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Characterization of the muscle proteome in cancer cachexia: Identification of novel targets for intervention

Duarte das Neves Afonso

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ABBREVIATIONS

2-DE	Two-dimensional electrophoresis
ACN	Acetonitrile
Apc	Adenomatous polyposis coli
APS	Ammoniumpersulfate
Bis	Bisacrylamide
BSA	Bovine Serum Albumin
CHAPS	(3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate)
CHCA	α-Cyano-4-hydroxycinnamic acid
CM	Conditioned medium
D0-D5	Day 0 – Day 5
Da	Dalton
Dex	Dexamethasone
DM	Differentiation medium
DMEM	Dulbecco´s modified eagle medium
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen (german collection of microorganisms and cell cultures)
DTT	Dithiothreitol
Erk1/2	Extracellular signal-regulated kinase 1 and 2
ESI	Electropspray ionization
FBS	Fetal Bovin Serum
GM	Growth medium
h	hour
HS	Horse serum

IEF	Isoelectric focusing
IGF-1	Insulin-like growth factor-1
IL	Interleukin
INF	Interferon
IPG	Immobilized pH gradient
LC	Liquid chromatography
MALDI -TOF	Matrix assisted laser desorption ionization - time-of-flight
Min	Multiple intestinal neoplasia
min	minute
M_r	Relative molecular mass
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MuRF-1	Muscle RING-finger protein-1
Na_3VO_4	Sodium orthovanadate
NF-kB	Nuclear factor ‘kappa-light-chain-enhancer’ of activated B cells
NH_4HCO_3	Ammonium bicarbonate
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
p*I*	Isoelectric point
PMF	Peptide mass fingerprint
SDS	Sodium dodecylsulfate
TEMED	N,N,N',N'-tetramethylethylendiamine
TEV	Tobacco etch virus

TNF	Tumor necrosis factor
TOF/TOF	Tandem time-of-flight

CHEMICALS, CONSUMABLES AND ANTIBODIES

Chemicals and consumables	*Company*
Amicon centrifugal filters	Millipore
BCA protein assay kit	Thermo Scientific
CHAPS	Serva Electrophoresis
CHCA	Bruker
Complete Mini protease inhibitor cocktail	Roche
Creatine kinase assay	BioAssay Systems
Dexamethasone	Merck
DTT	Serva Electrophoresis
DMEM	Gibco
ECL detection system	GE Healthcare
FBS	Gibco
Heat-inactivated horse serum	Invitrogen
HiLoad 16/60 Superdex 200 column	GE Healthcare
HiTrap Chelating HP column	GE Healthcare
Immobiline Drystrip	GE Healthcare
Iodoacetamide	Serva Electrophoresis
IPG buffer	GE Healthcare
Long-R3-IGF-1	GenScript
Microcon centrifugal filter	Millipore
PlusOne 2-D Quant kit	GE Healthcare

PlusOne silver staining kit	GE Healthcare
RAD001 (Everolimus)	Novartis
Thiourea	GE Healthcare
Urea	Serva Electrophoresis

Antibodies	*Company*
Rabbit polyclonal to galectin-1	Millipore
Rabbit polyclonal to galectin-1	Abcam
Mouse monoclonal to IGF-1	Abcam
Rabbit polyclonal to IGF-1	Millipore
Rabbit polyclonal to MuRF-1	Abcam
Rabbit polyclonal to myogenin	Abcam
Rabbit polyclonal to beta-tubulin	Abcam
Rabbit polyclonal to atrogin-1	Biotrend
Rabbit polyclonal to Erk1/2	Cell Signaling
ECL anti-rabbit 2nd horseradish peroxidase-conjugated	GE Healthcare
ECL anti-mouse 2nd horseradish peroxidase-conjugated	GE Healthcare

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1. INTRODUCTION

1. INTRODUCTION

1.1. Cancer cachexia and skeletal muscle wasting

1.1.1. Cancer cachexia

Cachexia has long been recognized as a syndrome associated with many illnesses (Fig. 1) and its prevalence varies from 5% -15% in chronic kidney disease (CKD) or chronic obstructive pulmonary disease (COPD) to 60% - 80% in advanced cancer and mortality rates of patients with cachexia range from 10% - 15% per year in COPD to 20% - 30% per year in CKD to 80% in cancer (von Haehling and Anker, 2010). Cancer cachexia accounts for nearly 30% of cancer-related deaths (Acharyya et al., 2005; Fearon, 2008; Tisdale, 2009) and is defined as an unintended weight loss of 10% during the previous 6 months (Bachmann et al., 2009) or 5% in 12 months or less (Evans et al., 2008). In the case of solid tumors, the likelihood of cancer cachexia is significantly higher than for cancers of the hematopoietic system (leukemias, myelodysplastic syndromes and other hematologic malignancies); an exception to solid tumors is breast cancer (Steiner et al., 2010). This syndrome correlates with a poor prognosis and severely compromises quality of life, physically weakens patients to a state of immobility and the response to standard treatment is usually poor (Acharyya et al., 2008).

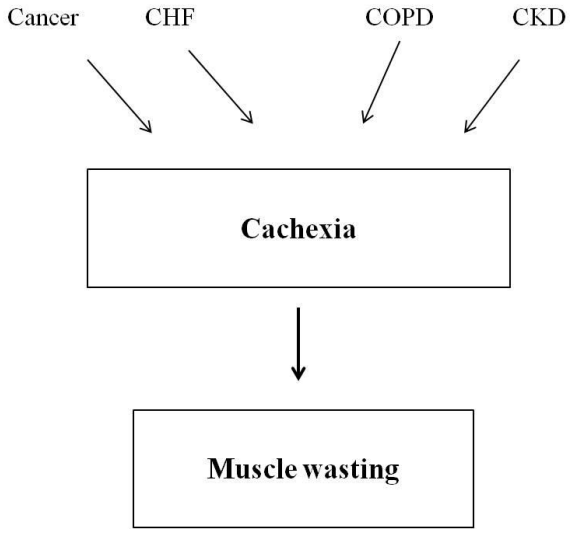

Figure 1. Main factors leading to cachexia and finally to muscle wasting. CHF: chronic heart failure; COPD: chronic obstructive pulmonary disease; CKD: chronic kidney disease.

Cancer cachexia is a severely debilitating syndrome, which is characterized by increased activity of intracellular proteolytic pathways, which is clinically manifest by loss of body weight, lack of appetite, but also, and especially, a loss of muscle mass, with depletion of fat deposits and deep metabolic changes (Beller et al., 1997; Tisdale, 2009). It adversely affects the quality of life of cancer patients and is seen as the final consequence of a series of metabolic and molecular alterations that occur very early, when it is seen as substantially non-reversible with common nutritional or metabolic treatment (Bartlett et al., 1995; Fearon, 2008). The pathogenesis of loss of muscle mass in the course of cancer has not yet been fully elucidated, since it depends on diverse factors, but it seems mainly due to an imbalance between the rate of synthesis and protein degradation in skeletal muscle (Meguid et al., 2000; Tisdale, 2009).

Typical patients suffering from malignant tumors (especially if located in the digestive tract) have complex etiology in which different mechanisms come into play: anorexia, abnormal glucose metabolism and the release of substances produced by the tumor or from the host are capable of influencing the metabolism back toward catabolism. These mechanisms are highly debilitating features in this disease process (Weindruch et al., 2001).

Although the definition and classification of cachexia have always been a matter of discussion, figure 2 presents a conceptual representation of the cachexia diagnosis criteria. However the diagnosis of cachexia is difficult because it is latent, and its development often proceeds for a long time before its clinical manifestation (Bruera et al., 2003).

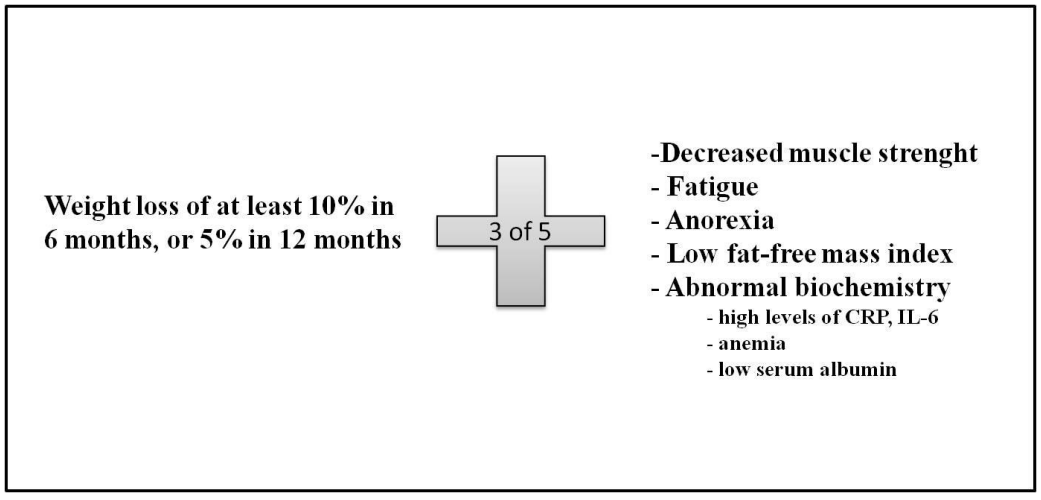

Figure 2. Cachexia diagnosis criteria (adapted from Evans et al., 2008; Tisdale, 2009).

Many times cachexia is associated with anorexia that may result from cancer treatment (chemotherapy, radiotherapy or immunotherapy), which often induces nausea and vomiting to varying degrees (Leppanen et al., 2005). It can also contribute to reduced intake alterations in perception of food and psychological reasons, such as depression. Sometimes anorexia can be attributed as a direct effect of the tumor, when it is located in the hypothalamus or in the digestive system (Beal et al., 1995). However, in most cases, the origin of the anorexia associated with cachexia appears to be the metabolic alterations experienced by the patient as a result of the presence of the tumor (Warmolts et al., 1975). Different factors, both humoral and source segregated by the host in response to tumor growth, or secreted by the tumor cells themselves may play an important role in the anorexic response (Fearon et al., 2006). In short, anorexia appears to be more an effect than a cause of weight loss and, in fact, decreased intake may manifest itself after there has been weight loss (Bhattacharyya et al., 2010). In any case, malnutrition due to reduced food intake only exacerbates the cachectic state, promoting a positive feedback mechanism that can eventually lead to death.

Treatments

The best approach to cure cachexia would be curing the cancer itself, however, if there were a therapy for cachexia, treating cancer would be easier. However, currently there are no effective treatments specifically targeting skeletal muscle cachexia. Being cancer cachexia a multi-factorial syndrome, multimodal approaches that address the different issues could be a key development in terms of therapy. Studies are being conducted, for example, in the following areas: stabilizing and improving the nutritional status, finding and validating novel biomarkers, developing better techniques for the estimation of muscle mass, and improving exercise training.

Exercise training is recognized as having obvious and important roles in skeletal muscle changes by acting in several pathways due to its multifactorial effects. Within the muscle, it has anabolic effects mediated by cytokines, it exerts anti-inflammatory effects inhibiting TNF-α *in vitro*, and it has antioxidative effects increasing the activity of radical scavenger enzymes (Lenk et al., 2010).

Because cachexia is closely associated with anorexia, the use of drugs such as steroids is administrated to help increase appetite (Zhou et al., 2011; Tisdale, 2009). Megestrol acetate

is the most frequently used stimulant in the treatment of cancer cachexia; it is a progestin used to treat the source of advanced breast cancer and is able to increase appetite and body weight in cancer patients by down-regulating the synthesis and release of proinflammatory cytokines. However, patients receiving megestrol acetate show an inferior response to chemotherapy and a trend for inferior survival duration (Tisdale, 2009). Corticosteroids such as dexamethasone are also used to stimulate appetite but only at late stages, since they enhance catabolism in skeletal muscle (Tisdale, 2009). Ghrelin, a neuropeptide released from the stomach in response to fasting, can also induce a positive energy balance by reducing fat and stimulating the appetite (Fearon et al., 2006).

Others agents have been studied for their anti-inflammatory action, such as melatonin, omega-3 polyunsaturated fatty acids, eicosapentaenoic acid, pentoxifylline and thalidomide, but the latter still have scarce clinical data for everyday use (Bhattacharyya et al., 2010). Table 1 provides examples of drugs currently in development (clinical trials) for the treatment of cachexia.

Table 1. **Examples of agents currently in clinical development for the treatment of cachexia** (adapted from Dodson et al., 2011).

Drug class	Example	Mechanism of action
Anti-TNF-α	Thalidomide	Inhibition of TNF-α, COX-2 and NF-kB
Anti-IL-6 antibodies	ALD-518	Inhibition of IL-6
Anabolic steroid analogs	Oxandrolone	Up-regulation of protein synthesis, production of IGF-1
Immunomodulators	AVR-118	Stimulation of proinflammatory cytokines
COX-2 inhibitors	Celecoxib	Inhibition of COX-2

Validated markers, significant to the underlying metabolic irregularities in cancer cachexia, are needed in order that interventions based on the understanding of its mechanisms can be generated. Some factors associated with cancer cachexia have already been found in

tumor tissue (Todorov et al., 1996), urine (Cariuk et al., 1997; Williams et al., 2004), serum (Beck et al., 1990; Felix et al., 2011) and adipose tissue (Bing et al., 2004), but because the skeletal muscle is one of the most affected tissues in cachexia, the identification of additional markers within this tissue (Stephens et al., 2010; Workeneh et al., 2006), which could improve diagnosis, prognosis or therapy of this cancer syndrome, is required.

1.1.2. Skeletal muscle wasting

Skeletal muscle atrophy or wasting refers to the decrease in the size of skeletal muscle. Changes in cell morphology may affect isolated cells or groups of them, therefore the modification of whole tissue. All stimuli that act on a cell are actually functional stimuli; when they exceed the physiological limits, they may injure the cell and cause it to reverse the processes of life, or cause significant regressive modifications (Warmolts et al., 1975). Moreover, atrophy must be distinguished from a disease that entails structural reduction of an organ, or part thereof, due to a necrotic destructive process, in which case there is massive cell death (Ryan et al., 2009). Skeletal muscle satellite cells, specialized cells residing beneath the basal lamina of myofibers, are usually in a quiescent state in adult muscle; but when minor damage or injury occurs, signals are generated within the muscle that activate these satellite cells into myoblasts and stimulate them to migrate to the site of an injury where they proliferate, differentiate, and fuse with the damaged fibers or form new fibers (Sharples and Stewart, 2011; Sakuma et al., 2010; Chargé et al., 2004).

There are several diseases and disorders that cause a decrease in muscle mass, including cachexia, extensive burns, liver failure, electrolyte disturbances, anemia and inactivity. Others can cause muscle wasting syndromes, such as malnutrition, denervation of motor neurons in spinal muscular atrophy of childhood, and the inflammatory myopathies and dystrophies, among others (Warmolts et al., 1975; Evans, 2010). In all cases of atrophy, the cytoplasm is the most affected and there is almost always a reduction in quantity of it - so much so that, observing the atrophic tissue under a microscope can distinguish a discrete cell densification caused by the reduction uniform cell volume. These changes are accompanied by profound alterations in cytoplasm, turbidity, presence of pigment granules and a decreased number of mitochondria (Ryan et al., 2009).

There are three important proteolytic pathways responsible for the degradation of proteins in skeletal muscle (Fig. 3). The calcium-activated system which includes calpains I and II, the lysosomal protease system, including cathepsins B, D, H and L, and finally the ATP-dependent ubiquitin-proteasome system. Although these systems may work as partners, the latter one is seen as having the most predominant role in the degradation of myofibrillar proteins, particularly in cachexia patients (Tisdale, 2009; Jackman and Kandarian, 2003). The process of ubiquitination, a labeling that can be fatal to the protein, involves the cooperative interaction of at least three classes of proteins termed E1 (ubiquitin activating), E2 (ubiquitin conjugating), and E3 (ubiquitin ligating) enzymes. Some of the most studied E3 ligases are MuRF-1 and MAFbx/Atrogin-1, which are found up-regulated in cancer cachexia (Glass, 2005; Jackman and Kandarian, 2004).

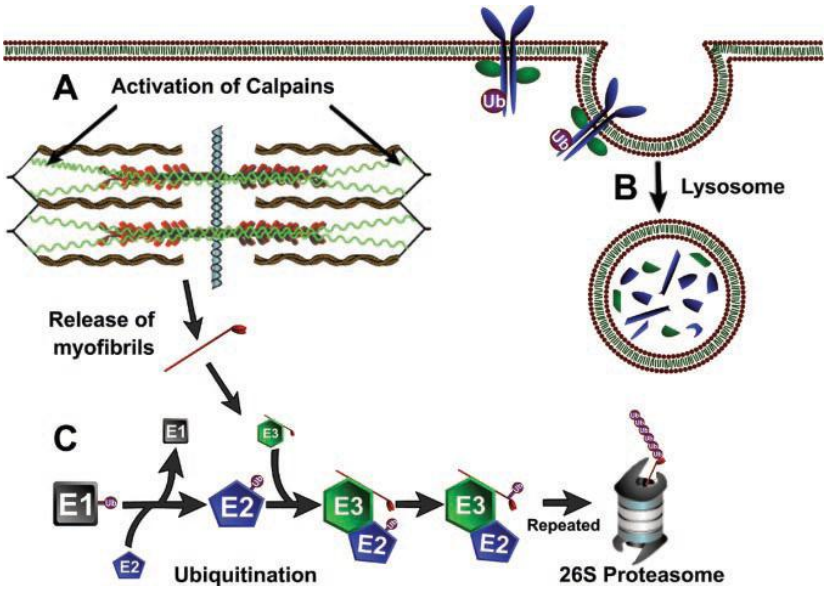

Figure 3. Three known proteolytic systems implicated in muscle atrophy resulting from disuse or disease: the calcium-dependent calpain system (A), the lysosomal protease system (cathepsins; B), and the ubiquitin-proteasome system (C) (Jackman and Kandarian, 2004).

