**FHL1 facilitates myostatin dependent muscle atrophy**

**FHL1 activates myostatin signalling in skeletal muscle and promotes atrophy**

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**ABSTRACT**

Myostatin is a TGFβ family ligand that reduces muscle mass. In cancer cells TGFβ signalling is increased by the protein FHL1. Consequently FHL1 may promote signalling by myostatin. We therefore tested the ability of FHL1 to regulate myostatin function. FHL1 increased the myostatin activity on a SMAD reporter and increased myostatin dependent myotube wasting. In mice, independent expression of myostatin reduced fibre diameter, whereas FHL1 increased fibre diameter both consistent with previously identified effects of these proteins. However, co-expression of FHL1 and myostatin reduced fibre diameter to a greater extent than myostatin alone. Together these data suggest that the expression of FHL1 may exacerbate muscle wasting under the appropriate conditions.

Key words: muscle wasting, fibre type, FHL1, myostatin, mouse

**Abbreviations used:** FHL1; four and a half LIM domain protein 1, COPD; chronic obstructive pulmonary disease, TGF-β; transforming growth factor beta, MHC; myosin heavy chain, GDF-15; growth and differentiation factor 15, PAI-1; plasminogen activator inhibitor 1, TA; tibialis anterior, VEGF-C; vascular endothelial growth factor C

**Highlights:**

Myostatin signals via SMADs to promote muscle wasting.
FHL1 normally promotes hypertrophy but can activate SMAD signalling
FHL1 promoted myostatin signalling in vitro
FHL1 promoted hypertrophy in the absence of myostatin but atrophy in its presence
**INTRODUCTION**

Voluntary movement is essential for a normal healthy life and the performance of daily activities. Such movement requires a sufficient quantity of skeletal muscle especially in the locomotor muscles and the appropriate proportions of the different fibre types. Different fibre-types have distinct rates of contraction and abilities to endure activity. The overall phenotype of a muscle is related to the relative proportions of the different fibres it contains. Muscle phenotype is plastic and the size and proportion of the individual fibres can change dependent on a number of factors including physical activity.

Changes in muscle mass and phenotype are important aspects of a number of chronic diseases such as chronic obstructive pulmonary disease (COPD), heart failure and cancer and have prognostic ability. Indeed exercise capacity and strength are better predictors of survival in patients with COPD than standard measures of pulmonary function[1]. Muscle mass is also lost in ageing and there is a marked change in phenotype again with prognostic implications [2, 3]. Consequently the factors that affect muscle mass are being intensively studied.

Not all fibres atrophy at the same rate and a number of studies have shown that type II fibres are more likely to atrophy than type I fibres in diseases as varied as COPD [4], heart failure [5] and osteoarthritis [6] as well as in normal human ageing [7]. As there is a shift towards type II fibres in the quadriceps muscles in chronic disease, this increased sensitivity of type II fibres to atrophy is likely to contribute to accelerated wasting. Under some conditions (e.g. starvation) where muscle acts as an emergency fuel store, this response may be important; however, in chronic disease it is likely to be detrimental.

Myostatin is one factor likely to be involved in the increased susceptibility of type II fibres to atrophy. This growth regulator is a member of the transforming growth factor-β (TGF-β) family that was identified from natural mutations in animals with a double muscled phenotype [8, 9]. Germ-line deletion of the myostatin gene from mice resulted in a similar hypermuscular phenotype suggesting that myostatin is an inhibitor of muscle growth. Deletion of myostatin also increased the proportion of the fastest type IIB fibres suggesting that the major effects of myostatin were on this fibre type [10, 11]. Furthermore myostatin expression is highest in type IIB fibres [12], is elevated in response to hind limb suspension and is a target for the type I fibre restricted microRNA, miR-499. Indeed it has been shown that increased myostatin mRNA and protein are associated with type II muscle atrophy[13].

