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Authors

Essawy, El Sayed El
Baar, Keith

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Rapamycin Insensitive Regulation of Engineered Ligament Structure and Function by IGF-1

El Sayed El Essawy[1,2], Keith Baar[2].

[1-] Department of Sport Psychology, Mansoura University, Dakahlia Governorate 35516, Egypt

[2-] Department of Neurobiology, Physiology and Behavior, University of California Davis, One Shields Ave, 195 Briggs Hall, Davis, CA 95616, USA

Email: dr.elsayedelessawy@gmail.com; kbaar@ucdavis.edu

Correspondence:

Keith Baar

University of California Davis, One Shields Ave, 195 Briggs Hall, Davis, CA 95616, USA

kbaar@ucdavis.edu

ABSTRACT

Following rupture, the anterior cruciate ligament (ACL) will not heal and therefore more than 400,000 surgical repairs are performed annually. Ligament engineering is one way to meet the increasing need for donor tissue to replace the native ligament; however, currently these tissues are too weak for this purpose. Treating engineered human ligaments with insulin-like growth factor-1 (IGF-1) improves the structure and function of these grafts. Since the anabolic effects of IGF-1 are largely mediated by mTORC1, we used rapamycin to determine whether mTORC1 was necessary for the improvement in collagen content and mechanics of engineered ligaments. The effect of IGF-1 and rapamycin were determined independently and interactions between the two treatments were tested. Grafts were treated for 6 days before mechanical testing and analysis of collagen content. Following 8 days of treatment, mechanical properties increased 34% with IGF-1 and decreased 24.5% with rapamycin. Similarly, collagen content increased 63% with IGF-1 and decreased 36% with rapamycin. Interestingly, there was no interaction between IGF-1 and rapamycin, suggesting that IGF-1 was working in a largely mTORC1-independent manner. Acute treatment with IGF-1 did not alter procollagen synthesis in growth media, even though rapamycin decreased procollagen 55%. IGF-1 decreased collagen degradation 15%, whereas rapamycin increased collagen degradation 10%. Once again, there was no interaction between IGF-1 and rapamycin on collagen degradation. Together, these data suggest that growth factor-dependent increases in collagen synthesis are dependent on mTORC1 activity; however, IGF-1 improves human engineered ligament mechanics and collagen content by decreasing collagen degradation in a rapamycin-independent manner. How the anti-catabolic effects of IGF-1 are regulated have yet to be determined.

NEW & NOTEWORTHY

- IGF-1 increases, and rapamycin decreases the mechanical and material properties of engineered human ligaments.

- IGF-1 increases, and rapamycin decreases the collagen content and concentration of engineered human ligaments.

- There was no interaction between IGF-1 and rapamycin, suggesting that IGF-1 and rapamycin are working independently.

- IGF-1 did not alter procollagen synthesis in growth media, whereas rapamycin decreased procollagen production.

- IGF-1 decreased collagen degradation and rapamycin increased collagen degradation in engineered human ligaments.

- Data suggest that IGF-1 improves human engineered ligament mechanics and collagen content by decreasing collagen degradation in a rapamycin-independent manner, whereas growth factor-dependent increases in collagen synthesis are blocked by rapamycin.

INTRODUCTION

The growth hormone (GH)/insulin-like growth factor (IGF-1) axis plays a central role in determining the size of the musculoskeletal system. A lack of GH/IGF-1 signaling results in proportionate short stature due to impaired growth of the long bones [1]. However, because of redundancy, simply not being able to signal from GH to IGF-1 is not sufficient to decrease size at birth [1]. For example, human offspring with an inactivating mutation of the GH releasing hormone receptor are normal size at birth because of the ability of maternal hormones to compensate [1]. Similarly, knocking out either IGF-1 or the analogous IGF-2 decreases birth weight ~40, whereas the double mutant is ~70% smaller at birth [2]. These data suggest that growth factors like IGF-1, and 2 and transforming growth factor (TGF) β are redundant in their ability to regulate overall musculoskeletal size through a generalized growth factor-inducible pathway.

