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CROSSTALK BETWEEN GLUCOCORTICOID RECEPTOR AND OBESTATIN/GPR39 SYSTEM IN SKELETAL MUSCLE: AN AVENUE FOR THE TREATMENT OF MUSCULAR ATROPHY

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Crosstalk between glucocorticoid receptor and obestatin/GPR39 system in skeletal mucle: an avenue for the treatment of muscular atrophy

Dña. Tania Cid Díaz

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CROSSTALK BETWEEN GLUCOCORTICOID RECEPTOR AND OBESTATIN/GPR39 SYSTEM IN SKELETAL MUSCLE: AN AVENUE FOR THE TREATMENT OF MUSCULAR ATROPHY

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ABSTRACT

Many pathological states characterized by muscle atrophy are associated with an increase in circulating glucocorticoids and poor patient prognosis. The development of treatments for glucocorticoid-induced wasting in skeletal muscle should be designed based on how the balance of muscle protein synthesis and degradation is deregulated. Here, we investigated whether the obestatin/GPR39 system, an autocrine/paracrine signaling system acting on myogenesis and with anabolic effects on the skeletal muscle, could protect against glucocorticoid-induced muscle cell atrophy.

Keywords: Obestatin signalling; Skeletal muscle; Skeletal muscle atrophy; Skeletal muscle cell atrophy

RESUMEN

Algunos estados patológicos caracterizados por atrofia muscular están asociados con un aumento de glucocorticoides circulantes y una mala prognosis para el paciente. El desarrollo de tratamientos para las patologías que cursan con atrofia muscular inducida por glucocorticoides debería basarse en la desregulación entre síntesis y degradación proteica en el músculo. En este trabajo hemos estudiado si el sistema Obestatina/GPR39, un sistema autocrino/paracrino con funciones sobre la miogénesis y con efectos anabólicos en el musculo esquelético, podría proteger al músculo frente de la atrofia inducida por glucocorticoides.

Palabras clave: Señalización Obestatina; Músculo esquelético; Atrofia del músculo esquelético; Atrofia de células del músculo esquelético

RESUMO

Algúns estadios patolóxicos caracterizados por atrofia muscular están asociados con un aumento de glucocorticoides circulantes e unha mala prognose para o doente. A búsqueda de tratamentos para as patoloxías que cursan con atrofia muscular inducida por glucocorticoides debería basearse na desregulación entre a síntese e degradación proteica no músculo. Neste traballo estudiamos se o

sistema Obestatina/GPR39, un sistema autocrino/paracrino con función sobre a mioxénese e con efectos anabólicos no músculo esquelético, podería protexer o músculo fronte a atrofia inducida por glucocorticoides

Palabras clave: Señalización Obestatina; Músculo esquelético; Atrofia do músculo esquelético; Atrofia de células do músculo esquelético

ABBREVIATIONS

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4E-BP1: eukaryotic translation initiation factor 4E (eIf4E)- binding protein 1

ALS: autophagy-lysosomal system

AMPK: AMP-activated protein kinase

BCAA: branched-chain amino acid

BCAT: branched-chain amino acid aminotransferase

bFGF: basic fibroblast growth factor

BSA: bovine serum albumin

CAMKII: calmodulin-dependent protein kinase II

CDK: cyclin-dependent kinase

CK: creatine kinase

CP: core particle

CSA: cross-sectional area

DAPI: 4',6-diamidino-2-phenylindole

Dexa: dexamethasone

DM: differentiation medium

DMD: Duchenne muscular dystrophy

ECL: enhanced chemiluminescence

ECM: extracelullar matrix

EDL: extensorium digitorium

EDTA: ethylenediamine tetra-acetic acid

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

eIF3-f: eukaryotic translation initiation factor 3 subunit f

ER: endoplasmic reticulum

ERK1/2: extracellular signal-regulated kinase-1/2

FAPs: muscle like fibroblast-adipogenic progenitors

FBS: fetal bovine serum albumin

FoxO: forkhead box protein O

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GHRL: ghrelin gene

GHSR1a: ghrelin receptor

GM: growth medium

GPCR: G-protein-coupled receptor

GPR38: motilin receptor

GPR39: G-protein 39-coupled receptor

GR: glucocorticoid receptor

GREs: glucocorticoid response elements

HDACs: histone deacetylases

HE: haematoxylin/eosin

HEK: human embryonic kidney 293

HS: horse serum

Hsc70: heat shock cognate 70

IFN **α**: interferon **α**

IGF1: insulin-like growth factor-1

IGFR: insulin-like growth factor receptor 1

IL10: interleukin 10

IL4: interleukin 4

IL6: interleukin 6

IRS1: insulin receptor substrate 1

KLF15: Krueppel-like factor 15

LC3: microtubule-associated protein light chain 3

MAFbx: F-box protein 32

MEF2: myocyte enhancer factor-2

MHC: myosin heavy chain

MRF4: myogenic regulatory factor 4

MRFs: myogenic regulatory factors

mTOR: mammalian target of rapamycin

mTORC1: mammalian target of rapamycin complex 1

MuRF1: muscle-Specific RING Finger Protein 1

MuSCs: muscular satellite cells

Myf5: myogenic factor 5

MyoD: myoblast determination factor

NF-kB: nuclear factor kappa B subunit p65

NFAT: nuclear factor of activated T-cells

NLS: nuclear localization sequence

NTSR1: neurotensin receptor

p62/SQSTM1: sequestosome-1

PAM: peptidyl glycine α-amidating monooxygenase

Pax3: paired box protein 3

Pax7: paired box protein 7

PBS: phosphate-buffered saline

PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PI3K: phosphoinositide 3 -kinase

PKC: protein kinase C

PKD: polycystic kidney disease protein

REDD1: protein regulated in development and DNA damage response 1

Rheb: Ras homolog enriched in brain

RP: regulatory particle

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM: standard error of the mean

siRNA: small interfering RNA

Sirt: sirtuins

SIX1: sine oculis homeobox homolog 1

SIX4: sine oculis homeobox homolog 4

SMAD: mothers Against Decapentaplegic Homolog 1

TA: tibialis anterior

TNF α: tumor necrosis factor α

ULK1: unc-51 like autophagy activating kinase 1

UPS: ubiquitin-proteasome system

ZnR: Zn^{+2}-sensing receptor

INTRODUCTION

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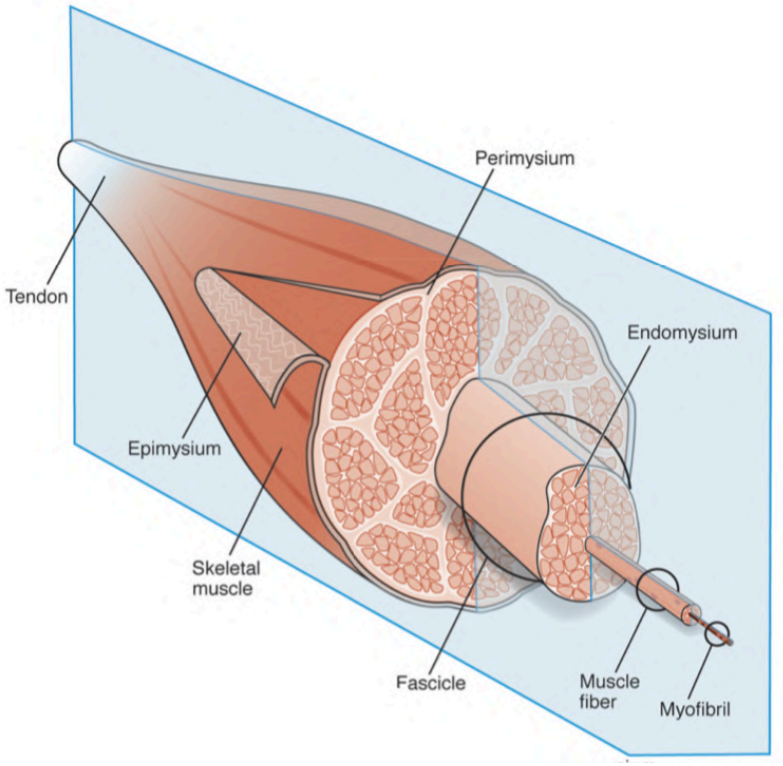
SKELETAL MUSCLE

Skeletal muscle is one of the most dynamic and plastic tissues of the human body. In humans, skeletal muscle comprises approximately 40 % of total body weight and contains 50–75 % of all body proteins. Skeletal muscle primary function was described as postural retention as well as locomotion but it is not only a simple scaffold but rather an endocrine organ that produces and releases various types of biological molecules (Frontera WR and Ochala J, 2015; Iizuka K et al., 2014) skeletal muscle produces and releases proteins with autocrine, paracrine, or endocrine functions, termed myokines, in response to contraction, which can influence metabolism. Endocrine functions of myokines are involved in body weight regulation, low-grade inflammation, insulin sensitivity, suppression of tumor growth, and improvement of cognitive function (Hoffmann C and Weigert C, 2017; Iizuka K et al., 2014; Pedersen BK, 2013).

Macroscopic organization of muscles show an exceptional level of organization, formed by nerves, blood vessels, conective tissue as well as contractile tissue. Skeletal muscle is attached to bone by tendons, epimysium, within is located the entire muscle composed by bundles of muscle fibers called fascicles surrounded by connective tissue, perimysium. Each fiber is nearby a layer of connective tissue, called endomysium, that includes the basal lamina that is over the sarcomere and where satellite cells are located. These are stem cells like, that play a key role in maintain the pool of myoblasts, the basic muscle cells, and in the repair process following a muscle injury. Fibers are multinucleated cells originated from the fusion of myoblasts and are formed of longitudinally units, myofibrils, surrounded by the sarcomere. The myofibrillar proteins, myosin (the thick filament), actin troponin, tropomyosin (the thin, filament) are enclosed by each sarcomere. These proteins allow the muscle contraction as a reaction to the calcium released from the

sarcoplasmic reticulum due to signals from motor neurons (Scheme 1) (Betts JG et al, 2013; Gilles AR and Lieber RL, 2011; Ömeroglu H and Ömeroglu S, 2016).



Scheme 1. Structural organization of skeletal muscle. From outside to inside, muscle is attached to tendons and covered by the epimysium, bundles of fibers, fascicle, are surrounded by the perimysium, each fiber is surrounded by endomysium and is cover by the sarcolemma where myofibrils are located, formed by thin and thick filaments of proteins. From Gillies AR et al, 2011. With permission of Willey.

FIBER TYPE HETEROGENITY AND SKELETAL MUSCLE PLASTICITY

Classification of myofiber types

Skeletal muscle fibers differ in their metabolic, physiologic and biomechanical parameters. This diversity of properties provides different muscles with different functions (Bassel-Duby R and Olson EN, 2006). The classification was reviewed in great detail by others (Pette and Staron RS, 2000; Schiaffino and Reggiani, 2011), the usual criteria employed includes: color of muscle fibers based on myoglobin content (red *vs.* white); contractile properties of the motor units in response to electrical stimulation; speed of shortening during a single twitch (fast *vs.* slow); degree of fatigability during sustained activation (fatigable *vs.* fatigue-resistant); predominance of certain metabolic or enzymatic pathways (oxidative *vs.* glycolytic); enzyme-histochemical stain reaction (based on ATPase or SDH staining techniques); calcium handling by the sarcoplasmic reticulum (slow *vs.* fast); and protein isoform expression, among others. One of the most extended and informative method of classification is based on specific myosin heavy chain isoform expression (MHC) (Pette and Staron RS, 2000). Owing this, myofibres can be cataloged as type I, also known as slow-twitch fibers, and type II, also termed fast-twitch fibers. Type II fibers can be also divided type IIa, type IIb and type IIx fibres. Type I exhibit an oxidative metabolism, have a large amount of mitochondria, have more capillaries, wield a slow contraction and have high fatigue resistance. This type of fibers is necessary for preservation of posture and tasks involving endurance. Type IIa fibers have, as well as type I, present oxidative metabolism while type IIb and IIx are mainly glycolytic. Type II fibers are involved in quick contractions and are easily fatigued. This type of fibers participates in strength and speed movements. (Bassel-Duby R and Olson EN, 2006; Pette and Staron RS, 2000; Schiaffino and Reggiani, 2011).

Skeletal muscle plasticity

In 1960 Buller et al described the muscle ability to be modified under environmental demands as a response to a diverse physiological and pathological signal. This capacity was described as plasticity (Buller AJ et al, 1960). This study revealed the significance of the neuromuscular activity in fibre type shifts, but other conditions such as exercise, mechanical loading or unloading, alteration in hormonal profiles, or ageing, can induce myofiber remodeling (Brendan E and Zierath JR, 2013; Leblanc et al., 2004; Pette and Staron RS, 2000; Phillips et al., 1996).

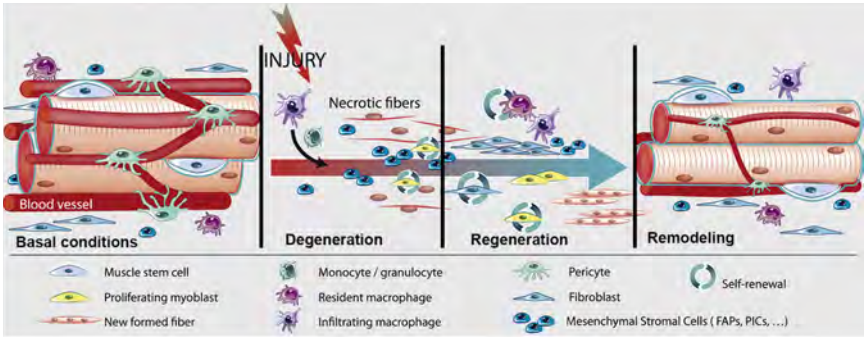
It have been studied several signaling pathways that regulates the remodeling of skeletal muscle phenotype, including calcineurin/ nuclear factor of activated T-cells (NFAT), myocyte enhancer factor-2 (MEF2), histone deacetylases (HDACs), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and AMP-activated protein kinase (AMPK) (Bassel-Duby R and Olson EN, 2006; Schiaffino and Reggiani, 2011).

This skeletal muscle plasticity not only offers different functionality but also brings susceptibility to pathological states (Ciciliot S, 2013). In fact, many myopathies, muscle wasting conditions or metabolic disorders affect to a kind of fiber types in a specific manner, like Duchenne muscular dystrophy (DMD), sarcopenia, cancer or obesity, among others (Schiaffino and Reggiani, 2011; Zierath JR and Hawley JA, 2004).

SKELETAL MUSCLE REGENERATION

In normal physiological conditions skeletal muscle is a stable tissue, it is estimated that in a normal adult muscle, no more than 1–2% of myonuclei are replaced every week (Schmalbruch H and Lewis DM, 2000). Nonetheless, skeletal muscle is able to perform a repair process in response to severe or mild injury for example, myopathies as DMD, exposure to myotoxic agents, contusion or punctures, ischemia, exposure to hot or cold temperatures and muscle contraction itself, due to extensive physical activity (Chargé SB and Rudniki MA, 2004; Karalaki M et al., 2009). The restoration of muscle goes from the molecular level to the reestablishment of the whole functional contractile apparatus. Muscle regeneration consists in three phases, degeneration, regeneration and remodeling (Baghdadi MB, Tajbakhsh S, 2018; Chargé SB and Rudniki MA, 2004; Karalaki M et al., 2009). Degeneration is characterized by necrosis and significant inflammation. In this event occurs a disturbance of sarcolemma integrity, leading to an increased myofiber permeability and a muscle proteins rise in the serum levels such as creatine kinase (CK), as well as increase of calcium influx that activates calcium-dependent proteolysis and drives tissue degeneration (Baird MF et al., 2012; Tu MK and Borodinsky LN et al., 2014). The inflammatory phase involves the recruitment to the damaged site of a numerous of inflammatory cell types. The first cells to be recruited are neutrophils, as they appear within 3 h following injury and they are no longer detectable after 3 d, their action relies on proteolysis, oxidation and phagocitosis (Baghdadi MB, Tajbakhsh S, 2018; Orimo S et al.,1991; Tidball and Villalta, 2010). Following neutrophil infiltration, macrophages are required for phagocytosis and release of cytokines, first a population of macrophages peak at 3 days, they are defined by the surface markers $CD38^{+}/CD163^{-}$ release pro-inflammatory molecules such as tumour necrosis factor α (TNFα), interferon α (INFα) or interleukin 6 (IL6). They account for the phagocytosis of cellular debris

and promotion of myoblast proliferation. The second population of macrophages undergo a phenotypical and functional switch toward an anti-inflammatory fate, characterized by the surface markers $CD38^{-}/CD163^{+}$, they secrete cytokines like IL4 or IL10 and promotes the proliferation and differentiation of satellite cells (Baghdadi MB, Tajbakhsh S, 2018;Lu et al., 2011; Merly F et al.1999). After degeneration and clearance of cellular debris occurs the activation of regeneration phase. Under physiological conditions satellite cells (MuSCs) remains dormant but after injury they are activated and enter in a proliferative state, they are the key of the reestablishment of muscle arquitecture (Sambasivan R et al., 2011), MuSCs differentiate into myoblast and fuse to new myofibers or repair the damaged ones. Finally, the remodeling phase go through hyperplasia and hypertrophy, the vasculature and innervation patterns are restored. The number of MuSCs and capillaries, as well as the timing of angiogenesis and myogenesis, are orchestrated during regeneration suggesting a reciprocal interaction between these cell types (Baghdadi MB, Tajbakhsh S, 2018) (Scheme 2).

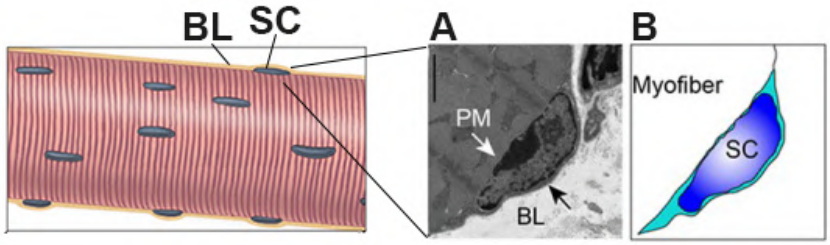


Scheme 2. Muscle regeneration in muscle and cells implicated in the process. In response to injury it occurs a necrosis of the myofibers and an inflammatory environment, resident myoblast starts to proliferate to fuse into the damage or new fibers while MuSCs leave their quiescent state starting to proliferate to recover the myoblast pool as well as the MuSCs pool. Finally, a complete remodeling of the vasculature and innervation take place and the full contractile apparatus is recovered. Figure modified from Baghdadi MB et al, 2018. With permission of Elsevier.

SATELLITE CELLS

Satellite cells (MuSCs) were first described by Mauro et al. in 1961 in electron micrographs of skeletal muscle, as a population of mononuclear cells situated over the outer boundary of myofibers. They were named as MuSCs owing to their anatomical position, between the sarcolemma and the basal lamina (Scheme 3) (Mauro A, 1961). In adult mucle, these cells represent around 2-10% of myonuclei, mostly derived from $PAX3^{+}$/$PAX7^{+}$ embryonic progenitor cells (Buckingham, 2007; Seale P et al., 2000; Seale et al., 2004). The ability of muscle to regenerate is governed significantly by MuScs (Scharner & Zammit, 2011) and its microenvironment (Lander, Kimble, Clevers, et al., 2012; Yin H and Rudniki M, 2013).

MuScs are a heterogenous population, differing in lineage potential, expression profile and proliferation/differentiation potential (Kuang S et al., 2007; Zammit PS, 2008). Most important parameter is that MuSCs are heterogeneus in stemness, it exists two subpopulations based on the expression of the myogenic factor 5 (Myf5). First one, $Myf5^-$, corresponds a low percentage of MuScs, the "true stem cells" and secondly, $Myf5^+$, cells commited to the myogenic program. After transplantation, these two populations diverge in regenerative potential, while Myf5+ cells undergo myogenic differentiation, Myf5- cells carry out muscle repair and participate in the renewal of the MuSC compartment (Kuang S et al., 2007). The MuSC remain uniform; however, reduces in population density and efficacy with age (Almada and Wagers, 2016).



Scheme 3. The satellite cells. MuSCs are located over the sarcolemma and under the basal lamina of the myofibers. A, Electron microscopy of the MuSC. B, schematic representation of MuSC in a myofiber. PM, plasma membrane; BL, basal lamina; SC, satellite cell. Figure modified from Fukada S et al. Isolation, characterization, and molecular regulation of muscle stem cells. Front Physiol. 2013;12(4):317 and Pearson Education, Inc, 2009. Is an open-access article

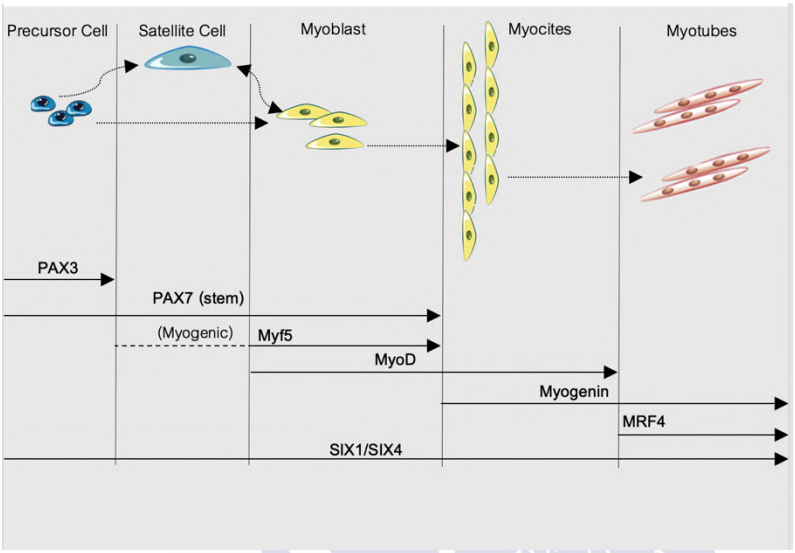
Quiescence, activation and differentiation of MuSCs

MuSCs resides between basal lamina and sarcolemma of the myofibers. This microenvironment, called niche, limit and orient the migration of the cells during

injury (Sanes JR, 2003). The niche has an important role in maintaining the quiescence and regulating the MuCS fate. It is composed by cellular and non-cellular components as, non-myogenic cells that resides in muscle like fibroblast-adipogenic progenitors (FAPs), macrophages, extracellular matrix and growth factors (Almada AE, Wagers AJ, 2016).

In resting adult muscles MuScs are in dormancy, they are non-mitotic, this state is also known as quiescent, most of them are derived from precursors $Pax3^{+}/Pax7^{+.}$ The expression of Pax7 and the absence of the myoblast determination factor (MyoD) characterize Quiescent MuScs. Quiescent MuSCs become activated following an injury due to several microenvironmental signals, to give rise to myoblast, these myogenic progenitors enter in the differentiation program and fuse to form multinucleated myotubes to fix damaged fibers. The activation process of MuSCs is regulated by basic helix-loop-helix transcription factors called myogenic regulatory factors (MRFs) as Myf5, MyoD, myogenin and myogenic regulatory factor 4 (MRF4). Myf5 and MyoD are decisive during muscle regeneration for the myogenic determination, activated MuSCs express Pax7 and MyoD at the same time, after several proliferation phases, these cells bring out to $Pax7^{-}/MyoD^{+}$ cells that are committed to proliferation while a few percentage of activated MuSCs derives in $Pax7^{+}/MyoD^{-}$, these ones return to state of quiescence which is critical to preserve the MuSCs pool (Collins CA et al., 2005; Dumont NA et al., 2015; Zammit PS, 2008). In this sense, the activation of Notch and Wnt signaling is essential for maintaining quiescence in MuSCs by inhibiting MyoD expression and inducing PAX7, respectively (Bjornson CR et al., 2012; Olguin HC and Olwin BB, 2004). Finally, the differentiation phase is marked by the downregulation of MyoD and Myf5 along with the increment in expression of MRF4 and myogenin in early stages (Yin H et al, 2013) whereas the terminal differentiation is marked by the expression of

myofibrillar proteins such as MHC (Scheme 4)(Almada AE and Wagers AJ, 2016; Wang YX et al., 2014).