Myostatin signals by binding to an activin IIB/alk4/5 receptor complex promoting the phosphorylation of SMAD-2/3. Consequently factors that modify SMAD-2/3 phosphorylation are likely to alter myostatin signalling. One protein that activates SMAD proteins is the four and a half LIM domain protein FHL1 which binds to CKδ and promotes SMAD phosphorylation [14]. In muscle cells such an activity would promote muscle atrophy but under normal conditions FHL1
FHL1 facilitates myostatin dependent muscle atrophy

appears to promote hypertrophy. For example, over-expression of FHL1 leads to muscle hypertrophy[15] and patients with mutations in FHL1 have a range of myopathic conditions including X-linked myopathy with postural muscle atrophy (XMPMA)[16]. However, a number of studies have shown that FHL1 can associate with atrophy. For example, denervation in mice increases FHL1 [17], long-term training in humans reduces FHL1 expression [18] and we observed that FHL1 was associated with weakness in COPD patients [19]. These observations raise the possibility FHL1 potentiates myostatin signalling in muscle cells so contributes to muscle wasting under a subset of conditions. Consequently in this study we determined the effect of FHL1 on SMAD reporter gene expression in response to myostatin, as well as the effect of FHL1 on myostatin induced myotube wasting. Finally we used electroporation of the *tibialis anterior* (TA) in mice to determine the effect of FHL1 on myostatin induced muscle wasting *in vivo*.

**MATERIALS AND METHODS**

**Cloning**

Full-length murine myostatin and FHL1 were sub-cloned into pGEMT by PCR from image clones (Source Bioscience) then shuttled into empty pCAGGS expression vector (containing a CMV-enhancer and chicken β-actin promoter) [20] and sequenced. Large-scale plasmid preparations were carried out using the EndoFree Mega kit (Qiagen) according to manufacturer’s instructions and plasmids were eluted in sterile dH2O.

**Cell culture, Luciferase reporter and myotube diameter assays**

For luciferase experiments, C2C12 myoblasts were cultured and transfected using lipofectamine as described in [21] scaled for culture in 24 well plates. A total of 0.4μg DNA was transfected per well in total: 0.2μg of (CAGA)12-luciferase plasmid[22], 0.1μg of pRLTK plasmid, 0.1μg of either FHL1-pcDNA or 0.1μg pcDNA as the control. After transfection the cells were cultured in DMEM supplemented with 10% (v/v) FBS. Twenty-four hours later, cells were serum starved for 7 hours then washed and treated with serum-free DMEM containing either TGF-β1 (Insight Biotechnology) or Myostatin (PromoKine) for 16 hours. Luciferase activity was assayed using the Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer’s instructions. Firefly luciferase activity was normalised to Renilla luciferase activity to account for transfection efficiency. All data were normalised to the mean of the pcDNA untreated (0ng/mL TGF-β) group for that set of transfections.

For myotube diameter measurements, C2C12 myoblasts were cultured and transfected as described in [23] with a total of 2μg DNA consisting of 0.5μg pCAGGS-GFP [24] and either 1.5μg FHL1-pcDNA or 1.5μg pcDNA. Thus, myoblasts that were successfully transfected with pCAGGS-GFP would also
be transfected with the FHL1 expression vector and allow identification of fluorescent ‘transfected’ myotubes. After 4.5h incubation, media was replaced with fresh DMEM + 10% FBS and the cells returned to the incubator. The medium was replaced every 2 days and allowed to become confluent (approximately 3 days). The media was replaced with DMEM supplemented with 2% horse serum (differentiation medium) to differentiate myoblasts into myotubes for 7 days, with fresh replacement every 2 days. Myotube formation was confirmed by the presence of multinucleate cells and parallel experiments looking at the expression of myogenic regulatory factors and MHCs showed an increase in the expression of all MHCs with an increase in myogenin expression consistent with previous observations [25, 26] (data not shown). After 7 days, the media was then replaced with differentiation medium supplemented with 20ng/ml myostatin (PromoKine) or vehicle control (0.1% BSA and 20mM HCl). After 48 and 96h of exposure, fluorescent myotubes were identified in randomly selected views at 10x magnification and captured using a Hamamatsu C4742-95-12ERG camera attached to a Zeiss Axiovert 200 microscope with the filter set 470nm/40nm. Average myotube diameter was ascertained by measuring the shortest distance across the myotube at five points along the length of fluorescent myotubes using Image J.

