract

Tendon rupture is a common injury. Inadequate endogenous repair often leaves patients symptomatic, with tendons susceptible to re-rupture. Administration of certain growth factors improves tendon healing in animal models, but their delivery remains a challenge. Here we evaluated the delivery of TGF- β 1 to tendon defects by the implantation of genetically modified muscle grafts. Rat muscle biopsies were transduced with recombinant adenovirus encoding TGF- β 1 and grafted onto surgically transected Achilles tendons in recipient animals. Tissue regenerates were compared to those of controls by biomechanical testing as well as histochemical and immunohistochemical analyses. Healing was greatly accelerated when genetically modified grafts were implanted into tendon defects, with the resulting repair tissue gaining nearly normal histological appearance as early as 2 weeks postoperatively. This was associated with decreased deposition of type III collagen in favour of large fibre bundles indicative of type I collagen. These differences in tendon composition coincided with accelerated restoration of mechanical strength. Tendon thickness increased in gene-treated animals at weeks 1 and 2, but by week 8 became significantly lower than that of controls suggesting accelerated remodelling. Thus localised TGF- β 1 delivery via adenovirus-modified muscle grafts improved tendon healing in this rat model and holds promise for clinical application.

Keywords: Transforming growth factor- β ; tendon healing; gene transfer; muscle graft; adenovirus

Introduction

Tendon ruptures are common sporting injuries (Schepisis *et al.*, 2002). Such injuries have a certain ability to heal spontaneously via endogenous biological processes that occur during three overlapping phases: inflammation, regeneration, and remodelling coupled to maturation. During the first two phases, inflammatory and then mesenchymal cells enter the lesion where they proliferate, differentiate into tenocytes and lay down a collagenous matrix (Kajikawa *et al.*, 2007). Initially, this matrix contains a high proportion of type III collagen, characterised by small, poorly cross-linked fibrils that provide limited mechanical strength. During remodelling and maturation, these are replaced by larger, highly cross-linked fibres of type I collagen that provide considerable tensile strength. Although a functional tendon regenerate forms within 6-8 weeks of rupture, complete regeneration of the tendon is never achieved, leading to re-rupture in up to 10 % of cases (Longo *et al.*, 2009; Majewski *et al.*, 2002). Advances in the treatment of tendon rupture, such as percutaneous suturing (Ma and Griffith, 1977) or functional treatment (Thermann *et al.*, 1995) have improved outcomes, but it has become increasingly clear that further improvements in the healing of tendon ruptures will require the implementation of novel, biological approaches (DeFranco *et al.*, 2004; James *et al.*, 2008; Rees *et al.*, 2009). It is particularly important to accelerate the initial stages of healing, as re-rupture of repaired tendons normally occurs early in the healing process.

A number of different growth factors have been shown to promote one or more of the biological processes involved in tendon healing (Evans, 1999; Molloy *et al.*, 2003). These include fibroblast growth factor-2 (FGF-2) (Chan *et al.*, 2000), platelet-derived growth factor (PDGF) (Thomopoulos *et al.*, 2007), bone morphogenetic protein (BMP)-12 (growth and differentiation factor (GDF)-7; cartilage-derived growth factor (CDGF)-3) (Lou *et al.*, 2001), BMP-13 (GDF-6; CDMP-2) (Forslund and Aspenberg, 2001), BMP-14 (GDF-5; CDMP-1) (Rickert *et al.*, 2001), and transforming growth factor- β (TGF- β) (Kashiwagi *et al.*, 2004). Problems in delivering proteins to sites of tendon injury in a sustained fashion restrain clinical application of such growth factors. Gene transfer provides the basis of a strategy for obviating this limitation (Evans *et al.*, 2004; Hildebrand *et al.*, 2004). In principle, the transfer to the injury site of cDNA encoding appropriate factors could lead to their sustained, local, endogenous production, thereby eliminating the problems associated with the exogenous delivery of proteins.

*Address for Correspondence:

Dr. med. Martin Majewski
Orthopädische Universitätsklinik Basel
Spitalstrasse 21
CH-4031 Basel, Switzerland

Telephone number: 0041 61 328 78 13

Fax number: 0041 61 328 78 09

E-mail: majewski01@yahoo.de

Data from several previous experimental studies confirm the merit of this approach for delivering potentially reparative cDNAs to injured tendons. Relevant genes include those encoding BMP-12 (Lou *et al.*, 2001; Majewski *et al.*, 2008), BMP-14/GDF-5 (Basile *et al.*, 2008; Bolt *et al.*, 2007; Rickert *et al.*, 2005), FGF-2 (Tang *et al.*, 2008), interleukin-10 (Ricchetti *et al.*, 2008), TGF- β 1 (Hou *et al.*, 2009b), insulin-like growth factor-1 (Schnabel *et al.*, 2009), vascular endothelial growth factor (Hou *et al.*, 2009a), and PDGF (Suwalski *et al.*, 2010). Most such studies have used viral vectors for gene transfer because of their high efficiency. These vectors can be used by direct, *in vivo* application or in an indirect, *ex vivo* fashion. Although promising data have been reported following *in vivo* delivery (Bolt *et al.*, 2007; Lou *et al.*, 2001; Ricchetti *et al.*, 2008; Rickert *et al.*, 2005; Suwalski *et al.*, 2010; Tang *et al.*, 2008) tendons have low cellularity, a circumstance that limits opportunities for *in vivo* gene transfer. Moreover, *in vivo* gene transfer raises greater safety concerns when clinical application is considered.

Ex vivo delivery strategies often use mesenchymal stem cells (MSCs) as vehicles (Hou *et al.*, 2009a; Hou *et al.*, 2009b; Schnabel *et al.*, 2009), but in a clinical setting such manipulations are cumbersome, expensive and invasive. In response to these considerations, we have been interested in developing expedited *ex vivo* gene delivery technologies that can be accomplished conveniently in a single operative session (Evans *et al.*, 2007). One such approach involves the genetic modification of autologous muscle biopsies (Evans *et al.*, 2009; Evans *et al.*, 2007). As well as providing a convenient tissue for expedited gene transfer, muscle contains progenitor cells with the potential to contribute to tendon healing (Pelinkovic *et al.*, 2003).