1.1.3 Factors influencing muscle mass in cancer cachexia

The mechanism by which cancer leads to the loss of skeletal muscle mass might be multifactorial. Several hormones, cytokines and tumor-derived factors have been shown to

influence the muscle protein balance in normal and disease states, through several signal transduction systems. While activation of the IGF-1/PI3K/Akt pathway is associated with muscle growth, in atrophying muscles there is an induction of distinct E3 ubiquitin ligases such as the ones already mentioned above MuRF1 and MAFbx/Atrogin-1 (Fig. 4) - which promote protein degradation (Tisdale, 2009; Glass, 2005). However, the exact role of these pathways in the pathogenesis of cancer cachexia is still unclear.

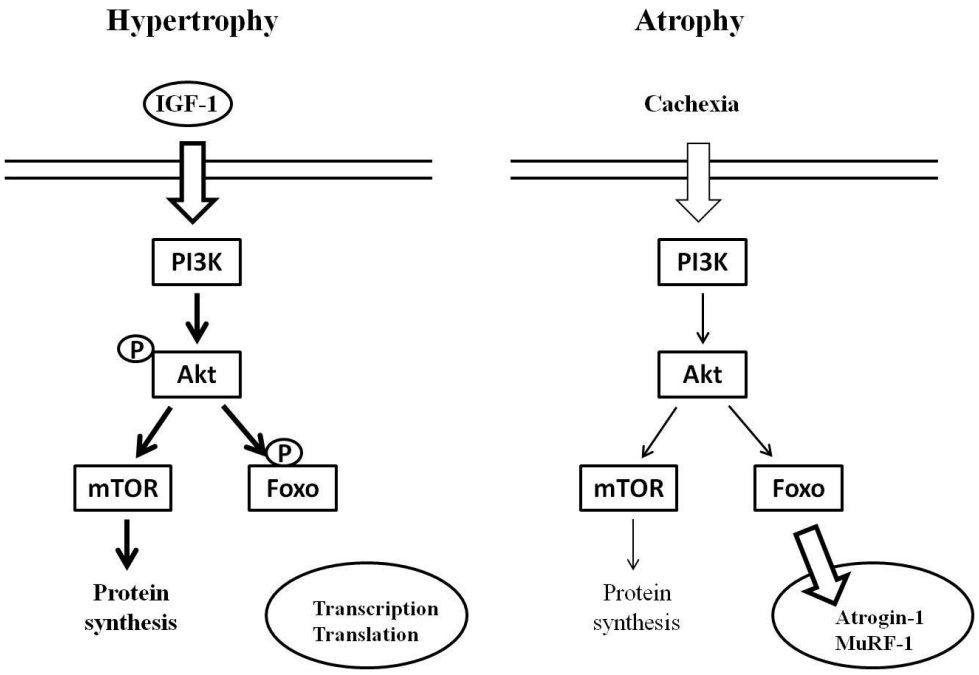

Figure 4. IGF-1/PI3K/Akt signaling in hypertrophy and atrophy. In hypertrophy, PI3K/Akt signaling is activated by IGF-1 promoting muscle growth and reducing breakdown by inhibiting Foxo, through phosphorylation, to translocate to the nucleus. In atrophy, PI3K/Akt activity is reduced by cachectic stimulus and Foxo is liberated to translocate to the nucleus, where it activates transcriptional genes such as atrogin-1 and MuRF-1.

Proteolysis-inducing factor (PIF), a well characterized tumor factor, which may be involved in the development of cachexia, is a small glycoprotein originally identified and purified from MAC16 tumor and urine samples from cachectic patients (Tisdale, 2009); it is suggested that one of its modes of action in cachexia is through the activation of NF-kB and regulation of its downstream genes (Acharyya and Guttridge, 2007). There is also experimental evidence for the involvement of certain proinflammatory cytokines (especially TNF-α and IL-6) in the pathogenesis of cachexia regarding muscle wasting. Like PIF, TNF-α induces activation of NF-kB, leading to the induction of the ubiquitin-proteasome pathway

(Tisdale, 2009), and its levels are elevated in patients with cancer cachexia, contributing to negative nitrogen balance (Glass, 2005). In cell culture, TNF-α up-regulates NF-kB, augments myofibrillar proteolysis and represses myosin synthesis, as well genetic inhibition of NF-kB via the expression of a dominant negative form of IkB blocks protein degradation (Jackman and Kandarian, 2004). Concerning IL-6, there is data showing that this cytokine is necessary for the onset of adipose and skeletal muscle wasting in the $Apc^{Min/+}$ mouse and that circulating IL-6 can regulate $Apc^{Min/+}$ mouse tumor burden (Baltgalvis et al., 2008). Also, muscle atrophy is observed in IL-6 transgenic mice that overexpress IL-6 (Tisdale, 2009). Angiotensin II, a hormone that plays a central role in cardiovascular homeostasis, induces the activation of PKR, being responsible ultimately for the increase of protein breakdown via the ubiquitin-proteasome pathway. Inhibition of PKR attenuates muscle atrophy increasing muscle mass in a mouse model and also inhibited its tumor growth (Eley et al., 2007).

Myostatin is a transforming growth factor (TGF)-β and functions as a potent negative regulator of muscle mass. Muscles from myostatin-null mice weigh ca. 2 to 4 times more than the control group (Acharyya and Guttridge, 2007) and when binding to activin type IIB receptor, myostatin is able to trigger the up-regulation of atrogin-1 and MuRF-1 and down-regulation of important myogenesis genes such as myoD and myogenin (Elkina et al., 2011). Also ActRIIB, a receptor that can be activated by myostatin and activin, has an important role in limiting muscle growth and its antagonism has been seen as a promising new approach for curing cancer cachexia. Using several models of cancer cachexia (cell lines and mice) and utilizing sActRIIB as an antagonist, ActRIIB pathway was blocked, inducing the abolishment of ubiquitin ligases and promoting muscle stem cell growth (Zhou et al., 2010).

In vitro studies have documented several molecules that can modulate satellite cell activity particularly, insulin-like growth factor-1 (IGF-1), whose expression has a role in regenerating muscle. IGF-1 positively regulated the proliferation and differentiation of satellite cells/myoblasts *in vitro* via different pathways (Jennische et al., 1992; Latres et al., 2005). In addition, IGF-1 system was shown to be down-regulated in experimental cancer cachexia using a rat model (Costelli et al., 2006).

1.2. Proteome research

1.2.1. Proteome and proteomics

Proteomics is a relatively recent science and the term was invented by Peter James in 1997 as an analogy with genomics: the word "proteome" is the fusion of **prote**in and gen**ome**. Proteomics is a large scale study of proteins, particularly their structure and function (Aebersold and Mann, 2003) and is considered, after genomics, the next step in the study of biological systems (Fig. 5). It is more dynamic than genomics and due to the number of proteins able to be synthesized, proteomics may allow a better understanding of the complexity of life and the process of evolution than the study of the genetic code alone (Sarto et al., 2002; Adams, 2008; Ohlendieck, 2011). When studying the proteome, complexity is revealed with alternative splicing, in which a single gene can produce multiple versions of a protein or with post-translational modifications, another source of protein variation (Tyers and Mann, 2003). More than 200 different types of post-translational modifications are known, and it is predicted that, on average, for each human gene three different modified proteins with different functions are produced (Brunet et al., 2003; Adams, 2008). The proteome is the full complement of proteins produced by a particular genome, including the modifications made to a particular set of proteins produced by an organism or system (Sarto et al., 2002). This varies with time and with different conditions (disease, stress, environment, etc), which a cell or organism undergoes (Himeda et al., 2004; Xiao et al., 2008). The description of the proteome allows a dynamic picture of all proteins expressed in a given time and under specified conditions of time and environment. In the case of proteomic analysis associated with specific diseases, it is possible to identify proteins that would diagnose disease or predict the evolution of it. These proteins are known under the generic name of biomarkers (Jung et al., 2000; Xiao et al., 2008).

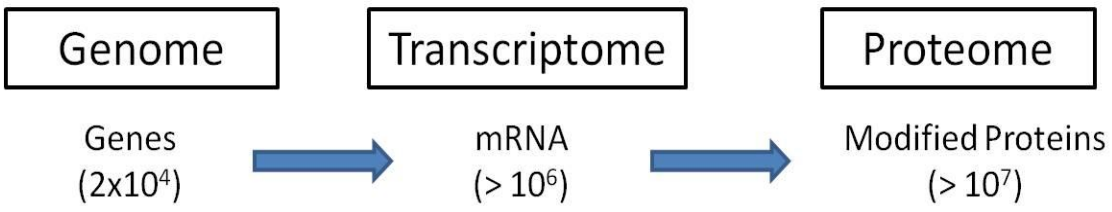

Figure 5. Complexity increases from genome to proteome. The estimated number of biomolecules in a typical cell is indicated in parentheses.

The main technologies under development include two-dimensional electrophoresis (2-DE), staining and analysis tools of the gels, technology for the identification of proteins, such as mass spectrometry, electronic management technologies, such as databases and search algorithms, and data recovery. The main challenge of proteomics is the automation and integration of such technologies (Ornstein et al., 2000; Aebersold and Mann, 2003; Walther and Mann, 2010). Mass spectrometry made possible the analysis of complex biological molecules and the exponential growth in the number of entries for genes and proteins in the databases (Sarto et al., 2002; Aebersold and Mann, 2003). This, combined with the use of powerful methods of fractionation and separation of peptides and proteins such as 2-DE and high resolution liquid chromatography (HPLC), has consolidated proteomics since the mid 1990s, as a science for massive protein analysis (Celis et al., 1999; Tyers and Mann, 2003; Xiao et al., 2008).

1.2.2. Two-dimensional electrophoresis and mass spectrometry (MS)

Electrophoresis is the central technique for analyzing large numbers of proteins at the same time. The electrophoretic technique was invented by Tiselius in the 1930s and further developed in the 1950s by Poulick and Smithies; the first two-dimensional gel was described in 1975 by O`Farrell. The streamlines of the process are still in use today (Rabilloud and Chevallet, 2000).

2-DE consists of two orthogonal dimensions along which different proteins are separated (Fig. 6). This technique is a type of electrophoresis used in the field of proteomics to separate complex protein mixtures such as a mixture of proteins extracted from a cell at any given time of the cell cycle (Rabilloud and Charmont, 2000; Dunn and Görg, 2000), and the main principles used are the isoelectric point and molecular weight (Görg and Weiss, 2000).

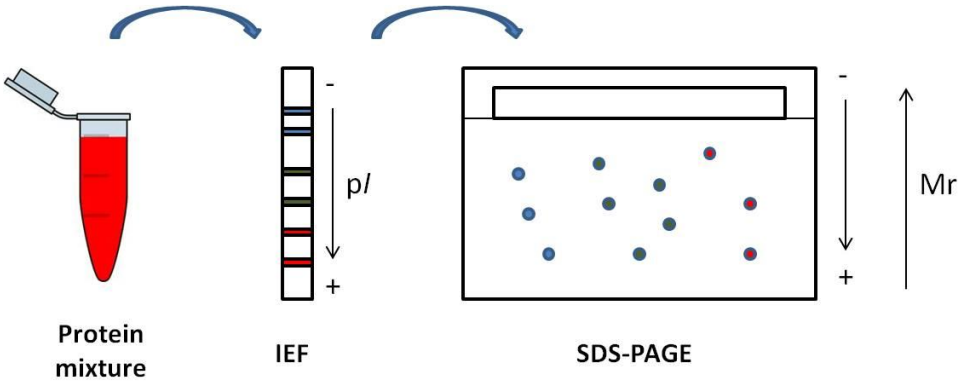

Figure 6. Scheme showing protein separation during the two dimensions of 2-DE.

The first dimension, isoelectric focusing (IEF), usually uses a gradient of pH obtained with amphoteric molecules being made to migrate within a gel of polyacrylamide placed in an electric field. This system is based on strips and provides more stability of the gradient and a higher experimental reproducibility (Rabilloud and Charmont, 2000; Görg et al., 2004). The sample is loaded on the gel and the applied electric field causes the proteins to move towards their isoelectric point, to which each molecule is in the zwitterionic form having a neutral global charge (Rabilloud and Chevallet, 2000; Görg et al., 2004). If in the first dimension the proteins are separated by their charge, in the second dimension, SDS-PAGE, the separation of proteins is done in the presence of SDS and the proteins are separated by their molecular mass. It is possible to separate thousands of proteins in one gel. Proper sample preparation is often the most important step to a successful outcome (Görg and Weiss, 2000; Görg et al., 2004). The spots on the gel are removed and each is treated with trypsin to produce a characteristic pattern of peptides. The peptides are identified by mass spectrometry, a technique that measures the mass of molecules (in this case the mass of the peptides produced by digestion with trypsin) with a high degree of accuracy (Görg and Weiss, 2000).

Mass spectrometry is a powerful analytical technique mainly used to identify constituents of a sample and to reveal chemical properties of different molecules. Mass spectrometers are constituted by an ion source producing ions from the sample such as Matrix Assisted Laser Desorption Ionization (MALDI), an analyzer such as the time-of-flight (TOF) where m/z ratio of ions is determined by measuring the time taken for the ions to travel through a fixed flight path and a detector, which provides data for determining the abundances

of each ion present (Dunn, 2011; Jung et al., 2000). The data coming out of mass spectrometry are fingerprints of peptides separated by mass and the identification of proteins is done by matching the identified particular patterns with a database (Fig. 7) (Himeda et al., 2004; Ornstein et al., 2000).

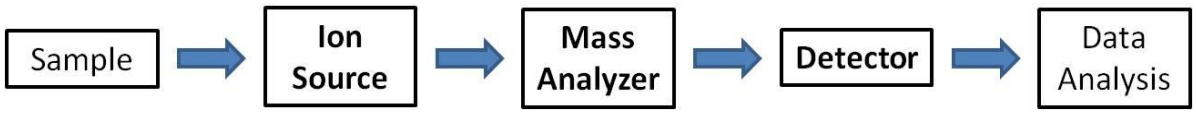

Figure 7. Scheme of MALDI-TOF mass spectrometry.

One of the goals of clinical proteomics is to provide an opportunity to identify new diagnostic, prognostic or therapeutic targets by discovering clinically important proteins which could become biomarkers. According to the U.S. Food and Drug Administration (FDA), a biomarker is a characteristic molecule that is objectively measured and evaluated as an indicator of normal biologic or pathogenic processes or pharmacological responses to a therapeutic intervention. Table 2 summarizes some recent clinical applications of proteomics in biomarker discovery projects in cancer research.

Table 2. Overview of recent clinical proteomics applications towards cancer biomarker discovery (adapted from Tambor et al., 2010).

Type of cancer and usefulness	Proteomic techniques	Candidate biomarker
Hepatocellular carcinoma – diagnosis	SDS-PAGE and western blotting	Autoantibodies against phospholipase A_2 receptor
Lung adenocarcinoma – prognosis	Affinity chromatography, 2-DE and western blotting	39 proteins including adiponectin, ceruloplasmin or cyclin H
Colorectal cancer – diagnosis	2-DE, western blotting	S100A8 and S100A9
Renal cell carcinoma – diagnosis	SELDI-TOF	Eukaryotic initiation factor 2B δ
Melanoma – prognosis	MALDI-TOF	Serum amyloid A
Pancreatic cancer – diagnosis	MALDI-TOF and ELISA	Platelet factor 4
Pancreatic cancer – diagnosis	2D-LC-MSMS (SILAP) and ELISA	ICAM-1 and BCAM

1.2.3. Proteomics in cancer cachexia and muscle wasting research

There is a lack of publications on the application of proteomics in cancer cachexia and muscle wasting or atrophy, which is evident by a simple search in PubMed or other searching tools. Using 1-D SDS-PAGE and MALDI-TOF MS, a preliminary study identified several candidate protein biomarkers of cancer cachexia in human urine (Skipworth et al., 2010). Concerning muscle wasting or atrophy there are more studies, but associated with other causes such as sarcopenia or aging (Doran et al., 2009; Gelfi et al., 2006; Piec et al., 2005) or due to disuse or unloading-induced atrophy (Ferreira et al., 2009; Moriggi et al., 2008).

1.3. Galectin-1

1.3.1 Galectins

Galectins are carbohydrate-binding proteins with an affinity for β-galactosides and include a total of 15 proteins sharing consensus of amino-acid sequences of about 130 amino acids (Camby et al., 2006), divided into three groups based on their protein structure (Fig. 8). The proto-type galectins, also called dimeric, contain one carbohydrate recognition domain (CRD) (galectins-1,2,5,7,10,11,13,14,15), the chimera-type galectin consists of a N-terminal domain connected to a CRD (galectin-3) and the tandem repeat-type galectins contains two distinct CRD (galectins-4,6,8,9,12) (Balan et al., 2010; Liu and Rabinovich, 2005).

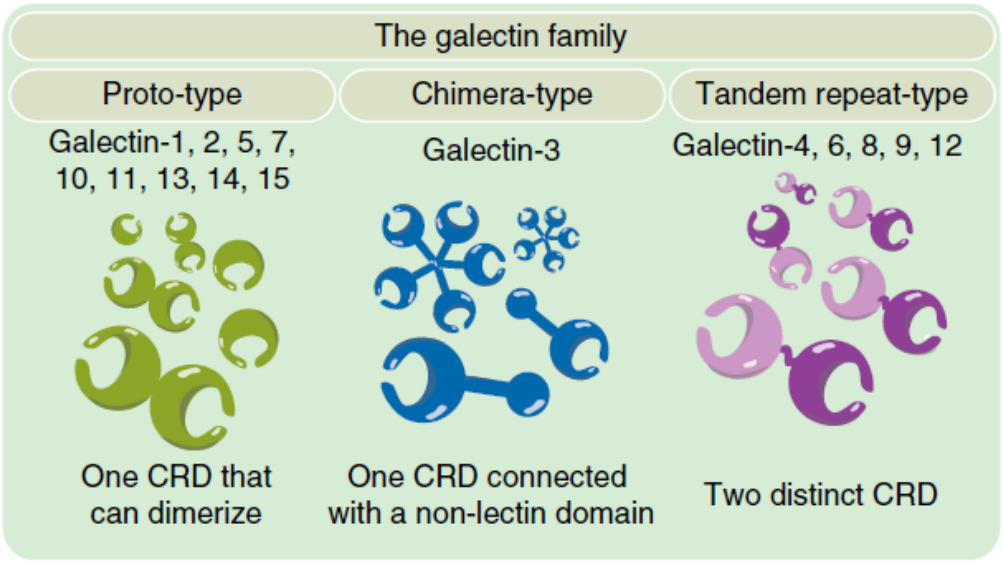

Figure 8. Subdivision of galectins in three groups based on their structure (Salatino et al., 2008).

