HSP70 drives myoblast fusion during C2C12 myogenic differentiation

Savant S. Thakur*, Kristy Swiderski, Victoria L. Chhen, Janine L. James, Nicki J. Cranna, A. M. Taufiqual Islam, James G. Ryall and Gordon S. Lynch‡

ABSTRACT

In response to injury, skeletal muscle stem cells (MuSCs) undergo myogenesis where they become activated, proliferate rapidly, differentiate and undergo fusion to form multinucleated myotubes. Dramatic changes in cell size, shape, metabolism and motility occur during myogenesis, which cause cellular stress and alter proteostasis. The molecular chaperone heat shock protein 70 (HSP70) maintains proteostasis by regulating protein biosynthesis and folding, facilitating transport of polypeptides across intracellular membranes and preventing stress-induced protein unfolding/aggregation. Although HSP70 overexpression can exert beneficial effects in skeletal muscle diseases and enhance skeletal muscle repair after injury, its effect on myogenesis has not been investigated. Plasmid-mediated overexpression of HSP70 did not affect the rate of C2C12 proliferation or differentiation, but the median number of myonuclei per myotube and median myotube width in differentiated C2C12 myotubes were increased with HSP70 overexpression. These findings reveal that increased HSP70 expression can promote myoblast fusion, identifying a mechanism for its therapeutic potential to enhance muscle repair after injury.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: Heat shock protein 70, Myogenesis, Fusion, C2C12, Skeletal muscle

INTRODUCTION

Skeletal muscle has a remarkable ability to regenerate in response to injury due to its resident population of adult muscle stem cells (MuSCs). Following injury, MuSCs are activated, enter the cell cycle and become specified to the myogenic lineage after which they proliferate rapidly, differentiate and ultimately undergo fusion and maturation (Yin et al., 2013). During myogenesis, dramatic changes occur in cell size, shape, metabolism, and motility, which alter proteostasis and cause cellular stress (Tang and Rando, 2014). Stressed cells produce heat shock proteins (HSPs) as an adaptive mechanism to survive the insult. Heat shock protein 70 (HSP70), the most well studied member of the highly conserved HSP family of molecular chaperones, protects against cellular stress and maintains proteostasis (Clerico et al., 2015; Thakur et al., 2018). Transgenic manipulation of HSP70 and pharmacological induction of Hsp72 (the inducible form of HSP70) have beneficial effects in muscle conditions including ageing, diabetes, obesity, disuse atrophy and Duchenne muscular dystrophy (Chung et al., 2016; Drew et al., 2014; Gehrig et al., 2012; Henstridge et al., 2014; Kennedy et al., 2016; McArdle et al., 2004; Senf et al., 2008). Furthermore, studies in HSP70−/− mice identified an important role for extracellular HSP70 in muscle repair through regulating the innate immune response and the activity of endogenous MuSCs at the site of damage (Senf et al., 2013). While HSP70 has well-established roles in regulating stress in skeletal muscle, comparatively little is known of its role in myogenesis (Liu et al., 2006; Senf et al., 2013).

HSP70 expression has been detected in slow type I fibres in mouse soleus and plantaris muscles at embryonic day 22 (Ogata et al., 2003), and muscle fibres in mice with a systemic deletion of HSP70 have a reduced cross-sectional area (Senf et al., 2013). In addition, whole transcriptome analyses of quiescent and activated MuSCs and proliferating and differentiating muscle cells revealed differential expression of the genes encoding HSP70 (Liu et al., 2013; Ryall et al., 2015). More recently, studies in C2C12 cells have identified a role for HSP70 in myoblast differentiation via stabilisation of p38MAPK (Fan et al., 2018). We and others have found that protein expression of HSP70 increases during the early stages of differentiation relative to that in proliferating myoblasts (Fan et al., 2018; Thakur et al., 2019), peaking at the onset of myoblast fusion, but the consequences of increased HSP70 expression during myogenesis have not been determined. Here we have overexpressed a GFP-HSP70 fusion protein in C2C12 myoblasts and investigated effects on proliferation, differentiation and fusion.