In previous experiments we have used a recombinant adenovirus vector to deliver BMP-12 cDNA to muscle biopsies that were then sutured to sites of Achilles tendon rupture in a rat model (Majewski *et al.*, 2008). BMP-12 was selected as a morphogen that promotes the differentiation of progenitor cells into tenocytes (Violini *et al.*, 2009; Wang *et al.*, 2005). In the present experiments, we have used adenovirus to deliver TGF- β 1 cDNA as a growth factor that promotes a different component of the healing process, the deposition of a collagenous matrix.

Materials and Methods

Study design

We used 133 adult male Sprague-Dawley rats, each weighing 400-425 g. Each experimental group consisted of 40 rats. The first group of 40 animals (surgical control) received no muscle graft after transection and suturing of the tendon. The second group (muscle control) received a muscle graft without modification by recombinant adenovirus. The third group (Ad.TGF- β 1 group) received a muscle graft transduced with a recombinant adenovirus carrying TGF- β 1 cDNA (Ad.TGF- β 1). Ten rats per timepoint were sacrificed after 1, 2, 4 and 8 weeks. For each time-point, 7 animals were used for biomechanical testing and 3 animals for histological examination. Four

additional rats were used for muscle harvesting and 9 rats were used to test for transduction after 1, 3 and 7 d *in vitro*. Approval for all procedures was obtained beforehand from the local Institutional Animal Care and Use Committee (Boston, MA, USA).

Vector preparation

A first generation (E1/E3-deleted), serotype 5, recombinant adenoviral vector carrying the cDNA for human TGF- β 1 (Ad.TGF- β 1) was constructed by Cre-lox recombination as described earlier (Hardy *et al.*, 1997). The transgene, under transcriptional control of the human cytomegalovirus immediate early promoter, was inserted into the E1 domain. Recombinant adenoviruses were propagated in 293/Cre8 cells. High-titer preparations were generated by amplifications in 293 cells, purification on CsCl gradients, and dialysis against 4 % sucrose with 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, and 10 mM MgCl₂.

Transduction of muscle graft

Skeletal muscle tissue was harvested with a round punch of 4 mm diameter from a donor rat 1 d before Achilles tendon surgery. To transduce the muscle discs, 5×10^{10} Ad.TGF- β 1 particles were dropped onto the surface of the muscle, and the grafts were incubated for 30 min at 37 °C and 5 % CO₂ without further dilution to enhance muscle cell modification. After this incubation, the muscle grafts were cultured in 1 mL DMEM medium containing 5 % foetal bovine serum in 24-well plates for 1 d in an incubator with 37 °C and 5 % CO₂. To confirm transgene expression, additional muscle grafts were transduced as described above, and conditioned media samples were tested after 1, 3 and 7 d for TGF- β 1 secretion by ELISA (R&D Systems, Minneapolis, MN, USA).

Surgical procedure

Rats were placed under general anaesthesia by the administration of isoflurane using a precision vapouriser. The animals then received intramuscular injections of 20 mg/kg cefazolin (antibiotic) and 0.06 mg/kg buprenorphine (analgesic) into the left thigh. Each animal's right hind leg was shaved and disinfected with Betadine®-Scrub 3x using aseptic techniques and rinsed with 70 % alcohol. Subsequently, it was transferred to a heated surgery table and covered with a sterile surgical drape such that only the prepared limb was exposed.

To generate the tendon defect, a 3 cm incision was first made above the right Achilles tendon and the superficial Achilles tendon was exposed. The peritendon was opened and the tendon transected with a no. 15 scalpel blade perpendicular to the collagen fibres, creating a gap 5 mm proximal to the calcaneal insertion of the tendons. The plantar tendon was transected to prevent an internal splint phenomenon. After transection, the Achilles tendon was sutured back together with PDS II 0-2 with a Kessler-Kirchmeyer stitch. The muscle graft for groups 2 and 3 was placed with two single stitches with PDS II 0-5 around the side of tendon injury after end-to-end suture repair. The subcutaneous layer and the skin were closed by continuous suture technique with Prolene 0-4.

Post-operative treatment

The animals woke up on a heating pad to aid the return to normal body temperature. During the next three days, animals received 20 mg/kg i.m. injections of cefazolin and 0.06 mg/kg i.m. injections of buprenorphine, each twice per day. For the duration of the experiment, all animals were monitored for signs of pain and infection. After the operation, no cast or dressing was applied. The animals were allowed unrestricted cage movement and provided rat chow and water *ad libitum*.

Euthanasia

Groups of animals were euthanised after 7, 14, 28 and 56 d by CO₂ asphyxiation under general anaesthesia and subsequent bilateral thoracotomy. Muscle–Achilles tendon–bone units were harvested by transection of the middle part of the gastrocnemius muscle and of the calcaneus.

Mechanical testing

The muscle-tendon-bone units were wrapped in cotton gauze soaked in lactated Ringer's solution and stored at -20 °C before testing. On the day of testing, the specimens were thawed in Ringer's solution for 4 h. Before testing, tendon thickness was measured with a precision calliper at the former site of tendon repair. The muscle-tendon-bone units were fastened in the clamping device by freezing the muscular segment between the cryo-jaws and by fixing the bony segment between the copper clamp (Wieloch *et al.*, 2004). The clamping device was attached to an electro-hydraulic materials testing machine (Fa. Zwick, Einsingen-Ulm, Germany). The tendons were not preconditioned or cyclically stretched prior to tensile testing. The room temperature was kept constant at 25 °C. To prevent the specimens from dehydrating they were kept moist with Ringer's solution. Both reservoirs of the cryo-jaws were filled with liquid nitrogen. The experiment was started as soon as the expansion of the freezing-zone reached the border of the metal clamp but did not extend into the tendon substance or into the repair site (registered manually with a metal-needle). The displacement rate was set at 1000 mm/min. Force-displacement curves were recorded and transferred to an IBM-compatible computer for subsequent data analysis. Load to failure (N, peak of the curve) and stiffness (N/mm) were measured. Load values were not corrected for area of tendon.