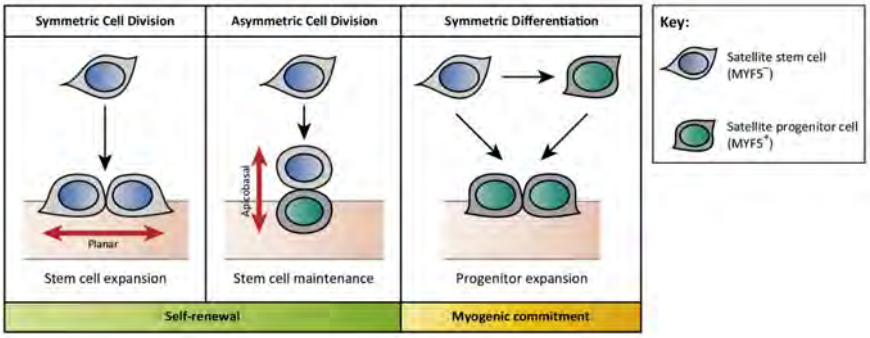
Scheme 4. Hierarchy of transcription factors regulating progression through the myogenic program. Pax3 and Pax7 are the early regulators of myogenic specification. Myf5 and MyoD commited cells to myogenic program. Myogenin and MRF4 are necessary for early and terminal differentiation respectively. Six1 and Six4 govern the regulation of several of named transcription factors during the myogenic program.

Self-renewal of MuScs

The balance between self-renewal and differentiation of MuScs is crucial for stem cell maintenance and muscle homeostasis as it maintains the stem cell population and supplies the myogenic progenitors needed for muscle regeneration (Kuang S et al., 2007) An error in self-renewal results in a depletion of stem cell population and the consecuent reduce in muscle regeneration. Self-renewal can be performed in two differenten types of cell division: Asymmetric or symmetric. Asymmetric divisions give rise two different doughter cells, one destined to self-renewal, to replenish the stem cell pool and the other, commited to differentiate. On the other

hand, symmetric division generates two identical daughter cells and this cells can adop different fates in a stocastic way. Some of them, exclusively generate two stem cell daughters, whereas alternaively, it could generate two cells commited to differentiation (Schema 5) (Almada AE and Wagers YX, 2016; Dumont NA et al., 2015; Motohashi N and Asakura A, 2014; Kuang S et al., 2008).

The fate of MuSCs in self-renewal is strongly influenced by the stem cell niche. It exists cell polarity within the niche, cell-cell and cell-ECM intractions. The basala lamina side of a satellite cell expreses integrin $\alpha 7\beta 1$ that interacts with laminin, whereas the apical side expresses M-cadherin to dock the satellite cell and transduce signalas from the fiber. In view of asymmetric distribution of apical-basal signals, we could predict that a planar division (symmetric) where the two daughter cells are exposed to both, apical and basal, signals it would be obtained two cells with the same fate. In contrast, an apical-basal-oriented cell division give rise two daughter cells exposed either to apical or basal signals, leading to a different cell fate (Kuang S et al., 2008). Importantly, the daughter thtat remains in contact with the basal lamina adopts a stem cell fate and the daughter that loses the basal lamina contact adopts a commited differentiation fate (Kuang et al., 2007).



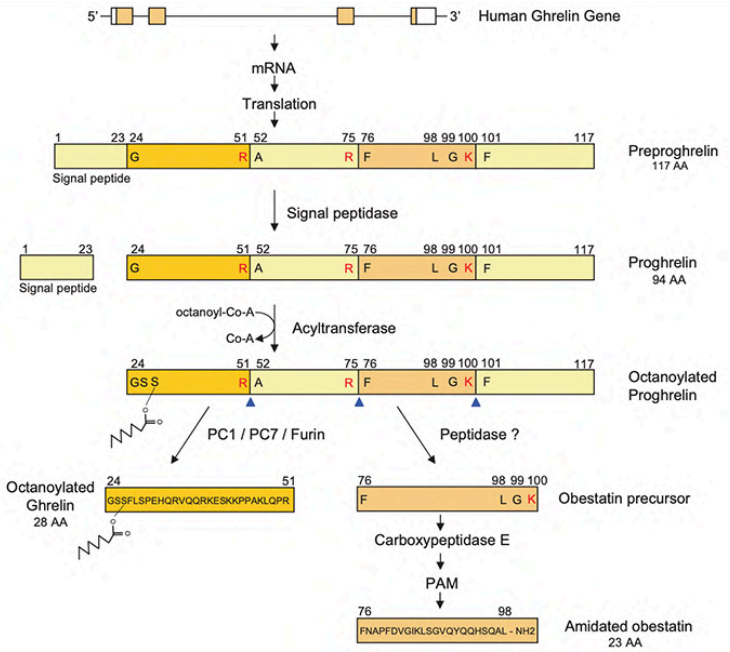
Scheme 5. Asymmetric and symmetric division of MuSCs. Asymmetric divisions give rise to one self-renewing cell (Myf5^{-} *blue*) and one committed cell (Myf5^{+}; *green*), it occurs perpendicular to the fiber axis. Symmetric divisions generate two identical (either stem or committed progenitor) daughter cells, it occurs parallel to the muscle fiber axis. From Chang NC et al., 2016. With permission of Elsevier

THE OBESTATIN/GPR39 SYSTEM

OBESTATIN

In 2005, based on bioinformatic predictions, Zhang et al. discovered a new peptide encoded by the ghrelin gene. It was named obestatin, from the Latin "obedere", that means devour, and statin, indicating suppression. At the first time it was thought to have the opposite physiological effect of ghrelin on food intake, weight and gastric emptying due to it was first isolated in rat stomach (Zhang JV. and Ren PG, 2005). Obestatin is 23-aminoacid hormone derived from the proteolic procesing of preproghrelin and encoded by the ghrelin gene (GHRL). Preproghrelin is composed of 117 amino acids, a 23-amino acid signal peptide and a 94-amino acid peptide called proghrelin which is cleaved in two sites, between arginine 75-phenyalanine 76 and lysine 100-phenyalanine 101 given rise to obestatin precursor. After the cleavage, the lysine residue at the carboxy terminal is cleaved by carboxypeptidase

E, being then amidated in the carboxy-terminal leucine reside by the peptidyl glycine α-amidating monooxygenase (PAM), the presence of a C-terminal amide group is essential for its biological activity (Alén BO et al., 2012; Garg A, 2007; Soares JB and Leite-Moreira AF, 2008 (Scheme 6).



Scheme 6. Post-translational processing of preproghrelin to mature ghrelin or obestatin. This polypeptide of 117 amino acids contains a signal peptide of 23 amino acids at the amino terminus, which is the first cleaved site, resulting in proghrelin, formed by 94 amino acids. Blue triangles indicate the sites of proteolytic section. Ghrelin is cleaved between arginine 51- alanine 52 and is susceptible to be amidated at serine 3. Obestatin results from proteolytic scission of proghrelin at arginine 75-phenyalanine 76 and lysine 100 and phenyalanine 101, is procesed at the carboyterminal by carbopeptidase E and then in leucine 23. Figure extracted form Garg A., 2007. With permission of Oxford University Press

Moreover, Zhang et al. indicate after a mass spectrometry analysis that a 13 amino acid peptide, obestatin (11-23), with biological active, may be also processed from obestatin (Zhang JV and Ren PG, 2005).

Rearding to obestatin distributions, as said, was found to be produced in rat stomach, in rat and human gastric mucosa (Dun SL et al., 2006; Zhang JV and Ren PG, 2005). However its expression was also demonstrated after in endocrine pancreas, adipose tissue, skeletal muscle, liver, lung, thyroid, mammary glands and male reproductive system, proposing an autocrine/paracrine functions in other tissues and organs (Gurriarán-Rodríguez U et al., 2012; Granata R et al., 2008; Granata R et al. 2010; Moretti E et al., 2014; Volante M et al., 2009; Zhao CM et al., 2008).

Obestatin receptor: GPR39

The GPR39 receptor was originaly isolated and cloned in 1997 by McKee et al. (McKee KK et al., 1997) and considered an orphan GPCR until Zhang et al. discovered obestatin, their data obtained from radiolabelled ligand binding assay, proposed the obestatin as a ligand of GPR39 (Zhang JV. and Ren PG, 2005). GPR39 receptor gene is located on chromosome 2, band q21-q22, comprising two exons, belongs to the same family as ghrelin (GHSR1a), motilin (GPR38) and neurotensin (NTSR1) receptors (McKee KK et al., 1997) and it is formed by 435 amino acids and seven transmembrane helices. First exon encodes the residues 1 to 285, conforminf the N-terminal region and the first transmembrane helices. Flowing the first exon exists an intron of 200kb that also appears in ghrelin receptor as well as in motilin receptor genes. The residues 286 to 435are encoded by the second exon and correspond to the last two transmembrane helices and the C-terminal region (Egerod KL et al., 2007).

Two splicing variants of the GPCR39 receptor was identified: a full-length receptor called GPR39 1-a, known as canonical form; and a truncated form of the receptor, GPR39 1-b, considered biologically inactive at the first time (Egerod KL et al., 2007).

GPR39 expression in the body is mainly circunscribe to the digestive system, but is also found in adipose tissue, liver, spleen, thyroid, lung, heart, reproductive tissues, brain and skeletal muscle (Dong XY et al., 2009; Gurriaran-Rodriguez et al. 2012). This widely expression of the receptor may suggest its role in the regulating diverse physiological functions, as myogenesis, adipogenesis, cell death, hormene secretion ot gastrointestinal motility. Dong XY et al., 2009; Gurriaran-Rodriguez et al. 2011; Gurriaran-Rodriguez et al. 2012)

Obestatin bioactivity

Despite obestatin was originally describe as a physiological opponent of ghrelin (Zhang JV. and Ren PG, 2005), this action was discarded by several groups (Green BD et al., 2007; Zhang JV et al., 2008). The central effects of obestatin remain unclear, it has been studied its role in peripheral cells and tissues, obestatin acts regulating adipogenesis, pancreatic homeostasis, cardiovascular function and myogenesis (Green BD and Grieve DJ, 2018; Trovato L et al., 2014). Moreover, obestatin regulates metabolic and cell differentiation functions, increasing cell survival and proliferation (Camiña JP et al., 2007; Granata R et al., 2008; Pazos Y et al., 2007) and inhibiting inflammation and apoptosis (Aragno M et al., 2012; Ceranowicz P et al., 2009; Granata R et al., 2012).

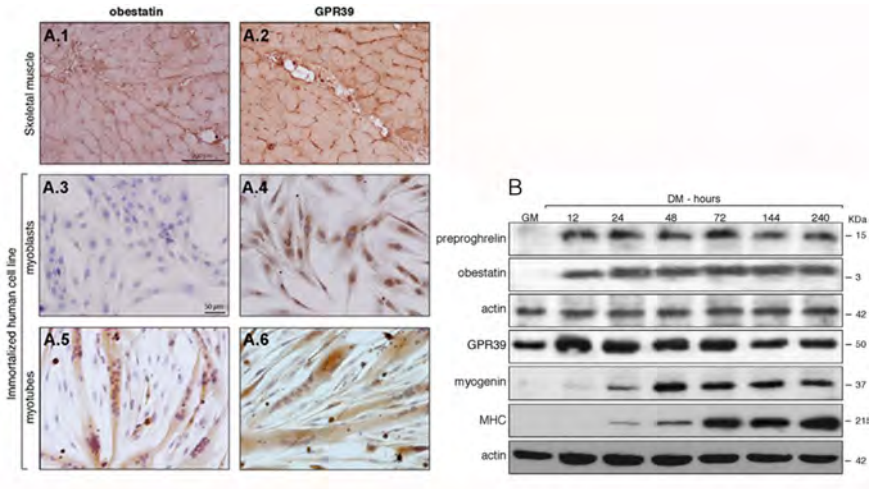
Obestatin exerts its activity through GPR39 as was demonstrated in several cell lines by knockdown of GPR39 by siRNA experiments producing the inhibition of obestatin signaling (Alén BO et al., 2015; Camiña JP et al., 2007; Gurriarán-Rodríguez U et al., 2011; Gurriarán-Rodríguez U et al., 2012; Santos-Zas I et al., 2016). Moreover, coimmunoprecipitation experiments demonstrated the binding of obestatin to

GPR39 in cultured C2C12 myoblasts (Gurriarán-Rodríguez U et al., 2015). Studies of GPR39 structure and activity related to mouse and human obestatin reveal conformational differences beyond their differences in primary structure. Mouse analogue adopts a different three-dimensional structure that cannot activate human GPR39 (Alén BO et al., 2012). Although there is a proof of the species-specific activity of the obestatin through its receptor, GPR39, it existed a controversy around if GPR39 is the obestatin receptor. First, independent groups perform experiments to elucidate binding and stimulatory function of obestatin on GPR39, but none was able to achieve this aim (Chartrel N et al., 2007; Lauwers E et al., 2006; Tremblay F et al., 2007). This was hardened by the inability to reproduce the binding of obestatin to GPR39 by Zhang's group as their original experiments(Zhang JV et al, 2007).Following years, it was demonstrate that the quality of obestatin supplied by some companies was deficient (De Spiegeller B et al., 2008), in fact, Zhang et al. pointed that the erratic binding of obestatin to GPR39 could be because of the bioactivity loss of obestatin after its poly-iodination (Zhang JV et al, 2007). Years later was revealed that mono-iodinated obestatin was efficient binding to human embryonic kidney 293 (HEK) cells transiently transfected with plasmids encoding GPR39 (Zhang JV et al., 2008). Other groups propose zinc ions (Zn^{+2}) as the endogenous ligand of GPR39, considering GPR39 as Zn^{+2}-sensing receptor (ZnR) and not the obestatin receptor (Besser L et al., 2009; Holst B et al., 2007).

The obestatin signaling an its role in myogenesis

Myogenesis is a multistep process. First, myoblast enter in a proliferative phase and then they exit the cell cycle to enter in a differentiation phase, in which they aliniate and fuse forming a multinucleated mature myotubes (Bentzinger CF et al., 2012; Zierath JR and Hawley JA, 2004). Obestatin is expressed in healthy skeletal muscle, in vitro experiments evidence that whether obestatin is up-regulated during early stages of myogenesis and sustained throught terminal differentiation, GPR39 is

expresses in myoblast as well as during differentiation (Scheme 7) (Santos-Zas I et al., 2016). The system is also increase following muscle injury (Gurriarán-Rodríguez U et al., 2012).

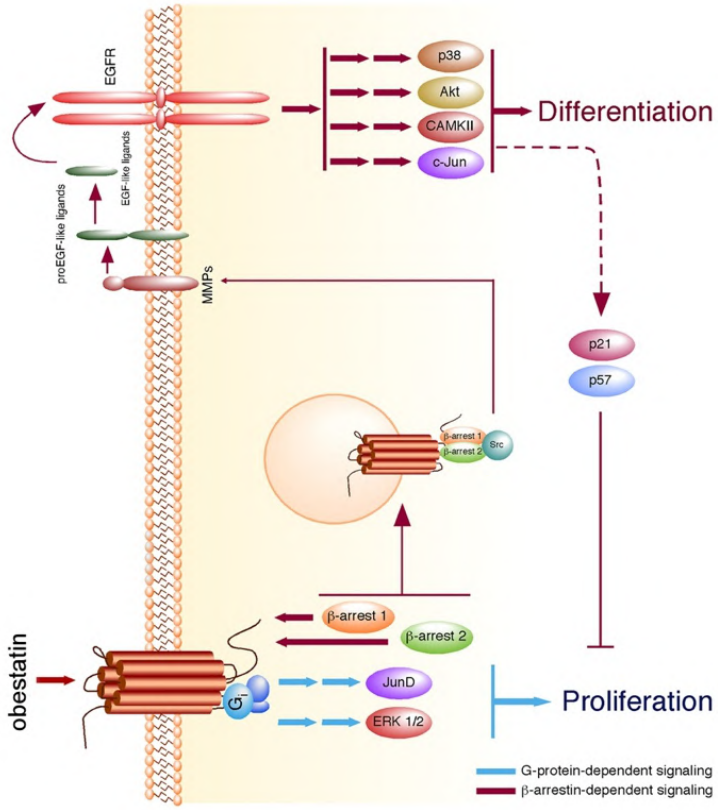


Schema 7. Obestatin and GPR39 expresion during myogenesis. A. Immunohistochemical detection of obestatin GPR39 in human skeletal muscle tissue and human skeletal muscle cell line. B. Immunoblot analysis of obestatin, GPR39 and myogenic markers in human skeletal muscle cell line. Figure extracted from Santos-Zas I et al. 2016. With permission of Springer Nature

After injury, intramuscular injection of obestatin rises regeneration by regulating multiple steps of myogenesis myoblast proliferation, cell cycle exit, differentiation, in order to fuse and form multinucleated myotubes (Gurriarán-Rodríguez U et al.2015). Lately, it was proved that oxidative phenotype of muscle fibre was favor by obestatin through both class II HDAC/MEF2 and PGC-1α mechanisms (Santos-Zas I et al., 2017). This properties of the obestatin GPR39 system suggest its clinical application in skeletal muscle.

Certanly, obestatin demonstrated not only enhance the efficiency of engraftment, but also facilitates the distribution of myoblasts within the host muscle in a cell transplantation therapy experiment, the experiment was based on myoblast-

based therapy by xenotransplanting primary human myoblasts into immunodeficient mice (Santos-Zas I et al., 2017). The obestatin/GPR39 system wields its action, in particular, by an interplay between G protein-dependent and β-arrestin-dependent mechanisms, governing myogenic program progression (Santos-Zas I et al., 2016). The first proliferation phase of the myogenic program is activated by an G protein-dependent mechanism that regulates the extracellular signal-regulated kinase-1/2 (ERK1/2) and JunD axis (Santos-Zas I et al., 2016). ERK1/2 exerts a crucial role in cell proliferation, migration and fusion (Leloup L et al., 2007). The ERK1/2 activation is concomitant to the first proliferation phase of myoblast, then this activity is decreased being a necessary stage for myoblast differentiation. Actually, ERK prevents the nuclear accumulation of members of MEF2 causing an inhibition of the differentiation (Winter B et al., 2000), it also blocks the expression of certain myogenic factors, including MyoD and the cyclin-dependent kinase (CDK) inhibitor p21 (Gredinger E et al., 1998; Wu Z et al., 2000). The myogenic action of obestatin is also regulated by the β-arrestin-dependent transactivation of epidermal growth factor receptor (EGFR), provoking the cell cycle exit and differentiation progression through a kinase cascade determined by the Akt, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), c-Jun, and p38 axes. Under obestatin stimulus, β-arrestins are recruited to the membrane, where they interact with GPR39 leading to Src activation and a later transactivation of EGFR via matrix metalloproteinases. These controls the mitotic arrest by regulation of p21 and p57 expression as well as mid-to-late stages of differentiation through JNK/c-Jun, CAMKII, Akt and p38 pathways aiming to the formation of multinucleated myotubes. Accordingly, the activation of β-arrestin-dependent signaling determines the expression of myogenic factors as myogenin among others and MHC (Scheme 8) (Santos-Zas I et al., 2017).



Scheme 8. Proposed model for the G-protein- and β-arrestin-mediated activation of myogenesis through the obestatin/GPR39 system. Obestatin binds to GPR39 receptor resulting in activation of Gi/o protein-dependent pathway, leading to the activation of ERK1/2 and JunD signalling nodes that regulates myoblast proliferation. Simultaneously, GPR39/β-arrestins/Src complex initiates the transactivation of EGFR through MMPs and subsequent downstream Akt, p38, CAMKII and JNK/c-Jun signaling to regulate cell cycle exit, p21 and p57, recruitment and fusion of myoblast to form myotubes. Figure extracted from Santos-Zas I et al., 2016. With permission from Springer Nature.

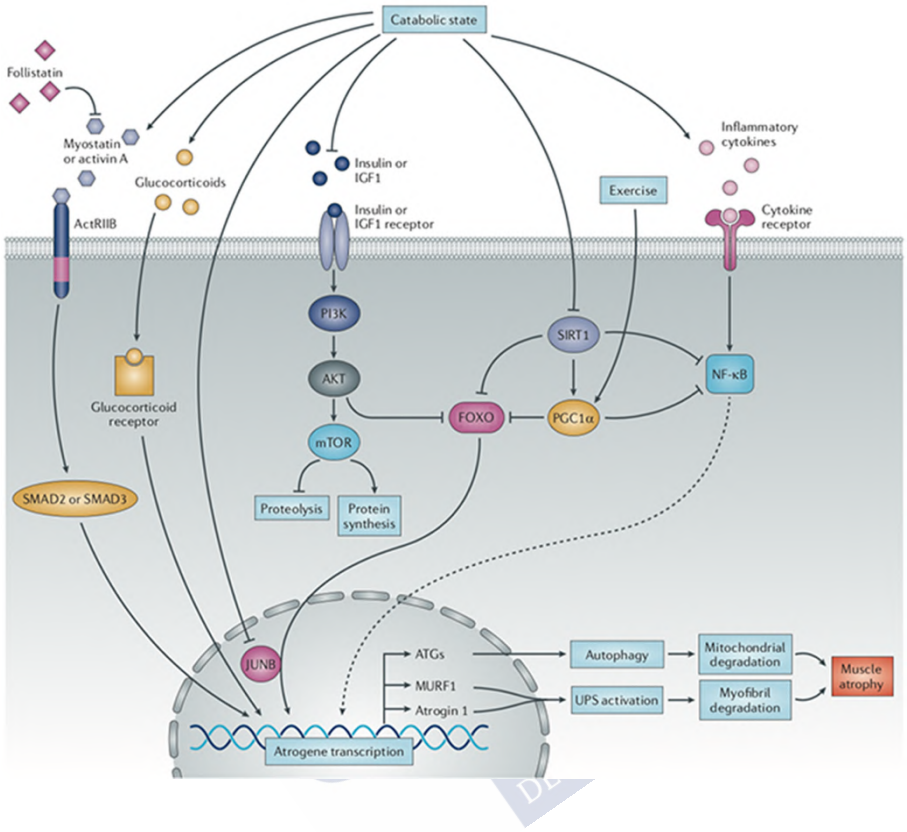
MUSCLE ATROPHY

Atrophy is defined as a decrease in the size of a tissue or organ due to cellular shrinkage, and it is caused by the loss of organelles, cytoplasm and proteins. In terms of muscle, this wasting is accompanied by a decrease in the cross-sectional area of the muscle fiber, the muscle volume, and the amount of muscle protein (Boonyarom O and Inui K, 2006; Jackman and Kandarian, 2004).

