Importance of functional and metabolic impairments in the characterization of the C-26 murine model of cancer cachexia

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SUMMARY
Cancer cachexia describes the progressive skeletal muscle wasting and weakness that is associated with many cancers. It impairs quality of life and accounts for >20% of all cancer-related deaths. The main outcome that affects quality of life and mortality is loss of skeletal muscle function and so preclinical models should exhibit similar functional impairments in order to maximize translational outcomes. Mice bearing colon-26 (C-26) tumors are commonly used in cancer cachexia studies but few studies have provided comprehensive assessments of physiological and metabolic impairment, especially those factors that impact quality of life. Our aim was to characterize functional impairments in mildly and severely affected cachectic mice, and determine the suitability of these mice as a preclinical model. Metabolic abnormalities are also evident in cachectic patients and we investigated whether C-26-tumor-bearing mice had similar metabolic aberrations. Twelve-week-old CD2F1 mice received a subcutaneous injection of PBS (control) or C-26 tumor cells. After 18-20 days, assessments were made of grip strength, rotarod performance, locomotor activity, whole body metabolism, and contractile properties of tibialis anterior (TA) muscles (in situ) and diaphragm muscle strips (in vitro). Injection of C-26 cells reduced body and muscle mass, and epididymal fat mass. C-26-tumor-bearing mice exhibited lower grip strength and rotarod performance. Locomotor activity was impaired following C-26 injection, with reductions in movement distance, duration and speed compared with controls. TA muscles from C-26-tumor-bearing mice had lower maximum force (–27%) and were more susceptible to fatigue. Maximum specific (normalized) force of diaphragm muscle strips was reduced (–10%) with C-26 injection, and force during fatiguing stimulation was also lower. C-26-tumor-bearing mice had reduced carbohydrate oxidation and increased fat oxidation compared with controls. The range and consistency of functional and metabolic impairments in C-26-tumor-bearing mice confirm their suitability as a preclinical model for cancer cachexia. We recommend the use of these comprehensive functional assessments to maximize the translation of findings to more accurately identify effective treatments for cancer cachexia.

INTRODUCTION
Cancer cachexia is a multifactorial syndrome characterized by an ongoing loss of skeletal muscle mass with or without loss of fat mass that leads to progressive functional impairment (Fearon et al., 2011). Cachexia is present in up to 80% of patients with advanced cancer and in 60-80% of individuals diagnosed with gastrointestinal, pancreatic and lung cancers (Bruera, 1997). It decreases mobility, physical activity and functional independence, leading to an overall reduction in quality of life (Dahele et al., 2007; Fouladiun et al., 2007). Cachexia can increase the risk of post-surgical complications and impair responses to chemotherapy and other anti-neoplastic treatments (Murphy and Lynch, 2009). As a consequence, more than 20% of all cancer-related deaths are attributable to cachexia (Bruera, 1997). Treatments are needed urgently to improve patient quality of life and reduce mortality.

Although the best way to treat cancer cachexia is to cure the cancer, this is rarely achieved and, even when successful, it typically occurs after the cachexia has worsened in the interim (Murphy and Lynch, 2009). Studies have therefore concentrated on treating conditions secondary to the cancer but, despite many investigations in this area, there is still no FDA-approved treatment for cancer cachexia. A lack of standard and appropriate primary end points for preclinical studies is one reason for this lack of progress (Murphy and Lynch, 2009). Despite the main outcome of cancer cachexia that affects patient quality of life and mortality being skeletal muscle function, many studies have failed to include functional assessments as a primary end point, and clinical trials have been initiated without this crucial information (Murphy and Lynch, 2009). It is imperative that animal models for preclinical studies closely mimic the human condition in order to maximize the translation of findings.

Mice bearing colon-26 (C-26) tumors are a commonly used animal model of cancer cachexia because they demonstrate reductions in body, muscle and fat mass, as well as showing muscle fiber atrophy and increases in the expression of inflammatory genes and ubiquitin ligases associated with protein degradation (Bonetto et al., 2009; van Norren et al., 2009; Asp et al., 2010; Aulino et al., 2010; Tian et al., 2010). In addition to exhibiting a reduction in muscle mass, these mice should also demonstrate a loss of muscle strength, reduced levels of physical activity and increased muscle fatigue in order to be suitably representative of the clinical condition. Because loss of muscle strength impairs functional independence and loss of diaphragm function might be implicated in respiratory failure, it is important that studies evaluating the
therapeutic potential of an intervention for cancer cachexia include assessments of limb and diaphragm muscle function. Several studies have investigated the peak strength and fatigability of limb muscles from cachectic tumor-bearing mice but these have utilized in vitro muscle preparations that are often limited by inadequate perfusion and they are not subject to the systemic changes found in cachectic tumor-bearing mice (Gorselink et al., 2006; van Norren et al., 2009; Aulino et al., 2010). The relevance of this preparation to in vivo muscle contractions is therefore uncertain. In situ analyses of muscle function preserve normal perfusion, and the presence of an intact nerve and blood supply more closely resembles that of contracting muscles in vivo. However, no study to date has determined whether in situ function of limb muscle from C-26-tumor-bearing mice is actually impaired. Furthermore, it is also unknown whether the function of diaphragm muscles from C-26-tumor-bearing mice is impaired. Thus, the primary aim of this study was to characterize the functional impairments in the C-26 murine model of cancer cachexia, with specific emphasis on the functional impairments of limb muscle in situ and of diaphragm muscle strips. A secondary aim was to identify a battery of tests that comprehensively assessed whole body and skeletal muscle function to provide suitable reference for future studies investigating the efficacy of potential treatments for cancer cachexia.