Histology

For each group 3 specimens were examined per time point per group, with 5 sections for each specimen, giving a total of 15 sections per time point per group. The tendons were immediately fixed in 4 % buffered formalin (pH 7.4) for 24 h, dehydrated and embedded in paraffin wax. Longitudinal sections (5 μ m) at the mid-substance of the tendon were stained with haematoxylin and eosin. Collagen III was specifically detected using the primary rabbit anti-rat collagen type III polyclonal antibody (1:300, AbD Serotec, Dusseldorf, Germany) and visualised with biotinylated goat anti-rabbit IgG antibodies (1:50, B6648, E8386, Sigma, Hamburg, Germany) and extavidin peroxidase. Collagen staining was quantified using a digital image

analysing software (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA). The results are expressed as percentage of the total area staining for type III collagen. Additional immunohistochemical staining for TGF- β 1 (rabbit polyclonal; Santa Cruz, CA, USA) was performed.

Cross polarisation microscopy

For cross polarisation microscopy, the same number of sections was used as for histology (15 per time point per group). 5 μ m sections were stained for 1 h in picosirius solution (0.1 % solution of Sirius Red F3BA in saturated aqueous picric acid, pH 2) according to the method of Junqueira (Junqueira *et al.*, 1979). The sections were washed for 2 min in 0.01 N HCl, dehydrated, cleared and mounted in synthetic resin. To analyse collagen ratios, tissue samples were observed under polarised light microscopy. Thicker mature collagen fibres, including type I collagen fibres, appeared red-orange, whereas thinner collagen fibres, including type III collagen appeared pale-green (Junqueira *et al.*, 1978).

For each sample 5 regions (centre of tendon, left border, right border, 2 mm above tendon centre, 2 mm below tendon centre) within the interface (400x, area 100 μ m x 100 μ m) were captured by a digital camera (Olympus C-3030, Hamburg, Germany). Collagen ratios were obtained by measuring the area of thicker mature red-orange versus thinner pale-green using a digital image analysing software (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA).

Semiquantitative histological analysis

For additional objective information, histological sections were analysed with a scoring system based on the Bonar score, validated by Maffulli *et al.* (Maffulli *et al.*, 2008). Sections were evaluated for tenocyte appearance (0 = normal to 3 = large round nucleus with abundant cytoplasm), collagen ordering (0 = normal to 3 = separation of fibres with complete loss of architecture) and number of cells. Two blinded investigators performed the evaluation. Mean tenocyte appearance and collagen ordering, as well as mean cell count divided by ten were averaged to give a score for each time point.

Statistics

The Mann Whitney test was used to test for statistically significant differences between the cohorts. The level of significance was set to $p < 0.05$.

Results

Consistent with previous findings (Evans *et al.*, 2009; Majewski *et al.*, 2008), the muscle biopsies were efficiently transduced with the adenovirus vector. When maintained in tissue culture, muscle discs secreted nanogramme quantities of TGF- β 1 into the medium for at least one week (data not shown). Sutures of the operated tendons had not yet resorbed completely by the time of mechanical testing. Mechanical testing revealed that defects treated with TGF- β cDNA had a much higher maximum load to failure than controls after 1 week. Indeed, at the 1 week

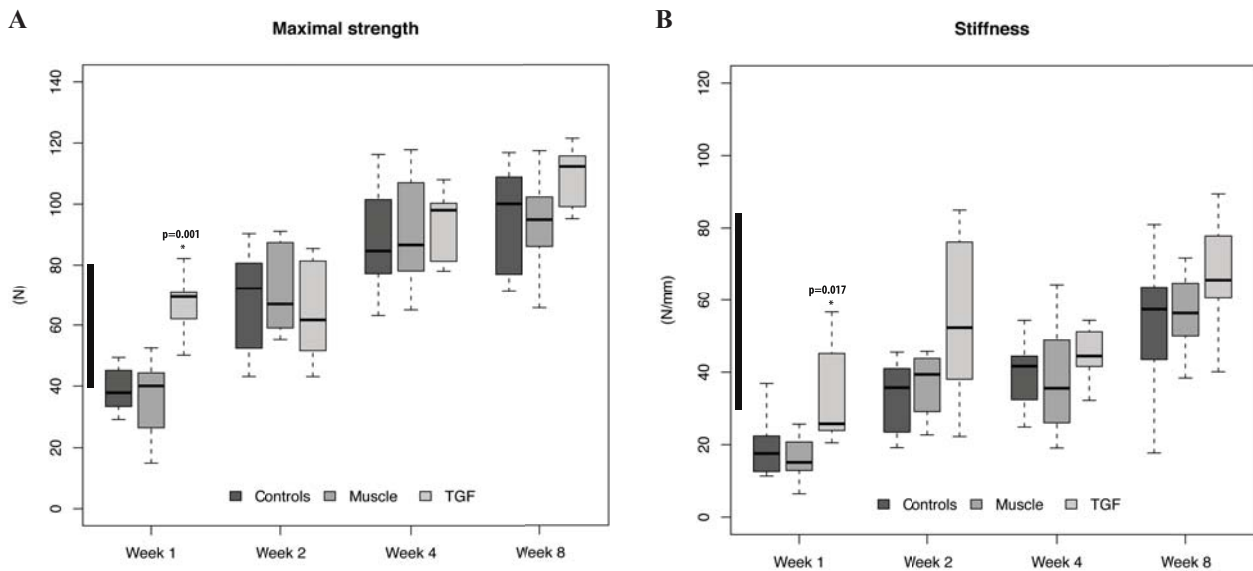


Fig. 1. Effect of TGF- β 1 cDNA delivery on the mechanical properties of the rat Achilles tendon at progressive times after transection. **(A)** Maximum load to failure and **(B)** stiffness were determined using explanted muscle-tendon-bone units. For each plot, the thick horizontal bars within each box indicate the median, the top and bottom of each box indicate the upper quartile and the lower quartile, and the outer bars show the highest and lowest values that were measured. Statistical significance is indicated by an asterisk. Range of normal tendon is indicated by the black bar on the y-axis.