In many tissues, growth factors modulate cell size and growth rates through the mechanistic target of rapamycin complex I (mTORC1). In mesenchymal stem cells, mTORC1 is activated by IGF-1 and transforming growth factor (TGF) β and this activity is required for normal tendon development [3]. Cong and colleagues have shown that mice with a specific ablation of mTOR in tendon cells showed a ~60% decrease in tendon size that resulted in a 50% decrease in material properties [3]. Similarly in humans, hypertrophy of the ligamentum flavum is induced by IGF-1 in an mTORC1-dependent manner [4]. Together, these data suggest that the redundant anabolic effect of growth factors on connective tissue structure and function occurs in an mTORC1-dependent fashion.

GH/IGF-1 signaling is thought to control body size is through the regulation of collagen synthesis [5]. In support of this hypothesis, systemic administration of IGF-1 improved ligament mechanical and material properties as well as collagen gene expression following injury regardless of loading status [6]. Further, culturing human fibroblasts with IGF-1 increases proliferation and

the expression of collagen mRNA [7,8] and in 3 dimensional (3D) cultures this results in an increase in both collagen content [9] and mechanical properties [10]. Interestingly, the increase in collagen content in 3D tendons/ligaments occurs in the presence of fetal bovine serum [9], even though the increase in collagen synthesis rates induced by serum and IGF-1 are similar [11]. Further, while IGF-1 levels correlate with collagen mRNA, there is not a relationship between IGF-1 and connective tissue collagen protein content [12]. Together, these data suggest that IGF-1 increases collagen synthesis using a generalized growth factor pathway and may influence the total amount of collagen within the matrix through a separate mechanism.

Since IGF-1 is closely related to tissue collagen content, many have hypothesized that this growth factor could promote tendon growth and repair. However, despite the success of IGF-1 *in vitro* and in model organisms, weekly injection of IGF-1 into tendinopathic tissues had no effect on either initial or prolonged recovery in patients [13]. The failure of IGF-1 to aide in tendon regeneration could reflect our poor understanding of the molecular mechanisms through which IGF-1 improves connective tissue collagen content. To address this gap in our understanding, the current work sought to determine the effects of IGF-1 and rapamycin on engineered human ligaments and then determine how these treatments affected collagen synthesis and degradation. Specifically, we tested the hypothesis that IGF-1 would increase collagen synthesis and improve engineered ligament material properties in a rapamycin-dependent manner.

EXPERIMENTAL PROCEDURES

Human ACL cell isolation: The University of California Davis Institutional Review Board approved all procedures and protocols involving human subjects and informed consent was obtained prior to tissue collection (IRB# 779755- A Tissue collection study for Patients undergoing Anterior Cruciate Ligament (ACL) Reconstruction). Briefly, human ACL cells were isolated from ACL remnants collected during surgical repair. Donor 1 was a 23-year-old female and donor 2 was a 25-year-old male. Both donors required surgery due to acute trauma and had no history of previous injury or metabolic disease. Tissue was digested overnight at 37°C in growth media (DMEM containing 10% fetal bovine serum and 1 x penicillin) containing 0.1% collagenase type II. Cells were freed from residual collagen by trituration, washed 3 times in growth media, resuspended, and plated on 15 cm tissue culture plates. Cells were expanded and frozen down at passage 2 and either stored in liquid nitrogen or thawed for experiments. All experiments were performed using cells before passage 5.

Engineered human ligaments: Ligament constructs were engineered as described previously [14–16]. Briefly, $2.5x10^5$ ACL fibroblasts were suspended in growth media containing 5.8 Units of thrombin (Lot No. 070M7351V, Cat. No. T4648-1KU), 20 µg aprotinin (Cat. No. A3428), and 2 µg 6-aminohexanoic acid (Fluka - Cat. No. 07260-100g Lot# 0001394302). 714 µL of this cell/thrombin solution was dispersed onto each Sylgard 184 (Silicone Elastomer, Dow Chemical, Midland, MI) coated 35mm plate containing 2 tear drop-shaped brushite cement anchors pinned 12 mm apart. A fibrin gel was formed by adding 286 µL of a 20 mg/mL fibrinogen solution (Sigma - Cat. No. F4883-5G - Lot No. SLBX7558) and agitating the dish to thoroughly combine the mixture. Fibrin gelation was apparent within one minute of the agitation step. The plates were then incubated at 37°C with 5% CO2 for 15 min to allow for complete gelation. Following gel formation, 2 mL of feed media (Dulbecco’s Modified Eagle’s Media (DMEM) supplemented