Galectins can bind to other proteins (Fig. 9) through intracellular protein-protein interactions and lectin-carbohydrate interactions. Their function can regulate signal transduction as well as epithelial morphogenesis via an effect on centrosome biology. Galectins may also bind glycans on the surface of potentially pathogenic microorganisms, and function as recognition and effector factors in innate immunity (Balan et al., 2010). Location studies of galectins have established that these proteins can segregate into multiple cell compartments in function of the status of the cells in question (Danguy et al., 2002; Liu and Rabinovich, 2005), although, in general, galectins do not have the signal sequence required

for protein secretion through the usual secretory pathway (Camby et al., 2006). Galectins also play a crucial role in the process of cell transformation and metastasis formation (Dias-Baruffi et al., 2010).

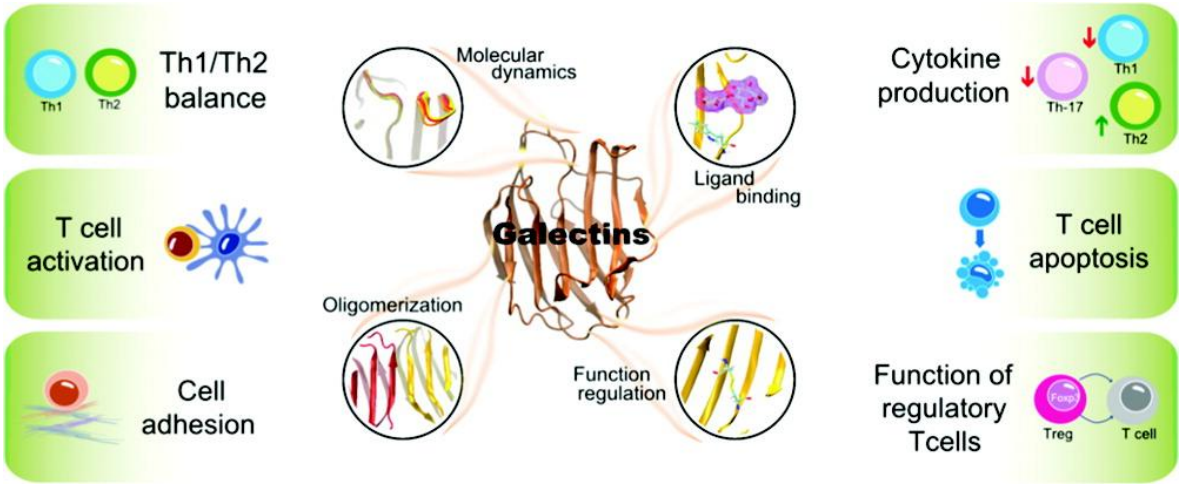

Figure 9. Biochemical features and structure–function relationships of galectins (Di Lella et al., 2011).

1.3.2 Galectin-1

Galectin-1 (Gal-1) is a 14.5 kDa protein which belongs to the proto-type galectins and can be found as a monomer or as a homodimer in multiple tissues (Camby et al., 2006; Rabinovich, 2005). Gal-1 is present both inside and outside cells having intracellular and extracellular functions (Fig. 10), which are associated with cancer biology, including tumor transformation, cell cycle regulation, apoptosis, cell adhesion, migrations and inflammation (Rabinovich, 2005). Galectins, on the whole, induce contrasting effects on cell growth, and the biological effect (proliferation or apoptosis) depends on the cell type and cell activation status. For instance, Gal-1 may play both a role in inhibition of proliferation of T cells and promote proliferation of vascular endothelial cells (Leppanen et al., 2005). Gal-1 together with co-receptors act as a potent regulator of homeostasis of the immune system, and the signals delivered by the various co-receptors may determine the nature of biological responses (Leppanen et al., 2005). For gal-1, immunoregulatory effects are related to induction of cytokines such as IL-27 and IL-10, to control T cell recruitment and to the regulation of the

functions of tolerogenic dendritic cells and CD4+CD25+Foxp3+regulatory T cells (Dias-Baruffi et al., 2010; Rabinovich et al., 2002).

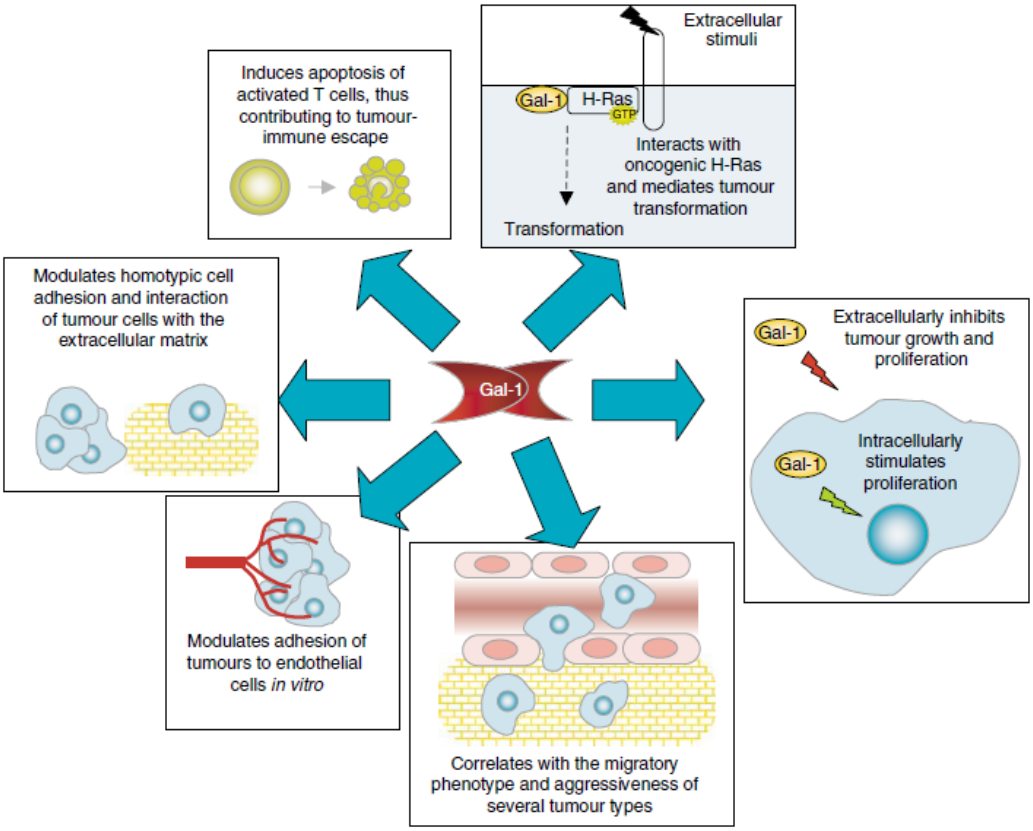

Figure 10. Example of galectin-1 multifunctional roles. In this case, the several contributions of galectin-1 to tumor progression (Rabinovich, 2005).

This galectin also plays an important role in the regeneration of the nervous system after injury (Camby et al., 2006; Svensson et al., 2009), and in the regulation of muscle development, homeostasis and repair (Cerri et al., 2008; Ahmed et al., 2009; Watt et al., 2004; Kami et al., 2005). In addition, Gal-1 may exert protective functions after tissue damage, possibly inhibiting mediators of the inflammatory response (Rabinovich et al., 2000).

Gal-1 null mice showed decreased myofiber formation and reduced regeneration of the same fibers after injury; *in vitro* this protein induces myogenic differentiation (Watt et al., 2004). Also, during early mouse myogenesis, it has an unique expression pattern and role being an early marker of myogenesis acting downstream of Myf5, a muscle determination

factor (Shoji et al., 2009). But gal-1 has an even more important role in adult myogenesis (Kami and Senba, 2005) and also contributes to muscle development in zebrafish (Ahmed et al., 2009).

In skeletal muscle, gal-1 exhibits an organized cytosolic staining pattern and colocalizes with sarcomeric actin (specialized for producing contractile forces) in the I bands (Fig. 11), which contribute to the striated pattern of the cells (Dias-Baruffi et al., 2010). Also in this tissue, one of the most important binding partners is the laminin-binding α7β1 integrin to which gal-1 binds (both laminin and integrin). Gal-1 can inhibit this laminin-integrin association, which promotes the detachment of differentiating myoblasts from the laminin-rich basement membrane and their fusion into myotubes, in this way regulating the myofibers development (Kami and Senba, 2005).

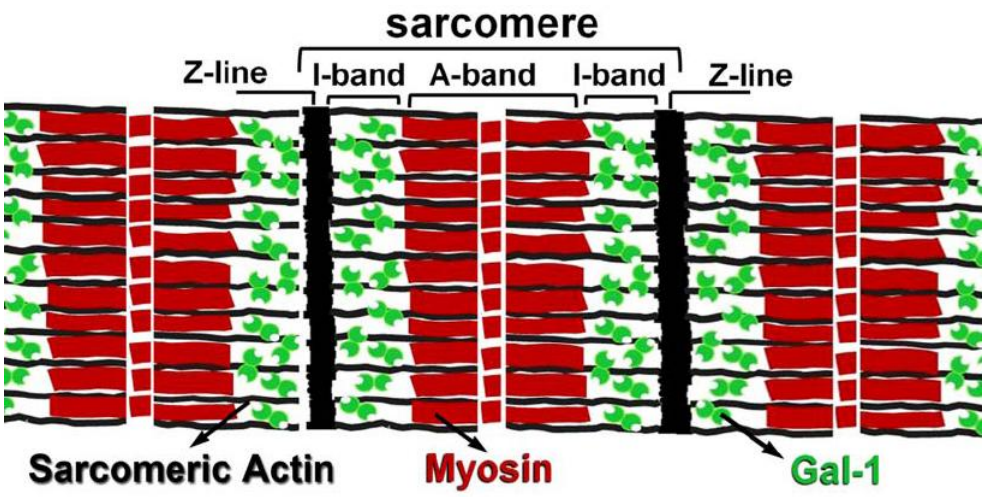

Figure 11. Scheme of skeletal muscle showing the colocalization of gal-1 with sarcomeric actin in the I band region (Dias-Baruffi et al., 2010).

Several studies proved that galectin-1 can be seen as a potential target for diagnosis, prognosis and/or therapies in diverse diseases (Table 3).

Table 3. Overview of the usefulness of galectin-1 in several diseases (Camby et al., 2006; Balan et al., 2010)

Human disease	Usefulness	Results
Multiple sclerosis	Therapy	Administration of recombinant human (rh) gal-1in mice: 63% protection against disease
Rheumatoid arthritis	Therapy	Fibroblast secreting mouse gal-1 or administration of rhGal-1in mice: therapeutic effects
T cell-mediated hepatitis	Therapy	Administration of rhGal-1in mice: prevention of liver injury
Peripheral nerve injury	Therapy	Administration of rhGal-1/oxidized in rats: functional recovery
Melanoma	Therapy	Transfection of anti-gal-1 antisense oligonucleotides: reduced mortality, tumor immune-escape
Colon cancer	Prognosis	Gal-1 expression levels in humans correlate with degree of dysplasia (malignant progression)
Pancreatic cancer	Diagnosis	Higher mRNA and protein levels of gal-1 in human cancer samples comparing with normal ones

1.4. Aim of the study

Cancer cachexia is a complex metabolic condition characterized by loss of skeletal muscle. Despite several years of collaborative efforts in basic and clinical research, as well as the existence of certain interventions that can increase muscle mass and appetite stimulation and also reduce inflammation, there are currently no drugs approved for the prevention or treatment of this cancer syndrome. Also, validated biomarkers relevant to the underlying pathogenesis, which could work as diagnostic tools, are much needed.

The aim of the study was to identify and validate new putative diagnostic, prognostic or therapeutic biomarkers for cancer cachexia. Two-dimensional electrophoresis, mass spectrometry and western blotting were applied on skeletal muscle samples from cancer patients with and without cachexia in order to find and, thereafter, validate the candidate protein galectin-1. By means of western blotting, microscopy and creatine kinase activity assays, a cancer cachexia mouse model ($Apc^{Min/+}$) and a cell line model of muscle wasting (C2C12) were used to follow the regulation of the putative biomarker during the process of skeletal muscle atrophy.

Furthermore, after cloning, expressing and purifying galectin-1, the addition of recombinant protein to atrophied C2C12 cells aimed to postulate a role of the protein in the regeneration of myotubes after atrophy.

2. MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Clinical Specimens

2.1.1. Preparation

Nineteen patients with a ductal adenocarcinoma of the pancreas, 10 without cachexia (definition: no weight loss or weight loss less than 10% of pre illness stable body weight within 6 months) and 9 with cachexia (definition: weight loss exceeding 10% of pre-illness stable body weight within 6 months). Nine patients were operated on in the Department of Surgery, Heidelberg, 10 patients in the Department of Surgery, Klinikum rechts der Isar, Munich. The patients were asked to give a written, informed consent for data acquisition and follow up data (Table 4); furthermore the patients were asked for muscle (rectus abdominis, Fig. 12) and fat tissue collection for research. Every patient was asked for his height and current weight. When a patient gave a history of weight loss, the corresponding time period was also documented.

Table 4. Characteristics of 19 patients with ductal adenocarcinoma of the pancreas.

all patients **N= 19**		N [%]
gender	male	13 [68.4]
	female	6 [31.6]
age	median [lq/uq]	71 [58/73]
cachexia	no	10 [52.6]
	yes	9 [47.4]
weight loss [%]	median [lq/uq]	7 [0/15]
Tumor resection	no	7 [36.8]
	yes	12 [63.2]

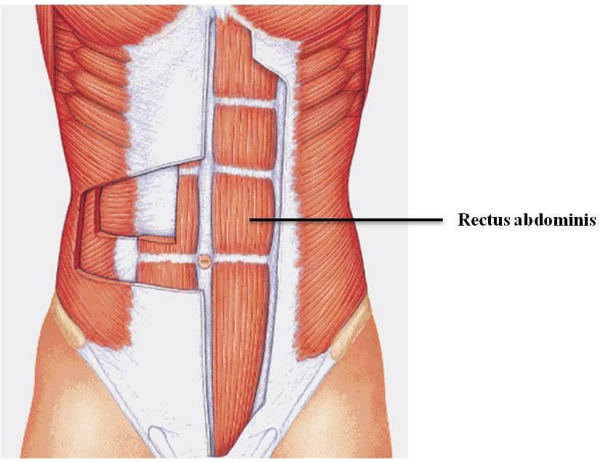

Figure 12. Localization of the rectus abdominis muscle within trunk of the human body (Addison Wesley Longman Inc.).

A patient was classified as cachectic when weight loss exceeded 10% of the stable pre-illness weight (Table 5). The study was performed according to the guidelines of the Declaration of Helsinki. Two independent pathologists from the University of Heidelberg, Department of Pathology and University of Munich, respectively, confirmed the diagnosis of the resected pancreatic tissue. After opening the skin and the subcutaneous layer, the fascia of the rectus abdominis muscle was opened and a specimen of about 1 cm^3 was resected with a scalpel. The tissue was immediately frozen in liquid nitrogen. Statistical analysis was performed using SPSS software, version 19 (SPSS Inc., Chicago, IL, USA). Results are displayed in median [with lower and upper quartile]. For testing significant differences between the examined groups, we used Student's t-test and the Mann-Whitney U test. A significance level < 0.05 was used.

Table 5. Characteristics of 10 patients with pancreatic cancer without cachexia and 9 patients with pancreatic cancer with cachexia.

