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ORIGINAL ARTICLE

β-Arrestin scaffolds and signaling elements essential for the obestatin/GPR39 system that determine the myogenic program in human myoblast cells

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Abstract Obestatin/GPR39 signaling stimulates skeletal muscle repair by inducing the expansion of satellite stem cells as well as myofiber hypertrophy. Here, we describe that the obestatin/GPR39 system acts as autocrine/paracrine factor on human myogenesis. Obestatin regulated multiple steps of myogenesis: myoblast proliferation, cell cycle exit, differentiation and recruitment to fuse and form multinucleated hypertrophic myotubes. Obestatin-induced mitogenic action was mediated by ERK1/2 and JunD activity, being orchestrated by a G-dependent mechanism. At a later stage of myogenesis, scaffolding proteins β-arrestin 1 and 2 were essential for the activation of cell cycle exit and differentiation through the transactivation of the epidermal growth factor receptor (EGFR). Upon obestatin stimulus, β-arrestins are recruited to the membrane, where they functionally interact with GPR39 leading to Src activation and signalplex formation to EGFR transactivation by matrix metalloproteinases. This signalplex regulated the mitotic arrest by p21 and p57 expression and the mid- to late stages of differentiation through JNK/c-Jun, CAMKII, Akt and p38 pathways. This finding not only provides the first functional activity for β-arrestins in myogenesis but also identify potential targets for therapeutic approaches by triggering specific signaling arms of the GPR39 signaling involved in myogenesis.

Keywords Skeletal muscle · Skeletal muscle regeneration · Obestatin signaling

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Introduction

Much attention has been recently focused on the understanding of the molecular and cellular mechanisms regulating skeletal muscle regeneration in different contexts, since such knowledge will be essential for the development of potential treatments for myopathies [1, 2]. Among the mechanisms involved, environmental influences suggest the action of cell-to-cell signaling [3, 4]. Furthermore, recent studies have identified human skeletal muscle as a secreting organ [5], a secretion that can be perturbed during pathological processes such as dystrophic conditions [6], emphasizing the importance of secreted molecules in orchestrating muscle regeneration. In this context, we reported that obestatin, a 23-amino acid

peptide derived from a polypeptide called preproghrelin, exerts an autocrine function through the G-protein-coupled receptor GPR39 to control the myogenic differentiation program in the rat cell line L6E9 [7]. Obestatin is expressed in healthy skeletal muscle, and this expression is strikingly increased upon muscle injury. In vitro, obestatin is coordinately up-regulated during the early stages of myogenesis, and its level remains sustained throughout terminal differentiation. Functionally, blocking obestatin during myogenesis induces a decrease in myogenic-associated markers, a process requiring the G-protein-coupled receptor GPR39. These data support the idea that the obestatin/GPR39 system is coordinately regulated as part of the myogenic program and operates as an autocrine signal regulating skeletal myogenesis. Notably, obestatin acts through a paracrine signaling on endothelial cells increasing the expression of VEGF and its receptor isoform VEGFR2 to provide the needed vascularization for developing skeletal muscle [7]. Obestatin was originally identified in the stomach as a physiological opponent of ghrelin [8]. Although obestatin has been known as a controversial ghrelin-associated peptide due to the lack of reproducible biological actions on feeding, this peptide displays a variety of cellular effects, by regulating metabolic and cell differentiation functions, increasing cell survival and proliferation, and inhibiting apoptosis and inflammation in different cell types [9]. In particular, obestatin regulates adipogenesis [10], pancreatic homeostasis [11] and is involved in gastric cancer [12]. In adipogenesis, for example, the obestatin/GPR39 system exerts a regulatory role on the expression of master regulators of the adipocyte fate and, consequently, lipid accumulation [10]. The relevance of obestatin as a regulator of adipocyte metabolism was further supported by GLUT4 translocation, and increased glucose uptake [10] being a mediator for insulin-induced adipogenesis [13]. Recent results have evidenced that the overexpression of the obestatin/GPR39 system in mouse skeletal muscle enhanced muscle regeneration after muscle injury [14]. Notably, the intramuscular injection of obestatin into acutely injured skeletal muscles improved the efficiency of muscle regeneration, as evidenced by the up-regulation of the expression of specific myogenic factors (MyoD, myogenin) and embryonic myosin heavy chain (eMHC), the decrease of serum CK levels, and the significant increase of the myofiber size and the number of myonuclei per myofiber. Obestatin induced the up-regulation of the specific satellite cells marker Pax7, which was associated with an increased number of activated satellite cells per myofiber. The obestatin action was due to a specific regulation on the different stages affecting the myogenesis: proliferation, migration, fusion and myofiber growth. Added to its promyogenic action, obestatin administration resulted in an increase in microvascularization, with no significant effect on collagen deposition in regenerating skeletal muscle. Finally, a potential inhibition of myostatin might contribute to the myogenic enhancement, thus further improving muscle growth and regeneration.

The ability of obestatin to regulate different stages of the myogenic program suggests its potential use as a therapeutic target for trauma-induced muscle injuries or skeletal muscle myopathies related to muscle regeneration. However, any proposition of therapeutic strategies requires extensive studies of the obestatin/GPR39 signaling in a human context. Although murine models have been extensively used to identify and test drug candidates for subsequent human trials, the assumption that murine models translate directly to human conditions has been challenged in different studies: Molecular behavior of key genes or key pathways of murine myogenesis differs substantially from those in humans, making such models weak predictors particularly in the context of disease [15] or therapy [16–18]. First, human skeletal muscle precursors elaborate a genetic program clearly distinct from that of the mouse [19–22]. Second, the differences in the temporal expression patterns of many skeletal muscle-related genes between mouse and human suggest a more precocious ability to differentiate in mouse than in human cells [15]. Third, the regulatory relationships between myogenic factors are not completely conserved between mouse and human [23–28]. Furthermore, inflammatory reactions in mouse may differ substantially from those in humans, since the mouse is a poor predictor of human immune mechanisms [29, 30]. Together, such differences may complicate the clinical translation of promising therapies from mouse models to humans. In this regard, the existence of human myoblast lines represents a powerful tool to assess signaling and/or functional regulation, particularly those in which these mechanisms have not yet been clearly elucidated. The existence of human cellular models provides powerful and valuable tools for validating gene or cell therapies and pharmacological strategies for muscular dystrophies, some of which might also be used to combat muscle weakness in the elderly. Using transduction with both telomerase-expressing and cyclin-dependent kinase 4-expressing vectors (hTERT and CDK-4), a battery of immortalized human muscle stem-cell lines from ‘normal’ individuals was generated [31–35]. These cell lines have extended proliferative life spans, and maintain their capacity to differentiate both in vitro and in vivo after transplantation into the regenerating muscles of immunodeficient mice [32], thus providing an ideal model for drug screening, and for biochemical, cellular and physiological characterization of their regenerative potential.

In the present study, we used a stable immortalized human myoblast cell line to investigate whether the

obestatin/GPR39 system, either by modulation of its expression or by exogenous administration, could have specific regulatory action on human myogenesis. Using several cellular strategies, we also analyzed the mitogenic and myogenic effect of this peptide and the associated intracellular signaling pathways. Our work shows novel mechanistic insights on how myogenesis is regulated by obestatin through the crosstalk between GPR39 and EGFR, and unravels the β-arrestins as key specific regulator of myogenic program. These findings support a link between both receptor systems that determine the steps of myogenic process in human myogenesis.

Methods

Materials

Human obestatin was obtained from California Peptide Research (Napa, CA, USA). Antibodies used are listed in Table S1. All other chemical reagents were from Sigma Chemical Co. (St. Louis, MO, US).

