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*Cell Injury, Repair, Aging and Apoptosis*

# Overexpression of MHC Class I Heavy Chain Protein in Young Skeletal Muscle Leads to Severe Myositis

## *Implications for Juvenile Myositis*

Charles Kwok-chong Li,* Paul Knopp,* Halima Moncrieffe,* Bhanu Singh,† Sonia Shah,‡ Kanneboyina Nagaraju,§ Hemlata Varsani,* Bin Gao,* and Lucy R. Wedderburn*

*From the Rheumatology Unit,* Institute of Child Health, and the Department of Bioinformatics,‡ University College London, London, United Kingdom; the Department of Pathology and Infectious Diseases,† the Royal Veterinary College, Hertfordshire, United Kingdom; and the Research Center for Genetic Medicine,§ Children's National Medical Center, Washington DC*

**Folding and transport of proteins, such as major histocompatibility complex (MHC) class I, through the endoplasmic reticulum (ER) is tightly regulated in all cells, including muscle tissue, where the specialized ER sarcoplasmic reticulum is also critical to muscle fiber function. Overexpression of MHC class I protein is a common feature of many muscle pathologies including idiopathic myositis and can induce ER stress. However, there has been no comparison of the consequences of MHC overexpression in muscle at different ages. We have adapted a transgenic model of myositis induced by overexpression of MHC class I protein in skeletal muscle to investigate the effects of this protein overload on young muscle fibers, as compared with adult tissue. We find a markedly more severe disease phenotype in young mice, with rapid onset of muscle weakness and pathology. Gene expression profiling to compare the two models indicates rapid onset of ER stress in young muscle tissue but also that gene expression of key muscle structural proteins is affected more rapidly in young mice than adults after this insult. This novel model has important implications for our understanding of muscle pathology in dermatomyositis of both adults and children.** ***(Am J Pathol 2009, 175:1030–1040; DOI: 10.2353/ajpath.2009.090196)***

The idiopathic inflammatory muscle diseases are a group of autoimmune conditions affecting patients of all age groups, characterized by proximal muscle weakness, skeletal muscle damage and systemic involvement. It has been observed that despite obvious similarities, there are also differences that distinguish myositis in adults and children. Childhood myositis may show a very rapid onset, is not associated with malignancy, is more often complicated by vasculitic systemic features, and carries a better prognosis of full muscle power and functional recovery if treatment is rapid and adequate.[1,2] Juvenile dermatomyositis is the most common of myositis of childhood onset, and is associated with considerable morbidity and even mortality.[3,4]

Class I major histocompatibility complex (MHC) protein is overexpressed in skeletal muscle from all types of idiopathic inflammatory muscle diseases, including juvenile dermatomyositis and also in other muscle pathologies including inclusion body myositis.[5–7] It is unclear whether MHC overexpression is directly causal in muscle damage. We and others have demonstrated that overexpression of MHC class I occurs early in both adult and juvenile dermatomyositis, even in the absence of inflammatory cell infiltrate.[7,8] The conditional transgenic model of myositis ('HT') where self MHC class I is overexpressed in skeletal muscle, exhibits clinical, biochemical, histological, and immunological features that parallel

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Current address of B.S., DuPont Haskell Global Center for Health and Environmental Sciences, Newark, Delaware.

Address reprint requests to Dr. Lucy R. Wedderburn, B.A., M.D., Ph.D., F.R.C.P., Rheumatology Unit, Institute of Child Health, UCL, 30 Guilford Street, London WC1N 1EH, UK. E-mail: l.wedderburn@ich.ucl.ac.uk.

some of those of human disease.[9] Following upregulation of transgenic self MHC class I at 5 to 6 weeks of age, female mice develop muscle histological changes from about 3 months of age, reduced locomotor activity, muscle enzyme (creatine kinase and glutamic-oxaloacetic transaminase) rise at 5 months, and histological features of muscle damage at 5 to 6 months.

MHC class I is expressed at low levels in healthy mature muscle fibers, but can be up-regulated by inflammatory cytokines such as tumor necrosis factor-$\alpha$ or interferons.[10–12] MHC class I is expressed at high levels in muscle progenitor cells, myoblasts. MHC class I expression is normally down-regulated during muscle differentiation from myoblast to myotube, and expression remains low in mature myofibers, a process that is tightly regulated by myoblast regulatory factors including MyoD, Myf5, and myogenin.[13]

The ER and Golgi of all cells, including skeletal muscle, have a stringent 'quality control' sensor system to detect and deal with abnormalities of protein expression, folding and assembly. Abnormal levels of expression, misfolding or abnormal glycosylation of ER resident proteins may lead to ER stress.[14,15] ER stress responses are classified into two major pathways: the unfolded protein response, and the ER overload response. The unfolded protein response reduces levels of misfolded or overexpressed proteins, by inducing transcription of ER chaperones, reducing protein synthesis, and enhancing degradation of misfolded proteins via an ubiquitin-proteasome system, ER-associated degradation. Prolonged unfolded protein response leads ultimately to cell death through apoptosis.

In skeletal muscle, ER stress plays a role in normal development and homeostasis. ER stress genes are upregulated during the transition from myoblast to myotube, when some cells die by apoptosis, and others survive and fuse to form multinucleate fibers.[16] Skeletal muscle has a highly specialized ER, the sarcoplasmic reticulum, where $Ca^{2+}$ binding proteins play a pivotal role in signals critical to muscle contraction and function, so may be highly sensitive to triggers that disrupt ER/sarcoplasmic reticulum function and $Ca^{2+}$ homeostasis. ER stress has been implicated in several muscle diseases, including inclusion body myositis, dermatomyositis and muscle disuse atrophy.[17–20] ER stress also has a role in the HT model where MHC class I protein is overexpressed in skeletal muscle.[19] However, there have been no previous studies comparing the response of skeletal muscle to specific stress triggers at different ages.

The aim of this study was to compare the consequences of upregulation of MHC class I protein in skeletal muscle of young and adult mice. Strikingly we find that the overexpression of MHC class I heavy chain protein at a young age leads to a more rapid and severe disease phenotype. This study provides new insights into age-specific differences in the responses of young and adult muscle to injury, as well as generating a novel model, which will be a highly efficient system for further investigation of the mechanisms of myositis.