Metabolic abnormalities are thought to be one of the main contributors to the pathogenesis of cancer cachexia (Tisdale, 2000). Compared with healthy controls, cachectic cancer patients have increased resting energy expenditure and fat oxidation, and reduced total energy expenditure and carbohydrate (CHO) oxidation (Hansell et al., 1986; Dahele et al., 2007). If similar metabolic changes are seen in C-26-tumor-bearing mice, then interventions can be tested in this model and results translated appropriately to human cancer patients at different stages of the condition. The third aim of this study was therefore to examine whole body metabolism in the C-26 murine model of cancer cachexia.

RESULTS

Tumor development and changes in body mass, muscle mass and muscle fiber size in C-26-tumor-bearing mice

Pair-fed (PF) control mice (injected with PBS alone and fed the same amount as eaten by the severely cachectic C-26-tumor-bearing group fed ad libitum) had a lower body mass compared with PBS control mice fed ad libitum (P<0.05; Fig. 1A). Despite a progressive increase in tumor size (Fig. 1C), there was no significant difference in relative body mass between mildly cachectic mice bearing C-26 tumors and PBS controls (Fig. 1A). Compared with PF controls and mildly cachectic tumor-bearing mice, severely cachectic C-26-tumor-bearing mice had a progressive reduction in body mass from day 10 (Fig. 1A) and a progressive increase in tumor size from day 9 (Fig. 1C). Over the 21-day period, severely cachectic C-26-tumor-bearing mice lost ~22% tumor-free body mass, whereas body mass of the other groups remained steady (Fig. 1B). Cumulative food intake was significantly lower from day 10 in PF controls compared with PBS mice fed ad libitum, and in severely cachectic C-26-tumor-bearing mice compared with mildly cachectic tumor-bearing mice (Fig. 1D).

In severely cachectic C-26-tumor-bearing mice, absolute mass of the extensor digitorum longus (EDL; ~21% versus PF and ~18% versus C-26 mild, P<0.001), tibialis anterior (TA, ~20% versus PF and ~13% versus C-26 mild, P<0.001), gastrocnemius (~19% versus PF and ~14% versus C-26 mild, P<0.001) and quadriceps (~21% versus PF and ~32% versus C-26 mild, P<0.05) muscles, as well as the heart (~11% versus PF and ~9% versus C-26 mild, P<0.001) and epididymal fat (~94% versus PF and ~95% versus C-26 mild, P<0.001), were reduced compared with PF controls and mildly cachectic tumor-bearing mice (Fig. 1E,F). There was no significant difference in muscle, heart or fat mass between PBS mice and either PF controls or mildly cachectic tumor-bearing mice (Fig. 1E,F).

When normalized for initial body mass, mass of the EDL (~20% versus PF and ~13% versus C-26 mild, P<0.03), TA (~19% versus PF and ~19% versus C-26 mild, P<0.01) quadriceps (~20% versus PF and ~27% versus C-26 mild, P<0.01) and epididymal fat (~94% versus PF and ~94% versus C-26 mild, P<0.001) were reduced in severely cachectic C-26-tumor-bearing mice compared with PF controls and mildly cachectic tumor-bearing mice (Table 1). Normalized mass of the gastrocnemius muscle (~18% versus PF, P<0.01) and heart (~15% versus PF, P<0.02) was only significantly different between PF controls and severely cachectic C-26-tumor-bearing mice (Table 1).

When normalized for final tumor-free body mass, mass of the epididymal fat (~93% versus PF and ~95% versus C-26 mild, P<0.001) remained lower but heart mass was higher (~15% versus PF and ~20% versus C-26 mild, P<0.001) in the severely cachectic C-26-tumor-bearing mice compared with PF controls and mildly cachectic tumor-bearing mice (data not shown). There was no difference between groups in the normalized mass of any of the muscles examined (data not shown).

Laminin staining of the sarcolemma to quantify TA muscle fiber cross-sectional area (CSA) revealed that median muscle fiber CSA was 7% smaller in PF controls compared with control mice fed ad libitum (Fig. 1G,H). Median muscle fiber CSA was 5% smaller in mildly cachectic C-26-tumor-bearing mice compared with PBS controls, and in severely cachectic C-26-tumor-bearing mice was 22% and 24% smaller than in PF controls and mildly cachectic tumor-bearing mice, respectively (Fig. 1G,H).

To further characterize the cachexia induced by the C-26 cells, expressions of the inflammatory gene IL-6 and the ubiquitin ligases atrogin-1 and MuRF-1 were assessed in TA muscles. IL-6 mRNA expression was 1.5-fold higher in mildly cachectic tumor-bearing mice compared with PBS controls, and in severely cachectic C-26-tumor-bearing mice was 3.6-fold and 1.0-fold higher than PF controls and mildly cachectic mice, respectively (Table 2). Atrogin-1 mRNA expression was 2.7-fold and 12.1-fold higher in severely cachectic C-26-tumor-bearing mice compared with PF controls and mildly cachectic mice, respectively (Table 2). Furthermore, MuRF-1 mRNA expression was 27.0-fold and 46.3-fold higher in severely cachectic C-26-tumor-bearing mice compared with PF controls and mildly cachectic mice, respectively (Table 2). There was no difference in IL-6, atrogin-1 and MuRF-1 mRNA expression between PBS controls fed ad libitum and PF controls (Table 2).

