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Dense Connective Tissue Repair: The Intricacies of Monitoring Actin Isoforms in Native Tissue Models In Vitro

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ABSTRACT

Dense Connective Tissue Repair: The Intricacies of Monitoring Actin Isoforms in Native Tissue Models In Vitro

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In preparation to quantify beta-actin and alpha-smooth muscle actin content in loaded and unloaded native rat tail tendon organ culture models, several methodologies were developed in the present paper. Since alpha-smooth muscle actin has not been monitored in tendon in vitro, developing the methods to culture and quantitatively detect alpha-smooth muscle actin, the protein marker for fibroblast to myofibroblast transition were developed. In addition, quantifying the amount of alpha-smooth muscle actin mRNA, using reverse transcription polymerase chain reaction (RT-PCR) assays was investigated and novel oligonucleotide primers were developed. A method to identify four highly conserved actin isoforms; beta cytoplasmic, gamma cytoplasmic, alpha-smooth muscle and gamma smooth muscle actin, in a tissue environment was created.

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Thank you for investing, time or money, in a young scientific investigator and furthering my passion for dense connective tissue.

Dense Connective Tissue Repair: The Intricacies of Monitoring Actin Isoforms in Native Tissue Models In Vitro

INTRODUCTION

Force is all around us (extracellular), acting on our tissues, and is necessary to develop and maintain fully functional tissue. This surrounding force, i.e. gravity, has been investigated extensively; however, the forces generated within a tissue are an important aspect to consider as well. The process of building dense connective tissue and tissue repair can be further understood by looking at this intercellular force generation. This has lead to an interest in the fibroblast to myofibroblast transition, as myofibroblasts are generally considered to produce greater force and contraction, being coined the, "muscle-fibroblast." Alpha-smooth muscle actin protein has been determined a key marker for the fibroblast to myofibroblast transition (Gabbiani, 2004). There have been methods established to monitor this transition; however a direct scheme looking at total actin protein composition has not been firmly developed. Developing this system to monitor the actin expression, through real time reverse transcription polymerase chain reaction (RT-PCR), seemed to be a reasonable approach for tracking the fibroblast to myofibroblast transition and total actin composition.

Fibroblasts use beta-actin microfilaments, myosin and other associated protein molecules to generate force. Myofibroblasts differentiate from fibroblasts and alpha-smooth muscle actin is expressed; myofibroblast being the key player in wound contraction. Wound repair is governed by this transition which has been shown to generate, "wound contraction and extracellular matrix remodeling" (Huet et al., 2007). Knowing the amount of beta-actin and alpha-smooth muscle actin during force

production could help to improve the understanding of the tissue healing process, and scar tissue formation.

Each normal, fully functional dense connective tissue has characteristic collagen fiber organization. In contrast, scar and fibrotic tissue is characterized by hypercellularity, alpha-smooth muscle actin expression, and deposition of disorganized extracellular matrix (Huet et al., 2007); scars being areas of fibrous tissue that can lead to adhesion formation. The myofibroblast phenotype is transiently expressed during wound healing; synthesizing collagen types I and III, disappearing at the conclusion of healing (Gabbiani, 2003). However, the continued expression of alpha-smooth muscle actin is characteristic of abnormal healing (Masur et al., 1996). In a fibrotic repair response, a myofibroblast produces an irreversible isometric contraction that, if expression persists, can lead to excessive scarring, with resulting functional impairment (Gabbiani, 2004). This excessive contraction and synthesizing activity can cause tissue abnormalities, i.e. hypertrophic scars and keloids, and may even lead to life-threatening fibrosis (Hinz, 2006). The final scar or fibrotic tissue formed is not the same as the original tissue being replaced.

The stronger retractile activity produced by myofibroblasts and its interaction with the surrounding extracellular matrix has a key role in the development of tissues (Gabbiani, 2004). This can be advantageous due to the decreased time for tissue contraction. However, aberrant activity can lead to excessive scarring, fibrosis or even adhesion formation. It needs to be established whether or not the amount of alpha-smooth muscle actin in fibroblasts and myofibroblasts correlate with force generation in weight bearing contracting native tendon organ culture models. It is proposed that the

increase in force production can lead to over contraction and subsequent out pouching; however, a stronger cross-link with increased tensile strength.