## Materials and Methods

### Mice

This project was performed with ethical approval, and a full UK Home Office License. The generation of conditional MHC class I transgenic mice was as described previously.[9] Briefly, overexpression of autologous MHC class I heavy chain in skeletal muscle was induced by expression of transgenic H-2K$^b$ controlled by the tetracycline/doxycyline transactivator (Tta), under the muscle-specific promoter, creatine kinase. Tissue-specific transgene expression occurs after withdrawal of doxycycline from the drinking water. Disease induction was performed in females only. For this study mice of the existing model were denoted HT-L. To mimic activation during juvenile stage of muscle development, doxycycline was given to female nursing mice only until weaning of offspring, inducing MHC class I heavy chain transgene induction from 21 days of age. Mice in this group were designated HT-E. HT-E mice were compared with the mice treated according to the original protocol (HT-L) in which doxycycline was withdrawn at 35 days, with consequent up-regulation of MHC class I heavy chain. Single transgenic females who received doxycycline for the same period of time, served as controls for histological analysis and gene expression analysis. Double transgenic (HT) littermates maintained on doxycycline served as controls for strength and mortality assessment. Mice were genotyped by PCR on genomic DNA by standard methods. Primers to detect the transgenes were H-2K$^b$ forward: 5′-TCGAGTTTACCACTCCCTATCAG-3′, H-2K$^b$ reverse: 5′-GATCTGACGGTTCACTAAACGAG-3′ and Tta forward: 5′-CGCTGTGGGGCATTTTACTTTAG-3′ and Tta reverse: 5′-CATGTCCAGATCGAAATCGTC-3′.

Mice were observed every other day for appearance, behavioral changes, and disease phenotype including muscle weakness. A scoring system of disease was devised according to veterinary regulations and subjects were culled when license regulations required this. To compare phenotype, histology, and gene expression, mice of each model (HT-E, HT-L, and age matched littermate controls) were sacrificed at pre-defined time points.

### Tissue Samples and Immunohistochemistry

Quadriceps femoris and gastrocnemius muscles were dissected immediately after culling. Tissues were either snap frozen in liquid nitrogen and cryopreserved, or fixed in 10% buffered formalin. Staining for morphological assessment was by standard H&E method (Sigma Haverhill, UK). Immunohistochemistry was performed on 8-$\mu$m cryosections after acetone fixation, by standard two-antibody method with visualization of avidin-peroxidase by 3,3′ diaminobenzidine, (Dako Glostrup, Denmark). Antibodies used were: monoclonal anti-MOMA-2, which recognizes cells of the macrophages/monocyte lineage (Dako), monoclonal anti-murine CD3 (eBioscience San Diego, CA), polyclonal rabbit anti-murine Ubiquitin (Dako), and isotype controls matching the primary antibody (Serotec, Raleigh, NC). Secondary antibodies were

biotinylated donkey anti-rabbit-IgG (Chemicon, Billerica, MA) or biotinylated rabbit anti-rat-IgG (Vector Laboratories, Burlingame CA).

Morphological features were scored by a veterinary pathologist blinded to identification or clinical status of the animals (B.S.) for features of degeneration, regeneration, and muscle fiber changes. Briefly, muscle degeneration was characterized by sarcoplasmic swelling, pallor, and vacuolization. Necrotic myofibers showed sarcoplasmic hypereosinophilia with loss of cross-striations, fragmentation, and nuclear pyknosis, karyorrhexis, and karyolysis.[21] A score of 0 was given for no lesion or changes, 1 for where scattered single myofibers were affected, 2 for where scattered small groups of myofibers affected, 3 for wide spread small groups of myofibers affected, and 4 for where confluent groups of myofibers were affected.

Inflammatory features consisted of inflammatory cells (predominantly macrophages, and fewer lymphocytes and plasma cells) infiltration. The microscopic features of regenerative changes include satellite cell activation/migration, myofibers with basophilic cytoplasm, nuclear internalization, and large nuclei with prominent nucleoli. A score of 0 was given for no changes, 1 for mild changes, 2 for moderate changes, 3 for marked changes, and 4 for severe/wide-spread changes. The scoring process for both degenerative and inflammatory features was performed twice at least 1 month apart for verification.

### *RNA Extraction and Gene Expression Profiling*

Mice from three groups (each $n = 3$), HT-E, HT-L, and controls, were selected for gene expression comparison. HT-E (early) were removed from doxycycline at 21 days, and sacrificed at 35 days of age, after 2 weeks of transgene expression. HT-L (late) mice were maintained on doxycycline until 35 days[9] and sacrificed at 49 to 50 days of age. Single transgenic littermate controls were age, sex-matched, maintained on doxycycline, and sacrificed as the HT-E group. Total RNA was extracted from 100 mg of quadriceps muscle using RNAB reagent (Campro Scientific, Berlin, Germany). RNA integrity and purity were confirmed by Nanoreader 600 Assay (Agilent, Palo Alto, CA). Five $\mu$g total RNA was used to prepare cRNA for each gene profile experiment. cRNA probe was generated by *in vitro* transcription, and fragmented biotinylated probe hybridized to murine MOE 430 v2.0 genechips using standard Affymetrix protocols (Santa Clara, CA). Genechip quality and experimental reproducibility were assessed using SimplyAffy package v2.6.0.

### *Statistical Analysis*

The statistical significance of the survival difference between groups of mice was calculated by Cox regression analysis of a Kaplan-Meier series. Quantitative data from histological scoring of muscle sections were expressed as the mean ± SD. Data from the scores of this muscle analysis were compared by the Mann Whitney test in SPSS. The raw mRNA gene expression data were normalized and summarized using GC Robust Microarray average.[22] Differentially expressed genes were identified using the Limma analysis package[23] in Bioconductor. Genes that had a Benjamini-Hochberg corrected $P$ value $<0.05$ were considered to be differentially expressed. The full raw gene expression profiling data are available on the Geo database (*http://www.ncbi.nlm.nih.gov/geo,* accession number GSE14997; last accessed August 14, 2009) and are compliant with minimum information about a microarray experiment (MIAME) guidelines.

## *Results*

### *Overexpression of MHC Class I Heavy Chain in Young Mice Leads to More Rapid Induction of Disease Phenotype, Compared with Adult Mice*

To investigate whether overexpression of MHC class I in skeletal muscle at a young age would alter either disease phenotype or kinetics of onset, we compared mice in which MHC class I induction was initiated at 21 days (HT-E) to those in which induction was at 35 days (HT-L). HT-E mice were found to develop weakness far more rapidly than HT-L mice. Behavioral changes including hunched posture and reduction in spontaneous movement such as climbing to the top of the cage, occurred at a much earlier stage in HT-E mice, typically at about 1 month, and in some cases as early as 14 days, after doxycycline withdrawal. The survival of mice of HT-E model ($n = 35$), mice of HT-L model ($n = 37$) and control mice ($n = 10$) were followed until death or until culling was required due to severe weakness (Figure 1). Survival time of transgenic mice was significantly reduced in HT-E compared with the HT-L group ($P < 0.0001$).