Muscle is the largest reservoir of protein in the body and serves as a source of amino acids for energy production during catabolic periods (Bonado P and Sandri M, 2013). In general, muscle mass depends on the balance between protein synthesis and degradation and both processes are sensitive to factors such as nutritional status, hormonal balance, physical activity, and injury or disease, among others (Frontera WR and Ochala J, 2015). Atrophy occurs in specific muscles due to inactivity or immobilization, denervation, systematically in old people (condition known as sarcopenia), as a response to fasting or malnutrition and in many diseases as chronic obstructive pulmonary disorder, cancer associated cachexia, diabetes, renal failure, Cushing syndrome, sepsis among others (Cohen S et al., 2015). However, muscle mass adapts to different pathophysiological conditions regulating the protein turnover, the excessive muscle mass loos aggravates the pathological state an increases morbidity and mortality. During catabolic conditions, four major proteolytic systems are activated: the ubiquitin-proteasome system (UPS) (Bonado P and Sandri M, 2013; Egerman MA and Glass DJ, 2014; Foletta VC et al., 2011), the autophagy-lysosomal system (ALS) (Bonado P and Sandri M, 2013; Sandri M, 2013; Schiaffino Set al., 2013), the calcium-dependent calpains (Bartoli M and Richard I, 2005) and caspases (Du J et al., 2004). By means of microarray analysis, it has been demonstrated that a set of 120 atrophy related genes are activated or repressed, contributing to the wasting process, these genes are known as

atrogenes (Jagoe RT et al., 2002; Lecker SH et al., 2004). On the one hand, myofibrillar components are degraded by the UPS contributing to the loss of the contractile machinery, reducing the muscle strength. The most studied proteins implicated in the UPS are the ubiquitin ligases MAFbx and MURF1, they are noticeably induced in most of the atrophy types (Bodine SC et al., 2001; Gomes MD et al 2001). On the other hand, mitochondria and other organelles are degraded by the ALS and these loss accounts for the decreased endurance capacity in atrophied muscles (Cohen S et al., 2015).

The mechanism whether a muscle atrophies or grows is governed by the phosphoinositide 3 -kinase (PI3K)-AKT-mTOR pathway (Bonado P and Sandri M, 2013). In differentiated muscle cells this pathway stimulates protein synthesis and inhibits protein degradation (Schiaffino S and Mammucari C, 2011). The inhibition of protein degradation is, in a great extent, due to the inhibition of forkhead box protein O (FoxO) transcription factors which control the expression of the atrogene program overall (Cid-Diaz T et al., 2017). During catabolic periods PI3K-AKT-mTOR signaling decrease, allowing FoxO transcription factors to exert its activity, mediating the expression of atrogenes (Cid-Diaz T et al., 2017; Latres E et al., 2005). Although FoxO transcription factor seems to be implicated and govern the activation of the atrophy program, it exists other transcription factors such as SMAD2, SMAD3, glucocorticoid receptor and NF-kB that play an important role in muscle wasting (Cai D et al., 2004; Menconi M et al., 2007; Sandri M et al., 2004; Sartori R et al., 2009), but all of them seem to collaborate or act through FoxO transcription factors (Scheme 9).



Scheme 9. Signaling pathways in muscle atrophy. During catabolic states, several signalling pathways that stimulates the expression of atrogenes are activated. The transcription factors that mediate the expression of the atrogenes are FOXO family proteins, NF-kB, SMAD2 and SMAD3. This transcription factors can be activated because of an external stimulus or due to decrease in PI3K-AKT-mTOR pathway. The disminish of protein synthesis together with the accelerated proteolysis leads to muscle atrophy. ActRIIB, activin A receptor, type IIB; ATGs, autophagy-related genes; IGF1, insulin-like growth factor 1; JUNB, transcription factor JunB; MURF1, muscle-specific RING-finger 1; PGC1α, peroxisome proliferator-activated receptor-γ coactivator 1α; SIRT1, NAD-dependent protein deacetylase sirtuin 1; UPS, ubiquitin–proteasome system. Figure extracted from Cohen S et al., 2015.

SKELETAL MUSCLE ATROPHY DUE TO GLUCOCORTICOIDS

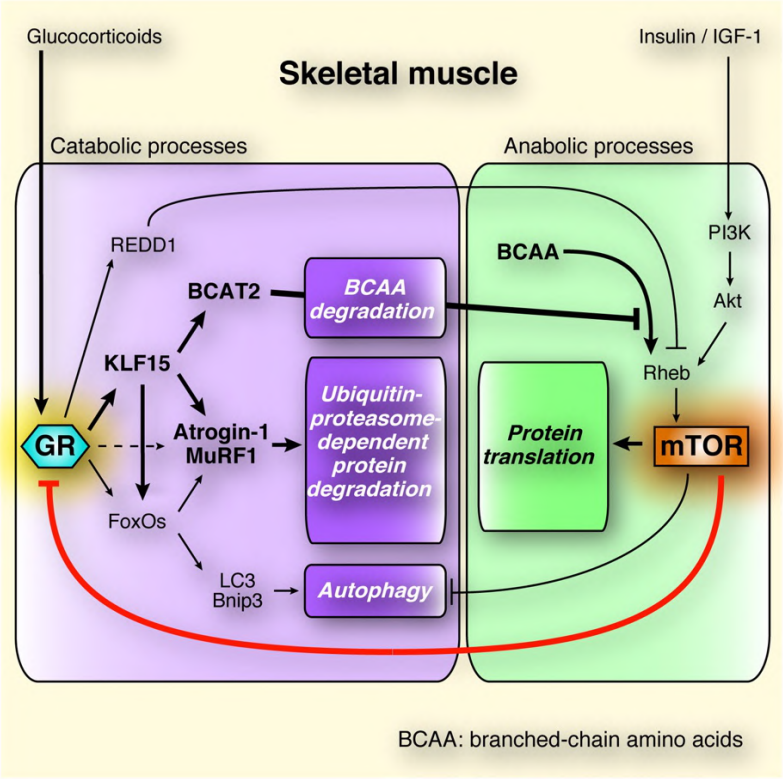
Glucocorticoid receptor

The catabolic effects of glucocorticoids are well known. Either used as drugs in medical conditions or increased in circulating levels due to a pathological state such as sepsis, cachexia, starvation, metabolic acidosis or Cushing disease, glucocorticoids are associated to muscle wasting (Schakman O et al., 2013). The effects of glucocorticoids are mediated by the glucocorticoid receptor (GR). Human GR gene is located in chromosome 5 and is composed of 9 exons, it is susceptible of alternative splicing in exon 9, near the end of the GR, that generates two receptor isoforms, GRα and GRβ. These two proteins are identical from amino terminus to amino acid 727 but then vary. GRα, the classic GR protein that mediates the actions of glucocorticoids, contains an additional 50 amino acids. Binding of glucocorticoids to GRα induces conformational changes in the receptor, dissociation from chaperone proteins, dimerization of the receptor, nuclear import, recruitment of cofactors and finally, binding to DNA. This complex binds to glucocorticoid response elements (GREs) in the promoter of target genes, resulting in the activation of the atrophy program. Two types of GREs exist: positive GREs, that command transcription induction and negative GREs that direct transcription repression. (Lu NZ and Cidlowski JA, 2004; Lu NZ and Cidlowski JA, 2006; Taiyi K et al., 2013). Whereas GRβ contains an additional, nonhomologous 15 amino acids and does not bind glucocorticoid agonists, it resides constitutively in the nucleus of cells, and by itself fails in activate gene transcription. From a functional point of view only GRα binds to DNA in response to glucocorticoid. The competition for GRE binding, for transcriptional coregulators, and formation of inactive GRα/GRβ heterodimers have been proposed to underlie the antagonism mediated by GRβ (Oakley RH and Cidlowski JA, 2013).

Dexamethasone in muscle atrophy

Dexamethasone (Dexa) is a synthetic glucocorticoid with 20 to 30 times the binding affinity for glucocorticoid receptors of endogenous cortisol. It has anti-inflammatory and immunosuppressant properties being widely used as a potent antiemetic, for the treatment of acute exacerbations of multiple sclerosis, allergies, cerebral edema, inflammation, and shocks as well as in patients with conditions such as asthma, atopic and contact dermatitis, and drug hypersensitivity reactions (Johnson DB, Kelley B, 2019; Whelan R and Apfel CC, 2013). Dexa acts through the glucocorticoid receptor and regulates the gene expression binding directly to GREs, known as genomic action (Oakley RH and Cidlowski JA,2009; Patel R et al., 2014; Zhao et al, 2009), or indirectly through direct interactions with cytosolic kinases or specific membrane-bound receptor named non genomic effects (Lowenberg et al, 2008; Stahn et al, 2008). However, is widely use in medicine, high dose of glucocorticoids may lead to muscle wasting, activating the protein degradation in muscle through the UPS and ALS overall (Menconi M et al., 2008; Schakman et al, 2009). The stimulation of the GR by Dexa leads to the activation of these two proteolytic systems increasing the expression of several atrogenes such as FoxO, E3 ubiquitin ligases, MAFbx and MuRF1, and LC3 and Bnip3 (Cid-Diaz T et al., 2017; Mammucari C et al., 2007; Sandri M et al., 2004; Stitt TN et al., 2004). Moreover, is was also described an inhibitory effect of glucocorticoids repressing mTORC1 signaling as a result of enhanced transcription of REDD1 and KLF-15, two genes that are direct targets of GR receptor in muscle. This fact suggests a coordinated interaction between the catabolic signal and the anabolic machinery. REDD1 represses mTORC1 by inhibiting Rheb, which is a positive effector of mTORC1, leading to decreased phosphorylation of both 4E-BP1 and S6K1. On the other hand, KLF15 stimulates the expression of branched-chain amino acid (BCAA) aminotransferase (BCAT), an enzyme which degrades BCAA, and accelerates the

intracellular catabolism of BCAA. Furthermore, KLF-15 cooperates with FOXO1 to upregulate the promoter activity of Atrogin-1 and MuRF1(Shimizu N et al., 2001; Tanaka H et al., 2017) (Scheme 10).



Scheme 10. Crosstalk between catabolic process due to glucocorticoids and anabolic process in skeletal muscle. Glucocorticoids exterts its atrophic effect directly promoting the expression of MURF1 or indirectly by the activation of the FOXO family transcription factors and KLF15 which foment MURF1, MAFbx and autophagy protein transcription. They also increase the expression of REDD1 which inhibits the analbolic effect of mTOR. Together these pathways promote muscle atrophy. Figure extracted from Shimizu N et al. 2011. With permission of Elsevier

Ubiquitin proteasome system

The UPS is an ATP-dependent proteolytic system that mediates the degradation of target proteins tagged with ubiquitin molecules in the 26S proteasome (Murton AJ et al.,2008). The ubiquitin is a 76 amino acid protein with an 8,5 kDa of molecular weight, it can be added to a lysine un the target protein as a single entity (monoubiquitin) or as a chain of variable length (polyubiquitin) (Kravtsova-Ivantsiv Y and Ciechanover A, 2012). The 26S proteasome is a macromolecular responsible for controlled degradation of ubiquitinated substrates. It is composed for 33 subunits at least, which are assemble in two subcomplexes: the 20S core particle (CP), responsible for proteolytic activities showing highly conserved "barrel"-like structure arranged into four heptameric rings stacked in the order of α7β7β7α7, and the 19S regulatory particle (RP), responsible of substrate recognition, deubiquitination, protein unfolding, and substrate translocation to the 20S CP for degradation (Wang X et al., 2017). The ubiquitylation is regulated by the activity of three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin protein ligases (E3). First, E1 adenylates the C-terminus of ubiquitin and forms a thioester bond between the ubiquitin C-terminus and a catalytic E1 cysteine residue, in an ATP-dependent process. Then, ubiquitin is transferred to a E2 enzyme, forming another thioester bond between ubiquitin and a cysteine residue of the E2. Finally, the ubiquitin molecule is transferred from the E2 to a lysine residue of the target protein by the action of a E3. E3 enzyme provides with specificity this process as they recognize and bind ubiquitin molecules to a specific target sequence (Bodine SC and Baehr LM, 2014; Rom O and Reznick AZ, 2016).

In the context of skeletal muscle, in 2001 two novel E3 ubiquitin-ligases were identified, which expression is significantly increased in response to multiple catabolic conditions characterized by muscle atrophy, MAFbx (*FBXO32*) and MURF1 (*Trim63*) (Bodine SC et al., 2001; Gomes MD et al., 2001). MAFbx belongs

to cullin- RING E3 ligase family, it has an F-box domain allowing it to bind both skp1 and cullin1, suggesting a function in a SCF ubiquitin ligase complex (Gomes MD et al., 2001). It is found in the cytoplasm but it also possesses a nuclear localization sequence suggesting a possible role in the nucleus. The primary targets of MAFbx in skeletal muscles are the myogenic regulatory factor MyoD and the eukaryotic translation initiation factor 3 subunit f (eIF3-f) (Lagirand-Cantaloube J et al, 2008; Tintignac LA et al., 2005). Further in vitro studies revealed that MAFbx and MyoD are regulated inversely during the differentiation of muscle cells, and overexpression of MAFbx suppressed the differentiation ability due to block the proper myoblast fusion for myotube formation (Tintignac LA et al., 2005). MURF1 is a RING E3 ligase with a zinc-finger domain and a leucine-rich coiled-coiled domain which allows it to form heterodimers with other MURF proteins, and an acidic C-terminus tail (Foletta VC et al., 2011). MURF-2 and MURF-3 are two proteins encoded by a different gene than MUFR-1 that have a high homology with it. They are found in the M-line of the sarcomere. Additionally, MURF1 and MURF-3 are in Z-lines. MURF1 and MURF-2 are also detected in the nucleus. Whereas MURF-2 and MURF-3 have been shown to be important for microtubule stability, just MURF1 have been associated with muscle atrophy (Bodine SC and Baehr LM, 2014). It was demonstrated the interaction of MURF1 with proteins involved in ATP generation and myofibrillar proteins such as nebulin, titin, MLC-2 and cTNI (Witt SH et al., 2005) and in vitro studies reveal that during muscle atrophy MuRF1 participates in the degradation on thick filament proteins following the degradation of MHC (Clarke BA et al., 2007; Cohen S et al., 2009). Knowing the role of this ubiquitin ligases under atrophy conditions, different in vitro studies using mouse cell lines under acute treatment of glucocorticoid demonstrate that both MAFbx and MURF1 undergo a significant upregulation (Cid-Diaz T et al., 2017; Evenson AR et al, 2005; Du J et al, 2000; Hong DH and Forsberg NE, 1995; Marinovic AC et al, 2006; Sacheck JM et

al, 2004; Stitt TN et al, 2004; Thompson MG et al, 1999; Wang L et al, 1998; Yang H et al, 2005).

In terms of regulation, both MAFbx and MURF1 are transcriptional regulated by the same transcription factors, the first described were FoxO family which includes FoxO1, FoxO3 and FoxO4 and can bind directly to MAFbx and MURF1 promoter (Waddell DS et al., 2008). One the one hand it was described that FoxO1 was necessary but not sufficient to increase the expression of MAFbx and MURF1 in vitro in response to dexamethasone (Stitt TN et al., 2004), other studies focus in FoxO3 demonstrating its ability to upregulate both ubiquitin ligases during atrophy conditions (Sandri M et al., 2004). However, it have been reported discordances in the regulation of FoxO transcription factors , MAFbx and MURF1 between human and mouse models, suggesting a complex regulation of this system depending on the type of atrophy and between species (Cid-Diaz T et al., 2017; Foletta VC et al., 2009; Gomes MD et al 2001; Gustafsson T et al., 1985; Harber MP et al., 2008; Larsen AE et al., 2006). Moreover, MURF1 has a glucocorticoid response element in the proximal region of the promoter that can bind directly the GR (Waddell DS et al., 2008), whereas MAFbx expression has to be activated indirectly since its promoter does not have direct binding sites to the GR (Shimizu N et al., 2011). Another transcription factor is responsible of the upregulation of both atrogenes, KLF15 is upregulated after dexamethasone treatment, it has the ability to upregulate the expression of FoxO1 and FoxO3, thus, promoting indirectly the expression of MAFbx and MURF1 as well as both atrogenes have KLF15-binding sites, allowing its direct expression by KLF15 (Shimizu N et al., 2011).

Autophagy Lysosomal system

Autophagy is a well conserved homeostatic mechanism used for degradation and recycling of bulk cytoplasm, long-lived proteins and organelles (Mizushima N and Komatsu M, 2011). There are three types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy is believed to be the major and the most studied type of autophagy, it needs the formation of a membrane called autophagosome, isolates a small portion of cytoplasm, this autophagosome fuses with the lysosome forming the autolysosome where the invaginated materials are degraded (Mizushima N and Komatsu M, 2011; Mizushima N et al., 2011). Autophagosomes are generated in the proximity to the endoplasmic reticulum (ER) and their formation is carried out by multiple Atg proteins (Nakatogawa H et al., 2009), more specifically, in muscle the autophagic response in atrophy conditions can be initiated via mTOR1 which activation leads to the autophagosome formation by the ULK1 complex (ULK1, ATG13, and FIP200)(Di Rienzo M, et al., 2019). However, autophagy is considered a non-selective degradation pathway, autophagosomes are able to recognize proteins such as p62, which binds target proteins and delivered them as a cargo inside the autophagosome. p62 directly interacts with LC3 on the isolation membrane through the LC3-interacting region being incorporated into the autophagosome to be degraded (Johansen T and Lamark T, 2011; Weidberg H et al., 2011). An impaired autophagy leads to an accumulation of p62 and ubiquitinated aggregates (Komatsu M et al., 2007). It also exists organelle directed autophagy, these include autophagy directed to organelles, as mitophagy, directed to mitochondria, pexophagy to peroxysomes and to intracellular bacteria, called xenophagy (Klionsky DJ et al. 2007).

Microautophagy is carried out in the lysosomes itself, the small components of the cytoplasm are invaginated for the lysosomal membrane and then degraded (Li WW

et al., 2012; Mizushima N and Komatsu M, 2011). In muscle is little known about microautophagy, is believe that it can participate in glycogen clearance (Raben N et al., 2008; Takikita S et al., 2010; Lim JA et al 2019)

The chaperone-mediated autophagy does not involve membrane reorganization. The chaperone protein heat shock cognate 70 (Hsc70) recognizes specific cytosolic proteins which must contain KFERQ-like pentapeptide, then Lamp-2a acts as a receptor in the lysosome allowing this target proteins to be translocated to the lysosome to be degraded (Mizushima N and Komatsu M, 2011; Mizushima N et al., 2011). This type of autophagy is essential for the muscle homeostasis in basal conditions, targeting proteins as filamin C which undergoes unfolding and refolding during muscle contraction (Arndt V et al., 2010). During the last years this type of autophagy has raised interest due to its role in aging, neurodegenerative disorders and lysosomal storage diseases (Kon M, Cuervo AM, 2010).

In terms of regulation, mTOR is the major metabolic sensor in muscle and coordinates the physiological processes depending on nutritional conditions (Neel BA, 2013). In presence of amino acids, mTOR is activated blocking autophagy via phosphorylation of the trimeric protein complex ULK1/FAK family kinase-interacting protein of 200ka (FIP200)/Atg13 (Neel Ba, 2013; Zachari M and Ganley G, 2017). Upon amino acid deprivation, mTOR located on lysosomal surface is no longer active leading to dephosphorylation of ULK1/FAK/Atg13 complex and concomitant autophagy induction (Zachari M and Ganley G, 2017). However, siRNA and inhibitor studies shown that inhibition of mTOR is not sufficient to alter the autophagic flux (Betzinger CF et al., 2008; Mammucari C et al., 2008; Sandri M, 2010). Moreover, AKT also plays an important role in autophagy regulation through FoxO3 phosphorylation. During catabolic conditions, AKT is not able to inhibit FoxO3 by phosphorylation, thus is translocated to the nucleus where it promotes the

expression of autophagy related genes such as LC3, Bnip3 and cathepsinL (Castets P and Rüegg MA, 2013; Cid-Diaz T et al., 2017; Neel BA, 2013).

AMPK is the other well-known regulator of autophagy. Opposing to mTOR activity, during catabolic conditions AMPK can inactivate mTOR blocking the inhibition of ULK1 as well as it can activate ULK1 by phosphorilating a different residue of ULK1 activating the autophagy initiation complex.

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OBJECTIVES

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High circulating glucocorticoid levels is a common issue observed in many pathological conditions. Glucocorticoids activate catabolic pathways such as ubiquitin-proteasome and autophagy-lysosomal systems, leading to deregulation of the balance of protein synthesis and degradation. Based on the anabolic action of the obestatin/GPR39 system, the aims proposed in this work are:

1. To determine whether the obestatin/GPR39 system counteracts the catabolic processes provoked by glucocorticoids.
2. To investigate the cross-talk between protein degradation and synthesis: proteasome- and autophagy-related factors and Akt/FoxO axis.

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MATERIAL AND METHODS

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MATERIALS

Rat/mouse obestatin was obtained from BCN Peptides (Barcelona, ES). Human obestatin was purchased from Biomedal (Sevilla, ES). Insulin was obtained from Novo Nordisk (Bagsvaerd, DK). All other chemical reagents were from Sigma Chemical (Missouri, US).

Antibodies

Table 1. Primary Antibodies

Primary antibody	Use	Dilution	Supplier	Reference
4E-BP1	WB	1:1000	Cell Signaling	9452
IGF-1	WB	1:500	Santa Cruz	Sc-9013
b-arrestin 1	WB	1:1000	Abcam	ab32099
b-arrestin 2	WB	1:1000	Cell Signaling	3857
SIRT1	WB	1:1000	Santa Cruz	Sc-15404
SIRT2	WB	1:1000	Santa Cruz	Sc-20966
SIRT3	WB	1:1000	Santa Cruz	Sc-49744
AC-FOXO1	WB	1:1000	Santa Cruz	Sc-49439
HDAC4	WB	1:1000	Cell Singaling	7628
pPDK (s744/748)	WB	1:1000	Cell Singaling	2054
pCAMKII	WB	1:1000	Cell Signaling	12716
pAMPK (T172)	WB	1:1000	Cell Singaling	2535
AMPK	WB	1:1000	Cell Singaling	2603
Ubiquitin	WB	1:1000	Cell Signaling	3933
S6K1	WB	1:1000	Santa Cruz	Sc-230
Glucocorticoid receptor	WB	1:1000	Santa Cruz	8992
Akt	WB	1:1000	Cell Signaling	9272
Cathepsin L	WB	1:1000	Abcam	ab133641
FKHR (H-128)	WB	1:1000	Santa Cruz	Sc-11350

FKHR (H-128)	IP	1:50	Santa Cruz	Sc-11350
FoxO1	WB	1:1000	Cell Signaling	2880
FoxO3a	WB	1:1000	Cell Signaling	12829
FoxO4	WB	1:1000	Cell Signaling	9472
GAPDH	WB	1:1000	Abcam	ab9485
HDAC4	WB	1:1000	Cell Signaling	7628
LC3A/B	WB	1:1000	Cell Signaling	12741
MAFbx	WB	1:1000	Santa Cruz	sc-166806
Murf1	WB	1:1000	Santa Cruz	sc-32920
Myogenin	WB	1:1000	Hibridoma Bank	F5D
Myosin heavy chain	WB	1:1000	Hibridoma Bank	MF20
Myosin heavy chain	IHF	1:200	Hibridoma Bank	MF20
p-S6 (S240/244)	WB	1:2000	Cell Signaling	2215
p-S6K1(T389)	WB	1:1000	Cell Signaling	9234
p4E-BP1 (Thr37/46)	WB	1:1000	Cell Signaling	9459
P4E-BP1 (Thr70)	WB	1:1000	Cell Signaling	9455
p62	WB	1:1000	Cell Signaling	5114S
pAkt(S473)	WB	1:1000	Cell Signaling	9275
pFoxO1 (Ser256)	WB	1:1000	Cell Signaling	9461
pFoxO1 (Thr24)/FoxO3a (Thr32)	WB	1:1000	Cell Signaling	9464
pFoxO1 (Thr24)/FoxO3a (Thr32)/FoxO4 (Thr28)	WB	1:1000	Cell Signaling	2599
pFoxO3a (Ser253)	WB	1:1000	Cell Signaling	13129
S6	WB	1:1000	Cell Signaling	2217
pHDAC4(S246)/HDAC5(S259)/ HDAC7(S155)	WB	1:1000	Cell Signaling	3443

Note: Relation of the primary antibodies used in the different analyses performed in this work. IF, immunofluorescence; WB, western blot.

