CACHEXIA: NOVEL PERSPECTIVES FOR AN OLD SYNDROME

Dario Coletti, Laura Belli, Sergio Adamo

Dipartimento di Istologia ed Embriologia Medica, Università La Sapienza, Roma and IIM, Istituto Interuniversitario di Miologia

Abstract
Cachexia, literally "bad condition", a syndrome reported even in the Old Testament, is associated to a variety of chronic diseases and to several kinds of traumatic injuries. Although an official definition of cachexia is still missing, this multi systemic syndrome is characterized by a massive loss of skeletal muscle and fat tissue. Such an important loss of body mass is often a direct cause of death, interferes with therapies and always represents a poor prognostic factor. In sharp contrast with the management of chronic disease states, a cure for cachexia is still missing. In recent years striking discoveries have cast light upon the molecular mechanisms underlying cachexia. Muscle specific targets for pharmacological, gene or cell therapy approaches have been identified and are summarized in this review. Although limited to cancer cachexia, the first phase I-III clinical trials have been recently terminated or are ongoing. These efforts are opening the possibility to prevent cachexia or treat it with novel approaches in the near future.
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Skeletal muscle, often considered a terminally differentiated, static tissue, actually undergoes continuous dynamic processes ensuring its homeostasis. The importance of skeletal muscle homeostasis becomes apparent following its deregulation in pathological conditions. These conditions include several forms of muscle atrophy, i.e. muscle wasting due to net myofibril protein loss following damage, disuse, ageing or illness [42]. A severe form of muscle atrophy is cachexia. Descriptions of cachexia date as far back as the Bible: King David was reported to suffer from cachexia, apparently secondary to a genitourinary cancer [6]. Nevertheless, an official definition for cachexia is still missing, although some key features of cachexia are widely accepted. Cachexia is a severe muscle atrophy whose etiology involves elevated levels of cytokines and other humoral factors associated to chronic diseases (such as cancer, AIDS, diabetes, renal or heart failure). Cachexia also represents a relevant clinical problem in intensive care units, since it significantly worsens the prognosis for traumas and burns. Cachexia is also characterized by an involuntary significant body weight loss due to both skeletal muscle and fat tissue loss. Cachexia may be associated to anorexia, anemia, asthenia and low albumin levels [82].

For decades the clinical importance of cachexia has been neglected. However, the relevance of such a devastating muscle mass wastage in relation to feasibility and efficacy of therapy, to patient's quality of life and survival, is now widely acknowledged. It has been calculated that cachexia directly accounts for the death of 20% of cancer patients [3]. This prevalence corresponds to almost 2 million deaths per year worldwide [24]. Given the increasing ageing of the population, as well as the gap between the management of chronic illness and its associated cachexia, it is evident that a better control of skeletal muscle homeostasis has a great social, economical and health relevance. During the last international conference on cachexia (3rd Cachexia Conference, Roma, Dec/8-10/2005) significant novel results were presented on the molecular mechanisms and clinical aspects of this syndrome. In this context a discussion forum has been set, to reach an agreement on a definition of cachexia, which is still missing. The lack of such a definition mirrors the global inattention to the syndrome of cachexia until the very last few years.

Inflammation and muscle wasting

Studies on animal experimental models have shown that pro-inflammatory cytokines (such as IL-1ß, IL-6, and TNF-a) play a causal role in eliciting cachexia. It is worth noting that the same cytokines are responsible for the production of the acute phase response (APR)

proteins, in particular the C-reactive protein. Cytokines also account for body weight loss, and elevated levels of cytokines represent a negative prognostic factor. In a study involving 102 pancreatic cancer patients the presence of APR > 10 mg/L was reported to be the most significant independent predictor of poor survival [31].