Immortalized human myoblast cell line, cell culture and differentiation

The muscle biopsy from gracilis muscle (donor age 25 years) was obtained from MYOBANK-BTR (Bank of Tissues for Research, a partner in the EU network Euro-BioBank) in accordance with European recommendations and French legislation. Surgical procedures were performed in accordance with the legal regulations in France and European Union ethical guidelines for animal research. Immortalized human myoblasts, KM155C25 Clone 48, were isolated and cultivated as described previously [32] in growth medium (GM) containing Medium 199:DMEM (1:4, v:v; Lonza, Pontevedra, SP) supplemented with 20 % FBS (v/v), 25 μg/μL fetuin, 5 ng/mL hEGF, 0.5 ng/mL bFGF, 0.2 μg/mL dexamethasone (Sigma–Aldrich; MO, USA) and 50 μg/mL gentamycin (Invitrogen). Differentiation into myotubes was initiated at 90 % confluence by switching to differentiation medium [DM; DMEM supplemented with either 10 μg/mL insulin or DM supplemented with 10 nM obestatin and 50 μg/mL gentamycin (Invitrogen)] for 7 days unless otherwise stated.

Proliferation assays

Cell proliferation of immortalized human myoblasts (KM155C25 Clone 48 cells) was determined using the BrdU incorporation-ELISA assay (Roche, Swiss) following manufacturer's instructions. In brief, cells (10×10^3/well) were cultured in GM supplemented with obestatin (0.01–100 nM) for 48 h and then were incubated with 10 mg/mL BrdU for 3 h before being fixed with FixDenat solution. The fixed cells were further treated with anti-BrdU-POD working solution, and rinsed with washing solution before substrate solution was added. The absorbance at 370 nm (reference wavelength at 492 nm) was measured using an ELISA plate reader (Reader VersaMaxPLUS).

Small interfering RNA (siRNA) silencing of gene expression

Chemically synthesized double-stranded siRNA duplexes targeting either preproghrelin, GPR39, β-arrestin 1 or β-arrestin 2 were selected from ON-TARGETplus SMART-pool siRNA from Thermo Fisher Scientific (Dharmacon; preproghrelin, GACAAGUGAUCGCCCACAA, GCCCU GAACACCAGAGAGU, CAGAGGAUGAACUGGAAGU, UCGAAGAAGCCACCAGCCA. GPR39, UCCAAUAUG UCCAUCUGUA, GCGCGAAACCAGCCAAUUC, GAGG CUGAUUGUUGUGACA, AACCAGAUUCGGAGGAU CA. β-Arrestin 1, UGGAUAAGGAGAUCUAUUA, AUG GAAAGCUCACCGUCUA, GAACUGCCCUUCACCCU AA, GAACGAGACGCCAGUAGAU. β-Arrestin 2, CGA ACAAGAUGACCAGGUA, CGGCGUAGACUUUGA-GAUU, GGGCUUGUCCUUCCGCAAA, UAGAUCA CCUGGACAAAGU. An ON-TARGETplus nontargeting siRNA was used as a control for all siRNA experiments. Human myoblasts were transfected with Lipofectamine 2000 (Invitrogen), following manufacturer's instructions.

Histology and immunofluorescence

Human myoblast and/or myotube cells cultured on gelatin-coated glass coverslips were fixed in 96 % ethanol for immunocytochemical analysis. Human skeletal muscle samples (muscle biopsy from gastrocnemius muscle) were mounted in tissue freezing medium (tragacanth paste) and snap-frozen in nitrogen-cooled isopentane. Sections, 10 μm thick, were mounted on HistoBond adhesion microslides (Marienfeld, Lauda-Königshofen, Germany). Cells or tissue samples were then consecutively incubated with: (1) primary antibody at a dilution of 1:100 in Dako ChemMate antibody diluent (Dako, Glostrup, Denmark); (2) EnVision peroxidase rabbit (Dako, Carpentaria, CA) used as the detection system; and (3) 3,3′-diaminobenzidine-tetrahydrochloride (Dako liquid DAB + substrate-chromogen system). For immunofluorescence analysis, cells were cultured on gelatin-coated glass coverslips and differentiated into myotubes in DM or DM supplemented with obestatin or insulin for 7 days. Differentiated cells were fixed with 4 % buffered paraformaldehyde–PBS for 15 min, washed, permeabilized, and blocked with PBT

(1 % Triton X-100, 1 % Tween 20, 5 % heat-inactivated normal goat serum, 0.2 % BSA in PBS) for 30 min and then incubated with anti-MHC antibody diluted in PBT (1:400) overnight at 4 °C. After three washes with PBS, cells were incubated with the secondary antibody (FITC-conjugate goat anti-mouse antibody) in PBT (1:1000) for 45 min at 37 °C. DAPI was used to counterstain the cell nuclei (Invitrogen). Digital images of cell cultures were acquired with a Leica TCS-SP5 spectral confocal microscope (Leica Microsystems, Heidelberg, Germany). Five fields from three independent experiments were randomly selected for each treatment. The differentiation grade was evaluated based on the fusion index [nuclei in MHC-positive cells (MHC^+)/total nuclei], myotube area (MHC^+) and number of nuclei per MHC^+ myotube (30–50 myotubes were scored). The digital images of the cell cultures were acquired with a Leica TCS-SP5 spectral confocal microscope (Leica Microsystems, Heidelberg, DE). For quantification of myotube area, ImageJ64 analysis software was used.

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 (Sigma Chemical Co, St. Louis, MO, US), phosphatase inhibitor cocktail (Sigma Chemical Co, St. Louis, MO, US)]. The lysates were clarified by centrifugation (14,000×*g* for 15 min at 4 °C) and the protein concentration was quantified using the QuantiPro™ BCA assay kit (Sigma Chemical Co, St. Louis, MO, USA). 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, Pierce, Rockford, IL, US). Densitometric measurements were performed using NIH ImageJ software from at least three replicates.

Phosphoproteome profiling

The control and obestatin-stimulated myoblast cells (10 nM, 10 min at 37 °C) were solubilized in NP-40 lysis buffer [20 mM Tris–HCl (pH 8.0), 1 % (v/v) NP-40, 137 mM NaCl, 10 % (v/v) glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 10 μg mL^{-1} aprotinin, 10 μg mL^{-1} leupeptin] by rocking the lysates gently at 4 °C for 30 min. Following microcentrifugation (14,000×*g*, 5 min), supernatants were transferred into a clean test tube and sample protein concentrations were determined using the QuantiPro™ BCA assay kit (Sigma Chemical Co, St. Louis, MO, USA). Lysates were diluted and incubated with Human Phospho-RTK and Human Phospho-MAPK Arrays (Proteome Profiler; R&D Systems; Minneapolis, MN, USA) as per the manufacturer's instructions. In this method, capture and control antibodies have been spotted in duplicate on nitrocellulose membranes. In brief, the membranes were blocked with 5 % (w/v) bovine serum album (BSA)/TBS [0.01 M Tris–HCl (pH 7.6)] for 1 h. Membranes were then incubated with 200 μg of total protein. After extensive washing with TBS including 0.1 % (v/v) Tween-20, three times for 5 min, to remove unbound materials, the membranes were then incubated with HRP-phospho-receptor tyrosine kinase (RTK) or phospho-MAPK antibodies for 2 h at room temperature. Unbound HRP antibody was washed out with TBS including 0.1 % (v/v) Tween-20. Finally, array data were developed on an X-ray film using a chemiluminescence detection system, and densitometry readings were carried out using the Image Studio Lite densitometry software from LICOR Biosciences.

Cell transfection and co-immunoprecipitation assays

Immortalized human myoblasts were transiently transfected with the plasmid construct expressing the HA-tagged β-arrestin 2 [β-arrestin 2-HA; pcDNA3 β-arrestin 2 HA was a gift from Robert Lefkowitz (Addgene plasmid n°14692)] using Lipofectamine 3000 (Life Technologies, Invitrogen, Gran Island, NY, US), according to the manufacturer's instructions. Pure cell lines were selected on the basis of resistance to neomycin. Cells expressing the β-arrestin 2-HA were cultured as described above. Following obestatin treatment, transfected 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 inhibitors). HA-tagged β-arrestin 2 were immunoprecipitated using anti-HA affinity matrix (Roche Applied Science, US) following the manufacture's instructions. 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 (s.e.m.). Student *t* test were performed to assess the statistical significance of two-way analysis. For multiple comparisons, ANOVA was employed. $P < 0.05$ was considered as statistically significant (*).