To assess whether the greater reductions in body and muscle mass in mice injected with C-26 cells causing severe cachexia compared with those injected with C-26 cells causing mild cachexia were simply due to the larger tumor size in the former, we examined body and muscle mass in a separate cohort of mice injected with the C-26 cells causing severe cachexia, as well as their PF controls, at 14 days post-inoculation (Fig. 2). At this time, tumor area (152±3
mm²) was similar to that at 21 days post-inoculation with the mild C-26 cells (149±15 mm²; compare Fig. 1C and Fig. 2A). At 14 days post-inoculation, tumor-bearing mice had a greater reduction in body mass compared with PF controls (P<0.02; Fig. 2B). They also had lower absolute mass of the plantaris (–21%, P<0.01), TA (–13%, P<0.01), gastrocnemius (–8%, P<0.02) and heart (–12%, P<0.01; Fig. 2C,D). When normalized for initial body mass, tumor-bearing mice had lower mass of the EDL (–11%, P<0.02), plantaris (–17%, P<0.01), TA (–10%, P<0.01) and heart (–8%, P<0.02; Fig. 2E,F).

Whole body strength and mobility in C-26-tumor-bearing mice
Mildly cachectic C-26-tumor-bearing mice had lower absolute grip strength (–22%) and grip strength normalized for body mass (–23%) compared with PBS controls (P<0.05; Fig. 3A). Absolute grip strength (–22%) and normalized grip strength (–23%) was also lower in severely cachectic C-26-tumor-bearing mice compared with PF controls (P<0.05; Fig. 3A). Latency-to-fall during the rotarod test was 55% lower in severely cachectic C-26-tumor-bearing mice compared with PF controls (P<0.05; Fig. 3C). There were no differences in grip strength or latency-to-fall during the rotarod test between PBS and PF controls, or between mildly cachectic and severely cachectic C-26-tumor-bearing mice (Fig. 3).

Locomotor activity in C-26-tumor-bearing mice
PF controls moved a greater distance than PBS control mice (fed ad libitum) when the light and dark cycles were combined, but there were no other differences between groups in locomotor activity (Fig. 4). There were also no differences in locomotor activity between control mice fed ad libitum and mildly cachectic mice. Severely cachectic C-26-tumor-bearing mice demonstrated...
Reduced movement distance and duration during 6 hours of the dark cycle and for the total of the light and dark cycles compared with PF controls (P < 0.05; Fig. 4A,B). As a consequence, average movement speed was lower in severely cachectic C-26-tumor-bearing mice compared with PF controls (P < 0.05; Fig. 4C). Severely cachectic C-26-tumor-bearing mice also rested for longer than PF controls (+9% for dark cycle and +5% for total light and dark cycles, P < 0.05; Fig. 4D). Compared with mildly cachectic mice, severely cachectic mice had increased movement distance and duration during the light cycle but reduced movement duration during the dark cycle (P < 0.05; Fig. 4A,B). Severely cachectic mice also had slower average movement speed and rested for longer during the dark cycle compared with mildly cachectic mice (P < 0.05; Fig. 4C,D).

Whole body metabolism in C-26-tumor-bearing mice

Average oxygen consumption (VO₂) and carbon dioxide production (VCO₂) and respiratory exchange ratio (RER) were higher during all cycles in PF controls compared with control mice fed ad libitum (Table 3). Absolute CHO oxidation was higher during the dark cycle, and fat oxidation was lower during all cycles in PF mice compared with PBS controls (Fig. 5A,B). Absolute energy expenditure was not different between control mice, but energy expenditure normalized for body mass was higher during all cycles in PF mice (Fig. 5C,D). Mildly cachectic C-26-tumor-bearing mice had higher average VO₂ and VCO₂ during the dark cycle compared with PBS controls (Table 3). They also had lower absolute CHO oxidation during the light cycle and average of the light and dark cycles, and lower absolute fat oxidation during the light cycle compared with PBS controls (Fig. 5A,B). CHO oxidation expressed as a percentage of total energy expenditure was lower during all cycles in mildly cachectic C-26-tumor-bearing mice compared with PBS controls (Fig. 5E,F). Average VO₂, VCO₂ and RER were lower during all cycles in severely cachectic C-26-tumor-bearing mice compared with PF controls (Table 3). Absolute CHO oxidation was lower during all cycles in severely cachectic C-26-tumor-bearing mice compared with PF controls (Fig. 5A), whereas absolute fat oxidation was only significantly higher in severely cachectic C-26-tumor-bearing mice in the dark cycle (Fig. 5B). Both absolute energy expenditure and energy expenditure normalized for body mass were lower in severely cachectic C-26-tumor-bearing mice during the dark cycle and when the light and dark cycles were averaged, compared with PBS controls (P < 0.05; Fig. 5C,D). CHO oxidation expressed as a percentage of total energy expenditure was lower during all cycles in severely cachectic C-26-tumor-bearing mice compared with PF controls (P < 0.01; Fig. 5E). Conversely, fat oxidation expressed as a percentage of total energy expenditure was higher during all cycles in severely cachectic C-26-tumor-bearing mice (P < 0.01; Fig. 5F).