Weight loss was reported to constitute an important prognostic factor in a retrospective study of the Eastern Co-operative Oncology Study Group involving 3047 patients, with a high statistic significance in breast cancer patients [28]. Deans and co-workers have studied about 200 patients with esophageal carcinoma: of this cohort 44,4% showed a weight loss <10% while 38,8% showed a weight loss >10% [26]. The amount of weight loss correlated with several negative prognostic parameters, including disease progression and reduced performance status, quality of life and survival. Although dysphagia may play a role in the effects of this particular tumor, the weight loss positively correlated with increased IL-8 and C-reactive protein serum levels as well as with decreased transferrin and albumin (i.e. APR negative proteins) serum levels in this clinical study, as well as in experimental studies on animals. Similar results were reported in a variety of solid tumors of the lung, the gastrointestinal apparatus, the kidney, the ovary, the skin, as well as in hematological tumors including lymphomas and multiple myeloma [31, 49]. Other studies highlighted the relationship between markers of systemic inflammation and factors of tumor origin, such as PIF and PTHRP [75, 76]. The latter is a circulating factor which is responsible for paraneoplastic hypercalcemia by mimicking the effects of the parathyroid hormone on kidney and bone; moreover, PTHRP can activate the pro-inflammatory cytokine cascade, in particular IL-6 and TNF-a. Another example of an important mediator of metabolic events associated to sepsis or tissue damage is IFN-.. Elevated IFN-. levels significantly affect nitrogen balance and lean body mass homeostasis [12].

Interestingly, cytokines and tumor-derived mediators characteristic of APR may lead to systemic, chronic inflammation in tumor patients. In turn, chronic inflammation is associated to increased catabolism, anorexia, weight loss, i.e. hallmarks of cancer cachexia. It has been hypothesized that the cytokine-APR protein network induces reprogramming of protein metabolism toward increased APR protein synthesis sustained by the aminoacids produced through skeletal muscle catabolism [61]. Thus, muscle wasting can be triggered by APR [86].

Effects of chronic inflammation on muscle homeostasis

Chronically elevated levels of pro-inflammatory cytokines are responsible for the vast majority of the cases of cachexia, as well as of several cases of anorexia-cachexia. The latter is beyond the purpose of this review and has been recently summarized by Laviano et al. [43]. TNF-a is necessary and sufficient to cause cachexia, a role mirrored by its original name of cachectin [18, 58, 73]. TNF-a is a peptide synthesized by several cell types (including macrophages, lymphocytes, Langerhans cells, etc.) as a response to a wide variety of stimuli inducing infection, inflammation and elevated levels of other cytokines [84]. IFN-. produced by lymphocytes T and NK mimics and potentiates TNF-a effects on muscle cells [2, 39]. IL-6 and leptin have also been shown to inhibit biosynthesis and stimulate breakdown of lipids and protein in adipocytes and myocytes, respectively [4]. A role of leptin in pathological conditions such as cancer cachexia has not been fully elucidated yet. Other cytokines, such as LIF, TGF-ß or IL-1, have been proposed to play a role in cachexia, although possibly secondary to the action of other mediators [3]. Great attention was triggered by myostatin, a protein of the TGF-ß superfamily, whose levels are increased in circulation upon muscle wasting. Myostatin mutations cause muscle hypertrophy in several species of mammals, while its overexpression is sufficient to induce cachexia [87, 89]. It may be of great therapeutic relevance that myostatin effects can be rescued in adults by antimyostatin antibodies, which trigger skeletal muscle hypertrophy in the absence of hyperplasia when delivered to animal models [87]. In cancer cachexia, factors of tumor origin can induce muscle wasting, either alone or in combination with host-derived cytokines. Among these, it is worth mentioning PIF and LMF, capable of inhibiting anabolism and stimulate catabolism in skeletal muscle tissue and fat tissue, respectively [83]. Noticeably, not all cytokines induce cachexia, some being actually potent anti-inflammatory agents. The evidence that IL-4, IL-10, IL-12 and IL-15 exert an anabolic action and ameliorate atrophy in tumor-bearing animals show that it is important to consider the network of circulating factors characterizing the inflammatory status of a patient as a whole [3], not to mention that cytokines can be synthesized by several organs including skeletal muscle and act locally [84].

