Mechanisms of chemotherapy-induced muscle wasting in mice with cancer cachexia

Kate T. Murphy1*, Kristy Swiderski1, James G. Ryall1, Jonathan R. Davey1, Hongwei Qian1, Séverine Lamon2, Victoria C. Foletta2, Jennifer Trieu1, Annabel Chee1, Suzannah J. Read1, Timur Naim1, Paul Gregorevic1 & Gordon S. Lynch1

1Centre for Muscle Research, Department of Anatomy and Physiology, The University of Melbourne, Melbourne, Victoria, Australia
2Institute for Physical Activity and Nutrition (IPAN), School of Exercise and Nutrition Sciences, Deakin University, Burwood, Victoria, Australia

Abstract

Background Cachexia is a debilitating complication of cancer characterized by progressive wasting and weakness of skeletal muscles that reduces quality of life and can compromise survival. Many anticancer treatments, such as chemotherapy, also cause muscle wasting, which impairs the response to treatment. Given that many cancer patients present with cachexia at the initiation of treatment, we investigated whether cachectic mice were susceptible to chemotherapy-induced muscle wasting and to investigate contributing mechanisms, including the dysregulation of microRNAs (miRs).

Methods Cachectic colon-26 (C-26) tumour-bearing mice were given 5-fluourouracil (5-FU) chemotherapy or vehicle treatment and analysed for muscle mass, fibre size and composition, and miR expression. Mechanisms were validated in vitro using C2C12 cell culture and miR mimics and inhibitors and were confirmed in vivo by injecting muscles of 5-FU-treated cachectic mice with recombinant adeno-associated viral (rAAV) vectors encoding a miR sponge.

Results In cachectic tumour-bearing mice, 5-FU chemotherapy exacerbated the loss of skeletal muscle mass compared with vehicle treatment (by −12% to −20%, P < 0.05). miR expression profiling, quantitative real-time PCR, and in vitro analyses revealed contributing mechanisms including miR-351-3p-dependent ERK2 inhibition. Intramuscular injection of rAAV vectors encoding a sponge to reduce miR-351-3p expression in 5-FU-treated cachectic mice enhanced ERK phosphorylation (+18%, P < 0.05) and increased muscle fibre size (+15%, P < 0.01). Hsa-miR-125a-3p shares similar predicted gene targets as mmu-miR-351-3p, and its inhibition in human muscle cells in vitro prevented 5-FU-induced atrophy (P < 0.001) and increased ERK phosphorylation (P < 0.001).

Conclusions The findings implicate miR-351-3p-mediated ERK2 inhibition as a contributing mechanism in chemotherapy-induced muscle wasting in mice with cancer cachexia and that its inhibition is a promising adjunct therapy for preserving muscles during cancer treatment.

Keywords ERK2; miR-351; Chemotherapy; Cancer cachexia; Muscle wasting

Received: 21 February 2021; Revised: 21 June 2021; Accepted: 27 July 2021

*Correspondence to: Kate T. Murphy, Centre for Muscle Research, Department of Anatomy and Physiology, The University of Melbourne, Melbourne, Victoria 3010, Australia.

Fax: +61 3 8344 5818, Email: ktmurphy@unimelb.edu.au

Introduction

Cachexia is the profound weight loss, frailty, and fatigue that affects many cancer patients. It is a multifactorial syndrome that manifests as progressive wasting of skeletal muscle (and typically fat) and is associated with significant functional impairments.1 In the worst cases, cachexia leads to metabolic, respiratory (diaphragm), and/or cardiac (heart) muscle failure and accounts for 20–30% of all cancer-related deaths.2,3 Indeed, weight loss and body mass index are

© 2021 The Authors. JCSM Rapid Communications published by John Wiley & Sons Ltd on behalf of Society on Sarcopenia, Cachexia and Wasting Disorders. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
predictors of survival in cancer patients. Cachexia affects 40–80% of all advanced cancer patients with the highest prevalence in those with pancreatic, gastric, oesophageal, colorectal, and lung cancer. It can also occur early in disease with weight loss present at diagnosis in >40% of patients with head and neck, gastrointestinal, pancreatic, colorectal, and lung cancers and is associated with reduced response to chemotherapy, increased treatment toxicity, and poorer survival.

Anticancer treatments, such as chemotherapy, can also cause muscle wasting and weakness, with fatigue being one of the most debilitating side effects. Preclinical studies in non-tumour-bearing mice have revealed that muscle wasting and weakness caused by 5-fluorouracil (5-FU)-based chemotherapy combinations result from muscle fibre atrophy, a shift from slow-to-fast fibre types, and mitochondrial dysfunction. Furthermore, muscle wasting persisted in the colon-26 (C-26) mouse model of cancer cachexia despite cisplatin chemotherapy reducing tumour size by nearly four-fold. Whether existing cachexia affects chemotherapy-induced muscle wasting and, if so, to identify the mechanisms responsible. To this end, we focused on dysregulated microRNAs (miRs) because they are important contributors regulating of muscle size and protein turnover, altered miR expression is associated with cancer cachexia and other conditions of muscle wasting, and miR-based therapies are effective and safe to use in vivo. We tested the hypothesis that mice with cancer cachexia would remain susceptible to chemotherapy-induced muscle wasting as a consequence of dysregulated miR expression. This information has important implications for the design of adjuvant therapies and the development of strategies that can attenuate chemotherapy-induced muscle wasting, reduce the risk of recurrence, and improve survival for cancer patients.

**Materials and methods**

**Experimental animals**

All experiments were approved by the Animal Ethics Committee of The University of Melbourne and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes as stipulated by the National Health and Medical Research Council (Australia). Male CD2F1 and Balb/c mice were obtained from the Animal Resources Centre (Canning Vale, Western Australia) and housed in the Biological Research Facility at The University of Melbourne under a 12:12 h light–dark cycle. Water and standard laboratory chow were available ad libitum, and consumption was recorded daily.

**Effect of 5-fluorouracil chemotherapy in cachectic tumour-bearing mice**

Frozen C-26 cells were kindly donated by Prof. Martha Belury (The Ohio State University, Columbus, OH, USA). We have previously described the procedures used to thaw and count the C-26 cells and have characterized the functional and metabolic impairments in mice injected with these cells in detail elsewhere.

On the day of inoculation (Day 1), 11- to 12-week-old CD2F1 mice were allocated randomly into one of three experimental groups: a control group injected with phosphate-buffered saline (PBS) alone (n = 8); a C-26 tumour-bearing group treated with DMSO vehicle (n = 6); and a C-26 tumour-bearing group treated with 5-FU (n = 7). Mice were anaesthetized via an intraperitoneal (i.p.) injection of a mixture of ketamine (100 mg·kg⁻¹) and xylazine (10 mg·kg⁻¹; VM Supplies, Chelsea Heights, VIC, Australia), such that they were unresponsive to tactile stimuli. Mice were shaved on the dorsal side and given a subcutaneous (s.c.) injection of 5 × 10⁵ C-26 cells suspended in 100 μL of sterilized PBS or 100 μL of sterilized PBS only (control). Mice recovered from anaesthesia on a heat pad and were given an i.c. injection of atipamezole (Antisedan; 1 mg·kg⁻¹; VM Supplies) to partially reverse the effects of xylazine and promote faster recovery from sedation. On Days 15, 18, and 21, lean body mass was assessed (EchoMRI™, Houston, TX, USA) and mice received an i.p. injection of either 60% DMSO vehicle (3.3 μL·g⁻¹ body mass; Sigma-Aldrich, Castle Hill, NSW, Australia) or 5-FU diluted in 60% DMSO (100 mg·kg⁻¹ body mass, 3.3 μL·g⁻¹ body mass; Sigma-Aldrich). The 5-FU dose was based on that used previously to reduce C-26 tumour size and predicted to be within the clinical range (50–154 mg·kg⁻¹ lean body mass). On Day 22, mice were anaesthetized with sodium pentobarbitone (Nembutal; 60 mg·kg⁻¹; Sigma-Aldrich) via i.p. injection and various hindlimb skeletal muscles [tibialis anterior (TA), extensor digitorum longus, soleus, and plantaris] as well as the epidymal fat, spleen, heart, and tumour were carefully excised, blotted on filter paper, and weighed on an analytical balance. The TA muscles were mounted in embedding medium and frozen in thawing isopentane, and the other tissues were frozen rapidly in liquid nitrogen. All tissues were then stored at −80°C for subsequent analyses. Mice were killed as a consequence of heart excision while still anaesthetized deeply.
Generation and intramuscular injection of rAAV6: eGFP-miR-351-3p sponge in 5-FU-treated mice with cancer cachexia