There were no differences in VO₂ and VCO₂ between mildly cachectic and severely cachectic C-26-tumor-bearing mice, but RER during the light cycle was significantly higher in severely cachectic mice (Table 3). Absolute and normalized energy expenditure, as well as absolute CHO oxidation, were similar between mildly cachectic and severely cachectic tumor-bearing mice (Fig. 5). Fat oxidation during the light cycle only was higher in severely cachectic C-26-tumor-bearing mice compared with mildly cachectic mice (Fig. 5B). CHO oxidation and fat oxidation expressed as a percentage of total energy expenditure were not significantly different between mildly and severely cachectic tumor-bearing mice (Fig. 5E,F).

Strength and fatigability of TA muscles in C-26-tumor-bearing mice

There was no statistically significant difference between groups in peak twitch force, time to peak twitch tension (TPT), one-half

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### Table 1. Normalized muscle, heart and epididymal fat mass analysis

| Tissue  | PBS         | PBS PF       | C-26 mild | C-26 severe |
|---------|-------------|--------------|-----------|-------------|
| Soleus  | 0.23±0.01   | 0.22±0.01    | 0.20±0.02 | 0.24±0.02   |
| EDL     | 0.37±0.01   | 0.39±0.01    | 0.36±0.03 | 0.31±0.01\,d |
| Plant   | 0.55±0.02   | 0.58±0.02    | 0.51±0.02 | 0.53±0.04   |
| TA      | 1.85±0.09   | 1.77±0.04    | 1.76±0.08 | 1.43±0.05\,d |
| Gastroc | 4.51±0.10   | 4.40±0.11    | 3.93±0.47 | 3.61±0.13\,d |
| Quad    | 6.57±0.14   | 5.70±0.34    | 6.26±0.14 | 4.56±0.27\,d |
| Heart   | 4.48±0.16   | 4.45±0.11    | 4.17±0.11 | 3.80±0.23\,d |
| Fat     | 16.85±1.01  | 15.59±2.14   | 15.82±3.59 | 0.96±0.64\,d |

*Muscle, heart and epididymal fat mass normalized for initial body mass of CD2F1 mice injected subcutaneously with C-26 cells inducing mild or severe cachexia, or with PBS and fed ad libitum (PBS) or pair-fed to the severe C-26 tumor-bearing mice (PBS PF). Values are expressed as mg/g initial body mass. EDL, extensor digitorum longus; Plant, plantaris; TA, tibialis anterior; Gastroc, gastrocnemius; Quad, quadriceps. Data are means ± s.e.m.; n = 6 for PBS; 14 for PBS PF; 6 for C-26 mild and 15 for C-26 severe. *P < 0.05 PBS versus C-26 severe; **P < 0.05 PBS PF versus C-26 severe.*

### Table 2. Gene expression of IL-6, atrogin-1 and MuRF-1 in TA muscles

| Gene  | PBS         | PBS PF       | C-26 mild | C-26 severe |
|-------|-------------|--------------|-----------|-------------|
| IL-6 mRNA | 0.03±0.00   | 0.03±0.00    | 0.07±0.02\,c | 0.13±0.01\,c,d |
| Atrogin-1 mRNA | 3.8±1.1     | 11.6±4.5     | 3.3±1.1   | 42.6±7.9\,d |
| MuRF-1 mRNA | 54.6±21.4   | 110.4±34.5   | 65.5±9.3 | 3095±585.7\,d |

*Gene expression of the inflammatory marker IL-6 and the ubiquitin ligases atrogin-1 and MuRF-1 in TA muscles from CD2F1 mice injected subcutaneously with C-26 cells inducing mild or severe cachexia, or with PBS and fed ad libitum (PBS) or pair-fed to the severe C-26 tumor-bearing mice (PBS PF). Values are expressed as mRNA/cDNA content (2^{-ΔΔCT}/ng/ml, x 10^{-3}). Data are means ± s.e.m.; n = 6 for PBS; 5 for PBS PF; 6 for C-26 mild and 6 for C-26 severe. *P < 0.05 PBS versus C-26 mild; **P < 0.05 PBS PF versus C-26 severe; ***P < 0.05 C-26 mild versus C-26 severe.*
relaxation time (1/2RT) or maximum rate of force development during a twitch (dP/dt) of TA muscles in situ (data not shown). The frequency-force relationship revealed that TA muscles from mildly cachectic C-26-tumor-bearing mice produced lower forces than PBS controls (P<0.001; Fig. 6C). TA muscles from severely cachectic C-26-tumor-bearing mice produced lower forces than both PF controls and mildly cachectic tumor-bearing mice (P<0.001; Fig. 6C). There were no differences in forces between PBS controls and PF mice (Fig. 6C). Peak tetanic force was 27% lower in TA muscles from severely cachectic C-26-tumor-bearing mice compared with PF controls (Fig. 6A), but there were no differences between groups in specific (normalized) force (Fig. 6B). A 4-minute intermittent fatiguing stimulation protocol revealed a group main effect for PBS mice fed ad libitum to produce lower forces than PF controls (Fig. 6D). Forces during intermittent fatiguing stimulation were also lower in severely cachectic C-26-tumor-bearing mice compared with PBS controls and mildly cachectic tumor-bearing mice (Fig. 6D).