By further defining the cellular changes of fibroblasts, a greater understanding of the tissue repair and building process could be attained. The long term goal is having the ability to manipulate the biological environment and establish an increased repair rate, by turning the myofibroblast 'on,' and decreased scar tissue formation, by turning them 'off.' The underlying protein expression during force production seems to be an appropriate means for monitoring the fibroblast to myofibroblast transition. This can be accomplished through designing oligonucleotide primers and employing the real time RT-PCR methodology. In order for viable tissue to form, being able to monitor the proteins expressed during repair and building is significant, and could have applicability in tissue engineering research.

Much research has been done on the regulation, differentiation and function of myofibroblasts (Hinz, 2006; Hinz et al., 2002; Hinz et al., 2001; Goffin et al., 2006). But, little has been done to quantify the amount of alpha-smooth muscle actin responsible for this force generation. Quantification of alpha-smooth muscle actin expression during force production, in a "native" tendon organ culture model, is the next step to be taken when looking to further elucidate the building of dense connective tissue and the tissue repair process. The expression of alpha-smooth muscle actin has been investigated in various models. In human models: articular cartilage (chondrocytes), (Kim and Spector, 2000; Qiu et al., 2000; Kinner and Spector, 2001; Zaleskas et al., 2001), meniscus (Ahluwalia et al., 2001; Zaleskas et al., 2001); canine chondrocyte models (Wang et al., 2000; Lee et al., 2000); bovine meniscal cells (Mueller et al., 1999); rabbit corneal

fibroblasts (Nakamura et al., 2002); and mouse fibroblasts (Saddiq et al., 2005). Kinner and Spector (2001), as well as Zaleskas et al., (2001) have compared the contractility of articular chondrocytes with the expression of alpha-smooth muscle actin. They found a causal relationship between alpha-smooth muscle actin expression and contraction of chondrocytes in a collagen-glycosaminoglycan matrix (Zaleskas et al., 2001). A common method to study these cells is through the use of cell populated collagen gels; not native tissue organ culture models. Also, tendon organ has not been investigated in depth despite its large concentration of tendon fibroblasts, tendency to form scar/fibrotic tissue and decreased metabolic activity.

Tendon is a dense connective tissue of predominately type I collage with 70% of tendon mass being composed of water (Dykyj and Jules, 1991). Tendon forms the connection between muscle and bone, connecting to bone at the fibro-osseous junction. On gross examination, rat tail tendon appears as bright-white cords exhibiting opalescence as light shines on them. Tendons main role is to withstand tensile loads with the collagen fibrils aligned in a liner fashion. Tendons exhibit decreased metabolic activity, with oxygen consumption being 7.5 fold lower than skeletal muscle (Vailas et al., 1978). This property is important as the extreme tensile loads that are placed on tendons can result in relatively ischemic conditions that would otherwise lead to tissue necrosis. However, metabolic rate and repair rate exhibit a causal relationship with the decreased metabolic activity resulting in a decreased repair rate (Williams, 1986). It is thought that the internal fibroblasts are responsible for the majority of collagen synthesis and the external cells provide the lubrication required for the gliding that occurs during movement (Slack et al., 1986). This lubricating effect being very important to minimize

the amount of frictional forces exhibited on the tendon; decreasing the shear stress placed on the tendon fibers.

The total composition of a tendon has been separated to microscopic levels (Figure 1.). The smallest level is composed of a triple-helix polypeptide chain that forms the "primary bundles" of tropocollagen (15 angstroms in diameter). Microfibrils (35 angstrom) are then composed of many small fibers of tropocollagen. Combined microfibrils form the subfibril (100-200 angstrom), and fibrils (500-5000 angstrom) are then formed when subfibrils group, which lead to the 50-300 micrometer fascicles. Each fascicle is covered by a fascicular membrane, and each bundle of tendon collagen (typically two-three fascicles) is covered by a reticular membrane (Kastelic et al., 1978).

