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REVIEW

Preclinical and clinical studies on cancer-associated cachexia

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BACKGROUND: Cancer cachexia is the wasting condition that is often seen in advanced stage cancer patients. This wasting is largely attributable to a systemic and progressive loss of skeletal muscle mass that greatly hinders performance of normal daily activities, resulting in reduced quality of life. Moreover, it negatively influences the prognosis of cancer patients. A general consensus in the field is that the loss of muscle mass is due both to an increase in protein degradation and a decrease in protein synthesis. Recent studies using preclinical models for studying cachexia have been useful in identifying the contribution of inflammatory cytokines (e.g. tumor necrosis factor-α and Interleukin-6), and myostatin receptors (e.g. the type IIB activin receptor) to cachexia development, and have led to several clinical trials. However, many questions remain about the molecular mechanisms thought to play a role in the development of cachexia.
METHODS: We conducted a literature search using search engines, such as PubMed and Google Scholar to identify publications within the cancer cachexia field.
RESULTS: We summarized our current knowledge of: 1) the driving mechanisms of cancer cachexia, 2) the preclinical models available for studying the condition, and 3) the findings of recent clinical trials.
CONCLUSION: Cancer cachexia is a complex and variable condition that currently has no standard effective therapeutic treatment. Further studies are desperately needed to better understand this condition and develop effective combination treatments for patients.

Keywords cancer cachexia, muscle wasting, bodyweight loss, metabolic changes, increased protein degradation, decreased protein synthesis

Introduction

The term ‘cachexia’ has Greek roots that translate to ‘bad condition’. Descriptions of the condition date back to Hippocrates, around 460 BC (Bennani-Baiti and Walsh, 2009, Lok, 2015). This condition arises frequently with many chronic illnesses, including chronic obstructive pulmonary disorder, chronic kidney disease, and the many forms of cancer (Lok, 2015). Historically, cachexia was characterized by the pale and wasted appearance of patients, but until recently, there were no formal set criterion for its clinical diagnosis (Bennani-Baiti and Walsh, 2009). In 2011, an international consensus was reached to formally establish diagnostic criterion of cancer cachexia for each of its three stages (Table 1). In the first stage, patients with precachexia have less than 5% premorbid bodyweight loss, but display signs of anorexia and/or metabolic changes. Patients that have progressed to the second stage, cachexia, have weight loss greater than 5% of the their premorbid bodyweight, or alternatively weight loss greater than 2% with signs of sarcopenia or a body mass index under 20. Cachexia patients in this stage are likely to also have systemic inflammation, which may be indicated by high levels of C-reactive protein (CRP) in the serum (Penafuerte et al., 2016). Cachexic patients in the final stage, refractory cachexia, have a life expectancy of less than three months, and their cancer is no longer responsive to treatment (Fearon et al., 2011).

Cachexia is the result of progressive muscle wasting and impaired muscle function. Although one defining character-

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Table 1 Established stages of cancer cachexia.

	Precachexia	Cachexia	Refractory cachexia
Weight loss [a]	$< 5\%$	$> 5\%$	$> 5\%$
Other symptoms	Altered metabolism	Inflammation	Cancer unresponsive to treatment

[a] When Body mass index (BMI) is > 20 and there are no signs of sarcopenia related muscle atrophy.

istic of cachexia is weight loss, it differs significantly from illnesses such as anorexia (Table 2). Unlike anorexia, where fat is the main source of initial weight loss, skeletal muscle wasting is a key feature of cachexia occurring early on in the condition (Mueller et al., 2016). Additionally, changes in diet and an increase in nutrient intake do not consistently result in weight gain and increased muscle function in cachexic patients (Onesti and Guttridge, 2014, Lok, 2015).

Table 2 Differences between cachexia and anorexia.