**Electroporation**

Mouse experiments were approved by the Royal Veterinary College Ethical Review Process (ERP-A-2010-WS01) and were licensed by the UK Secretary of State for the Home Office under Project License PPL 70/6797. Twelve female CD1 mice (7.5 weeks old) were anaesthetised with Hypnorm (VetaPharma) and Hypnovel (Roche), both lower legs were shaved and 10U (25μl) of bovine hyaluronidase (Sigma) was injected percutaneously into each TA to increase transfection efficiency.[27, 28]. Mice were allowed to partially recover at 37°C and after 1.5 hours they were re-anaesthetised using 5% isoflurane and maintained at 2% isoflurane. The TA muscles were injected with 25μl of the appropriate plasmid at 1mg/mL. Immediately following the plasmid injection electro-conductive cream was applied to electrodes which were placed either side of TA, separated by approximately 5mm. Electroporation was performed using 10 pulses of 85V each for 20 msec, at a frequency of 1 Hz. Following electroporation, mice recovered and were left for 2 weeks, after which the mice were sacrificed and TA muscles were harvested and placed upright onto small pieces of cork with a small amount of OCT at the bottom to fix the bottom of the TAs onto cork and flash frozen in liquid nitrogen cooled iso-pentane.

Muscle samples were sectioned as previously described [27] to obtain tissue for histology and RNA analysis from defined levels within the muscle. Muscle sections were stained with haematoxylin and eosin, and by immunofluorescence for fibre type as previously described [29]. Random fields were captured at 20x magnification using an Olympus CKX41 camera and Cell^D software (Olympus Europe).
RNA extraction from tissue

Muscle sections from regions adjacent to the histology samples were placed into CK-14 ceramic beaded tubes containing 500µl of TRIzol® (Invitrogen) and homogenised with the Precellys 24 (Stretton Scientific) for 2 x 15 second cycles at 5500rpm. The samples were centrifuged at 8000rpm for 3 minutes at 4°C and the supernatant transferred to fresh micro-centrifuge tubes and RNA extracted according to manufacturer’s instructions (Qiagen). The RNA was resuspended in 30µl RNase-free dH2O and stored at -80°C. RNA concentration was quantified using a Nanodrop.

Quantitative real-time PCR (QPCR)

cDNA was synthesised from 150ng RNA and amplified by qPCR as previously described [30]. The PCR primers used have been described previously[31]. Data was normalised to a geometric mean of ribosomal protein large P0 (RPLPO) and β2 microglobulin using the ΔΔCt method.

Statistical analysis

Data are presented as mean ±SEM for data with a parametric distribution and median (interquartile range) for non-parametric data. Differences were determined by t-test for parametric data and by Mann-Whitney test for non-parametric data. To establish differences in muscle fibre profiles data were compared by one-way ANOVA for each point. Significance was set at a 2 tailed p value = 0.05).

RESULTS

Effect of FHL1 on SMAD reporter gene expression in response to TGFβ ligands

Treatment of the cells with 10ng/mL TGFβ alone increased CAGA12 luciferase reporter gene expression by ~4 fold and this was increased further to ~7 fold by expression of FHL1 (Fig. 1a) consistent with an effect of FHL1 on TGF-β signalling. FHL1 expression had no effect on luciferase activity in the absence of added TGF-β. In the absence of transfection with an FHL1 expression plasmid (i.e. in the cells transfected with pCDNA), myostatin had no effect on luciferase activity at concentrations below 100ng/mL. At 100ng/mL myostatin alone increased luciferase activity ~1.4 fold (p=0.025). However, in the presence of FHL1, myostatin caused a detectable increase in luciferase activity at all concentrations above 20ng/mL (20ng/mL 1.4 fold, p=0.003, 50ng/mL, 1.8 fold p<0.001, 100ng/mL 1.7 fold p=0.001). Furthermore, at all doses myostatin caused a larger increase in mean luciferase activity in the presence of FHL1 than in its absence (Fig. 1b) which reached statistical significance for 50ng/mL (20ng/mL 1.3 fold, p=0.065, 50ng/mL, 1.5 fold p=0.001, 100ng/mL 1.2 fold p=0.221). These data show that FHL1 increases the activity of myostatin signalling in myoblasts. The lack of a significant effect of FHL1 on luciferase activity at 100ng/mL
myostatin raises the possibility that FHL1 increases the sensitivity of the cell to myostatin rather than the size of the response. Alternatively it may reflect the weakness of the response of the reporter system to myostatin compared to TGF-β.