RESULTS

GFP-HSP70 overexpression does not alter subcellular localisation of HSP70

Before assessing the effect of HSP70 overexpression on myoblast functions, we first confirmed expression and subcellular localisation of the GFP-HSP70 fusion protein. C2C12 cells were either left untransfected (control), or transfected with GFP or GFP-HSP70, incubated for 24 h, then lysed for protein extraction. Western immunoblotting of C2C12 cell lysates probed with HSP70 antibody confirmed the presence of a 99 kDa band corresponding to GFP-HSP70 (Fig. 1A). No GFP-HSP70 was detected in either untransfected (CON) or GFP-transfected cells. Endogenous HSP70 (72 kDa) was expressed at similar levels across all groups (Fig. 1A).

We next examined the subcellular localisation of HSP70 in control (Fig. 1B), GFP-transfected (Fig. 1C), and GFP-HSP70-transfected (Fig. 1D) C2C12 myoblasts under control conditions and in response to heat shock for 2 h at 42°C. Endogenous HSP70/72 localised...
diffusely throughout the cytoplasm and the nucleus of both control
(Fig. 1B; top) and GFP-transfected (Fig. 1C; top) cells, and
translocated to the nucleus after heat shock (Fig. 1B,C; bottom). In
GFP-transfected C2C12 myoblasts, GFP was localised throughout
the cytoplasm and nucleus and its localisation did not change after
heat shock (Fig. 1C). In GFP-HSP70-transfected cells, the GFP-
HSP70 transgene was present mainly in the cytoplasm under control
conditions (Fig. 1D; top) but became concentrated in the nucleus after
heat shock, similar to endogenous HSP70/72 (Fig. 1D; bottom).
Together, these data demonstrate that the GFP-HSP70 fusion protein
is expressed in transfected C2C12 cells and retains the ability of
endogenous HSP70 to translocate to the nucleus after heat shock.

**GFP-HSP70 overexpression does not alter the rate of C2C12
cell proliferation or differentiation**

To determine whether HSP70 overexpression alters C2C12 cell
proliferation, cells were seeded at a low density and raw cell counts
were taken at 24, 48 and 72 h post-transfection. Blue boxes
representing the number of GFP transfected cells and red triangles
representing GFP-HSP70 transfected cells overlapped almost
completely at each timepoint and the growth curves were identical
(Fig. 2A). No difference was observed in the mean doubling times
of C2C12 myoblasts transfected with either GFP or GFP-HSP70
(Fig. 2B). Therefore, HSP70 overexpression does not alter the
proliferative capacity of C2C12 myoblasts.
The ability of C2C12 cells transfected with either GFP or GFP-HSP70 to undergo differentiation was next assessed by western immunoblotting. Expression of endogenous HSP70 in GFP-transfected and GFP-HSP70 transfected cells was similar at D2, D3 and D4 of differentiation (Fig. 2C,D). The GFP-HSP70 fusion protein was detected in all GFP-HSP70 transfected cells resulting in a 12-, 9-, and 6-fold overexpression of HSP70 at D2, D3, and D4 of differentiation, respectively. At D4 GFP-HSP70 overexpression was decreased relative to D2 (Fig. 2C,D), consistent with previous reports of HSP70 expression levels during C2C12 differentiation.

Fig. 2. See next page for legend.
**C2C12 cells transfected with GFP-HSP70 undergo enhanced fusion during differentiation**

As HSP70 overexpression had no impact on proliferation or differentiation of C2C12 myoblasts, we next examined the effect on myoblast fusion and myotube formation. C2C12 cells were transfected with either GFP or GFP-HSP70 and differentiated for 2, 3, or 4 days after which the size and number of nuclei per myotube was determined. The median width of myotubes was increased in GFP-HSP70 transfected cells relative to GFP-transfected cells at D3 (Fig. 3A,C) and D4 (Fig. 3A,D) but not D2 (Fig. 3A,B) of differentiation. In addition, the median number of nuclei per myotube was increased in GFP-HSP70 transfected cells relative to GFP-transfected cells at D2 (Fig. 3A,E), D3 (Fig. 3A,F) and D4 (Fig. 3A,G) of differentiation.

We next analysed the number of nuclei per myotube, which was significantly reduced in GFP-HSP70-transfected myotubes containing less than 50 myonuclei; significantly increased in myotubes with 51–200 nuclei; and unchanged in myotubes with more than 200 nuclei relative to GFP-transfected myotubes at D3 (Fig. 3I) and D4 (Fig. 3J) but not D2 (Fig. 3H) of differentiation. Together, these findings indicate that HSP70 overexpression enhances myoblast fusion to increase myotube size.