	Cachexia	Anorexia
Classification	Metabolic disorder	Eating disorder
Initially present with	Loss of skeletal muscle mass Possible loss of fat	Loss of adipose tissue No loss of muscle mass
Nutritional supplementation	Does not reverse condition	Reverses condition

Cancer-associated cachexia affects roughly 50% of all cancer patients regardless of their cancer type (Onesti and Guttridge, 2014, Lok, 2015, Fukawa et al., 2016). This percentage increases to about 80% when the cancer is classified as advanced (Onesti and Guttridge, 2014, Lok, 2015, Fukawa et al., 2016). The prevalence of patients developing cachexia does vary between cancer types, but is most common in pancreatic and gastrointestinal cancer patients. For example, roughly 80% of pancreatic or gastrointestinal cancer patients will be classified as cachexic at some point throughout the course of their disease, while a slightly smaller percentage (50-60%) of prostate and lung cancer patients will develop cachexia (Del Ferraro et al., 2012; Argilés et al., 2014). Problematically, cancer patients displaying signs of cachexia are faced with a worsened prognosis and decreased quality of life (Al-Majid and Waters, 2008, Onesti and Guttridge, 2014). However, whether there is a correlation between tumor burden and the development of cachexia is still subject to debate in the field (Consul et al., 2016, Petruzzelli and Wagner, 2016, Shiono et al., 2016). As muscle wasting progresses, patient fatigue and weakness also grow. Performing normal daily activities becomes a constant struggle for cachexia patients. In addition to this, cachexia makes it substantially more difficult to treat their cancer, since chemotherapy treatments are often too toxic for cachexic patients to endure, and major surgeries are too high risk for their weakened state (Lok, 2015). To date, roughly one third of all cancer related deaths are being attributed to cachexia (Onesti and Guttridge, 2014). Moreover, there is no definitive treatment or cure for cachexia. While ongoing research strives to find an effective therapeutic for cachexia patients and much progress has been made in the field, the molecular mechanisms behind its development are not completely understood, and findings from the models used to study cachexia do not always apply to humans.

In this review, we therefore provide a concise overview of cancer cachexia, encompassing: key molecular mechanisms involved in its development, frequently used preclinical models for its study, and the current standing of recent clinical trials.

Molecular mechanisms contributing to the development of cancer cachexia

Although muscle wasting in cancer cachexia results from the volatile combination of increased protein degradation and decreased protein synthesis within the skeletal muscle, the exact molecular mechanisms that affect both of these processes have yet to be completely discerned. Systemic inflammation has been widely implicated for its role in the development of the cachexic condition, although the systemic levels of inflammatory cytokines have not been consistently correlated with cachexia development in humans (Maltoni et al., 1997; Fearon et al., 2011; Suzuki et al., 2013). Cytokines of the innate immune system such as tumor necrosis factor (TNF)-α and Interleukin 6 (IL-6) have been previously connected to increased muscle deterioration and fat store depletion, which was interpreted as cachexia development in rodent models (Narsale and Carson, 2014; Onesti and Guttridge, 2014; Mueller et al., 2016). These inflammatory cytokines increase activity of the ubiquitin proteasome system which has been suggested to have the most critical role in the breakdown of skeletal muscle proteins contributing to cachexia, and has also been implicated in the cardiac atrophy that can occur with cancer cachexia (Al-Majid and Waters, 2008; Onesti and Guttridge, 2014; Bilodeau et al., 2016; Porporato, 2016). The normal function of the ubiquitin proteasome system is to breakdown poly-ubiquitin tagged protein substrates. To briefly summarize the process, enzymes E1, E2, and E3, the latter of which controls specificity of the targeted substrate, target a specific substrate to tag with a polyubiquitin chain. The ubiquitinated protein is then degraded by the proteasome (Pagan et al., 2013). TNF-α signaling through IκB kinase (IKK) activates the nuclear transcription factor-κB (NFκB) pathway to increase transcription of the ubiquitin proteasome genes, muscle RING-finger protein-1 (MuRF1), which codes for an E3 ligases specific to myofibrillar proteins, such as troponin I, myosin heavy and light chains, and actin (Sandri, 2013; Winbanks et al., 2016), and contributes to increased ubiquitination and consequently proteasome degradation of these myofibrillar proteins (Li and Reid, 2000; Sakuma and Yamaguchi, 2012;