with, 5 ng/mL TGF-β1 (Peprotech-Lot No. 0122209, Cat. No. 100-21), 200 μM L-Ascorbic acid 2-Phosphate Sesquimagnesium salt hydrate, and 50 μM L-proline) was added to each plate. The constructs were cultured for 14 days with media changes every other day from day 0 to day 6. At day 8, constructs were assigned to one of four groups: 1) control feed media; 2) feed media containing 300ng/ml IGF-1 (Peprotech - Cat. No. 100-11); 3) feed media containing 50nM rapamycin; and 4) feed media containing a mixture of both of IGF-1 and rapamycin.

Mechanical Testing:

On day 14, the length and the width of the grafts were measured using a digital caliper. Graft depth was set to 0.5mm for the calculation of cross-sectional area (CSA). Maximum tensile load (MTL) was determined by placing the brushite anchors into 3D printed reverse molded grips secured into the test space of a Model 68SC-1 single column tensile tester (Instron, Norwood, MA) containing a 10N load cell, submerging the sample in 37°C saline within the temperature controlled BioBath, and mechanically testing the grafts using 10 cycles of preconditioning to 0.10 N at a rate of 0.3 mm/s prior to loading to failure at a constant rate of 0.3mm/s. MTL was the maximal load measured prior to failure in Newtons. Ultimate tensile strength (UTL) was calculated by normalizing the MTL by CSA and the Young's modulus was calculated as the maximal slope of the stress-strain (displacement divided by the initial length) curve.

Collagen content:

Collagen content of the grafts was measured using a hydroxyproline assay as first described by Woessner [17]. Briefly, following mechanical testing and removal of the brushite anchors, engineered ligaments were dried on a glass plate for 30 minutes at 120°C and dry mass of the grafts was measured after cooling. Dried grafts were hydrolyzed in 200 μL of 6N HCl at 120˚C for 90 minutes. The lids of the tubes were opened and the HCl evaporated in a lamellar flow hood. The dried pellet or a standard curve of hydroxyproline

was suspended in 200µL of hydroxyproline buffer (173 mM citric acid, 140 mM acetic acid, 588 mM sodium acetate, 570 mM sodium hydroxide), 150 µL of Chloramine T solution was added to each sample, before mixing, and incubating for 20 min at room temperature. Next, 150 µL aldehyde-perchloric acid containing 60% 1-propanol, 5.8% perchloric acid, and 1M 4-(dimethylamino) benzaldehyde was added to each tube and the samples incubated at 60°C for 15 min. Samples were cooled for 5 minutes and 200µl of the solution was read at a wavelength of 550 nm on an Epoch Microplate Spectrophotometer (BioTek Instruments Limited, Winooski, VT). Hydroxyproline content was calculated against the standard curve and then converted to collagen assuming hydroxyproline contributes to 13.7% of the dry mass of collagen.

Collagen Degradation

To estimate the amount of collagen degradation, ligaments were engineered and fed as described above except using phenol red free DMEM. Phenol red needed to be removed from the growth media since the dye interfered with the colorimetric hydroxyproline assay. To determine hydroxyproline in the media, 1 ml of spent media was collected from each 35mm plate just prior to feeding on days 10, 12, and 14. The media was dried using a Speed Vac SC110 and the dried pellet was hydrolyzed for an hydroxyproline assay as described above. The growth media contains some hydroxyproline, so all controls had the same basal media as the treated groups.