Results

The obestatin/GPR39 as an autocrine system during myogenesis

As seen in Fig. 1a, the expression of the obestatin/GPR39 system was detected in skeletal muscle (gastrocnemius) by immunohistochemistry (Fig. 1A.1, A.2, respectively). To elucidate and validate the role of this system in human myogenesis, an in vitro cell culture model that closely recapitulates the formation and maintenance of human skeletal muscle was utilized: the immortalized human muscle stem-cell lines from a control individual (KM155C25 Clone 48 cells; for details see "Methods"). Upon reaching confluence, serum was withdrawn to induce the differentiation of immortalized KM155C25 from proliferative myoblasts into terminally differentiated multinucleated myotubes that express markers of terminal skeletal muscle differentiation such as MHCs. As shown in Fig. 1a, KM155C25 myotubes, obtained after a 10-day differentiation period, showed a stronger obestatin expression when compared with that observed in myoblasts (Fig. 1A.5 when compared with Fig. 1A.3 for KM155C25 cells). In contrast, GPR39 was detected in both myoblast and myotube cells (Fig. 1A.4, A.6, respectively). Preproghrelin expression, as the source of obestatin, was examined at the protein level by immunoblot, from samples taken at the proliferative state and throughout

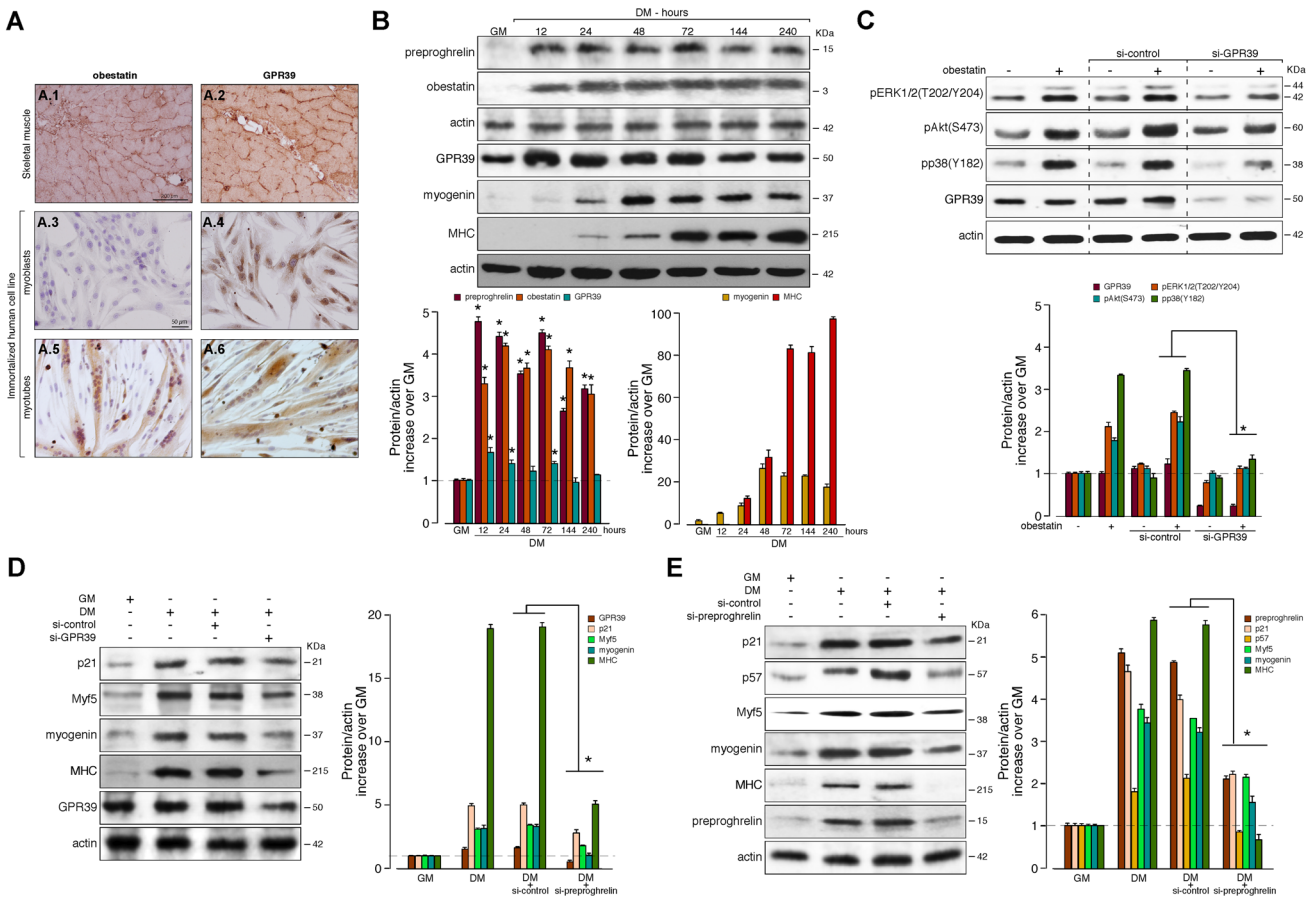


Fig. 1 The obestatin/GPR39 system as an autocrine system during myogenesis. **a** Immunohistochemical detection of obestatin (*A.1*) and GPR39 (*A.2*) in human skeletal muscle (gastrocnemius; objective magnification ×10). Immunocytochemical detection of obestatin and GPR39 in human myoblast (*A.3* and *A.5*) and myotube (*A.4* and *A.6*) KM155C25 cells (objective magnification ×40). **b** Immunoblot analysis of preproghrelin, obestatin, GPR39, myogenin, and MHC expression in the course of KM155C25 myogenesis. Protein level was expressed as a fold over control cells in GM ($n = 3$). **c** Effect of GPR39 knockdown by siRNA on obestatin-activated pERK1/2(T202/Y204), pAkt(Ser-473) and pp38(Tyr-182) in KM155C25 myoblast cells (10 nm, 5 min). GPR39 was expressed as a fold of its level to control siRNA-transfected cells ($n = 3$). Activation of ERK1/2, Akt and p38 was expressed relative to control cells. **d** Effect of siRNA depletion of GPR39 on myogenesis. KM155C25 cells were transfected with GPR39 siRNA prior to induction of myogenesis for 96 h. Expression of p21, Myf5, GPR39, myogenin and MHC was expressed as a fold of the respective levels to control GM cells ($n = 3$). **e** Effect of siRNA depletion of preproghrelin on myogenesis. KM155C25 cells were transfected with preproghrelin siRNA prior to induction of myogenesis for 96 h. Expression of p21, p57, Myf5, preproghrelin, myogenin and MHC was expressed as a fold of the respective levels to control GM cells ($n = 3$). In **b**–**e**, immunoblots are representative of the mean value. Data were expressed as the mean ± s.e.m. obtained from intensity scans of independent experiments. $^*P < 0.05$ versus control values

differentiation (Fig. 1b). Preproghrelin is expressed as a biphasic pattern: a rapid increase at 12 h of differentiation (~4.8-fold) followed by a second maximal within 72 h of differentiation (~4.6-fold) remaining at reduced but sustained levels during terminal differentiation (~2.7-fold at 144 h), still higher than in myoblast cells (~3.2-fold at 240 h, Fig. 1b). Obestatin peptide expression closely paralleled the expression of preproghrelin (~3.4- and 4.1-fold at 12 and 72 h, respectively; Fig. 1b). The myogenic transcription factor myogenin showed maximal expression levels 48 h after induction (~28.4-fold; Fig. 1b). The expression of MHC, a late marker of differentiation, showed maximal levels at 240 h, which marked the progression of terminal differentiation (~227-fold; Fig. 1b).