time point, the strength of defects treated with TGF- β cDNA was already well within the normal range of 60-80 N (Fig. 1A). Defects treated with TGF- β cDNA also had much greater stiffness at weeks 1 and 2 (Fig. 1B) and were within the 30-85 N/mm range of normal tendon at 2 weeks. Genetic delivery of TGF- β also increased tendon thickness at weeks 1 and 2, after which time thickness declined. By 4 weeks, there was no significant difference from controls. At 8 weeks, the regenerate formed in response to the TGF- β cDNA was less thick than the healing controls, although it was still thicker than normal tendon (Fig. 2).

Histological examination of the repair tendon tissue showed that by 2 weeks delivery of TGF- β cDNA led to the formation of well-organised, tendinous tissue with large collagen fibres. Control specimens, in contrast, showed more tissue with a disorganised matrix and greater cellularity (Fig. 3). Scoring of the histological slides displayed better scores for the TGF group from 2 weeks onwards (Fig. 4). Quantitative immunohistochemistry (Fig. 5) confirmed that delivery of TGF- β cDNA suppressed the expression of type III collagen that is associated with repair tissue. The collagenous components of the matrix were further investigated by polarised light microscopy in conjunction with sirius red staining (Fig. 3). Two weeks post-operatively the majority of the collagen fibres in the defects treated with TGF- β cDNA stained orange, indicating the presence of large fibres indicative of type I collagen. The fibres of control defects, in contrast, contained a considerable proportion of smaller fibres staining green, indicative of type III collagen (Fig. 6).

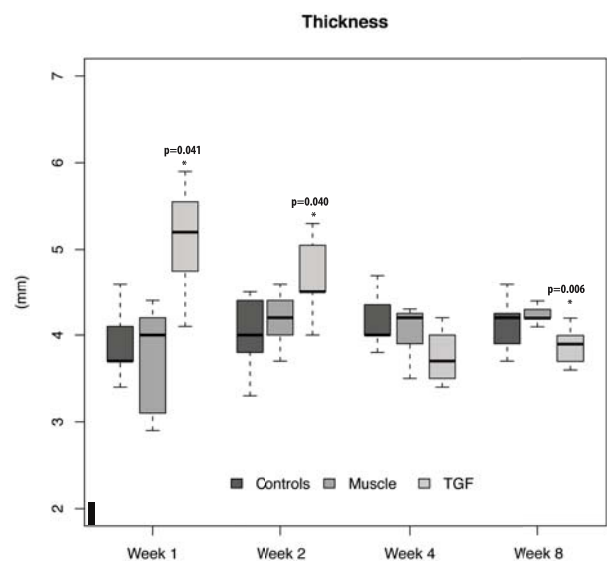


Fig. 2. Effect of TGF- β 1 cDNA delivery on the thickness of the repair site in the rat Achilles tendon. Thickness of the explanted tendons was measured in mm. The thick horizontal bars within each box indicate the median, the top and bottom of each box indicate the upper quartile and the lower quartile and the outer bars show the highest and lowest values that were measured. Statistical significance is indicated by an asterisk. Range of normal tendon is indicated by the black bar on the y-axis.