**Correlations**
A summary of the progression and consequences of functional and metabolic impairments in C-26-tumor-bearing mice is presented in Fig. 8. When results for all groups were pooled (n=23), there was a significant positive correlation between CHO oxidation (% total energy expenditure) and grip strength when expressed in both absolute (r=0.42, P<0.05) and relative (normalized) values (r=0.52, P<0.02). There was a significant negative correlation between atrogin-1 mRNA expression and the percentage change in tumor-free body mass (r=-0.72, P<0.0001), TA muscle mass (r=0.54, P<0.01), epididymal fat mass (r=-0.72, P<0.001), heart mass (r=0.46, P<0.03) and average locomotor speed (r=0.47, P<0.04). There was also a...
significant negative correlation between MuRF-1 mRNA expression and the percentage change in tumor-free body mass ($r=0.74$, $P<0.0001$), TA muscle mass ($r=0.45$, $P<0.04$), epididymal fat mass ($r=0.76$, $P<0.001$) and average locomotor speed ($r=0.54$, $P<0.02$). A significant positive correlation was found between latency-to-fall during a rotarod test and both atrogin-1 mRNA expression ($r=0.47$, $P<0.03$) and MuRF-1 mRNA expression ($r=0.54$, $P<0.01$).

**DISCUSSION**

We characterized the functional and metabolic changes in mice bearing C-26 tumors to determine their suitability as a preclinical model for cancer cachexia. Severely cachectic C-26-tumor-bearing mice exhibited reductions in whole body strength and mobility, levels of physical activity, and both reductions in maximum strength and increased fatigability of TA muscles and diaphragm muscle strips. The mice exhibited increased fat oxidation, and reductions in carbohydrate oxidation and total energy expenditure. Comparison of these findings with those obtained in mildly cachectic C-26-tumor-bearing mice indicate that the cachexia per se accounts for the reductions in mobility, levels of physical activity, and maximum strength of TA muscles and diaphragm muscle strips. These functional and metabolic impairments are similar to those observed in patients with cancer cachexia and therefore verify the suitability of this model for preclinical studies.

**Standard approaches to assess whole body and skeletal muscle function in murine models of cancer cachexia**

One of the bottlenecks limiting development of effective treatments for cancer cachexia has been a lack of consensus regarding primary endpoint measures for the assessment of muscle function in preclinical models (Murphy and Lynch, 2009). Patients with cancer cachexia exhibit functional impairments, including reductions in whole body strength and mobility, and in levels of physical activity (Guo et al., 1996; Dahele et al., 2007). They also experience severe fatigue (Curt et al., 2000; Ahlberg et al., 2003). Specifically, loss of function of limb muscles impairs functional independence and loss of diaphragm function is implicated in respiratory failure (Murphy et al., 2011). Here we describe a battery of tests performed in murine models of cancer cachexia that comprehensively assess the same functional impairments experienced by cachectic patients. These tests include: grip strength as an assessment of whole body strength; rotarod performance as a general assessment of whole body mobility; locomotor activity to assess levels of physical activity; contractile properties of TA muscles in situ to assess maximum strength and fatigability of limb muscles; and contractile properties of diaphragm muscle strips in vitro to assess maximum strength and fatigability of respiratory muscles. Adoption of these standard approaches of assessment will facilitate more accurate comparisons of data generated by different laboratories and assist with the development and optimization of preclinical treatments for translation into clinical therapies (Grounds et al., 2008).

**Assessment of cachexia in our model of C-26-tumor-bearing mice**

Because different sources of C-26 cell lines and implantation by different laboratories can produce varying degrees of cachexia, it was important that we initially characterized the cachexia induced with our two C-26 cell lines. We found that injection of the C-26 cells obtained from the NCI Frederick Cancer DCT Tumor Repository induced very mild cachexia, with no significant change in tumor-free body mass, a small (5%) decrease in median fiber CSA, a small increase in IL-6 mRNA but not in atrogin-1 or MuRF-1 mRNA, and no change in food intake. Conversely, injection with C-26 cells kindly donated by Martha Belury (The Ohio State University, Columbus, OH) induced anorexia and severe cachexia, with a large loss of tumor-free body mass, muscle, heart and fat mass (absolute and normalized for initial body mass), muscle fiber atrophy, and a large increase in mRNA expression of IL-6, atrogin-1 and MuRF-1. These findings were not due to differences in tumor size, because body and muscle mass was still lower in mice bearing C-26 cells inducing cachexia compared with PF control mice at 14 days post-inoculation, when tumor size was similar to that in mice injected with C-26 cells inducing only mild cachexia. These findings...
were also not due to anorexia because there were no significant differences in most of these parameters between PF control mice and control mice fed ad libitum. These results are consistent with previous studies using the same source of C-26 cells (Asp et al., 2010; Tian et al., 2010) and also with studies using a different source of C-26 cells (van Norren et al., 2009; Benny Klimek et al., 2010; Bonetto et al., 2011). We did not assess insulin tolerance in our mice because we have found that the measurement compromises the ability of the severely cachectic mice to successfully complete subsequent functional analyses. However, others using the same source of C-26 cells have demonstrated insulin resistance in severely cachectic tumor-bearing mice (Asp et al., 2010).