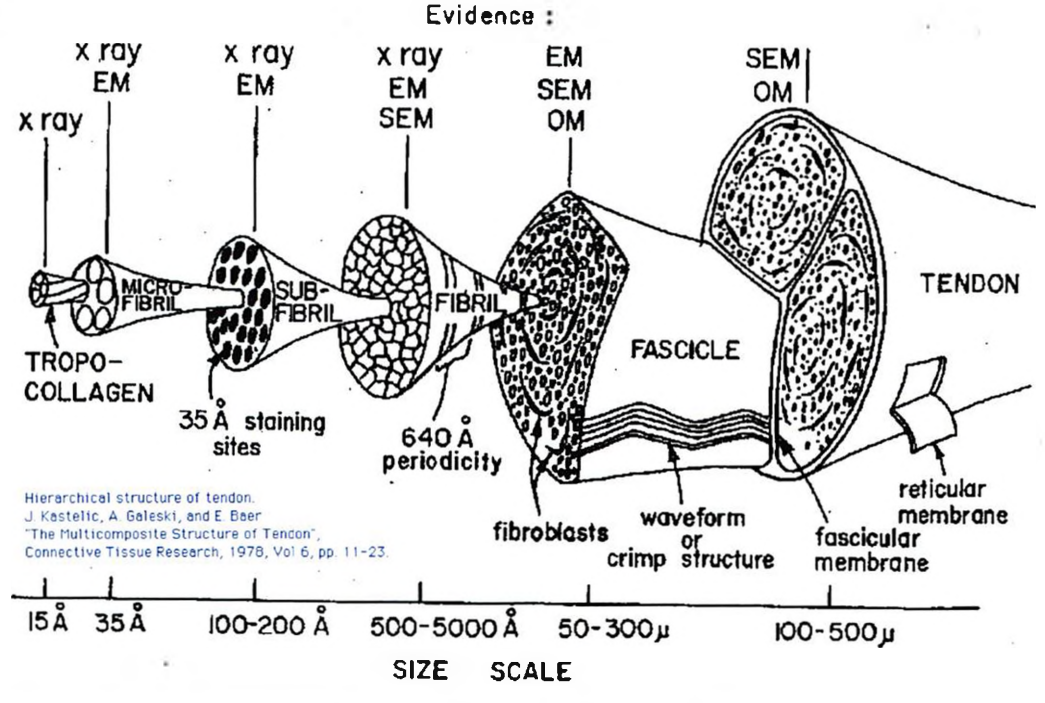

Figure 1. Total composition of tendon separated to the microscopic level

There is also a distinct wave-like or "crimp" within the fascicular structure of different topographies (Kastelic et al., 1978); forming a spring-like structure capable of stretching and returning to the original position. This is capable while still maintaining extreme tensile strength, being able to withstand forces equivalent to thousands of Newtons (Wilson et al., 1999). This hearty tissue is possible due to the cellular subtypes that make up its composition.

Previously established data on tendon fibroblasts took place in Dr. Johnson-Wints laboratory. This looked at 1) beta-actin in a loaded and unloaded collagen gel matrix 2) beta-actin in osteocytes and tendon fibroblasts and rate of collagen gel contraction. Both were setup using a collagen gel contraction assay, with either compressive or tensile loads. It has been established that tendon fibroblasts in a compressive weight bearing environment contain 100-300 million beta-actin molecules per cell. Also, tendon fibroblasts contain two-three times the number of beta-actin molecules as osteocytes, and can lift four times as much weight per cell as osteocytes in cell populated collagen gels. This indicates a correlation between force generation and total actin content. Tendon fibroblasts also contract cell populated collagen gels faster than osteocytes when comparing equal cell numbers.