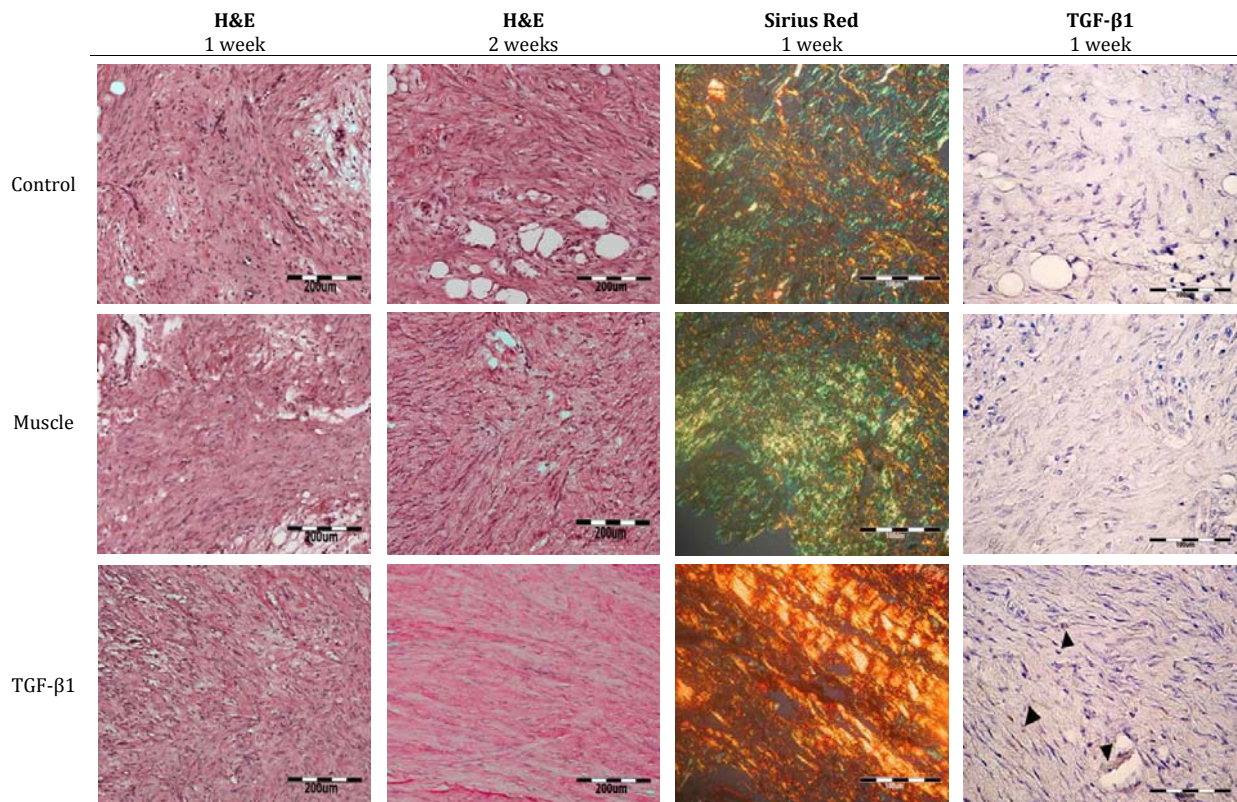


Fig. 3. Effect of TGF- β 1 cDNA delivery on the histological appearance of the repair site in the rat Achilles tendon. H&E sections: Compared to untreated lesions (Control) and lesions receiving untransduced muscle (Muscle), the TGF- β 1 group (TGF- β 1) showed larger collagen bundles in a parallel orientation, generally reduced number of cells between the collagen bundles and less scar tissue. Sirius Red sections under polarised light: large collagen fibres stain orange-red and small fibrils stain green. Remodelling of the matrix was much slower in untreated lesions (Control) and lesions receiving untransduced muscle (Muscle) than in the TGF- β 1 group (TGF- β 1), as indicated by the greater fraction of thinner, green-staining fibrils relative to red-orange fibres. Immunohistochemical staining for TGF- β 1: the TGF- β 1 group displays the greatest amount of stained TGF- β 1 compared to untreated lesions (Control) or lesions receiving untransduced muscle (Muscle). Black and striped bars in the H&E images represent 200 μ m, whereas black and striped bars in the sirius red and immunohistochemical images represent 100 μ m.

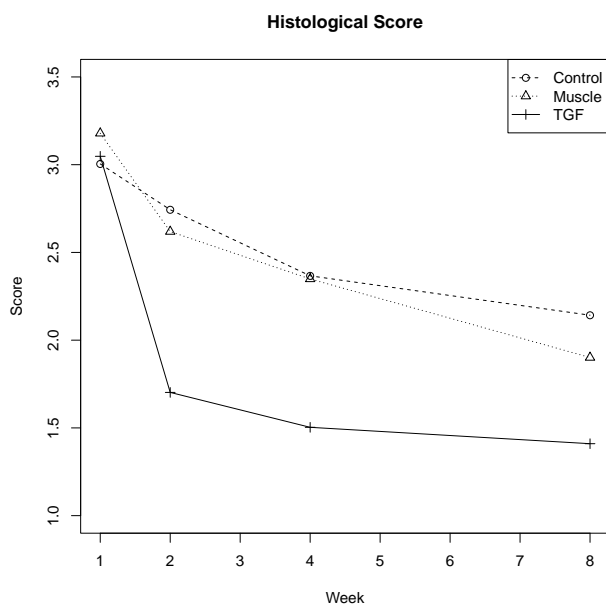


Fig. 4. Semiquantitative scoring of histological slides. Semiquantitative scoring for tenocyte appearance, collagen organisation and cell count was performed for all histological slides. Mean values are displayed for each time point and group.

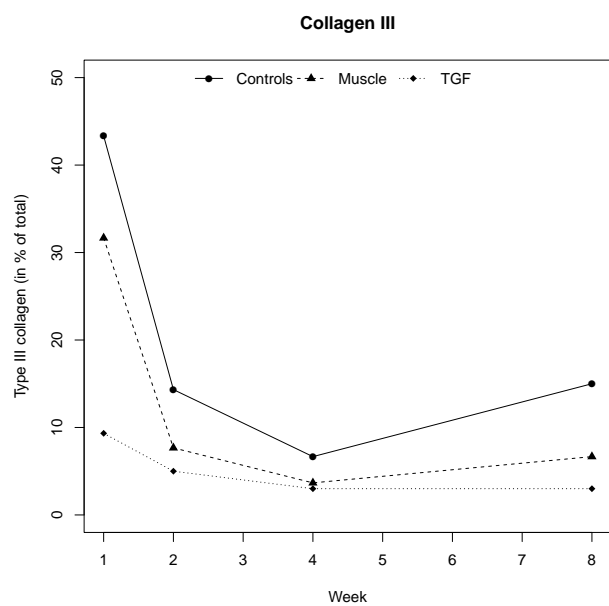


Fig. 5. Effect of TGF- β 1 cDNA delivery on the amount of collagen type III within the tendon defect. Quantitative immunohistochemistry was used to determine the percentage of type III collagen within the collagenous matrix of the defects. Collagen staining was calculated using a digital image analysing software.

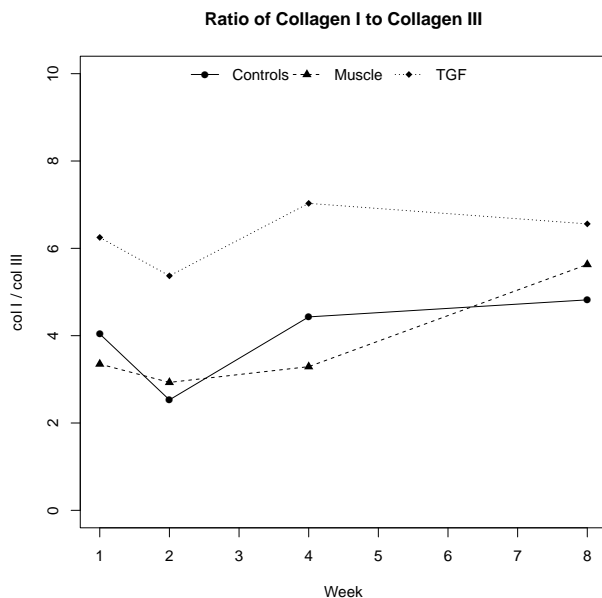


Fig. 6. Ratio of collagen I to collagen III as measured with Sirius red and polarised light microscopy. The ratio of thick fibres (stained red-orange under sirius red-polarised light microscopy) to thin fibres (stained green under Sirius red-polarised light microscopy) was calculated at each time point. The analysis was performed using a digital image analysing software.