### *Pathological Changes in Skeletal Muscle of Young Mice Develop More Rapidly Following Overexpression of MHC Class I Heavy Chain*

To compare the features and kinetics of muscle pathology induced in the 'early' mouse model of myositis, HT-E,

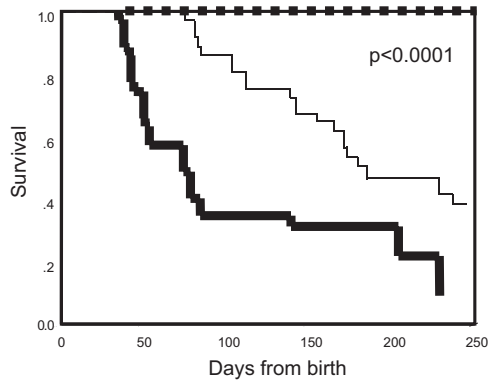


**Figure 1.** Early induction of self MHC class I in murine skeletal muscle leads to rapid onset of weakness and reduced survival. Kaplan-Meier plot comparing two groups of mice (HT-E, HT-L) that differ only in time of transgene activation. Doxycycline was removed at mean 21.2 days (SD ± 1.3 days) for HT-E mice (bold line, $n = 35$ mice), and 37.4 days (SD ± 8 days) for HT-L mice (fine line, $n = 37$ mice). Control HT mice were maintained on doxycycline at all times (dotted line, $n = 10$ mice).

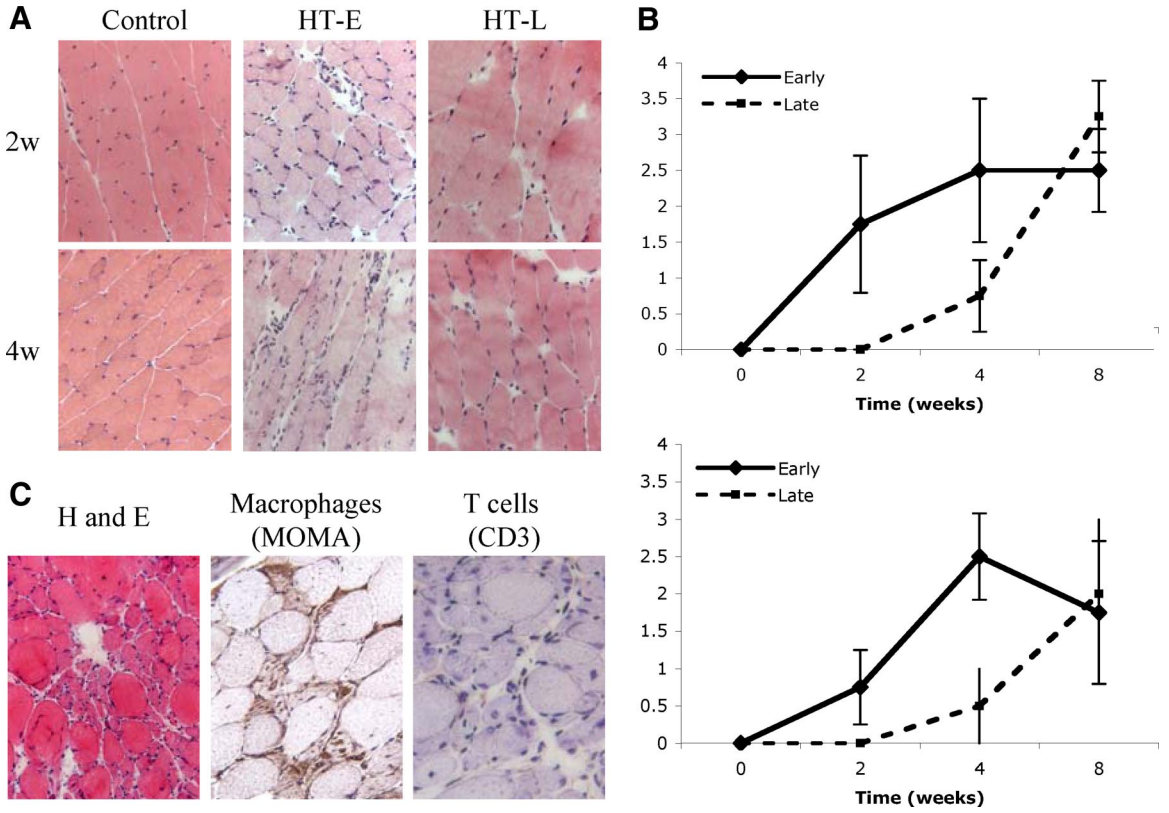


**Figure 2. A:** H&E-stained sections of quadriceps femoris muscle from control, HT-E, and HT-L models 2 and 4 weeks after transgene expression or no transgene expression in controls, as shown. **B:** Quantitation of abnormality of histological findings for quadriceps femoris (**top graph**) and gastrocnemius (**lower graph**) muscles from HT-E (bold line), and HT-L mice (fine line) at 0, 2, 4, and 8 weeks after transgene expression. Data points represent mean values from groups (each group $n = 4$), error bars $\pm 1$ SD. **C:** Quadriceps femoris sections from HT-E mice stained with H&E (**left**), and by immunostaining for macrophages, identified by MOMA positivity (**middle**), and T cells (CD3, **right**) as shown. All panels $\times 200$ final magnification.

HT-L, and control mice were culled at different times after transgene induction, (2, 4 and 8 to 12 weeks) $n = 4$ mice per group. This timed analysis confirmed that changes in skeletal muscles were observed more rapidly after overexpression of MHC class I in the HT-E than the HT-L model. Microscopic changes observed included early vacuolar degeneration, segmental fiber necrosis, variable fiber size, centralized myonuclei, and an inflammatory cell infiltration (composed of cells of myeloid origin) detectable in skeletal muscle of HT-E mice (Figure 2A). Results from scoring of the degenerative features confirmed an early difference in histological changes in HT-E compared with HT-L model, Figure 2B. Four weeks after transgene induction, differences in severity scores between HT-E and L gastrocnemius muscle were statistically significant ($P = 0.028$), and showed a similar trend in quadriceps ($P = 0.0578$). By 8 weeks, there was no difference between HT-E and HT-L models in severity scores in either gastrocnemius or quadriceps scores. As described in the original myositis model, the inflammatory infiltrate in the HT-E model was predominantly of a macrophage/myeloid lineage with no detectable T or B cell lymphocytic component (Figure 2C). Postmortem examinations were performed 6 weeks after transgene activation on those HT-E mice that had demonstrated behavioral deterioration such as reduced spontaneous activity and hunched posture. These postmortem examinations revealed skeletal muscle degeneration and necrosis compared with controls, but no abnormalities were found in other organs including diaphragmatic muscles, lungs, kidneys, liver, and spleen (data not shown).