Table 2. Secondary Antibodies

Secondary antibody	Use	Dilution	Supplier	Reference
Goat Anti-Mouse IgG (H&L) Alexa Fluor 488	IF	1:1000	Invitrogen	A11029
Goat Anti-Rabbit IgG Fc Alexa Fluor 488	IF	1:1000	Abcam	ab150089
Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L)	WB	1:10000	Jackson ImmunoResearch	111-035-003
Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L)	WB	1:10000	Jackson ImmunoResearch	115-035-003
Peroxidase AffiniPure Donkey Anti-Goat IgG (H+L)1	WB	1:10000	Jackson ImmunoResearch	705-035-003

Note: Relation of the primary antibodies used in the different analyses performed in this work. IF, immunofluorescence; WB, western blot

METHODS

Cell culture and differentiation. Mouse C2C12 (ECACC, Whiltshire, UK) myoblasts were cultured as described by the supplier (ECACC, Whiltshire, UK). Briefly, C2C12 myoblasts were maintained in growth medium (GM) containing DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin. For routine differentiation, the cells were grown to ~80% confluence and GM was replaced with differentiation medium (DM) DMEM

supplemented with 2% horse serum (HS), 100 U/mL penicillin, and 100 U/mL streptomycin) for 7 days unless otherwise stated.

Myogenic primary, C25 cells, and clonal line, KM155C25 Clone 48 (KM155C25 cells), were obtained from the platform for immortalization of human myoblasts of the Center for Myogenic Research in Myology in Paris (Paris, FR) who developed the isolation and immortalization from a biopsy obtained through MYOBANK, a partner in the EU network EuroBioBank (gracilis muscle, donor age 25 years). Primary myogenic cells isolated from biopsies were purified by magnetic activated cell sorting using anti-CD56 (a specific marker of myoblasts) beads (MACS, Miltenyl Biotech). Purity before and after cell sorting was determined by immunolabelling (anti-desmin and anti-mouse IgG1 AlexaFluor 488 antibodies) following the protocols previously described (Mamchaoui K, et al., 2011). Myogenic primary line, C25 cells, were cultured in GM containing Medium 199 (1:4, v/v; Lonza, Pontevedra, ES) supplemented with 20% FBS (v/v), 50 µg/mL gentamicin (Invitrogen, Thermo Fisher Scientific; Massachusetts, US), 25 µg/µL fetuin, 5 ng/mL hEGF, 0.5 ng/mL bFGF, 0.2 µg/mL Dexa (Sigma Chemical, Missouri, US) and 50 µg/mL gentamycin (Invitrogen, Thermo Fisher Scientific; Massachusetts, US) on matrigel-covered cell culture dishes (0.1 mg/mL, 1 mL per 19.5 cm^2 dish area, 45 min, 37 °C), incubated at 37 °C, 5% CO_2. C25 cells were differentiated in DM, Medium 199: DMEM (1:4, v/v) supplemented with 2% FBS and 1 µg/mL gentamicin on matrigel-covered 12 well multiplates for 7 days unless otherwise stated, incubated at 37 °C, 5% CO_2. Stable immortalized cell line from C25, KM155C25 cells was carried out as previously described (Mamchaoui K, et al., 2011). In brief, primary C25 cells were co-transduced with two retroviral vectors expressing hTERT and CDK-4 cDNA (Mamchaoui K, et al., 2011). Co-transduced cells were selected by neomycin and puromycin and then purified using magnetic beads coupled to antibodies directed against the myogenic marker CD56. Following culture at clonal density, individual

myogenic clones with extended proliferative lifespans, as compared to the untransduced cells, were isolated from each population. Immortalized human myoblasts, KM155C25 Clone 48 (KM155C25 cells), maintain their capacity to differentiate both in vitro and in vivo after transplantation into the regenerating muscles of immunodeficient mice (Mamchaoui K, et al., 2011; Thorley M et al., 2016). KM155C25 cells were cultured in GM containing Medium 199:DMEM (1:4, v/v; Lonza, Pontevedra, SP) supplemented with 20% FBS (v/v), 50 µg/mL gentamicin (Invitrogen), 25 µg/µL fetuin, 5 ng/mL hEGF, 0.5 ng/mL bFGF, 0.2 µg/mL Dexa (Sigma Chemical, Missouri, US) and 50 µg/mL gentamycin (Invitrogen, Thermo Fisher Scientific; Massachusetts, US) in cell culture dishes incubated at 37 °C, 5% CO_2. KM155C25 cells were differentiated in DM Medium 199: DMEM (1:4, v/v) supplemented with 1 µg/mL gentamicin in 12 well multiplates for 7 days unless otherwise stated, incubated at 37 °C, 5% CO_2.

Murine and human myotubes were treated with Dexa at concentrations from 0.05 to 100 µM for 24 hours in the presence or absence of obestatin from 5 to 100 nM, or with insulin 100 nM as a positive control of atrophy protection.

Protein synthesis analysis. We used Click-iT™ Plus OPP Alexa Fluor™ 488 Protein Synthesis Assay Kit (Thermo Fisher Scientific; Massachusetts, US), according to instruction provided by manufacturer. In brief OPP reagent is a puromycin analog containing an alkyne moiety. When added to culture media, OPP is readily taken up by actively growing cells. OPP inhibits protein synthesis by disrupting peptide transfer on ribosomes causing premature chain termination during translation. Addition of the Alexa Fluor® picolyl azide leads to a chemoselective ligation between the picolyl azide dye and the alkyne OPP, allowing the modified proteins to be detected by imaged-based analysis. We treated C2C12 an KM155C25 cells with Dexa 1 µM, Dexa 1 µM + obestatin 10 nM or Dexa 1 µM+ Insulin 100 nM during

24 h. After this treatment we added 20 μM of Click-iT® OPP reagent for 30 min. Intact cells were washed once with PBS, fixed with 96% ethanol 15 min at room temperature, washed twice with PBS, permeabilized with 0.5% Triton® X-100 in PBS and incubate for 15 minutes at room temperature. Cells were washed twice with PBS and incubated with Click-iT® Plus OPP reaction cocktail for 30 min, washed twice with PBS incubated and with HCS NuclearMask™ Blue Stain for 30 min more. Digital images of cell cultures were acquired with a Leica TCS-SP5 spectral confocal microscope (Leica Microsystems, Heidelberg, DE) and then fluorescence intensities were analyzed with Image J.

Immunofluorescence. Myoblast cells were cultured on coverslips and differentiated into myotubes under DM for 7 days until a full mature differentiation was visible. Myotubes were then treated with Dexa for 24 h in the presence or absence of obestatin or insulin as indicated above. Intact cells were fixed with 96% ethanol for 15 min at room temperature, washed twice with ice cold PBS, permeabilized with PBST (PBS, 0,3% Triton X-100, 0,1M Glycine) 10 min at room temperature, washed three times with ice cold PBS for 5 min and blocked with 1% BSA in PBST for 30 min at room temperature. Then incubated with primary antibody diluted in 1% BSA in PBST over night at 4°C. After three washes with ice cold PBS for 5 min each, cells were incubated with the secondary antibody (FITC-conjugate goat anti-mouse antibody or FICT-conjugated goat anti-rabbit antibody) in 1% BSA in PBST (1:1000) for 1 h at room temperature, then washed three times in ice cold PBS for 5 min each in dark. Topro or DAPI was used to counterstain the cell nuclei (Invitrogen Thermo Fisher Scientific; Massachusetts, US). Digital images of cell cultures were acquired with a Leica TCS-SP5 spectral confocal microscope (Leica Microsystems, Heidelberg, DE) and Zeiss Axio Vert.A1 (Zeiss, DE). Myotube areas (MHC^+ cells, ≥

3 nuclei) were quantified by measuring a total of >100 myotube areas from 5 random fields in 3 replicates using ImageJ64 analysis software.

Small interfering RNA (siRNA) silencing of gene expression. Chemically synthesized double-stranded siRNA duplexes targeting FoxO4 was selected from ON-TARGETplus SMARTpool siRNA (Dharmacon, Colorado, US). Human FoxO4, AGUCAUGCCUGGAAGCUUU; GAGAAGCGACUGACACUUG; GGAAAUACCAGCUUCAGUC; CAACGAGGCCACCGGCAAA).

Chemically synthesized double-stranded siRNA duplexes targeting IGF-1 was a pool of three target sequences (Santa Cruz): 1. CAGACUUUGUACUUCAGAAtt; UUCUGAAGUACAAAGUCUGtt. 2. GCAUGGGUGUUGUAUAGAUtt; AUCUAUACAACACCCAUGCtt. 3.GACUAGAGUUUCAGUUGAAtt; UUCAACUGAAACUCUAGUCtt

Chemically synthesized double-stranded siRNA duplexes targeting β-arrestin 1 and β-arrestin 2 was selected from ON-TARGETplus SMARTpool siRNA (Dharmacon, Colorado, US) Human β-arrestin 1, UGGAUAAGGAGAUCUAUUA; AUGGAAAGCUCACCGUCUA; GAACUGCCCUUCACCCUAA; GAACGAGACGCCAGUAGAU; Human β-arrestin 2, CGAACAAGAUGACCAGGUA, CGGCGUAGACUUUGAGAUU, GGGCUUGUCCUUCCGCAAA, UAGAUCACCUGGACAAAGU.

Chemically synthesized double-stranded siRNA duplexes targeting KLF15 was a pool of three target sequences (Santa Cruz): 1. CAGCAAGAUGUACACCAAAtt; UUUGGUGUACAUCUUGCUGtt. 2. CCAAGGCCAGAACUUUAGUtt; ACUAAAGUUCUGGCCUUGGtt. 3. CAUAGGUCCAUCCACAUAAtt; UUAUGUGGAUGGACCUAUGtt.

An ON-TARGETplus nontargeting siRNA (Dharmacon, Colorado, US) was used as a control for all siRNA experiments. Human KM155C25 myotubes were transfected

at day-6 post-differentiation with Lipofectamine 2000 (Invitrogen Thermo Fisher Scientific; Massachusetts, US), following manufacturer's instructions with 50nM of SiRNA targeting FoxO4, IGF-1, β-arrestin 1 or β-arrestin 2 for 24 h, after block the protein of interest cells were stimulated with Dexa, obestatin or a combination of both as was indicated above.

SDS-PAGE and western blot analysis. The cell samples were directly lysed in ice-cold RIPA buffer [50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 0.25% (w/v) Na-deoxycholate, protease inhibitor cocktail, phosphatase inhibitor cocktail (Sigma Chemical, Missouri, US)]. The lysates were clarified by centrifugation (14,000xg for 15 min at 4°C) and the protein concentration was quantified using the QuantiPro™ BCA assay kit (Sigma Chemical, Missouri, US). For immunoblotting, equal amounts of protein were fractionated by SDS-PAGE and transferred onto nitrocellulose membranes. Immunoreactive bands were detected by enhanced chemiluminescence (Pierce ECL Western Blotting Substrate; Thermo Fisher Scientific; Massachusetts, US).

Co-immunoprecipitation assays. Following treatment [DM, DM+Dexa (1 μM, 24 h), DM+Dexa (1 μM, 24 h)+obestatin (10 nM, 24 h)], KM155C25 myotubes cells were washed twice with ice-cold PBS and lysed in co-immunoprecipitation lysis buffer (50 mM Tris, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 10 mM glycerol phosphate, 200 μM, sodium orthovanadate, 2.5 mM sodium pyrophosphate plus protease and phosphatase inhibitors). FoxO1 was immunoprecipitated using rabbit anti-FoxO1 antibody coupled to protein A-Dynabeads according to instruction provided by manufacturer (Thermo Fisher Scientific; Massachusetts, US). The washed immunoprecipitates were subjected to western blot analysis using the indicated antibodies.

Data analysis. All values are presented as mean ± standard error of the mean (SEM). Student *t* test were performed to assess the statistical significance of 2-way analysis. For multiple comparisons, ANOVA was employed. P <0.05 was considered as statistically significant (*).

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RESULTS

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THE OBESTATIN/GPR39 SIGNALING CONTROLS THE UBIQUITIN-PROTEASOME AND AUTOPHAGY-LYSOSOME SYSTEMS IN GLUCOCORTICOID INDUCED MUSCLE CELL ATROPHY IN MOUSE AND HUMAN

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Glucocorticoid receptor has same levels of expression in C2C12 KM155C25 cell lines and in C25 primary cells

Since the cellular level of glucocorticoid receptor is a critical determinant of glucocorticoid sensitivity and resistance (Oakley H and Cidlowski A, 2013), we investigated by immunoblot analysis the glucocorticoid receptor levels in our studied cell models; C2C12 mouse myoblast cell line, KM155C25 human myoblast cell line and C25 human myogenic primary cells. As we shown in Figure 1.1 these three myogenic cell models expressed comparable protein levels of glucocorticoid receptor alpha as well as glucocorticoid receptor beta.

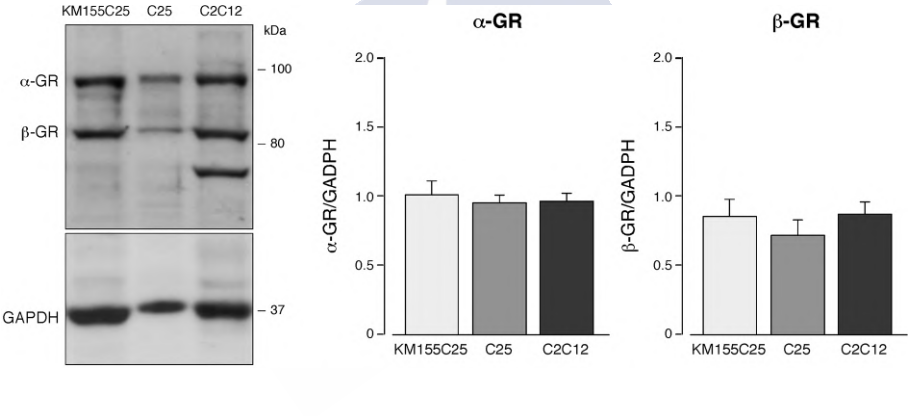

Figure 1.1. Expression of glucocorticoid receptor. Immunoblot analysis of GR on C2C12 mouse myoblast cell line, KM155C25 human myoblast cell line and C25 human myogenic primary cells. Data were expressed as mean±SEM obtained from intensity scans (n=3)

The obestatin/GPR39 system regulates ubiquitin-proteasome and autophagy-lysosomal signaling in dexamethasone treated C2C12 mouse myotubes

We first defined a dose-response effect for the synthetic glucocorticoid Dexamethasone (Dexa: 0.05-100 µM) in order determine the concentration in which the maximal response in the expression of the ubiquitin-ligases MuRF1 and MAFbx would be obtained in fully differentiated C2C12 myotubes. After 24 h of Dexa treatment, a maximal effect on MuRF1 and MAFbx protein content was detected at 1 µM of Dexa [~4.5-4.9-fold of control cells, respectively (Fig. 1.2)], this concentration was used for the subsequent assays.

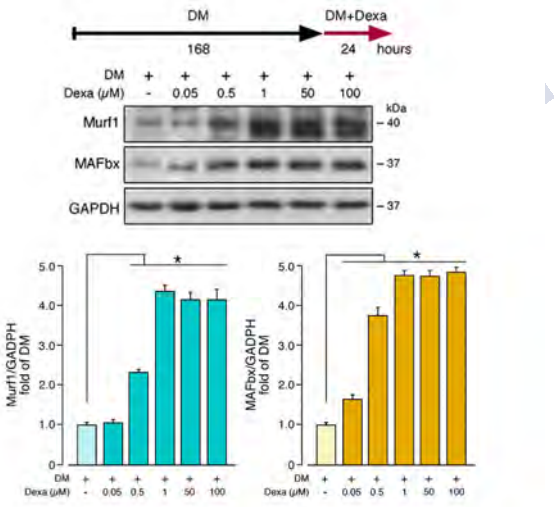

Figure 1.2. Dose response of Dexa in MURF1 and MAFbx expression. Immunoblot analysis of MuRF1 and MAFbx on differentiated C2C12 mouse myotubes under Dexa treatment (0,05-100 µM). Data were expressed as mean±SEM obtained from intensity scans (n=3; * *P*< 0.05 *versus* DM values. Student t-test).

We performed an immunofluorescence assay for MHC, as it is the structural protein of skeletal muscle, to found out the morphological changes suffered after Dexa treatment. Under Dexa treatment, C2C12 myotubes showed a 40±2% decrease in area (Fig. 1.3a). This decrease was reversed, observing a marked rescue of myotube area when we co-administrated obestatin at the range of concentrations tested (5, 25 and 100 nM: 39±2%, 36±4% and 36±2% increase over Dexa treated myotubes, respectively; Fig. 1.3a). Furthermore, it should be noted that obestatin-treated cells achieved ~27% more recovery than insulin-treated cells (12±3% increase over Dexa treated myotubes; Fig. 1.3a), used as positive control of muscle atrophy. A detailed area analysis at 5 nM obestatin revealed a significant increase in the percentage of myotubes with larger area compared to Dexa treated myotubes ~91% versus 4% of myotubes in a range between 2x103-8x102 μm2, and ~9% versus 96% in the range between 8-2x102 μm2, respectively, (Fig. 1.3b).

We analyzed the effects of obestatin treatment (5-100 nM, 24 h) at a molecular level by immunoblot analysis. In mature differentiated myotubes, obestatin treatment combined with Dexa treatment, decreased the expression of the ubiquitin E3-ligases MuRF1 and MAFbx compare with Dexa treated cells by 45-69% and 44-58%, respectively. Insulin (100 nM, 24 h) decreased similarly MuRF1 and MAFbx expression by 62±2% and 93±2%, respectively (Fig. 1.4). Concomitant with the results obtained in the previous immunofluorescence analysis, it is important to highlight that obestatin signaling was able to restore MHC expression levels, showing 32-108% above those reached in Dexa treated C2C12 myotubes (Fig. 1.4b). Moreover, it should be noted that the results achieved for MHC expression in response to obestatin (100 nM) were superior to those reached with insulin (108±6% versus 35±3%, respectively).

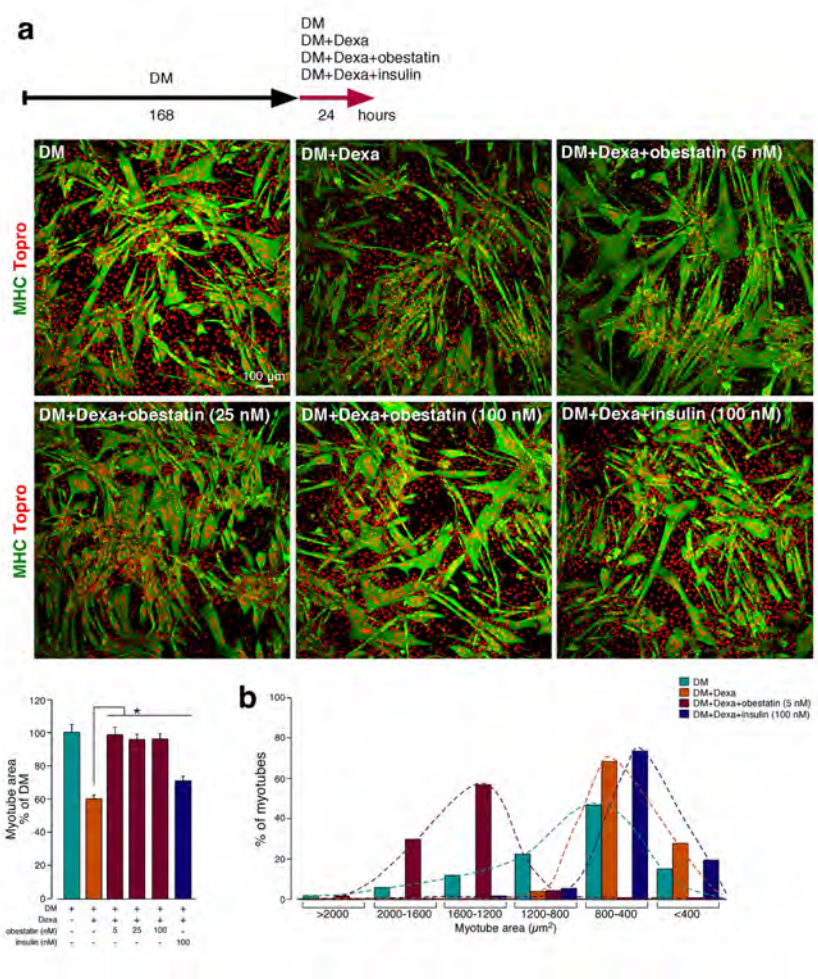

Figure 1.3. Obestatin restores myotube area in Dexa atrophied myotubes. (a) Immunofluorescence detection of MHC and Topro on differentiated C2C12 mouse myotubes under DM, DM+Dexa 1 µM, DM+Dexa 1 µM+obestatin 5nM, DM+Dexa 1 µM+obestatin 25nM, DM+Dexa 1 µM+obestatin 100nM DM+Dexa 1 µM+insulin 100nM during 24h. (b) Evaluation of myotube area and distribution. Data were expressed as mean±SEM (n=3; $P< 0.05$ *versus* *DM or # DM + Dexa1µM values. Student t-test)

Furthermore, when we analyzed the muscle-specific transcription factor myogenin. Myogenin was upregulated following Dexa treatment as well as after obestatin and insulin co-treatments by ~24% compared with control cells (Fig 1.4), this result was consistent with the dual role of myogenin in skeletal muscle, not only promoting the expression of the MuRF1 and MAFbx under catabolic conditions, but also counteracting the atrophy phenotype favoring muscle growth (Moresi, et al., 2010). We studied autophagy as one of the mayor degradative pathways in catabolic conditions. The levels of microtubule-associated protein 1 light chain 3 isoform I (LC3-I) did not change in Dexa treated myotubes, whereas the lipidated form (LC3-II), a reliable marker of autophagosome formation, was significantly elevated (Fig. 1.4c), evidenced by an increase in the LC3II/LC3I ratio observed in Dexa treated C2C12 myotubes. This increase was reversed by 18-34% upon obestatin treatment consistent with decreased LC3-II levels (Fig. 1.4). Furthermore, significant accumulation of p62 protein was seen in obestatin-stimulated cells as compared to Dexa treated C2C12 myotubes (144-169%) indicating a block in autophagy (Fig. 1.4c). By contrast, obestatin stimulation was associated with a reduction in the expression of the lysosomal enzyme Cathepsin L by 31-29% as compared to Dexa treated myotubes, further supporting, at least, a partial inactivation of autophagy. Insulin treatment decreased LC3-II/LC3-I ratio and increased p62 accumulation, although it failed to modify Cathepsin L levels (Fig. 1.4c).