In preparation to quantifying beta-actin and alpha-smooth muscle actin content in loaded and unloaded native rat tail tendon organ culture models, several methodologies were developed in the present paper. Since alpha-smooth muscle actin has not been monitored in tendon in vitro, developing the methods to culture and quantitatively detect alpha-smooth muscle actin, the protein marker for fibroblast to myofibroblast transition were developed. In addition, quantifying the amount of alpha-smooth muscle actin

mRNA, using reverse transcription polymerase chain reaction (RT-PCR) assays was investigated and novel oligonucleotide primers were prepared. A differential approach to actin isoform identification in a tissue environment was created.

MATERIALS AND METHODS

Tissue Preparation–

For tissue cultures the Rat Tail Tendon (RtTT) was obtained from Sprague-Dawley rats. All animal care complied with the universities acceptable animal care and use protocols for laboratory animals. The rats were sacrificed with an overdose of carbon dioxide. Using sterile technique RtTT was removed from the host and placed in 150 mm Petri dishes containing HBSS (Figure 3.). The tendon samples were cut into 30 mm samples, for a total of 20-48 pieces per specimen depending on culture used. Time zero samples were placed individually in microfuge tubes and frozen down, the other samples were placed in appropriate tissue culture.

Isolation of Rat Aorta-

Using sterile technique, rat aorta was isolated from the host after one sagittal and three transverse incisions were made along the ventral aspect of the rat. Further dissection required removing a number of structures that impeded entry into the thoracic cavity as well as down the descending aortas' track. The aorta was removed in one piece, including the ascending and descending, and was placed in a 150 mm Petri dish containing HBSS. The aorta sample was further cleaned of most non-vascular smooth muscle tissue and placed in a microfuge tube and frozen down. The sample was later extracted in reducing sample buffer for SDS-PAGE /Western blotting and was used as a source of alpha smooth muscle actin.

Tissue Cultures-

Unloaded Tendon Organ Culture Model- The RtTT was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 5-10% NBCS and 2X Penicillin/streptomycin

species (100 U/mL) in 10 ml glass tubes. Samples were incubated at 37 °C, 95 % air, 5% CO2. The tissue fragments were placed in individual microfuge tubes and frozen down.

Loaded Tendon Organ Culture Model- A weight bearing contracting native tissue culture, experiencing tensile loads, provided our environment to quantify (myo)-fibroblast contractile ability. This culture is similar to the unloaded tendon organ culture model with the addition of a 'U' shaped weight hanging from the end of the tendon fragment.

Plate Tendon Organ Culture Model- The RtTT were cultured in DMEM with 5-10% NBCS and 2X Penicillin/streptomycin species (100 U/mL) in Costar 24 well culture plates. Plates were incubated at 37 °C, 95 % air, 5% CO2, for five days, with digital photographs taken on days 1, 2, 3 and 5 of culture.

Tension Tendon Organ Culture Model- A tension dependent native tissue culture, experiencing isometric load, provided our environment to quantify (myo)-fibroblast contractile ability. This culture is similar to the plate tendon organ culture model with the exception that the ends of the tendon fragment were pinned down. The tendon fragment wraps over another pin at the top of the dish forming a 'V' shape.

Western Blot Analysis-

Resident fibroblast from native RtTT and rat aorta were extracted and suspended in 200 micro-liters 1X reducing sample buffer and solubilized by heating for 5 minutes at 70 C. Following heating, a series of three freeze thaw cycles took place. Equal amounts of proteins were loaded to SDS-PAGs (7.5% stacking, 10% running), (Bio-Rad Laboratories LSG, Hercules, California), separated by PAGE (Laemmli, 1970) at room temperature, 150 V, for 45 minutes, and transferred to nitrocellulose membrane (Towbin

et al., 1979) at room temperature, 100 volts, for one hour. Membranes were then probed with primary antibody, either anti-beta-actin (SIGMA) or anti-alpha-smooth muscle actin (SIGMA). Incubations were followed by secondary antibody (goat anti-mouse IgG Biotin Conjugated), with streptavidin and biotinylated-alkaline phosphates to follow. Signals were detected by color development (9.6 mL Q-H2O, 0.4 mL 25x stock color development buffer solution, 100 uL Reagent A, 100 uL Reagent B), (Bio-Rad Laboratories LSG, Hercules, California)

Bands were digitized with scanner; scanning program used was "ScanSoft," and using Adobe Photoshop, the color image was converted to black and white with brightness/contrast modified and the ratio between all band densities calculated by computer software (SCION Image, Frederick, Maryland).