Western blotting

Human ACL (hACL) fibroblasts were expanded and plated into 24-well plates and allowed to reach 100% confluence before treatment. Cells were treated with ascorbic acid free feed media (DMEM + 50 µM Proline + 5 ng/mL TGF-β), so that procollagen could not be processed and exported from the cells, and collected at 0, 1.5, 3, 6, 24h, and 48h. At the appropriate collection time, the cells were placed on ice, media was aspirated, and the wells washed twice with 500µL ice cold phosphate-buffered saline (PBS). 75 µL of Laemmli

sample buffer (LSB) was added to each well and, cells were lysed on shaker for one minute. The lysates were collected, sonicated, and denatured at 100°C for 5 minutes. Equal aliquots of protein were loaded on 4%-20% Criterion TGX Stain-free gels (Bio-Rad), run for 45 minutes at 200V and visualized after a UV-induced reaction to fluorescently quantify total protein. Proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane at constant voltage of 100V for 30 minutes, after the membrane was activated in methanol and equilibrated in transfer buffer. Membranes were Ponceau stained to confirm proper transfer, washed in TBST (Tris-buffered saline w/ 0.1% Tween) and blocked in 1% Fish Skin Gelatin (FSG) in TBST for 60 minutes. Membranes were then rinsed and incubated overnight at 4°C with the appropriate primary antibody. All primary antibodies used were diluted in TBST at 1:1,000. The next day, membranes were washed with TBST (3 x 5 minutes), and subsequently incubated at room temperature with peroxidase-conjugated secondary antibodies in a 0.5% Nonfat Milk TBST solution at 1:10,000 before washing again with TBST (3 x 5 minutes). Prior to imaging, Immobilon Western chemiluminescent HRP substrate (Millipore, Hayward, CA, USA) was applied to the membranes for protein visualization via chemiluminescence. Image acquisition and band quantification was determined using the ChemiDoc MP System and Image Lab 5.0 software (Bio-Rad). The antibodies used were as follows: procol1a1 primary antibody (the SP1.D8 mouse monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank where it was deposited by H. Furthmayr) at a concentration of 1:250, p-S6 (Ser240/244) (Cell Signaling technology – Cat. No.5364S- Lot No.8; 1:1000), p-AKT (Ser473) (Cell Signaling technology – Cat. No.4060S- Lot No.19; 1:1000),

Statistical analysis

For all assays, a technical replicate was a single engineered ligament or tissue culture well within a group at a given time. Biological replicates reflect that the experiments were repeated using either a new donor or a separate

vial of cells from the same donor. Each biological replicate was analyzed independently using a two-way ANOVA with IGF-1 as factor 1 and rapamycin as factor 2 using GraphPad Prism v9. Where statistical differences were detected, a Tukey's honestly significant difference test was used for post-hoc analysis since all groups demonstrated equal variance. Statistical analyses and the type I error was maintained at $\alpha < 0.05$ for all comparisons.

RESULTS

IGF-1 increases, and rapamycin decreases graft mechanical and material properties

Maximum tensile load was increased 34% by IGF-1, decreased 24.5% by rapamycin, and the mixture of rapamycin and IGF-1 was no different than control (Figure 1). Statistical analysis indicated a main effect of IGF-1 ($p < 0.05$) and rapamycin ($p < 0.01$); however, there was no interaction between IGF-1 and rapamycin ($p = 0.34$). Ultimate tensile strength was increased 43% by IGF-1, decreased 30% by rapamycin, and the mixture of rapamycin and IGF-1 was no different than control. Statistical analysis indicated a main effect of IGF-1 ($p < 0.05$) and rapamycin ($p < 0.01$); however, there was no interaction between IGF-1 and rapamycin ($p = 0.14$). Modulus was increased 95% by IGF-1, decreased 38% by rapamycin, and the mixture of rapamycin and IGF-1 was no different than control. Statistical analysis indicated a main effect of IGF-1 ($p < 0.01$) and rapamycin ($p < 0.01$); however, there was no interaction between IGF-1 and rapamycin ($p = 0.30$).