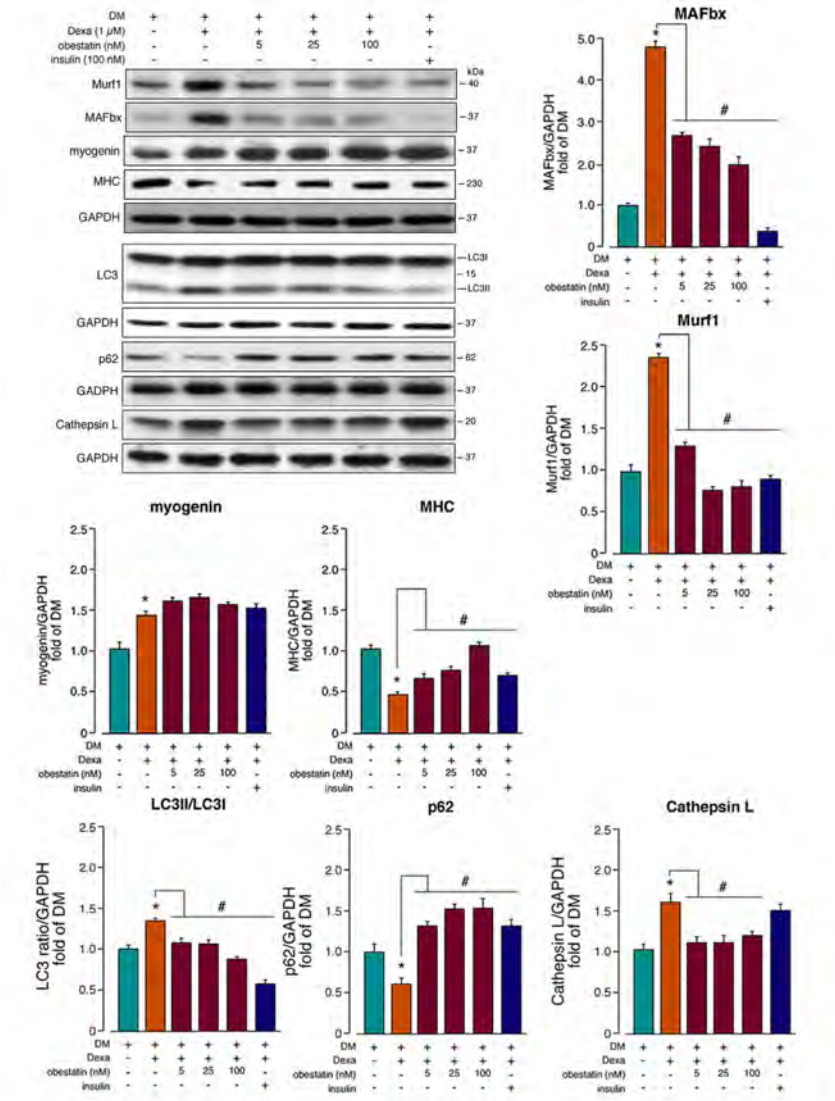

Figure 1.4. Obestatin counteract the atrophy pathways activated during Dexa treatment. Immunoblot analysis of MuRF1, MAFbx, myogenin, MHC, LC3I/II, p62 and cathepsinL on differentiated C2C12 mouse myotubes under DM, DM+Dexa1μM, DM+Dexa1μM+obestatin 5nM and DM+Dexa1μM+insulin 100nM during 24h. Data were expressed as mean±SEM obtained from intensity scans (n=3; $P< 0.05$ *versus* *DM or # DM + Dexa1μM values. Student t-test)

The obestatin/GPR39 system restored protein synthesis in dexamethasone treated C2C12 mouse myotubes

Given that we saw a restored level of MHC we decided to study whether or not protein synthesis pathway could be altered after Dexa treatment and if the obestatin/GPR39, as an anabolic signal, could be restoring this pathway.

By immunoblot analysis we found a 1.8- to 2.9-fold increase in S6K1 phosphorylation at S371 [pS6K1(S371) related to Dexa treated cells; Fig. 1.5a] and consequently a 2.5- to 2.6-fold increase in phosphorylation of its downstream target, the ribosomal protein S6 at S240/244 [pS6(S240/244); Fig. 1.4]. In insulin-treated cells, pS6K1(S371) and pS6(S240/244) were also significantly increased 1.9- and 2.5-fold, respectively (Fig. 1.5a).

Furthermore, obestatin markedly promoted 4E-BP1 hyperphosphorylation at T70 [p4E-BP1(T70)], especially concerning the γ form (2.0- to 2.9-fold), altering the basal phosphorylation of β and γ forms at T37/T46 [p4E-BP1(T37/46); 1.3- to 1.6-fold and 2.0- to 2.9-fold, respectively] in Dexa treated cells (Fig. 1.5a). The p4E-BP1(T70) γ form was also increased by insulin (1.9-fold related to Dexa treated cells). Insulin did not appreciably alter basal T37/T46 phosphorylation (β or γ forms).

To further assess the protein synthesis status, we performed a protein synthesis assay in presence or not of cycloheximide, a protein synthesis inhibitor (Bonifacio A et al., 2017). As shown in Fig 1.5b, a significant increase in protein synthesis was observed after obestatin treatment related to Dexa-treated cells (Fig 1.5b).

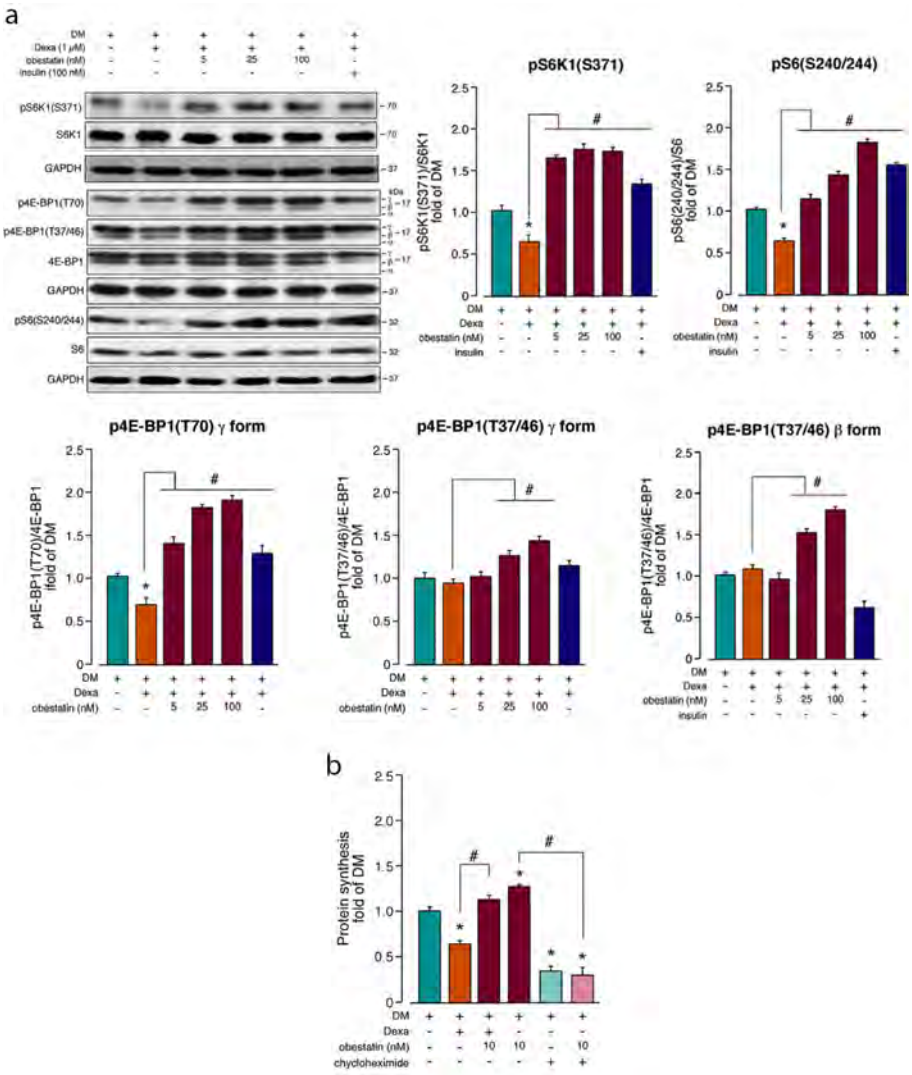

Figure 1.5. Obestatin restores protein synthesis after Dexa treatment. (a) Immunoblot analysis of S6K1, S6 and 4EBP1 on differentiated C2C12 mouse myotubes under DM, DM+Dexa1µM, DM+Dexa1µM+obestatin5nM and DM+Dexa1µM+insulin 100nM during 24h. (b) Evaluation of protein synthesis on differentiated C2C12 mouse myotubes under DM, DM+Dexa1µM, DM+Dexa1µM+obestatin5nM and DM+Dexa1µM+insulin 100nM during 24h using Click-iT™ Plus OPP Alexa Fluor™ 488 Protein Synthesis. Data were expressed as mean±SEM obtained from intensity scans (n=3; *P*< 0.05 *versus* *DM or # DM + Dexa1µM values. Student t-test)

The obestatin/GPR39 system regulates the interplay between AKT and FoxO signaling in dexamethasone-induced atrophy in C2C12 mouse myotubes

The down-regulation of ubiquitin E3-ligases and autophagy and up-regulation of protein synthesis seen after obestatin treatment suggested the implication obestatin/AKT-mTOR signaling pathway. Indeed, the regulatory residue S473 [pAKT(S473)] of AKT was analyzed showing an increase of 2.5- to 3.8-fold in obestatin-stimulated cells as compared to Dexa treated C2C12 myotubes. This up-regulation in AKT activity was concomitant with a phosphorylation at S2448 residue [pmTOR(S2448)] of mTOR, 2.6- to 2.8-fold increase in obestatin treated cells related to Dexa treated cells (Fig 1.6).

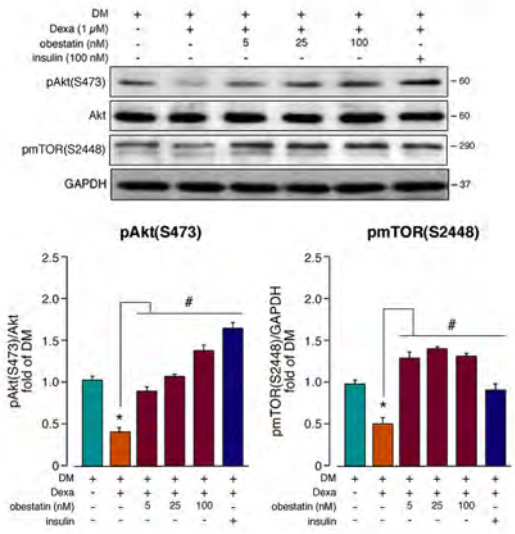

Figure 1.6. Obestatin restores anabolic signaling after Dexa treatment. Immunoblot analysis of AKT and mTOR on differentiated C2C12 mouse myotubes under DM, DM+Dexa1μM, DM+Dexa1μM+obestatin5nM and DM+Dexa1μM+insulin 100nM during 24h. Data were expressed as mean±SEM obtained from intensity scans (n=3; *P*< 0.05 *versus* *DM or # DM + Dexa1μM values. Student t-test)

One mechanism by which AKT reduces the expression of the ubiquitin E3-ligases is the phosphorylation and subsequent nuclear exclusion of FoxO family members. Since mTOR is a suppressor of the autophagy-lysosome system, while FoxO is an inducer of autophagy-dependent degradation, we decided to study the FoxO family status under the obestatin/GPR39 system activation. As shown in Figure 1.7 obestatin markedly increased FoxO4 phosphorylation at T28 [pFoxO4(T28)] by 1.9- to 3.4-fold related to Dexa treated C2C12 myotubes but did not change FoxO3a and FoxO1 phosphorylation (Fig. 1.7). In contrast, insulin treatment did appreciably increase phosphorylation of FoxO1 at T24 [pFoxO1(T24) 3.8-fold related to Dexa treated C2C12 myotubes] but had no effect at S256 [pFoxO1(S256)] (Fig. 1.7). Furthermore, insulin increased FoxO3a phosphorylation at T32 and S253 [pFoxO3a(T32) (S253) 2.2-and 3.2-fold, respectively] with minor effect on FoxO4 phosphorylation (1.4-fold; Fig. 1.7).

Taken together these results, our data support a model where the interplay between AKT, mTOR and FoxO4 regulates two of the main proteolytic systems, the ubiquitin-proteasome and the autophagy-lysosome systems, and the signaling associated with protein translation in response to the obestatin/GPR39 system.

Effect of dexamethasone treatment in KM155C25 human myotubes

In order to validate whether the obestatin/GPR39 signaling is conserved between human and mouse, we used an in vitro cell culture model of human skeletal muscle: the human muscle stem cell line immortalized from a control individual, KM155C25 cells (Mamchaoui K, et al., 2011; Thorley M et al., 2016). Treatment of this cell line with Dexa resulted in dose-dependent increases in the MuRF1 and MAFbx protein content with a maximal effect at 1 μM of Dexa [5.4–5.9-fold, respectively (Fig1.8)].

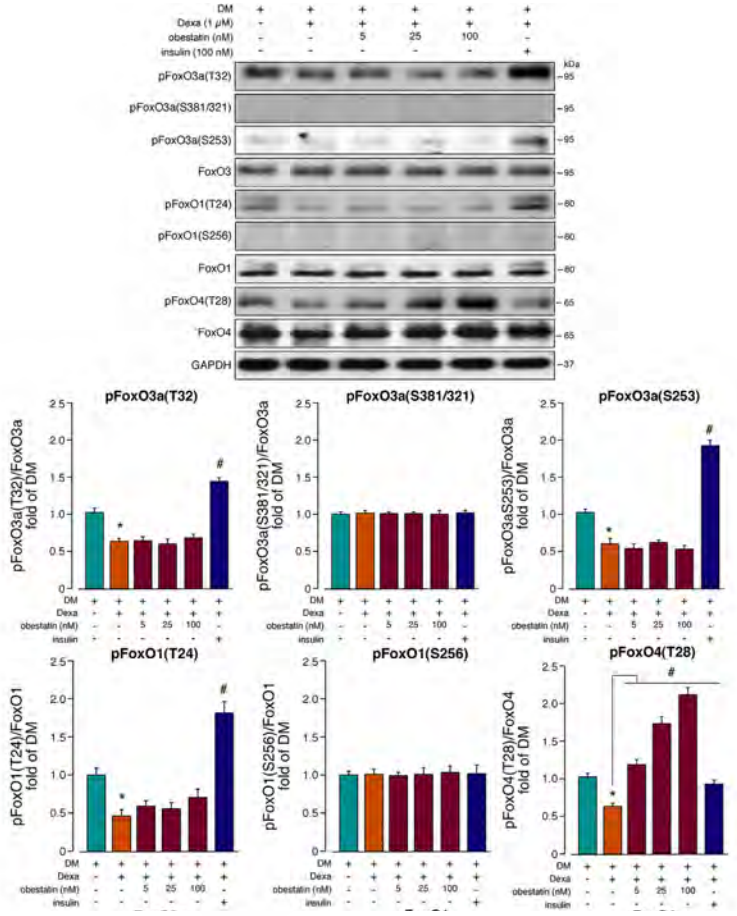

Figure 1.7. Obestatin controls through FOXO4 inhibition. (a) Inmunoblot analysis of FOXO1, FOXO3a and FOXO4 on differentiated C2C12 mouse myotubes under DM, DM+Dexa1μM, DM+Dexa1μM+obestatin5nM and DM+Dexa1μM+insulin 100nM during 24h. Data were expressed as mean±SEM obtained from intensity scans (n=3; *P*< 0.05 *versus* *DM or # DM + Dexa1μM values. Student t-test)

Up-regulation of ubiquitin E3-ligases was concomitant with a decrease in AKT activity and MHC expression (Fig1.8). Intriguingly, myogenin expression was down-

regulated following Dexa treatment, contrary to what was observed in C2C12 myotubes, possibly related to the fact that myogenin is up-regulated during the initial phases of differentiation, and the kinetics of differentiation may differ between C2C12 and KM155C25 (Fig1.8).

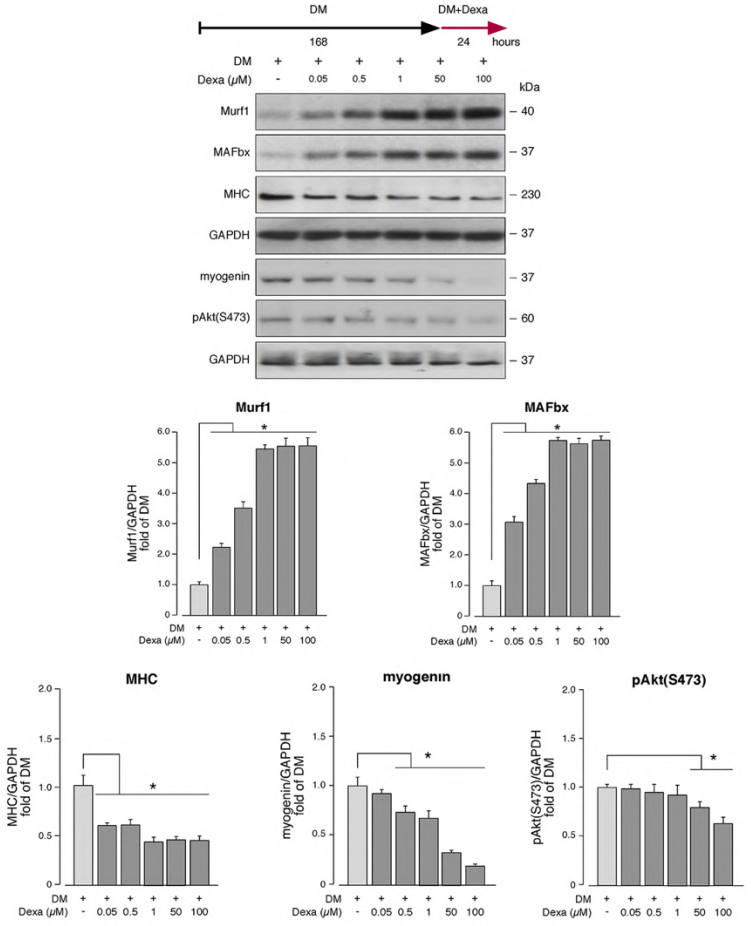

Figure 1.8. Dose response of Dexa activates atrophy program in humans. Immunoblot analysis of MuRF1, MAFbx, MHC, myogenin and AKT on differentiated KM155C25 human myotubes under Dexa treatment (0,05-100 μM) during 24h. Data were expressed as mean±SEM obtained from intensity scans (n=3; *P*< 0.05 *versus* *DM values. Student t-test).

The obestatin/GPR39 system attenuates atrophy-related gene expression by targeting FoxO4 in dexamethasone-induced atrophy in human KM155C25

Having evaluated the effect of Dexa under basal conditions, the action of obestatin/GPR39 signaling was determined on Dexa related myotubes at 1 μM concentration, based on the maximal effect on the MuRF1 and MAFbx expression at that dose.

Consistent with the up-regulation of the studied atrogenes after Dexa treatment, myotubes showed a decrease in area of 63 ± 3% in response to Dexa (Fig1.9). These changes were reversed by obestatin (10 nM, 24 h) as revealed by a marked increase in myotube area (~100 ± 12% over Dexa-treated myotubes, Fig1.9). Furthermore, the myotube areas were ~40% larger in the obestatin treated cells than insulin treated cells (60 ± 8% increase over Dexa-treated myotubes; Fig1.9), used as positive control.

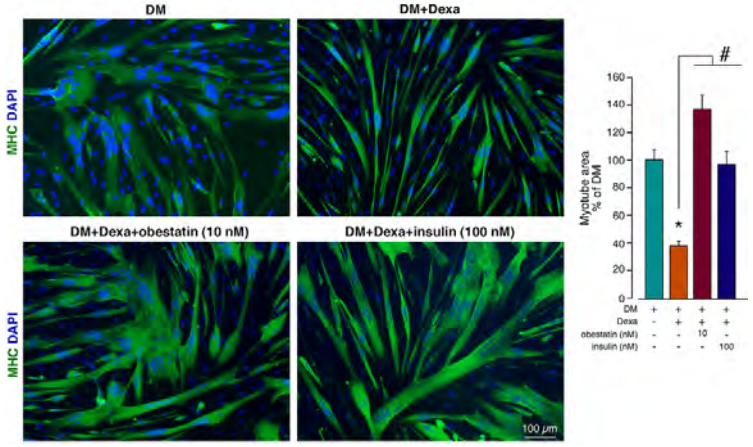

Figure 1.9. Obestatin restores myotube area in Dexa atrophied myotubes. Immunofluorescence detection of MHC and DAPI on differentiated KM155C25 human myotubes under DM, DM+Dexa 1 μM, DM+Dexa 1 μM+obestatin 10nM, DM+Dexa 1 μM+insulin 100nM during 24h. Data were expressed as mean±SEM (n=3; *P*< 0.05 *versus* *DM or # DM + Dexa1μM values. Student t-test)

At a molecular level, the immunoblot analysis demonstrate that obestatin treatment (10–100 nM, 24 h) decreased the expression of the ubiquitin E3-ligases MuRF1 and MAFbx by 65–80% and 35–42% referring to Dexa treated cells, respectively. Insulin (100 nM, 24 h) similarly decreased the MuRF1 and MAFbx expression by 58 ± 2% and 18 ± 3%, respectively (Fig1.10), curiously, its effect was lower than the obestatin, at the contrary of what we saw in the mouse cell model. The obestatin-activated signaling is able to restore MHC levels, showing 185–222% over those reached in Dexa-treated KM155C25 myotubes (Fig1.10).

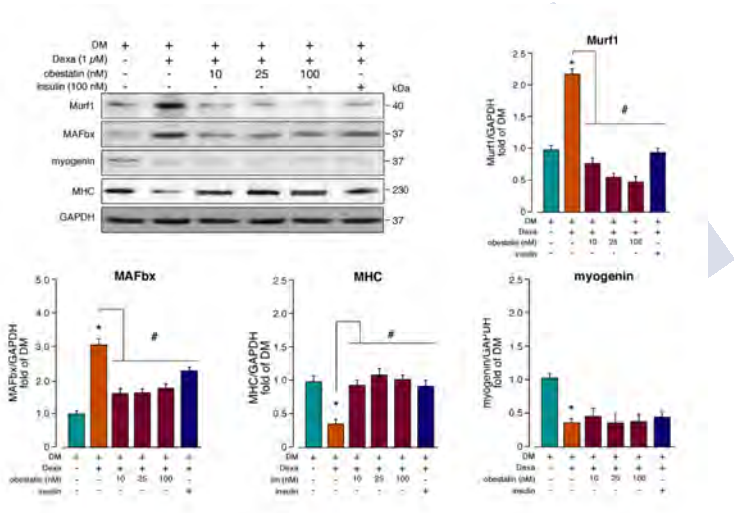

Figure 1.10. Obestatin counteract the atrophy pathways activated during Dexa treatment. Immunoblot analysis of MuRF1, MAFbx, myogenin and MHC on KM155C25 human myotubes under DM, DM+Dexa 1 μM, DM+Dexa 1 μM+obestatin 10nM, DM+Dexa 1 μM+insulin 100nM during 24h. Data were expressed as mean±SEM obtained from intensity scans (n=3; *P*< 0.05 *versus* *DM or # DM + Dexa1μM values. Student t-test).

The results achieved for MHC were similar to those reached with insulin (192 ± 9% vs. 222 ± 5% for 100 nM obestatin). However, myogenin was not up-regulated following obestatin- or insulin-treated myotubes (Fig1.10). A protein synthesis assay, using the protein synthesis inhibitor cycloheximide, revealed that obestatin

treatment (10 nM, 24 h) increased protein synthesis by 91 ± 5% in Dexa-treated cells (Fig1.10).

To assess how ubiquitin E3 ligases were regulated by the obestatin/GPR39 signaling, the phosphorylation code of FoxO transcription factors was evaluated. As shown in Fig 1.11, Dexa treatment, as opposed in the C2C12 mouse cells, failed in activate FoxO1 at any of its phosphorylation sites (S256 and T24) and surprisingly FoxO3a appeared to be inhibited after Dexa treatment. Nonetheless FoxO4 was activated after Dexa treatment. When we co-administrate obestatin we saw a significantly increased amount of phosphorylated FoxO4 at T28 and FoxO1 at S256 by 2.1- and 1.4-fold related to Dexa-treated KM155C25 myotubes, respectively. Obestatin stimulation failed to modify the phosphorylation levels of FoxO1 phosphorylation at T24. Moreover, FoxO3a phosphorylation at T32, S381 and S321 sites was not modified by obestatin maintaining Dexa-stimulated levels (Fig1.11) keeping its inhibition state.