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Assays-

Initial primer selection of 'actin specific' primers occurred through using the National Center for Biotechnology Information Primer-BLAST software. This was followed by Integrated DNA Technologies IDT SciTools OligoAnalyzer 3.1, to check Tm, self-dimer formation delta G, and performed second NCBI BLAST. The Kalign software (European Bioinformatics Institute) multiple sequence alignment algorithm, provided a means to analyze regions of similarity between actins, primers, and plasmids without actin sequences present. The cDNA plasmids (Open Biosystems, Huntsville, AL) that were analyzed for each actin were selected on basis of homology and specificity, with most being full-length cDNAs.

Photography and Image Preparation–

Digital photographs were taken each day to monitor tissue contraction, Adobe Photoshop and MSWord were used to manipulate and coordinate the images.

RESULTS

Tissue cultures-

Plate Tendon Organ Culture Model- The morphological changes of native tendon fragments were visualized in an unloaded plate culture model; showed a distinct contractile mechanism (Figure 2.), since they went from 3 cm strands to compact tissue balls or shortened thickened strands. Over a five day period digital photographs were taken to represent the change in conformation of the RtTT. From Figure 2, compare column 'a' with column'd' for each fragment (1-4) to see the morphological changes.

The contractions of tendon fragments in an unloaded native tendon tissue fragment culture have been shown to occur over a five day period. The morphological changes were visualized and showed a distinct contractile mechanism commonly attributed to fibroblast and their beta-actin microfilaments, myosin motor protein interaction. The tendon fragments underwent rapid contraction progressing from a linear normal morphological state, to a balled up contracted state, further illustrating the importance of tissue being loaded to maintain its linearly organized state and functionality. There was a continuous contraction for all the sample tendon fragments, with varying degrees of full contraction over the five day period.

Culture well microscopic analysis showed that the wells contained sub-confluent islands of cells. This indicates that some fibroblasts leave the RtTT fragment during the five day period and attach to and grow in the culture well.

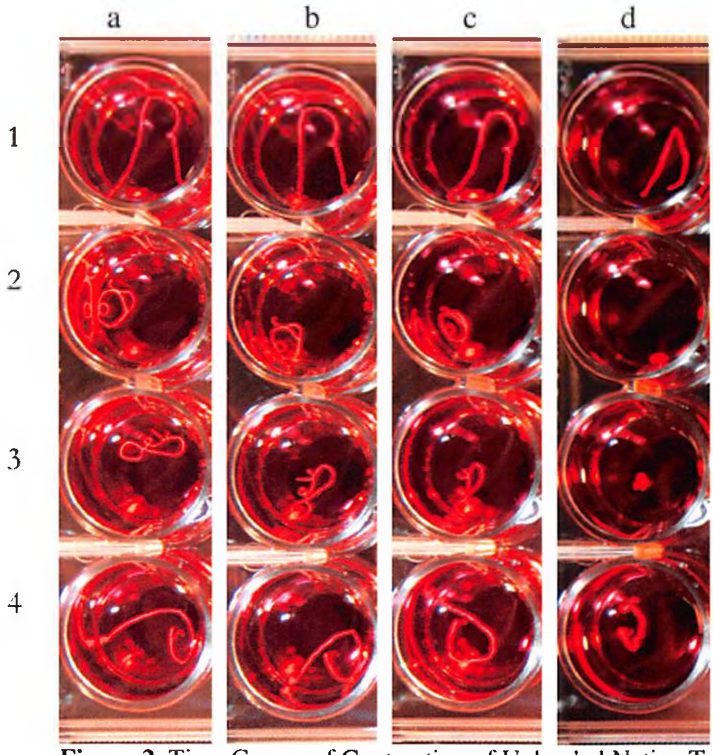

Figure 2. Time Course of Contraction of Unloaded Native Tendon Fragments during culture – Four of the ten tendon fragments (top to bottom) from the same animal over five days (left to right) are shown. 1-4 are 4 individual tendon fragments; a = day 1, b = day 2, c = day 3, d = day 5.

