Section: Regenerative Medicine and Tissue Engineering

Acute mechanical overload increases IGF-I and MMP-9 mRNA in 3D tissue-engineered skeletal muscle

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Abstract

Skeletal muscle (SkM) is a tissue that responds to mechanical load following both physiological (exercise) or pathophysiological (bed rest) conditions. The heterogeneity of human samples and the experimental and ethical limitations of animal studies provide a rationale for the study of SkM plasticity *in vitro*. Many current *in vitro* approaches of mechanical loading of SkM disregard the three-dimensional (3D) structure *in vivo*. Tissue engineered 3D SkM, that displays highly aligned and differentiated myotubes, was used to investigate mechano-regulated gene transcription of genes implicated in hypertrophy/atrophy. Static Loading (STL) and Ramp Loading (RPL) at 10% strain for 60 min were used as mechano-stimulation with constructs sampled immediately for RNA extraction. STL increased IGF-I mRNA compared to both RPL and CON (Control, $p = 0.003$ and 0.011 respectively) whilst MMP-9 mRNA increased in STL and RPL compared to CON (both $p < 0.05$). IGFBP-2 mRNA was differentially regulated in RPL and STL compared to CON ($p = 0.057$), whilst a reduction in IGFBP-5 mRNA was found for STL and RPL compared to CON (both $p < 0.05$). There was no effect in the expression of putative atrophic genes, myostatin, MuRF-1 and MAFBx (all $p > 0.05$). These data demonstrate a transcriptional signature associated with SkM hypertrophy within a tissue-engineered model that more greatly recapitulates the *in vivo* SkM structure compared previously published studies.

Introduction

Skeletal muscle mass plays a pivotal role in overall health and wellbeing. A decline in skeletal muscle mass and contractile proteins with disease (cachexia, Lenk et al. 2010) and sarcopenia (Pillard et al. 2011), contributes to an increased susceptibility to metabolic diseases including; obesity, diabetes and associated cardiovascular disease (Degens et al. 2006; Narici et al. 2010). Moreover a decrement in muscle mass and strength with age contributes to increased morbidity and mortality (Carmeli et al. 2002; Rantanen et al. 2003).

Skeletal muscle is a highly adaptable tissue, responding to situations of increased (exercise) or decreased (bed rest, aging, injury, zero gravity in spaceflight) mechanical loading (Sandri 2008). Delineating the cellular and molecular mechanisms that are responsive to mechanical loading, may contribute to effective molecular or pharmacological therapies for the treatment of sarcopenia and other skeletal muscle diseases as well as elucidating the molecular regulation of exercise induced skeletal muscle adaptation.

The mechanisms that govern the response of skeletal muscle hypertrophy have stemmed from the seminal work of Goldberg who demonstrated the rapid hypertrophic response after only 24 hrs post tenotomy of a synergistic muscle (Goldberg 1967). This method of inducing work overload-induced hypertrophy has received much attention (for example: Armstrong et al. 1979; Esser et al. 1995; Kandarian et al. 1992; Linderman et al. 1996) and has resulted in a far greater understanding of the molecular mechanisms that regulate this response. Increases in amino acid transport (Goldberg et al. 1969; Henriksen et al. 1993) and satellite cell proliferation (McCarthy et al. 2007; O'Connor et al. 2007; Rosenblatt et al. 1992; Schiaffino et al. 1972) have been shown in part to form the basis of hypertrophy. A global increase in protein synthesis (Goldspink 1977) along with increases in protein content of the sub-fractions within skeletal muscle (Cuthbertson et al. 2006), also play a significant role in the increase of skeletal muscle mass to overload or stretch.