The localization of endogenous FoxO transcription factors was further visualized by fluorescence microscopy in KM155C25 myotubes, and the ratio of nuclear to cytoplasmic fluorescence was calculated (Fig1.12; Fig1.13; Fig 1.14). As depicted in the representative images, FoxO1, FoxO3a and FoxO4 were present both in the nucleus and in the cytoplasm in control conditions. FoxO1 was localized predominately to the nucleus in Dexa treated cells, indicating that despite not being phosphorylated, could be another post-translational modification allowing it to be active and translocated to the nucleus. In response to obestatin FoxO1 showed increased localization to the cytoplasm (Fig1.12). FoxO3a was mainly localized in the cytoplasm in response to Dexa or obestatin (Fig1.13), this result correlates with its phosphorylation state saw by immunoblot excluding FoxO3a of having a role in human muscle atrophy due to glucocorticoids.

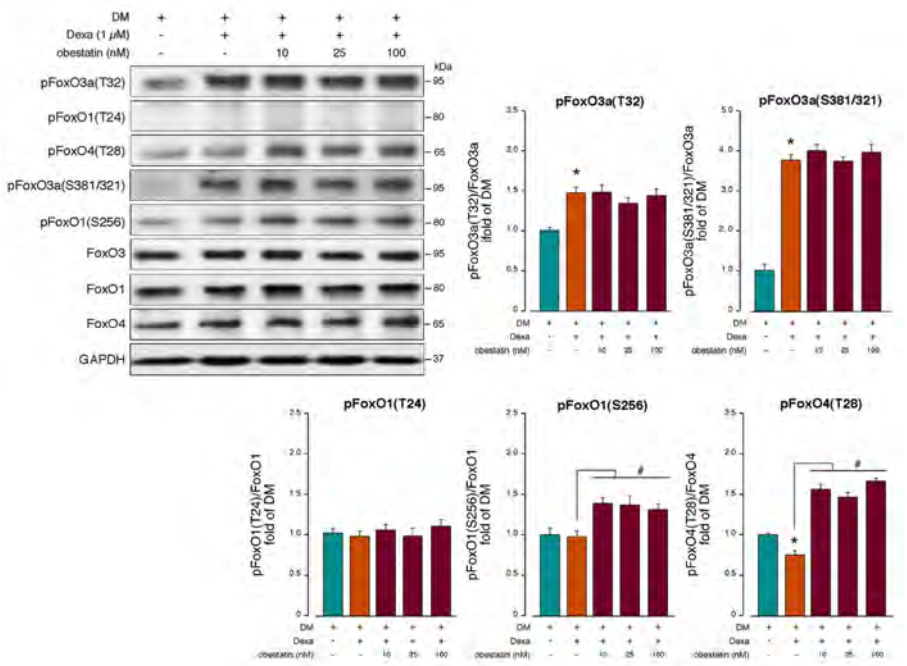

Figure 1.11. Obestatin controls through FOXO4 and FOXO1 inhibition. Immunoblot analysis of FOXO1, FOXO3a and FOXO4 on KM155C25 human myotubes under DM, DM+Dexa 1 μM, DM+Dexa 1 μM+obestatin 10nM, DM+Dexa 1 μM+insulin 100nM during 24h. Data were expressed as mean±SEM obtained from intensity scans (n=3; *P*< 0.05 *versus* *DM or # DM + Dexa1μM values. Student t-test)

FoxO4 localized in both the nucleus and cytoplasm in response to Dexa treatment, but an increased cytoplasmic translocation was induced by obestatin (Fig1.14). These results suggested that obestatin signaling induced FoxO4 T28 phosphorylation, inactivation and nuclear exclusion, contributing to the regulation of transcription of FoxO target genes, such as MuRF1 and MAFbx.

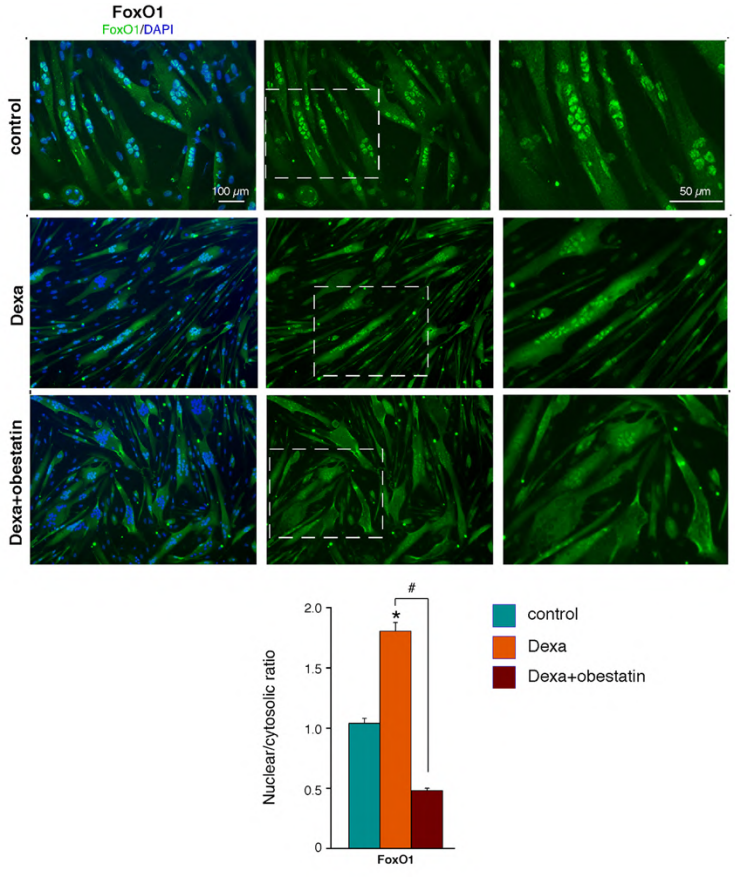

Figure 1.12. FOXO1 localization under Dexa and Dexa+obestatin treatment. Immunofluorescence detection of MHC and DAPI on differentiated KM155C25 human myotubes under DM, DM+Dexa 1 µM, DM+Dexa 1 µM+obestatin 10nM during 24h. Data were expressed as mean±SEM (n=3; *P*< 0.05 *versus* *DM or # DM + Dexa1µM values. Student t-test)

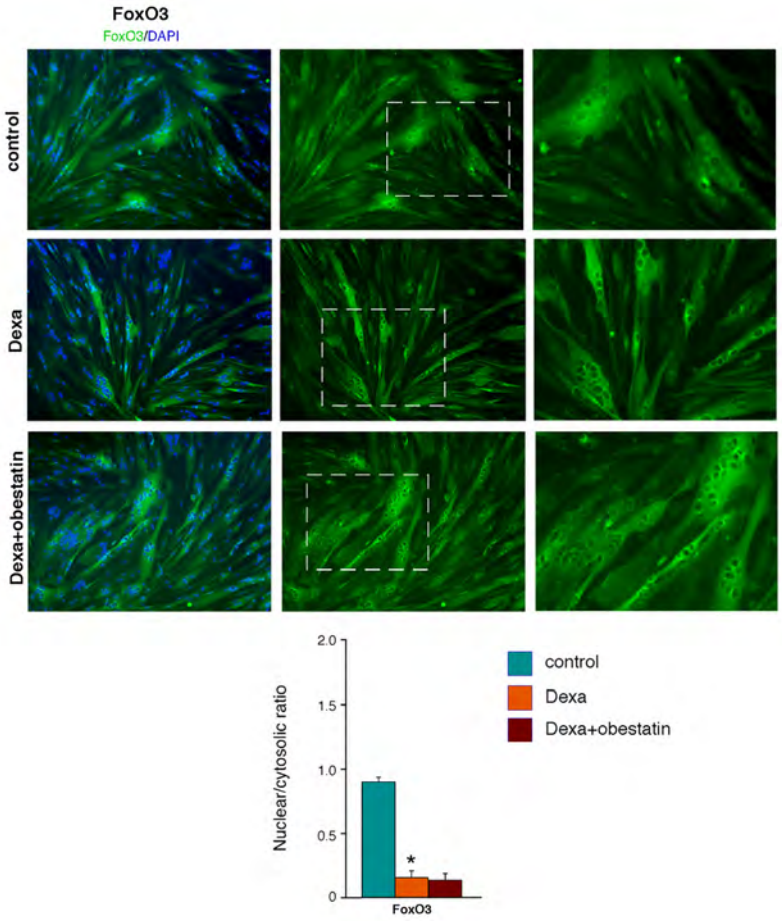

Figure 1.13. FOXO3a localization under Dexa and Dexa+obestatin treatment. Immunofluorescence detection of MHC and DAPI on differentiated KM155C25 human myotubes under DM, DM+Dexa 1 µM, DM+Dexa 1 µM+obestatin 10nM during 24h. Data were expressed as mean±SEM (n=3; $P< 0.05$ *versus* *DM values. Student t-test)

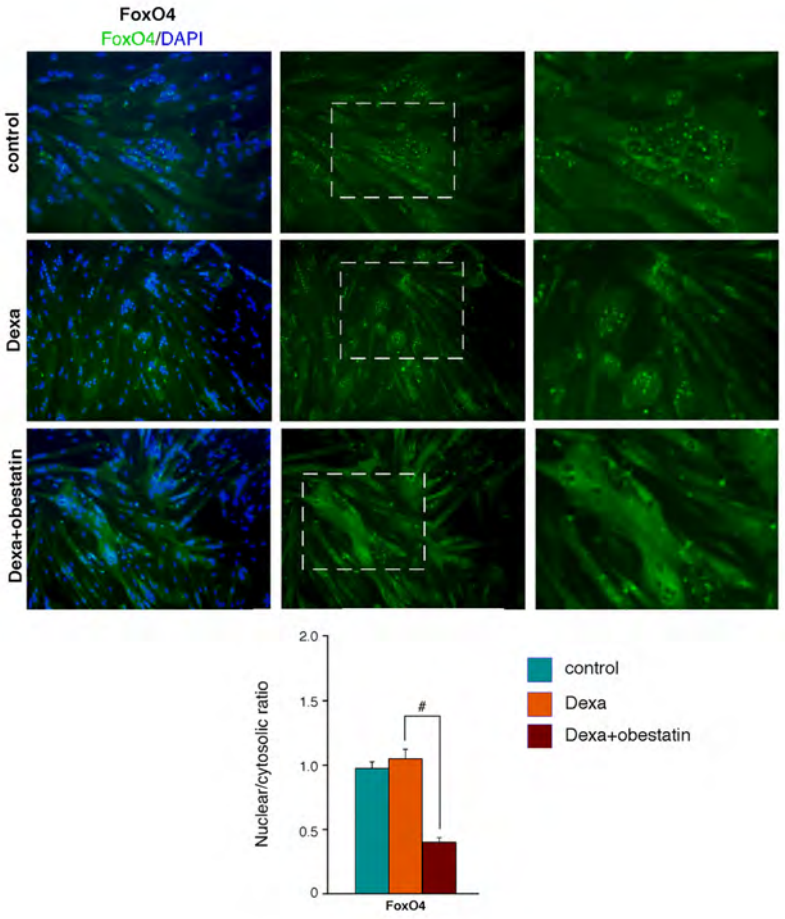

Figure 1.14. FOXO4 localization under Dexa and Dexa+obestatin treatment. Immunofluorescence detection of MHC and DAPI on differentiated KM155C25 human myotubes under DM, DM+Dexa 1 µM, DM+Dexa 1 µM+obestatin 10nM during 24h. Data were expressed as mean±SEM (n=3; *P*< 0.05 *versus* # DM + Dexa1µM values. Student t-test)

As KM155C25 cells are a no commercial immortalized cell line, we decided to investigate whether the pathways activated after Dexa treatment and the signaling of obestatin/GPR39 system was also maintained on the primary human cells. As shown in Fig1.15, in a immunofluorescence assay for MHC, Dexa treatment in C25 myotubes was able to reproduce muscle atrophy phenotype by reducing the myotube area (62,7±2% referred to control cells, (Fig1.15), whether obestatin treatment restored the myotube area by 185,5±2% over Dexa treated cells. In addition, by a immunoblot analysis we confirmed that obestatin treatment not only reversed Dexa-induced myotube atrophy decreasing MuRF1 and MAFbx expression (Fig1.16) but also increased the amount of phosphorylated FoxO4 at T28 which correlated with decreased in the atrogenes expression. Moreover, FoxO3 and FoxO1 remain inhibited and unaltered respectively after Dexa or obestatin treatments (Fig1.16).

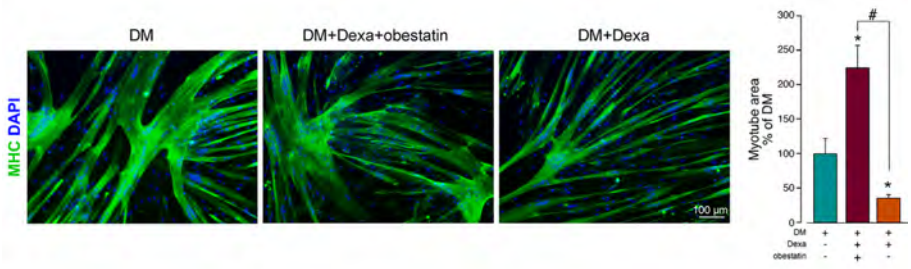

Figure 1.15. Obestatin counteract Dexa atrophy in C25 primary cells. Immunofluorescence detection of MHC and DAPI on differentiated C25 primary human myotubes under DM, DM+Dexa 1 µM, DM+Dexa 1 µM+obestatin 10nM during 24h. Data were expressed as mean±SEM (n=3; *P*< 0.05 *versus* *DM or # DM + Dexa1µM values. Student t-test)

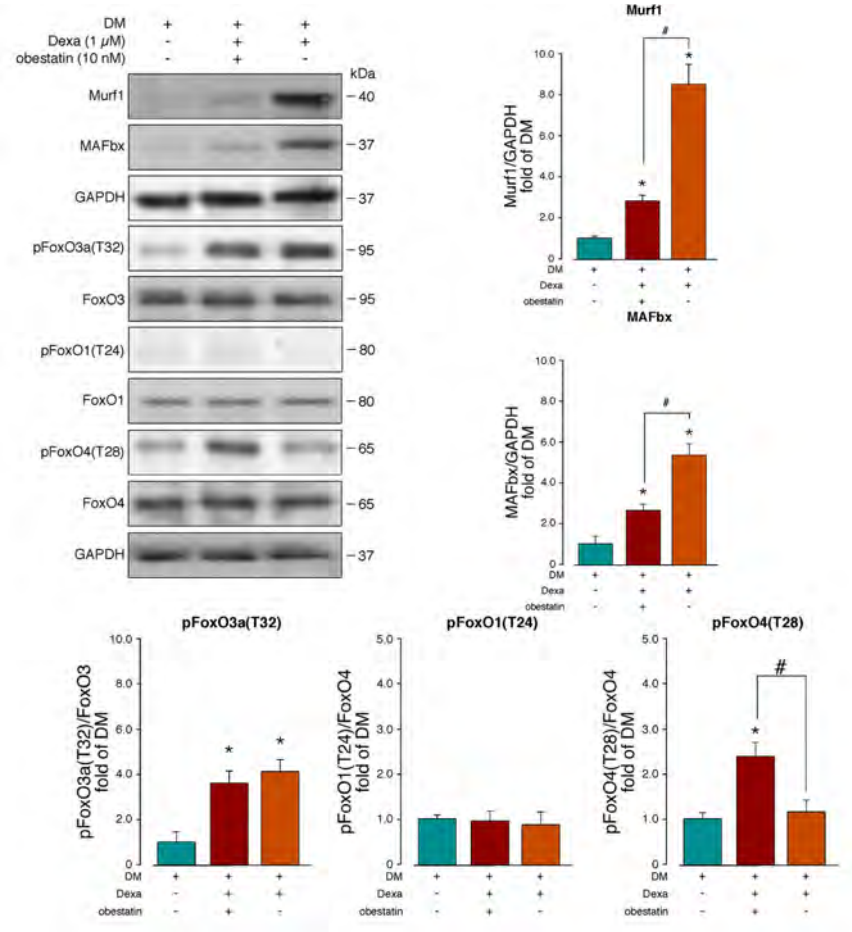

Figure 1.16. Obestatin counteract the atrophy pathways activated during Dexa treatment in C25 primary cells. Immunoblot analysis of MURF1, MAFbx, myogenin and MHC on KM155C25 human myotubes under DM, DM+Dexa 1 μM, DM+Dexa 1 μM+obestatin 10nM, DM+Dexa 1 μM+insulin 100nM during 24h. Data were expressed as mean±SEM obtained from intensity scans (n=3; *P*< 0.05 *versus* *DM or # DM + Dexa1μM values. Student t-test)

We further tested by siRNA experiments the role of FoxO4 governing the activation of muscle atrophy genes. siRNA targeting FoxO4 (81 ± 4% reduction relative to si-control) significantly decreased the MuRF1 and MAFbx expression in Dexa stimulated cells by 55 ± 1 and 61 ± 1% relative to si-control, respectively (Fig1.17).

Under these conditions, si-FoxO4 experiments increased the MuRF1 and MAFbx expression in obestatin stimulated cells by 88 ± 12 and 82 ± 6% relative to si-control, respectively. Furthermore, the FoxO4 knockdown showed deregulations of autophagy related proteins. The acute FoxO4 deficiency on Dexa treated cells increased the expression levels of p62 by 69 ± 3% relative to si-control (Fig1.17). In contrast, the Cathepsin L levels and the LC3II/LC3I ratio were decreased by 52 ± 6 and 57 ± 5%, respectively. Silencing of FoxO4 increased the p62 and Cathepsin L levels as well as the LC3II/LC3I ratio in obestatin treated myotubes by 94 ± 6, 564 ± 15 and 86 ± 3% relative to si-control, respectively (Fig1.17). These results demonstrate not only the role of FoxO4 in triggering the atrophy program, but also that its activity is tightly controlled by the obestatin/GPR39 system.

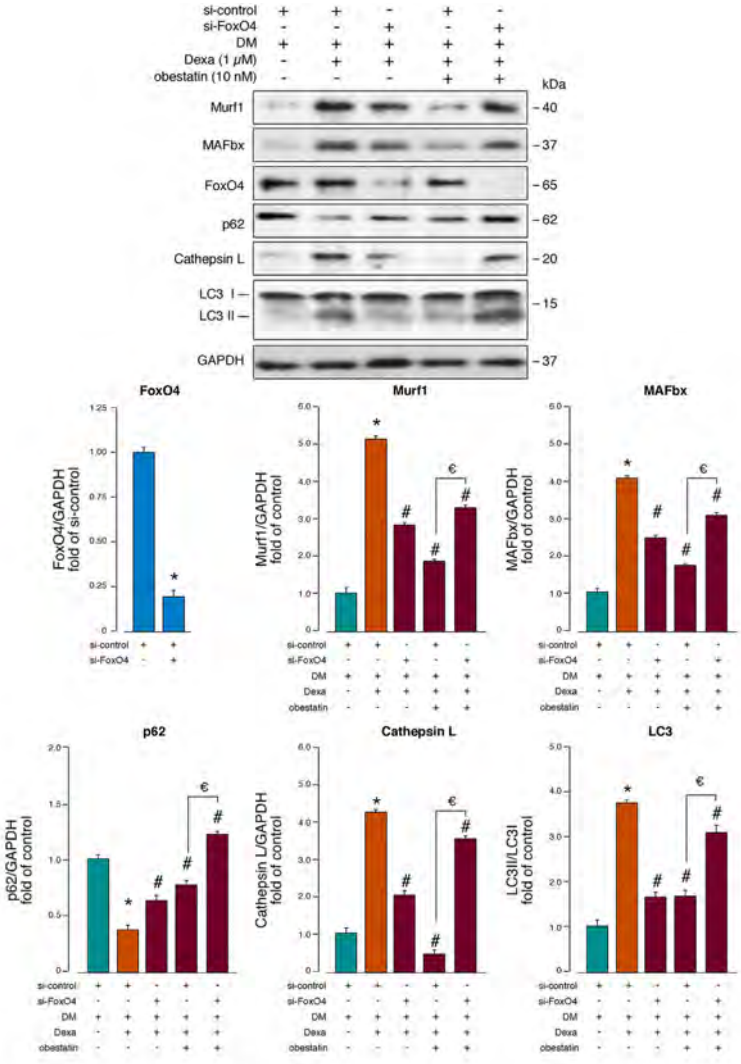

Figure 1.17. Obestatin counteracts the atrophy effect of Dexa treatment through FoxO4. Immunoblot analysis for MAFbx, MuRF1, p62, cathepsinL and LC3 in KM155C25 human myotubes under DM, DM+Dexa 1 μM, DM+Dexa 1 μM+obestatin 10nM during 24h. Data were expressed as mean±SEM obtained from intensity scans (n=3; *, #, € $P < 0.05$ vs. control. Student t-test)

We further investigated the role of obestatin on FoxO family regulation by KLF15. siRNA targeting KLF15 (44±6% reduction relative to si-control) significantly decreased the expression of FoxO3, FoxO1 and FoxO4 in Dexa-treated cells related to si-control (34±1, 31±2, and 26±1 %, respectively). MAFbx and MuRF1 expression was decreased by 33±3 and 55±2 %, respectively (Fig 1.18). Under obestatin treatment, FoxO4 was clearly downregulated related to Dexa treatment in both si-control and si-KLF15 treated cells (Fig1.18). By contrast, neither FoxO3 nor FoxO1 expression showed differences related to Dexa treatment neither si-control nor si-KLF15 treated cells (Fig1.18). These results suggested the involvement of alternative signaling nodes in the regulation of FoxO4 expression. Indeed, MAFbx and MURF1 expression was affected to a greater extent by obestatin (Fig1.18).

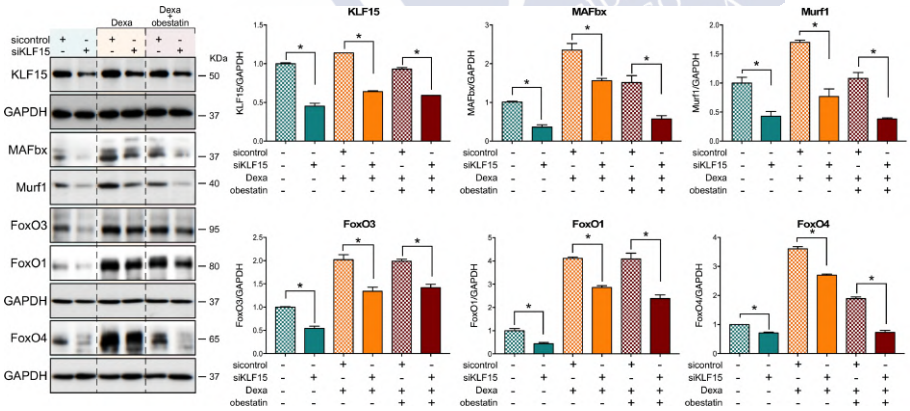

Figure 1.18. Obestatin regulates FoxO4 independent of KLF15. Immunoblot analysis for MAFbx, MURF1, FoxO1, FoxO3 and FoxO4 in KM155C25 human myotubes under DM, DM+Dexa 1 μM, DM+Dexa 1 μM+obestatin 10nM during 24h. Data were expressed as mean±SEM obtained from intensity scans (n=3; *P*< 0.05 *versus* *si control values. Student t-test).