Western blot analysis-

When looking to quantify alpha-smooth muscle actin through the Western blot procedure it was determined to be inadequate. The two antibodies used in the preliminary study of rat aorta (Figure 3.), thought to be a rich source of alpha-smooth muscle actin, did not detect any alpha-smooth muscle actin through Western analysis (Figure 4.). Through further investigation for new antibodies it was found that there are not many commercially available rat alpha-smooth muscle actin specific antibodies. Most detect gamma- and alpha-smooth muscle actin, which is unsuitable for the current project. This led to the development of the real time RT-PCR methodology.

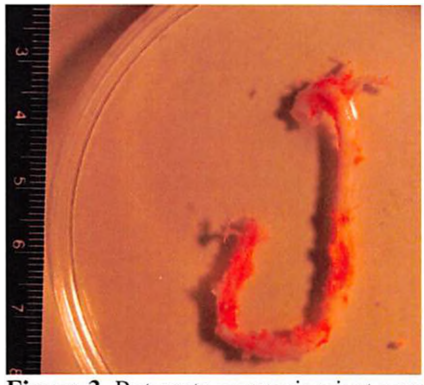

Figure 3. Rat aorta measuring just over 95 mm in total length

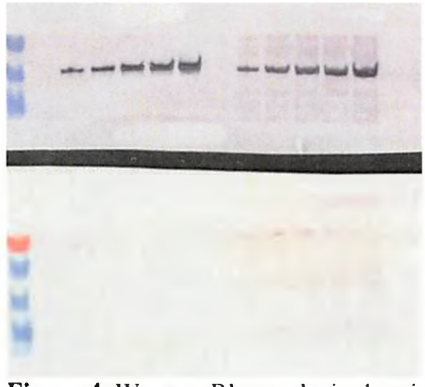

Figure 4. Western Blot analysis showing: on top blot (beta-actin detection), beta-actin standard (left samples n=5), aorta sample (right sample n=5); on bottom blot (alpha-smooth muscle actin detection), beta-actin standard (left samples n=5), aorta sample (right sample n=5).

Primer Constructs for RT-PCR-

The following tables show the oligonucleotide primers (Table1.) designed and products formed (Table 2.) upon RT-PCR assays. The beta-actin (Actb) forward primer Tm of 69.7 ºC, reverse primer Tm of 70.2 ºC; smooth muscle alpha (Acta2) forward primer Tm of 66.7 ºC, reverse primer Tm of 70.2 ºC; gamma 2, smooth muscle, enteric (Actg2) forward primer Tm of 66.7 ºC, reverse primer Tm of 67.3 ºC; and cytoplasmic gamma 1 (Actg1) forward primer Tm of 67.9 ºC, reverse primer Tm of 68.4 ºC. Self-dimer formation was evaluated and each primer had a delta G in the acceptable range of greater than -5 kcal/mole, or no 3' involvement. Each forms a product of acceptable size: 330, 262, 238, and 340 base pairs. Since actin cDNA containing plasmids will be used as

controls to validate the specific primers developed to identify specific actin cDNAs in the PCR reactions, the plasmids themselves were evaluated for potential sequence homology with the primers. The evaluation through Kalign for similar sequence areas between actin primers and plasmids alone (no actin cDNA insert) produced some concern. The most sequence similarity came from both forward (72%) and reverse (75%) primers for beta-actin and Vector pExpress-1. This could potentially produce a product by reaction with the plasmid itself, not the actin cDNA, however, a much larger product (2101 base pairs) would form. This would be easily discernable from expected product and disregarded. The beta-actin cDNA; however, is not in the pExpress-1 vector, instead it was inserted into the pDNR-LIB vector. This caused more concern, as with the beta-actin primers and pDNR-LIB vector showed a forward (62.5%) and reverse (68.4%) sequence similarity, with a product of 326 base pairs. This product is rather close in size to the expected beta-actin cDNA product, and is not as discernable. The possible "plasmid" PCR reaction would also compete with and change the kinetics of the beta-actin PCR reaction and is not desirable.