Skeletal muscle hypertrophy is characterised by an increase in gene transcript expression, whether following *in vitro* stretch or stimulation (Carson et al. 2002) or *in vivo* exercise (Bickel et al. 2005). The necessity for an increase in RNA to initiate a hypertrophic response following tenotomy has been demonstrated by the addition of a DNA-dependent RNA synthesis inhibitor, actinomycin D, which ablated any hypertrophy from occurring (Goldberg et al. 1969). Specific gene transcript evidence has demonstrated roles for myogenic and hypertrophic genes; Insulin-like growth factor I (IGF-I, Adams et al. 1996, 1999; Awede et al. 1999, 2002) and matrix metalloproteinase-9 (MMP-9, Dahiya et al. 2011; Lewis et al. 2000; Mehan et al. 2011), along with the regulation of genes involved in negatively regulating muscle mass including myostatin and ubiquitin ligases,

muscle RING finger protein 1 (MuRF-1) and muscle atrophy factor box (MAFBx, McPherron et al. 1997a, b; Trendelenburg et al. 2009; Welle 2009).

Developing a model of overload-induced hypertrophy *in vitro* would overcome many of the experimental issues surrounding human and animal studies. Only recently have authors demonstrated the molecular response associated with multiple biopsy sampling (Caron et al. 2011; Friedmann-Bette et al. 2012), providing evidence in support of the use of an alternative system to investigate the responses to exercise or overload. Furthermore, the isolated nature of *in vitro* systems also allows for the analysis of intrinsic mechanisms that are activated in response to mechanical stimuli, controlling for the influence of neural and systemic factors. Moreover, the use of *in vitro* stretch systems has been shown to recapitulate many of the adaptations of *in vivo* SkM to loading and exercise (Passey et al. 2011). Previously published *in vitro* models have demonstrated a stretch-induced hypertrophic response (Vandenburg et al. 1979, 1988); however, *in vitro* models often lack bio-mimicry with regards to the cellular architecture, the dimension in which the cells are grown and the extra-cellular matrix (ECM) composition. As such, the development of 3D culture systems has tried to address these issues (Cheema et al. 2005, 2003; Powell et al. 2002), including work from our laboratory (Auluck et al. 2005; Martin et al. 2013; Mudera et al. 2010; Sharples et al. 2012; Smith et al. 2012).

Recent advancements have allowed for the development of a system mimicking *in vivo* SkM in terms of histologic structure and myogenic gene expression, allowing for the longer term culture and hence enhanced maturation of the constructs (Smith et al. 2012). To date, there is minimal data surrounding increased mechanical loading of such constructs following the development of aligned myotubes. This model provides an ideal format to investigate mechano-transcription of candidate hypertrophic genes within a representative type-1 collagen-based 3D matrix. To this end, the aim of the current investigation was to examine the effect of distinct mechanical overload paradigms on mediating the transcription of known hypertrophic transcriptional regulation.

Materials and methods

Cell culture

The C2C12 murine muscle myoblast cell line (Blau et al. 1985) were routinely sub-cultured and passaged when they reached 80% confluence. For all experimentation cells were used between passage three and eight. Cells were cultured in Growth Media (GM) [High Glucose Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine supplemented with 20% (v/v) foetal bovine serum Gold (FCS; PAA Laboratories, Somerset, UK), penicillin (100 U/ml) and streptomycin (100 µg/ml)]. To induce differentiation, cells were switched to

Differentiation Medium (DM) consisting of DMEM supplemented with 2% (v/v) FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml) and insulin-like growth factor-I (IGF-I; 10ng/ml; Sigma-Aldrich).

Cell seeded 3D compliant collagen constructs

The method used is based upon that of Smith et al. (2012). Briefly, 0.3 ml 10x Minimal Essential Media (MEM; Gibco) was added to 2.6 ml type 1 rat-tail collagen (First Link, Birmingham, UK; dissolved in 0.1 M acetic acid, protein at 2.035 mg/ml). NaOH was used to neutralise the solution for polymerisation. Following neutralisation, 12×10^6 cells in 0.1ml GM were added to the collagen solution and the collagen solution was pipetted into a custom-made glass chamber (Soham Scientific, Cambridgeshire, UK, Fig 1 a) between two custom-made polyethylene mesh flotation bars. The resulting construct was allowed to polymerise for 30 min at 37°C, 5% (v/v) CO₂. Once set, the compliant construct was physically detached from the chamber and floated in 4 ml GM. Custom made stainless steel wire 'A-frames' (wire, Scientific Wire Company, Great Dunmow, UK) attached to the flotation bars (Fig 1 a) facilitated resistance against the glass chamber. The resistance allowed for the generation of longitudinal tension for cell alignment and fusion in a single plane. Following 4 days of culture in GM, the constructs were transferred to DM to promote myoblast differentiation. A further 10 days of culture allowed for the maturation of the constructs and the differentiation of myoblasts into multinucleated myotubes (Fig 1 b).