		Ø cachexia N= 10	cachexia N= 9	P value
weight loss	% median [lq/uq]	0 [0/3]	15 [13/17]	<0.001
Resection rate		7 [70]	5 [55.6]	0.526
Body mass index	Median [lq/uq]	23.8 [21.7/25.1]	25.4 [24.5/26.8]	0.191

2.1.2. Two-dimensional electrophoresis and MALDI-TOF/TOF

Two-Dimensional Electrophoresis (2-DE) and Data Analysis

Samples were frozen in liquid nitrogen, crushed and then homogenized in a solubilization buffer (7 M urea, 2M Thiourea, 4% (w/v) CHAPS, 40mM DTT, and 2% (v/v) IPG buffer). The samples were vortexed and sonicated (30% of power) on ice, three times for 10 s and then centrifuged for 30 min at 14,000 x g at 4°C. The supernatants were stored at -80°C until analysis and protein concentration was measured using PlusOne 2-D Quant Kit. Rehydration of Immobiline Dry Strips with 30 μg protein sample was carried out with an IPGbox (GE Healthcare) according to the manufacturer's instructions and IPG strips (pH 3−10), 13 cm, were used. The rehydrated strips were then subjected to isoelectric focusing which was performed using Multiphor II electrophoresis unit (GE Healthcare) at 20°C. Briefly, 13 cm strips were focused at 300 V for 30 min, 600 V for 30 min, 1000 V for 1 h, 1500 V for 1h and 3500 V for 4 h. After focusing, the strips were stored at -80 °C for later use. Prior to the second dimension SDS-PAGE, IPG strips were equilibrated for 20 min in a 10 mL equilibration solution containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, and traces of bromophenol blue with 1% DTT. A second step was carried out for 15 min by adding 2.5% iodoacetamide instead of DTT in the equilibration solution. Second dimension SDS-PAGE was performed using 12.5% acrylamide gels (20 x 20 cm) in a large vertical unit (Biostep). Electrophoresis was performed at a constant voltage of

100 V for 30 min followed by 130-170 V for about 5 h until the bromophenol band had exited the gel (Fig. 13).

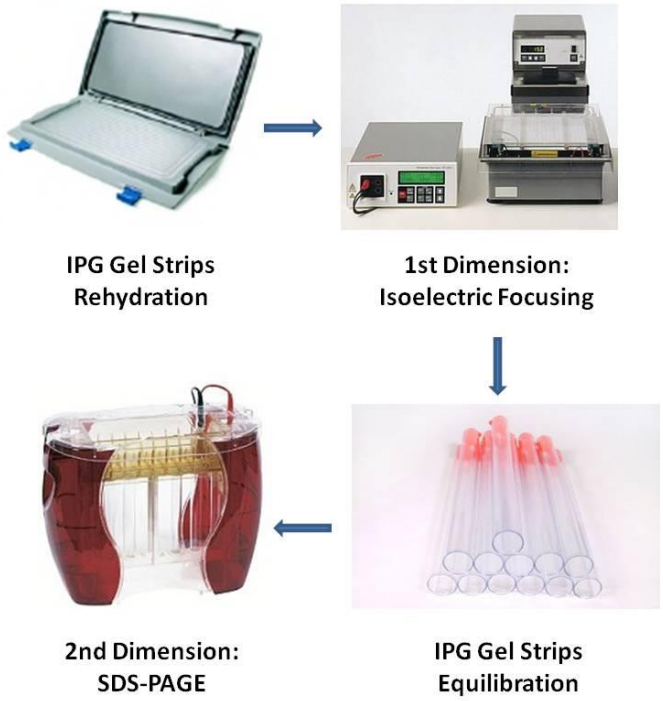

Figure 13. Main steps during 2-DE procedure. IPG gel strips are rehydrated in a IPGbox, thereafter 1st dimension (IEF) takes place in a Multiphor II electrophoresis unit. IPG gel strips are then equilibrated in tubes containing equilibrating solution and then 2nd dimension (SDS-PAGE) is done in a large vertical unit (www.gelifesciences.com and www.serva.de).

After this, gels were stained using a MS-compatible silver staining (Table 6) and the corresponding images were obtained with a scanner (Epson Perfection 4990) in gray-scale mode. Image analyses were performed with Redfin 3 software (Ludesi) and a combination of $p < 0.05$ and a 1.5-fold expression change were used as cut-off criteria. At least three gels were performed for each sample.

Table 6. MS-compatible silver staining protocol (adapted from Shevchenko et al., 1996).

Steps	Components and quantity (500 mL)	Procedure
Fixation	Methanol 250 mL Glacial acetic acid 60 mL Formaldehyde (37% w/v) 0.25 mL Water to 500 mL	2 x 30 min.
Washing	Ethanol 250 mL Water to 500 mL	3 x 20 min.
Sensitizing	Sodium thiosulphate (5% w/v) 5 mL Water to 500 mL (take 12,5 mL)	1 min.
Washing	Water 500 mL	fast
Silver reaction	Silver nitrate sol.(2.5% w/v) 75 mL Formaldehyde (37% w/v) 0,375 mL Water to 500 mL	20 min.
Washing	Water 500 mL	fast
Developing	Sodium carbonate 30 g Sensitizing sol. 12,5 mL Water to 500 mL Stir vigorously Formaldehyde (37% w/v) 0.250 mL	0-10 min.
Stopping	Methanol 250 mL Glacial acetic acid 60 mL Water to 500 mL	10 min.

Protein Identification by MALDI-TOF/TOF

Protein spots were manually cut, destained and washed with buffer containing 50 mM NH_4HCO_3 in 30% ACN and equilibrated in 10 mM NH_4HCO_3 prior to proteolytic digestion. Gel pieces were shrunk with 100% v/v ACN and rehydrated in 10 mM NH_4HCO_3. This treatment was repeated, followed by the addition of 0.1-0.2 µg of modified trypsin (Sigma, Germany) per piece. Digestion was carried out overnight at 37°C. The supernatant was collected and combined with the eluates of subsequent elution steps with 80% v/v ACN, 1% v/v TFA. The combined eluates were dried in a SpeedVac centrifuge. The dry samples were dissolved in 20 µl 50% v/v ACN, 0.1% v/v TFA for the subsequent MALDI preparation. Therefore 0.5µl of a 1:1 mixture of sample and a matrix solution consisting of 5 mg/mL CHCA were spotted on a MALDI target. Mass spectra were acquired using a Proteomics Analyzer 4700 (MALDI-TOF/TOF) mass spectrometer (Applied Biosystems) (Fig. 14).

Figure 14. Proteomics Analyzer 4700 (MALDI-TOF/TOF) mass spectrometer from Applied Biosystems (www.helmholtz-muenchen.de/proteomics).

Measurements were performed with a 355 nm Nb:YAG laser in positive reflector mode with a 20 kV acceleration voltage. For each MS and MS/MS spectrum, 3000 shots were accumulated. For each spot on a MALDI plate the eight most intense peptides were selected for additional MS/MS analysis. The acquired MS/MS spectra were searched against the UniRef100 databases using an in-house version of Mascot with the following parameters: As taxon human was chosen and as enzyme trypsin. Carbamidomethylation was set as fixed modification and oxidized methionine as variable modifications. The GPS Explorer 2 software reports two different scores: The Mascot best ion score, the highest score of a single

peptide, and a total ion score, the sum of all peptide scores of one protein. The significance level for a peptide score is usually higher than 20 and for a protein score higher than 50-60. Because different database searches have different Mascot significance levels, due to different databases sizes and different numbers of masses submitted for a search, scores cannot be compared directly. For this reason, the software calculates a confidence interval from Mascot protein scores or ion scores, and the Mascot significance level for each search is defined as the 95% confidence level. Therefore, the total ion score confidence level is a reliable and comparable parameter for the significance of a database search.

2.1.3. Immunoblotting

Skeletal muscle samples were homogenized in a protein lysis buffer (20 mM Hepes, 1 mM EDTA, 50 mM β-glycerophosphate, 10% glycerol and 1% triton X-100, pH 7.4). For myotubes lysis, SDS lysis buffer was used (50 mM Tris-HCl and 2% SDS, pH 7.4). To both lysis buffers were freshly added 0.1 mM DTT, 0.1 mM Na_3VO_4 and Complete Mini protease inhibitor cocktail. The mixtures were kept on ice for 30 min, centrifuged at 14,000 g for 15 min at 4°C and the supernatant was kept. Protein concentration was measured by BCA protein assay kit. Aliquots containing 10 µg of protein were heat-denaturated in sample-loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol and traces of bromophenol blue) separated by SDS-PAGE and blotted onto nitrocellulose membranes. The membranes were blocked with phosphate buffered saline (PBS), containing 0.1% Tween and 5% non-fat dry milk, and incubated overnight with primary antibody at 4°C. Membranes were then incubated with secondary horseradish peroxidase-conjugated antibody for 1 h. The membrane-bound immune complexes were detected by an ECL system. Protein loading was normalized according to beta-tubulin expression and quantification of the bands was performed using Quantity One (Bio-Rad).

2.2. Mouse model

2.2.1. Skeletal muscle samples

Apc$^{Min/+}$ mice muscle tissues were kindly provided by Dr. E. Burgermeister from the 2nd Medical Department, Klinikum rechts der Isar, TU München, Munich (current affiliation: 2nd Medical Department, Universitätsmedizin Mannheim).

Apc$^{Min/+}$ mice were sacrificed at the ages of 4, 5 and 6 months and vastus lateralis muscle (Fig. 15) was excised, snap frozen in liquid nitrogen and stored at -80°C until further analysis.

Figure 15. Localization of the vastus lateralis muscle within the upper leg and an *Apc*$^{Min/+}$ mouse (www.vastuslateralis.com and www.jax.org).

2.2.2. Immunoblotting

See section 2.1.3.

2.3. *In vitro* models

2.3.1. Cell culture

C2C12 mouse myoblast cell line was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Myoblasts were grown in DMEM supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37ºC in the presence

of 5% CO_2 and all cells were used until passage 20. To stimulate differentiation, growth medium (GM) was replaced by differentiation medium (DM) consisting of DMEM, 2% heat-inactivated horse serum, penicillin (100 U/mL) and streptomycin (100 µg/mL). DM was replaced every day and the time point at which differentiation is induced is referred to as day 0 (D0). (Fig. 16).

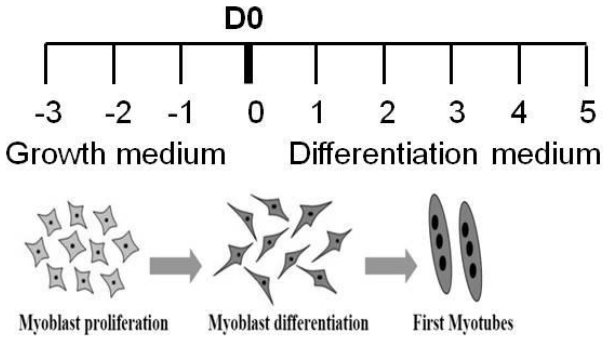

Figure 16. Experimental overview of differentiation: C2C12 myoblasts grow to about 80-90% confluence (D0), and differentiation into myotubes is induced when GM is replaced with DM.

For atrophy and regeneration experiments, the concentration of chemicals used was 10 ng/ml IGF-1 and 100 µM Dex. IGF-1 was administered on D2 post-fusion, while Dex on D3 (Fig. 17). Ten ng/ml IGF-1 induces hypertrophy in C2C12 myotubes after 48 h of IGF-1 treatment (D2:48h) and 100 µM Dex induces atrophy in C2C12 myotubes after treatment for 24 h (D3:24h) (Latres et al., 2005).

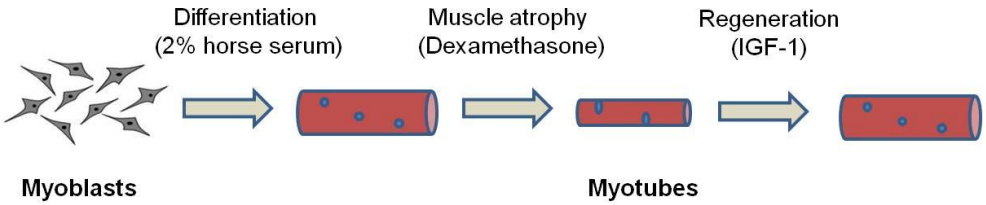

Figure 17. Experimental overview of induced-atrophy and -hypertrophy: C2C12 myoblasts differentiate into myotubes and these are atrophied after exposure to Dex. When myotubes are exposed to IGF-1, hypertrophy or regeneration takes place.

For mTOR signaling inhibition using RAD001, 100 nm RAD001 was added to DM every day starting at D0 (Willett et al., 2009) and for experiments using recombinant galectin-1, 100 µM of this protein was added to the medium.

To quantify the myotube diameter, 10 fields were chosen randomly and 10 myotubes were measured per field at ×100 magnification. The average diameter per myotube was the mean of 10 measurements taken along the length of the myotube.

All experiments were performed at least three times with three replicates, except for the experiments using conditioned medium and recombinant galectin-1, where two replicates were performed.

2.3.2. Immunoblotting

See section 2.1.3.

2.3.3. Protein extraction from Conditioned Medium

The conditioned medium (CM) was collected and filtered with a 0.2 µm syringe filter. Proteins in the CM were precipitated by acetone for 60 min at -20°C and pelleted by centrifugation at 14,000 g for 10 min. The pellet was air-dried to allow acetone to evaporate and dissolved in SDS-loading buffer. Then it was concentrated using a Microcon YM-3 centrifugal filter at 14,000 g for 30 min at RT and protein concentration was determined using the BCA assay.

2.3.4. Creatine kinase (CK) activity

CK activity was determined in cell lysates following the protocol supplied by the manufacturer (BioAssay Systems). This assay is based on enzyme-coupled reactions in which creatine phosphate and ADP is converted to creatine and ATP by CK. The generated ATP is used to phosphorylate glucose by hexokinase to generate glucose-6-phosphate, which is then oxidized by NADP in the presence of glucose-6-phosphate dehydrogenase. The resulting NADPH, measured at 340 nm, is proportionate to the CK activity in the sample (Fig. 18). Unit (U) is defined as one unit of CK that transfers 1 µmole of phosphate from phosphocreatine to ADP per minute at pH 6.0.

All experiments were performed at least three times with three replicates.

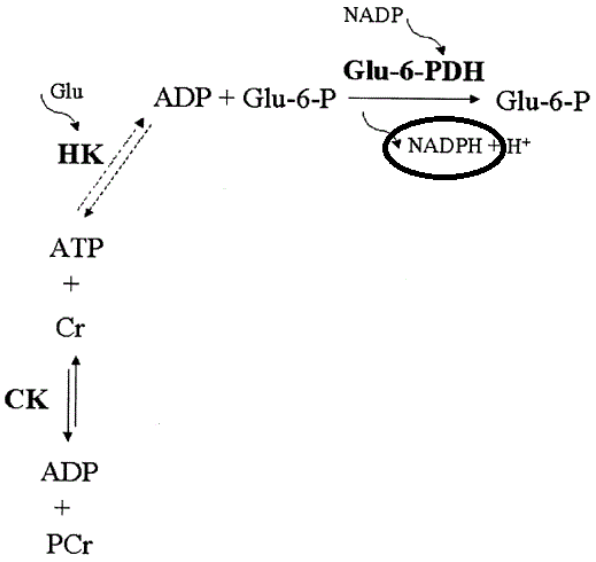

Figure 18. Scheme of the enzyme-coupled reactions taken place at the CK assay showing when NADPH is produced (http://journals.cambridge.org).

2.4. Cloning, expression and purification of galectin-1

Cloning

The open reading frame of the mouse galectin-1 gene was PCR amplified using Pfu polymerase and E.coli chromosomal DNA as the template (Table 7). The following primers were used: *gal1*-forward containing a *Bsa*I restriction site (underlined): 5'-GATCGGTCTCACATGGCCTGTGGTCTGGTCG-3' and *gal1*-reverse containing an *Xho*I restriction site (underlined) and two STOP codons (in bold): 5'-GATCCTCGAG**TTATCA**CTCAAAGGCCACGCAC-3'.

Table 7. Reaction mixture used in the PCR amplification of the insert.

Volume (µl)	Reagent
5	10X Pfu polymerase buffer
0.5	5'-primer (100 pmol/µl)
0.5	3'-primer (100 pmol/µl)
1	dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP)
1	DNA template
1	Pfu DNA polymerase (2.5 units/ µl)
41	Sterile water

The thermal cycle was as follows: denaturation at 95°C for 2 min; 30 amplification cycles of denaturation 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C of 10 min. The PCR product was digested with *Bsa*I (1 h at 50°C) and *Xho*I (2 h at 37°C) and ligated into the *Nco*I/*Xho*I digested and dephosphorylated pETM-11 (Fig. 19). The resulting construct, pETM-11/gal1, was checked by double stranded DNA sequencing.

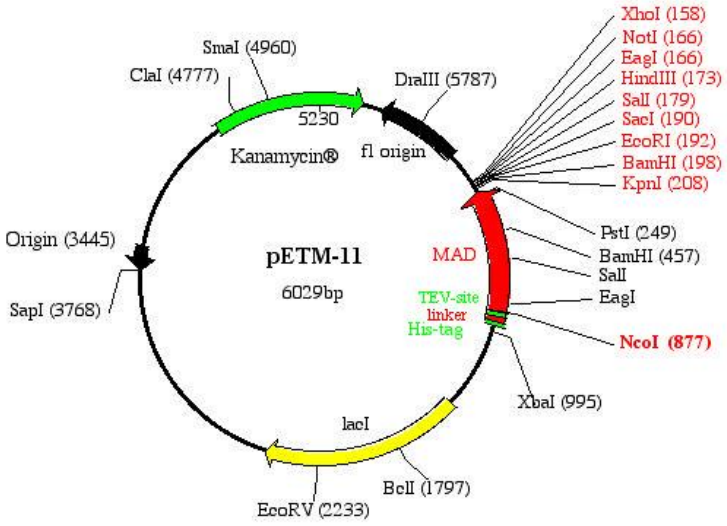

Figure 19. pETM-11, an EMBL-made expression vector harbouring an N-terminal His_6-tag followed by a TEV protease cleavage site. Kindly provided by Dr. A. Geerlof, Institute of Structural Biology, HelmholtzZentrum München, Munich.