Functional impairments in cachetic C-26-tumor-bearing mice
Hand grip strength is reduced by more than 25% in patients with cancer cachexia (Guo et al., 1996). In addition to affecting the ability to perform even the simplest tasks such as rising from a chair, cooking or maintaining personal hygiene, reductions in grip strength in cancer patients is strongly correlated with postoperative complications (Guo et al., 1996). In the current study, we found that grip strength was reduced by 22% in severely C-26-tumor-bearing mice compared with PF controls. This impairment is remarkably similar to that in cachectic cancer patients. A similar reduction in grip strength was seen in mildly cachectic tumor-bearing mice, indicating that the tumor rather than cachexia per se was responsible for the reduction in grip strength.

Patients suffering from cancer cachexia have reduced mobility and levels of physical activity, taking up to 43% fewer steps per day compared with healthy controls (Dahele et al., 2007; Fouladiun et al., 2007). As well as reducing functional independence, the reduction in physical activity levels has been negatively correlated with weight loss (Fouladiun et al., 2007). We found that severely cachectic C-26-tumor-bearing mice exhibited a 55% reduction in

Table 3. Selected metabolic parameters from CD2F1 mice injected subcutaneously with C-26 cells inducing mild or severe cachexia, or with PBS and fed ad libitum (PBS) or pair-fed to the severe C-26-tumor-bearing mice (PBS PF).

| Time       | PBS       | PBS PF    | C-26 mild | C-26 severe |
|------------|-----------|-----------|-----------|-------------|
| Light      | 60.3±1.6  | 79.0±2.5* | 65.2±1.4  | 63.0±6.5*   |
| Dark       | 64.6±4.2  | 83.0±3.0* | 77.6±2.9  | 63.9±6.0*   |
| Average    | 62.5±2.8  | 81.0±2.7* | 71.4±1.4  | 63.5±6.1*   |
| Dark       | 42.9±1.6  | 68.9±2.2* | 46.8±1.7  | 51.5±6.3*   |
| Dark       | 49.1±5.2  | 73.2±2.7* | 62.4±2.0  | 51.7±5.9*   |
| Average    | 46.0±3.3  | 71.0±2.4* | 54.6±0.8  | 51.6±5.8*   |
mobility and a 34-78% reduction in locomotor activity compared with PF controls. Interestingly, the reductions in locomotor activity were significantly different from controls in the dark cycle, when mice are most active, but not in the light cycle. These findings are consistent with a previous study reporting reductions in physical activity levels in C-26-tumor-bearing mice compared with controls in the dark but not the light period (van Norren et al., 2009). Because mobility and locomotor activity were not different between mildly cachectic C-26-tumor-bearing mice and PBS controls, it seems that the impairments in the severely cachectic tumor-bearing mice were due to the cachexia per se.

Cancer cachexia is associated with an impaired functional capacity of the limb muscles, with patients exhibiting a 33-40% lower isometric and isokinetic strength of the quadriceps muscles compared with healthy controls (Weber et al., 2009). Impaired functional capacity of the limb muscles reduces the ability of affected individuals to perform the activities of daily living and might lead to a loss of independence and premature retirement (Murphy and Lynch, 2009; Murphy and Lynch, 2012). Previous studies have reported lower maximal tetanic forces and lower forces during fatiguing stimulation in EDL muscles in vitro from C-26-tumor-bearing mice than controls (Gorselink et al., 2006; van Norren et al., 2009). Our study assessed contractile properties of the TA muscle in situ because this allows the nerve and blood supply of the muscle to remain intact, facilitating analysis in a setting that closely resembles in vivo muscle contraction (Grounds et al., 2008). Consistent with the results for the EDL muscle in vitro, we found that TA muscles assessed in situ from severely cachectic C-26-tumor-bearing mice exhibited lower submaximal and maximal tetanic forces, and lower forces during intermittent fatiguing stimulation, compared with PF controls. Because specific force (normalized to muscle CSA) was not different between groups, the reduction in muscle mass is the main mechanism responsible for the reduction in force. Using a technique closely mimicking in vivo contractions to assess muscle function, we found that severely cachectic C-26-tumor-bearing mice exhibited reductions in strength and increases in fatigability of their limb muscles, similar to that reported for cachectic cancer patients (Weber et al., 2009).

One of the major causes of death in cancer cachexia is respiratory muscle failure (Tan and Fearon, 2008) and it is therefore important to determine whether respiratory muscle function is also impaired in the C-26 model of cancer cachexia. Diaphragm muscle strips from severely cachectic C-26-tumor-bearing mice produced lower submaximal and maximal specific (normalized) forces, and forces during fatiguing electrical stimulation were also lower, compared...
with PF controls. The impairments in respiratory muscle function in the severely cachectic C-26 model of cancer cachexia are therefore analogous to those seen in cachectic cancer patients. Maximal tetanic force of TA muscles and diaphragm muscle strips from mildly cachectic C-26-tumor-bearing mice was similar to controls, indicating that cachexia was responsible for the impairments seen in the severely cachectic tumor-bearing mice. By contrast, both severely cachectic and mildly cachectic mice had lower forces during fatiguing stimulation than controls, suggesting that the tumor rather than cachexia was responsible for the decrement in force.