Fig. 1

Experimentation

Following the 14 day maturation period, constructs were removed from the chambers and transferred to polyethylene moulds, floated in DM and mounted upon the Tensioning Culture Monitor (t-CM MK2, a modification to the system of Cheema et al. 2005) for mechanical overload. Constructs were used for each condition with constructs mounted upon the t-CM subjected to no mechanical stretch for 60 min acting as controls (CON, n = 4). Overload regimes were programmed as follows; Rapid Static Load (STL, n = 5) 10% strain for 60 min, Ramp Load (RPL, n = 3) continuous increasing load to achieve 10% strain over 60 min (Fig 2 b). The RPL condition employed, equated to a 50% total reduced gross workload compared to the STL condition (Fig 2 b). In order to assess the acutely activated transcriptional mechanisms associated with mechanical overload, constructs were sampled immediately at 60 min for RNA. The STL regime was designed to mimic the increased load that would be achieved following a synergistic ablation or overload protocol utilised

Fig. 2

in vivo or ex vivo, whilst the RPL condition aimed to model a continuously increasing load to ensure maximal mechanical strain upon the myotubes.

Lactate analysis

Conditioned media was sampled immediately post stretch for both regimes along with CON. An Analox Analyser P-GL5 (Analox, London, UK) was appropriately calibrated and used to measure media lactate. In order to assess whether the collagen matrix was acting as a ‘sponge’ preventing lactate accumulation in the conditioned media, a-cellular constructs were also tested for the regimes described. Media lactate was not different to basal media control confirming no effect (data not shown).

Construct sampling and extraction protocol

Immediately following the end of the stretch protocol, the constructs were removed from the t-CM and submerged in TRIzol. The tissue was homogenised using a hand-held homogeniser (IKA T-10, IKA, Straufen, Germany) before the extraction of RNA using TRIzol: RNA concentration and purity was assessed at 260 and 280 nm using a Nanodrop 3000 spectrophotometer.

Primer design and synthesis

Primers (Supplementary Table 1) were designed and synthesised by Sigma-Aldrich (Haverhill, UK). Primer sequences were analysed for GC nucleotide content as well as being analysed using a BLAST search (www.ncbi.nlm.nih.gov/tools/primer-blast/) to check for target sequence specificity. Melt curve analysis revealed single peaks for each tested gene, confirming single product amplification.

Quantitative reverse transcriptase Real-Time PCR (qRT-PCR)

A one step reaction chemistry was used for the qRT-PCR for all experiments (Quantifast, Qiagen, Crawley, UK). The Qiagen (Crawley, UK) Qiagility Robot was used to perform all ‘master mix’ and sample preparations, using associated programmable software. The reaction, in 20 µl, consisted of; 9.5 µl RNA (7.3ng/µl; a total of 70 ng template RNA for each sample), 0.15 µl forward primer, 0.15µl reverse primer (100 µM), 10µl Quantifast SYBR Green Mix (Qiagen, Crawley, UK) and 0.2µl of Reverse Transcriptase (RT)-Mix (Qiagen, Crawley, UK).