Skeletal muscle signal transduction in response to regulatory stimuli of metabolism

The great attention dedicated in the very last years to the characterization of signal transduction pathways activated by cachexia-inducing factors stems from the goal of finding targets, possibly tissue specific ones, for novel therapeutic approaches against cachexia.

TNF-a-dependent signaling pathways are exemplificative of cellular response to a cytokine playing a pivotal role in cachexia. Following TNF-a binding to its receptors (TNF-R1 and TNF-R2) at least two groups of proteins are recruited to the receptors: binding proteins characterized by a Death Domain (e.g. TRADD or

FAD), and kinases (e.g. IKK or MAPKKK). The recruitment and interaction of these two groups of proteins is mediated by other adaptors (e.g. TRAF2 and RIP), which ultimately determine the formation of a multifactorial complex which starts an enzymatic cascade [15]. The latter may involve many different proteins, including: kinases regulating activation and nuclear translocation of transcription factors (e.g. Jun, NF-.B, see below); cysteine proteases, known as caspases, degrading structural and regulatory proteins.

It has been recently brought to light that important mediators of atrophic signals in muscle cells are proteins regulating Nitric Oxide (NO) synthesis, such as Cox2 and iNOS – the expression of the latter being NF-.B -dependent. AMP-dependent kinases are also involved in the activation of the iNOS-NO pathway. This pathway leads to loss of MyoD (a pivotal myogenic transcription factor) at the mRNA level, ultimately leading to muscle wasting [29].

Several studies focus on effector proteins with hypertrophic effects, since hypertrophy induction is widely considered as a potential therapeutic approach for atrophic/cachectic conditions. Indeed, IGF-I-mediated hypertrophic signals counteract several muscular degenerative conditions [5, 69]. In response to activation of Tyr-kinase receptors, such as IGF-I-R, Akt is activated by PI3K-mediated phosphorylation. In turn, Akt activates a phosphorylation cascade whose final output is a coordinated inhibition of protein catabolism, activation of protein anabolism, and assembly of newly synthesized myofilaments. Overall, these phenomena lead to an increase in fiber size and force [34]. Among the factors involved in Akt-dependent pathways, it is worth mentioning: mTOR, whose inhibition elicits muscle atrophy in vitro and in vivo [9]; GSK3ß, whose repression allows the synthesis of eIF, factors essential for protein synthesis [63]. Calcineurin, a Ca2+-dependent phosphatase, is another important intracellular mediator of hypertrophic signals. Calcineurin directly regulates transcriptional processes by controlling the nuclear translocation of NFAT. The latter is responsible of the hypertrophic response, as shown by a genetic approach exploiting in vitro and in vivo models expressing ipo- or iper-active forms of NFAT [55, 77].

Work from our laboratory contributed to characterize the role of Ca2+ and Ca2+-dependent pathways (e.g. calcineurin) in muscle differentiation and hypertrophy [27, 71, 72]. In particular, we have recently reported that vasopressin, a pleiotropic hormone with myogenic potential, activates both the calcineurin and the CaMK pathways: the combined activation of these two pathways leads to the formation of multifactorial transcriptional complexes necessary for the onset of terminal differentiation [72]. Accordingly, vasopressin not only is sufficient to induce muscle differentiation but also increases intracellular Ca2+ concentration in myogenic cells in culture [78]. Beside inducing muscle differentiation by activating pro-myogenic genes [50], vasopressin removes signals known to negatively affect myogenesis, such as elevated cAMP levels [57]. The latter effect represents one of the many examples of cross-talk between intracellular pathways exerting opposite effects on the muscle phenotype. Unpublished data from our laboratory suggest that vasopressin may also have an in vivo role in maintaining muscle homeostasis, not only by stimulating muscle regeneration following injury but also by inducing muscle fiber hypertrophy (Scicchitano B.M. et al., manuscript in preparation).