The effect of acute GPR39 deficiency on obestatin signaling was first determined by treatment of KM155C25 myoblast cells with a siRNA targeting GPR39 (si-GPR39). Under these conditions, the constructs decreased GPR39 expression by 80 ± 1 % (Fig. 1c), while in the presence of si-control, no significant change in expression of GPR39 was detected, and obestatin-activated ERK1/2(T202/Y204), Akt(S473) and p38(Y182) phosphorylation was similar to levels observed with untransfected cells. Silencing of GPR39 decreased subsequent pERK1/2(T202/Y204), pAkt(S473) and pp38(Y182) with respect to si-control by 90 ± 2, 86 ± 2 and 83 ± 3 % with treatment of obestatin (10 nM, 10 min; Fig. 1c), respectively. The effect of this acute GPR39 deficiency on myogenesis was further evaluated on myogenic differentiation. Under these conditions, the GPR39 expression was decreased by 57 ± 0.3 % (Fig. 1d) during a 96-h differentiation period. The GPR39 knockdown decreased the expression levels of Myf5, myogenin and MHC by 50 ± 2.1, 62 ± 1.1 and 75 ± 1.0 %, respectively, as compared to si-control (Fig. 1d). In addition, the cell cycle arrest protein p21 was decreased by 46 ± 0.5 % in the presence of si-GPR39 (Fig. 1d). On the other hand, the effect of acute obestatin deficiency was determined by preproghrelin knockdown by siRNA prior to induction of myogenic differentiation. Under these conditions where preproghrelin level was reduced by 72 ± 3 % as compared to si-control (Fig. 1e) during a 96-h differentiation period, the expression of cell cycle arrest proteins p21 and p57 was decreased by 56 ± 2 and 83 ± 1 %, respectively (Fig. 1e). Myf5, myogenin and MHC were also decreased with respect to si-control by 51 ± 1, 70 ± 1 and 95 ± 0.1 %, respectively (Fig. 1e).

The obestatin action is mediated by both G-protein-dependent and β-arrestin-dependent mechanisms in human myoblasts

We utilized a phospho-kinase array to investigate the intracellular signaling pathways activated downstream of GPR39 in KM155C25 myoblast cells. Eighteen proteins, Akt1, Akt2, CREB, ERK1, ERK2, GSK3α, HSP27, JNK1, JNK2, JNK3, MKK3, p38α, p38β, p38γ, p53, S6K1, mTOR and RSK1, exhibited increased phosphorylation following obestatin treatment (10 nM, 10 min; Fig. 2a). An additional protein, MSK2, was partially inhibited in response to obestatin. These data supported roles for obestatin/GPR39 system in multiple processes including cell growth, proliferation, survival, and myogenic differentiation. Previous works from our group demonstrated that obestatin signaling functions are partially mediated by the ability of β-arrestin to serve as an adaptor or scaffold molecule, bringing elements of diverse signaling pathways into proximity with one another and with the receptors and thereby facilitating their activation. In this way, the epidermal growth factor receptor (EGFR) transactivation is the link between obestatin receptor and Akt signaling pathway, whereas $G_{i/o}$ proteins regulate ERK1/2 pathway [12]. We used a phospho-receptor tyrosine kinase (RTK) array to detect the relative phosphorylation of different RTKs activated downstream of GPR39. The most robust response to obestatin stimulation (10 nM, 10 min) was a phosphorylation of EGFR at the time points tested (Fig. 2b). Next, we determined the role of β-arrestin as interplay between obestatin receptor and EGFR. The effect of acute β-arrestin 1 or β-arrestin 2 deficiencies by siRNA (si-β-arrestin 1 or si-β-arrestin 2) was investigated in KM155C25 myoblast cells. siRNA experiments targeting β-arrestin 1 or β-arrestin 2 reduced its expression by 59 ± 2 or 70 ± 2 %, respectively (Fig. 3a). Under these conditions, si-β-arrestin 1 decreased phosphorylation of several EGFR sites that are involved in its activation, Y1068, Y1045 and Y 992, after 10 min of obestatin stimulation (10 nM) with respect to si-control (69 ± 4 % at Y1068; 88 ± 1 % at Y1045; 89 ± 1 % at Y 992). On the other hand, β-arrestin 2 siRNA reduced the EGFR phosphorylation by 55 ± 3 % at Y1068; 89 ± 1 % at Y1045; and, 93 ± 1 % at Y 992 after obestatin stimulation (Fig. 3a). Phosphorylation of EGFR at Y845, the Src phosphorylation site, was not affected by obestatin stimulation (Fig. 3a).

The interplay between G-protein and β-arrestin for obestatin signaling network led us to determine the role of β-arrestins as signal transducers in human myoblasts. siRNA experiments targeting β-arrestin 1 (58 ± 3 % reduction relative to si-control) significantly decreased the obestatin-induced activation of Akt [pAkt(S473)], p38 [pp38(T180/Y182)], c-Jun [pc-jun(S63)], and CAMKIIα, β and γ [pCAMKII(T286)] by 49 ± 2, 61 ± 1, 40 ± 2, 30 ± 1, 51 ± 1 and 34 ± 1 % relative to si-control, respectively (Fig. 3b). The β-arrestin 1 siRNA had no significant effect on ERK1/2 [pERK1/2(T202/Y204)] or JunD [pJunD(S100)] phosphorylation, ruling out the

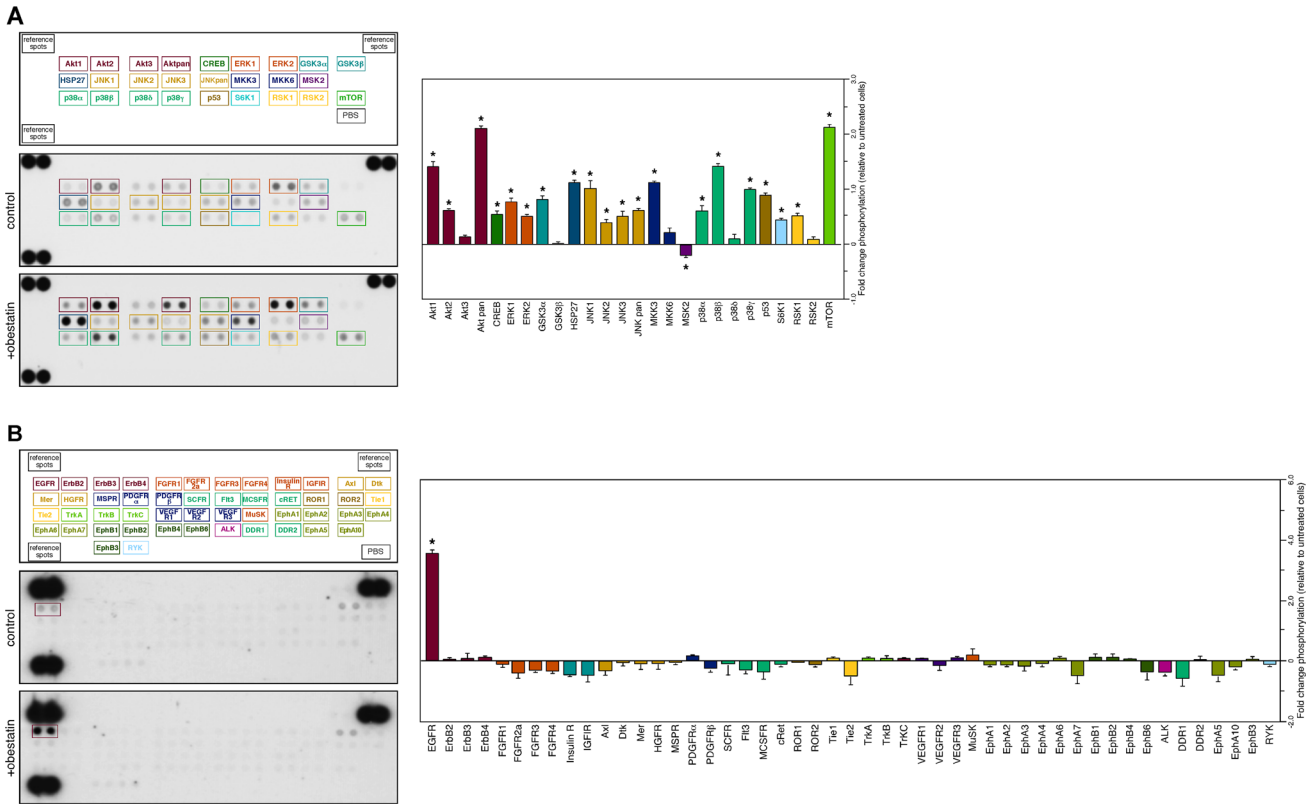


Fig. 2 Screening of intracellular targets associated to the activation of the obestatin/GPR39 system in KM155C25 myoblasts. **a** Phosphoproteomic analysis of obestatin-treated KM155C25 myoblasts. Upper template showing the location of MAPK antibodies spotted onto the Human Phospho-MAPK Array Kit (Proteome Profiler™; R&D Systems). Lower template shows the detection of obestatin-modulated intracellular kinases (10 nM, 10 min). **b** Phosphoproteomic analysis of obestatin-treated KM155C25 myoblasts. Upper template showing the location of RTK antibodies spotted onto the Human Phospho-RTK Array Kit (Proteome Profiler™; R&D Systems). Lower template shows the detection of obestatin-modulated phosphoRTKs (10 nM, 10 min). Array data were developed on X-ray films following exposure to chemiluminescent reagents. **a**, **b** show representative phosphoproteome analysis obtained from three independent experiments. Data were expressed as a fold of the respective levels to control cells. Data were expressed as the mean ± s.e.m. obtained from intensity scans of independent experiments. $*P < 0.05$ versus control values