Rattus Norvegicus Actin Primers				
			length	Tm (°C)
beta	Forward	GGTCCACACCCGCCACCAGTTCGCC	25	69.7
(Actb)	Reverse	GGGTGCTCCTCAGGGGCCACACGC	24	70.2
GI:42475962				
smooth muscle alpha	Forward	TGGAGAAGCCCAGCCAGTCGCCATC	25	66.7
(Acta2)	Reverse	GGATGCTCCTCTGGGGCCACGCGAAGCTCGTTATAGAAGG	40	70.2
GI:148298812				
gamma 2, enteric	Forward	CATGTGTGAAGAAGAGACCACCGCCCTTGTGTGTG	35	66.7
(Actg2)	Reverse	GTTCGTGATGATGCCGTGCTCAATGGGGTATTTGAGGG	38	67.3
GI:6978440				
cyto. gamma 1	Forward	CTTACACTGCGTTTCTTTCCGCTGCTCCGTCGTCC	35	67.9
(Actg1)	Reverse	ACACGCAGCTCGTTGTAGAAGGTGTGGTGCCAGATC	36	68.4
GI:188536081				

Table 1. Oligonucleotide primers

Rattus Norvegicus Actin Primer Products
actin, beta (Actb), mRNA
57 ggtc
61 cacacccgcc accagttcgc catggatgac gatatcgctg cgctcgtcgt cgacaacggc
121 tccggcatgt gcaaggccgg cttcgcgggc gacgatgctc cccgggccgt cttcccctcc
181 atcgtgggcc gccctaggca ccagggtgtg atggtgggta tgggtcagaa ggactcctac
241 gtgggcgacg aggcccagag caagagaggc atcctgaccc tgaagtaccc cattgaacac
301 ggcattgtca ccaactggga cgatatggag aagatttggc accacacttt ctacaatgag
361 ctgcgtgtgg cccctgagga gcaccc 386
product length = 330
smooth muscle alpha-actin (Acta2), mRNA
7 tgga gaagcccagc cagtcgccat caggaacctc gagaagctgc tccagctatg
61 tgtgaagagg aagacagcac agctctggtg tgtgacaatg gctccgggct ctgtaaggcg
121 ggctttgctg gtgatgatgc tcccagggct gttttcccat ccatcgtggg acgtcccaga
181 caccagggag tgatggttgg aatgggccaa aaggacagct atgtggggga cgaagcgcag
241 agcaagagag ggatcctgac cctgaagtat ccgatagaac acggcatcat caccaactgg
301 gacgacatgg aaaagatctg gcaccactcc ttctataacg agcttcgcgt ggccccagag
361 gagcatccga 368
product length = 262
actin, gamma 2, smooth muscle, enteric (Actg2), mRNA
15 catgtg tgaagaagag accaccgccc ttgtgtgtga caatgggtct
61 ggcctgtgca aggcaggctt tgcaggagac gacgctccca gggctgtctt tccctccatt
121 gtgggccgcc ctcggcatca gggcgtgatg gtgggaatgg gccagaaaga cagctatgtg
181 ggggacgaag cccagagcaa gcgtgggatc ctgaccctca aataccccat tgagcacggc
241 atcatcacga ac 252
product length = 238
actin, gamma 1 (Actg1), mRNA
22 cttacactg cgtttctttc cgctgctccg tcgtcccgtc
61 ctctgccgat cgcaatggaa gaagaaatcg ccgccctcgt cattgacaat ggctccggca
121 tgtgcaaagc tggctttgct ggggacgacg cccccagggc cgtgtttcct tccatcgtcg
181 ggcgcccccg acaccagggt gtcatggtgg gcatgggcca gaaagactcg tacgtgggtg
241 atgaggccca gagcaagagg ggtattctga ccctgaagta ccctattgag cacggcattg
301 tcaccaactg ggacgacatg gagaagatct ggcaccacac cttctacaac gagctgcgtg
361 t 361
product length = 340