## *HT-E Model Reveals Significant Alterations in Early Gene Expression Induced by MHC Up-Regulation in Young Mice*

Gene expression analysis was performed on muscle tissue from three groups of mice: HT-E and HT-L mice (each after only 14 days of transgene expression), and age matched littermate controls. Unsupervised hierarchical clustering of samples revealed that gene expression in the standard myositis model (HT-L) was largely unaltered from control mice (Figure 3A). In contrast gene expression of the HT-E mice was noted to cluster separately from both control mice and the HT-L model. Transcripts of MHC class I H-2K, detected with probes that do not distinguish between the endogenous and transgenic MHC class I expression, were up-regulated in both HT-E and HT-L mice, as compared with that in controls, as expected. There was no significant difference in H-2K expression between HT-E or HT-L models, confirming that the transgene is equally expressed in both models (data not shown). There was no significant difference in expression of creatine kinase, an important validation,

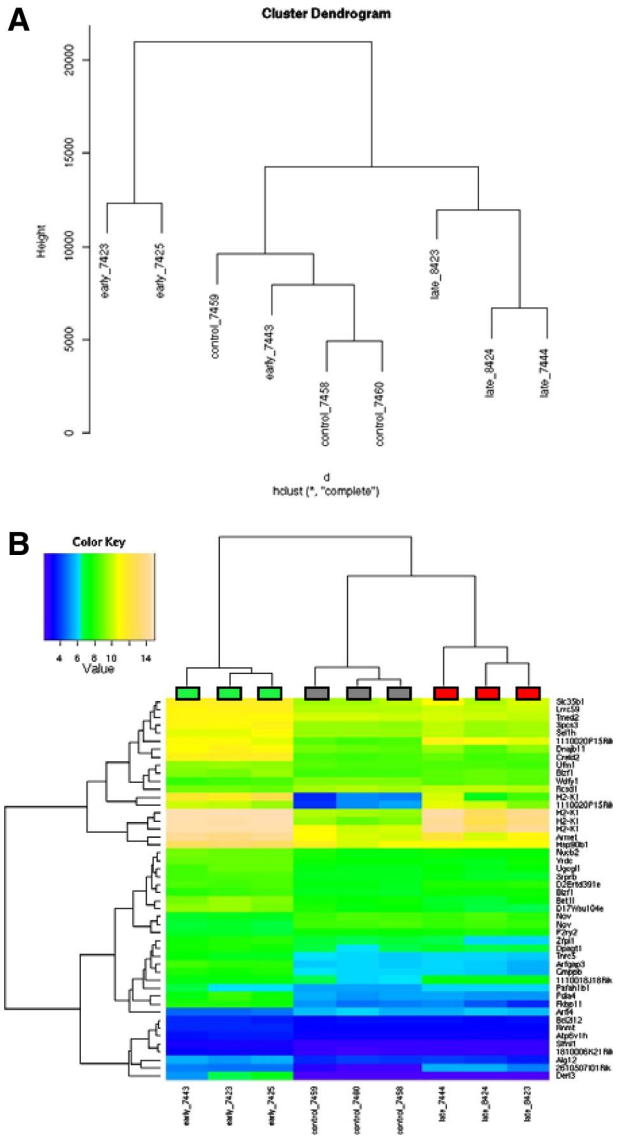


**Figure 3. A:** Cluster dendrogram indicating relationships of gene expression overall between the nine samples analyzed by gene expression profiling. Each sample is indicated by a number and designations (early indicating HT-E mice; control; or late indicating HT-L mice). **B:** Heat map of the top 48 probes across all nine samples represented in log2 intensity, dark blue indicating low expression and yellow indicating high expression. The dendrogram of the nine samples above the heat map is clustered using these 48 probes only; green: HT-E samples; grey: controls; red: HT-L samples. Affymetrix probe IDs and names of the 48 probes are detailed in Table 1.

since the transgene is regulated by this muscle specific promoter.

Analysis of gene expression data from the three groups of mice revealed many genes with significantly altered expression to a level of $P < 0.05$, after correction for multiple testing. Analysis of genes that were significantly altered in the HT-E model compared with their age matched controls revealed 48 probe sets from 41 genes that were differentially expressed (Table 1). Of these 48 'top' differentially expressed probes, 36 were also significantly altered in HT-E when compared with HT-L model, with parallel direction and fold changes. Of those 12 probes that were not significantly different between HT-E and HT-L mice, but did differ between HT-E and controls, 4 were specific for MHC class I H-2K (equally up-regulated in HT-E and HT-L). Figure 3B shows hierarchical clustering analysis (using Euclidian distance and complete linkage) of these 48 differentially expressed probes in the HT-E model compared with HT-L and to control mice. Gene expression in the HT-L model clustered closely to control mice while gene expression in HT-E mice clustered apart from either HT-L mice or controls.

Analysis of significantly altered genes in the HT-E compared with HT-L model revealed 549 probes (representing 504 genes) that were significantly altered ($P < 0.05$), of which 273 probes demonstrated a ≥twofold change in expression. These 273 probes are listed in the Supplementary Data Table (please see Supplemental Table at *http://ajp.amjpathol.org*). Analysis of genes that differed between the HT-E and HT-L models suggested several functional pathways that were altered after only two weeks of MHC class I overexpression in young muscle.

## *Alterations in ER Golgi and Protein Folding and Transport Pathways in Young Mice after Overexpression of MHC Class I*

The first group of genes altered in HT-E compared with HT-L mice were genes intrinsic to protein transport, folding, processing, glycosylation, or other ER and Golgi functions (Table 2). These included proteins that ensure correct folding such as protein disulphide isomerases,[24] $Ca^{2+}$ dependent chaperones calreticulin, Tra1 (also known as Grp94),[15] and members of the Hsp40 family, binding partners of Hsp70 chaperones. Many of these are known to be increased during ER stress.[25] The demonstration of Hsp expression parallels our own previous findings in patients with juvenile dermatomyositis, in whom Hsp60 and Hsp70 proteins are highly expressed in muscle.[26]

Other differentially expressed genes included those coding for derlin proteins (Derl1, Derl2, and Derl3) involved in degradation of misfolded proteins in the ER,[27–29] molecules critical to protein transport/export (such as Sec proteins Sec22, Sec61), those involved in Golgi trafficking (such as coatomer proteins), and constituents of the SNARE complex including Bet1[30] and syntaxin5.[31] In addition several genes involved in protein ubiquitination were over-expressed including E2 conjugating enzymes (Ube2g2),[32] E3 ligases such as Zfpl1, and peptidases such as Usp28. Analysis of ubiquitin expression in quadriceps muscle after transgene upregulation demonstrated protein expression in HT-E mice, with a punctate pattern of staining within fibers, markedly earlier than in the HT-L mice after MHC class I expression (Figure 4), confirming increased activation of the ubiquitin pathway suggested by the gene expression profiling.