Discussion

These data suggest that *ex vivo* delivery of TGF- β 1 cDNA to sites of tendon rupture using muscle biopsies accelerates the early repair response of rat Achilles tendon. This was seen as an early recovery of mechanical strength which, in turn, is likely to reflect the precocious deposition of type I collagen and the attenuated deposition of type III collagen. Histologically, the matrix showed greater organisation, lower cellularity and an emerging crimp pattern. A rapid increase in mechanical strength is important clinically, because re-rupture of a tendon commonly occurs early in the repair process.

Although we did not measure the duration of TGF- β 1 transgene expression *in vivo*, genetically modified muscle continued to express high levels of TGF- β 1 for at least one week *in vitro*. This suggests that TGF- β 1 expression persists locally within the lesion during the critical early stages of healing, which is consistent with the early gains in mechanical strength and thickness noted here. Under *in vivo* conditions, first generation adenovirus vectors typically express transgenes at high levels for about 2 weeks. In this context, it is worth noting that no adhesions were observed at the time of explantation in the treatment group. Adhesions form as a result of prolonged exposure to TGF- β 1. If exposure to TGF is short, as is likely to be the case here, it may improve biomechanical stability without increasing adhesion formation. Exposure to TGF- β 1 during this time-frame would also explain the rapid increase in thickness of the genetically-treated regenerate, accompanied by increases in stiffness and strength. By 8

weeks these differences have largely disappeared, probably due to tapering of TGF- β 1 expression and catching up by the innate healing process. The important point, however, is the dramatic early increase in these properties, during the time when the tendon is most liable to re-rupture.

These data are in qualitative agreement with those of Hou *et al.* (Hou *et al.*, 2009a), who employed adenovirus to deliver TGF- β cDNA in an *ex vivo* fashion to injured rabbit Achilles tendon using MSCs. Like us, they found that TGF- β cDNA accelerated healing, based upon histology, collagen analysis and mechanical strength. In a subsequent study, the same group (Hou *et al.*, 2009b) confirmed that delivery of TGF- β cDNA promoted the synthesis of type I collagen and improved the ratio of type I to type III collagen. In these studies, TGF- β expression was detected up to 56 d. In the present study, TGF- β expression in transfected muscle was measured for a period of 7 d and might be increased beyond this time point. However, immunohistochemical staining for TGF- β confirmed its elevated presence only in the first week.

Compared to the genetic delivery of BMP-12 in this model, which we examined in an earlier study (Majewski *et al.*, 2008), TGF- β gives a larger increase in mechanical strength during the first week, but in other respects the results are strikingly similar. Given that BMP-12 and TGF- β are likely to act on different components of the healing process, they have the potential to act synergistically when co-administered. This possibility has not yet been examined experimentally. Alternative isoforms of TGF- β also remain to be evaluated. Transfer of cDNAs encoding BMP-14/GDF-5 (Bolt *et al.*, 2007; Rickert *et al.*, 2005), PDGF (Suwalski *et al.*, 2010), and FGF-2 (Tang *et al.*, 2008) have also shown promise in treating animal models of tendon damage, but differences in the models, methods of gene delivery, and other variables preclude direct comparison with the present work.

The precise mechanisms through which TGF- β expression accelerates healing in our model are unknown. From what is known about the properties of TGF- β we can suggest that it enhances collagen synthesis and the differentiation of mesenchymal progenitor cells. Muscle is a rich source of such cells, and could thus provide progenitors to the healing tendon. Histology did not provide evidence for this, but it should be a subject of future research.

A limitation of the present study is the use of transected rat Achilles tendon, which does not accurately reflect human Achilles tendon tears that arise as a result of tendinopathy (Lui *et al.*, 2011). There is a struggle to find an appropriate model for the human condition, which has not yet produced satisfying results (Longo *et al.*, 2011). However, it is crucial to understand this vastly complex system of healing on a simplified level, for which precisely this simplified injury model has been chosen.

A further limitation was that sutures had not dissolved completely by the time of biomechanical testing. However, since all tendons were sutured in the exact same way, differences in load bearing capacity were only due to the different tissue properties at the site of repair.

Regardless of the transgene that is used, successful translation of these concepts into the clinic will require careful attention to additional matters such as cost, safety and practicality (Evans *et al.*, 2007). As noted, *ex vivo* delivery using expanded, autologous cells and the *in vivo* delivery of most viral vectors are likely to be problematic. In response to these constraints, Basile *et al.* (Basile *et al.*, 2008) have developed a technology whereby recombinant adeno-associated virus (AAV) vectors are freeze-dried onto allograft tendon, thus forming a construct that can be implanted into a defect. Because AAV is relatively stable, such constructs have the potential to provide an off the shelf product. Proof of principle was demonstrated in a murine, flexor digitorum longus tendon model with GDF-5 as the transgene. The muscle biopsy method we used in the present work provides an additional strategy. In a clinical setting, the autologous muscle biopsy could be harvested, genetically modified and implanted in a single operative session, thereby reducing cost and complexity. Before contemplating human application, it is necessary to consider the limitations of rodent models such as the one used here, and the need for large animal studies (Evans, 2011). Moreover, the use of gene therapy always raises safety concerns (Evans *et al.*, 2011).

Acknowledgments

We are grateful to Mrs H. Schaller and Mrs C. Pilapil for histology preparation, Mrs E. Krott for assistance during histology examination. Dr. L. Dürselen for help during mechanical testing, and Mr A. Todorov for proofreading and help with graphics. This work was supported by NIH grant number R01 AR052809 to CHE from NIAMS.