Table 2. Oligonucleotide primer products

DISCUSSION

Normal connective tissue, tendon or bone, is found in a loaded environment within the human body. The importance of this environment can be illustrated by looking at the fibroblast response in our plate tendon organ culture model. The tissue has been shown to contract and lose its linear relationship, with the beta-actin molecules still actively contracting, pulling the tendon into an unorganized state. Healthy connective tissue is found in a loaded state as this helps establish a well organized functioning tissue. Observations in this lab showed that tendon balls itself up in culture; a fashion similar to cut or torn tendon in situ. This observation suggests that intact tendon organ culture may be an excellent model for studying tendon healing.

Through experiments (not provided) it was determined that Dulbecco's Modified Eagle's Medium (DMEM) with 10% new born calf serum with 2X Penicillin/streptomycin species (100 U/mL) incubated at 37 °C, 95 % air, 5% CO2, provided the best environment to induce contraction, and possibly the myfibroblast transition with alpha-smooth muscle actin expression. This culture setup has now become the standard when looking to induce contraction in RtTT and can be used with any of the three tendon organ culture models. Total actin composition in culture can now be analyzed using our RT-PCR oligonucleotide primers.

Various methods of PCR have been described, with real time RT-PCR being the most sensitive for detecting low-abundance mRNA (Bustin, 2000). Through the development of the oligonucleotide primers much consideration was given to the cross reaction that could occur between actins. The rat actin amino acid isoforms show >90% overall sequence homology, but only 50-60% homology in their 5' untranslated region

(Skalli et al., 1986). For this reason great care was taken in developing each of the four primers to be 'actin specific.' The oligonucleotide primers were prepared in consultation with Dr. W. Scott Grayburn, Department of Biological Sciences, and a Tm of 65 °C or greater, and a primer product length between 150-400 base pairs was recommended. This now provides a theoretical method to verify and track the fibroblast to myofibroblast transition, through their distinguishing protein marker alpha-smooth muscle actin. The beta-actin primer could serve as an internal standard (Bustin, 2000), as it was one of the first RNAs to be used for this, and it is still considered a quantitative reference for RT-PCR assays (Kreuzer et al., 1999). Whether or not specific actin (alpha-smooth muscle actin) messages are present in the RT-PCR analysis gives indication as to what proteins are in the samples and if the transition has taken place. These experimental methodologies; however, have in no way been validated outside of processing the primers through computerized software analysis which provide theoretical products. Dakhama et al., (1996) has stated that frequently beta-actin mRNA primers are designed in a fashion to also amplify DNA. Therefore, there may be experimental error once procedures are run, i.e. amplifying DNA (Bustin, 2000; Dakhama et al., 1996), and pseudogenes. With over 20 human (Ng et al., 1985), and up to 10 mouse and rat (Zhang et al., 2004) processed pseudogenes having been described for beta-actin, the employment of probes may be another step to take in providing more actin specificity. As amplifying pseudogenes and DNA can skew results and cause interpretation errors (Dirnhofer et al., 1995; Raff et al., 1997; Mutimer et al., 1998; Bustin, 2000).

Future studies will look at the loaded and unloaded tendon organ culture model of RtTT and the kinetics associated with the contraction using photographs to provide a

qualitative and quantitative measurement. The determination of cell number and beta-actin concentration per tendon fragment will provide data to potentially correlate with quantitative measurements of tendon contraction. RtTT beta-actin concentration and cell number may reveal the amount of beta-actin/fibroblast required to induce contraction of a loaded tendon tissue fragment, and will establish the change in beta-actin concentration over a five day period. Alpha-smooth muscle actin quantification methodology will be finalized and potentially produce data on the number of molecules involved in the fibroblast/myofibroblast force generation. The RT-PCR assay will also be run, and total actin composition in culture determined through use of the oligonucleotide primers. Further primer considerations will include the use of probes, as well as additional genomic and plasmid analysis to ensure primers span exon-exon boundaries and do not cross react with plasmids alone. All further considerations geared toward increasing 'actin specificity.'

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