Methods for the design, cloning, and in vitro validation of the AAV:eGFP-miR-351-3p sponge and scrambled controls are provided in the Supporting Information. Transfection of the AAV:eGFP-miR-351-3p sponge and AAV:eGFP-Scrambled control plasmids with the pDGM6 packaging plasmid into HEK 293 cells generated type 6 pseudotyped viral vectors that were harvested and purified as described previously. Briefly, HEK 293 cells were plated on a 10 cm culture dish, 8–16 h prior to transfection with 10 μg of a vector genome-containing plasmid and 20 μg of the packaging plasmid pDGM6, using the calcium phosphate precipitate method to generate pseudotype 6 vectors. Seventy-two hours after transfection, the media and cells were collected and homogenized through a microfluidizer (Microfluidics) prior to 0.22 μm clarification (Millipore). Purified vector was generated from the clarified lysate by affinity chromatography over a HiTrap heparin column (Amersham) and ultracentrifuged overnight prior to resuspension in sterile Hank’s balanced salt solution.

For this experiment, we used the more readily available Balb/c mouse model that has an endpoint of 18 days after the injection of C-26 cells, at which time mice exhibit a similar percentage decrease in tumour-free body mass and loss of muscle and heart mass as C-26 tumour-bearing CD2F1 mice at endpoint (21 days after C-26 injection). On the day of inoculation (Day 1), 15–20-week-old male Balb/c mice (n = 8) were anaesthetized with isoflurane (induction, 3–4% oxygen–isoflurane at 0.5 L min⁻¹; maintenance, 2–3% at 0.5 L min⁻¹), such that they were unresponsive to tactile stimuli. They were then shaved on the dorsal side and given an s.c. injection of 5 × 10⁶ C-26 cells suspended in 100 μL of sterilized PBS. Four days later (Day 5), mice were again anaesthetized with isoflurane, both hindlimbs shaved and the right TA muscle was injected with rAAV6:eGFP-miR-351-3p sponge while the left TA muscle was injected with rAAV6:eGFP-Scrambled sponge at a dose of 5 × 10⁵ vector genomes (determined by optimization pilot studies) in 40 μL of sterilized Hank’s balanced salt solution. Mice were given an s.c. injection of buprenorphine (0.05 mg·kg⁻¹) for analgesia and recovered on a heat pad. To match the timing of 5-FU treatments to the CD2F1 model where injections were given every 3 days with the final injection given the day before endpoint, on Days 12, 15, and 18, mice received an i.p. injection of 5-FU diluted in 60% DMSO (100 mg·kg⁻¹ body mass). On Day 19, mice were anaesthetized with sodium pentobarbitone (Nembutal; 60 mg·kg⁻¹) via i.p. injection and the right TA and left TA muscles were carefully excised, blotted on filter paper, and weighed on an analytical balance. The TA muscles were mounted in embedding medium, frozen in thawing isopentane, and stored at −80°C for subsequent analyses. Mice were killed by cardiac excision while still anaesthetized deeply.

Culture, transfection, and treatment of C2C12 cells

Murine C2C12 myoblasts (American Type Culture Collection, Manassas, VA, USA) were seeded in 12-well plates and cultured in growth media containing Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Scoresby, VIC, Australia) supplemented with 10% FBS and 1% l-glut (DMEM/10% FBS/1% l-glut) at 37°C ± 5% CO₂. Upon confluency, the media were changed to DMEM containing 2% (v/v) horse serum (HS, Life Technologies) and 1% l-glut (DMEM/2% HS/1% l-glut, differentiation media) for 4 days to induce differentiation during which time the media were changed every 48 h. For experiments assessing the effect of treatment with various chemotherapeutics, and as per our standard procedures, treatment was initiated on Day 4 of differentiation, at which point we observe fully differentiated myotubes. Cells were then incubated for an additional 48 h at 37°C ± 5% CO₂ in fresh DMEM/2% HS/1% l-glut containing 5-FU (5 μM in 0.0013% DMSO) or vehicle (0.0013% DMSO). Pilot dose–response experiments showed that 5 μM was the lowest dose of 5-FU to induce significant and reproducible atrophy.

For miRNA mimic and inhibitor experiments, murine C2C12 myoblasts were seeded in six-well plates and cultured in growth media at 37°C ± 5% CO₂. When myoblasts reached ~75% confluency, they were transfected with 25 pmol of miRNA mimic negative control [Applied Biological Materials Inc. (ABM), Richmond, BC, Canada]; mmu-miR-351-3p mimic (ABM); miRNA inhibitor negative control (ABM); or mmu-miR-351-3p inhibitor (ABM) using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA), according to manufacturer’s instructions. At 24 h after transfection, growth media were changed. At 48 h after transfection, the media were changed to differentiation media for 6 days, with media refreshed every 48 h. In these experiments, myotubes were incubated at 37°C ± 5% CO₂ for 48 h (Days 4–6) in fresh differentiation media with vehicle control (0.0013% DMSO) or 5-FU (5 μM); vehicle control (0.2% DMSO), oxaliplatin (40 μM, Sigma-Aldrich), or cisplatin (50 μM, Sigma-Aldrich); and leucovorin (10 μg·ml⁻¹, Sigma-Aldrich) or irinotecan (20 μg·ml⁻¹, Sigma-Aldrich).

Statistics

All values are expressed as mean ± SEM unless stated otherwise. Groups were compared using Student’s t-test or a one-way or two-way ANOVA, where appropriate. Bonferroni’s post hoc test was used to determine significant differences between individual groups. The level of significance was set at P < 0.05 for all comparisons except global miRNA expression profiling where cut-offs for significant changes were a fold-change >1.5 and a P value ≤0.01.

DOI: 10.1002/rco2.50
Figure 1  Cachectic mice are susceptible to chemotherapy-induced muscle wasting. (A) Schematic of experimental design showing means of relative total (including tumour mass) body mass (% initial). Grey bars represent days of vehicle (DMSO) or 5-FU treatment. At the end of the experimental period, tumours were excised and weighed, allowing for calculation of (B) relative tumour mass (normalized for body mass) and (C) absolute mass of various hindlimb muscles, including the extensor digitorum longus (EDL), tibialis anterior (TA), plantaris, and soleus, as well as the (D) heart. (E) Representative images of TA muscle cross-sections reacted for laminin (red) to indicate all muscle fibres and myosin IIa (N2.261, green) to indicate type IIa fibres. Because mouse TA muscles express very few type I fibres, those fibres not reacting with N2.261 (i.e. black fibres in the merge) were assumed to be of type IIX/b. Quantification of N2.261 and laminin based on reaction intensity facilitated determination of (F) the proportion of type IIa and type IIX/b fibres (non-reacting with N2.261), (G) average cross-sectional area (CSA) of the type IIa and type IIX/b fibres and all muscle fibres, and (H) histogram analysis of the distribution of fibre size in vehicle-treated and 5-FU-treated cachectic C-26 tumour-bearing mice (B–D, F–G, unpaired t-test; H, two-way ANOVA; *P < 0.05; **P < 0.01; ***P < 0.001; n = 5–6, ±SEM). Scale bar, 50 μm.
Further details about the Materials and methods section are provided in the Supporting Information.