References

Basile P, Dadali T, Jacobson J, Hasslund S, Ulrich-Vinther M, Soballe K, Nishio Y, Drissi MH, Langstein HN, Mitten DJ, O'Keefe RJ, Schwarz EM, Awad HA (2008) Freeze-dried tendon allografts as tissue-engineering scaffolds for Gdf5 gene delivery. *Mol Ther* **16**: 466-473.

Bolt P, Clerk AN, Luu HH, Kang Q, Kummer JL, Deng Z-L, Olson K, Primus F, Montag AG, He T-C, Haydon RC, Toolan BC (2007) BMP-14 gene therapy increases tendon tensile strength in a rat model of Achilles tendon injury. *J Bone Joint Surg Am* **89**: 1315-1320.

Chan BP, Fu S, Qin L, Lee K, Rolf CG, Chan K (2000) Effects of basic fibroblast growth factor (bFGF) on early stages of tendon healing: a rat patellar tendon model. *Acta Orthop Scand* **71**: 513-518.

DeFranco MJ, Derwin K, Iannotti JP (2004) New therapies in tendon reconstruction. *J Am Acad Orthop Surg* **12**: 298-304.

Evans CH (1999) Cytokines and the role they play in the healing of ligaments and tendons. *Sports Med* **28**: 71-76.

Evans CH (2011) Barriers to the clinical translation of orthopedic tissue engineering. *Tissue Eng Part B Rev* **17**:437-441.

Evans CH, Ghivizzani SC, Robbins PD (2004) The 2003 Nicolas Andry Award. Orthopaedic gene therapy. *Clin Orthop Relat Res* **429**: 316-329.

Evans CH, Palmer GD, Pascher A, Porter R, Kwong FN, Gouze E, Gouze JN, Liu F, Steinert A, Betz O, Betz V, Vrahas M, Ghivizzani SC (2007) Facilitated endogenous repair: making tissue engineering simple, practical, and economical. *Tissue Eng* **13**: 1987-1993.

Evans CH, Liu FJ, Glatt V, Hoyland JA, Kirker-Head C, Walsh A, Betz O, Wells JW, Betz V, Porter RM, Saad FA, Gerstenfeld LC, Einhorn TA, Harris MB, Vrahas MS (2009) Use of genetically modified muscle and fat grafts to repair defects in bone and cartilage. *Eur Cell Mater* **18**: 96-9111.

Evans CH, Ghivizzani SC, Robbins PD (2011) Orthopaedic gene therapy – lost in translation? *J Cell Physiol* **227**:416-420.

Forslund C, Aspenberg P (2001) Tendon healing stimulated by injected CDMP-2. *Med Sci Sports Exerc* **33**: 685-687.

Hardy S, Kitamura M, Harris-Stansil T, Dai Y, Phipps ML (1997) Construction of adenovirus vectors through Cre-lox recombination. *J Virol* **71**: 1842-1849.

Hildebrand KA, Frank CB, Hart DA (2004) Gene intervention in ligament and tendon: current status, challenges, future directions. *Gene Ther* **11**: 368-378.

Hou Y, Mao Z, Wei X, Lin L, Chen L, Wang H, Fu X, Zhang J, Yu C (2009a) Effects of transforming growth factor-beta1 and vascular endothelial growth factor 165 gene transfer on Achilles tendon healing. *Matrix Biol* **28**: 324-335.

Hou Y, Mao Z, Wei X, Lin L, Chen L, Wang H, Fu X, Zhang J, Yu C (2009b) The roles of TGF-beta1 gene transfer on collagen formation during Achilles tendon healing. *Biochem Biophys Res Commun* **383**: 235-239.

James R, Kesturu G, Balian G, Chhabra AB (2008) Tendon: biology, biomechanics, repair, growth factors, and evolving treatment options. *J Hand Surg Am* **33**: 102-112.

Junqueira LC, Cossermelli W, Brentani R (1978) Differential staining of collagens type I, II and III by Sirius Red and polarization microscopy. *Arch Histol Jpn* **41**: 267-274.

Junqueira LC, Bignolas G, Brentani RR (1979) Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem J* **11**: 447-455.

Kajikawa Y, Morihara T, Watanabe N, Sakamoto H, Matsuda K, Kobayashi M, Oshima Y, Yoshida A, Kawata M, Kubo T (2007) GFP chimeric models exhibited a biphasic pattern of mesenchymal cell invasion in tendon healing. *J Cell Physiol* **210**: 684-691.

Kashiwagi K, Mochizuki Y, Yasunaga Y, Ishida O, Deie M, Ochi M (2004) Effects of transforming growth factor-beta 1 on the early stages of healing of the Achilles tendon in a rat model. *Scand J Plast Reconstr Surg Hand Surg* **38**: 193-197.

Longo UG, Ronga M, Maffulli N (2009) Acute ruptures of the achilles tendon. *Sports Med Arthrosc* **17**: 127-138.

Longo UG, Forriol F, Campi S, Maffulli N, Denaro V (2011) Animal Models for Translational Research on

Shoulder Pathologies: From Bench to Bedside. Sports Medicine and Arthroscopy Review **19**: 184-193

Lou J, Tu Y, Burns M, Silva MJ, Manske P (2001) BMP-12 gene transfer augmentation of lacerated tendon repair. J Orthop Res **19**: 1199-1202.

Lui PPY, Maffulli N, Rolf C, Smith RKW (2011) What are the validated animal models for tendinopathy? Scand J Med Sci Sports **21**: 3-17.

Ma GW, Griffith TG (1977) Percutaneous repair of acute closed ruptured achilles tendon: a new technique. Clin Orthop Relat Res **128**: 247-255.

Maffulli N, Longo UG, Franceschi F, Rabitti C, Denaro V (2008) Movin and Bonar scores assess the same characteristics of tendon histology. Clin Orthop Relat Res **466**: 1605-1611.

Majewski M, Widmer KH, Steinbruck K (2002) [Achilles tendon ruptures: 25 year's experience in sport-orthopedic treatment]. Sportverletz Sportschaden **16**: 167-173.