**Effect of FHL1 on myotube diameter in response to myostatin**

To determine whether FHL1 enhanced myostatin dependent myotube wasting, we determined the effect of myostatin on myotube size in the presence or absence of FHL1 expression. Treatment of the cells with 20ng/mL myostatin alone did not alter the size of the myotubes 2 days or 4 days after treatment (2 days-myostatin 29.6μm (24.1, 33.4) Control 30.1μm (22.8, 36.2, Fig.2a and 2b), 4 days-myostatin 29.3μm, (24.9, 37.4) Control 30.4μm, (25.3, 36.0), Fig. 2), an effect consistent with the lack of CAGA12 activation seen in myoblasts at this dose. Transfection of FHL1 into C2C12 cells caused a small but significant increase in myotube size (FHL1 31.1μm (25.5, 39.8) compared to control transfection 30.1μm (22.8, 36.2) p<0.05, Fig. 2), consistent with previously described effects [15] In contrast to the lack of effect of myostatin on control cells, in cells expressing FHL1, 20ng/mL myostatin caused a significant reduction in myotube size both 2 and 4 days after treatment (2 days-FHL1 31.1μm (25.5, 39.8μm), vs FHL1 + myostatin 27.1μm (23.9, 31.6) p<0.001, 4 days- (FHL1 31.5μm, (27.5, 36.2), FHL1 + myostatin 28.5μm, (23.9, 31.1) p<0.001) Fig. 2).

**Effect of FHL1 on myostatin induced muscle wasting in vivo.**

To determine whether FHL1 also increased myostatin dependent atrophy in vivo, we over-expressed myostatin and FHL1, alone or in combination in the tibialis anterior (TA) muscles of mice by electroporation. To determine the effect of myostatin or FHL1 alone, mice were injected in the right TA with pCAGGS-myostatin (M1) or pCAGGS-FHL1 (F) and in the left TA with empty pCAGGS as a control (C1 and C2 respectively). To determine the effect of FHL1 on myostatin activity, mice were electroporated in the right TA with both pCAGGS-myostatin and pCAGGS-FHL1 (M+F) and in the left TA with pCAGGS-myostatin (M2). Electroporation with pCAGGS-myostatin increased myostatin mRNA expression in M1 compared to the contralateral C1 (Fig. 3A). Similarly, electroporation of pCAGGS-FHL1 increased FHL1 expression in F and F+M muscles compared to their respective controls (C2 and M2) but this only reached statistical significance in F+M vs M2 (Fig. 3B). However, there was no effect of myostatin on FHL1 expression and FHL1 mRNA was significantly higher in the F and F+M groups combined than in all other groups combined (2 fold, p<0.01, Fig. 3B). Myostatin expression in the muscle electroporated with both plasmids (F + M) did not differ from that in the contralateral muscle electroporated with pCAGGS-myostatin alone (M2) (Table 3).
FHL1 facilitates myostatin dependent muscle atrophy

Sections from each electroporated muscle were stained with H&E to analyse the effect of electroporation on muscle fibre diameter (Fig 4). Consistent with previous studies, myostatin expression alone caused a ~10% decrease in fibre diameter [32] (Fig. 4A and F, from 37.8±0.9 µm (n=8) to 33.1±0.9 µm (n=8) p<0.001) whereas FHL1 expression increased fibre diameter by 10% compared with the control TA (Fig. 4B and F to 42.7±0.5 µm (n=4) p<0.001). Co-expression of FHL1 with myostatin in the same TA caused a larger decrease in fibre diameter than expression of myostatin alone (8.7% further reduction, to 30±1.2 µm (n=4), Fig. 4C and F, p=0.033). Comparing the diameter of myofibres overexpressing FHL1 in the presence or absence of myostatin showed that myostatin decreased fibre diameter from 42.7±5µm to 30±1.2 µm a reduction of approximately 25% (p<0.001).