Results

Cachectic mice are susceptible to chemotherapy-induced muscle wasting

As depicted in Figure 1A, cachectic CD2F1 mice bearing C-26 tumours received three intraperitoneal injections of vehicle (DMSO) or 5-FU (Days 15, 18, and 21) and, 1 day after the final injection, were anaesthetized deeply, and the tumour, various skeletal muscles, and organs were carefully excised and weighed. Lean body mass was assessed prior to each treatment, and calculations revealed that mice received an average 5-FU dose of 117 ± 2 mg·kg⁻¹ lean body mass. No signs of 5-FU-related toxicity such as diarrhoea, palmar plantar erythematous, mucositis, or ataxia were observed. Cumulative food intake was not different between vehicle-treated and 5-FU-treated mice (Figure S1A), but 5-FU increased cumulative water intake from Day 16 (P < 0.0001; Figure S1B). 5-FU had no effect on the percentage change in tumour-free body mass from the start of the experiment (Figure S1C) but reduced relative tumour mass by 42% compared with vehicle (P < 0.001, Figure 1B). Compared with vehicle, 5-FU reduced mass of the soleus (by −13%, P < 0.01), extensor digitorum longus (by −14%, P < 0.05), plantaris (by −9%, P < 0.05), TA (by −11%, P < 0.05, all Figure 1C), and heart (by −8%, P < 0.03, Figure 1D) but had no effect on mass of the epididymal fat or spleen (Figure S1D).

Tibialis anterior muscle cross-sections were reacted for myosin Ila (N2.261, green) and laminin (red) to identify type Ila fibres and to visualize all fibres (Figure 1E), respectively, for assessment of fibre-type proportions and cross-sectional area. Because mouse TA muscles contain very few detectable type I fibres,31 those fibres not reacting to N2.261 were assumed to be type IIX/b fibres. 5-FU-induced a fibre-type shift towards more type Ila fibres (+22%, P < 0.05) and fewer type IIX/b fibres (−22%, P < 0.05, Figure 1F) and reduced cross-sectional area of the type IIX/b fibres (−15%, P < 0.05) and average of all muscle fibres compared with vehicle-treated controls (−16%, P < 0.05, Figure 1G). A histogram analysis confirmed that 5-FU reduced the proportion of larger muscle fibres and increased the proportion of smaller muscle fibres in cachectic mice (P < 0.05, Figure 1H).

Chemotherapy-induced muscle wasting is associated with miR-351-3p-dependent ERK2 inhibition

Given that recent studies have demonstrated that cancer cachexia is associated with dysregulated miR-regulated networks,20,32,33 we performed global miR expression profiling analysis to identify whether miR expression alters with chemotherapy-induced wasting in cachectic mice. Of the 641 miRNAs measured, 313 (49%) were considered not expressed in mouse plantaris muscle and were therefore excluded from further analysis. Using a cut-off for significant changes of a fold-change >1.5 and P value ≤0.01 (q value <0.05), five miRs were differentially expressed (three up-regulated and two down-regulated) in plantaris muscles from 5-FU-treated mice compared with DMSO vehicle-treated mice (Figure 2A). Of the five differentially regulated miRs, only miR-351-3p was significantly increased with 5-FU when analysed by quantitative real-time PCR (qPCR, by 7.6-fold, P < 0.05, Figure 2B). Gene expression of the three up-regulated miRs was also examined in PBS mice and compared with DMSO vehicle-treated C-26 mice to differentiate between effects of cancer cells and chemotherapy. There were no differences between groups, indicating that the increase in miR-351-3p mRNA expression in 5-FU-treated cachectic mice was due to the chemotherapy and not the cancer cells (data not shown).

The gene targets of mouse miR-351-3p (mmu-miR-351-3p) were predicted on the basis of 3’UTR sequence homology, with 2370 potential targets identified, and these were submitted to KEGG analysis of signalling pathways (Table S1). Of known pathways typically associated with regulation of muscle size,34 the MAPK signalling pathway was the top pathway enriched with predicted gene targets of mmu-miR-351-3p, including Mapk1 (ERK2), Mapk14 (p38), and Mapk8 (JNK1) (Figure 2C and Table S1), and so we investigated the phosphorylation status of these proteins in muscles from vehicle-treated and 5-FU-treated cachectic mice (Figure 2D). 5-FU reduced ERK1/2 phosphorylation by 37% (P < 0.05) but had no effect on phosphorylation of p38 or p54/p46 SAPK/JNK (Figure 2E).

We used the C2C12 cell culture system to examine the direct effects of miR-351-3p modulation on myotube size and/or the phosphorylation status of ERK1/2. C2C12 myoblasts transfected with a miR-351-3p mimic and differentiated into mature myotubes resulted in a modest but significant increase in miR-351-3p expression (Figure 2F) and caused a 23% reduction in myotube size (P < 0.0001, Figure 2G) and a 43% decrease in ERK1/2 phosphorylation (P < 0.05, Figure 2H and 2I).

To further investigate the involvement of miR-351-3p in 5-FU-induced muscle atrophy, C2C12 myoblasts were transfected with a miR-351-3p inhibitor or negative control and, after 4 days of differentiation into myotubes, were treated for a further 48 h with 5-FU or vehicle control. The miR-351-3p inhibitor induced a trend for reduced miR-351-3p expression (P < 0.06, Figure 3A) and completely prevented atrophy induced by 5-FU (P < 0.01, Figure 3B). Furthermore, the miR-351-3p inhibitor prevented the 5-FU-induced decrease in ERK1/2 phosphorylation in C2C12...
Increased miR-351-3p expression is associated with chemotherapy-induced muscle wasting in cachectic mice, and a miR-351-3p mimic induces myotube atrophy in vitro. (A) Comparative miRNA profiling analysis identified five miRNAs differentially expressed in plantaris muscles from 5-FU-treated cachectic mice compared with DMSO vehicle-treated cachectic mice (unpaired t-test; **P < 0.01; ***P < 0.001; ****P < 0.0001; n = 4–5, ±SEM). (B) Quantitative real-time PCR (qPCR) analysis of the genes altered with miRNA profiling confirmed significant up-regulation of miR-351-3p in plantaris muscles from 5-FU-treated cachectic mice compared with DMSO vehicle-treated cachectic mice (unpaired t-test; *P < 0.05; n = 4–6, ±SEM). (C) KEGG pathways enriched with predicted gene targets of mmu-miR-351-3p. Values next to the bars represent the number of enriched genes. (D, E) Western blot analysis of phosphorylated and total ERK1/2, p38 and p54/p46 SAPK/JNK, and total protein in plantaris muscles from DMSO vehicle-treated and 5-FU-treated cachectic mice (unpaired t-test; *P < 0.05; n = 5–6, ±SEM). C2C12 myoblasts were transfected with a miRNA mimic negative control (-ve control) or miR-351-3p mimic and, after 6 days of differentiation, were taken for (F) qPCR analysis of miR-351-3p expression, (G) myosin and DAPI staining (top) to enable quantification of myotube diameter (bottom), and (H, I) western blot analysis of phosphorylated and total ERK1/2 and total protein (unpaired t-test; *P < 0.05; ****P < 0.0001; E, n = 5; F, n = 5; G, n = 9–10; I, n = 4, ±SEM).
myotubes ($P < 0.05$, Figure 3C and 3D). To assess whether
the findings reflected effects on proliferation, raw cell counts
were taken at 24, 48, and 72 h after transfection and revealed
very similar growth curves for C2C12 myoblasts transfected
with the miR-351-3p inhibitor and inhibitor negative control
(Figure S2A and S2B) and no difference between groups for
the mean doubling time (Figure S2C). These findings
demonstrate that miR-351 inhibition does not affect the pro-
liferative capacity of C2C12 myoblasts.