IGF-1 increases, and rapamycin decreases graft collagen

Collagen content was increased 63% by IGF-1, decreased 36% by rapamycin, and the mixture of rapamycin and IGF-1 was not different than control (Figure 2). Statistical analysis indicated a main effect of IGF-1 ($p < 0.01$), and rapamycin ($p < 0.01$); however, there was no interaction between IGF-1 and rapamycin ($p = 0.30$). The concentration of collagen, expressed as µg of collagen per mg of dry mass was increased 20% by IGF-1, decreased 28% by rapamycin, and the mixture of rapamycin and IGF-1 was not different than control. Statistical analysis indicated a main effect of IGF-1 ($p < 0.01$), and rapamycin ($p < 0.01$); however, there was no interaction between IGF-1 and rapamycin ($p = 0.63$).

IGF-1 does not change, whereas rapamycin decreases procollagen synthesis

To determine how IGF-1 was improving ligament mechanics, we treated 24-well plates containing confluent human ACL fibroblasts with feed media (DMEM containing 10% FBS and 5ng/mL TGFβ) in the presence or absence of 300 ng/mL of IGF-1 and measured Akt/mTORC1 signaling and procollagen synthesis. As expected, feed media increased Akt, S6K1, and s6 phosphorylation in a time-dependent manner and this was augmented by IGF-1 (Figure 3). In contrast, IGF-1 did not augment procollagen Ia1 production at any time point.

To determine how rapamycin was impairing ligament mechanics, we treated 24-well plates containing confluent human ACL fibroblasts with feed media (DMEM containing 10% FBS and 5ng/mL TGFβ) in the presence or absence of 50 nM rapamycin and measured Akt/mTORC1 signaling and procollagen synthesis. As expected, feed media increased Akt and s6 phosphorylation in a time-dependent manner and Akt phosphorylation was augmented and s6 phosphorylation was prevented by rapamycin (Figure 4). Rapamycin decreased ~50% of procollagen Ia1 synthesis between 12 and 48 hours after feeding.

IGF-1 decreases, and rapamycin increases collagen degradation

Since IGF-1 increased collagen content in the grafts but had no effect on collagen synthesis, we next developed an assay to determine the rate of collagen degradation in engineered ligaments (Figure 5). For this assay, the amount of hydroxyproline, a modified amino acid only found in collagen, in the media of engineered ligaments was determined. Collagen degradation (hydroxyproline in the media) was decreased ~15% by IGF-1, tended to increase with rapamycin (10%), and rapamycin and IGF-1 was no different than control (2.4%). Statistical analysis showed main effect of IGF-1 ($p = 0.0011$) to decrease degradation, of rapamycin to increase degradation ($p < 0.001$), and no interaction between IGF-1 and rapamycin ($p = 0.24$).

DISCUSSION

IGF-1 is known to increase tendon/ligament collagen content and mechanics. Here we confirm that IGF-1 increases collagen content and further demonstrate that *in vitro* the positive effect of IGF-1 is mediated not by an increase in collagen synthesis, but rather from a decrease in collagen degradation. Using rapamycin, we demonstrate that mTORC1 is necessary for approximately half of the collagen synthesis in response to feeding and that inhibition of mTORC1 increased collagen degradation. Together, these data suggest that growth factors both increase collagen synthesis and decrease degradation resulting in increased accrual of collagen within tendons/ligaments.

Herchenhan and colleagues have previously shown that IGF-1 can increase the content of collagen in engineered ligaments without affecting collagen fibril diameter [9]. Similarly, we have previously shown that even in a media containing the growth factor rich fetal bovine serum and the anabolic transforming growth factor (TGF)β we were able to demonstrate a dose-dependent increase in collagen content and mechanics that peaked at 600 ng/ml and 1200 ng/ml, respectively [10]. In fact, when designing an optimal growth factor environment for engineered ligaments we had shown that media containing 50 ng/ml TGFβ1, IGF-1, and GDF-7 produced the strongest ligaments [14], suggesting that these three growth factors worked in a complimentary fashion. Similarly, Kobayashi and colleagues found a dose-dependent effect of IGF-1 on collagen content in cultured rabbit anterior cruciate ligament cells that was further augmented by fibroblast growth factor (FGF) [18]. Here, we confirm these previous experiments by showing a main effect of 300 ng/ml of IGF-1 to increase graft collagen content and mechanical and material properties in FBS enriched media. Even though IGF-1 increases collagen content in engineered tissues, three weekly intratendinous injections of IGF-1 had no effect on collagen mRNA following 12 weeks of heavy slow resistance training and did not further improve VISA-