Metabolic impairments in cachectic C-26-tumor-bearing mice

Patients with cancer cachexia demonstrate metabolic abnormalities that are thought to contribute to the pathogenesis of the condition (Tisdale, 2000). Specifically, cachectic cancer patients exhibit increased resting energy expenditure (+19%) but reduced total energy expenditure (–8%), lower oxygen uptake (–24%) and CHO oxidation (–25%), and higher fat oxidation (+27%) compared with healthy controls (Hansell et al., 1986; Dahele et al., 2007; Travers et al., 2008). Much of the increase in resting energy expenditure is due to the energy demands of the tumor, whereas the decrease in total energy intake reflects, at least in part, reduced physical activity levels (Dahele et al., 2007). Similar to cachectic cancer patients, we found that severely cachectic C-26-tumor-bearing mice exhibited reductions in total energy expenditure, oxygen uptake and CHO oxidation, and increases in fat oxidation, compared with PF controls. Consistent with the reduction in CHO oxidation and increase in fat oxidation, the RER was lower in severely cachectic C-26-tumor-bearing mice. The changes in substrate oxidation support a previous finding of insulin resistance in C-26-tumor-bearing mice (Asp et al., 2010). Insulin resistance is also present in patients with cancer cachexia and is thought to be one of the main contributors to the development of cachexia in this population (Yoshikawa et al., 1999). Mildly cachectic C-26-tumor-bearing mice did not exhibit impairments in VO2 and VCO2 compared with PBS controls, but they had a reduced CHO oxidation and increased fat oxidation. These findings suggest that cachexia per se was responsible for the reduction in VO2 and VCO2 in the severely cachectic mice, whereas the tumor was responsible for the alterations in substrate oxidation. Severely cachectic C-26-tumor-bearing mice exhibited metabolic abnormalities similar to the clinical condition and thus represent a suitable model for testing therapeutic interventions for these metabolic abnormalities in cancer cachexia.

Progression and consequences of functional and metabolic impairments in C-26-tumor-bearing mice

As summarized in Fig. 8, changes occurring during early cachexia in C-26-tumor-bearing mice included increased inflammation, metabolic alterations, enhanced muscle fatigability and reduced grip strength. Correlation analyses supported a significant relationship between CHO oxidation and whole body strength. The enhanced fatigability and reductions in strength could reduce functional independence, necessitating a change in work duties and an impairment in overall quality of life. Changes occurring later in the progression of cachexia included anorexia, increased mRNA expression of the ubiquitin ligases atrogin-1 and MuRF-1, reductions in muscle, heart and fat mass, weight loss, decreased strength of limb muscles and respiratory muscles, and impairments in oxygen uptake, mobility and average locomotor speed. Most of these alterations were significantly correlated with atrogin-1 and MuRF-1 mRNA expression, supporting a major role of the ubiquitin ligases in the pathogenesis of cancer cachexia (Glass, 2010). These changes could increase the risk of falls and fall-related injuries, reduce independence, impair quality of life and, in extreme cases, cause death.
Conclusions

The characterization of functional and metabolic impairments in C-26-tumor-bearing mice confirms their suitability as a preclinical model for cancer cachexia. Future investigations using the C-26 murine model to assess the efficacy of potential treatments for cancer cachexia should include comprehensive evaluations of functional and metabolic parameters to appropriately translate relevant findings to the clinical condition and identify better treatments for cancer cachexia. The use of mildly and severely cachectic C-26-tumor-bearing mice is useful for delineating the effects between cachexia and the tumor.

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). Twelve-week-old male CD2F1 mice were allocated randomly into one of four experimental groups: a mildly cachectic C-26-tumor-bearing group ($n=6$), a severely cachectic C-26-tumor-bearing group ($n=15$), a control group injected with PBS alone and fed ad libitum ($n=6$), and a control group injected with PBS alone and pair-fed to the severely cachectic C-26-tumor-bearing group (PF; $n=15$) to account for the reduction in food intake due to anorexia. Pair-feeding was conducted to ensure that any functional and/or metabolic impairments in severely cachectic C-26-tumor-bearing mice were not simply due to a reduction in caloric intake.

A separate cohort of severely cachectic C-26-tumor-bearing mice (C-26 severe 14d; $n=8$) and a control group injected with PBS and pair-fed to the severely cachectic C-26-tumor-bearing group (PBS PF 14d; $n=14$) were used to examine tissue mass at a time when tumor size is similar to that at 21 days post-injection of C-26 cells inducing mild cachexia. All 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-hour light-dark cycle. Water was available ad libitum, and standard laboratory chow was provided, changed and monitored daily. The amount of food consumed per mouse per day was determined and expressed as cumulative food intake.
C-26 cell line and inoculation
Frozen C-26 cells inducing mild cachexia were kindly donated by Paul Gregorevic (Baker IDI, Melbourne, Australia) via the NCI Frederick Cancer DCT Tumor Repository (Frederick, MD), and frozen C-26 cells inducing severe cachexia were kindly donated by Martha Belury (The Ohio State University, Columbus, OH). Frozen cells were thawed rapidly in a 37°C water bath and transferred to a 100-mm culture plate (Corning, Corning, NY) containing growth media consisting of RPMI Medium 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% (vol/vol) FBS (Invitrogen), 1% L-glutamine (Invitrogen) and 1% penicillin/streptomycin (Invitrogen), and incubated at 37°C with 5% CO₂. Cells were maintained in growth media and passaged when 70-80% confluent. Before the injection of C-26 cells into mice (day 1), cells were counted using a hemocytometer (Bright Line, Hauser Scientific, Horsham, PA), pelleted via centrifugation (1600 g for 5 minutes at 25°C) and resuspended at 5×10⁶ cells/ml of sterilized PBS. All mice were anesthetized via an intraperitoneal (i.p.) injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg; 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 injection of either 5×10⁵ C-26 cells suspended in 100 μl of sterilized PBS, or 100 μl of sterilized PBS only (control). Mice recovered from anesthesia on a heat pad and were given an i.p. injection of atipamezole (Antisedan; 1 mg/kg body weight; VM Supplies, Melbourne, VIC, Australia), such that they were unresponsive to tactile stimuli. Mice were still anesthetized deeply.