Given that the MAPK pathway has been associated with in-
creased expression of the ubiquitin ligases MuRF-1 and
atrogin-1, their expression was investigated in the experi-
ments described previously. There was no difference in
MuRF-1 ($P < 0.44$) or atrogin-1 mRNA expression

Figure 3  miR-351-3p inhibition prevents 5-FU-induced reductions in myotube size and ERK1/2 phosphorylation, and Mapk1 (ERK2) is a direct target
gene of miR-351-3p. C2C12 myoblasts were transfected with a miRNA inhibitor negative control (-ve control) or miR-351-3p inhibitor and, (A) after
6 days of differentiation into myotubes, were assessed for miR-351-3p expression using qPCR or, after 4 days of differentiation into myotubes, were
treated for another 48 h with DMSO vehicle or 5-FU and then taken for (B) myosin and DAPI staining (left) for quantification of myotube diameter
(right) or (C, D) western blot analysis of phosphorylated and total ERK1/2 and total protein (A, unpaired t-test; B & D, two-way ANOVA; *$P < 0.05$;
**$P < 0.01$; ***$P < 0.001$; A, $n = 6$; B, $n = 6–9$; D, $n = 6–10$, ±SEM). (E) NanoLuc luciferase activity was assessed in HEK 293 cells after co-transfection with
150 ng pNanoglo2 vector containing the full-length Mapk1 3’UTR and either 5 nM miR-351-3p mimic (+) or 5 nM of an irrelevant miRNA (negative
control, −) (unpaired t-test; ***$P < 0.0001$, experiments repeated three times using $n = 6$ wells per group, ±SEM). (F) Top, schematic of the putative
binding site between miR-351-3p and the Mapk1 3’UTR at position 1426 (WT, blue font). Bottom, NanoLuc luciferase activity was assessed in HEK 293
cells following co-transfection with 150 ng pNanoglo2 vector containing either the putative miR-351-3p binding site of Mapk1 3’UTR (WT) or its mu-
tant control and either 5 nM miR-351-3p mimic (+) or 5 nM of an irrelevant miRNA (negative control, −) (two-way ANOVA; ****$P < 0.0001$, exper-
iments repeated three times using $n = 6$ wells per group, ±SEM).
cachectic mice (P < 0.48) in muscles from vehicle-treated and 5-FU-treated cachetic mice (Figure S2D). There was also no effect on MuRF-1 and atrogin-1 mRNA expression of the miR-351-3p mimic (Figure S2E) or of either 5-FU or the miR-351-3p inhibitor (Figure S2F).

To determine whether miR-351-3p could directly bind and regulate the Mapk1 3’UTR, HEK 293 cells were co-transfected with a reporter plasmid containing the full-length mouse Mapk1 3’UTR alongside either a miR-351-3p mimic or an irrelevant miRNA lacking the predicted binding site on the Mapk1 3’UTR (negative control). HEK 293 cells were used for this purpose due to their high transfection efficiency. At 24 h after co-transfection, there was a 33% reduction in luciferase activity in the cells containing the miR-351-3p mimic compared with the negative control (P < 0.001, Figure 3E). We then investigated the miR-351-3p putative binding site on the Mapk1 3’UTR by co-transferring HEK 293 cells with the miR-351-3p mimic or negative control as well as a reporter plasmid with a shorter version of the Mapk1 3’UTR containing only the putative miR-351-3p binding site (WT, Figure 3F). At 24 h after co-transfection, there was a 31% reduction in luciferase activity (P < 0.0001) in cells containing the miR-351-3p mimic compared with the negative control (Figure 3F). However, this reduction was completely prevented when cells were co-transfected with a shorter Mapk1 3’UTR reporter plasmid in which the miR-351-3p binding site was mutated (Mut, P < 0.13, Figure 3F). Taken together, these data indicate that Mapk1 is a direct target of miR-351-3p.

miR-351-3p inhibition increases muscle fibre size in chemotherapy-treated cachetic mice

To test the therapeutic potential of miR-351-3p inhibition for attenuating chemotherapy-induced muscle atrophy, we generated a AAV:eGFP-miR-351-3p sponge (Figure S3A) and AAV:eGFP-Scrambled (control) sponge plasmid. Function of the AAV:eGFP-miR-351-3p sponge was confirmed with transduction of C2C12 myotubes preventing the 5-FU-associated decrease in myotube size observed with the scrambled sponge (−26%, P < 0.0001; Figure S3B). These findings also confirm that effects of the miR-351-3p inhibitor were directly on myotubes and not myoblasts.

Having confirmed the function of the vector in vitro, we generated AAV6 preparations to test whether AAV6-mediated inhibition of miR-351-3p expression could increase muscle fibre size in 5-FU-treated mice with cancer cachexia. For this experiment, we used the more readily available Balb/c mouse model, which, after the injection of C-26 cells, develops tumours at a similar rate and size as the CD2F1 model (Figure S3C) and, at endpoint (Day 19), exhibits a similar percentage decrease in tumour-free body mass (Figure S3D) and loss of muscle and heart mass as C-26 tumour-bearing CD2F1 mice (Figure S3E). A schematic of the experimental timeline is shown in Figure 4A, with intramuscular injection of AAV6 on Day 5 (4 days after C-26 injection) and 5-FU treatments on Days 12, 15, and 18 to match the timing in the CD2F1 model where injections occurred every 3 days with the final injection given the day before endpoint. At 14 days after intramuscular injection into the TA muscles of cachetic C-26 tumour-bearing mice (Day 19), western blotting for GFP confirmed effective transduction of AAV6:eGFP-Scrambled sponge and AAV6:eGFP-miR-351-3p sponge vectors (Figure 4B), and injection of the AAV6:eGFP-miR-351-3p sponge increased ERK1/2 phosphorylation (+18%, P < 0.05, Figure 4B and 4C). H&E staining revealed no evidence of toxicity after intramuscular injection of AAV6:eGFP-miR-351-3p sponge or AAV6:eGFP-Scrambled control sponge (Figure 4D). Immunohistochemistry was used to differentiate between transduced (GFP positive) and non-transduced (GFP negative) fibres and revealed that the average size of fibres transduced with the AAV6:eGFP-miR-351-3p sponge was greater than those transduced with AAV6:eGFP-Scrambled sponge (+15%, P < 0.01, Figure 4E and 4F), due to a reduced proportion of smaller muscle fibres and an increased proportion of larger muscle fibres (P < 0.05, Figure 4G).