P scores in these tendinopathic individuals [13]. These data suggest either that IGF-1 has no effect in clinical situations or that it requires more frequent treatments due to the mechanism of action.

To determine the mechanism of action, this study investigated the effects of IGF-1 and the mTORC1 inhibitor rapamycin on collagen synthesis, degradation, and mechanics. Rapamycin had a main effect to decrease collagen content and concentration by approximately 40%. As a result, both mechanical (MTL) and material properties (UTS and modulus) were decreased in the presence of rapamycin. Interestingly, IGF-1 and rapamycin showed no interaction, meaning that rapamycin did not alter the effect of IGF-1 on engineered ligaments grown in growth factor rich media. These data suggest that the positive effect of IGF-1 on collagen content and ligament mechanics was occurring in an mTORC1 independent manner. Consistent with this hypothesis, IGF-1 did not alter the production of procollagen in the presence of fetal bovine serum in human ACL fibroblasts (Fig. 3). This finding is similar to Abrahamsson and colleagues [11] who showed that IGF-1 stimulated procollagen synthesis *ex vivo* in rabbit flexor tendons using L-[^{3}H]proline tracing. As with the current work, Abrahamsson showed that IGF-1 and fetal calf serum (FBS) increased collagen synthesis identically, suggesting that IGF-1 increases collagen synthesis using a general growth factor-stimulated pathway that can be mimicked using FBS and is inhibited by rapamycin. The additive effect of FBS and IGF-1 seen in the current study in terms of collagen content and mechanics therefore is likely independent of the growth factor-stimulated increase in collagen synthesis.

Since collagen content is the sum of collagen synthesis and degradation, we next determined whether IGF-1 could alter collagen degradation. To determine collagen degradation rate, we developed a novel assay that measured the hydroxyproline in the media. Since hydroxyproline is only present in collagen, we hypothesized that the amount of hydroxyproline in

the media would be a good measure of how much collagen had been made and then degraded since the last feeding. Consistent with our hypothesis, the hydroxyproline in the media decreased between days 10 and 14 as the collagen content of the engineered ligaments increased (data not shown). Further, there was a main effect of IGF-1 to decrease, and of rapamycin to increase, hydroxyproline in the media. These data are consistent with an IGF-1-dependent decrease in collagen degradation. Similarly, Hui and colleagues found that IGF-1 decreased collagen degradation in cartilage cells treated with TNFα [19]. These authors showed that IGF-1 decreased collagenolytic activity by ~30% in part through the inhibition of matrix metalloproteinases 1, 3, and 13. We hypothesized that one protein that could modulate collagen degradation was Akt, since Akt is activated by IGF-1 and can regulate protein degradation through the phosphorylation of the forkhead transcription factors (FOXOs; 10). In skeletal muscle, stimulation of Akt by IGF-1 blocks protein degradation and the expression of Atrogens like MURF and MaFBx [20]. Arguing against this hypothesis, IGF-1 and rapamycin increased Akt phosphorylation at Ser473 similarly. An increase in Ser473 phosphorylation with IGF-1 or FBS has been reported in human periodontal ligament fibroblasts [21]. However, since both IGF-1 and rapamycin increased Akt Ser473 phosphorylation yet altered protein degradation in opposing manners, our data suggest an Akt-independent mechanism controls protein degradation in human ligaments.