Samples were loaded into a Rotogene 3000Q (Qiagen, Crawley, UK) for a one step RNA-CT reaction. The PCR thermo-cycling followed the manufacturers recommendations as follows; reverse transcription step (hold at 50°C for 10 min), transcriptase inactivation and initial denaturation (hold 95°C for 5 min) and PCR Step consisting of 40 cycles; 95°C for 10 s for denaturation and 60°C for 30 s for annealing and extension. Fluorescence at the end of each extension step was measured using at excitation 470 nm and emission 510 nm. Samples were run in duplicate and the mean C_T (threshold cycle) values were used to calculate relative mRNA expression of the genes of interest (GOI). An experimental control (CON, tethered to the t-CM without stretch) along with an endogenous control gene (RNA polymerase II, polypeptide B [RPII-B], were used to calculate the mRNA expression of each GOI. The mean C_T for RPII-B was 21.15 ± 0.51 . The relative gene expression levels were calculated using the comparative C_T ($\Delta\Delta C_T$) equation (Schmittgen et al. 2008) for determining normalised expression ratios, where the relative expression is calculated as $2^{-\Delta\Delta C_T}$. All individual samples were normalised to a single designated CON sample (CON N1), in order to provide an indication as to the intrinsic variation within CON samples (represented by standard deviation [SD] in CON data bars).

Statistical analyses

All statistical analysis was performed using SPSS software version 17 (SPSS INC, Chicago, IL). Significant differences between variables were analysed using a one way ANOVA. A pair-wise comparison *post-hoc* test was performed with a Bonferroni adjustment for any data with significant F values. The alpha value of significance was set at $p < 0.05$ and statistical analysis was performed using SPSS software. All data is represented as the mean $\pm$ standard deviation.

Results

Effect of mechanical loading on growth media lactate concentrations

To measure how the gross mechanics employed for each regime contributed to an increase in cellular metabolic work, media lactate was quantified immediately post experimentation. Lactate concentrations were significantly increased in both RPL (6.43 ± 0.12 mM) and STL (4.8 ± 0.46 mM) conditions compared to CON (1.73 ± 0.31 mM, RPL vs CON $p = 0.003$, STL vs CON $p < 0.001$, Fig 3). In addition the RPL condition produced an increased lactate concentration that was significantly higher than that produced in the STL condition ($p < 0.001$). Together these data provide the evidence for an increased metabolic activity following mechanical overload, suggesting a stretch response-induced at the biochemical substrate level.

Fig. 3

Modulation of hypertrophic genes, IGF-I and MMP-9, following mechanical overload

To investigate whether the differential gross and metabolic workloads contributed to a molecular transcriptional response associated with hypertrophy, the role of candidate mechanical work-dependent hypertrophic genes IGF-I and MMP-9, were quantified. Static loading (STL) induced a striking induction of IGF-I mRNA expression (71.42 ± 16.42), above that seen with both RPL (17.4 ± 7.77 , $p = 0.011$ vs STL) or non-loaded CON constructs (3.94 ± 3.88 , $p = 0.003$ vs STL, Fig. 4 a). No significant difference was observed between RPL and CON samples ($p = 0.603$).

Fig. 4

A significant increase in MMP-9 mRNA was also observed (main effect, $p = 0.013$) with increases induced by both static load (16.37 ± 6.69 , $p = 0.027$ vs. CON) and RPL (22.7 ± 2.7 , $p = 0.005$ vs. CON) compared to unloaded CON constructs (4.06 ± 2.37 , Fig 4 b). Increases in both IGF-I and MMP-9 mRNA for both STL and RPL compared to CON suggests the acute regulation of these genes following mechanical overload.

A reduction in IGFBP-5 mRNA following mechanical overload

Following the quantitation of increased transcription of IGF-I induced by mechanical loading regimes in the 3D muscle constructs, we next wished to ascertain changes in the expression of IGF binding protein genes induced in response to mechanical overload. The response of IGFBP-2 and IGFBP-5 was quantified, as the modulation of these binding proteins has been implicated in hypertrophic and atrophic phenotypes during basal and exercise conditions, along with *in vitro* observations (Bickel et al. 2003; Rehfeldt et al. 2010; Severgnini et al. 1999; van den Beld et al. 2003). Furthermore, data demonstrates an alternate regulation of IGFBP-2 mRNA within *in vitro* observations of hypertrophic and atrophic phenotypes (Sharples et al. 2010).