Relevance to other chemotherapeutic drugs and human cancer

To gain a better understanding of the therapeutic potential of miR-351-3p inhibition, we examined the ability of the miR-351-3p inhibitor to attenuate muscle wasting in response to other chemotherapeutic agents. These included leucovorin and oxaliplatin, which are often used in combination with 5-FU (Folfox) as well as irinotecan, used in combination with 5-FU and leucovorin (Folfiri). Cisplatin was also examined as it is used to treat numerous human cancers including lung, ovarian, breast, bladder, head and neck, and testicular cancer. None of the chemotherapeutic agents increased endogenous miR-351-3p mRNA expression (Figure S4A and S4B). When added to C2C12 myotubes in vitro, oxaliplatin and cisplatin reduced myotube size by 23% (P < 0.0001) and 28% (P < 0.0001), respectively; effects were completely prevented by miR-351-3p inhibition (Figure S4C and S4D). Irinotecan increased ERK1/2 phosphorylation (by 1.77-fold, P < 0.01), but neither leucovorin nor irinotecan induced C2C12 myotube atrophy (Figure S4E and S4F) and so were not combined with the miR-351-3p inhibitor. Thus, miR-351-3p inhibition may have broad therapeutic implications in human cancer treatments.
Figure 4  Intramuscular injection of AAV6:eGFP-miR-351-3p sponge increases muscle fibre size in chemotherapy-treated mice with cancer cachexia. (A) Experimental timeline showing that at 4 days (Day 5) after injection of C-26 cells, the right TA (RTA) muscles of Balb/c mice were injected with AAVs encoding for GFP-tagged miR-351-3p sponge and the left TA (LTA) muscles were injected with equivalent doses of GFP-tagged scrambled control sponge, and mice were given an i.p. injection of 5-FU on Days 12, 15, and 18. On Day 19, the RTA and LTA muscles were taken for western blot assessment of (B) GFP expression and (B, C) phosphorylated and total ERK1/2 expression. TA muscle sections were stained (or reacted for): (D) H&E to examine general muscle morphology and signs of toxicity and (E) wheat germ agglutinin (red) to indicate all muscle fibres and facilitate determination of (F) average CSA of the transduced fibres (GFP positive, green) and (G) histogram analysis of the size of transduced (GFP positive) fibres in muscles injected with AAV6:eGFP-Scrambled sponge (LTA) or AAV6:eGFP-miR-351-3p sponge (RTA) in 5-FU-treated cachectic mice. (H) qPCR analysis of the mRNA expression of MHCIIa, MHCIIb, and MHCIIx in muscles injected with AAV6:eGFP-Scrambled sponge or AAV6:eGFP-miR-351-3p sponge in 5-FU-treated cachectic mice. (C, F, H, unpaired t-test; G, two-way ANOVA; *P < 0.05; **P < 0.01; n = 6–8, ±SEM). Scale bar, 100 μm.
potential across multiple and specific chemotherapy regimens.

miR-351-3p is not expressed in the human genome, and so we sought to identify a human miRNA targeting similar genes and pathways. Using miRWalk software and KEGG pathway analysis, we found that 16 of the 27 pathways enriched in predicted gene targets of human miR-125a-3p (hsa-miR-125a-3p) were shared with mouse miR-351-3p (mmu-miR-351-3p, Table S2). Furthermore, the MAPK signalling pathway was the top-ranked pathway enriched in genes predicted to be targets of hsa-miR-125a-3p (Figure 5A and Table S2), including MAPK1, which contains four potential miR-125a-3p binding sites (Figure 5B). To assess the relevance of our findings to humans, we tested whether hsa-miR-125a-3p inhibition could attenuate 5-FU-induced atrophy of human primary skeletal muscle cells (Figure 5C and 5D). Transfection of the hsa-miR-125a-3p inhibitor had no effect on the size of DMSO-treated myotubes (P < 0.69) but completely prevented the 5-FU-induced reduction in muscle cell size and ERK1/2 phosphorylation (P < 0.001; Figure 5C and 5D). Therefore, inhibition of hsa-miR-125a-3p, which is predicted to target similar genes and pathways as mmu-miR-351a-3p, has therapeutic potential for attenuating chemotherapy-induced muscle wasting in patients with cancer cachexia.

Figure 5 miR-125a-3p targets similar genes and pathways as miR-351-3p, and inhibition of human miR-125a-3p prevents 5-FU-induced atrophy and reduction in ERK1/2 phosphorylation in human muscle cells in vitro. (A) Top-ranked KEGG pathways enriched with predicted gene targets of hsa-miR-125a-3p. Values next to the bars represent the number of predicted gene targets. (B) Human hsa-miR-125a-3p has four predicted binding sites to the human Mapk1 3’UTR. (C, D) Primary human skeletal muscle cells were transfected with a miRNA inhibitor negative control (-ve control) or hsa-miR-125a-3p inhibitor and, after 8 days of differentiation, were treated for 48 h with DMSO vehicle or 5-FU and then taken for (C) myosin and DAPI staining (left) for quantification of myotube diameter (right) or (D) western blot analysis of phosphorylated and total ERK1/2 (two-way ANOVA; ***P < 0.001; n = 6, ±SEM).
Discussion

Given that many cancer patients present with cachexia at diagnosis and initiation of anticancer treatment, and because chemotherapy itself can cause muscle wasting and weakness, we investigated whether mice with existing cachexia were susceptible to chemotherapy-induced muscle wasting. Here, we show that in mice with cancer cachexia, 5-FU chemotherapy treatment increased loss of skeletal muscle and heart mass despite reducing tumour mass. As summarized in Figure 6, mechanisms mediating the chemotherapy-associated muscle wasting in cachetic mice include miR-351-3p-dependent ERK2 inhibition. Reducing miR-351-3p levels with an AAV-based sponge in skeletal muscle of 5-FU-treated mice with cancer cachexia enhanced ERK phosphorylation and increased muscle fibre size. These findings identify potential therapeutic targets and strategies to preserve muscle fibres during chemotherapy in patients with cancer cachexia.

Because more than 40% of patients with gastrointestinal, pancreatic, colorectal, and lung cancer exhibit cachexia upon diagnosis and the initiation of anticancer treatment, it is clinically relevant to determine whether these patients are susceptible to chemotherapy-induced wasting and, if so, identify potential mechanisms and treatments to preserve skeletal muscle mass and quality of life in an already fragile population. We chose 5-FU-based chemotherapy as it is the common drug in combination therapies such as Folfox (5-FU, leucovorin, and oxaliplatin) and Folfiri (5-FU, leucovorin, and irinotecan) that are used frequently to treat cancers associated with early cachexia. Using 5-FU alone enables identification of its specific effects that could not be discerned with combination therapy. Importantly, the dose received normalized to lean body mass (~117 mg·kg⁻¹) was within the clinical range (50–154 mg·kg⁻¹).27 We found that 5-FU treatment initiated after C-26 tumour-bearing mice had already begun to exhibit cachexia exacerbated chemotherapy-induced wasting of skeletal muscles and the heart. Although cardiac function was not assessed in the present study, it is well known that chemotherapeutic agents cause cardiotoxic side effects, with >20% of patients receiving 5-FU exhibiting cardiotoxicity.36 The increased loss of heart mass in 5-FU-treated mice highlights the need for non-5-FU-based chemotherapeutics for cachectic patients in order to reduce toxic effects on the heart. Although 5-FU and other chemotherapeutics are frequently associated with anorexia, this was not observed in the present study, which is likely due to cachetic C-26 tumour-bearing mice already exhibiting anorexia compared with PBS controls.25