In contrast to our previous analysis of genes that are altered in the adult HT mice with established disease,[19] expression of classical downstream genes in the ER stress response such as ATF6, caspase12 or NFkB-induced genes were not differently expressed between the HT-E and HT-L models at this early time point. However

**Table 1.** Differentially Expressed Genes in the HT-E Model Compared with Controls and with the HT-L Model

| | | Early (HT-E) vs. control | | | | Early (HT-E) vs. late (HT-L) | | |
|---|---|---|---|---|---|---|---|---|
| Probe set ID | Symbol | Log fold change | Fold change | *P* value | Log fold change | Fold change | *P* value | |
| 1452754_at | *5730592L21Rik* | ↑ 2.93 | 7.64 | 0.0042 | ↑ 2.77 | 6.81 | 0.0063 | |
| 1426852_x_at | *Nov* | ↓ −1.59 | −3.02 | 0.0060 | ↓ −1.47 | −2.77 | 0.0071 | |
| 1426534_a_at | *Arfgap3* | ↑ 2.11 | 4.32 | 0.0089 | ↑ 2.21 | 4.63 | 0.0071 | |
| 1427746_x_at | *H2-K1* | ↑ 5.10 | 34.27 | 0.0138 | | | NS | |
| 1421018_at | *1110018J18Rik* | ↑ 1.02 | 2.03 | 0.0138 | | | NS | |
| 1425348_a_at | *Srprb* | ↑ 1.29 | 2.44 | 0.0138 | ↑ 1.42 | 2.68 | 0.0091 | |
| 1418974_at | *Blzf1* | ↑ 1.10 | 2.14 | 0.0138 | ↑ 0.55 | 1.46 | 0.0367 | |
| 1418899_at | *Ufm1* | ↑ 0.87 | 1.83 | 0.0138 | ↑ 0.98 | 1.98 | 0.0091 | |
| 1425336_x_at | *H2-K1* | ↑ 4.14 | 17.69 | 0.0138 | | | NS | |
| 1424948_x_at | *H2-K1* | ↑ 5.39 | 41.87 | 0.0138 | | | NS | |
| 1439030_at | *Gmppb* | ↑ 1.54 | 2.90 | 0.0138 | ↑ 1.89 | 3.70 | 0.0091 | |
| 1453749_at | *2610507I01Rik* | ↑ 2.40 | 5.26 | 0.0138 | | | NS | |
| 1440002_at | *Rnmt* | ↑ 0.49 | 1.40 | 0.0153 | ↑ 0.51 | 1.43 | 0.0104 | |
| 1418355_at | *Nucb2* | ↑ 1.42 | 2.68 | 0.0153 | ↑ 1.33 | 2.52 | 0.0112 | |
| 1434341_x_at | *1110020P15Rik* | ↑ 2.24 | 4.73 | 0.0176 | | | NS | |
| 1416234_at | *AA959742* | ↑ 1.36 | 2.56 | 0.0199 | ↑ 1.12 | 2.17 | 0.0167 | |
| 1416497_at | *Pdia4* | ↑ 2.24 | 4.74 | 0.0199 | 2.36 | 5.12 | 0.0104 | |
| 1423793_at | *D2Ertd391e* | ↑ 1.13 | 2.19 | 0.0199 | | | NS | |
| 1451175_at | *Spcs3* | ↑ 1.62 | 3.07 | 0.0199 | ↑ 1.34 | 2.54 | 0.0167 | |
| 1434744_at | *Yrdc* | ↑ 1.36 | 2.56 | 0.0226 | ↑ 1.47 | 2.77 | 0.0104 | |
| 1424194_at | *Rcsd1* | ↓ −0.76 | −1.69 | 0.0233 | ↓ −0.99 | −1.99 | 0.0091 | |
| 1434340_at | *1110020P15Rik* | ↑ 5.16 | 35.74 | 0.0233 | | | NS | |
| 1455839_at | *Ugcgl1* | ↑ 1.43 | 2.69 | 0.0233 | ↑ 1.26 | 2.39 | 0.0167 | |
| 1453677_a_at | *Derl3* | ↑ 4.07 | 16.78 | 0.0262 | ↑ 4.06 | 16.66 | 0.0145 | |
| 1425242_at | *1810006K21Rik* | ↑ 0.67 | 1.59 | 0.0271 | ↑ 0.61 | 1.53 | 0.0167 | |
| 1450318_a_at | *P2ry2* | ↓ −0.64 | −1.56 | 0.0278 | ↓ −0.76 | −1.69 | 0.0104 | |
| 1418973_at | *Blzf1* | ↑ 0.97 | 1.95 | 0.0278 | | | NS | |
| 1426851_a_at | *Nov* | ↓ −1.28 | −2.43 | 0.0321 | ↓ −1.12 | −2.17 | 0.0212 | |
| 1423151_at | *Dnajb11* | ↑ 2.04 | 4.12 | 0.0323 | ↑ 1.75 | 3.36 | 0.0217 | |
| 1437358_at | *Wdfy1* | ↓ −0.79 | −1.73 | 0.0352 | | | NS | |
| 1428112_at | *Armet* | ↑ 3.58 | 11.99 | 0.0371 | ↑ 2.83 | 7.10 | 0.0265 | |
| 1438040_a_at | *Tra1* | ↑ 2.14 | 4.40 | 0.0371 | ↑ 2.28 | 4.85 | 0.0157 | |
| 1448549_a_at | *Dpagt1* | ↑ 1.24 | 2.36 | 0.0384 | ↑ 1.03 | 2.04 | 0.0242 | |
| 1436774_at | *Sel1 hours* | ↑ 1.14 | 2.20 | 0.0403 | ↑ 0.83 | 1.78 | 0.0343 | |
| 1456947_at | *Pafah1b1* | ↑ 0.98 | 1.97 | 0.0415 | | | NS | |
| 1440549_at | *Atp6v1 hours* | ↑ 0.45 | 1.36 | 0.0415 | ↑ 0.46 | 1.37 | 0.0167 | |
| 1450534_x_at | *H2-K1* | ↑ 7.29 | 156.37 | 0.0415 | | | NS | |
| 1453718_at | *Bcl2l12* | ↑ 0.40 | 1.32 | 0.0428 | ↑ 0.36 | 1.28 | 0.0225 | |
| 1426479_a_at | *Tnrc5* | ↑ 1.12 | 2.17 | 0.0428 | ↑ 1.21 | 2.32 | 0.0167 | |
| 1422980_a_at | *Bet1l* | ↑ 1.59 | 3.00 | 0.0428 | ↑ 2.11 | 4.33 | 0.0104 | |
| 1448769_at | *Slc35b1* | ↑ 1.63 | 3.10 | 0.0428 | ↑ 1.21 | 2.32 | 0.0371 | |
| 1417267_s_at | *Fkbp11* | ↑ 2.68 | 6.42 | 0.0428 | ↑ 3.31 | 9.89 | 0.0123 | |
| 1424818_at | *Alg12* | ↑ 1.62 | 3.07 | 0.0428 | ↑ 1.67 | 3.19 | 0.0181 | |
| 1455838_at | *4933406A14Rik* | ↑ 0.40 | 1.32 | 0.0428 | ↑ 0.36 | 1.28 | 0.0232 | |
| 1417119_at | *Zfpl1* | ↑ 1.15 | 2.22 | 0.0428 | ↑ 1.54 | 2.91 | 0.0104 | |
| 1420868_s_at | *Tmed2* | ↑ 0.74 | 1.67 | 0.0436 | ↑ 0.83 | 1.78 | 0.0164 | |
| 1416696_at | *D17Wsu104e* | ↑ 1.72 | 3.29 | 0.0462 | ↑ 2.56 | 5.89 | 0.0104 | |
| 1418250_at | *Arfl4* | ↓ −1.23 | −2.35 | 0.0494 | ↓ −1.19 | −2.28 | 0.0225 | |