Regulation of protein metabolism in skeletal muscle

Deviations from normal muscle size share an unbalance in muscle protein net levels. Virtually all the cases of muscle atrophy are characterized by reduced protein synthesis and enhanced protein breakdown. The altered metabolism results in a decrease in fiber dimension and muscle mass (Fig. 1). The opposite is observed during muscle hypertrophy, following increased exercise or hormonal stimulation. The convergence of atrophic and hypertrophic stimuli on protein metabolism is commonly viewed as a sign that the two pathways share common mechanisms [48].

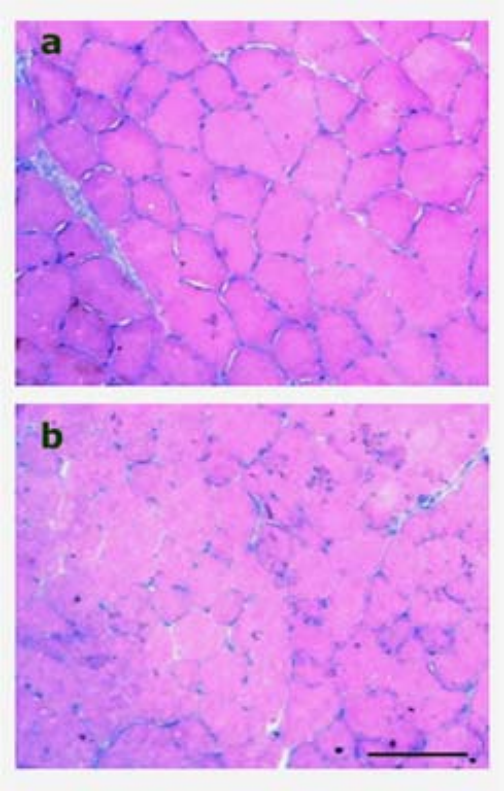

Figure 1.

It has been recently reported that different models of muscle atrophy share increased gene expression of proteins involved in protein degradation, such as cathepsin and ubiquitin (Ub)-ligases [44]. Besides, other proteins never before associated to muscle atrophy (e.g. metallothionein) have been reported to be upregulated during muscle wasting [44]. Ub-ligases are enzymes catalyzing Ub binding to proteins targeted to proteasome-mediated degradation. Thus, ubiquitination represents one of the main pathways for protein degradation. So far, in atrophy conditions, identified Ub-ligase targets include not only structural and sarcomeric proteins, such as myosin, troponin and titin [88], but also factors involved in signal transduction or gene transcription, such as calcineurin and MyoD [80]. Therefore, it is significant that the mRNAs of some Ub-ligases are potently induced in atrophic muscle. The discovery of two skeletal muscle specific Ub-ligases, i.e. atrogin-1/MAFbx and MuRF1 [8,36] has propelled the studies on muscle atrophy because of two distinct implications. These two proteins can indeed be seen as: i) possible early markers of muscle atrophy, since their synthesis precedes myofibrillar protein disruption ultimately leading to muscle atrophy; ii) potential specific targets for therapeutic approaches, since their expression is muscle specific and it is necessary for cachexia induction.

Transcriptional regulation of the atrogenes following atrophying stimuli depends on transcriptional factors such as NF-.B and Foxo. On one hand, NF-.B and Foxo activate Murf and atrogin-1, respectively; on the other hand they are fundamental mediators of cachexia: in fact, their inhibition abrogates the cachectic phenotype of skeletal muscle in experimental animal models [10, 66]. Translocations of both these factors is regulated by phosphorylation. In particular, IKK a/ß/. -mediated phosphorylation causes the degradation of I.Ba, a NF-.B ligand responsible for NF-.B retention in the cytoplasm. On the contrary, direct phosphorylation of Foxo inhibits its translocation into the nucleus [48]. Foxo phosphorylation is catalyzed by the above mentioned Akt. This protein represents the first example of cross-talk between opposite pathways regulating muscle homeostasis to be identified. In fact, factors stimulating protein synthesis (and hypertrophy) in skeletal muscle are also able to block, in a Akt-dependent fashion, Foxo translocation into the nucleus, i.e. to repress atrogene transcription[10, 66].