To analyse further the effects on muscle fibre size, we determined the proportion of fibres within 5µm bins and the proportion of fibres below a given fibre diameter again in 5µm steps (Fig. 5). Comparison of the two sets of control muscles with each other and the two sets of myostatin muscles with each other, showed no significant difference at any point, validating the technique and showing that expression of myostatin or FHL1 in the contralateral TA did not affect fibre size (Fig. 5E and F). This approach also allowed us to pool the data from the control and the data from the myostatin treated muscles. Myostatin caused a significant shift to smaller fibres compared to control electroporated muscles (Fig. 5A and G). FHL1 caused a significant increase in the proportion of larger fibres compared to control fibres. (Fig. 5B, E and G). In FHL1 expressing muscles, myostatin caused a greater increase in the smallest fibres than myostatin alone with a significant increase in the fibres below 25µm (Fig 5C, F and G).

Fibre proportions were analysed by immunofluorescence (Fig. 6 and 7). Comparison of the proportion of fibres in each muscle showed that myostatin expression reduced the proportion of type IIB fibres and increased the proportion of IIA fibres compared to the controls. FHL1 alone did not affect the fibre proportion compared to controls and in combination with myostatin appeared to cause an increase in type IIB/type IIX fibres (p=0.037, Fig. 6).

Gene expression within the groups was then examined to identify changes in pathways associated with muscle wasting. Given that myostatin over-expression alone caused wasting, downstream TGF-β signalling was analysed, revealing a significant increase in PAI-1 (1.49 fold, M1 compared to C1, p<0.05, Table 1), suggesting activation of the TGF-β signalling pathway. To identify the atrophy pathway induced by the myostatin signalling, expression of components of proteasomal degradation, autophagy and apoptosis were measured. No significant differences were observed in the expression of any of these genes but there was a trend to a decrease in expression of ATG4B (0.73 fold, p=0.059) and ATG12l (0.58 fold, p=0.076), genes that are associated with autophagy. Changes in the expression of the myosin heavy chains were identified; with an increase in MHCII (13.1 fold, p<0.01) and a decrease in expression of MHCIIIB (0.34, p<0.05, consistent with the observed
FHL1 facilitates myostatin dependent muscle atrophy

reduction in type IIB fibres) and a trend towards a reduction in MHCIIX (0.64, p=0.06). There was no apparent increase in the expression of MHCHIA even though there was an increase in type IIA fibre proportion possibly due to the small number of samples analysed. There was also a significant increase in myogenin expression (1.82, p<0.05).

Expression of FHL1 alone did not significantly alter the expression of any of the genes tested (F compared to C2, Table 2). However, there was a trend to an increase in VEGF-C (1.2 fold, p=0.085). Additionally there was a trend to a decrease in BAX expression (0.81 fold, p=0.075).

Expression of FHL1 and myostatin in the same muscle (F + M compared to M2, Table 3) caused a significant increase in the expression of genes associated with autophagy (ATG12l; 1.46 fold, p<0.05 with a trend to an increase in ATG4B; 1.41 fold, p=0.052) and apoptosis (BAD; 1.36 fold, p<0.05 and BAX; 1.29 fold, p<0.01) as well as a trend to an increase in MuRF1 (1.27 fold, p=0.056, Table 3) compared to the expression of myostatin alone. There were no significant differences in the expression of myosin heavy chains in between muscles expressing myostatin alone and myostatin and FHL1 but there was a trend towards an increase in the expression of MHCIIX (1.88 fold, p=0.058) and MHCI (2.99 fold, p=0.075).