pETM-11

Full length: 6029 bp

Composition: 1388 A; 1571 C; 1598 G; 1472 T; 0 OTHER

Percentage: 23% A; 26% C; 27% G; 24% T; 0% OTHER

Molecular Weight (kDa): ssDNA: 1861.69 dsDNA: 3717.0

ORIGIN

1 ATCCGGATAT AGTTCCTCCT TTCAGCAAAA AACCCCTCAA GACCCGTTTA GAGGCCCCAA

61 GGGGTTATGC TAGTTATTGC TCAGCGGTGG CAGCAGCCAA CTCAGCTTCC TTTCGGGCTT

121 TGTTAGCAGC CGGATCTCAG TGGTGGTGGT GGTGGTGCTC GAGTGCGGCC GCAAGCTTGT

181 CGACGGAGCT CGAATTCGGA TCCGGTACCA CTAGTTAGAG ACCAAGACAC GCCTTGTGAC

241 TGTCCTGCAG CTTTATTCTC TTGATGCTGG TGCTGGAATA GCCCTCATCA CTGCCGAGGC

301 TCTGCATGCT GCCCCGCTCG TCAGAGTCGC TCACACTGCT GCTGCTCCAG TCCAGATCAC

361 CTGTGAGATA GTCCGTGCTC TCCACGTCAA CGTCGATTTC TTCCCTGTCG GAGTCGGAGC

421 GCTCCGAGGA GACGGTGGAG CCGATGCTGT CCATCCGGAT CCTCTCAATG CCCAGCTTCT

481 CCAGCTGCCT CTTCAGGTGT CGCTGCTCTC GCTGAAGCTG GTCGATTTGG TGAACGGCTT

541 TTCTGTCACA ATCTTCAAGT TTCTTTATGT GCAATTTGGC TTTTGTTAAT AAACTCAACG

601 TAGTGTGTCG ACTTGATTCG GGTCCCAGTG GCACCAGCCC CTTCAACTTC TCCAGGCACA

661 AGCGAAGATG AGCCCGTCTA TTCTTCTCCA TTTCATTGTG AGTTGATCTG CTACTGCTGT

721 TATTCTTTTT GGATTTGTTC CTCCGTTTTA AGGCATCTCT GTCCTTGTTT TTGTATGGTA

781 ACATGGAGGC ATAACCATGT TCAGCTTCTC TCTCCCGCCG CTCCAGATAG TCGGCCGCCT

841 CCAGCAGCAT CTGGATGTTC ATCCGAACCG CCGCCGCCAT GGCGCCCTGA AAATAAAGAT

901 TCTCAGTAGT GGGGATGTCG TAATCGCTCA TGGGGTGATG GTGATGGTGA TGTTTCATGG

961 TATATCTCCT TCTTAAAGTT AAATCAAAAT TATTTCTAGA GGGGAATTGT TATCCGCTCA

1021 CAATTCCCCT ATAGTGAGTC GTATTAATTT CGCGGGATCG AGATCTCGAT CCTCTACGCC

1081 GGACGCATCG TGGCCGGCAT CACCGGCGCC ACAGGTGCGG TTGCTGGCGC CTATATCGCC

1141 GACATCACCG ATGGGGAAGA TCGGGCTCGC CACTTCGGGC TCATGAGCGC TTGTTTCGGC

1201 GTGGGTATGG TGGCAGGCCC CGTGGCCGGG GGACTGTTGG GCGCCATCTC CTTGCATGCA

1261 CCATTCCTTG CGGCGGCGGT GCTCAACGGC CTCAACCTAC TACTGGGCTG CTTCCTAATG

1321 CAGGAGTCGC ATAAGGGAGA GCGTCGAGAT CCCGGACACC ATCGAATGGC GCAAAACCTT

1381 TCGCGGTATG GCATGATAGC GCCCGGAAGA GAGTCAATTC AGGGTGGTGA ATGTGAAACC

1441 AGTAACGTTA TACGATGTCG CAGAGTATGC CGGTGTCTCT TATCAGACCG TTTCCCGCGT

1501 GGTGAACCAG GCCAGCCACG TTTCTGCGAA AACGCGGGAA AAAGTGGAAG CGGCGATGGC

1561 GGAGCTGAAT TACATTCCCA ACCGCGTGGC ACAACAACTG GCGGGCAAAC AGTCGTTGCT

1621 GATTGGCGTT GCCACCTCCA GTCTGGCCCT GCACGCGCCG TCGCAAATTG TCGCGGCGAT

1681 TAAATCTCGC GCCGATCAAC TGGGTGCCAG CGTGGTGGTG TCGATGGTAG AACGAAGCGG

1741 CGTCGAAGCC TGTAAAGCGG CGGTGCACAA TCTTCTCGCG CAACGCGTCA GTGGGCTGAT

1801 CATTAACTAT CCGCTGGATG ACCAGGATGC CATTGCTGTG GAAGCTGCCT GCACTAATGT

1861 TCCGGCGTTA TTTCTTGATG TCTCTGACCA GACACCCATC AACAGTATTA TTTTCTCCCA

1921 TGAAGACGGT ACGCGACTGG GCGTGGAGCA TCTGGTCGCA TTGGGTCACC AGCAAATCGC

1981 GCTGTTAGCG GGCCCATTAA GTTCTGTCTC GGCGCGTCTG CGTCTGGCTG GCTGGCATAA

2041 ATATCTCACT CGCAATCAAA TTCAGCCGAT AGCGGAACGG GAAGGCGACT GGAGTGCCAT

2101 GTCCGGTTTT CAACAAACCA TGCAAATGCT GAATGAGGGC ATCGTTCCCA CTGCGATGCT

2161 GGTTGCCAAC GATCAGATGG CGCTGGGCGC AATGCGCGCC ATTACCGAGT CCGGGCTGCG

2221 CGTTGGTGCG GATATCTCGG TAGTGGGATA CGACGATACC GAAGACAGCT CATGTTATAT

2281 CCCGCCGTTA ACCACCATCA AACAGGATTT TCGCCTGCTG GGGCAAACCA GCGTGGACCG

2341 CTTGCTGCAA CTCTCTCAGG GCCAGGCGGT GAAGGGCAAT CAGCTGTTGC CCGTCTCACT

2401 GGTGAAAAGA AAAACCACCC TGGCGCCCAA TACGCAAACC GCCTCTCCCC GCGCGTTGGC

2461 CGATTCATTA ATGCAGCTGG CACGACAGGT TTCCCGACTG GAAAGCGGGC AGTGAGCGCA

2521 ACGCAATTAA TGTAAGTTAG CTCACTCATT AGGCACCGGG ATCTCGACCG ATGCCCTTGA

2581 GAGCCTTCAA CCCAGTCAGC TCCTTCCGGT GGGCGCGGGG CATGACTATC GTCGCCGCAC

2641 TTATGACTGT CTTCTTTATC ATGCAACTCG TAGGACAGGT GCCGGCAGCG CTCTGGGTCA

2701 TTTTCGGCGA GGACCGCTTT CGCTGGAGCG CGACGATGAT CGGCCTGTCG CTTGCGGTAT

2761 TCGGAATCTT GCACGCCCTC GCTCAAGCCT TCGTCACTGG TCCCGCCACC AAACGTTTCG

2821 GCGAGAAGCA GGCCATTATC GCCGGCATGG CGGCCCCACG GGTGCGCATG ATCGTGCTCC

2881 TGTCGTTGAG GACCCGGCTA GGCTGGCGGG GTTGCCTTAC TGGTTAGCAG AATGAATCAC

2941 CGATACGCGA GCGAACGTGA AGCGACTGCT GCTGCAAAAC GTCTGCGACC TGAGCAACAA

3001 CATGAATGGT CTTCGGTTTC CGTGTTTCGT AAAGTCTGGA AACGCGGAAG TCAGCGCCCT

3061 GCACCATTAT GTTCCGGATC TGCATCGCAG GATGCTGCTG GCTACCCTGT GGAACACCTA

3121 CATCTGTATT AACGAAGCGC TGGCATTGAC CCTGAGTGAT TTTTCTCTGG TCCCGCCGCA

3181 TCCATACCGC CAGTTGTTTA CCCTCACAAC GTTCCAGTAA CCGGGCATGT TCATCATCAG

3241 TAACCCGTAT CGTGAGCATC CTCTCTCGTT TCATCGGTAT CATTACCCCC ATGAACAGAA

3301 ATCCCCCTTA CACGGAGGCA TCAGTGACCA AACAGGAAAA AACCGCCCTT AACATGGCCC

3361 GCTTTATCAG AAGCCAGACA TTAACGCTTC TGGAGAAACT CAACGAGCTG GACGCGGATG

3421 AACAGGCAGA CATCTGTGAA TCGCTTCACG ACCACGCTGA TGAGCTTTAC CGCAGCTGCC

3481 TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG GAGACGGTCA

3541 CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG TCAGGGCGCG TCAGCGGGTG

3601 TTGGCGGGTG TCGGGGCGCA GCCATGACCC AGTCACGTAG CGATAGCGGA GTGTATACTG

3661 GCTTAACTAT GCGGCATCAG AGCAGATTGT ACTGAGAGTG CACCATATAT GCGGTGTGAA

3721 ATACCGCACA GATGCGTAAG GAGAAAATAC CGCATCAGGC GCTCTTCCGC TTCCTCGCTC

3781 ACTGACTCGC TGCGCTCGGT CGTTCGGCTG CGGCGAGCGG TATCAGCTCA CTCAAAGGCG

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4081 CTGCCGCTTA CCGGATACCT GTCCGCCTTT CTCCCTTCGG GAAGCGTGGC GCTTTCTCAT

4141 AGCTCACGCT GTAGGTATCT CAGTTCGGTG TAGGTCGTTC GCTCCAAGCT GGGCTGTGTG

4201 CACGAACCCC CCGTTCAGCC CGACCGCTGC GCCTTATCCG GTAACTATCG TCTTGAGTCC

4261 AACCCGGTAA GACACGACTT ATCGCCACTG GCAGCAGCCA CTGGTAACAG GATTAGCAGA

4321 GCGAGGTATG TAGGCGGTGC TACAGAGTTC TTGAAGTGGT GGCCTAACTA CGGCTACACT

4381 AGAAGGACAG TATTTGGTAT CTGCGCTCTG CTGAAGCCAG TTACCTTCGG AAAAAGAGTT

4441 GGTAGCTCTT GATCCGGCAA ACAAACCACC GCTGGTAGCG GTGGTTTTTT TGTTTGCAAG

4501 CAGCAGATTA CGCGCAGAAA AAAAGGATCT CAAGAAGATC CTTTGATCTT TTCTACGGGG

4561 TCTGACGCTC AGTGGAACGA AAACTCACGT TAAGGGATTT TGGTCATGAA CAATAAAACT

4621 GTCTGCTTAC ATAAACAGTA ATACAAGGGG TGTTATGAGC CATATTCAAC GGGAAACGTC

4681 TTGCTCTAGG CCGCGATTAA ATTCCAACAT GGATGCTGAT TTATATGGGT ATAAATGGGC

4741 TCGCGATAAT GTCGGGCAAT CAGGTGCGAC AATCTATCGA TTGTATGGGA AGCCCGATGC

4801 GCCAGAGTTG TTTCTGAAAC ATGGCAAAGG TAGCGTTGCC AATGATGTTA CAGATGAGAT

4861 GGTCAGACTA AACTGGCTGA CGGAATTTAT GCCTCTTCCG ACCATCAAGC ATTTTATCCG

4921 TACTCCTGAT GATGCATGGT TACTCACCAC TGCGATCCCC GGGAAAACAG CATTCCAGGT

4981 ATTAGAAGAA TATCCTGATT CAGGTGAAAA TATTGTTGAT GCGCTGGCAG TGTTCCTGCG

5041 CCGGTTGCAT TCGATTCCTG TTTGTAATTG TCCTTTTAAC AGCGATCGCG TATTTCGTCT

5101 CGCTCAGGCG CAATCACGAA TGAATAACGG TTTGGTTGAT GCGAGTGATT TTGATGACGA

5161 GCGTAATGGC TGGCCTGTTG AACAAGTCTG GAAAGAAATG CATAAACTTT TGCCATTCTC

5221 ACCGGATTCA GTCGTCACTC ATGGTGATTT CTCACTTGAT AACCTTATTT TTGACGAGGG

5281 GAAATTAATA GGTTGTATTG ATGTTGGACG AGTCGGAATC GCAGACCGAT ACCAGGATCT

5341 TGCCATCCTA TGGAACTGCC TCGGTGAGTT TTCTCCTTCA TTACAGAAAC GGCTTTTTCA

5401 AAAATATGGT ATTGATAATC CTGATATGAA TAAATTGCAG TTTCATTTGA TGCTCGATGA

5461 GTTTTTCTAA GAATTAATTC ATGAGCGGAT ACATATTTGA ATGTATTTAG AAAAATAAAC

5521 AAATAGGGGT TCCGCGCACA TTTCCCCGAA AAGTGCCACC TGAAATTGTA AACGTTAATA

5581 TTTTGTTAAA ATTCGCGTTA AATTTTTGTT AAATCAGCTC ATTTTTTAAC CAATAGGCCG

5641 AAATCGGCAA AATCCCTTAT AAATCAAAAG AATAGACCGA GATAGGGTTG AGTGTTGTTC

5701 CAGTTTGGAA CAAGAGTCCA CTATTAAAGA ACGTGGACTC CAACGTCAAA GGGCGAAAAA

5761 CCGTCTATCA GGGCGATGGC CCACTACGTG AACCATCACC CTAATCAAGT TTTTTGGGGT

5821 CGAGGTGCCG TAAAGCACTA AATCGGAACC CTAAAGGGAG CCCCCGATTT AGAGCTTGAC

5881 GGGGAAAGCC GGCGAACGTG GCGAGAAAGG AAGGGAAGAA AGCGAAAGGA GCGGGCGCTA

5941 GGGCGCTGGC AAGTGTAGCG GTCACGCTGC GCGTAACCAC CACACCCGCC GCGCTTAATG

6001 CGCCGCTACA GGGCGCGTCC CATTCGCCA

Expression and purification

pETM-11/gal1 was transformed into the *E. coli* expression strain Rosetta2 (DE3) pLysS and cultured at 20°C in 2-L flasks containing 500 ml ZYM 5052 auto-induction medium (Studier, 2005), 30 µg/ml kanamycin and 33 µg/ml chloramphenicol. Cells were harvested by centrifugation (9,000 x g for 20 min) after reaching saturation, resuspended in 30 ml lysis buffer (50mM Tris-HCl, 300mM NaCl, 20mM imidazole, 1mM AEBSF.HCl (serine protease inhibitor), 0.2% (v/v) NP-40, 0.02% (v/v) 1-thioglycerol, pH 8.0), and lysed by sonication 2 times for 3 min on ice-water using a Bandelin Sonopuls HD2200 (duty cycle 30%, amplitude 60%). The lysate was clarified by centrifugation at 40,000 x g and filtration over a 0.22 µM filter. The supernatant was applied to a 5 ml HiTrap Chelating HP column (GE Healthcare), equilibrated in binding buffer (50 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, 0.01% (v/v) 1-thioglycerol, pH 8.0) using an Äkta Explorer (GE Healthcare) (Fig. 20). The column was washed with binding buffer and binding buffer containing 50mM imidazole until a stable baseline was reached (monitored at 280nm). Bound proteins were eluted with elution buffer (50 mM Tris-HCl, 300 mM NaCl, 300 mM imidazole, 0.01% (v/v) 1-thioglycerol, pH 8.0) and fractions containing protein pooled and dialyzed overnight at 4°C against 1-L GF buffer (50mM Tris-HCl, 150 mM NaCl, and 0.01% (v/v) 1-thioglycerol, pH 8.0) in the presence of His_6-tagged TEV protease in a 1:50 molar ratio (TEV:protein). TEV-cleaved protein was further purified by affinity chromatography, as described above, and the flow-through and protein containing wash fractions were pooled and concentrated to less than 5 ml. This was subsequently subjected to size exclusion chromatography using a HiLoad 16/60 Superdex 200 column (GE Healthcare), equilibrated in GF buffer. The main elution peak containing galectin-1 was collected, concentrated to 1.90 mg/ml using centrifugal concentrators with a 3-kDa cut-off (Amicon), and stored in several aliquots at 4°C and 80ºC in 8 mM DTT.

Figure 20. Äkta Explorer with fixed columns (www.gelifesciences.com).

2.5. Data processing and statistical analysis

Data are represented as mean and standard deviation. Differences between experimental conditions were tested with one-way ANOVA (differences among at least three groups) or t test (differences among two groups) using Prism 5 software (GraphPad, San Diego, California, USA). Significances: * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$. Concerning 2-DE and MALDI-TOF/TOF data, see section 2.1.2.

3. RESULTS

3. RESULTS

3.1. Differentially expressed proteins in cancer cachexia and cancer non-cachexia human muscle tissue

Two-dimensional electrophoresis (2-DE) was performed with cachexia and non-cachexia skeletal muscle from 10 patients with pancreatic cancer (Fig. 21); but, prior to that, several conditions had to be optimized, especially regarding sample preparation and IEF. In terms of sample preparation it was very important to keep in mind throughout the whole process that the goal was a 2-DE result; this way, possible contaminations, lysis buffer and detergents choices were always taken into account. Considering IEF the most important aspect was to achieve optimal and reproducible focusing conditions (voltage and time). Although silver staining is a more complex and time-consuming procedure, it was used due to its higher sensitivity (comparing with coomassie staining), since the amount of sample was limited.

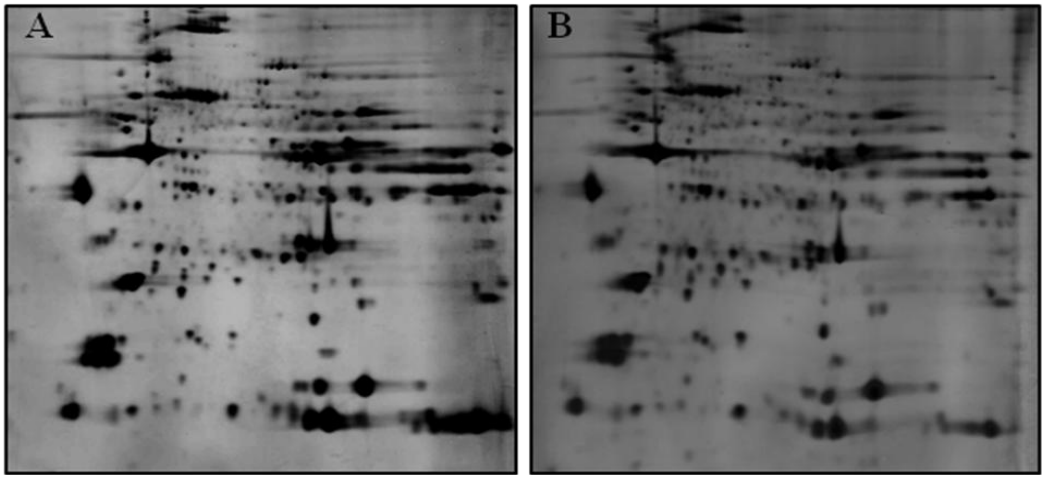

Figure 21. Representative 2-DE gels after silver-staining procedure. (A) Cancer cachexia. (B) Cancer non-cachexia.

After the optimization process and several analytical 2-DE (at least 3 replicates per sample), image analysis was performed using REDFIN 3 software, obtaining reproducible protein profiles (Fig. 22). Also, a Principal Components Analysis (PCA) plot was obtained

showing the good separation between the two groups and the inexistence of significant outliers (Fig. 23).