A moderate but non-significant up-regulation in IGFBP-2 mRNA expression was found in the STL condition, with a non-significant down-regulation in RPL, compared to CON ($p = 0.057$, CON = 1.03 ± 0.03 , STL = 1.51 ± 0.52 , RPL = 0.44 ± 0.03 , Fig 5 a). In contrast, a down-regulation in IGFBP-5 mRNA expression occurred following both overload regimes (RPL 0.34 ± 0.12 , $p = 0.027$ vs non-loaded controls, CON = 1.01 ± 0.01 ; STL = 0.46 ± 0.23 , $p = 0.059$ vs CON, Fig. 5 b). No difference in IGFBP-5 mRNA was found between overload conditions ($p = 1$). The down-regulation in IGFBP-2 in the RPL condition and IGFBP-5 in both the RPL and STL conditions, provides evidence for the regulated control of IGFBPs in this system leading to a potential increased effect of IGF-I action following mechanical overload in this model.

Fig. 5

Mechanical overload has no effect on the mRNA expression of Myostatin, MuRF-1 or MAFBx.

With evidence to suggest an activated hypertrophic transcriptional signal following overload, the role of candidate atrophic genes was investigated. The defined mechanisms for the role of these genes in driving an atrophic phenotype are yet to be fully elucidated, however it is hypothesised that Myostatin has a role in actively inhibiting protein synthesis (McPherron et al. 1997a; Sharples et al. 2011; Wang et al. 2012), whilst MuRF-1 and MAFBx are thought to act through the ubiquitin proteasome pathway (UPP, Glass 2003; Gumucio et al. 2012; Yang et al. 2006). Both overload regimes contributed to small mean reductions in myostatin mRNA compared to CON, despite no statistical difference ($p = 0.572$, CON = 0.45 ± 0.35 , STL = 0.15 ± 0.08 , RPL = 0.08 ± 0.06 , Fig 6 a). No significant changes were apparent in MuRF-1 mRNA (CON = 1.24 ± 1.00 , STL = 1.03 ± 0.22 , RPL = 1.87 ± 0.47 , $p = 0.469$) and MAFBx mRNA expression (CON = 1.07 ± 0.34 , STL = 1.37 ± 0.39 , RPL = 1.13 ± 0.23 , $p = 0.632$ respectively, Fig 6 b and c), for any regime confirming the overload regimes employed were not sufficient to contribute to modulation of these key genes in hypertrophic phenotypes.

Fig. 6

Discussion

The aim of the current investigation was to address the extent to which mechanical signals mediate acute transcriptional mechanisms associated with a gene transcript signature that would provide relevant downstream signals to induce SkM adaptation. Particularly, we sought to address the effect of mechanical signals transduced in a 3D *in vitro* model incorporating a representative extra-cellular matrix.

In order to test whether the mechanical overload regimes were in fact eliciting a cellular metabolic response above control and to assess the extent to which the different regimens moderated this response, construct media was analysed for lactate post experimentation. The measurement of this metabolite provides an indication as to the translation of gross mechanical stimuli to cellular metabolic output within the collagen constructs. Despite a reduced total gross workload in the RPL condition, the enhanced metabolic response in this condition (demonstrated by increases in peak media lactate compared to STL) could be explained in terms of an intricate cellular response to the mechanical strain. It has previously been postulated that cells in 3D matrices respond to minimise the perceived strain placed upon the ECM during tension (Eastwood et al. 1998a,b). This data may provide evidence for the enhanced metabolic response in RPL, as the cells will be continually reorganising themselves with the continuous increased mechanical load.