**DISCUSSION**

Evidence to date supports a positive influence for HSP70 on myoblast fusion during muscle differentiation. Enhanced myoblast fusion through increased HSP70 expression supports its therapeutic potential for treating muscle injury and disorders associated with muscle atrophy.

**MATERIALS AND METHODS**

**Plasmids**

To examine the effect of HSP70 overexpression on C2C12 cell proliferation and differentiation, the pEGFP-C3 plasmid containing the murine HSP70 cDNA was used [pCMV-EGFP-HSP70 (GFP-HSP70)] was a gift from Lois Greene [Addgene plasmid #15215, http://n2t.net/addgene:15215; RRID:
Fig. 3. See next page for legend.
Cell culture
Proliferating C2C12 cells (American Type Culture Collection, ATCC, Manassas, VA, USA) were cultured at 60–70% confluency in growth media [Dulbecco’s Modified Eagle Medium (DMEM, 4.5 g/l D-glucose, 4.0 mM L-glutamine, no sodium pyruvate; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% foetal bovine serum (FBS; Thermo Fisher Scientific) and penicillin (100 units/ml)/streptomycin (100 μg/ml) (Pen/strep; Thermo Fisher Scientific)]. Cells were maintained in a humidified chamber at 37°C, in 95% air and 5% CO₂. To induce myogenic differentiation, C2C12 cells were grown to 100% confluency and switched from growth media to low serum differentiation media [DMEM–70% confluency in growth media, replaced with differentiation media to induce myogenic differentiation. The cells were then allowed to differentiate for 1, 2, or 3 days with media changes every 24 h. At 1, 2, or 3 days post-differentiation, cells were fixed with 4% paraformaldehyde (PFA; Aesar, Ward Hill, MA, USA) in phosphate buffered saline (PBS; Thermo Fisher Scientific) for 15 min, washed in PBS once and then permeabilised with 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 10 min. Non-specific binding sites were blocked with 3% bovine serum albumin (BSA, Sigma-Aldrich) in PBS for 45 min at room temperature. Target proteins were detected by incubating coverslips in blocking solution containing primary antibodies against mouse-α-myosin (IgG1 (1:200); MA5-11486; Thermo Fisher Scientific) and mouse-α-sarcomeric myosin heavy chain IgG2b (1:50; deposited to the University of Iowa Developmental Studies Hybridoma Bank by Fischman, D.A. (DSHB Hybridoma Product MF 20) at 4°C overnight. On the following day, coverslips were washed in PBS (3×5 min) and incubated in blocking solution containing Alexa Fluor 555-conjugated goat-α-mouse IgG1 (1:100; A21127; Thermo Fisher Scientific) and Alexa Fluor 555-conjugated goat-α-mouse IgG2b (1:1000; A21147; Thermo Fisher Scientific) for 2 h at room temperature in the dark. The coverslips were then washed with PBS (3×5 min) and counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 1:1000, Thermo Fisher Scientific) in PBS. Finally, coverslips were mounted onto glass slides using a water based mounting medium (Fluoro-Gel; ProSciTech Pty. Ltd., Kirwan, QLD, Australia) and coated in nail polish.

Western immunoblotting
Cells were washed twice in 2 ml PBS per well, PBS was aspirated from the wells and intact six-well plates were stored at ~80°C until the day of lysis and protein extraction. Whole cell lysates were prepared as described previously (Park et al., 2016). Briefly, cells were washed with ice-cold PBS containing phenylmethylsulfonyl fluoride (PMSF, 1 mM, Thermo Fisher Scientific) and subsequently lysed in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer [50 mM TrisHCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% Na-Deoxycholic acid and 0.1% sodium dodecyl sulphate (SDS); Millipore, Billerica, MA, USA] containing protease and phosphatase inhibitor cocktail (PIC, 1 μM, Sigma-Aldrich) using a cell scraper (Corning Costar; Sigma-Aldrich). Cell lysates were sonicated for 15 s using a Microson XL-2000 sonicator (Misonix, NY, USA) to disrupt the nuclear membrane, centrifuged for 10 min at 10,000 rpm and 4°C to remove cellular debris, and stored at ~80°C until further analysis. Total protein concentration was determined through a DC protein assay (Bio-Rad Laboratories) by measuring absorbance at 750 nm using a Multiskan Spectrum spectrophotometer (Thermo Fisher Scientific). All samples were equalised to a protein concentration of 1 μg/ml with RIPA lysis buffer. Laemmli buffer [4×; 0.25 M TrisHCl (pH 6.8), 6% SDS, 40% glycerol, 0.04% bromophenol blue, 16% Dithiothreitol (DTT)] was then added and samples were incubated for 5 min at 95°C. Identical amounts of each sample (10 μg) were loaded onto pre-cast SDS-polyacrylamide gels (4–15% Criterion TGX Stain-Free Precast Gels, Bio-Rad Laboratories) in Tris/Glycine/SDS (TGS) buffer (Bio-Rad Laboratories) and resolved using SDS-PAGE for 45 min at 200 V. Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes using a Trans-blot Turbo Blotting System (Bio-Rad Laboratories) by applying a constant current (1 amp) for 7 min. Efficient protein transfer was confirmed using post-transfer stain free image of gel and membrane. Non-specific binding sites were blocked by incubating the PVDF membrane in 3% BSA in Tris-buffered saline containing Tween 20 (TBST; 50 mM TrisHCl, 150 mM