Argilés et al., 2014; Onesti and Guttridge, 2014; Patel and Patel, 2017). Consistent with this notion, TNF-α serum levels are reported to be higher in pancreatic cancer patients with a poor nutritional status (Karayiannakis, Syrigos et al., 2001). IL-6 activates the janus kinase (JAK) (Bonetto et al., 2012)/ signal transducer and activator of transcription (STAT)-3 pathway and mitogen-activated protein kinases (MAPK) cascades (Na et al., 2007) within muscle cells, to promote increased caspase activity, resulting in increased cellular apoptosis (Argilés et al., 2014; Onesti and Guttridge, 2014; Puppa et al., 2014). Additionally, TNF-α, IL-6, and IL-1β have also been implicated in lipolysis, potentially contributing to fat loss in cancer cachexia (Narsale and Carson, 2014; Petruzzelli and Wagner, 2016; Patel and Patel, 2017). Browning of white fat cells to 'beige' cells has been a focus of cachexia studies involving adipose tissue. In beige cells, there is a higher population of uncoupling proteins that decrease the amount of adenosine triphosphate (ATP) synthesized in the cells' mitochondrion and increases energy expenditure through thermogenesis (Petruzzelli et al., 2014; Petruzzelli and Wagner, 2016). These cytokines likely come from the host's innate immune response to the tumor. Macrophages have been examined as a major source for TNF-α and IL-1 in particular (Onesti and Guttridge, 2014). IL-6 is known to be released from both contracting skeletal muscle and activated innate immune cells (Carson and Baltgalvis, 2010).

Tumor-derived proteolysis inducing factor has also been identified in mouse cachexic models and is thought to play a critical role in the breakdown of skeletal muscle proteins through the ubiquitin proteasome pathway in these models. However, whether the human homolog of this factor is capable of eliciting a cachexic response is currently subject to controversy (Monitto et al., 2004; Wieland et al., 2007; Onesti and Guttridge, 2014). On the other hand, the type IIB activin receptor (ActRIIB) which binds the myokine myostatin, has also been implicated as a potential cachexia target for its role in regulating the transcription of the muscle protein targeting E3 ligases of the ubiquitin pathway. Blocking ActRIIB signaling has shown promising results reducing muscle loss and increasing survival in mice, and has also been reported to improve cardiac atrophy (Zhou et al., 2010; Argilés et al., 2014; Onesti and Guttridge, 2014; Petruzzelli and Wagner, 2016). A recent study evaluated the adeno-associated virus delivery of the gene for the endogenous ActRIIB inhibitor, similar to mothers against decapentaplegic homolog 7 (Smad7), to combat ActRIIB driven muscle atrophy in mice. This strategy shows initial promise for its ability to specifically target striated muscle signaling, potentially cutting down on dangerous off target effects seen with other antagonists (Winbanks et al., 2016). For example, one study using an ActRIIB antagonist in humans with muscular dystrophy was canceled due to unanticipated bleeding in the participants (NCI, 2011; Winbanks et al., 2016). The safety of these strategies for use in patients will therefore have to be carefully evaluated.

While the above cellular pathways may partially explain the wasting of skeletal muscle, they are not entirely sufficient to explain the loss of protein synthesis that accompanies protein degradation during cachexia. Other factors such as the reduced phosphorylation of the mechanistic target of rapamycin (mTOR) and p70 have been shown to inhibit skeletal muscle protein synthesis in mice with murine adenocarcinoma 16 (MAC16) tumors, contributing to cachexia by preventing repair of weakening muscles, such as the gastrocnemius muscle in the hind limbs of mice (Eley et al., 2007; Al-Majid and Waters, 2008). Additionally, heightened levels of angiotensin II, commonly known for its role as a vasoconstrictor peptide regulating blood pressure as part of the renin-angiotensin system, have been associated with the inhibited synthesis of new proteins in murine myotubes, impaired regeneration of wasting skeletal muscle, and atrophied skeletal muscles as shown in excised muscles and primary satellite cell culture from mice with a cardiotoxin induced injury (Russell et al., 2006; Yoshida et al., 2013). Angiotensin II may simultaneously contribute to cachexia by both impairing protein synthesis and increasing activity of the ubiquitin proteasome system through increased transcription of key muscle protein specific E3 enzymes, as evidenced in murine myotube models (Al-Majid and Waters, 2008). It was even suggested that angiotensin II may be useful as a biomarker for cancer cachexia, based on plasma and whole blood measurements from 122 patients with seven types of cancer (Penafuerte et al., 2016). Although all of the pathways discussed here may contribute to cachexia development, the exact driving mechanisms behind individual cases of cancer cachexia are complex and can differ between patients. For this reason, it is important to have models that can adequately represent cachexia in all of its complexities.