Post-translational regulation of FoxO1 by the obestatin/GPR39 system in dexamethasone induced atrophy in human KM155C25 myotubes

While the obestatin/GPR39 signaling clearly controlled the FoxO4 activity by phosphorylation, altering its subcellular location, its role in regulating FoxO1 was not so clear. Nuclear/cytoplasmic shuttling of FoxO1 suggested the implication of posttranslational modifications beyond protein phosphorylation, i.e. acetylation and ubiquitination. By immunoblot analysis we saw that in response to Dexa, FoxO1 was acetylated (Ac-FoxO1), and the Ac-FoxO1 level was significantly decreased by 59–76% in response to obestatin (Fig 1.19).

The acetylation status of FoxO transcription factors is generally balanced by histone acetylases and histone deacetylase (HDAC), including the NAD+-dependent sirtuins. As shown in Figure FoxO1 acetylation was accompanied by Dexa induced phosphorylation of HDAC4 [pHDAC4(S246), 1.7-fold], but this phosphorylation was clearly decreased 29–61% in response to obestatin. In addition, Dexa treatment increased Sirt1 expression by 1.7-fold, and this expression was decreased in obestatin-treated cells (36–60% inhibition related to Dexa treated cells; Fig 1.19). Sirt2 was also increased 1.6-fold in response to Dexa and this expression was decreased in obestatin-treated cells (36–42% inhibition related to Dexa treated cells; Fig 1.19). No significant modification of Sirt3 expression was observed in any condition (Fig 1.19).

Depending on the cell/tissue type and upstream stimulus, multiple kinase families including the Ca^{+2}/CaM-dependent protein kinases (CAMKs), protein kinase D (PKDs) and AMPK can phosphorylate and regulate the localization of the class IIa HDACs (Mihaylova & Shaw, 2013)

The activation of PKD [pPKD/PKCμ(S916)] was up-regulated 1.7-fold in the presence of Dexa, but this action was reduced in response to obestatin (35–50%

inhibition related to Dexa treated cells; Fig 1.19). Dexa treatment increased 2.0-fold the activation of CAMKII, estimated by the phosphorylation of CAMKII at T286 [pCAMKII(T286)], being decreased by obestatin stimulation (33–45% inhibition related to Dexa treated cells; Fig 1.19). Finally, activation of AMPK, estimated by the phosphorylation of AMPK at T172 [pAMPK(T172)], was increased by 2.4-fold in response to Dexa but decreased by 34–73% under obestatin stimulation (Fig1.19). Therefore, HDAC4 activity was regulated by interplay among PKD, CAMKII and AMPK, which suggests some mechanisms for the modulation of FoxO1 acetylation.

Next, we looked for evidence of potential FoxO1-interacting proteins in control, Dexa (1 μM) and Dexa (1 μM)/obestatin (10 nM) treated cells. Proteins of myotube cells were immunoprecipitated with anti-FoxO1 and probed with anti-Ac-FoxO1, anti-Sirt1, anti-Sirt2, anti-Sirt3, anti-HDAC4 antibodies or ant-Ubiquitin. Figure shows that Sirt1 interacted with FoxO1, but this interaction was clearly decreased by 59 ± 1% in response to Dexa. In addition, the interaction between Sirt1 and FoxO1 was clearly increased by obestatin stimulation by 105 ± 2% as compared with Dexa treated cells. In contrast, a significant decrease in Sirt2 was detected in Dexa treated or obestatin treated cells (Fig 1.20), excluding the direct involvement of this NAD+-dependent histone deacetylase in regulating FoxO1 acetylation through obestatin. No detectable changes in Sirt3 were observed after the treatments. However, we found that HDAC4 interacted with FoxO1 and this interaction was decreased by 20 ± 3% in response to Dexa but increased by 57 ± 10% in obestatin treated cells. We also investigated whether Dexa or obestatin signaling could modify the ubiquitination of FoxO1. Dexa treatment decreased by 54 ± 2% the ubiquitination of FoxO1 related to control levels (Fig 1.20). This effect was partially counteracted by obestatin resulting in 47 ± 3% increase in the level of ubiquitination when compared with Dexa treated cells. Thus, obestatin/GPR39 signaling controls

FoxO1 activity by altering an intricate combination of post-translational modifications, such as phosphorylation, acetylation and ubiquitination.

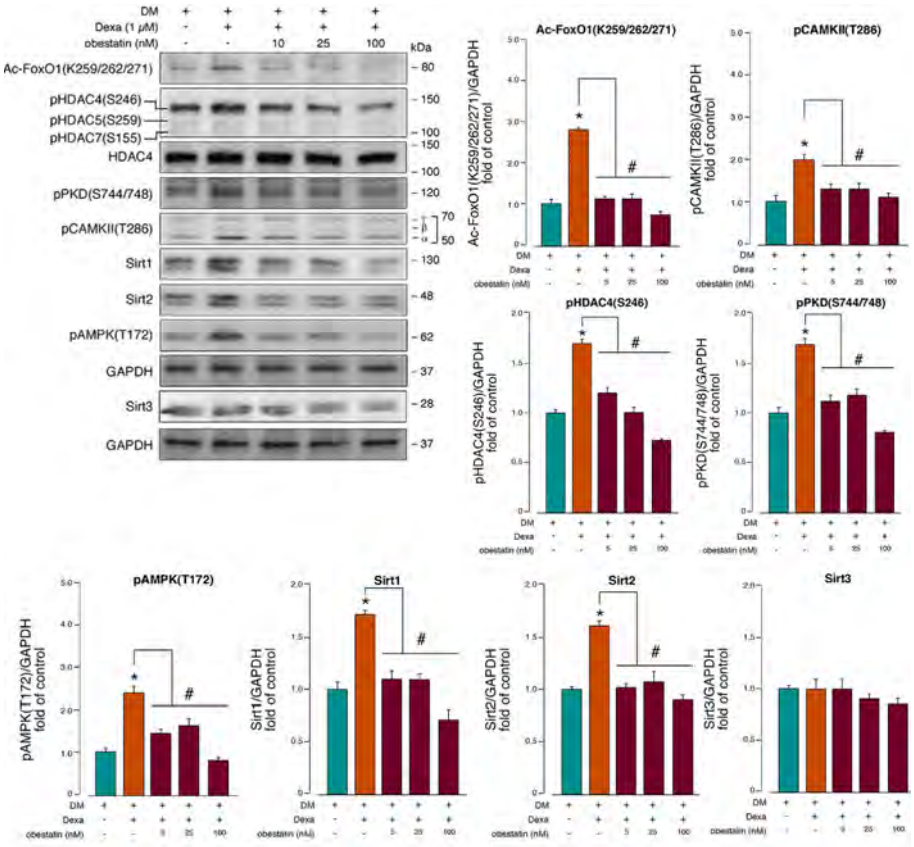

Figure 1.19. Obestatin counteracts the atrophy effect of Dexa treatment through FoxO1 posttranslational modifications. Immunoblot analysis of Ac-FoxO1, CAMKII, HDAC, PKD, AMPK, Sirt1, Sirt2 and Sirt3 in KM155C25 human myotubes under DM, DM+Dexa 1 µM, DM+Dexa 1 µM+obestatin 10nM during 24h. Data were expressed as mean±SEM obtained from intensity scans (n=3; *P*< 0.05 *versus* *DM or # DM + Dexa1µM values. Student t-test).

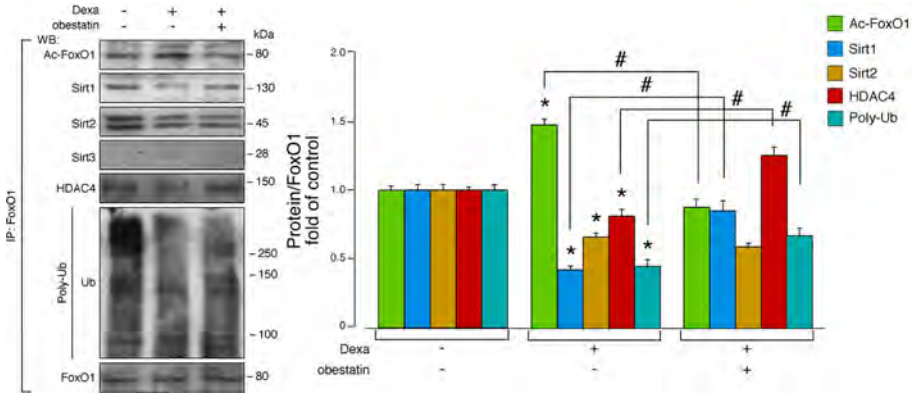

Figure 1.20. Obestatin promotes FoxO1 acetylation and ubiquitination during Dexa atrophy. Immunoblot analysis of immunoprecipitation assay of Ac-FoxO1, Sirt1, Sirt2, Sirt3 and Ubiquitin in KM155C25 human myotubes under DM, DM+Dexa 1 μM, DM+Dexa 1 μM+obestatin 10nM during 24h. Data were expressed as mean±SEM obtained from intensity scans (n=3; *P*< 0.05 *versus* *DM or # DM + Dexa1μM values. Student t-test).

Obestatin/GPR39 system induce negative regulation of FoxO transcription factors through β-arrestin signal complex and it is independent of IGF signaling

Having shown that obestatin induced negative regulation of FoxO transcription factors, we investigated the involvement of the β-arrestin signal complex (Santos-Zas I, et al., 2016)

The effect of down-regulation of β-arrestin 1 and β-arrestin 2 by specific siRNA was evaluated in KM155C25 myotubes. The siRNAs decreased β-arrestin 1 and β-arrestin 2 expressions by 58 ± 3 and 61 ± 4%, respectively (Fig 1.21). In these conditions, obestatin stimulated phosphorylation of AKT in S437, FoxO3a in T32, S318 and S321, FoxO4 in T32 and FoxO1 S256 but not in T24. This effect were reduced by depletion of β-arrestin 1 or 2 [pAKT(S473): 66±1 or 72±2%; pFoxO3a(T32): 68±1 or 67±3%; pFoxO4(T28): 78±2 or 67±2%; pFoxO1(T256): 47±2

or 53 ±3%; pFoxO3a(S318/321): 66±2% or 63±3%, respectively Fig1.21]. This finding provides a functional activity for β-arrestin dependent signalplex being specific signaling arm to activate AKT/FoxO signaling in myotubes.

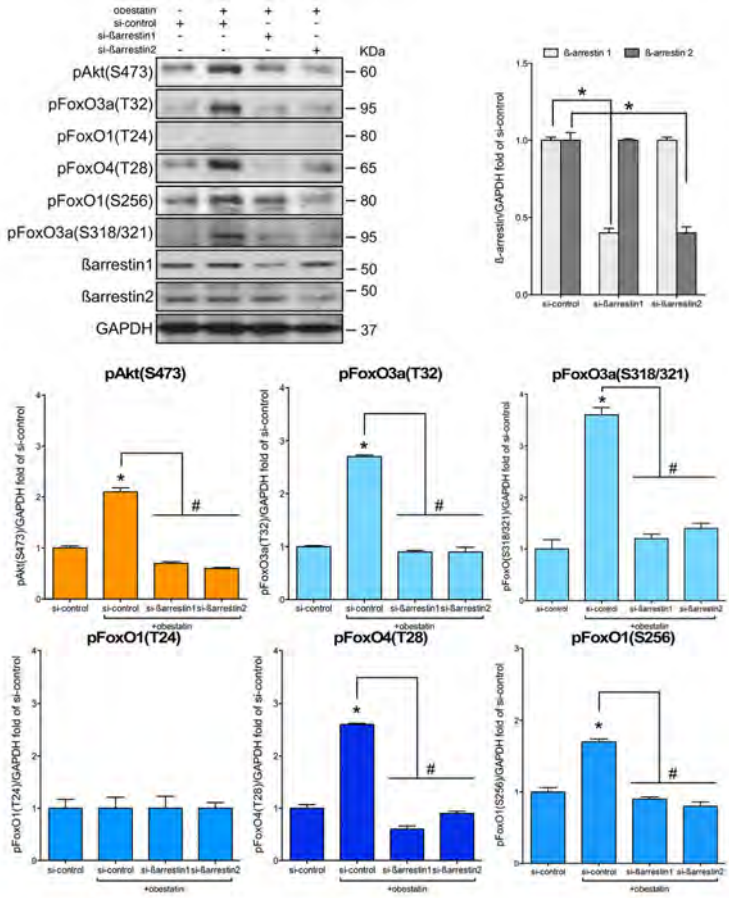

Figure 1.21. The obestatin/GPR39 system controls the atrophy program through b-arrestin signaling. Immunoblot analysis of β-arrestin 1, β-arrestin 2, AKT, FoxO1, FoxO3 and FoxO4 in KM155C25 human myotubes under DM, DM+Dexa 1 µM, DM+Dexa 1 µM+obestatin 10nM during 24h. Data were expressed as mean±SEM obtained from intensity scans (n=3; *P*< 0.05 *versus* *DM or # DM + Dexa1µM values. Student t-test).

Even though was previously demonstrated that obestatin regulates myogenesis by limiting IGFR/IRS1 activity (Gurriarán-Rodriguéz U, et al., 2015) and signals to activate the AKT/mTOR pathway through crosstalk with EGFR (Santos-Zas I, et al., 2016) we tested if IGF-1 production might mediate the anticatabolic effect of obestatin in KM155C25 human myotubes. We performed a siRNA experiment for prepro-IGF-1, (68±2% reduction relative to si-control) did not modify MuRF1 and MAFbx expression in obestatin co-stimulated cells relative to si-control cells (Fig 1.22). These results rule out the implication of IGF-1 in the anabolic control by the obestatin/GPR39 system of muscle atrophy.

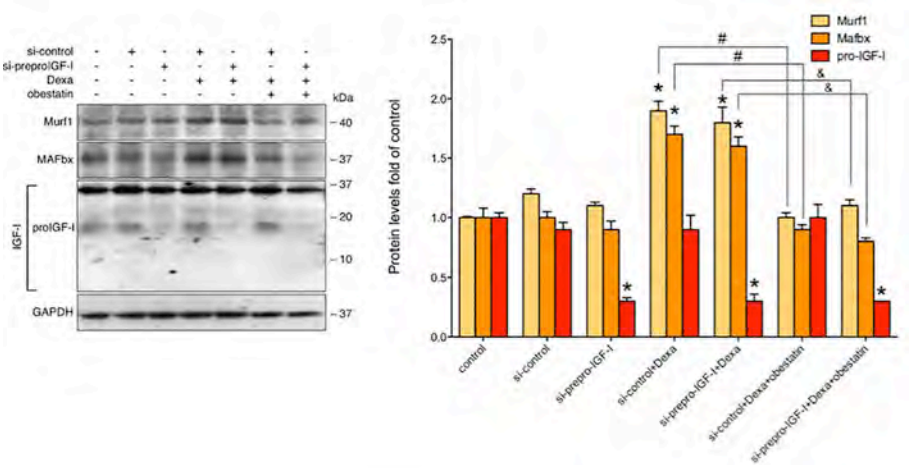

Figure 1.22. Obestatin counteracts Dexa atrophy independent of IGF-1 signaling. Immunoblot analysis of IGF1, MURF1 and MAFbx in KM155C25 human myotubes under DM, DM+Dexa 1 μM, DM+Dexa 1 μM+obestatin 10nM during 24h. Data were expressed as mean±SEM obtained from intensity scans (n=3; *P*< 0.05 *versus* *DM, # DM + Dexa1μM or & si-prepro-IGF1+ Dexa1μM values. Student t-test).

DISCUSSION

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In the present study, we have shown that obestatin specifically regulates protein synthesis, autophagy and ubiquitin–proteasome systems in a coordinated manner involving AKT-, PKD/PKCμ-, CAMKII- and AMPK- dependent mechanisms with distinct sets of effector proteins that ultimately affect FoxO transcription factors. Additionally, a specific pattern of FoxO post-translational modifications, including FoxO4 phosphorylation and FoxO1 deacetylation, is critical in the regulation of autophagy and ubiquitin–proteasome. These observations demonstrated that the obestatin/GPR39 system is able to counteract deregulations in the proteostasis, e.g. those associated to glucocorticoid-induced myotube atrophy, and to restore efficient basal homeostasis.

In the C2C12 myotubes used in this study, the regulation of the ubiquitin–proteasome machinery partly derives from the activation of the AKT pathway. AKT controls both protein degradation *via* FoxO family transcription factors, and protein synthesis *via* mTOR (Bonaldo P and Sandri M, 2013; Schakman O et al., 2013). AKT has been previously shown to phosphorylate members of the FoxO family, which keeps FOXO proteins from translocating to the nucleus (Calnan DR and Brunet A, 2008). Our results show that neither FoxO1 nor FoxO3 is involved in the response to obestatin *via* phosphorylation. In contrast, FoxO4 appears to be the obestatin-responsive isoform, thus suggesting an isoform specific mode of regulation. On the other hand, the implication of the mTOR pathway is demonstrated by the activation of S6 K1 and by eIF4E availability, through phosphorylation of 4E-BP1, which finally promotes protein synthesis (Bonaldo P and Sandri M, 2013; Laplante M and Sabatini DM, 2012). Thus, the activation of the obestatin/GPR39 system results in an anti-atrophic effect as evidenced by an increase in protein content in myotube, e.g. MHC, and an increase in the size of these myotubes in the presence of Dexa. Synthesis and degradation of proteins are two processes that are intimately connected and regulated by pathways that can affect both (Bonaldo P and Sandri

M, 2013). The activation of the obestatin/GPR39 system blocks Dexa-induced autophagy. Reduction of LC3 lipidation (LC3II) and accumulation of p62 together with a reduced Cathepsin L protein level support the hypothesis that autophagy flux was slowed down in Dexa-treated myotubes. Thus, the obestatin/GPR39 system controls both protein synthesis and protein degradation by specific modulation of anabolism and proteasomal activity.

Although murine models sometimes can translate directly to human conditions, we saw in our results what is concerning to the molecular behavior, some key genes or key pathways, or even potentially at genome-wide level, murine myogenesis differs substantially from human myogenesis((Bachrach E et al., 2004; Boldrin L et al., 2012).Although the obestatin/GPR39 system reverts Dexa-induced atrophy in human myotubes by regulating both ubiquitin–proteasome and autophagy–lysosome systems, we show here that the molecular response in the cellular mouse model C2C12 reflects only partly the mechanisms involved in human muscle cells. First, transcriptional and post-translational regulation of FoxO3 by Dexa differs between murine and human cells. Dexa activates FoxO3 at the transcriptional level in human myotubes. Indeed, induction of FoxO3 transcripts was demonstrated to require glucocorticoid receptor binding steroids (Lützner N et al., 2012). Consistent with the stimulation of FoxO3 being a primary response to glucocorticoids, analysis of genomic DNA as well as ChIP assays revealed two functional glucocorticoid responsive elements within the FoxO3 promoter (Lützner N et al., 2012). Furthermore, we provide evidence for a phosphorylation switch by which Dexa induces transcriptional activation of the FoxO3 gene but subsequently inactivates the corresponding protein by site-specific phosphorylation, i.e. T32, S381 and S321. Thus, glucocorticoid appears to exert a dual action in activating FoxO3 target gene expression by combining transcriptional and post-translational levels of regulation. Recent evidence indicated that FoxO3 transcription factor is activated in response

to metabolic stress, characterized by low intracellular energy and high glucocorticoid levels (Foss ML et al., 1971; Wasserman DH, 1955). Under these conditions, FoxO3 is activated on the transcriptional level and phosphorylated by AMPK. This results in an increased transcription of the FoxO3 target gene Liver Kinase B1 (LKB1), which in turn can phosphorylate and thereby activate AMPK promoter (Lützner N et al., 2012). Once activated, AMPK switches off anabolic pathways while simultaneously activating catabolic pathways, which result in the production of ATP (Bachrach E et al., 2004). When cellular energy levels are restored, AMPK is then allosterically inactivated by ATP (Scott JW et al., 2004; Yeh LA et al., 1980). Under such conditions, the serum and glucocorticoid-inducible kinase 1 (SGK-1) phosphorylates FoxO3 at T32, inactivating its transcriptional activity (Lützner N et al., 2012). Furthermore, the fact that the induction of FoxO3 transcripts was enhanced by AMPK activation under Dexa treatment, but inhibited by the obestatin related AKT signalling, suggests that AKT and the AMPK pathways can have opposite biological effects. While the obestatin related AKT pathway stimulates the anabolic process, the AMPK pathway promotes catabolic process. FoxO3 is one of the intersections between the two pathways, being post-translationally inhibited by downstream effectors of the AKT pathway and post-translationally upregulated by AMPK. Thus, the AMPK signaling pathway may coordinate a series of transcriptional and post-transcriptional changes that allow cells to adapt to changes in the energy status. Second, FoxO1 activity is not only regulated by phosphorylation at AKT phosphorylation site S256, (Aoki M et al., 2004; Matsuzaki H et al., 2003) but also the acetylation of FoxO1 is involved in human myotubes. AKT phosphorylation site S256 is a requisite to promote ubiquitination and degradation of FoxO1 (Huang H et al., 2005). Additionally, the acetylation status of FoxO1 is associated to specific dephosphorylation of class II HDAC4 on a series of conserved serine residues. Indeed, HDAC phosphorylation provides docking

sites for the 14-3-3 chaperone protein leading to nuclear export and inactivation of HDACs (McKinsey TA et al., 2000; McKinsey TA et al., 2002). The increase HDAC4 activity occurred together with significant changes in the activation of PKD/PKCμ and CAMKII, enzymes known to regulate class II HDAC activation via phosphorylation (Backs J et al., 2006; Vega RB et al., 2006). This is further supported by results of immunoprecipitation assays that demonstrate that HDAC4 interacts with FoxO1, and that this interaction is clearly decreased in response to Dexa. We also observed an increased interaction between FoxO1 and Sirt1, and not Sirt2, under obestatin stimulation. Taking into account the role of acetylation on FoxO1 activity on autophagy, (Brunet A et al., 2004; Zhao Y et al., 2010)FoxO1 could function in eliciting autophagy in response to Dexa by directly binding to the promoter region of autophagic genes (Qiang L et al., 2010; Liu J et al., 2015) or by specific interaction with Atg7, an E1-following protein, to influence the autophagic process (Zhao Y et al., 2010). On the other hand, the lack of AKT specific phosphorylation of FoxO1 at S256 prevents its degradation (Huang H et al., 2005). Thus, we propose that the obestatin/GPR39 signaling has exerted its specific function by reducing FoxO1 activity by deacetylation and phosphorylation. Deacetylation of FoxO1 occurs as a result of its association to Sirt1 and HDAC4, and the deacetylated FoxO1 fails to induce the autophagic process. Obestatin related AKT signaling promotes the ubiquitin dependent degradation via FoxO1 phosphorylation, thereby inhibiting its transcriptional function. This is further supported by our results showing that obestatin can increase the ubiquitination status of FoxO1, and thus accelerate its degradation. Third, the Dexa-induced expression pattern of myogenin is different between mouse and human myotubes. Myogenin plays a dual role as both a regulator of muscle development and an inducer of neurogenic atrophy by directly activating the expression of MuRF1 and MAFbx in mice (Moressi V et al., 2010; Tang H and Goldman D, 2006). These

contrasting activities reflect differential modulation by signaling pathways that enable myogenin to regulate distinct sets of target genes (Tang H and Goldman D, 2006). Although myogenin was upregulated in C2C12 myotubes following Dexa treatment, at no time, this transcription factor did show an increase in human myotubes. This might be ascribed to differences in kinetics of myogenin expression between cellular models. The precise mechanisms underlying this phenomenon are unclear; however, the fact that MuRF1 and MAFbx were downregulated under obestatin treatment in the absence of myogenin upregulation appears to exclude its implication as inducer of the E3 ubiquitin ligases in glucocorticoid-induced atrophy. In this regard, myogenin is not induced in response to other forms of atrophy such as cancer, fasting or diabetes (Lecker SH et al., 2004; Sacheck JM et al., 2007). Together, these findings highlight the existence of fundamental differences between the regulatory circuitry in human and mouse muscle wasting and their modulation. Further experimentation will be required to fully elucidate the functional consequences of these differences.