involvement of this scaffold protein in these pathways. Similarly, the β-arrestin 2 knockdown (59 ± 2 % relative to si-control) inhibited pAkt(S473), pp38(T180/Y182), pc-jun(S63), and pCAMKII(T286)α, β and γ by 50 ± 2, 59 ± 1, 51 ± 1, 37 ± 1, 44 ± 1 and 38 ± 1 % relative to si-control, respectively, in response to obestatin with no relevant effect on pERK1/2(T202/Y204) or pJunD(S100) (Fig. 3b).

The mitogenic action of obestatin is mediated by G-protein-dependent mechanisms in human myoblasts

First, mitogenic action of obestatin was explored by dose–effect experiments performed on KM155C25 myoblasts treated with a range of obestatin concentrations (0.1–100 nm) in GM (proliferating conditions; Fig. 3c). Maximal effect in the BrdU incorporation was observed at 10 nM obestatin (∼2.5-fold). Next, to explore the functionality of β-arrestin-dependent signaling pathway on proliferation, the effect of β-arrestin knockdown on cell proliferation was determined in myoblast cells. Despite siRNA experiments targeting β-arrestin 1 or β-arrestin 2 significantly decreased the β-arrestin levels (58 ± 3 or 54 ± 5 decrease relative to si-control, respectively), we found that both scaffolding proteins were not essential molecules for the mitogenic action of obestatin (10 nM) measured as BrdU incorporation (Fig. 3d). Thus, G-protein-dependent signaling (i.e., G_i/PKCε/Src/ERK1/2 pathway) is critical and sufficient for obestatin-associated mitogenic effect.

Obestatin enhances the expression of differentiation markers without changing their kinetics

To assess the activity of obestatin as a promoter of the differentiation process, KM155C25 myoblast cells were switched to DM supplemented with obestatin at a range of concentrations (0.1–100 nM) for 10 days. Insulin

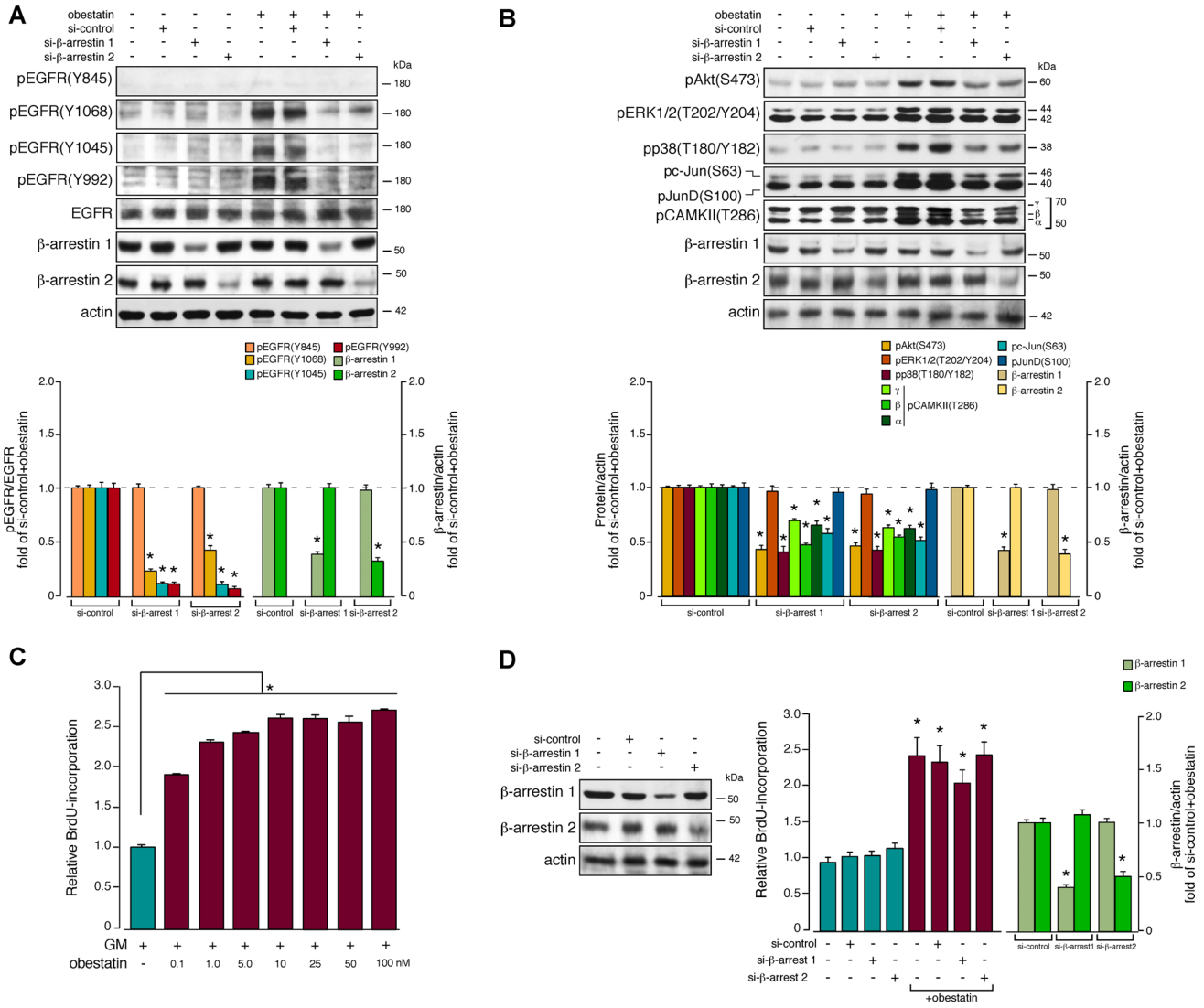


Fig. 3 Differential activation of the obestatin/GPR39 by β-arrestins in human KM155C25 myoblasts. **a** Effect of siRNA depletion of β-arrestins on obestatin-induced EGFR transactivation. After transfection with specific β-arrestin 1 or 2 siRNAs, KM155C25 myoblasts were stimulated with obestatin (10 nM) and EGFR phosphorylation at Y845, Y992, Y1045 and Y1068, was analyzed by immunoblot. **b** Immunoblot analysis of the effect of obestatin on the activation of Akt, ERK1/2, p38, c-Jun and CAMKII in the absence or presence of β-arrestin 1 or 2 siRNAs in KM155C25 myoblasts. **c** Dose–response effect of obestatin (0.1–100 nM) on KM155C25 myoblast proliferation (48 h, $n = 6$). Results were expressed as a fold of the BrdU incorporation relative to control cells maintained 48 h in GM. **d** Effect of siRNA depletion of β-arrestins on the obestatin-induced proliferation of KM155C25 myoblasts. The cells were transfected with specific β-arrestin 1 or 2 siRNAs and then stimulated for 48 h with obestatin (10 nM, $n = 6$). Results were expressed as a fold of the BrDU incorporation relative to control cells in GM. In **a**, **b** and **d**, immunoblots are representative of three independent experiments. The data are expressed as the mean ± s.e.m. The *asterisk* (*) denotes $P < 0.05$ when comparing the treated control siRNA group with the control siRNA group