Preclinical models to study cancer cachexia

Currently, the models available for studying cancer cachexia consist of a combination of *in vitro*, *in vivo*, and e*x vivo* models. There are effective established cell lines for developing an *in vivo* animal model of cachexia. For example, both the cultured colon-26 (C26) carcinoma cell line, and lewis lung carcinoma (LLC) cell line (Choi et al., 2013) are known to induce weight loss and increase mortality when used in a mouse model, providing an effective model of cachexia (Onesti and Guttridge, 2014; Fukawa et al., 2016). The delivery of the cancer cells and the resulting symptoms of cachexia may vary between individual cell lines. C26 colon carcinoma causes significant loss of bodyweight and increased skeletal muscle protein degradation in mice when injected subcutaneously as a cell suspension, or implanted subcutaneously as a solid fragment of a C26 tumor from a

donor mouse (Aulino et al., 2010). LLC cells are typically delivered intramuscularly, and in rarer instances subcutaneously, to cause cachexia in a mouse model (Deboer, 2009; Choi et al., 2013). Tumor-free body mass, as well as excised extensor digitorum longus mass, have been shown to decrease in C57BL/6 mice injected intramuscularly with LLC cells when compared to media-injected mice, without any notable change in diet. The loss in muscle mass corresponded with a loss in measurable tetanic muscle force in an *ex vivo* measurement (Choi et al., 2013). The usefulness of these cell lines as a model for specific studies may depend on the end result that is being measured. For example, high levels of TNF-α are seen in models using LLC cells, but not C26 or MAC16 cells (Mueller et al., 2016). For the C26 model, IL-6 is traditionally viewed as a key mediator of cachexia development (Aulino et al., 2010; Bonetto et al., 2012). Conversely, tumor-derived proteolysis inducing factor (PIF) and lipid-mobilising factor (LMF) have been shown to play a larger role in MAC16 muscle proteolysis, adipolysis, and cachexia development than immune-derived inflammatory cytokines (Bing et al., 2001; Islam-Ali et al., 2001).

Changes in whole bodyweight, daily food intake, total body lean and fat mass composition, cardiac muscle, individual skeletal muscle weights, muscle fiber cross sectional area, and muscle function are key measurements of interest in these *in vivo* models (Deboer, 2009; Winbanks et al., 2016). Key muscles of interest in cachexia studies include the extensor digitorum longus (EDL) and tibialis anterior (TA) muscles located in the hindlimbs (Aulino et al., 2010). These muscles represent multiple muscle fiber types, as the EDL contains predominantly fast twitch fibers and the TA contains a mixture of fast and slow twitch fibers, allowing studies to address how cachexia models may affect specific muscles differently (Aulino et al., 2010; Choi et al., 2013). Repeatable *in vivo* measurements of systemic muscle function include grip strength and force transduction measurements. The former measurement utilizes a grip strength meter (Fig. 1). To do this measurement with the forelimbs, the mice are held by their tails and allowed to grab onto a wire mesh square or bar with only their forepaws. Then they are pulled away from the mesh horizontally by their tails until they release their grip on the mesh or bar. The grip strength meter will then read out the peak force generated from the mouse's grip on the mesh. A second readout uses a force transducer to measure the maximum contractile force able to be elicited from specific muscles or muscle groups stimulated with electrodes (Fig. 2). This technique allows for measurement of both contractile forces and time to fatigue. Individual muscles may also be excised upon sacrifice, weighed, and contractile forces measured as an *ex vivo* model using electrical stimulation and a force transducer (Aulino,et al., 2010; Waning et al., 2015).