With evidence to suggest a differential metabolic demand related to cellular workload in the RPL condition compared to STL, it was logical to investigate whether the cellular metabolic workload contributed to differential expression of workload related genes. STL significantly increased IGF-I mRNA above CON and RPL (both $p < 0.05$), demonstrating the augmented acute regulation of this gene in response to this specific mode of overload. Numerous *in vivo* models of mechanical overload have demonstrated increases in IGF-I, including models of synergistic ablation (Adams et al. 1996, 1999), mechanical overload (Awede et al. 1999) or *in vitro* stretch (Goldspink et al. 1995; McKoy et al. 1999) induced hypertrophy.

IGF-I is a growth factor that has been implicated in the hypertrophic process of skeletal muscle and works at the endocrine, paracrine and autocrine levels with variations in protein structure and function arising from the alternate splicing of the IGF-I gene (Goldspink 2005). Despite the liver supplying a large proportion of circulating IGF-I, it has been demonstrated that liver deficient IGF-I mice display similar gains in skeletal muscle strength following resistance exercise compared to control wild type mice (Matheny et al. 2009). Moreover, localised infusion of IGF-I induces skeletal muscle hypertrophy in rats (Adams et al. 1998). These findings provide support for the use and production of intrinsic IGF-I in the hypertrophic response in basal and exercise conditions *in vivo* (Aguiar et al. 2012; Kim et al. 2005). However, the use of *in vitro* experiments provides a means to investigate the role of intrinsically produced IGF-I in response to mechanical overload or stretch. Data presented here further supports the notion of the intrinsic regulation of IGF-I in the response to acute mechanical overload.

Matrix metallo-proteinases (MMPs), and particularly MMP-9 play a pivotal roles in hypertrophy, satellite cell activation and migration, and the maintenance of skeletal muscle mass (Dahiya et al. 2011; Lewis et al. 2000; Mehan et al. 2011). The mechanical overload regimes used in the current investigation increased the expression of MMP-9 mRNA compared to CON (both, $p < 0.05$), with a larger increase in response to RPL (a condition of continuous increased load) supporting the notion of MMP gene expression being mechano-regulated via ECM-integrin-mediated mechano-transduction (Mudera et al. 2000; Nagase et al. 1999; Riikonen et al. 1995). MMPs are secreted in a pro-enzyme form that is not active until the pro-peptide is removed by proteolysis, (Liu et al. 2010). Not only does this degradation of the ECM allow for the enhanced action of growth factors (such as IGF-I), MMPs have been shown to enhance the bio-availability of IGF-I through the proteolysis of IGF binding proteins (Coppock et al. 2004). Furthermore, overexpression of an active mutant of MMP-9 in mice leads to increases in phenotypic (fibre cross-sectional area and contractile proteins) and functional (isometric force)

characteristics of skeletal muscle (Dahiya et al. 2011). In this respect, the increases in the transcription of MMP-9 established in this investigation suggest an activated mechanism to induce subsequent hypertrophy. The exact molecular mechanism for the mechano-activation of MMP-9 is yet to be fully elucidated, with the current model providing a platform to investigate the translation of extra-cellular matrix forces to gene transcription.

IGFBPs-2 and -5 regulate SkM hypertrophy and differentiation *in vivo* and *in vitro*. IGFBPs regulate the action of IGF-I, working to inhibit or potentiate its action (Clemmons 1998). IGFBPs control growth and development through governing the bio-availability of IGF-I and the affinity to the IGF-I receptor, inhibiting growth in excess quantities (Wolf et al. 2005). Consequently, in a transgenic mouse model overexpressing IGFBP-2, myofibre size is reduced and the ability for proliferating nuclei is hindered (Rehfeldt et al. 2010). Furthermore, IGFBP-2 is differentially regulated in hypertrophic and atrophic phenotypes *in vitro* (Sharples et al. 2010), demonstrating its important role in modulating a hypertrophic outcome. Future experiments should seek to identify whether the mean observed increase in IGFBP-2 mRNA in the static loading condition presented here would contribute to a physiological response. Such interaction between IGF-I and IGFBP-2 proteins, has been investigated in monolayer culture relating to differentiation and hypertrophy (Sharples et al. 2013) and warrants investigation in this model. The increase in IGFBP-2 mRNA in the static load condition warrants investigation, to identify whether consequential IGF-I signalling is inhibited whilst also identifying the cytomolecular mechanism leading to increased expression of this gene.