**DISCUSSION**

Our data indicate that exogenous expression of FHL1 increases myostatin activity in skeletal muscle cells *in vitro* and exacerbates muscle wasting in the presence of elevated myostatin *in vivo*. The accepted role for FHL1 is as a promoter of hypertrophy, as shown by the effects of overexpression of FHL1 in mice and postural muscle atrophy in patients with mutations in FHL1 [15, 16]. In the absence of myostatin we observed an increase in both myotube and myofibre diameter in response to FHL1 consistent with the prior data. However, in the presence of myostatin we found that FHL1 enhanced the effects of myostatin both *in vitro* and *in vivo*. These data are consistent with FHL1 potentiating the effects of TGF-β as identified in hepatic carcinoma cells[14]. However, in muscle cells FHL1 did not increase SMAD signalling in the absence of a TGF-β ligand as observed by Ding *et al* in tumor cells [14]. The reason for this difference is not clear but differences in cell type with different relative levels of expression or localisation of CK1δ or SMAD proteins may provide an explanation.

The activation of myostatin by FHL1 may help to explain the increase in FHL1 following sciatic nerve section[17] and the association of FHL1 with weakness in COPD patients[19] as myostatin expression increases in both situations[33, 34]. Furthermore, FHL1 is expressed at higher levels in type II fibres than in type I fibres raising the possibility that it contributes to the greater sensitivity of type II fibres to myostatin.

Myostatin has previously been shown to increase the expression of the atrogenes MuRF-1 and atrogin-1 and we have previously shown that myostatin increases the expression of autophagy-
**FHL1 facilitates myostatin dependent muscle atrophy**

associated genes *in vitro* [31, 35]. Whilst there was no detectable increase in the expression of genes associated with these pathways in the muscles treated with myostatin alone in the presence of both myostatin and FHL1, the expression of genes associated with autophagy and apoptosis were increased consistent with muscle atrophy. The lack of increase in the presence of myostatin alone may have been the result of the time point studied thus any earlier increase in the expression of these genes may have been missed. Such an explanation is consistent with the small increases observed in the presence of myostatin and FHL1 where the atrophy was greater.

**Critique of the experimental approach**

The data present a consistent argument that FHL1 increases the functional effects of myostatin *in vitro* and *in vivo*. This observation is also consistent with activation of SMAD signalling by FHL1 in other cells. However, there are a number of potential confounding factors in the data that need to be considered. First it should be noted that the experimental approach relies on over-expression and it is possible that this causes an artefact. Against this suggestion is the consistency of the responses in myoblasts, myotubes and muscle *in vivo* as FHL1 increases myostatin activity measured by 3 different assays (luciferase based SMAD reporter assays, myotube wasting *in vitro* and myofibre wasting *in vivo*). The second potential confounding issue is that the *in vivo* response occurs on the background of a regeneration response to electroporation injury complicating any picture. However, our observations on the effects of the single agents (myostatin and FHL1) are consistent with previous studies using transgenic over-expression, which does not cause injury and regeneration [15, 36]; with myostatin alone causing atrophy and FHL1 alone causing hypertrophy. Furthermore, the effects we observe *in vitro* are the same as those we observe *in vivo* (i.e. FHL1 alone increases myotube size whereas in combination with myostatin it causes a greater reduction in myotube size than myostatin alone). Together these observations suggest that the combined response *in vivo* is not caused by the experimental approach. The third confounding factor is that we did not observe large changes in gene expression in response to either FHL1 or myostatin. Indeed only the increase in myostatin (which we over expressed) and MCH-I exceeded a 2 fold change although a number of other changes reached statistical significance. These small changes are likely to result from the time-point chosen for the analysis which is 2 weeks after the electroporation and the likely reduction in the expression of the transfected genes with time. Such small changes and the fact that many trends did not reach statistical significance may account for the apparent opposite direction of change observed for a number of the genes between myostatin compared to control (Table 1) and myostatin + FHL1 compared to myostatin (Table 3). However, as FHL1 also interacts with a number of transcription factors including NFATC1 [15] it is also possible that these differences in direction of change reflect a different time course that results from the interaction between FHL1 and myostatin or some other factor within the experimental system.
Conclusion

In conclusion we demonstrate that exogenous FHL1 expression exacerbates the atrophic effects of myostatin in vitro and in vivo. These observations together with the relative restriction of FHL1 to type II fibres observed in the literature [16], suggest that FHL1 may contribute to type II fibre atrophy under the appropriate conditions. However, further experiments are required to confirm that FHL1 contributes to the increased sensitivity of type II fibres to myostatin dependent atrophy.

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