Muscle precursor cells, regeneration and homeostasis

Muscle regeneration is compromised during cachexia. Muscle regeneration is very sensitive to extracellular factors, which represent the signals triggering its onset. It is worth noting that these factors are not necessarily present in circulation but may be released by the damaged muscle itself. Some of these factors (such as IL-4, LIF, and IL-6) are chemotactic signals for monocytes and macrophages [14], while others directly affect differentiation of muscle and non-muscle cells. For instance, it has been demonstrated that Wnt is able to convert to the myogenic lineage stem cells (CD45+) in regenerating muscle [60]; IL-4 recruits myoblasts and enhances their fusion with pre-differentiated cells, thus contributing to muscle growth [41]. Some of the cytokines clearly involved in cachexia are not necessary for muscle regeneration: TNF-a knock out mouse muscles regenerate similarly to controls [20]. Recent evidence demonstrate that TNF-a, IL-6 and other cytokines which are able to induce cachexia, also inhibit muscle differentiation in vitro as well as muscle regeneration [17, 19, 38]. Accordingly, the pharma-cological blockade of TNF-a helps resolving inflammation and accelerate regenerating muscle recovery [37]. The fact that numerous factors affect both muscle differentiation and atrophy suggests that these two processes may be linked and that muscle regeneration may be important for muscle homeostasis in vivo. The first evidence toward this hypothesis stems from Pavlath's laboratory. Her group has demonstrated that unloading-induced muscle atrophy determines in vivo a reduction of muscle precursor cells, which in turn are necessary for muscle recovery after the end of the atrophying stimulus [51, 52]. In our laboratory, we have demonstrated in vivo that cachexia is associated to defective muscle regeneration capacity [17]. In this context, it is interesting to note that negative regulation of the myogenic transcription factor MyoD has been reported to occur in many experimental models of cachexia. Induction of MyoD transcription is a hallmark of muscle precursor cell activation after muscle damage as well as of muscle differentiation in culture. While the role played by regeneration in the maintenance of muscle homeostasis in specific pathological conditions, such as Duchenne's dystrophy [16, 85] is evident, the role a compromised regenerative capacity may play in the muscle mass loss observed in cachexia is not clear yet.

A major source of muscle precursor cells is represented by satellite cells, muscle fiber attendant cells that are activated, proliferate and contribute to fiber regeneration following muscle damage. Asymmetric cell division allows the maintenance of a reservoir of undifferentiated cells, while the major part of the satellite cell population differentiate fusing with damaged fibers or form muscle fibers de novo [21]. Recently, the involvement in muscle regeneration of cell population other than those already committed to the myogenic lineage has been reported. Stem cell populations, of muscular and non-muscular origin, are able to significantly contribute to muscle regeneration in the presence of proper stimuli. Such stem cells either reside into the skeletal muscle, or they can be recruited to the muscle from other compartments, such as the

bone marrow [7, 13]. The importance of stem cell recruitment in muscle homeostasis is shown by IGF-I-triggered mechanisms involved in muscle trophism. On one hand, IGF-I has a real hypertrophic effect, on the other hand it induces proliferation of satellite cells and enhance stem cell (including hematopoietic stem cells) recruitment to muscle [53]. Current, intriguing proposals for cell-based therapy against cachexia are based on the possibility of making more efficient the stem cell recruitment process.