Modulation of IGFBP-5 has also been shown in a model of overload induced hypertrophy where IGFBP-5 mRNA was reduced to a third of control levels (Awede et al. 1999). Awede et al. (1999) also established a temporal relationship between decreasing IGFBP-5 mRNA and increasing IGF-I mRNA, suggestive of a particular interaction between the transcriptional control of these two genes. These data together, surrounding the influence of IGFBPs on IGF-I action and hypertrophy are compelling, with for their transcriptional regulation following mechanical overload *in vitro*.

When investigating the molecular regulation of skeletal muscle mass, it is important to consider the mechanisms involved in inhibiting protein synthesis or increasing proteolysis. The effect of mechanical loading *in vivo* demonstrates a clear bi-phasic regulation pattern of proteolytic associated genes, MuRF-1, MAFBx and Myostatin, related to acute and chronic exercise (Louis et al. 2007; Yang et al. 2006; Zanchi et al. 2009). Modest, but non-significant, reductions in myostatin mRNA were found in both of the overload conditions compared to control, whilst no effect in MuRF-1 or MAFBx transcription was evident. Such data may reflect the

short time-frame that was utilised in the current investigation, with prolonged overload periods contributing to reductions in the expression of these key genes in the hypertrophic response as evident with *in vivo* investigations (Louis et al. 2007; McPherron et al. 1997b; Roth et al. 2003).

The current investigation demonstrates the acute hypertrophic transcriptional response of an *in vitro* model of SkM, following two mechanical overload regimes. These data provide further evidence for the role of mechanical signals in mediating increases in intrinsic IGF-I and MMP-9 mRNA. Furthermore, this rapid increase in transcription illustrates a significant role for these genes in the acute adaptive response to SkM overload. This study demonstrates for the first time, the intrinsic role of IGF-I and the IGF-BPs -2 and -5 in response to mechanical overload and augments the potential for IGF-I signalling to induce compensatory hypertrophy. Further experiments are required to address the mechanisms pertaining to the diverse IGF-BP-5 mRNA response presented and the extent to which such a response contributes to altered IGF-I protein signalling.

The transcriptional response identified here shows many similarities to *in vivo* investigations (Table 1), demonstrating the potential for the use of this model as a pre-clinical model of exercise therapies in a model that more closely represents *in vivo* SkM. Hence, the data presented herein is a significant advancement on previously published models with respect to the application of tissue engineered models to further understand *in vivo* physiology. Furthermore, this establishes the conserved and intrinsic nature of the mechanisms investigated in an isolated engineered system. In addition, this study has provided evidence for the use of an animal-free bio-mimetic *in vitro* model to investigate the intrinsic mechanistic response of SkM to mechanical overload. Finally, future experiments will seek to delineate the intra-cellular mechanisms that regulate the interaction between mechanical forces and the transcriptional observations presented herein.

Table 1

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519 **Table 1 Findings relating to *in vivo* investigations in the response to mechanical load and exercise**

Gene	Response	<i>In Vivo Response</i>	Effect	References
IGF-I	↑	↑	Potential for increased protein synthesis and satellite cell activation (SC)	Kim et al. (2005), Aguiar et al. (2012)
MMP-9	↑	↑	Increase potential for matrix remodelling-SC activation, growth factor availability and consequential hypertrophy	Rullman et al. (2009), Dahiya et al. (2011)
IGFBP-2	↔	↓	Increased potential for bio-availability and signalling through IGF-I and IGF-IR	Transgenic model-Rehfeldt et al. (2010)
IGFBP-5	↓	↓	Increased potential for bio-availability and signalling through IGF-I	Awede et al. (1999)
MuRF-1	↔	Acute ↑ Chronic ↓	Suppression of ubiquitin proteolytic activity	Louis et al. , (2007) Yang et al. (2006) Zanchi et al. (2009)
MAFBx	↔	Acute ↑ Chronic ↓	Suppression of ubiquitin proteolytic activity	Louis et al. (2007) Yang et al. (2006) Zanchi et al. (2009)
Myostatin	Small ↓	↓	Suppression of ubiquitin proteolytic activity	Louis et al. (2007)