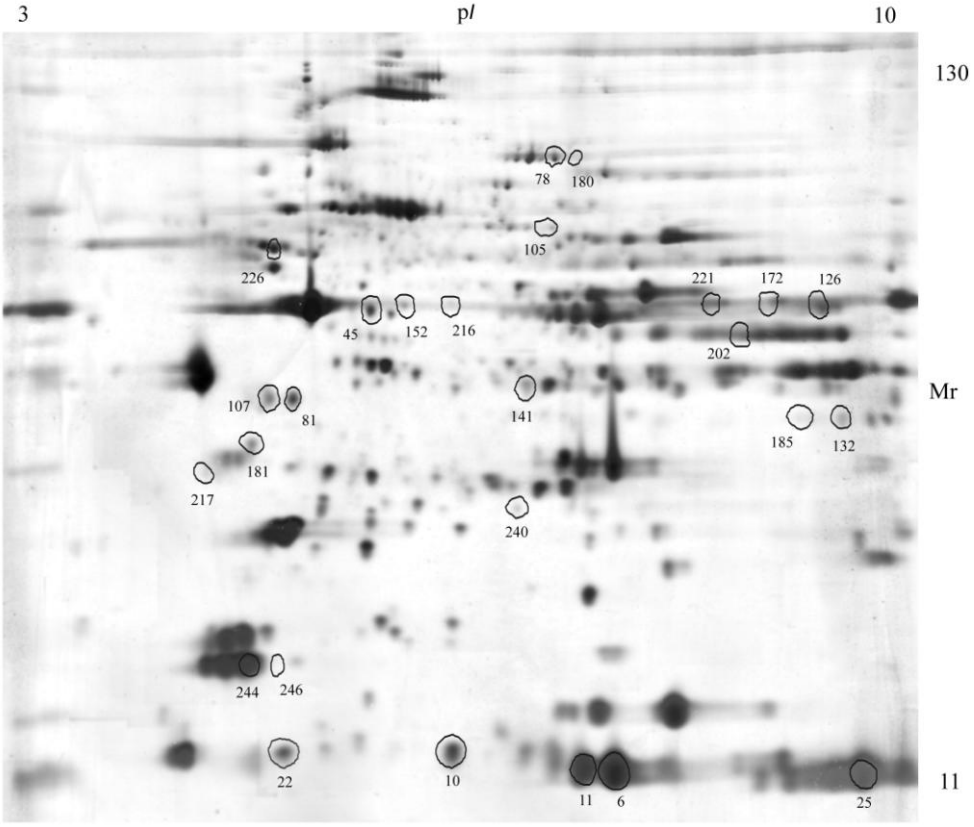

Figure 22. Representative 2-DE gel generated by REDFIN3 software highlighting differentially expressed proteins (p < 0.05) from cancer cachexia *versus* cancer non-cachexia patients. Protein extracts were resolved by 2-DE on a pH 3-10 non-linear gradient. Proteins were visualized by silver staining and twenty-six differentially expressed spots were excised for further identification.

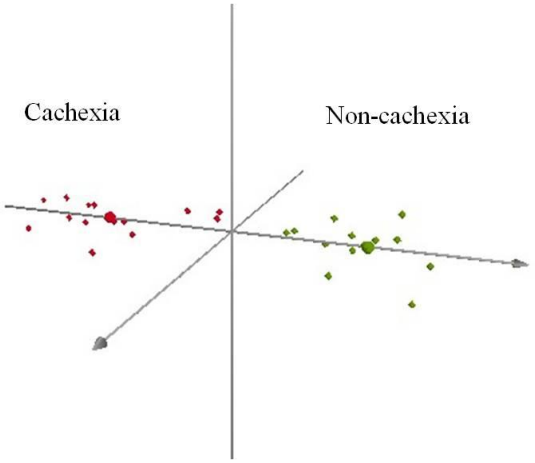

Figure 23. PCA of 2-DE data shows evidence of separation between cachexia and non-cachexia groups and the inexistence of significant outliers.

Differentially expressed proteins were defined as statistically significant based on the intensity alterations (>1.5-fold, Anova $p < 0.05$) and on the presence of each spot at least in 90% of the gels. Following these criteria, 26 spots were selected and analyzed using MALDI-TOF/TOF and from these, a total of 17 proteins were identified with statistical significance regarding the protein scores. They are listed in table 8.

Table 8. List of the identified differentially expressed proteins after 2-DE coupled with mass spectrometry analysis. P < 0.05 corresponds to Mascot Score > 63.

Gel Spot ID	Access. No.	Mw (kDa)	p*I*	Protein score	Sequence coverage (%)	(Unique) peptides matched	Highest Ion Score (MS/MS)	Fold change	Protein Description
226	P06576	56525	5.26	508	38	19	78	1.7	ATP synthase subunit beta
244	Q96A32	19116	4.91	370	51	12	75	2.2	Myosin regulatory light chain 2
22	P09382	15048	5.34	220	46	6	85	1.7	Galectin-1
10	Q6IBD7	14878	6.29	296	69	9	63	1.6	FABP3 protein
11	Q9UK54	14069	6.46	332	71	9	60	1.5	Hemoglobin beta subunit variant
6	Q4TWB7	11537	5.90	610	84	11	158	1.9	Beta globin (Fragment)
25	Q96T46	8386	6.63	244	82	4	136	1.7	Hemoglobin alpha 2 (Fragment)
180	B4DRW6	96356	7.94	255	36	27	24	-2	Phosphorylase
45	A8K3K1	42362	5.23	466	31	11	77	-1.7	cDNA FLJ78096
152	A8K3K1	42362	5.23	503	31	10	101	-1.6	cDNA FLJ78096
216	A8K3K1	42362	5.23	177	25	8	37	-1.9	cDNA FLJ78096
221	P22695	48584	8.74	52	21	7	16	-1.7	Cytochrome b-c1 complex subunit 2
202	P04075	39851	8.30	154	33	12	51	-1.7	Fructose-bisphosphate aldolase A
132	O75112-6	30980	9.20	145	30	7	68	-1.9	Isoform 6 LIM domain-binding protein 3
217	Q5VU59	27386	4.77	161	37	12	40	-2	Tropomyosin 3
240	P47985	29934	8.55	79	14	5	43	-2.4	Cytochrome b-c1 complex subunit 11
246	Q96A32	19116	4.91	93	51	9	40	-2.6	Myosin regulatory light chain 2

3.2. Overexpression of galectin-1 in human cancer cachexia

Examining table 8, gal-1 was up-regulated 1.7-fold in cancer cachexia compared with cancer non-cachexia and MS/MS analysis revealed six uniquely matched peptides with 46% sequence coverage and a Mascot score of 220. This protein was identified as gal-1 and chosen for further analysis, since it plays a role in muscle homeostasis and due to its putative protective action after tissue damage. In addition, there have been no studies, until now, relating this protein with muscle wasting in cancer cachexia.

Restoring the image analysis software, in order to get more information concerning gal-1, the statistically significant difference is shown graphically (Fig. 24A), as well as the 3-D images from the protein-corresponding spots (Fig. 24B) illustrating higher peaks in the cachexia group.

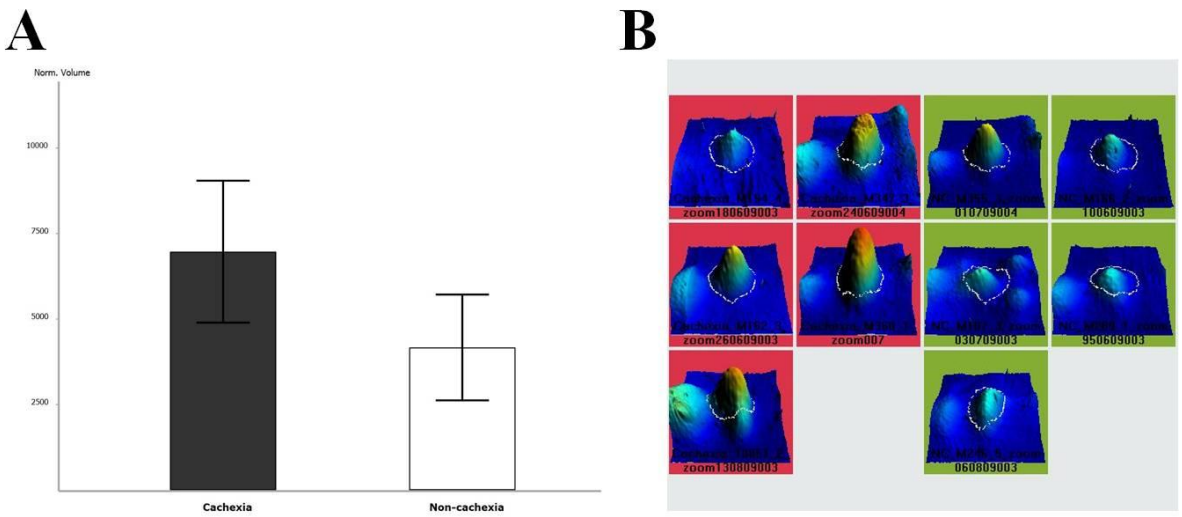

Figure 24. Gal-1-corresponding spots obtained during image analysis. (A) Comparing the two groups (cachexia and non-cachexia), a 1.7-fold (p-value 0.02, statistically significant) was obtained for gal-1. (B) 3-D images of representative gal-1 spots from the two groups (red background: cachexia; green background non-cachexia).

In order to confirm the overexpression of galectin-1 in cancer cachexia, western blotting analyses were performed using the samples that had been previously analyzed by 2-DE (Fig. 25A) plus nine additional patient samples, four corresponding to cachexia and five

to non-cachexia (Fig. 25B). These data show that galectin-1 is overexpressed in cancer cachexia muscle tissues, consistent with the observation made in the 2-DE analysis. The protein levels of the E3 ubiquitin ligase MuRF1, a mediator of muscle atrophy, were increased in cachectic patients, while expression of IGF-1 was decreased in cancer cachexia, results consistent with previous reports (Costelli et al., 2006).

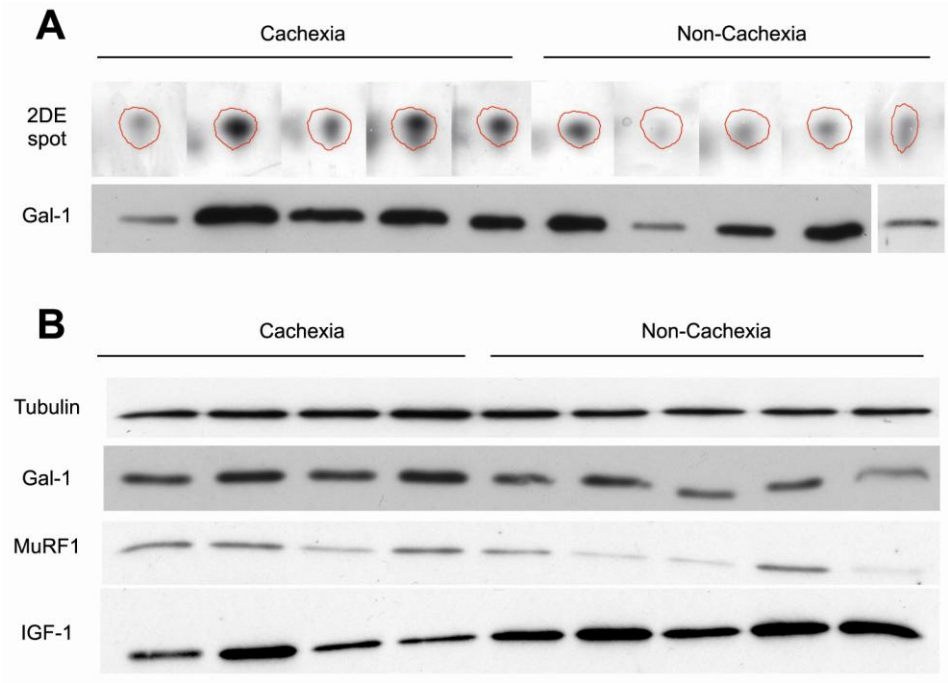

Figure 25. Validation of galectin-1 overexpression in human skeletal muscle by western blotting. (A) In human samples, galectin-1 2-DE spots expression matches with the one revealed by western blotting. (B) Further human samples confirm galectin-1 overexpression in cachectic patients, as also over- and underexpression of MuRF1 and IGF-1, respectively.

3.3. Galectin-1 overexpression in a cancer cachexia mouse model (*Apc*$^{Min/+}$)

Apc$^{Min/+}$ mice are heterozygotes for a mutation in the *Apc* tumor suppressor gene and spontaneously develop intestinal polyps at 4 weeks of age. Since the APC gene is mutated in a large percentage of human colon cancer cases, this is a common model for studying environmental factors that influence a genetic predisposition for colorectal cancer occurrence (Moser et al., 1990). The advantages of using the *Apc*$^{Min/+}$ mouse as a model of cachexia are the ratio of tumor mass to body mass, lack of anorexia, chronic inflammation and slow rate of

wasting, which are important characteristics of cachectic humans. These mice also demonstrate muscle weakness, fatigue, decreased volitional activity, and elevated circulating IL-6 levels. They develop this syndrome between 3 and 6 months of age (Baltgalvis et al., 2008).

Western blotting analyses were performed to evaluate galectin-1 expression in muscle tissue from these mice (Fig. 26).

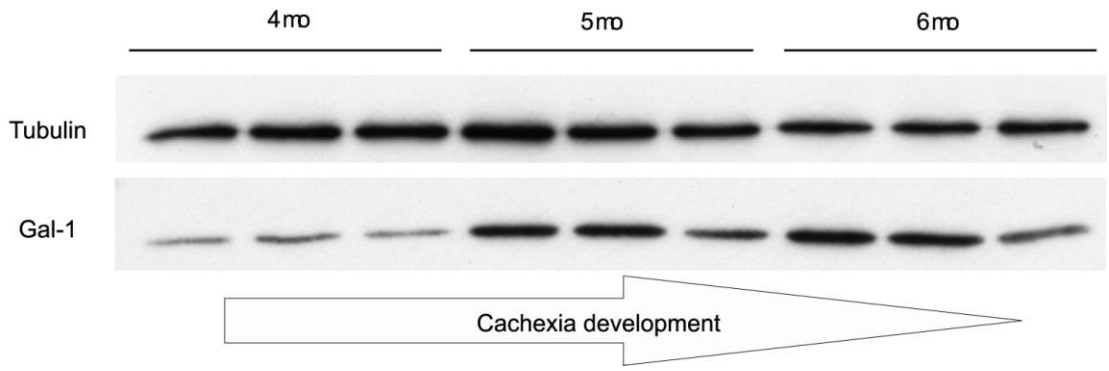

Figure 26. Galectin-1 protein overexpression observed in skeletal muscle tissue of a mouse model (*Apc*$^{Min/+}$), which develops cachexia between 3 and 6 months of age. Beta-tubulin was used as loading control.

These data show an increasing expression of galectin-1 as cachexia develops. Nine mice ranging from four to six months of age were used and their body weight changes were calculated (Table 9).

Table 9. Body weight and respective change in *Apc*$^{Min/+}$ mice grouped by age and cachexia development. 4 month-old mice were considered weight stable for this analysis. Body weight values are mean and standard deviation.

Group		Body Weight (g)	Change (%)
4 months (weight stable)	N = 3	25,30	0,00
5 months	N = 3	21,57	+ 14,74
6 months	N = 3	19,60	+ 22,53

3.4. Galectin-1 up-regulation after atrophy in a wasting cell line model

Dexamethasone-treated C2C12 myotubes are a well characterized model of muscle wasting (Latres et al., 2005). This model was used in order to follow the expression of galectin-1 throughout the development of muscle atrophy. Dex was used at day 3 for 24 hours (D3:24h). Galectin-1 was shown to be up-regulated in the first days of differentiation from myoblasts to myotubes (Fig. 27A). This up-regulation was observed immediately after Dex-induced atrophy at day 4 post-differentiation, but was more obvious at day 5 (Fig. 27B). As a biochemical control for this atrophy, MuRF1, a mediator of muscle atrophy, was assessed and its level increased after 24 hours of Dex treatment and decreased again the next day. Consistent with these results, IGF-1 levels were decreased after Dex treatment.

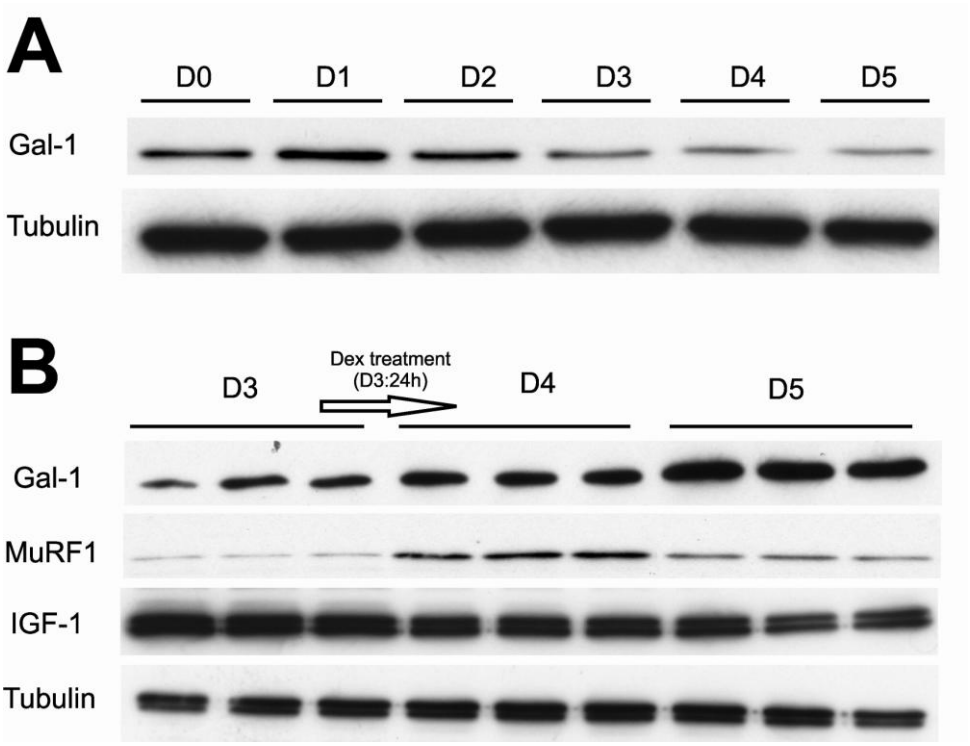

Figure 27. Regulation of galectin-1 during myotubes differentiation and dexamethasone treatment. (A) When C2C12 myoblasts differentiate into myotubes, galectin-1 is up-regulated in the first days of myogenesis. D0 is the day where growth medium is replaced by differentiation medium. (B) Galectin-1 is up-regulated at D4 after Dex-induced atrophy (D3 for 24 h) but especially at D5 after atrophy peak (at D4 revealed by MuRF1 expression). Dex treatment also down-regulates IGF-1 expression.