To further study the molecular mechanisms where by cancer affects muscle functions, *in vitro* culture systems using primary muscle cell or muscle cell lines can be used.

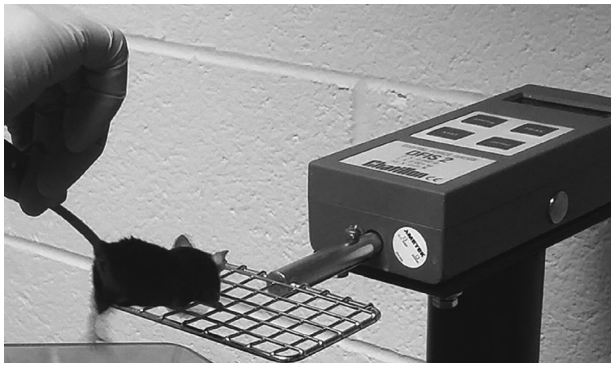

Figure 1 Grip strength measurement. Mouse is held by its tail as it grips the wire mesh bar attached to the grip strength meter. As the mouse is pulled horizontally away from the mesh and loses its grip, the peak force generate from the mouse's grip on the mesh is measured.

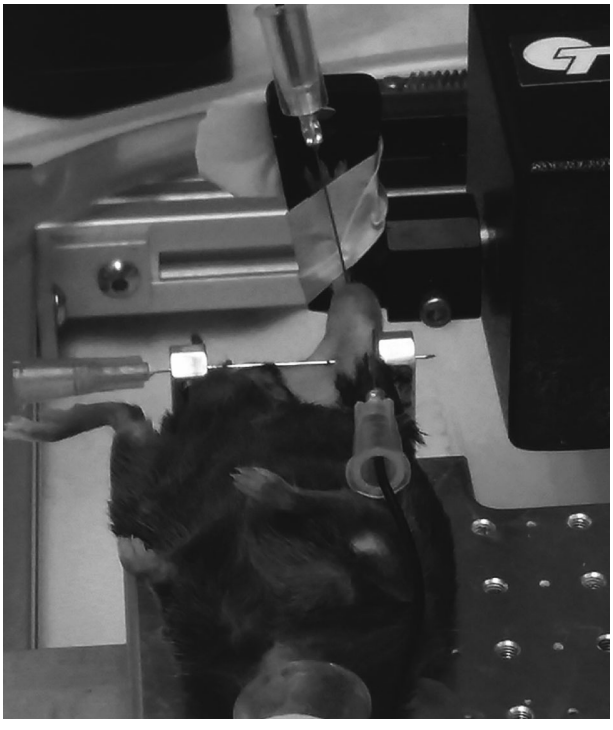

Figure 2 Force transducer measurement. The mouse is anesthetized and its foot attached to the pedal of the force transducer. An electrode is placed over the tibialis anterior muscle. A second electrode is placed at the base of the tendon. An electrical current stimulates the muscle to contract. As the muscle contracts, the mouse pushes the pedal, and the resulting force is recorded.

Commonly, both murine and human myotubes are cultured *in vitro*, and their responses to potential cachectic factors added to the media are observed, such as changes in tubule size or diameter, oxidative stress, and protein content (McLean et al., 2014; Bowen et al., 2015; Fukawa et al., 2016). The C2C12 mouse myoblast cell line is able to differentiate into myotubes when its media is supplemented with horse serum. Primary human and mouse myotubes can be generated using isolated

muscle stem cells or satellite cells from individual muscle fibers (Pasut et al., 2013; Fukawa et al., 2016). This provides an alternative that allows for human tissues to be studied and potential differences between the mouse and human muscle regeneration processes to be recorded.