Experimental models for the study of cachexia

Deriving conclusions directly applying to clinical practice from results obtained on specific animal models requires caution. Many experimental approaches have been adopted for the study of basic mechanisms underlying cachexia. One of the most common approaches consists in the subcutaneous implant of a solid tumor. This approach mimics cancer in its complexity. However, when compared to human analogs, it has the characteristics of a fast and abnormal tumor growth (up to 50% of the body weight in rodents), and of being limited to isogenic animals in respect to the tumor. The features of this experimental approach have been reviewed in [30]. Despite its limitations, the use of this experimental approach has allowed to elegantly demonstrate novel mechanisms of cachexia. For instance, the C26 colon carcinoma tumor model [67] has been used to address striking, new roles of muscle-specific components in the etiology of cachexia. Guttridge and co-workers have shown that cancer cachexia is also due to loss of muscle dystrophin, an event shared by cachexia and dystrophy [1]. With the same experimental model we have recently shown the involvment of p53 and its effector Peg3/PW1 in mediating cachexia, possibly by regulating the number of satellite cells and their differentiation potential in the presence of cytokines [70].

Alternatively to tumor implant, cytokine administration to animals has been exploited to induce muscle wasting, given the importance of cytokines in triggering cachexia. Cytokines have been delivered to rodents in various ways: from i.p. injection of recombinant factors to gene delivery by transplant in nude mice of cells engineered to produce specific cytokines [58]. Experimental models of sepsis are induced by i.p. injection of bacterial endotoxins or yeast extracts capable of inducing an acute inflammatory response [11]. To chronically increase the production of a specific cytokine (TNF-a) in wild type or transgenic mice, regardless of their strain, we set up a novel gene delivery protocol consisting in the injection, into selected muscles, of an expression vector coding for the secreted form of murine TNF-a, followed by electroporation to help DNA enter muscle cells [17]. In this model, the electroporated muscle secretes TNF-a in an amount sufficient to induce a systemic reaction, consisting in muscle atrophy and impairment of regeneration. Other laboratories have created transgenic animals mimicking chronic inflammation, in a muscle-specific and inducible fashion (e.g. the above mentioned mouse with muscle-specific, inducible NF-kB expression) [10].

These approaches allow to clarify at the molecular level the mechanisms underlying muscle atrophy in both pathological and physiological conditions. However, the potential of cell cultures as models of regenerating muscle (which is recapitulated by in vitro muscle differentiation) or adult muscle (mimicked by myotube cultures) must be kept in mind. By combining these approaches, important mechanisms at the basis of TNF-a biological effects on muscle cells have been elucidated. For example, novel mechanisms at the basis of the inhibitory effects on both differentiation of myoblasts and sarcomeric myosin content in differentiated cells have been recently uncovered [19, 45].

Therapeutic perspectives for cachexia

The following conclusions can be drawn from the above: i) cachexia, a severe complication of many pathologies, should be therapeutically counteracted; ii) even though incomplete, current knowledge on the mechanisms of cachexia allows to set the goal of a therapy against cachexia, possibly acting at different levels of intervention; this is not in contrast with the fact that (iii) the therapy of cachexia also consists in the primary disease therapy; iv) ideally, cachexia should be prevented rather than cured. An overview of the therapies of the primary diseases commonly associated to cachexia is beyond the scope of this article. We will rather examine the therapeutic perspectives specifically focused on cachexia *per se*.

Since the transcription factors AP-1 and NF-.B play a pivotal role in the pathogenesis of cachexia, it is possible to hypothesize to interfere with their pathways at the level of either the circulating factors (TNF-a, IL-6) or downstream the receptors triggering their signaling. On the other hand, IGF-I stimulation could be exploited to inhibit signaling pathways downstream of NF.B, through Akt and Foxo [66]. Among IGF-I effects on muscle it is worth mentioning stimulation of protein anabolism and fiber hypertrophy [35, 54, 77]. Myostatin and its effectors are also taken into account as therapeutic targets for treatments against cachexia as well as senile atrophy [62]. An additional, intriguing approach is the inhibition of early events in muscular protein degradation pathways, such as calpain-mediated hydrolysis [33, 42, 79, 81]. Several studies have been performed exploiting the ability of clenbuterol and analogues to interference with ß2-adrenergic signaling [22, 23, 40]. These compounds have been shown to have a positive effect on hypertrophy (i.e. they are anti-atrophic), however the systemic effects, including those on the cardiovascular system, cannot be avoided.