Metabolism and locomotor activity
Whole body mobility and coordination was assessed on day 19 by rotarod performance (Rotamex-5, Columbus Instruments). Following a 15-minute acclimatization in the test room, mice were placed on the rod, which was rotating at an initial speed of 4 rpm. The speed was increased gradually by 1 rpm every 8 seconds and latency-to-fall on to a soft pad was recorded. The test was repeated twice more, with 15 minutes between tests. Average latency-to-fall was calculated over the three trials.

Skeletal muscle histology
Serial sections (5 μm) were cut transversely through the TA muscle using a refrigerated (~20°C) cryostat (CTI Cryostat; IEC, Needham Heights, MA). Sections were reacted for laminin for determination of median myofiber CSA (Murphy et al., 2010b). Digital images of stained sections were obtained using an upright microscope with camera (Axio Imager D1, Carl Zeiss, Wrek, Göttingen, Germany), controlled by AxioVision AC software (AxioVision AC Rel. 4.7, Carl Zeiss, Germany).
Genetic analysis was performed as described previously (Murphy et al., 2010a). The cDNA stored at –20°C for subsequent analysis. Real-time RT-PCR determined spectrophotometrically at 260 nm, and the samples (PureLink RNA Mini Kit, Invitrogen). RNA concentration was commercially available kit, according to the manufacturer's instructions. Total RNA was extracted from 10-20 mg of TA muscle using a commercially available kit. Images were quantified using AxiolabVision 4.7 software.

**Real-time RT-PCR analyses**

Total RNA was extracted from 10-20 mg of TA muscle using a commercially available kit, according to the manufacturer's instructions (PureLink RNA Mini Kit, Invitrogen). RNA concentration was determined spectrophotometrically at 260 nm, and the samples were stored at –80°C. RNA was transcribed into cDNA using the Invitrogen SuperScript Vilo cDNA Synthesis Kit, and the resulting cDNA stored at –20°C for subsequent analysis. Real-time RT-PCR was performed as described previously (Murphy et al., 2010a). The forward and reverse primer sequences used were: 5'-CCG-GAGGAGGACCTCAGC-3' and 5'-TCCACGATTTCCCCAGAGAC-3'; atrogin-1, 5'-GGTGGGCGACGCGGAAG-3' and 5'-TTGCCAGAGAAGGCCTATG-3'; and MuRF-1, 5'-AGGTGTCAGCAGGAAGCAGT-3' and 5'-CCTCCTTTGTCCCTCTTGCTG-3', respectively. The content of single-stranded DNA (ssDNA) in each sample was determined using the Quanti-IT OligoGreen ssDNA Assay Kit (Molecular Probes, Eugene, OR), as described previously (Lundby et al., 2005; Murphy et al., 2010a).

**Statistical analyses**

All values are expressed as mean ± s.e.m., unless stated otherwise. Groups were compared using a one-way or two-way ANOVA, where appropriate. Bonferroni’s post hoc test was used to determine significant differences between individual groups. Correlations were determined by least squares linear regression. The level of significance was set at P<0.05 for all comparisons. Muscle fiber CSA was not normally distributed (Anderson Darling Normality test), and so data for CSA were presented as 95% confidence intervals of the median. Differences were considered significant when no overlap existed between the 95% confidence interval of the median (Sim and Reid, 1999).

**Implications and future directions**

These data indicate that the functional and metabolic impairments exhibited by C-26-tumor-bearing mice mimic those in patients with the condition, highlighting their suitability as an appropriate animal model of cancer cachexia. Mildly and severely cachectic C-26-tumor-bearing mice can be used to delineate the relationship between cachexia and tumors, and for studying different stages of cancer cachexia. Preclinical studies using these mice should include comprehensive assessments of whole-body and skeletal muscle function and metabolism. Employing these standard and clinically relevant endpoints will enhance translation and help to identify therapeutic interventions for cancer cachexia that will improve patient quality of life and reduce mortality.

**Competing interests**

The authors declare that they do not have any competing or financial interests.

**ACKNOWLEDGEMENTS**

We thank Martha Belury (Department of Human Nutrition, The Ohio State University) for kindly donating the C-26 cells and Donna McCarthy (College of Nursing, The Ohio State University) for arranging the shipment of these cells. We thank Graham R. Robertson (ANZAC Research Institute, University of Sydney) for arranging animal availability. We thank René Koopman (Department of Physiology, The University of Melbourne) for assisting with the setup of the Open Field Metabolic Chamber.

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