Figure 6  In cachectic tumour-bearing mice, 5-FU-based chemotherapy induces miR-351-3p-mediated ERK2 inhibition in skeletal muscle, leading to increased wasting and poor prognosis. Inhibition of miR-351-3p using AAV-based gene therapy is a potential adjunct therapy for attenuating chemotherapy-induced muscle wasting and improving prognosis in cancer cachexia.
The mechanisms underlying chemotherapy-induced muscle wasting in cachectic mice remain largely unknown with most studies having been conducted in non-tumour-bearing rodents, limiting their clinical relevance. The few studies comparing chemotherapy treatment in tumour-bearing and non-tumour-bearing mice have found that despite treatment inducing similar reductions in muscle mass, mechanisms vary considerably between groups. It was therefore essential in the present study to investigate mechanisms underlying the susceptibility of cachectic muscles to chemotherapy-induced wasting, and in this regard, we focused on dysregulated miRs because they are important contributing regulators of muscle size, altered miR expression is associated with numerous muscle wasting conditions including cancer cachexia, and miR-based therapies are effective and safe to use in vivo. Global miR expression profiling and qPCR analyses revealed mechanisms of 5-FU-induced muscle wasting to include increased expression of miR-351-3p, and using luciferase reporter assays, we demonstrated that ERK2 is a direct gene target of miR-351-3p. Supporting these results, ERK1/2 phosphorylation was decreased in muscles from 5-FU-treated cachectic mice and associated with selective atrophy and loss of the large, glycolytic type IIx/b muscle fibres. These findings are consistent with previous reports of ERK1/2 inhibition inducing muscle atrophy and a fibre-type shift. However, they contrast with those implicating increased ERK1/2 phosphorylation in the pathophysiology of cachexia in tumour-bearing mice and those in healthy mice where ERK1/2 activation was associated with Folfox- and Folfiri-induced muscle wasting. The discrepancies provide further evidence that different mechanisms are responsible for chemotherapy-induced muscle wasting in tumour-bearing and non-tumour-bearing mice. They may also reflect effects of irinotecan, which increased ERK1/2 phosphorylation in the present study but did not induce C2C12 myotube atrophy. However, they are unlikely to involve effects of leucovorin (used in both Folfox and Folfiri), which did not affect myotube size or ERK1/2 phosphorylation.

This is the first report of miR-351-3p as a mediator of chemotherapy-induced wasting and adds to the growing body of evidence implicating dysregulated miR-regulated networks in cancer cachexia and the regulation of skeletal muscle MAPK signalling. There is a dearth of information on the role of miR-351-3p in skeletal muscle, but our in vitro experiments revealed that its overexpression reduced C2C12 myotube size and its inhibition did not alter the size of healthy myotubes but completely prevented myotube atrophy induced by chemotherapeutics including 5-FU, oxaliplatin, and cisplatin. Proliferation experiments confirmed that miR-351-3p inhibition did not affect C2C12 proliferation, and transduction of myotubes with an AAV:eGFP-miR-351-3p sponge confirmed direct effects on myotube size. Taken together, these findings suggest that endogenous miR-351-3p expression is low with little effect on the regulation of healthy muscle size but that its expression can increase significantly with some cytotoxic drugs such as 5-FU and contribute to muscle wasting in these conditions. Importantly, inhibition of miR-351-3p can also be beneficial for reducing the muscle wasting associated with other cytotoxic drugs such as oxaliplatin and cisplatin even without an increase in its endogenous expression.

We used intramuscular injection of an AAV-based therapeutic expressing a miRNA sponge to specifically inhibit miR-351-3p expression in a single skeletal muscle and investigate its therapeutic potential for protecting cachectic muscle from chemotherapy-induced wasting. Advantages of this system include that miRNA sponges are plasmid constructs containing multiple high-affinity miRNA antisense binding sites that can inhibit specific miRNAs and prevent their binding to endogenous target genes. Because of tissue limitations, we were unable to assess the unlikely possibility that other miRs were altered with the miR-351-3p sponge, but combining AAV with a tissue-specific promoter and a miRNA sponge limits regulation to a specific organ and avoids potential off-target effects typically associated with systemic delivery of miRNA sponges. These benefits have enhanced the likelihood of vector-based strategies manipulating miRNA activity entering the clinic. Because we used a dose of AAV that reduces miR-351-3p levels without inducing toxicity and not transducing all muscle fibres, the addition of a GFP tag to the sponge constructs revealed that transduced (GFP-positive) fibres were significantly larger with the miR-351-3p sponge compared with the scrambled control sponge in 5-FU-treated cachectic mice. These findings demonstrate the utility of our approach for inhibiting miR-351-3p activity in striated muscle and provide proof-of-principle evidence of its therapeutic potential for protecting cachectic muscles from chemotherapy-induced wasting. A limitation of the constructs containing a GFP tag is that we were unable to assess whether the miR-351-3p sponge attenuated the 5-FU mediated fibre-type shift because the filter for immunohistochemical detection of GFP is the same as that for identification of the type IIa fibres. However, qPCR analysis of the mRNA expression of the MHC isoforms expressed in mouse TA muscles supported this notion by revealing that the miR-351-3p sponge increased MHCIIb expression.

Although miR-351-3p is not expressed in the human genome, we identified that human miR-125a-3p shares similar predicted target genes and pathways, including MAPK1 (ERK2) and other members of the MAPK signalling pathway, and is part of the miR-125 family that has been implicated in carcinogenesis and chemoresistance. Furthermore, miR-125a-3p inhibition completely prevents 5-FU-induced decreases in ERK1/2 phosphorylation and atrophy of primary human skeletal muscle cells, establishing the relevance of our findings to humans. Thus, local inhibition of miR-125a-

DOI: 10.1002/rc2.50
3p could help prevent chemotherapy-induced wasting in patients with cancer cachexia. Further development of the viral vector approach for manipulating miRNA activity could include intravascular injection to achieve widespread transduction of striated musculature, including the heart, which exhibits susceptibility to 5-FU-induced wasting in cachetic mice.

In conclusion, mice with cancer cachexia remain susceptible to chemotherapy-induced muscle wasting, with mechanisms including miR-351-3p-dependent ERK2 inhibition. We have identified inhibition of mmu-miR-351-3p/hsa-miR-125a-3p as a potential adjunct therapy for attenuating chemotherapy-induced muscle wasting in patients with cancer cachexia. These findings highlight the importance of initiating strategies to combat cachexia prior to treatment to enhance the effectiveness of chemotherapy and improve cancer prognosis.

Acknowledgements

The authors thank Prof. Martha Belury (Department of Human Nutrition, The Ohio State University) for donating the C-26 cells and Prof. Donna McCarthy (College of Nursing, The Ohio State University) for arranging the shipment of these cells, Dr Marissa Caldow (Centre for Muscle Research, Department of Anatomy and Physiology, The University of Melbourne) for donating the primary human skeletal muscle cells, and Associate Professor Peter Crack (Department of Biochemistry and Pharmacology, The University of Melbourne) for use of the FlexStation 3 machine. The MF 20 monoclonal antibody, developed by D. A. Fischman (Weill Cornell Medical College), and the N2.261 monoclonal antibody, developed by H. M. Blau (Stanford University School of Medicine), were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242.

Funding

This study was supported by project grants from the National Health and Medical Research Council (NHMRC, Australia, GNT1041865, to G.S.L. and K.T.M.), Victorian Cancer Agency (16852, to K.T.M.), and Cancer Council Victoria (1120752, to K.T.M. and G.S.L.). K.T.M. was supported by a Career Development Fellowship from the NHMRC (1023178). K.S. was supported by an Early Career Fellowship from the NHMRC (16852, to K.T.M.), and an Early Career Seed grant from The University of Melbourne.

Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. (A) Cumulative food intake and (B) water intake in DMSO vehicle and 5-FU treated cachetic C-26 tumor-bearing CD2F1 mice. Grey bars represent days of DMSO or 5-FU treatment (two-way ANOVA; ****P < 0.0001; n = 5–7, ±SEM). At the end of the experimental period, (C) the percentage change in tumor-free body mass compared with initial (pre-inoculation) was calculated and (D) epididymal fat and spleen mass determined (unpaired t-test, no significant differences were found; n = 5–6, ±SEM).