Finally, the cells were assayed for changes in myotubes diameters and the addition of Dex clearly resulted in an atrophic phenotype (Fig. 28).

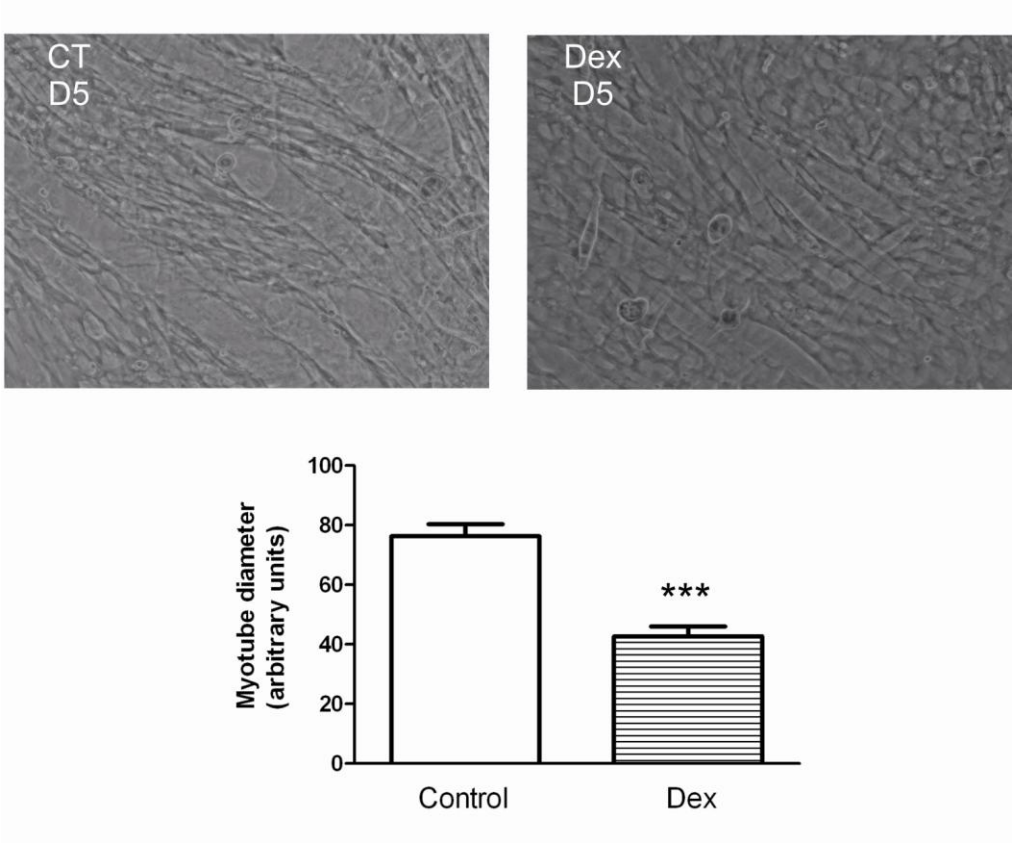

Figure 28. Microscope pictures taken from D5 myotubes cultivated in differentiation medium (control) and from myotubes treated with Dex at D3 for 24h. The diameters were measured and represented in the graph. Magnification of ×100.

3.5. Galectin-1 is down-regulated in hypertrophy and in IGF-1-induced regeneration after atrophy

IGF-1 was added to the medium at day 2 for 48 h (D2:48h) to cause hypertrophy in C2C12 myotubes (Latres et al., 2005). In this setting, galectin-1 expression decreased at day 5 compared with control cells, though not significantly. However, when compared with the already mentioned expression after Dex-induced atrophy (Fig. 27B), gal-1 levels decreased significantly (Fig. 29A). Myogenin expression, a marker for muscle differentiation, increased upon IGF-1 hypertrophic treatment**.** IGF-1 plays a central role in muscle regeneration. Thus,

in order to follow galectin-1 expression in IGF-1-induced regeneration after atrophy, myotubes were treated with Dex (D3:24h) and after this drug-induced atrophy, the same myotubes were treated with IGF-1 at day 5 for an additional 48 h. According to the expression of myogenin, the process of muscle differentiation decreased with Dex-induced atrophy but was re-established with IGF-1 treatment (Fig. 29B). Galectin-1 protein levels significantly decreased after IGF-1 treatment.

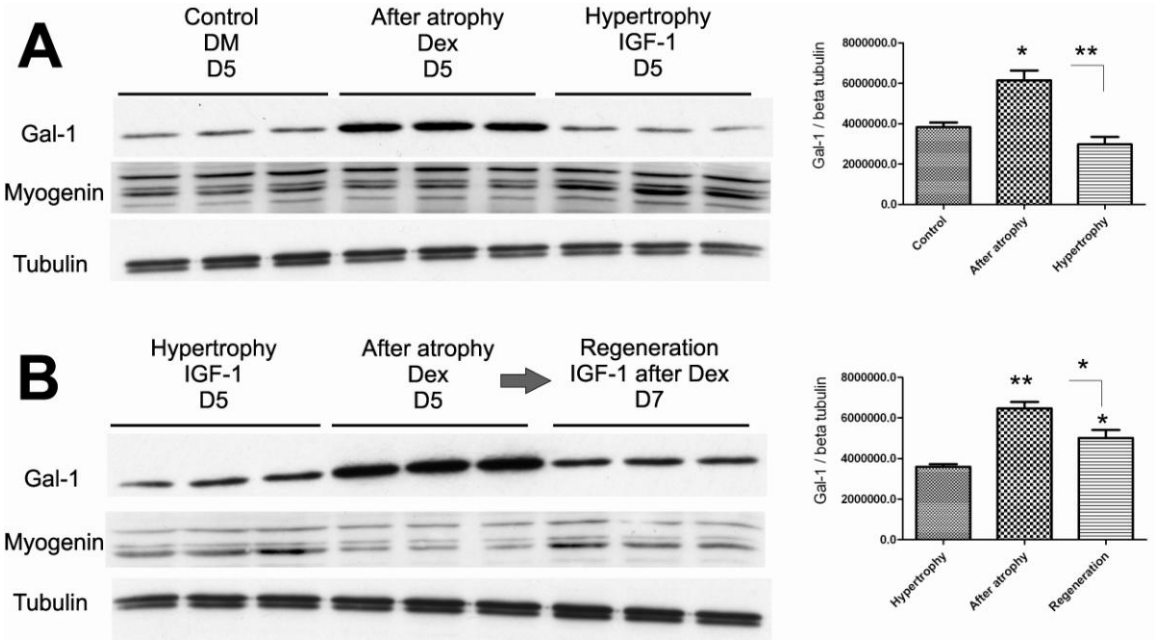

Figure 29. Regulation of galectin-1 in myotubes atrophy, hypertrophy and regeneration using IGF-1. (A) Galectin-1 is up-regulated at D5 after Dex-induced atrophy and down-regulated at D5 after IGF-1-induced hypertrophy in relation to control myotubes at the same day of post-differentiation. Myogenin expression reveals that IGF-1 stimulates myogenic differentiation. (B) At D5 after Dex-induced atrophy, 10 ng/ml IGF-1 was added to the medium for 48 h and at D7 the cells were harvested showing an up-regulation of myogenin and a down-regulation of galectin-1. The amount of galectin-1 was quantified by densitometric analysis and normalized by the amount of beta-tubulin.

3.6. Regulation of galectin-1 secretion during Dex-induced muscle atrophy

In order to follow how galectin-1 secretion is regulated during muscle atrophy, the conditioned medium of C2C12 myotubes was collected on several days post-differentiation and the expression levels of the secreted proteins was assessed (Fig. 30). Galectin-1 started to be detectable on day 4, both in the control medium and in the Dex-treated one. During Dex

treatment (D3:24h) gal-1 expression was very low on day 4, but considerably increased at day 5, consistent with the previously observed cells lysates (Fig. 27B). Since RAD001 inhibits C2C12 differentiation from myoblasts to myotubes (Willett et al., 2009), Gal-1 was not secreted when the myoblasts were treated with this drug because this protein is only secreted during the differentiation process.

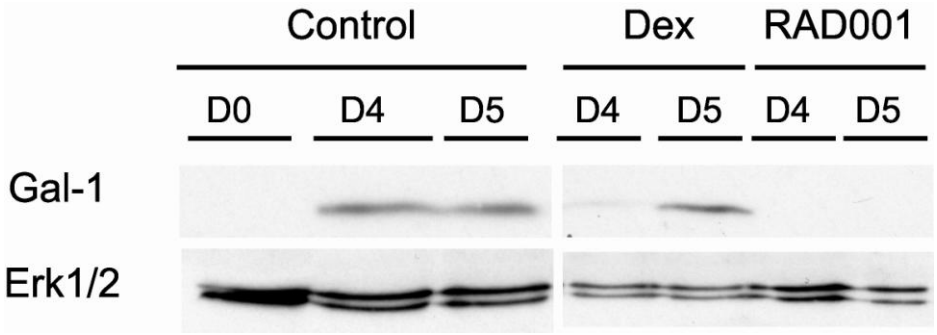

Figure 30. Regulation of galectin-1 secretion. Galectin-1 is not secreted at the beginning of myotubes differentiation and was only detected starting at D4. After Dex-induced atrophy, galectin-1 secretion was decreased at D4 and only increased again 24 h after Dex treatment (D5). When RAD001 was used, galectin-1 was not detected in the conditioned medium because this drug prevents myogenic differentiation of C2C12 myoblasts. Erk1/2 was used as a loading control.

3.7. Cloning, expression and purification of galectin-1

In order to further understand the role of gal-1 during muscle wasting and regeneration, cloning, expression and purification of this protein was undertaken. During the procedure of transformation of the expression plasmids into *E. coli* expression hosts, two different chemically competent cell lines were used: Rosetta2(DE3)pLysS and BL21 Star(DE3); this was done to co-express genes encoding for rare codon tRNAs that might lower the protein expression yield when they are not present. After transformation, the cells were cultivated in an auto-induction medium. Figure 31 shows the SDS-PAGE outcome of the protein expression after lysis of the cells.

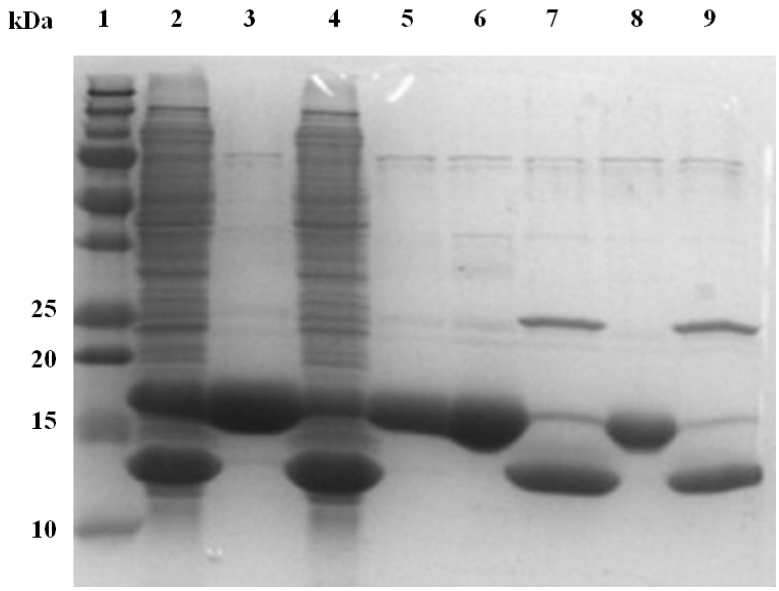

Figure 31. SDS-PAGE showing protein expression. The molecular weight of galectin-1 is 14.5 kDa and the TEV protease molecular weight is about 27 kDa. **Lanes**: 1 – MWM; 2 – RS supernatant; 3 – RS elute; 4 – R supernatant; 5 – R elute; 6 – RS elute; 7 – RS elute + TEV; 8 – R elute; 9 – R elute + TEV. **RS**: Rosetta2(DE3)pLysS strain; **R**: BL21 Star(DE3) strain.

It was decided to continue the further expression and purification of galectin-1 using the *E. coli* strain Rosetta2(DE3)pLysS because it showed a higher expression yield (lane 7, Fig. 31). This strain encodes for the following rare codon genes: AGG/AGA (arginine), CGG (arginine), AUA (isoleucine) CUA (leucine)CCC (proline), and GGA (glycine). After cleaving galectin-1 from TEV, the target protein was then purified by affinity chromatography

and the flow-through and protein containing wash fractions were pooled, concentrated and ran into a SDS-PAGE (Fig. 32).

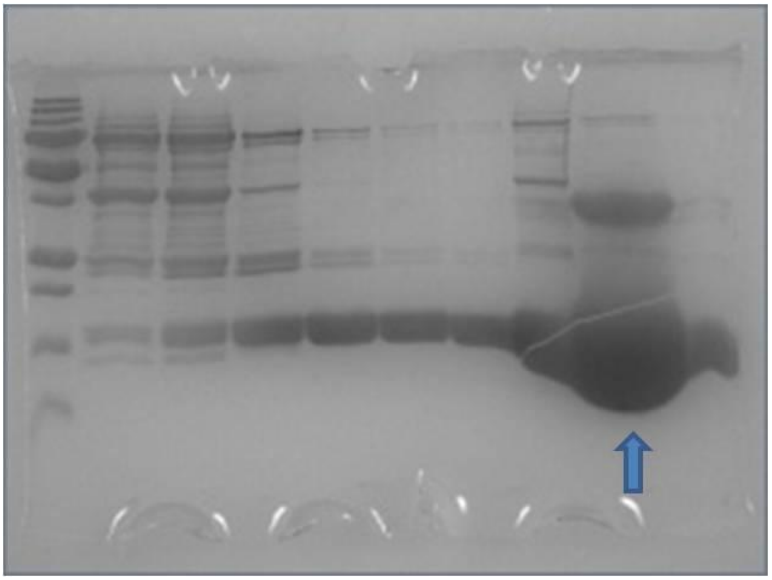

Figure 32. Galectin-1 purification by affinity chromatography. The expression was checked by means of SDS-PAGE followed by Coomassie Blue staining. The arrow indicates the fraction with the highest yield of purified galectin-1.

This was subsequently subjected to size exclusion chromatography to obtain an improved purification and the main elution peaks containing galectin-1 (Fig. 33) were collected and kept for further analysis.

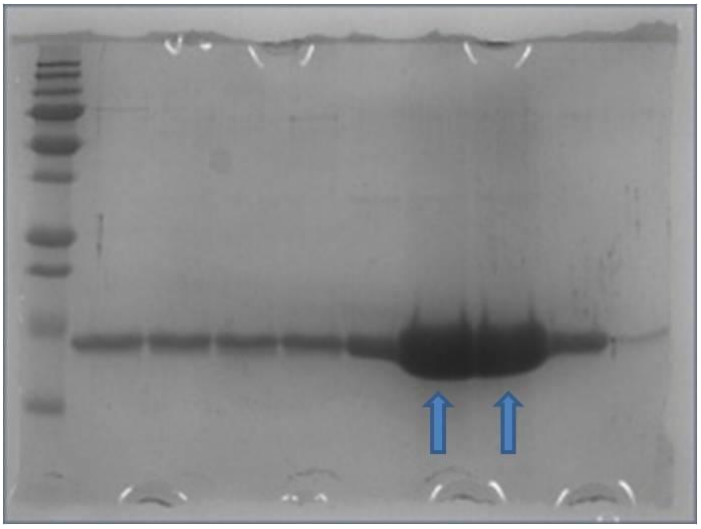

Figure 33. Galectin-1 purification by size exclusion chromatography. The expression was checked by means of SDS-PAGE followed by Coomassie Blue staining. The arrows point to the main elution peaks containing the target protein.

3.8. Recombinant galectin-1 up-regulates myogenin and CK activity after atrophy

To investigate the effect of recombinant galectin-1 after induction of atrophy, the protein levels of myogenin and the activity of creatine kinase were measured, since these proteins have an important role in the formation of new myotubes.

Initial experiments characterized the time frame of differentiation of C2C12 cells after replacement of GM with DM, by analysis of the activity of CK, an enzyme expressed in skeletal muscle whose levels increase with differentiation (Fig. 34). A small increase in CK activity was revealed in day 2, and a prominent growth observed after this day.

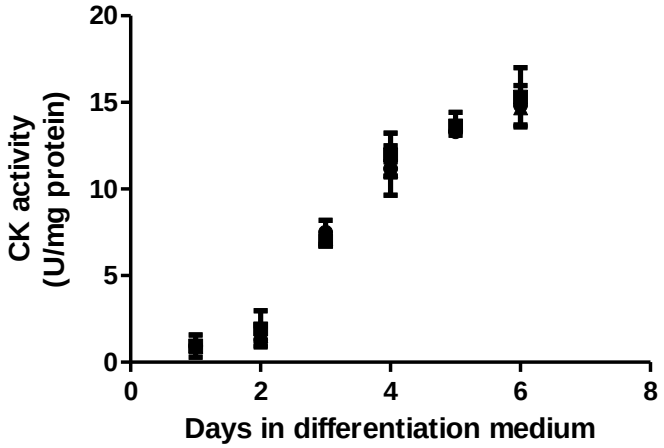

Figure 34. Assessment of the differentiation state of C2C12 cells by CK activity. Myoblasts were allowed to differentiate by switching GM to DM.