Clinical studies regarding cancer cachexia

Human based studies have been completed for various types of solid tumors, but are relatively small in number when compared with the number of cachexia studies performed using animal models (Mueller et al., 2016). Clinical studies seeking to identify a biomarker for cachexia development have been met with variable results that are difficult to reproduce between multiple patient populations. A recent study attempted to account for this inherent variability by first characterizing their cohort population into three separate groups. The patients were from seven types of cancer and were divided into a 'no cachexia', 'pre-cachexia', or 'cachexia' group. This study complied with the international consensus definition, and cachexia was defined as loss of 5% or more of total bodyweight within 6 months, or loss of more than 2% of bodyweight with a BMI less than 20. Patients in the 'pre-cachexia' group had elevated levels of CRP without cachexic levels of weight loss, patients in the 'no cachexia' group did not present with cachexia or elevated CRP, and patients in the 'cachexia' group had both elevated CRP levels and significant weight loss (Penafuerte et al., 2016). The study then determined the plasma levels of angiotensin II using an ELISA, and the whole blood levels of neutrophil-derived proteases, such as cathepsin B, for each group, using a chip array to measure mRNA expression. This study found significantly higher mRNA expression of both angiotensin II and cathepsin B in the precachexia and cachexia groups when compared to the no cachexia group (Penafuerte et al., 2016). Based on these results angiotensin II and cathepsin B, or similar neutrophil-derived proteases, may serve as usable biomarkers for cachexia, providing the patients are accurately categorized. This characterization of patients based on their stage of cachexia may also be useful in determining which treatments will be most beneficial for individual cancer cachexia patients.

In addition to the work being done to identify suitable biomarkers for cancer cachexia, clinical trials are currently underway to test potential therapeutics for cachexia. To date, these trials have been met with mixed results. Attempts to treat cachexia through exercise and limited bed rest have been largely unsuccessful (Onesti and Guttridge, 2014). The selective androgen receptor modulator, enobosarm is a candidate therapeutic that increased lean body mass in a double blind phase II trial with patients diagnosed with non-small cell lung cancer (NSCLC), colorectal cancer, non-Hodgkin lymphoma, chronic lymphocytic leukemia, or breast cancer (Dobs et al., 2013). Two international double-blind phase III trials, dubbed the POWER 1 and 2 trials, measured benefits in muscle mass preservation in NSCLC patients receiving enobosarm orally with and without a taxane and chemotherapy, but the results from these trials have not yet been fully published (Crawford et al., 2016). Anamorelin, a mimic of the appetite regulator ghrelin, was found during two phase III trials to increase lean body mass in patients with NSCLC but had no effect on muscle function, measured with handgrip strength (Lok, 2015; Temel et al., 2016). A phase I/II trial with the TNF-α blocker etanercept with gemcitabine did not improve overall survival of participants with advanced cancer, and a phase III study with incurable cancer patients presenting weight loss or impaired appetite showed no benefit in weight or appetite from subcutaneous etanercept injections (Jatoi et al., 2007; Wu et al., 2013; Onesti and Guttridge, 2014; Mueller et al., 2016).

Examples of therapeutic agents targeting inflammation that are being evaluated for their effects on cancer cachexia include Clazakizumab and MABp1. Clazakizumab, or ALD518, is a humanized anti-IL6 monoclonal antibody that has been tested in preclinical trials in NSCLC patients and improves lean body mass preservation (Bayliss et al., 2011). MABp1, also known as Xilonix, is an antibody against IL-1α. This has been tested in a double-blind phase III clinical trial with advanced colorectal cancer patients. The primary endpoint for this trial was measured as either an overall improvement in lean body mass preservation or a combination of improved pain, fatigue, or anorexia when compared to the initial baseline measurements. Results from this study showed 33% of MABp1 treated patients showed improvement, while only 19% of patients receiving the placebo showed improvement (Hickish et al., 2017). Further evaluation for these agents as cachexia therapeutics will be needed, but indicate that anti-inflammatories are a viable avenue to pursue for cancer cachexia therapy. Much work clearly remains to be done to establish an effective therapeutic strategy for cancer cachexia patients.