Figure S2. To assess effects on proliferation, C2C12 myoblasts were transfected with a miRNA inhibitor negative control (–ve control) or miR-351-3p inhibitor and at 24, 48 and 72 h post-transfection, (A) representative bright field images were taken and (B) total cell counts were used to generate exponential growth curves and (C) mean doubling time (TD); no significant differences were found; n = 4, mean ± SEM for B and mean ± 95% confidence interval for C). (D) qPCR analysis of MuRF-1 and atrogin-1 mRNA expression in plantaris muscles from 5-FU compared with DMSO vehicle treated cachetic mice (unpaired t-test; no significant differences were found; n = 5–6, ±SEM). (E) C2C12 myoblasts were transfected with a miRNA mimic negative control (–ve control) or miR-351-3p mimic and after 6 days of differentiation, were taken for qPCR analysis of MuRF-1 and atrogin-1 mRNA expression (unpaired t-test; no significant differences were found, n = 5, ±SEM). (F) C2C12 myoblasts were transfected with a miRNA inhibitor negative control (–ve control) or miR-351-3p inhibitor and after 4 d differentiation into myotubes, were treated for another 48 h with DMSO vehicle or 5-FU and then taken for qPCR analysis of MuRF-1 and atrogin-1 mRNA expression (two-way ANOVA; no significant differences were found, n = 6, ±SEM).

Figure S3. (A) Schematic showing design of the miR-351-3p sponge including four repeats of: two miR-351-3p binding sites (8 nt and 9 nt); a 3 nt bulge at nucleotide position 12; and 4 nt spacer sequence between the individual sponges, under the control of the CMV promoter. Inset shows nucleotide sequence of each sponge repeat (red)–13; and 4 nt spacer sequence between the individual sponges, under the control of the CMV promoter. Inset shows nucleotide sequence of each sponge repeat (red) that sequesters the target miRNA (black). (B) C2C12 myotubes that had been differentiated for 1 d were transduced with a AAV:eGFP-Scrambled (control) sponge or AAV: eGFP-miR-351-3p sponge and after an additional 3 d differentiation, were treated for 48 h with DMSO vehicle or 5-FU and then taken for: (B) imaging of GFP to assess transduction efficiency (left) and then myosin and DAPI staining to enable quantification of myotube diameter (right, two-way ANOVA; ****P < 0.0001; n = 3, ±SEM). Comparison of
Chemotherapy-induced muscle wasting in cachectic mice

(C) tumor growth, (D) the percentage change in tumor-free body mass from the start of the experiment to end-point (day 22 in CD2F1 model and day 19 in Balb/c model) and (E) the percentage change in mass of the extensor digitorum longus (EDL), tibialis anterior (TA) and heart compared with PBS controls in CD2F1 and Balb/c mice injected subcutaneously with C-26 cells (C, two-way ANOVA; D-E, unpaired t-test; no significant differences were found; n = 5–6, ± SEM).

Figure S4. C2C12 myotubes that had been differentiate

for 4 d were treated for another 48 h with: (A) DMSO vehicle (control), oxaliplatin or cisplatin; or (B) leucovorin or irinotecan and then taken for qPCR analysis of miR-351-3p mRNA expression (one-way ANOVA; no significant differences were found; n = 4, ± SEM). (C, D) C2C12 myoblasts were transfected with a miRNA inhibitor negative control (-ve control) or miR-351-3p inhibitor and after 4 d differentiation into myotubes, were treated for another 48 h with DMSO vehicle or (C) oxaliplatin or (D) cisplatin and were then stained for myosin and DAPI (left) to enable quantification of myotube diameter (right; two-way ANOVA; ****P < 0.0001; n = 4, ± SEM). C2C12 myotubes treated without (control, Con) or with leucovorin (LV) or irinotecan (IR) were also: (E) stained for myosin and DAPI (left) to enable quantification of myotube diameter (right); or (F) taken for western blot analysis of phosphorylated and total ERK1/2, and total protein (E, F; one-way ANOVA; **P < 0.01; n = 12, ± SEM).

Table S1. Supporting Information

Table S2. Supporting Information

Conflict of interest

None declared.

References

1. Fearon K, Strasser F, Anker SD, Bosaeus I, Bruera E, Fainsinger RL, et al. Definition and classification of cancer cachexia: an international consensus. Lancet Oncol 2011;12:489–495.
2. Pwers SK, Lynch GS, Murphy KT, Reid MB, Zijdevind W. Disease-induced skeletal muscle atrophy and fatigue. Med Sci Sports Exerc 2016;48:2307–2319.
3. Tisdale MJ. Cachexia in cancer patients. Nat Rev Cancer 2002;2:862–871.
4. Martin L, Senesse P, Gioulbasanis I, Antoun S, Bozetti F, Deans C, et al. Diagnostic criteria for the classification of cancer-associated weight loss. J Clin Oncol 2015;33:90–99.
5. Renfro LA, Loupakis F, Adams RA, Seymour MT, Heinemann V, Schmoll HJ, et al. Body mass index is prognostic in metastatic colorectal cancer: pooled analysis of patients from first-line clinical trials in the ARCAD database. J Clin Oncol 2016;34:144–150.
6. Sun I, Quan XQ, Yu S. An epidemiological survey of cachexia in advanced cancer patients and analysis on its diagnostic and treatment status. Nutr Cancer 2015;67:1056–1062.
7. Blum D, Stene GB, Solheim TS, Fayers P, Hjermstad MJ, Baracos VE, et al. Validation of the Consensus-Definition for Cancer Cachexia and a classification model—a study based on data from an international multicentre project (EPCRC-CSCA). Ann Oncol 2014;25:1635–1642.
8. O’Donoghue N, Shrotriya S, Aktas A, Hullihen B, Ayvaz S, Estfan B, et al. Clinical significance of weight changes at diagnosis in solid tumours. Support Care Cancer 2019;27:2725–2733.
9. Dewys WD, Begg C, Lavin PT, Band PR, Bennett JM, Bertino JR, et al. Prognostic effect of weight loss prior to chemotherapy in cancer patients. Eastern Cooperative Oncology Group. Am J Med 1980;69:491–497.
10. Prado CM, Baracos VE, McCargar LJ, Reiman M, Tourtzikas M, Tonkin K, et al. Sarcopenia as a determinant of chemotherapy toxicity and time to tumor progression in metastatic breast cancer patients receiving capecitabine treatment. Clin Cancer Res 2009;15:2920–2926.
11. Barret M, Antoun S, Dalban C, Malka D, Mansourbakht T, Zaanan A, et al. Sarcopenia is linked to treatment toxicity in patients with metastatic colorectal cancer. Nutr Cancer 2014;66:583–589.
12. Gilliam LA, St Clair DK. Chemotherapy-induced weakness and fatigue in skeletal muscle: the role of oxidative stress. Antioxid Redox Signal 2011;15:2543–2563.
13. Kimura M, Naito T, Kennotsu H, Taira T, Wakuda K, Oyakawa T, et al. Prognostic impact of cancer cachexia in patients with advanced non-small cell lung cancer. Support Care Cancer 2015;23:1699–1708.
14. Barreto R, Mandilli G, Witzmann FA, Novelli F, Zimmers TA, Bonetto A. Cancer and chemotherapy contribute to muscle loss by activating common signaling pathways. Front Physiol 2016;7:472.
15. Barreto R, Waning DL, Gao H, Liu Y, Zimmers TA, Bonetto A. Chemotherapy-related cachexia is associated with mitochondrial depletion and the activation of ERK1/2 and p38 MAPKs. Oncotarget 2016;7:43442–43460.
16. Damrauer JS, Stadler ME, Acharya S, Baldwin AS, Couch ME, Guttridge D. Chemotherapy-induced muscle wasting: association with NF-κB and cancer cachexia. Basic Appl Myol 2008;18:139–148.
17. Alexander MS, Kunkel LM. Skeletal muscle microRNAs: their diagnostic and therapeutic potential in human muscle diseases. J Neuromuscul Dis 2015;2:1–11.
18. Zacharewicz E, Kalanon M, Murphy RM, Russell AP, Lamon S. MicroRNA-99b-5p downregulates protein synthesis in human primary myotubes. Am J Physiol Cell Physiol 2020;319:C432–C440.
19. Lee DE, Brown JL, Rosa-Caldwell ME, Blackwell TA, Perry RA Jr, Brown LA, et al. Cancer cachexia-induced muscle atrophy: evidence for alterations in microRNAs important for muscle size. Physiol Genomics 2017;49:253–260.
20. Freire PP, Fernandez GJ, Cury SS, de Moraes D, Oliveira JS, de Oliveira G, et al. The pathway to cancer cachexia: microRNA-regulated networks in muscle wasting based on integrative meta-analysis. Int J Mol Sci 2019;20:1962.
21. Chen R, Lei S, Jiang T, She Y, Shi H. Regulation of skeletal muscle atrophy in cachexia by microRNAs and long non-coding RNAs. Front Cell Dev Biol 2020;8:577010.
22. Lamon S, Zacharewicz E, Butchart LC, Orellana L, Mikovic J, Grounds MD, et al. MicroRNA expression patterns in
post-natal mouse skeletal muscle development. *BMC Genomics* 2017;18:52.