many genes that were over expressed in the HT-E model are clearly identified as part of the unfolded protein response or ER overload response pathways. Examples include Sdf2L1 and Armet, up-regulated 22.9- ($P$ = 0.029) and 7.1- ($P$ = 0.027) fold respectively, in HT-E mice. Sdf2L1 is constitutively expressed at low levels in muscle but up-regulated in ER stress[33]; Armet is expressed during the unfolded protein response of ER stress, in a pattern parallel with the expression of Grp78.[34]

Interestingly several genes that we found to be differentially expressed in the HT-E model are homologues of known human autoantigen targets of myositis-related autoantibodies, including Ufm1, (Ubiquitin modifier [SUMO] like),[35] and Srp (Signal recognition particle, which targets nascent chains to the ER), both also ER stress induced.[36,37]

## *Alteration in Muscle-Specific Proteins or Muscle Regulatory Genes, in 'Young' Muscle on Up-Regulation of MHC Class I*

A second set of genes specifically altered in the early myositis model compared with the standard model were those that are structural muscle proteins intrinsic to muscle function, or genes critically involved in muscle development (Table 3). Thus myosin heavy chain (Myh11), myosin light chain kinase (Mylk2), myopallidin (Mypn), muscleblindlike2 (Mbnl2), and fukutin, were all signifi-

**Table 2.** Genes Up-Regulated in the HT-E Model that Relate to ER Function, Protein Transport, Golgi Function, the SNARE Complex, or Ubiquitination

| Functional role | Symbol (alternative) | Name | HT-E vs. HT-L | | HT-E vs. control | |
|---|---|---|---|---|---|---|
| | | | Fold change | *P* value | Fold change | *P* value |
| ER protein transport | *Srprb* | Signal recognition particle receptor, B subunit* | 3.42 | 0.0225 | 2.44 | 0.0138 |
| ER protein transport | *Tmed9* | Transmembrane emp24 protein transport domain containing 9* | 3.42 | 0.0167 | | NS |
| ER protein transport | *Tmed2* | Transmembrane emp24 domain trafficking protein 2* | 1.78 | 0.0164 | 1.67 | 0.0436 |
| ER protein transport | *Tmed3* | Transmembrane emp24 domain containing 3 | 3.30 | 0.0225 | | NS |
| ER transporter regulation | *Yrdc* | yrdC domain containing (*E. coli*) | 2.77 | 0.0104 | 2.56 | 0.0226 |
| ER glycosylation | *Ugcgl1* | UDP-glucose ceramide glucosyltransferase-like 1 | 2.39 | 0.0167 | 2.69 | 0.0233 |
| ER glycosylation | *Alg12* | Asparagine-linked glycosylation 12 homolog (yeast, alpha-1,6-mannosyltransferase) | 3.19 | 0.0181 | 3.07 | 0.0428 |
| ER sugar transport | *Slc35b1* | Solute carrier family 35, member B1 | 2.32 | 0.0371 | 3.10 | 0.0428 |
| ER clearance of signal peptides | *Spcs3* | Signal peptidase complex subunit 3 homolog (*S. cerevisiae*) | 2.54 | 0.0167 | 3.07 | 0.0199 |
| ER protein involved in protein quality control | *Sdf2l1* | Stromal cell-derived factor 2-like 1 | 22.98 | 0.0295 | | NS |
| ER protein induced by UPR | *Armet* | Arginine-rich, mutated in early stage tumors | 7.10 | 0.0265 | 11.99 | 0.0371 |
| ER retention of proteins | *KDELR2* | KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2 | 3.84 | 0.0170 | | NS |
| Ca dependent ER chaperone | *Calr* | Calreticulin | 2.87 | 0.0461 | | NS |
| ER Chaperone | *Tra1* | Tumor rejection antigen gp96 | 4.85 | 0.0157 | 4.40 | 0.0371 |
| hsp40 family chaperones | *dnaJb11* | DnaJ (Hsp40) homolog, subfamily B, member 11 | 3.36 | 0.0217 | 4.12 | 0.0323 |
| hsp40 family chaperones | *dnaJc3* | DnaJ (Hsp40) homolog, subfamily C, member 3 | 6.29 | 0.0256 | | NS |
| Protein folding in ER | *PDIa 4 (ERp72)* | Protein disulfide isomerase associated 4 | 5.12 | 0.0104 | 4.74 | 0.0199 |
| Protein folding in ER | *PDIa 6 (CaBP1)* | Protein disulfide isomerase associated 6* | 5.56 | 0.0225 | | NS |
| Protein folding in ER | *PDIa 3 (ERp57)* | Protein disulfide isomerase associated 3 | 2.80 | 0.0267 | | NS |
| Isomerase activity protein folding | *Fkbp11* | FK506 binding protein 11 | 9.89 | 0.0123 | 6.42 | 0.0428 |
| Protein recycling | *Rab4b* | RAB4B, member RAS oncogene family | 4.08 | 0.0212 | | NS |
| Degradation of misfolded proteins | *Derl3* | Der1-like domain family, member 3 | 16.66 | 0.0140 | 16.76 | 0.0262 |
| Degradation of misfolded proteins | *Derl2* | Der1-like domain family, member 2 | 2.96 | 0.0230 | | NS |
| Degradation of misfolded proteins | *Derl1* | Der1-like domain family, member 1 | 2.94 | 0.0450 | | NS |
| Golgi coatamer proteins | *Copa* | Coatomer protein complex subunit alpha | 4.80 | 0.0343 | | NS |
| Golgi coatamer proteins | *Copg* | Coatomer protein complex, subunit gamma | 3.35 | 0.0225 | | NS |
| Golgi coatamer proteins | *Cope* | Coatomer protein complex, subunit epsilon | 2.24 | 0.0406 | | NS |
| Golgi resident Ca binding protein | *Nucb2* | Nucleobindin 2 | 2.52 | 0.0112 | 2.68 | 0.0153 |
| Golgi transport and assembly | *Golt1b* | Golgi transport 1 homolog B* | 5.96 | 0.0343 | | NS |
| Golgi transport | *Gorasp2* | Golgi reassembly stacking protein 2* | 4.16 | 0.0258 | | NS |
| Vesicle trafficking | *Arfgap3* | ADP-ribosylation factor GTPase activating protein 3 | 4.63 | 0.0071 | 4.32 | 0.0089 |
| SNARE proteins | *Bet1* | Blocked early in transport 1 homolog (*S. cerevisiae*) like | 4.33 | 0.0100 | 3.00 | 0.0428 |
| SNARE proteins | *Stx5a* | Syntaxin 5A | 2.15 | 0.0167 | | NS |
| Protein export | *Sec61* | Sec61 alpha 1 subunit* | 4.88 | 0.0091 | | NS |
| Protein export | *Sec22* | SEC22 vesicle trafficking protein-like 1 | 3.68 | 0.0167 | | NS |
| Ubuquitin pathway | *Ube2g2* | Ubiquitin-conjugating enzyme E2G 2 | 5.86 | 0.0456 | | NS |
| Ubuquitin pathway | *Ube1dc1* | Ubiquitin-activating enzyme E1-domain containing 1 | 2.52 | 0.0337 | | NS |
| Ubuquitin pathway | *Zfpl1* | Zinc finger like protein 1 | 2.91 | 0.0104 | 2.22 | 0.0428 |
| Ubuquitin pathway | *Usp28* | Ubiquitin specific peptidase 28 | 2.03 | 0.0406 | | NS |
| Ubuquitin modifier (SUMO like) | *Ufm1* | Ubiquitin-fold modifier 1 | 1.98 | 0.0091 | 2.03 | 0.0138 |