Future directions and conclusion

Cachexia is a very complex metabolic syndrome with the capacity to target multiple pathways and organs at once. Much like the cancer that drives the development of cachexia, it is unlikely two cachexic patients will present with their disease in the same way (Mueller et al., 2016). For this reason, it is also unlikely that a single targetable therapeutic will be equally effective in all cachexic patients. If this statement is true, then ongoing clinical trials will help determine why certain patients benefit from specific therapeutics while others do not. Due to this inherent variability among cancer cachexia patients, and the multiple molecular mechanisms involved in its development (Fig. 3), individualized treatment plans and multiple therapeutic options are likely needed to adequately devise effective treatment options

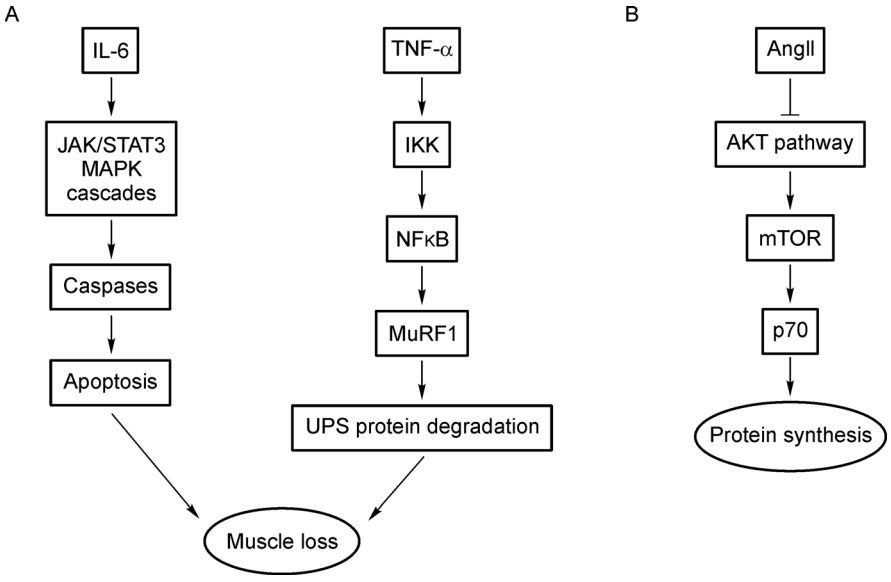

Figure 3 A schematic model of molecular pathways contributing to cancer cachexia. (A) In response to the tumor, the host's immune system releases inflammatory cytokines that can promote cachexia development. Tumor necrosis factor (TNF)-α signaling activates IκB kinase (IKK) and the nuclear transcription factor-κB (NFκB) pathway to upregulate transcription of the E3 ligase, muscle RING-finger protein-1 (MuRF1), increasing ubiquitin mediated degradation of muscle proteins, and leading to muscle loss. Interleukin 6 (IL-6) activates the janus kinase (JAK)/signal transducer and activator of transcription (STAT)-3 pathway and mitogen-activated protein kinases (MAPK) cascades, upregulating caspase activity, and resulting in increased cellular apoptosis in the muscle. (B) Angiotensin II (AngII) can inhibit protein synthesis by inhibiting the Protein Kinase B (AKT) pathway and reducing phosphorylation of mechanistic target of rapamycin (mTOR) and p70.

for cancer cachexia. Combination therapies encompassing multiple therapeutics may also be further explored to uncover an effective treatment for all cachexic patients. Animal models and *in vitro* studies have helped to identify key pathways and potential targets in this complex disease, but unfortunately they are unable to completely mimic all the complexities and variables that are often seen in humans with cachexia. As a result, the findings from these models do not always translate to humans, as evidenced by the unexpected side effects of the ActRIIB inhibitor in clinical trial (NCI, 2011). To overcome these issues, more studies involving human muscle tissue and clinical trials will clearly be needed in order to confirm the translatability of *in vitro* and *in vivo* findings. Combining the knowledge gained from multiple preclinical models with the findings of diverse clinical trials will further knowledge on the complex mechanisms driving cancer cachexia and lead to effective therapeutic options for its treatment.

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Compliance with ethics guidelines

D. Brooke Widner, D. Clark Files, Kathryn E. Weaver, Yusuke Shiozawa declare that they have no conflict of interest. All institutional and national guidelines for the care and use of laboratory animals were followed. This article does not contain any studies with human subjects performed by any of the authors.

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