(1.72 μM) was used as the positive control based on its role in the control of skeletal muscle growth and in the regulation of muscle mass. As shown in Fig. 4a, the protein levels of myogenin and MHC, as detected by immunoblot, were dose-dependent and both were up-regulated at a maximal level in the presence of 10 nM obestatin (~25- and 100-fold, respectively). The possibility that obestatin accelerates differentiation was also investigated (DM + 10 nM obestatin for 10 days). During the differentiation process, the immunoblot analysis of Ki67, a cell proliferation marker, and p21, revealed elevated protein expression in obestatin-treated cells as compared to untreated cells (DM; Fig. 4b). At 12 h, Ki67 showed maximal expression levels, followed by an increase in the expression of p21 that reached maximal levels at 72 h, reflecting growth arrest to promote cellular differentiation. The protein levels of myogenin and MHC were up-regulated, displaying maximal levels at 72 and 96 h,

respectively (Fig. 4b). Of note, both proliferating and myogenic markers did not show any significant change in their kinetics in obestatin-treated cells as compared to untreated cells. To investigate whether obestatin stimulated hypertrophic growth, the KM155C25 myoblasts were treated with obestatin in DM (Fig. 4c). The myotube areas were 311 % larger in the obestatin-treated cells (DM + 10 nM obestatin; 3401.6 ± 29 μm^2) as compared to control conditions (DM; 827.4 ± 10 μm^2) and 68 % larger than insulin-treated cells (DM + insulin; 2016 ± 18 μm^2) at 240 h after differentiation (Fig. 5c). The fusion index was increased by 27 and 9 % in the obestatin-treated cells as compared to untreated control and insulin-treated cells, respectively. Furthermore, myonuclei per MHC-positive myotube cell (MHC^+) were 19 ± 0.4 in obestatin-treated cells versus 8.4 ± 1 in control cells or 11 ± 0.5 in insulin-treated cells (Fig. 4c). This effect represents an increase of ~2.3-fold as compared to control cells.

Obestatin/GPR39 signaling in differentiated myotubes

To establish the molecular events activated by obestatin to drive the myogenic process, the obestatin signaling pathways were analyzed in differentiating KM155C25 cells during 10 days (Fig. 5). The obestatin treatment (10 nM) resulted in markedly elevated levels of the cell cycle arrest protein p57 and that reached maximal levels at 72 h, reflecting cell cycle exit to promote cellular differentiation. The activation of Akt [pAkt(S473)] was markedly elevated during the differentiation reaching maximal levels at 12 and 72 h. The obestatin treatment resulted in increased levels of phosphorylated S6K1 [pS6K1(S371)] during differentiation showing maximal levels at 48 and 144 h. The increased Akt activity was also evinced by the rising in phosphorylated GSK3 [pGSK3α/β(S2/S9)] with consequent inhibition of GSK3 activity. Obestatin increased ERK1/2 activity [pERK1/2(T202/Y204)] in the early steps of differentiation. The levels of phosphorylated p38 [pp38(T180/Y182)] were markedly increased during differentiation showing maximal levels at 48 h. The phosphorylation levels of c-Jun [pc-Jun(S63)] showed differences following the obestatin treatment compared to the control cells reaching maximal levels at earlier time points (24–48 h). When the phosphorylation pattern of JunD [pJunD(S100)] was compared to control cells, obestatin was much more responsive. The phosphorylation patterns of CAMKII isoforms [pCAMKII(T286)] showed differences under the obestatin treatment. An increase of phosphorylated CAMKIIα and γ isoforms was observed at early points (24–48 h) maintaining high levels throughout differentiation. By contrast, CAMKIIβ isoform showed a rapid increase at earlier time points (12 h) followed by a gradual rise to reach maximal levels 240-h post-differentiation.

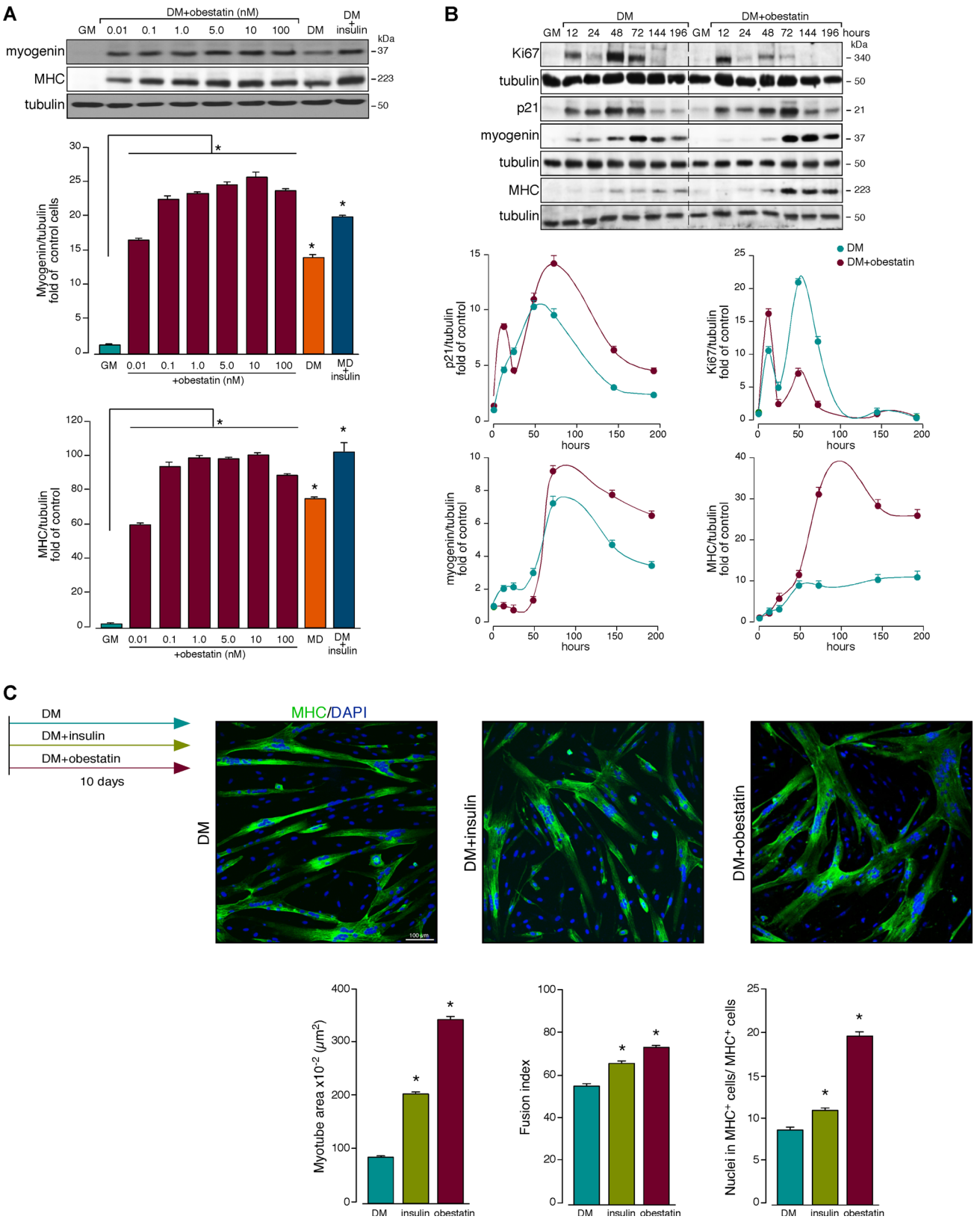
The β-arrestin-scaffolding complex regulates GPR39-EGFR transactivation to control myogenic differentiation