*Genes had 2 or more differentially expressed probe sets. For these genes the data for the probe set with the highest fold change is shown.

cantly down-regulated. Most of these proteins have been associated with human muscle diseases, such as dystrophies, or altered muscle function in mouse models, when they are altered, lost, or mutated.[38,39] Fukutin is a Golgi protein, in which mutations are associated with limb girdle dystrophies.[40]

Interestingly in relation to myoblast differentiation, the myoblast specific transcription factor myogenin,[41,42] and Tnfrsf12a (also called Fn14), a receptor that is required for differentiation of myoblast to myotubes,[43] were highly up-regulated in HT-E muscle, suggesting a robust attempt at new muscle fiber differentiation. In contrast, several genes in the Wnt signaling pathway, including Nov[44] and Fzd4,[45] shown to be important to muscle development, and of the Notch/delta/numb pathway, such as bone morphogenetic protein-4, known to be critical in satellite cell to myoblast transition[46] were down-regulated in the HT-E mice.

Since few of the muscle-specific genes also reached significance of $P < 0.05$ in the comparison of HT-E to their own age matched controls, than in the comparison of HT-E to HT-L murine models, the data comparing HT-E to

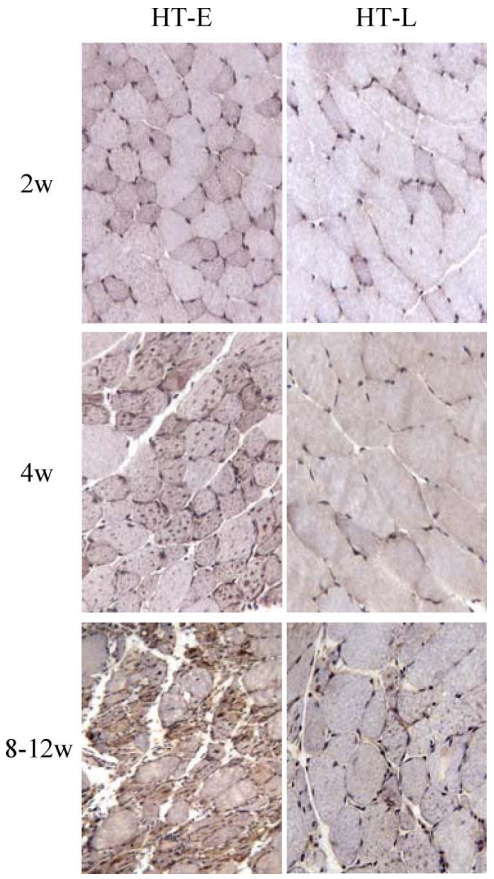


**Figure 4.** Immunohistological analysis of Quadriceps femoris sections for Ubiquitin complex, in HT-E (**left**) and HT-L (**right**) models at 2, 4, and 8 to 12 weeks after transgene overexpression as shown. All panels ×200 final magnification.

HT-L could suggest that there are some age-specific, rather than disease-specific, differential expression effects in these muscle specific genes. However, the expression of these muscle specific genes was not significantly different between HT-L and controls, indicating that differences seen between HT-L and HT-E mice were unlikely to be due to age alone for those probe sets. To illustrate this, the raw data expression levels for all three groups of mice for three representative probes of these genes, Mbnl2, Tnfrsf12a, and Tbc1d4 are shown in Figure 5.

## *Discussion*

In this study, we have adapted our previous model of myositis, in which self MHC class I heavy chain is overexpressed in muscle, by overexpressing the MHC class I protein from a younger age. In this adapted model (HT-E), we have seen rapid onset of weakness and even early death. This phenotype is paralleled by signs of muscle fiber changes and inflammatory myositis. The model will be a valuable tool in the analysis of effects of MHC overexpression because it facilitates rapid experimental approaches in the laboratory. The speed of onset of weakness and myositis is intriguing, given the clinical parallel of rapid onset of disease in some children with juvenile dermatomyositis.

Histological analysis of the HT-E model, in comparison with HT-L over a series of time points after transgene expression showed that the changes in the models were broadly similar, and did not suggest a different type of inflammatory process in the HT-E model, but instead showed more rapid kinetics. Therefore we suggest that the phenotype seen in the HT-E model suggests that in younger muscle there is a more rapid response to MHC overexpression, leading to muscle damage, rather than the activation of fundamentally different pathways in younger mice. To further investigate this we have performed gene expression in muscle tissue at an early time point after transgene expression. As expected, in the original model, muscle obtained only 2 weeks after MHC class I overexpression revealed few significant differences from control muscle. In contrast, in the young mice, muscle tissue analyzed only 2 weeks after transgene expression revealed marked differences of gene expression. We found changes of expression in multiple proteins involved in ER function including many chaperone proteins, as well as proteins regulating protein folding and glycosylation, those central to protein transport, and ER to Golgi traffic. In addition proteins whose role it is to deal with increased protein load such as the derlins,