Alternative therapeutic approaches against muscle atrophy are based on the interference with chronic inflammation. Several drugs have been tested at the experimental level: steroid and nonsteroid anti-inflammatory drugs [25, 46]; omega-3 fatty acids, including eicosapentaenoic acid, possibly in association with vitamin E and/or anti-inflammatory agents, due to their effects on APR proteins [32, 47]; several other drugs known to inhibit cytokine synthesis, release or action, such as pentoxyfillin, thalidomide, statins etc.[56, 59, 74]. The recent availability of drugs designed to inhibit the action of TNF-a (Remicade/ Infliximab, a chimeric monoclonal antibody, and Enbrel, a soluble receptor) has made possible to test their effect in an acute muscle atrophy mouse model, showing that these drugs block the early inflammatory cell response in vivo without inhibiting muscle regeneration [37].

Finally, it is possible to speculate on therapeutic approaches for cachexia based: (i) on the increased number or activity of myogenic precursor cells (satellite cells and extra-muscular precursors); (ii) on the blockade of mechanisms hampering their myogenic potential in different conditions of atrophy [17, 51, 52]; (iii) on the use of factors (IGF-I, AVP) stimulating muscle precursor cell proliferation, recruitment and regeneration potential. The most recent anti-cachexia gene therapy reports exploiting the expression of growth factors with anabolic affects have recently been reviewed by Schakman and Thissen [68]. It is established now that factors such as IGF-I exert their anabolic effects involving stem cell recruitment phenomena [53]. For pathologies specific of the skeletal muscle tissue (e.g. muscle dystrophy) striking results have been obtained from stem cell transplants. Cossu and co-workers have morphologically and functionally rescued the muscle phenotype in a murine model of hindlimb girdle dystrophy by transplant of mesangio-blasts, stem cells of mesoderm origin with myogenic potential[64,65]. The success of experimental protocols based on stem cell transplants, currently in the course of validation for species different from mouse, opens new perspectives about gene and cell-based therapy of cachexia.

Abbreviations

Akt, protein kinase B; APR, Acute Phase Response; AVP, Arg-VasoPressin; Cox2, cicloxigenase 2; eIF, Eukaryotic Initiation Factor; FADD, Fas Associated Death Domain protein; Foxo, Forkhead Box O; GSK3ß, Glycogen Sinthase kinase 3ß; IGF-I, Insulin-like Receptor I; IFN-., Interferon-gamma; I.Ba, Inhibitor of NF-kB; IKK a/ß/., Inhibitor of NF-kB Kinase; IL, Interelukin; iNOS, inducible Nitric Oxide Synthase; LIF, Leukemia Inhibitory Factor; LMF, lipid mobilizing factor; MAPKKK, MAP Kinase Kinase Kinase; MyoD, one of the Myogenic regulatory Factors; MuRF1, Muscle Ring Finger1; MAFbx, Muscle Atrophy F-box; mTOR, muscle Target Of Rapamycin; NFAT, Nuclear Factor of Activated T cells; NF-kB, Nuclear Factor-kappa B; PIF, proteolysis-inducing factor; PTHRP, parathyroid hormone - related peptide; RIP, Receptor-Interacting Protein; TGF-ß, Transforming Growth factor-ß; TNF-a, Tumor Necrosis Factor-a TNF-R 1 and 2, TNF Receptor 1 and 2; TRADD, TNF R1 Associated Death Domain protein; TRAF, Tumor Necrosis Factor-a Associated Factor; Ub, Ubiquitin.

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Address correspondence to:

Sergio Adamo, Dipartimento di Istologia ed Embriologia Medica, Via Scarpa 16, 00161 Rome;
Ph.: 0649766756 Fax: 0649766643;
E-mail: sergio.adamo@uniroma1.it

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