To determine the β-arrestin-dependent signaling mechanism in the regulation of myogenesis, the effect of down-regulation of β-arrestin 1 and β-arrestin 2 by specific siRNA was evaluated during myogenic differentiation. The siRNAs decreased β-arrestin 1 and β-arrestin 2 expressions by 74 ± 1 and 78 ± 2 %, respectively, during a 96-h differentiation period (Fig. 6a). In these conditions, the expression levels of myogenin were inhibited by 55 ± 2 and 78 ± 1 % by β-arrestin 1 and β-arrestin 2 knock-downs, respectively. The MHC expression was also inhibited by 56 ± 3 and 64 ± 1 % with respect to si-control indicating the importance of β-arrestins for the obestatin/GPR39 signaling on the differentiation program. Further, obestatin-stimulated phosphorylation of EGFR [pEGFR(Y1068)], Akt, p38, c-Jun and CAMPKII isoforms were reduced by depletion of β-arrestin 1 or 2 [pEGFR(Y1068): 64 ± 2 and 78 ± 1 %; pAkt(S473): 82 ± 1 and 71 ± 2 %; pp38(T180/Y182): 65 ± 2 and 11 ± 1 %; pc-Jun(S63): 63 ± 1 and 67 ± 2 %; pCAMPKII(T286)γ: 74 ± 1 and 53 ± 2 %; pCAMPKII(T286)β: 64 ± 1 and 45 ± 2 %; pCAMPKII(T286)α: 50 ± 1 and 48 ± 1 %, respectively) but no significant effect was observed on the activation of ERK and JunD (Fig. 6a). Phosphorylation of EGFR at Y845 was not affected by obestatin stimulation (Fig. 6a). Furthermore, the immunoblot analyses revealed a diminution in p21 and p57 expression levels by si-β-arrestin 1 or 2 in obestatin-treated cells (p21: 45 ± 1 and 43 ± 2 %; p57: 52 ± 1 and 47 ± 1 %; Fig. 6a).

β-Arrestin-scaffolding complex regulates EGFR phosphorylation through Src and MMP

Because emerging evidence indicates that β-arrestins organize and scaffold an active signaling complex with Src, leading to EGFR transactivation, we evaluated the recruitment of β-arrestins to GPR39 in myoblast cells overexpressing β-arrestin 2-HA. The overexpressed HA-tagged target protein was co-immunoprecipitated by anti-HA antibody showing the association with the activated form of Src [pSrc(Y416)], GPR39 and β-arrestin 1 (Fig. 6b), indicating that obestatin induced the formation of a GPR39/β-arrestin-1/β-arrestin-2/Src signaling complex, or signalplex.

In myoblast cells, obestain induced rapid Src and EGFR activation (Fig. 6c). The selective non-receptor tyrosine



kinase Src inhibitor PP2 (5 µM, 30 min) inhibited the obestatin-induced Src and EGFR phosphorylation [pSrc(Y416): 100 ± 1 %; pEGFR(Y1068): 136 ± 1 %]. This inhibition was specific, since pretreatment with PP3 (5 µM, 30 min), a negative control for PP2, had no effect on the obestatin-induced EGFR phosphorylation at Y1068

◀**Fig. 4** The obestatin/GPR39 system activates early, mid- and late myogenesis program. **a** Dose–response effect of obestatin (0.01–100 nM) or insulin (1.72 μM) on differentiating KM155C25 cells. Levels of myogenin and MHC were represented as a fold of respective expression in GM. **b** Effect of obestatin (10 nM) on Ki67, p21, myogenin and MHC expression on differentiating KM155C25 cells. **c** *Upper panel*, immunofluorescence detection of MHC and DAPI (objective magnification ×20) in KM155C25 myotube cells under DM (control), DM + insulin (1.72 μM) or DM + obestatin (10 nM) at the 10-day point after stimulation. *Lower panel*, the differentiation grade was evaluated based on the myotube area, the fusion index and the mean number of myonuclei in MHC-positive cells (MHC^{+}). In **a**, **b**, immunoblots are representative of six independent experiments. The data are expressed as the mean ± s.e.m. The *asterisk* (*) denotes $P < 0.05$

residue. The Zn^{2+}-activated metalloproteinases (MMP) inhibitor, GM6001 (1 μM, 1 h), inhibited the obestatin-induced EGFR phosphorylation although did not affect the Src activation [pEGFR(Y1068): 138 ± 1 %; pSrc(Y416): 99 ± 1 %], demonstrating that this process is MMP dependent (Fig. 6b). Furthermore, pSrc(Y416) was inhibited by 46 ± 1 and 50 ± 4 % by β-arrestin 1 and β-arrestin 2 knockdowns (Fig. 6d) indicating the role of both scaffolding proteins in the activation of Src.

The obestatin/GPR39 system regulates fusion and hypertrophy of myotubes

Having shown that obestatin induced hypertrophy with enhanced nuclei addition, we investigated whether this was partly due to enhanced proliferation in the first steps of differentiation, and so whether the administration of obestatin 3 days after the initiation of differentiation also triggered hypertrophy in these cultures (Fig. 7). Furthermore, the myotubes were treated with obestatin after administration of cytosine arabinoside (AraC), an inhibitor of DNA replication to abolish the de novo fusion of the myoblasts. The cells in these experimental conditions stopped proliferating, as evidenced by lack of BrdU incorporation (Fig. 7a). The immunoblot analyses

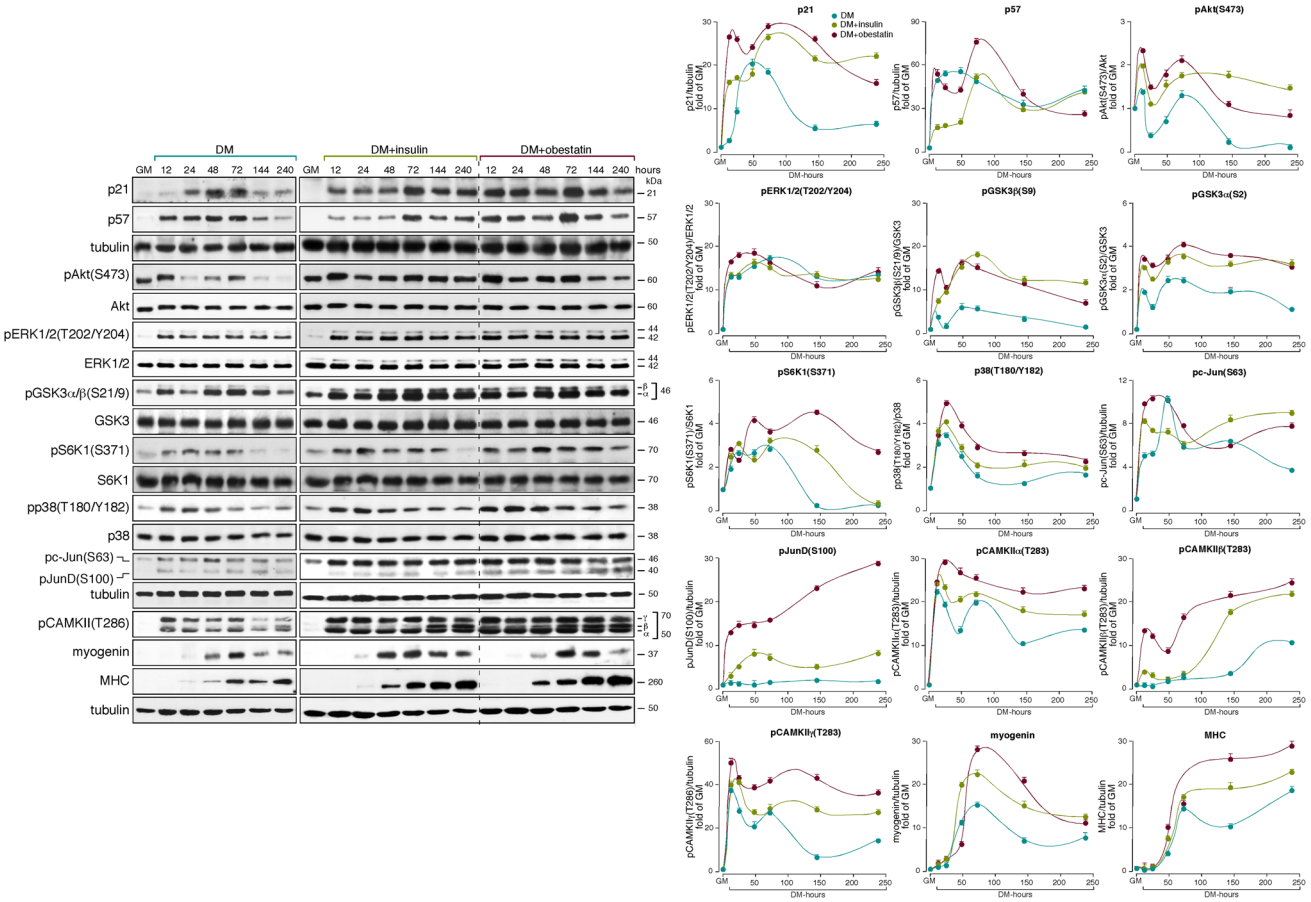


Fig. 5 The obestatin/GPR39-activated intercellular networks involved during myogenic program in human KM155C25 cells. Immunoblot analysis of p21, p57, pAkt(S473), pERK1/2(T202/Y204), pGSK3α/β(S21/9), pS6K1(S6371), pp38(T180/Y182), pc-Jun(S63), pJunD(S100), pCAMKII(T286), myogenin and MHC in the course of myogenesis under DM, DM + insulin (1.72 μM) or DM + obestatin (10 nM). Protein level was expressed as fold of control cells in GM ($n = 5$). Immunoblots are representative of the mean value. Data were expressed as mean ± s.e.m. obtained from intensity scans