**Table 3.** Genes Relating to Muscle Development or Structure/Function that Were Significantly Over- or Underexpressed in the HT-E Compared with the HT-L Model

| Functional role | Symbol | Name | Direction of Change | HT-E vs. HT-L Fold change | HT-E vs. HT-L *P* value |
|---|---|---|---|---|---|
| Muscle development Akt substrate | *Tbc1d4* | TBC1 domain family, member 4 | ↓ | −4.15 | 0.0457 |
| Muscle development; TWEAK receptor | *Tnfrsf12a (Fn14)* | Tumor necrosis factor receptor superfamily, member 12a | ↑ | 8.64 | 0.0371 |
| Muscle development | *Myog* | Myogenin | ↑ | 5.32 | 0.0474 |
| Splice regulator in muscle tissue | *Mbnl2* | Muscle blind like | ↓ | −2.39 | 0.0402 |
| Sarcomere function; model of dystrophy | *Mypn* | Myopalladin | ↓ | −2.00 | 0.0279 |
| Myosin muscle protein | *Myh11* | Myosin heavy polypeptide 11 | ↓ | −2.42 | 0.0143 |
| Myosin muscle protein | *Mylk2* | Myosin light polypepetide kinase | ↓ | −2.28 | 0.0406 |
| Notch/delta signalling pathway | *Bmp6* | Bone morphogenetic protein6 | ↓ | −2.75 | 0.0307 |
| Wnt signalling pathway | *Fzd4* | Frizzled homolog 4 (Drosophila) | ↓ | −6.03 | 0.0104 |
| Regulator of skeletal muscle | *Nov* | Nephroblastoma overexpressed gene | ↓ | −2.77 | 0.0071 |

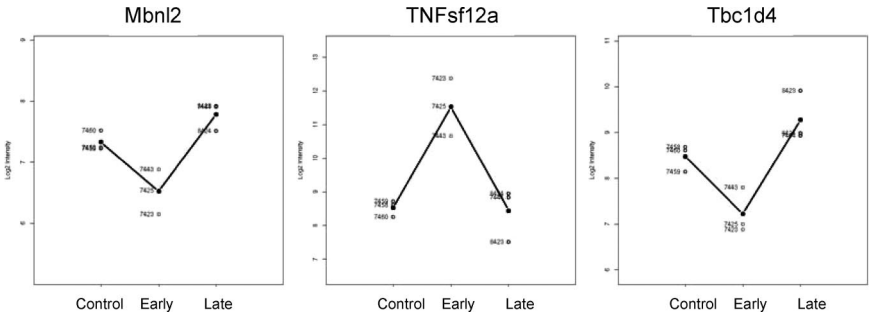


**Figure 5.** Gene expression in 3 of the muscle specific genes whose expression was altered in the HT-E model, **left**-Mbnl2; **middle**-TNFsf12a; and **right**-Tbc1d4. Each panel shows data points for control, HT-E (early) and HT-L (late) samples as marked, plotted as log2 intensity values.

and components of the ubiquitination pathway, were altered, suggesting an attempt to 'clear' the increase of a ER resident protein, MHC class I.

Most of the ER and Golgi proteins have a role in maintaining homeostasis of protein folding and traffic, and many have been noted to increase during ER stress, in particular the unfolded protein response and ER-associated degradation pathways. There is striking overlap between the gene expression altered in our HT-E model and other models where ER stress is implicated in myopathy or muscle damage. Thus, during transgenic overexpression of MCP1 in cardiac muscle leading to a cardiomyopathy in which ER stress is implicated, genes that were up-regulated included Ufm1, DNAJ (Hsp40), protein disulphide isomerases, Sec61 and ubiquitin.[36] Mice that lack the enzyme hexose-6-phosphate dehydrogenase (H6PD) develop skeletal muscle myopathy; gene expression profiling in these H6PD null mice revealed ER stress pathways, and among the differentially expressed genes, 31 were in common between that model and our HT-E model.[47]

The link between ER stress and muscle damage is not confined to mouse models of muscle disease but has also been implicated in human myositis, including inclusion body myositis[18] and in our own previous studies in dermatomyositis,[19] as well as in some genetic myopathies. The ubiquitin-proteosome pathway is increasingly being recognized as critical to muscle physiology, muscle protein turnover, and degradation of unwanted proteins. Ubiquitin ligases have been shown to be overexpressed in inclusion body myositis. Our demonstration of overexpression of ubiquitin protein in HT-E mice soon after MHC overexpression, closely resembles that shown in a model of over expression of the ring finger protein RNF5, in which mice develop a degenerative myopathy.[48]

Since ER of skeletal muscle (sarcoplasmic reticulum) is highly specialized, in which many calcium binding proteins reside, and since calcium flux plays a critical role in muscle function, changes in expression of calcium binding proteins such as calreticulin and others may disrupt muscle function, and ultimately lead to muscle damage.[17] Together this emerging theme suggests that muscle may be highly sensitive to ER stress pathways, and to damage as a consequence thereof, even though ER stress itself plays a homeostatic role in healthy muscle.[16] Thus caspase 12 is highly expressed in myoblasts[16]; during fusion of myoblasts to become multinucleate myofibers, some cells die, while others survive and ER stress leading to apoptosis has a role in this apparent 'selective' process.[49]

The transition from myoblast to myofiber normally involves a change in levels of MHC class I expression from high to low, and interestingly overexpression of MHC class I in myoblasts driven by interferon-$\gamma$ blocks their ability to differentiate to myotubes and myofibers *in vitro*.[50] Therefore we suggest that increased MHC expression *in vivo* may alter or inhibit healthy development of myoblasts to myofibers. Given that satellite cells and myoblast activity may be higher in younger muscle,[51] we suggest that this pathway may be more rapidly affected in the HT-E model. In this context, many muscle-specific genes were differentially expressed in the HT-E model. These included myogenic regulatory factors such as myogenin, receptors required for muscle differentiation, and muscle structural proteins. It is possible that rapid growing muscle at a young age is more sensitive to disturbances in the balance of satellite cell/myoblast to mature myofiber and therefore changes in these pathways may lead more rapidly to overall muscle damage.

It is of interest that we have seen increased expression in the HT-E model of at least two proteins that are related to known targets of myositis-specific antibodies in patients with inflammatory myositis, namely Ufm1 and Srp. Proteins that are the targets of myositis-related autoantibodies can be overexpressed in the muscles of myositis patients.[52] It would therefore appear that in this novel model, we have recapitulated this phenomenon.

In humans several lines of evidence have suggested that a type 1 interferon 'signature' is expressed in muscle from both adult and pediatric patients with inflammatory myositis.[53,54] In our HT-E model an interferon signature was not observed. This difference may be due to the very early time point of muscle sampling (2 weeks after induction) since it is also known that time of disease before muscle gene expression profiling makes a significant difference in signature observed.[55] Alternatively this may be because overexpression of MHC class I alone in a model system does not fully recapitulate human disease.

In conclusion, we present data on a novel murine model of muscle damage in which overexpression of MHC class I protein at a very young age leads to a rapid myopathy and myositis. Our data suggest that the overexpression of MHC in skeletal muscle at a young age leads to a dramatic up-regulation of proteins that implicate ER stress as one of the mechanisms of damage, and also of genes that indicate dysregulation of the homeo-

static balance of developing muscle fibers. This model will shed new insights into the mechanisms of damage to muscle in patients with myositis and ultimately may assist in generating new targets for novel therapies.

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