Dex was added to the medium (D3:24h) inducing muscle atrophy and down-regulated myogenin, at days 4 and 5 (Fig. 35A). After onset of atrophy, at day 4, recombinant galectin-1 was added for 12 and 24 hours. Addition of gal-1 increased expression of myogenin to the levels observed before Dex treatment. The protein levels of MuRF1 were decreased after galectin-1 treatment, as well. The addition of recombinant galectin-1 after atrophy also increased creatine kinase activity (Fig. 35B), indicating induction of regeneration.

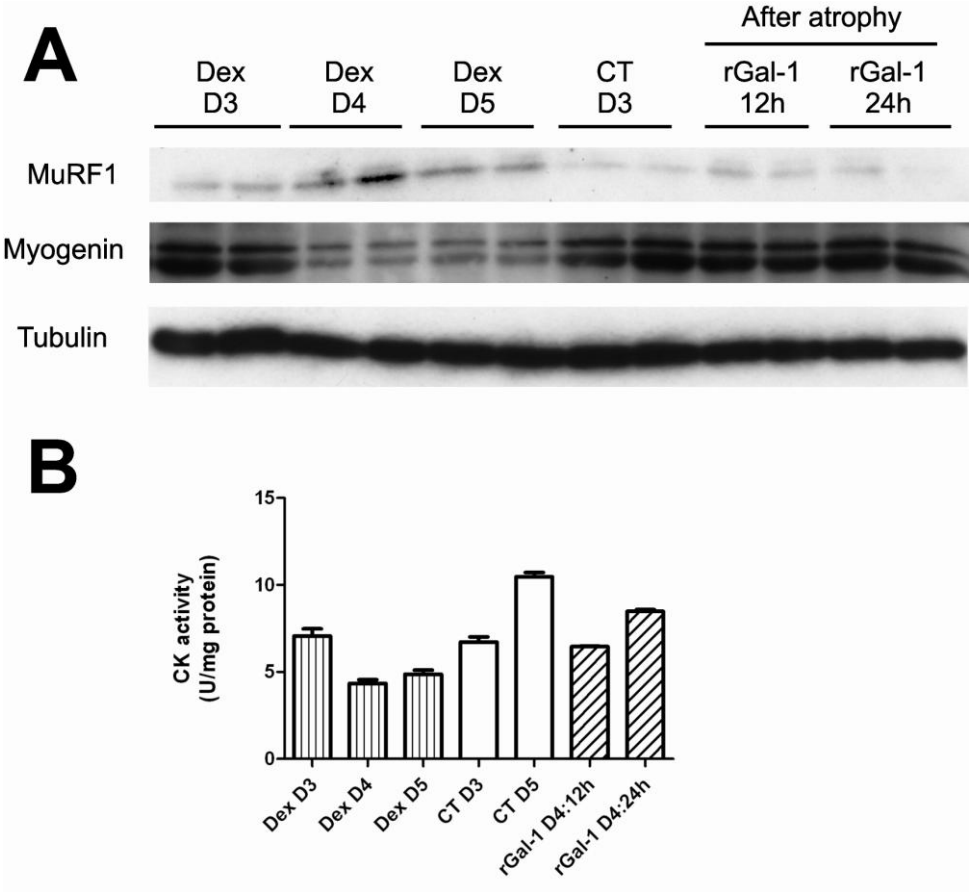

Figure 35. Effect of recombinant galectin-1 on myotubes differentiation after atrophy. (A) Dex was added to the medium at D3 for 24 h and induced the down-regulation of myogenin. When recombinant galectin-1 was added after atrophy at D4 for 12 and 24 h, MuRF1 was down-regulated and myogenin was up-regulated. (B) The addition of recombinant galectin-1 resulted in a stimulation of creatine kinase (CK) activity, indicating the induction of differentiation that was decreased after Dex-induced atrophy.

4. DISCUSSION

4. DISCUSSION

4.1. Skeletal muscle in cancer cachexia: overexpression of galectin-1

For the first time, a proteomic approach was applied in skeletal muscle tissue from cancer patients with and without cachexia, since the atrophy of this tissue is a hallmark of this cancer syndrome, with the aim to search for new putative diagnostic markers or therapeutic targets.

Since the introduction of two-dimensional electrophoresis, this method went through several modifications; after becoming a significant and widely used separation technique, it was found to be useful in detection of disease markers, monitoring therapies, drug discovery, cancer research and in general for monitoring differential protein expression in cells and tissues, since it enables screening a large number of proteins at the same time. Several biomarkers have already been discovered using this technique (Tambor et al., 2010).

For this study, it was first necessary to optimize the technique, especially concerning sample preparation, IEF conditions and silver staining. This was effectuated in order to achieve a higher resolution and reproducibility of the 2D gels. Subsequently, using 2-DE, 26 differentially expressed spots were found in muscle tissue from patients with cancer cachexia and cancer non-cachexia. As seen in the introduction above, the definition and classification of cachexia have always been and still are a matter of discussion. However, during this study, the definition of cachexia taken into account was "weight loss exceeding 10% of pre-illness stable body weight within 6 months," since it is the one used by the surgery departments whence the samples originally came.

Performing a MALDI-TOF/TOF analysis, 17 of these proteins were identified and galectin-1 was chosen for further evaluation mainly because (a) the expression of this protein was significantly higher in cancer cachexia tissues; (b) there is evidence of its role in the regeneration of the nervous system after injury (Camby et al., 2006; Svensson et al., 2009), and in the regulation of muscle development, homeostasis and repair (Cerri et al., 2008; Ahmed et al., 2009; Watt et al., 2004; Kami et al., 2005); and (c) there is a lack of knowledge on the relation of galectin-1 to cancer cachexia. Furthermore, several studies have proved that galectin-1 can work as a potential target for diagnosis, prognosis and/or therapies in several diseases (Camby et al., 2006; Balan et al., 2010).

Occasionally, when using 2-DE and western blotting to compare and validate the expression pattern of a protein, differences can be observed due to post-translational modifications. For instance, if a protein is modified after its translation, its several isoforms with different isoelectric points and different molecular weights can be visualized as several spots with different expression levels within a large two-dimensional gel. However, when running this same sample in a smaller one-dimensional gel in order to immunoblot the target protein, the post-translational modifications might be not as obvious as in the 2-DE expression pattern, due to a lower resolution of the 1-DE technique. In our case, when the expression levels of galectin-1 in muscle tissue from patients (revealed by 2-DE) were assayed by western blotting, it was observed that the expression pattern was maintained. This can be estimated comparing the 2-DE spots and western blotting bands from each patient's sample (Fig. 25A).

The level of expression of this protein in a mouse model of cancer cachexia ($Apc^{Min/+}$) was also analyzed. This mouse strain represents a suitable cancer cachexia model because it develops the wasting syndrome between three and six months of age (Baltgalvis et al., 2008). Although using a limited number of mice, galectin-1 was also found to be overexpressed in the mice that showed a significant decrease in body weight (more than 10%). The levels of expression of the protein seem to increase as cachexia develops.

Skeletal muscle fibers are classified in two major groups: the slow-twitch and fast-twitch fibers (Schiaffino et al., 1996). Their contractile properties are determined by myosin heavy chain (MyHC) isoforms: the slow MyHC type I and the fast MyHC types II. This grouping is also related with their myosin-ATPase activity. Slow-twitch types have a low level, while fast-twitch have a high level of this activity.

For analysis, rectus abdominis was used as human muscle tissue, but vastus lateralis was utilized as mouse tissue because the latter is a more identifiable muscle tissue in mice (in order to minimize the contamination of other tissues that could affect sample analysis) and it is also classified as fast-twitch type II fiber along with rectus abdominis muscle. This was taken into account because muscle wasting is more pronounced in fast-twitch than in slow-twitch containing muscles (Acharyya et al., 2004) and because there are already studies

stating that the effects of cachexia are similar in these two different muscle fibers in different performed assays (Ramamoorthy et al., 2009; Stephens et al., 2010). For comparison, soleus muscle is a typical slow-twitch type I fiber (Table 10).

Table 10. Percentages of slow-twitch type I fibers in rectus abdominis, vastus lateralis and soleus muscles (Colling, 1997).

Muscle	% slow-twitch type I fiber
Rectus abdominis	46.1
Vastus lateralis	42.3
Soleus	87.7

4.2. Regulation of galectin-1 in an *in vitro* muscle wasting model

Several studies show that the degeneration of injured skeletal muscles induces high expression of galectin-1 (Cerri et al., 2008), that the galectin-1 is involved in skeletal muscle differentiation and regeneration (Watt et al., 2004), or that this protein has a role in facilitating reinnervation of denervated skeletal muscle, improving muscle regeneration (Svensson et al., 2009). However, a model that could mimic the process of muscle wasting that occurs in cancer cachexia was also analyzed, using the well-characterized dexamethasone (Dex)-treated C2C12 cells as an *in vitro* muscle wasting model. This model involves treatment of myotubes with the cachectic glucocorticoid Dex, which inhibits Akt phosphorylation, inducing the up-regulation of MuRF-1 and atrogin-1, a decrease in myotube size and a decrease in total protein content in the myotubes.

Using this model, galectin-1 is shown to be up-regulated not only immediately after dexamethasone-induced atrophy, but especially after atrophy peak, suggesting a more important role for this protein in the early post-injury/regenerative process. Its function in the regeneration process may be similar to the one it has during the early development of myofibers, where this protein has an important role in the fusion of the differentiating

myoblasts (Kami and Senba, 2005; Watt et al., 2004) dissociating them from their substrates, such as laminin, in order for them to fuse and differentiate into myotubes. Also, in chick myogenesis, the highest level of gal-1 expression is observed when the fusion of myoblasts reaches its peak (Nowak et al., 1976).

In has already been shown that, through IGF-1, the hypertrophy-inducing PI3K/Akt pathway can dominantly block the atrophic effects of Dex via Akt-mediated phosphorylation and subsequent inhibition of the ubiquitin ligases (Latres et al., 2005; Glass, 2005). Further exploring the regulation of galectin-1 in the Dex-treated C2C12 cells model, it was observed that this protein is not significantly regulated under IGF-1-induced-hypertrophy, but down-regulated when IGF-1 rescues muscle regeneration after atrophy. The addition of IGF-1 *in vitro* to differentiated myotubes promoting myotube hypertrophy supports the idea that IGF-1 is sufficient to induce hypertrophy (Latres et al., 2005). Since IGF-1 is down-regulated in cancer cachexia (Costelli et al., 2006), it can be postulated that galectin-1 plays a role in muscle regeneration in the absence of IGF-1.

Galectin-1 expression and its cellular localization, following muscle atrophy and during regeneration, change significantly. This galectin can be found distributed diffusely throughout the cytoplasm, but can also be secreted interacting with laminin in the basement membrane (Svensson and Tågerud, 2009). Thus, how its secretion is regulated during muscle atrophy was analyzed. Collecting and analyzing the conditioned medium, it was observed that: (a) the level of secretion of gal-1 was very low, almost undetectable, immediately after Dex-induced atrophy; and (b) the gal-1 secretion reached its maximum 24 h after Dex-treatment, consistent with the previous results obtained with the cell lysates. This may attribute a more direct/functional role to galectin-1 in the muscle regeneration process, rather than being merely an epiphenomenon of the muscle atrophy progression.

4.3. Contribution of recombinant galectin-1 to muscle regeneration

It is has been reported that exposure of C2C12 cells to galectin-1 have resulted in an increase of myoblasts fusion into myotubes, and myoblasts derived from galectin-1 null

mouse showed reduced fusion (Watt et al., 2004); there is also evidence that the stimulation of laminin receptors of C2C12 cells increases myoblasts proliferation, myotubes formation and the expression levels of positive myogenic factors, such as myoD and myogenin (Grossi et al., 2007). Mechanical stimulation via laminin receptors is also reported to induce myoblasts differentiation and to promote an increase of galectin-1 expression (Grossi et al., 2011).

Therefore, whether the addition of exogenous galectin-1 to the Dex-induced atrophied cells would have a positive effect was verified. For expressing the recombinant galectin-1, the *E. coli* strain Rosetta2(DE3)pLysS was used because it showed a higher expression yield than the other strains. Two purification steps were taken: affinity chromatography and size exclusion chromatography. In order to maintain the carbohydrate-binding activity of the recombinant protein, the purification and storage of galectin-1 were carried out in the presence of a thiol-reducing agent (DTT) to prevent oxidative inactivation due to the formation of intramolecular disulfide bonds.

Using the recombinant galectin-1 at day 4 for 12 and 24 h, the induction of differentiation after muscle atrophy could be restarted. Although both treatments had positive results, the second one (24 h) resulted in higher levels of the differentiation markers. Besides the fact of longer exposure to the protein, this might also have to do with the fact that galectin-1 could have a more important role not right after the muscle injury, but at a later time. As observed previously, galectin-1 expression and secretion were always higher on day 5, 24 h after Dex-induced atrophy, pointing to a more important function during this time. Although cancer cachexia is a complex metabolic condition, the present findings suggest that galectin-1 could be taken into account as a potential therapeutic target in muscle wasting, which is the hallmark of this cancer syndrome.

4.4. Future perspectives

Currently there are no drugs approved for the prevention or treatment of cancer cachexia and specific diagnosis tools are needed to differentiate this cancer syndrome from others, such as sarcopenia or anorexia.

To validate galectin-1 as a diagnostic tool, due to its overexpression in skeletal muscle of pancreatic cancer patients with cachexia, it would be necessary to screen more samples from this type of patient to check whether this overexpression is maintained within a larger number of samples. Also, it would be interesting to screen patients suffering with cachexia due to other types of cancer, such as gastric or colon cancer (which also have a high prevalence of cachexia) to see if gal-1 is also overexpressed. Moreover, it would bring greater knowledge if other types of fast-twitch type II muscle, such as vastus lateralis, could be analyzed. This way it would be possible to foresee the specificity or ambiguity of galectin-1, concerning type of cancer and type of skeletal muscle within cachexia. Furthermore, a larger number of more $Apc^{Min/+}$ mice could be analyzed as well as other cachexia animal models, such as the murine adenocarcinoma (MAC) 16 tumor model or the rat Yoshida AH-130 model.

Using recombinant gal-1, the induction of differentiation after muscle atrophy could be restarted. These results indicate that a suitable application of gal-1 might have great potential in the therapy of muscle wasting. The administration of recombinant gal-1 in cachexia mouse models at different stages of development would be a good next step to check its effect on putative muscle regeneration.

At a fundamental scientific level, more functional studies should be carried out in order to improve our understanding of gal-1 functions in muscle atrophy and regeneration, as well as its interaction with key players, such as IGF-1 or laminin and other receptors.

5. SUMMARY AND CONCLUSIONS

5. SUMMARY AND CONCLUSIONS

Cancer cachexia is defined as an unintended weight loss of 10% within 6 months and is a complex metabolic condition that affects up to 80% of patients with advanced cancer and accounts for nearly 30% of cancer-related deaths. Loss of skeletal muscle is one of the most important components of the pathophysiology of this condition.

In the present thesis, a proteomic approach shows that skeletal muscle from patients with cancer cachexia, as well as from a mouse model ($Apc^{Min/+}$) of this cancer syndrome, express higher levels of galectin-1. Using the well-characterized dexamethasone-treated C2C12 cells as an *in vitro* muscle wasting model, it was observed that galectin-1 is up-regulated after muscle atrophy when IGF-1 signaling is down-regulated, which is characteristic for cancer cachexia.

Adding recombinant galectin-1, atrophied C2C12 cells started to redifferentiate. It is postulated that galectin-1 could have a role in the regeneration process after muscle wasting induced by cancer cachexia, and that this protein could be a potential diagnostic biomarker and a therapeutic target in the treatment of muscle wasting due to this cancer syndrome.

ZUSAMMENFASSUNG

Tumorkachexie ist eine komplexe Stoffwechselstörung, bei der der Verlust von Skelettmuskulatur eine der wichtigsten pathophysiologischen Komponenten ist. Mittels eines proteomischen Ansatzes wurde gezeigt, dass die Skelettmuskulatur von Patienten mit Tumorkachexie und die des Mausmodells höhere Mengen des Proteins Galectin-1 exprimieren. In einem *in vitro* Muskelverlustmodell zeigte sich, dass Galectin-1 bei Muskelatrophie hochreguliert wird, wenn der IGF-1 Signalweg herunterreguliert ist, was bei Tumorkachexie typischerweise der Fall ist. Bei Zugabe von rekombinanten Galectin-1 begannen atrophierte C2C12 Zellen zu redifferenzieren. Galectin-1 könnte eine Rolle im Regenerationsprozess nach durch Kachexie induziertem Muskelverlust spielen und sowohl einen diagnostischer Marker als auch ein therapeutisches Ziel in der Behandlung von Muskelverlust durch Kachexie darstellen.

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CURRICULUM VITAE

Persönliche Daten:

Duarte das Neves Afonso

Helmut-Käutner-Str. 25, 81739 München

Tel: 0151 54697781

afonso.dn@gmail.com

Geburtsdatum und –ort: 06.02.1981 in Mönchengladbach

Staatsangehörigkeit: Portugiesisch

Ausbildung:

2009-2012 Promotion an der Technischen Universität München,

Lehrstuhl für Molekulare Ernährungsmedizin

2006-2008 Masterstudium Entwicklungsbiologie,
Universidade Lusófona de Humanidades e Tecnologias, Lissabon, Portugal

2002-2006 Licenciatura (Hochschulabschluss) Biochemie,
Universidade de Lisboa, Lissabon, Portugal

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