Majewski M, Betz O, Ochsner PE, Liu F, Porter RM, Evans CH (2008) *Ex vivo* adenoviral transfer of bone morphogenetic protein 12 (BMP-12) cDNA improves Achilles tendon healing in a rat model. Gene Ther **15**: 1139-1146.

Molloy T, Wang Y, Murrell G (2003) The roles of growth factors in tendon and ligament healing. Sports Med **33**: 381-394.

Pelinkovic D, Lee JY, Engelhardt M, Rodosky M, Cummins J, Fu FH, Huard J (2003) Muscle cell-mediated gene delivery to the rotator cuff. Tissue Eng **9**: 143-151.

Rees JD, Maffulli N, Cook J (2009) Management of tendinopathy. Am J Sports Med **37**: 1855-1867.

Ricchetti ET, Reddy SC, Ansoorge HL, Zgonis MH, Van Kleunen JP, Liechty KW, Soslowsky LJ, Beredjikian PK (2008) Effect of interleukin-10 overexpression on the properties of healing tendon in a murine patellar tendon model. J Hand Surg Am **33**: 1843-1852.

Rickert M, Jung M, Adiyaman M, Richter W, Simank HG (2001) A growth and differentiation factor-5 (GDF-5)-coated suture stimulates tendon healing in an Achilles tendon model in rats. Growth Factors **19**: 115-126.

Rickert M, Wang H, Wieloch P, Lorenz H, Steck E, Sabo D, Richter W (2005) Adenovirus-mediated gene transfer of growth and differentiation factor-5 into tenocytes and the healing rat Achilles tendon. Connect Tissue Res **46**: 175-183.

Schepsis AA, Jones H, Haas AL (2002) Achilles tendon disorders in athletes. Am J Sports Med **30**: 287-305.

Schnabel LV, Lynch ME, van der Meulen MCH, Yeager AE, Kornatowski MA, Nixon AJ (2009) Mesenchymal stem cells and insulin-like growth factor-I gene-enhanced mesenchymal stem cells improve structural aspects of healing in equine flexor digitorum superficialis tendons. J Orthop Res **27**: 1392-1398.

Suwalski A, Dabboue H, Delalande A, Bensamoun SF, Canon F, Midoux P, Saillant G, Klatzmann D, Salvétat J-P, Pichon C (2010) Accelerated Achilles tendon healing by PDGF gene delivery with mesoporous silica nanoparticles. Biomaterials **31**: 5237-5245.

Tang JB, Cao Y, Zhu B, Xin K-Q, Wang XT, Liu PY (2008) Adeno-associated virus-2-mediated bFGF gene

transfer to digital flexor tendons significantly increases healing strength. an *in vivo* study. J Bone Joint Surg Am **90**: 1078-1089.

Thermann H, Frerichs O, Biewener A, Krettek C, Schandelmeier P (1995) [Functional treatment of acute rupture of the Achilles tendon. An experimental biomechanical study]. Unfallchirurg **98**: 507-513.

Thomopoulos S, Zaegel M, Das R, Harwood FL, Silva MJ, Amiel D, Sakiyama-Elbert S, Gelberman RH (2007) PDGF-BB released in tendon repair using a novel delivery system promotes cell proliferation and collagen remodeling. J Orthop Res **25**: 1358-1368.

Violini S, Ramelli P, Pisani LF, Gorni C, Mariani P (2009) Horse bone marrow mesenchymal stem cells express embryo stem cell markers and show the ability for tenogenic differentiation by *in vitro* exposure to BMP-12. BMC Cell Biol **10**: 29-29.

Wang Q-W, Chen Z-L, Piao Y-J (2005) Mesenchymal stem cells differentiate into tenocytes by bone morphogenetic protein (BMP) 12 gene transfer. J Biosci Bioeng **100**: 418-422.

Wieloch P, Buchmann G, Roth W, Rickert M (2004) A cryo-jaw designed for *in vitro* tensile testing of the healing Achilles tendons in rats. J Biomech **37**: 1719-1722.

Discussion with Reviewers

Reviewer I: The thickness of the TGF- β 1 treated tendon never returns close to normal but is similar to untreated tendon by week 8, suggesting an inadequate heal. Why does TGF- β 1 not continue to "improve" the thickness? This has not been discussed at all. Is TGF- β 1 the "wrong" isoform, and where does TGF- β 3 come into play in the healing process? Do these need to be applied sequentially to improve the heal further?

Authors: The precise mechanisms through which TGF- β expression accelerates healing in our model are unknown. From what is known about the properties of TGF- β , we can suggest that it enhances collagen synthesis and the differentiation of mesenchymal progenitor cells. Muscle is a rich source of such cells, and could thus provide progenitors to the healing tendon. Histology did not provide evidence for this, but it should be a subject of future research. The literature suggests that all isoforms of TGF- β stimulate the synthesis of types I and III collagen by tendon fibroblasts to a similar degree. There are a few *in vivo* studies in which the ability of the different isoforms to promote tendon healing has been directly compared. Such data do not suggest large quantitative or qualitative differences between the effects of the different isoforms. However, this matter deserves further experimental investigation. The sequential application of different growth factors as healing progresses is an attractive general concept. However, much more research is needed to determine which factors to add, how much to add, when to add them, and how to add them.

Reviewer II: Do the transfected muscle biopsies continue to produce TGF- β for the duration of the experiment? Are

there any quantitative differences in the fibril diameter and organisation at 8 weeks, between the controls and the TGF- β transfected biopsies?

Authors: When maintained in tissue culture, muscle discs secreted nanogramme quantities of TGF- β 1 into the medium for at least one week. The immunohistochemistry shown in the present article confirms that TGF- β 1

expression persists locally within the lesion during the critical early stages of healing, which is consistent with the early gains in mechanical strength and thickness. The percentage of collagen III did not differ significantly between the 3 groups after 4 and 8 weeks. Also, after 8 weeks there was no significant difference in fibril diameter and organisation noted in our semiquantitative analysis.