**Table 1.** Western Blotting Results

| Protein | Control | GFP-HSP70 |
|---------|---------|-----------|
| MyoG    | 2.1 ± 0.3 | 3.2 ± 0.4 |
| MyHC    | 1.8 ± 0.2 | 2.1 ± 0.3 |

**Note:** Data are presented as mean±s.e.m and compared with a t-test and a two-way ANOVA and compared with a two-way ANOVA and compared with a two-way ANOVA.
NaCl, 0.05% Tween 20) for 2 h at room temperature. Target proteins were detected by probing the PVDF membrane with either rabbit-α-HSP70 (1:5000; ADI-SPA-812, Enzo Life Science, Farmingdale, NY, USA), mouse-α-myoglobin (1:400; sc12732, Santa Cruz Biotechnology, Rockford, IL, USA), mouse-α-MF20 (1:5000; DSHB Hybridoma Product MF 20), or rabbit-α-β-actin (1:1000; Cell Signaling Technology, Danvers, MA, USA) diluted in 3% BSA/TBST overnight at 4°C. The following day, PVDF membrane was washed in TBST (1×2 min, 3×10 min) and incubated with 3% BSA/TBST containing either HRP-α-mouse or HRP-donkey-α-rabbit IgG (1:5000; GE Life Sciences, Buckinghamshire, UK) for 1 h at room temperature. PVDF membrane was then washed in TBST (1×2 min, 3×10 min) and immunodetection was performed using Supersignal West Femto luminescent substrate (Thermo Fisher Scientific). The membrane was visualised and imaged on a Chemidoc System (Bio-Rad Laboratories). The protein bands were analysed using ImageLab software (Bio-Rad Laboratories) and the intensity band of interest was normalised to total protein.

Statistical analysis
Data are presented as mean±SEM unless indicated otherwise. Unpaired Student’s t-test was used to compare differences between GFP and GFP-HSP70 groups. When the assumption of Gaussian distribution was not met, a non-parametric Mann–Whitney U-test was used for comparisons. For comparisons between more than two groups, a one- or two-way ANOVA was performed using Supersignal West Femto luminescent substrate (Thermo Fisher Scientific) and the intensity band of interest was normalised to total protein.

Author contributions
Conceptualization: S.S.T., K.S., J.G.R., G.S.L.; Methodology: S.S.T., V.L.C., J.L.J., N.J.C., A.M.T.I.; Validation: S.S.T., K.S., J.G.R., G.S.L.; Formal analysis: S.S.T., K.S., J.G.R., G.S.L.; Investigation: S.S.T., V.L.C., J.L.J., N.J.C., A.M.T.I.; Resources: K.S., J.G.R., G.S.L.; Data curation: S.S.T., K.S., J.G.R.; Writing - original draft: S.S.T., K.S., G.S.L.; Writing - review & editing: K.S., J.G.R., G.S.L.; Supervision: K.S., J.G.R., G.S.L.; Project administration: K.S., J.G.R., G.S.L.; Funding acquisition: G.S.L.

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Author/s:
Thakur, SS; Swiderski, K; Chhen, VL; James, JL; Cranna, NJ; Islam, AMT; Ryall, JG; Lynch, GS

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