23. Moraes LN, Fernandez GJ, Vechetti-Junior IJ, Freire PP, Souza RWA, Villacis RAR, et al. Integration of miRNA and mRNA expression profiles reveals microRNA-regulated networks during muscle wasting in cardiac cachexia. *Sci Rep* 2017;7:6998.

24. Bernardo BC, Ooi JY, Lin RC, McMullen JR. miRNA therapeutics: a new class of drugs with potential therapeutic applications in the heart. *Future Med Chem* 2015;7:1771–1792.

25. Murphy KT, Chee A, Trieu J, Naim T, Lynch GS. Importance of functional and metabolic impairments in the characterization of the C-26 murine model of cancer cachexia. *Dis Model Mech* 2012;5:533–545.

26. Van Laar JA, Rustum YM, de Witt CLV, Smit K, Kuiper CM, Pinedo HM, et al. Tumor size and origin determine the antitumor activity of cisplatin or 5-fluorouracil and its modulation by leucovorin in murine colon carcinomas. *Cancer Chemother Pharmacoil* 1996;39:79–89.

27. Williams GR, Deal AM, Shachar SS, Walko CM, Patel JN, O’Neil B, et al. The impact of skeletal muscle on the pharmacokinetics and toxicity of 5-fluorouracil in colorectal cancer. *Cancer Chemother Pharmacol* 2018;81:413–417.

28. Blankinship MJ, Gregorevic P, Allen JM, Harper SQ, Harper H, Halbert CL, et al. Efficient transduction of skeletal muscle using vectors based on adeno-associated virus serotype 6. *Mol Ther* 2004;10:671–678.

29. Thakur SS, James JL, Cranna NJ, Chhen VL, Swiderski K, Ryall JG, et al. Expression and localization of heat-shock proteins during skeletal muscle cell proliferation and differentiation and the impact of heat stress. *Cell Stress Chaperones* 2019;24:749–761.

30. Murphy KT, Hossain MI, Swiderski K, Chee A, Naim T, Trieu J, et al. Mus receptor activation slows tumor growth and attenuates muscle wasting in cancer. *Cancer Res* 2019;79:706–719.

31. Murphy KT, Chee A, Gleeson BG, Naim T, Swiderski K, Koopman R, et al. Antibody-directed myostatin inhibition enhances muscle mass and function in tumor-bearing mice. *Am J Physiol Regul Integr Comp Physiol* 2011;301:R716–R726.

32. Acunzo M, Croce CM. MicroRNA in cancer and cachexia—a mini-review. *J Infect Dis* 2015;212:574–577.

33. Narasimhan A, Ghosh S, Stretch C, Greiner R, Bathe OF, Baracos V, et al. Small RNAome profiling from human skeletal muscle: novel miRNAs and their targets associated with cancer cachexia. *J Cachexia Sarcopenia Muscle* 2017;8:405–416.

34. Vainshtein A, Sandri M. Signaling pathways that control muscle mass. *Int J Mol Sci* 2020;21:4759.

35. Murphy KT. The pathogenesis and treatment of cardiac atrophy in cancer cachexia. *Am J Physiol Heart Circ Physiol* 2016;310:H466–H477.

36. Pai VB, Nahata MC. Cardiotoxicity of chemotherapeutic agents: incidence, treatment and prevention. *Drug Saf* 2000;22:263–302.

37. O’Connell TM, Pin F, Couch ME, Bonetto A. Treatment with soluble activin receptor type IIb alters metabolic response in chemotherapy-induced cachexia. *Cancers (Basel)* 2019;11:1222.

38. Sirago G, Conte E, Fracasso F, Cormio A, Grossberg AJ, Zhu X, Agarwal A, et al. The impact of JNK/MAPK signaling from human skeletal muscle of cancer-bearing mice. *J Infect Dis* 2017;216:334–343.

39. Shi H, Scheffler JM, Zeng C, Pleitner JM, Hannon KM, Grant AL, et al. Mitogen-activated protein kinase signaling is necessary for the maintenance of skeletal muscle mass. *Am J Physiol Cell Physiol* 2009;296:C1040–C1048.

40. Shi H, Scheffler JM, Pleitner JM, Zeng C, Park S, Hannon KM, et al. Modulation of skeletal muscle fiber type by mitogen-activated protein kinase signaling. *FASEB J* 2008;22:2990–3000.

41. Penna F, Costamagna D, Fanzani A, Bonelli G, Baccino FM, Costelli P. Muscle wasting and impaired myogenesis in tumor-bearing mice are prevented by ERK inhibition. *PLoS One* 2010;5:e13604.

42. Quan-Jun Y, Yan H, Yong-Long H, Li-Li W, Jie L, Jin-Lu H, et al. Selumetinib attenuates skeletal muscle wasting in murine cachexia model through ERK inhibition and Akt activation. *Mol Cancer Ther* 2017;16:334–343.

43. Brown JL, Lee DE, Rosa-Caldwell ME, Brown LA, Perry RA, Haynie WS, et al. Protein imbalance in the development of skeletal muscle wasting in tumour-bearing mice. *J Cachexia Sarcopenia Muscle* 2018;9:987–1002.

44. Xie SJ, Liu JH, Chen HF, Tan YY, Liu SR, Zhang Y, et al. Inhibition of the JNK/MAPK signaling pathway by myogenesis-associated miRNAs is required for skeletal muscle development. *Cell Death Differ* 2018;25:1581–1597.

45. Baumann V, Winkler J. miRNA-based therapies: strategies and delivery platforms for oligonucleotide and non-oligonucleotide agents. *Future Med Chem* 2014;6:1967–1984.

46. Wang JK, Wang Z, Li G. MicroRNA-125 in immunity and cancer. *Cancer Lett* 2019;454:134–145.