The obestatin/GPR39 system arises as one of the autocrine systems for coordinating muscle growth, enhancing muscle repair and increasing muscle mass through regulatory role on proliferation, differentiation and hypertrophy of muscle cells (Gurriaran-Rodriguéz U et al., 2012; Santos-Zas I et al., 2016). If one adds to this a role in regulating muscle atrophy through regulation of ubiquitin E3-ligases expression and autophagy, this system displays clear functional similarities with the IGF and/or insulin signaling, but acts using distinct receptors and signaling pathways. The major has been the identification of key anti-atrophic signaling nodes in humans, in particular FoxO4 and FoxO1, in response to obestatin. It is worth underlining that, siRNA targeting FoxO4 suppress the ability of obestatin to regulate E3 ubiquitin ligases and autophagy during glucocorticoid treatment. Furthermore, FoxO1 is regulated by post-translational modifications, for example

acetylation/deacetylation and ubiquitination. Interestingly, the obestatin action was not due to an effect on the FoxO3, the most critical factor for the atrophy program (Mammucari C et al., 2007a; Milan G et al., 2015; Zhao J et al., 2007). Indeed, we provide functional evidence for a FoxO3 phosphorylation switch that explains how glucocorticoids, *per se*, regulate transcriptional activation of the gene but subsequently inactivate the corresponding protein by site-specific phosphorylation and nuclear export. Therefore, FoxO4 and FoxO1 are both required for the optimal regulation of the proteostasis in response to the obestatin/GPR39 system, at least during glucocorticoid-induced atrophy.

In skeletal muscle, GR activates a transcription network driven by KLF15 that induces the expression of atrogenes such as MAFbx and Murf1 as well as regulate the expression of FoxOs (Shimizu N et al., 2011). In this case in particular, we demonstrated the implication of KLF15 in the expression of FoxOs and atrogenes in human myoblasts. The functional cooperativity of GR, FoxOs, and KLF15 in the expression of the atrogenes denote the molecular basis for the involvement of GR in muscle atrophy. Intriguingly, GR-mediated transcription through KLF15 of FoxO3 and FoxO1 was not repressed by obestatin but did so for FoxO4. This support that obestatin signaling regulates FoxO3 and FoxO1 activities via post-transcriptional modification. However, obestatin modulates FoxO4 activity by both transcriptional and post-transcriptional modification. Although it is well stablished the functional cooperativity of GR and KLF15 in the expression of FoxOs, obestatin appears to modulate FoxO4 expression independently to KLF15 axis. Indeed, cotreatment of Dexa and obestatin downregulated to a greater extent the FoxO4 expression under siRNA against KLF15 conditions related to Dexa treatment. Since this action represses to a further extent the expression of atrogenes, it would be of particular interest to identify the molecular mechanisms involved for the development of

treatments for glucocorticoid-induced and wasting disorder-related skeletal muscle atrophy. Further studies are clearly needed to clarify this issue.

In summary, these results demonstrate that the activation of obestatin/GPR39 system impaired proteolysis via specific inactivation of FoxO4 and FoxO1 and efficiently counteracts the catabolic processes provoked by glucocorticoids. Notably, AKT/mTOR activation combined with PKD/CAMKII/AMPK inactivation by the obestatin signaling is required to inhibit FoxO dependent atrogenes and autophagy in human muscle cells. This crosstalk between glucocorticoid receptor and the obestatin/GPR39 signaling is a coordinated interaction between an anabolic signal and the catabolic machinery. Modulation of the activity of this system may represent a new strategy to ameliorate the debilitating effects of the muscle atrophic response to glucocorticoids.

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CONCLUSIONS

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Conclusions

1. The obestatin/GPR39 system regulates protein synthesis, ubiquitin-proteasome and autophagy systems through a coordinated regulation involving Akt-, PKD/PKCμ-, CAMKII-, AMPK- and p38-dependent mechanisms with different sets of effector proteins that eventually affect FoxO transcription factors.

2. Some key regulators in glucocorticoid induced muscle atrophy process differ between human and mouse. Although the final effect is the same, in order to design pharmacological treatment, the targets must be assessed.

3. A specific pattern of FoxO post-translational modifications, including FoxO4 phosphorylation and FoxO1 deacetylation is critical in the regulation of autophagy and the ubiquitin-proteasome system.

4. These results demonstrate that the obestatin/GPR39 signaling pathways not only promote myogenesis but is able to counteract deregulations in the proteostasis, e.g. those associated to glucocorticoid-induced myotube atrophy, and to restore efficient basal homeostasis.

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RESUMEN

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El músculo esquelético es uno de los tejidos más dinámicos y con mayor capacidad de adaptación del cuerpo humano y comprende aproximadamente el 40% del peso total del cuerpo. A pesar de atribuírsele como única función la retención postural y locomoción, actualmente se considera un órgano endocrino, en el que se producen y liberan moléculas biológicas llamadas mioquinas, éstas se producen en respuesta a la contracción, a diferentes condiciones fisiológicas o patológicas que finalmente influenciarán en el metabolismo.

En condiciones fisiológicas normales el músculo esquelético es un tejido estable, con una tasa de renovación de núcleos del 1-2% por semana, sin embargo, en respuesta a un daño ya sea leve o severo, el músculo esquelético sufre un proceso altamente coordinado de renovación, que va desde el nivel molecular al restablecimiento de un aparato contráctil funcional. En este sentido, las células satélite, las células madre del músculo esquelético, juegan un papel crucial en la renovación del musculo. En condiciones fisiológicas normales, estas células permanecen quiescente, situadas entre el sarcolema y la lámina basal de las miofibras, comprendiendo una población heterogénea caracterizada por la expresión de factor de transcripción Pax7. Después de dañó muscular, las células satélite quiescentes, se activan y entran en un estado proliferativo, dando lugar a las células precursoras miogénicas, denominadas mioblastos. Estos mioblastos entran también en un ciclo proliferativo para terminar saliendo del ciclo celular, entrando posteriormente en una fase de diferenciación y fusionándose para formar nuevas fibras musculares o reparar aquellas que estuviesen dañadas. Este proceso de regeneración muscular culmina finalmente con una fase de restauración de la vasculatura además de los patrones de inervación. Un aspecto clave en el proceso de regeneración muscular es la capacidad de las células satélite de autorrenovarse, proporcionando los precursores migénicos necesarios para el proceso de regeneración y a su vez manteniendo una población basal de células

madre. Esta regulación de la activación y posterior diferenciación de las células satélite esta mediada por una familia de factores de transcripción llamada factores de regulación miogénica (MRF), Myf5, MyoD, miogenina y MRF4. El proceso de autorrenovación de las células satélite se produce realizando dos tipos de división: división simétrica, en la que la célula madre da lugar a dos células hijas que, de manera estocástica, serán dos células madre o dos células comprometidas a entrar en el programa da diferenciación. Por otro lado, las células satélite pueden sufrir una división asimétrica, por la cual, se generan dos células hijas diferentes, una destinada a mantener la población de células madre y la otra comprometida a entrar en el programa de diferenciación.

La atrofia muscular se define como la disminución del tamaño de un tejido u órgano debido a la merma del tamaño celular, causada por la perdida de orgánulos, citoplasma y proteínas. Generalmente la masa muscular depende del balance entre la síntesis y la degradación proteica, ambos factores muy sensibles al estatus nutricional, balance hormonal, actividad física, entre otros. La atrofia muscular ocurre en músculos específicos debido a situaciones como traumas, inmovilización, denervación, sarcopenia (atrofia asociada a personas de avanzada edad), en respuesta al ayuno o malnutrición y a diferentes estados patológicos como enfermedad pulmonar obstructiva crónica, caquexia asociada a cáncer, diabetes, fallo renal, síndrome de Chusing, sepsis, entre otros. Esta atrofia se verá reflejada en la disminución del área de las fibras musculares, así como el volumen y proteínas musculares y pese a que el músculo es un órgano con gran capacidad de adaptación, la excesiva perdida de masa muscular no hace más que agravar el estado patológico además de aumentar la mortalidad y morbilidad. Existen cuatro sistemas proteolíticos activados durante los estados catabólicos que se caracterizan por la atrofia muscular. El sitema ubiquitin-proteasoma, el autofagia-lisosomal, siendo estos dos los más relevantes, las calpainas dependientes de

calcio y las caspasas. Por una parte, los componentes miofibrilares son degradados mayoritariamente por el sistema ubiquitin-proteosoma contribuyendo a la perdida de la maquinaria contráctil, siendo las ubiquitin-ligasas MAFbx y MuRF1 las más implicadas en el proceso, encargadas de marcar las proteínas con molecula de ubiquitina para su posterior degradación. Por otra parte, el sistema autofagia-lisosomal es el responsable de la degradación de mitocondrias, orgánulos y grandes agregados proteicos, generando así una disminución en la resistencia del musculo en los músculos atrofiados. Los mecanismos por los cuales se controla si un musculo crece o se atrofia están controlados por la vía de PI3K-AKT-mTOR, que en condiciones normales estimula a la síntesis proteica y además mantiene inhibida una de las principales vías de activación de genes relacionados con la atrofia (atrogenes), la familia de factores de transcripción FOXO. Se ha demostrado que otros factores de transcripción como SMAD2, SMAD3, el receptor de glucocorticoides o NF-kB están implicados en la activación de atrogenes, pero la mayor parte de ellos parecen colaborar o actuar a través de los factores de transcripción FOXO. En condiciones catabólicas la señalización de la vía PI3K-AKT-mTOR se encuentra reducida, permitiendo a los factores de transcripción FOXO ejercer su actividad promoviendo la expresión de atrogenes, desencadenando la degradación de proteínas y estructuras musculares, generando finalmente atrofia.

Algunos estados patológicos caracterizados por atrofia muscular están asociados con un aumento de glucocorticoides circulantes y una mala prognosis para el paciente. El efecto catabólico de los glucocorticoides en el músculo ha sido muy bien estudiado, estos, son usados bien como tratamiento en ciertas dolencias o también se pueden encontrar aumentados en sangre debido a estados patológicos como la sepsis, el cáncer, la acidosis metabólica o el síndrome de Cushing. El efecto de los glucocorticoides en el músculo se ejerce a través del receptor de

glucocorticoides. Una vez los glucocorticoides se unen al receptor, se produce un cambio conformacional provocando finalmente su translocación al núcleo, donde se une a elementos de respuesta a glucocorticoides (GREs) en los promotores de los genes diana, resultando en la activación del programa atrófico. Más concretamente, la dexametasona es un glucocorticoide sintético ampliamente utilizado en la clínica con efectos antiinflamatorios e inmunosupresivos, es usado como antiemético, en alergias, en edema cerebral, en patologías inflamatorias, entre otros. La dexametasona, actúa a través del receptor de glucocorticoides, regulando directamente la expresión génica a través de los GREs, llamada acción genómica, o a través de interacciones con quinasas citosólicas, llamada acción no genómica. Aunque es muy común su uso en la clínica, se sabe que una elevada dosis de dexametasona puede producir atrofia muscular, debido a la activación de los sistemas ubiquitin-proteasoma y autofagia-lisosomal. Se ha desmostrado que la activación del receptor de glucocorticoides a través de la dexametasona es capaz de producir un aumento de la expresión de atrogenes, como los factores de transcripción FOXO, genes del sistema ubiquitin-proteasoma como MAFbx, MURF1, genes implicados en el proceso autofágico como LC3 y catepsinaL. Además, no solo promueve la expresión de genes implicados en el programa atrófico si no que también es capaz de inhibir en cierta medida la señalización mediada por mTOR, como resultado de un aumento en la actividad del factor de transcripción REDD1 y KLF15, este último a su vez, es capaz de cooperar con los factores de transcripción FOXO promoviendo a su vez la expresión de MAFbx y MuRF1.

En este contexto, la obestatina, un péptido de 23 aminoácidos derivado de escisión proteolítica de la preproghrelina y descubierto por Zhang y colaboradores en 2005 en tejido gástrico. Fue descrito por nuestro grupo, por ejercer una acción autocrina en el músculo a través de su receptor, el receptor acoplado a proteínas G, GPR39.

Este sistema regula múltiples etapas de la miogénesis como: proliferación de mioblastos, salida del ciclo celular y las diferentes fases del programa de diferenciación, reclutamiento y fusión de los mioblastos para la formación de miotubos polinucleados. La activación del GPR39 a través de la obestatina, activa una cascada de señalización dependiente de proteínas G y β-arrestinas. Por un lado, la activación del sistema obestatina/GPR39, ejerce una acción mitogénica a través de el eje de señalización EKR1/2 y JunD, activando el primer paso de proliferación del programa miogénico. Por otro lado, a través de un mecanismo mediado por β-arrestinas y transactivación del EGFR, se promueve la salida del ciclo celular provocando el arresto mitótico a través de p21 y p57 y la progresión hacia el programa de diferenciación, a través de una cascada de señalización de quinasas determinada por los ejes AKT, CAMKII,c-Jun y p38. La obestatina se expresa en músculo sano, en estudios *in vitro* se demostró que la obestatina comienza a expresarse en estadios tempranos de la miogenesis y se mantiene durante la diferenciación terminal, mientras que el GPR39 se expresa tanto en mioblastos como durante la diferenciación. A nivel funcional, se observó que la inyección de obestatina promovía la regeneración después de sufrir un daño muscular, además de controlar el fenotipo oxidativo de las fibras musculares más resistente al daño mediante HDACII/Mef2 y PGC1α. Por otro lado, estudios de terapia celular, en lo que se implantaron mioblastos humanos en el músculo de ratones inmunodeficientes, demostraron que, el sistema obestatina/GPR39 no solo aumentaba la eficiencia del transplante de células si no que también facilitaba la distribución de nuevos mioblastos en el musculo hospedador. Las propiedades de este sistema hacen que, la obestatina, sea un candidato terapéutico para el tratamiento de enfermedades que cursan con degeneración muscular. En este sentido los objetivos de este trabajo fueron: 1) Determinar si el sistema obestatina/GPR39 podría contrarrestar el proceso catabólico desencadenado por

el tratamiento con glucocorticoides. 2) Estudiar la relación entre la degradación y síntesis proteica y mostrar evidencias de que el sistema obestatina/GPR39 actua a través del eje AKT/FOXO controlando los sistemas ubiquitin-proteosoma y autofagia-lisosomal. 3) Dilucidar las posibles diferencias en la regulación de las vías de señalización catabólicas entre humanos y ratón.

Para el desarrollo de este trabajo empleamos dos modelos *in vitro* de células musculares. La línea celular de mioblastos de ratón C2C12, la línea celular de mioblastos humanos KM155C25 y el cultivo primario de mioblastos humanos C25. Sometimos a los mioblastos a un proceso de diferenciación durante 7 días, una vez obtuvimos miotubos maduros, procedimos al tratamiento de los mismos con dexametasona o con dexametasona más obestatina, con la finalidad de dilucidar si la activación del sistema obestatina/GPR39 podría contrarrestar el efecto catabólico de los glucocorticoides en ambos modelos y establecer las posibles diferencias en cuanto a regulación de estos.

Los resultados de este trabajo demostraron que, en miotubos de ratón C2C12 tanto la regulación de la maquinaria proteolítica como síntesis proteica, era llevada a cabo por la activación de la vía de señalización de AKT/mTOR a través del sistema obestatina/GPR39. Pese a que la activación de AKT es capaz de regular la familia de factores de transcripción FOXO, en este caso bajo el tratamiento con obestatina, no se observó una inhibición de FOXO3 ni FOXO1 por fosforilación, al contrario que FOXO4, sugiriendo que la obestatina regula de manera específica esta isoforma. De esta manera, bajo el tratamiento con obestatina se produjo una inhibición del sistema ubiquitin-proteosoma, tal y como pudimos determinar por la inhibición de las ubiquitin-ligasas MAFbx y MuRF1, a la par que el flujo autofágico se vio frenado tal y como demostró la redución en la forma lipidada de LC3, la disminución en la expresión de catepsina L y la acumulación de p62. En cuanto a la síntesis proteica la activación de la vía mTOR fue demostrada a través de la activación de sus dianas

S6K1, S6 y 4EBP1, de manera concomitante observamos un efecto antiatrófico tanto a nivel fenotípico, con una recuperación del área de los miotubos tratados con obestatina, a la vez que observamos un aumento en el contenido proteico de los mismos con una recuperación de la expresión de MHC. Estos resultados demostraron que la obestatina controla tanto la síntesis como la degradación proteica, regulando de manera especifica tanto el anabolismo como la actividad proteolítica en miotubos de ratón.

Pudimos comprobar que, de la misma manera, la obestatina, fue capaz de revertir el fenotipo atrófico en miotubos humanos. Tanto la actividad proteasomal, analizada a través de la expresión de las ubiquitin-ligasas MAFbx y MuRF1, como el flujo autofágico, a través del análisis de LC3II, catepsina L y p62, se vieron reducidos bajo el tratamiento con obestatina, a la par que era capaz de restaurar la síntesis de MHC y recuperar el área de los miotubos. Sin embargo, es sabido que, entre humanos y ratón, ciertos genes clave y vías de señalización pueden diferir sustancialmente en el proceso miogénico. Nuestros resultados demostraron que el modelo celular de ratón C2C12, reflejaba únicamente de manera parcial los mecanismos involucrados en la señalización en células humanas. En primer lugar, observamos diferencias en la regulación de los factores de transcripción FOXO. FOXO3 fue activado de manera transcripcional tras el tratamiento con dexametasona a la vez que se vio inhibido por fosforilación en el residuo T32 y S318/321 bajo el mismo tratamiento, en este sentido los glucocorticoides parecen ejercer un papel dual en la regulación de FOXO3 en humanos, combinando una regulación transcripcional y post-traduccional, haciendo esta isoforma inactiva en el proceso atrófico por corticoides. FOXO1, no solo se vio regulado por fosforilación en el residuo S256 a través de la activación de AKT tras el estímulo con obestatina, si no que también se vio regulado por acetilación en los miotubos humanos. Los resultados obtenidos parecen demostrar que, la diminución en la acetilación de

FOXO1 tras el tratamiento con obestatina, fue llevado a cabo a través de la activación de HDAC4 por defosforilación. A la par de el aumento de actividad de HDAC4, también se observó una disminución de la actividad de PKF/PKCμ y CAMKII, todas ellas enzimas conocidas por regular la actividad de histonas deacetilasas de clase II. Estos resultados fueron apoyados además por los ensayos de inmunoprecipitación de FOXO1, donde se pudo observar una interacción directa entre FOXO1 y HDAC4 además de una interacción con SIRT1 en las células control, que se vio claramente disminuida tras el tratamiento con dexametasona; tras el tratamiento con obestatina se observó una recuperación de la interacción entre FOXO1 HDAC4 y SIRT1. Además, pudimos observar un aumento del estado de ubiquitinación de FOXO1 tras el tratamiento con obestatina, evidenciando su marcaje para ser degradado en el sistema ubiquitin-proteasomal. Tanto en el modelo humano como en el de ratón, FOXO4 parece ser la isoforma regulada de manera específica por fosforilación tras el tratamiento con obestatina. De esta manera, en ensayos de siRNA contra FOXO4, observamos que, el bloqueo de este factor de transcripción era suficiente para regular de manera negativa la expresión de las ubiquitin-ligasas MAFbx y MURF1, además de reducir el flujo autofágico. Más allá de la regulación post-traduccional de los factores de transcripción FOXO por fosforilación o acetilación en miotubos humanos, observamos tras un ensayo de siRNA contra KLF15, un regulador clave de la expresión de los factores de transcripción FOXO, que la obestatina no era capaz de regular la expresión de FOXO1 ni FOXO3, si embargo si fomentaba la disminución de la expresión de FOXO4 independientemente de KLF15. Los resultados demostraron que FOXO1 y FOXO4 son necesarios para la regulación de la proteostasis en respuesta al estímulo del sistema obestatina/GPR39, en el modelo de atrofia muscular inducida por glucocorticoides, mientras que FOXO3, a pesar de ser descrito como un factor clave en el desarrollo de este tipo de programa atrófico, fue regulado

transcripcionalmente de manera positiva, al mismo tiempo que se inhibia por fosforilación tras el tratamiento con corticoides, excluyendo su implicación promoviendo la expresión de atrogenes.

La estimulación de miotubos humanos con obestatina, demostró ser capaz de frenar el programa atrófico a través de la señalización mediada por β-arrestinas e independientemente de la señalización de IGF1, así fue demostrado en dos ensayos de siRNA, de manera que, bloqueando la señalización de β-arrestinas, la obesatina no fue capaz de promover la fosforilación de FOXO3, FOXO1 ni FOXO4, además de no fomentar la activación de AKT por fosforilación en su residuo S473. En cuanto al bloqueo de IGF1, la ausencia de está proteína no tuvo ningún efecto, ni en el desarrollo del programa atrófico mediado por glucocorticoides, ni en la capacidad de la obestatina de contrarrestar los efectos catabólicos de los mismos. Otra de las diferencias que encontramos entre la señalización de humanos y ratón fue el patrón de expresión de el factor de transcripción miogenina, ha sido descrito en la bibliografía que ejerce un papel dual en el músculo, regulando el proceso miogénico a la par que fomenta la expresión de atrogenes en ratones. En el modelo de ratón observamos que la miogenina no se encontraba activada, mientras que, si lo estaba en el modelo humano, este hecho, junto a la disminución de la expresión de MAFbx y MURF1 tras el tratamiento con obestatina, nos hacen pensar que se podría tratar, simplemente, de diferencias en la cinética de expresión de esta proteína entre humanos y ratón.

El sistema obestatina/GPR39 demostró ser un sistema autocrino, regulando el crecimiento muscular y promoviendo la regeneración. En el proceso atrófico inducido por glucocorticoides, la obestatina fue capaz de regular la expresión de atrogenes tales como las ubiquitin-ligasas MAFbx y MURF1, además del flujo autofágico, mostrando claras similitudes con el sistema IGF1 o insulina pero actuando a través de otras vías de señalización, implicando la regulación de

FOXO4 y FOXO1 bajo una regulación post-traducional específica de fosforilación, acetilación y ubiquitinación, contrarrestando de manera eficiente el proceso catabólico. De manera notable, la activación de la vía de señalización a través de la obestatina, de AKT/mTOR, combinada con la inhibición de PKD/CAMKII/AMPK, es necesaria para la inhibición de la actividad de los factores de transcripción FOXO y la expresión de atrogenes dependiente de los mismos. Cabe destacar que existen diferencias en algunos reguladores clave del programa atrófico entre humanos y ratón, que hace que, a pesar de que el resultado final sea el mismo, a la hora de diseñar fármacos destinados al tratamiento de la atrofia, las dianas moleculares han de ser comprobadas.

Finalmente, con este trabajo se sugiere que la señalización entre el receptor de glucocorticoides y el sistema obestatina/GPR39 se trata de una interacción entre la señalización anabólica y la maquinaria catabólica. La capacidad de modular la actividad de este sistema hace que represente una buena estrategia para paliar los efectos de la atrofia muscular inducida por glucocorticoides.

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Dña.: Tania Cid Díaz

DECLARA:

Esta tesis está basada en el artículo "Cid-Diaz T, Santos-Zas I, Gonzalez-Sanchéz J, Gurriarán-Rodriguez U, Mosteiro CS, et al. Obestatin controls the ubiquitin–proteasome and autophagy–lysosome systems in glucocorticoid-induced muscle cell atrophy. J Cachexia Sarcopenia Muscle 2017; 8(6): 974-990" el cuál ha sido publicado en formato "*open access article* under the terms of the Creative Commons Attribution-NonCommercial License*"* permitiendo así su libre uso siempre que no sea con fines comerciales.

En Santiago de Compostela, 17 de Diciembre de 2019.

Fdo.: Tania Cid Díaz