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528 **Figure Legends**

Fig 1 a. Macroscopic contraction of a construct seeded with 4×10^6 cells/ml at 14 days. Note the floatation bars and A-frames at either end of the glass chamber, providing the tension required to generate lines of principle strain throughout the matrix which facilitates cell alignment and fusion. The remodelling of the matrix contributes to increased incidence of cell-cell contact required for fusion (scale bar = 10 mm).

b. Representative fluorescence micrographs of un-stimulated collagen constructs seeded with 4×10^6 cells/ml for 14 days. Note the high level of orientation and density of the cells (scale bar = 20 μ m).

Fig 2 a. Schematic of the tensioning Culture Monitor (t-CM) used for mechanical overload. i. The fixed point which provides uniaxial tension applied to the constructs. ii. Opposing force transducer and fixed point. iii. PTFE mould used to suspend the constructs in DM. iv. The programmable stepper motor used to control the overload regimes.

b. Graphical representation of the mechanical overload regimes used in this investigation. $n = 3+$ constructs were used for each condition with constructs subjected to no mechanical stretch acting as controls (CON). Overload regimes were programmed as follows; Static Load (STL, square and solid line) 10% strain for 60 mins, Ramp Load (RPL, round and dashed line) continuous increasing load to achieve 10% strain at 60 min.

Fig 3 Conditioned media lactate analysis. Conditioned media was sampled immediately post mechanical overload for the analysis of lactate. Significant increases in media lactate were observed for STL and RPL compared to CON (both $*p < 0.001$). A significant increase in Lactate was found between RPL compared to STL ($\#p < 0.001$), suggesting an increased metabolic demand in this condition. Bars = mean $\pm$ SD.

Fig 4 a. Modulation of IGF-I mRNA following mechanical overload ($p = 0.002$), with STL significantly increasing IGF-I mRNA compared to both CON ($*p = 0.003$) and RPL ($\#p = 0.011$). A moderate but non-significant increase in IGF-I mRNA was observed in the RPL condition, compared to CON ($p = 0.603$). All data is normalised to the expression of RP-IIB and made relative to $N = 1$ CON construct. $N = 3+$ per condition. Bars = mean $\pm$ SD.

b. Change in MMP-9 mRNA following mechanical overload ($p = 0.013$). Increases were evident for both STL ($*p = 0.027$) and RPL ($\#p = 0.005$) compared to CON, whilst RPL moderately increased MMP-9 mRNA compared to STL ($p = 0.234$). All data is normalised to the expression of RP-IIB and made relative to $N = 1$ CON construct. $N = 3+$ per condition. Bars = mean $\pm$ SD.

Fig 5 a. Regulation of IGFBP-2 mRNA in the STL condition, with a non-significant reduction in RPL, compared to CON ($p = 0.057$).

b. IGFBP-5 mRNA expression following overload regimes ($p = 0.018$), with significance in the RPL condition compared to CON, and approaching significance in STL ($*p = 0.027$ and $\#p = 0.059$ respectively). No difference in IGFBP-5 mRNA was found between overload conditions ($p = 1$). All data is normalised to the expression of RP-IIB and made relative to $N = 1$ CON construct. $N = 3+$ per condition. Bars = mean $\pm$ SD.

Fig 6 a. Myostatin mRNA following STL and RPL compared to CON ($p = 0.572$).

b. MuRF-1 mRNA following either mechanical overload regime ($p = 0.469$).

c. MAFBx mRNA expression following either mechanical overload regime ($p = 0.632$). All data is normalised to the expression of RP-IIB and made relative to $N = 1$ CON construct. $N = 3+$ per condition. Bars = mean $\pm$ SD.

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