While outside the scope of the current work, the IGF binding proteins (IGFBPs) can modulate the action of IGF-1. IGFBPs modulate IGF-1 action by increasing its half-life and inhibiting or potentiating growth factor binding to its receptor [22]. Interestingly, exercise, which increases the production of IGF-1 also regulates the expression of the IGFBPs by tendon cells. Specifically, IGFBP-1, 2, and 4 increase, whereas IGFBP-3 and 5 appear to decrease after exercise [23,24]. However, the role that the IGFBPs play in the regulation of collagen in tendons and ligaments remains uncertain.

Together, the data presented in this report suggest that FBS is sufficient to increase collagen synthesis in an mTORC1-dependent manner, whereas IGF-1 can further increase the collagen content of grafts cultured in growth media by decreasing collagen degradation. If the primary, unique, effect of IGF-1 is to decrease degradation, it should not be surprising that intratendinous injections of IGF-1 did not improve symptoms in tendinopathic individuals since collagen degradation is likely necessary for remodeling and repair of the damaged tissue [13].

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FIGURE LEGENDS

Figure 1. Mechanical and Material Properties of Engineered Human Ligaments are Improved by IGF-1 and Impaired by Rapamycin. (A) Maximum tensile load was increased 34% by IGF-1, decreased 24.5% by rapamycin, and the mixture of rapamycin and IGF-1 was no different than control. (B) Graft cross-sectional area (CSA) was not altered by either IGF-1 or rapamycin. (C) Ultimate tensile stress (UTS) was increased 44% by IGF-1, decreased 29.1% by rapamycin, and the mixture of rapamycin and IGF-1 was no different than control. (D) Modulus was increased 95% by IGF-1, decreased 37.4% by rapamycin, and the mixture of rapamycin and IGF-1 was no different than control. * indicates a main effect of IGF-1, † indicates a main effect of rapamycin ($p < 0.05$). Data are representative of 5 experiments using at least two different cell donors.

Figure 2. Collagen Content and Concentration of Engineered Human Ligaments are Improved by IGF-1 and Impaired by Rapamycin. Collagen content was increased 34% by IGF-1, decreased 30% by rapamycin, and the mixture of rapamycin and IGF-1 was not different than control. (B) Mass was increased by IGF-1 and was not affected by rapamycin. (C) Collagen concentration was increased by IGF-1, decreased by rapamycin, and the mixture of rapamycin and IGF-1 was no different than control. * indicates a main effect of IGF-1, † indicates a main effect of rapamycin ($p < 0.05$). Data are representative of 5 experiments using at least two different cell donors.

Figure 3. IGF-1 Augments Akt/mTORC1 Signaling Without Altering Procollagen Production in Engineered Human Ligaments. Western blots were used to show that adding 300 ng/mL IGF-1 resulted in (A) an increase in Akt and (B) s6 phosphorylation compared with standard growth media without (C) increasing procollagen Ia1 protein. A second finding is that

procollagen 1a1 production decreases early in feeding followed by increasing production that peaks ~48 hours after feeding. The key finding here is that IGF-1 had no effect on procollagen levels at any time point. Data are representative of 2 experiments using different donors.

Figure 4. Rapamycin Increases Akt Signaling, Blocks mTORC1 Activity, and Decreases Procollagen Production in Engineered Human Ligaments. Rapamycin (A) increases Akt phosphorylation, while (B) blocking s6 phosphorylation (mTORC1 activity) and (C) decreasing procollagen Ia1 synthesis ~50% between 12 and 48 hours after feeding. The important finding here is that inhibiting mTORC1 only blocks 50% of the increase in procollagen Ia1 and this correlates with the decrease in collagen content in engineered ligaments treated with rapamycin for 8 days. Data are representative of 2 experiments using different donors.

Figure 5. IGF-1 Decreases Collagen Degradation in Engineered Human Ligaments. Collagen degradation was decreased ~17% by IGF-1, tended to increase with rapamycin alone (10%), and rapamycin and IGF-1 was no different than control. * indicates a main effect of IGF-1, † indicates a main effect of rapamycin ($p < 0.05$). Data are representative of 2 experiments using different donors.