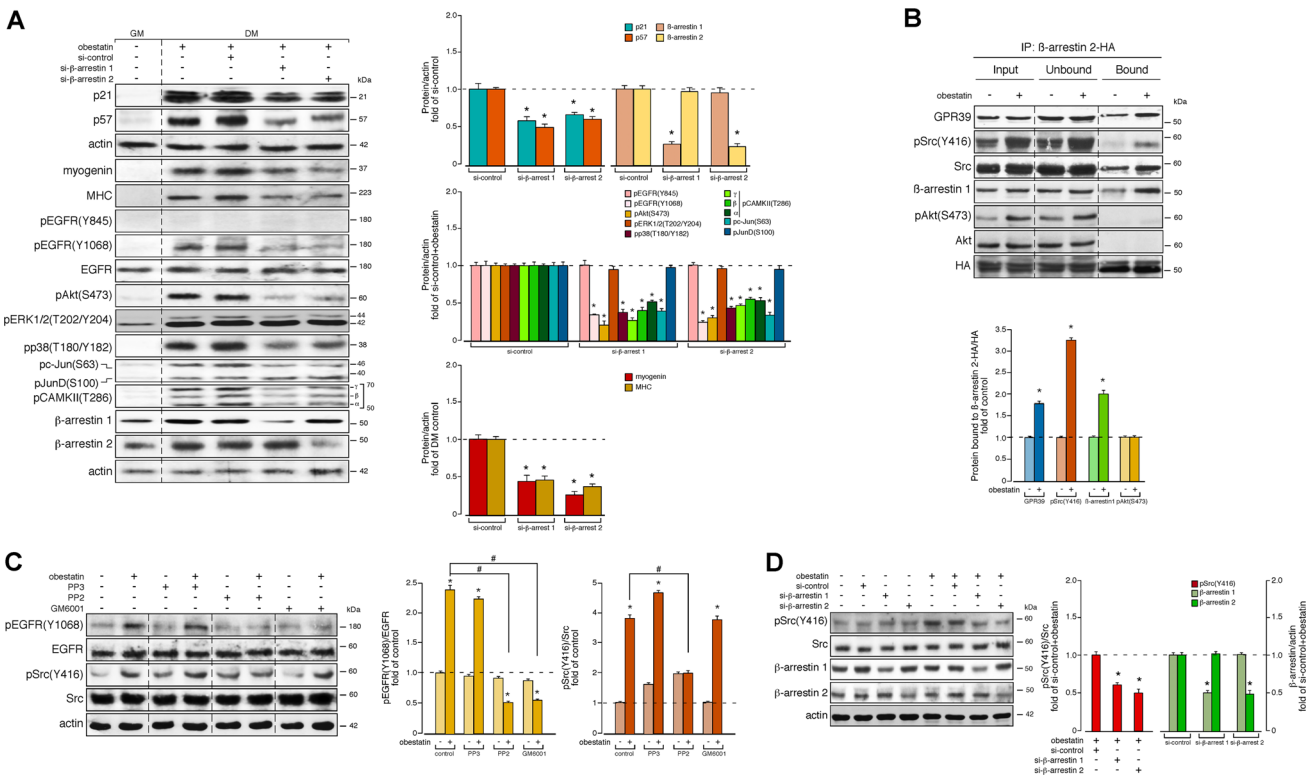


Fig. 6 β-Arrestins determine the mid- and late myogenic stages associated to the obestatin/GPR39 system. **a** After transfection with specific β-arrestin 1 or 2 siRNAs, KM155C25 myoblasts were induced to differentiate with obestatin (10 nM) for 96 h. The effect of β-arrestin 1 or 2 siRNAs was evaluated on obestatin-activated signaling network, pEGFR(Y845), pEGFR(Y1068), pAkt(S473), pERK1/2(T202/Y204), pp38(T180/Y182), pc-Jun(S63), pJunD(S100) and pCAMKII(T286). Furthermore, p21, p57, myogenin and MHC expression was determined in the course of myogenesis. Protein level was expressed as fold of si-control cells. **b** Co-immunoprecipitation assays used to validate the β-arrestin 2-interacting proteins under obestatin (10 nM, 10 min) stimulation. KM155C25 myoblasts were transfected with pcDNA3-β-arrestin 2-HA. The β-arrestin 2-scaffolding complexes were co-immunoprecipitated with anti-HA affinity beads and analyzed by Western blot using anti-GPR39, anti-pSrc(Y416), anti-Src, anti-β-arrestin1, pAkt(S473), anti-Akt and anti-HA antibodies. **c** Effects of the Src inhibitor PP2 and the Zn^{2+}-activated metalloproteinases inhibitor GM6001 on the obestatin-induced EGFR and Src phosphorylations. KM155C25 myoblasts were pretreated with PP2 (5 μM, 30 min), PP3 (5 μM, 30 min) or GM6001 (1 μM, 1 h) previous to obestatin stimulation (10 nM, 10 min). **d** Effect of siRNA depletion of β-arrestins on obestatin-induced Src activation. After transfection with specific β-arrestin 1 or 2 siRNAs, KM155C25 myoblasts were stimulated with obestatin (10 nM) and pSrc(Y416) was analyzed by immunoblot. In **a**–**d**, data show representative blots from one of the three independent experiments. Immunoblots are representative of the mean value. Data were expressed as mean ± s.e.m. obtained from intensity scans. $^*P < 0.05$ versus control values

revealed an increase in myogenin and MHC levels between both groups: control (DM) and obestatin-treated cells (DM for 72 h followed by DM + 10 nM obestatin for 96 h; Fig. 7b). In this case, the myotube areas were ~3.99-fold larger in the obestatin-treated cells (DM for 72 h followed by DM + 10 nM obestatin for 96 h; 2983 ± 58 μm^2) than in the control cells (DM; 746 ± 11 μm^2) at 168 h after differentiation. Under these conditions, the fusion index was increased by ~22 % in the obestatin-treated cells compared to the control cells. The myonuclei per MHC^+ cell were ~4.2-fold higher in obestatin-treated cells than in control cells. Of note, the obestatin-treated cells displayed ~4.6-fold larger myotubes areas under AraC treatment (DM for 72 h followed by DM + AraC (50 μM) + obestatin (10 nM) for 96 h; 2686 ± 38 μm^2; Fig. 7c) than in the control cells (DM; 580 ± 7 μm^2) at 168 h. The myonuclei per MHC^+ cell were ~3.3-fold higher in obestatin-treated cells than in control cells. The immunoblot analyses showed clear increased myogenin and MHC levels between both groups: DM + obestatin and DM + AraC + obestatin (Fig. 7b). Interestingly, the obestatin stimulation under AraC treatment (DM + AraC + obestatin) resulted in markedly elevated levels of pAkt(S473), pS6K1(S371), pCAMPKII(T286) and pJunD(S100) in relation to the AraC treatment alone (DM + AraC). No significant effects were observed on the activation of p38 and c-Jun (Fig. 7b).

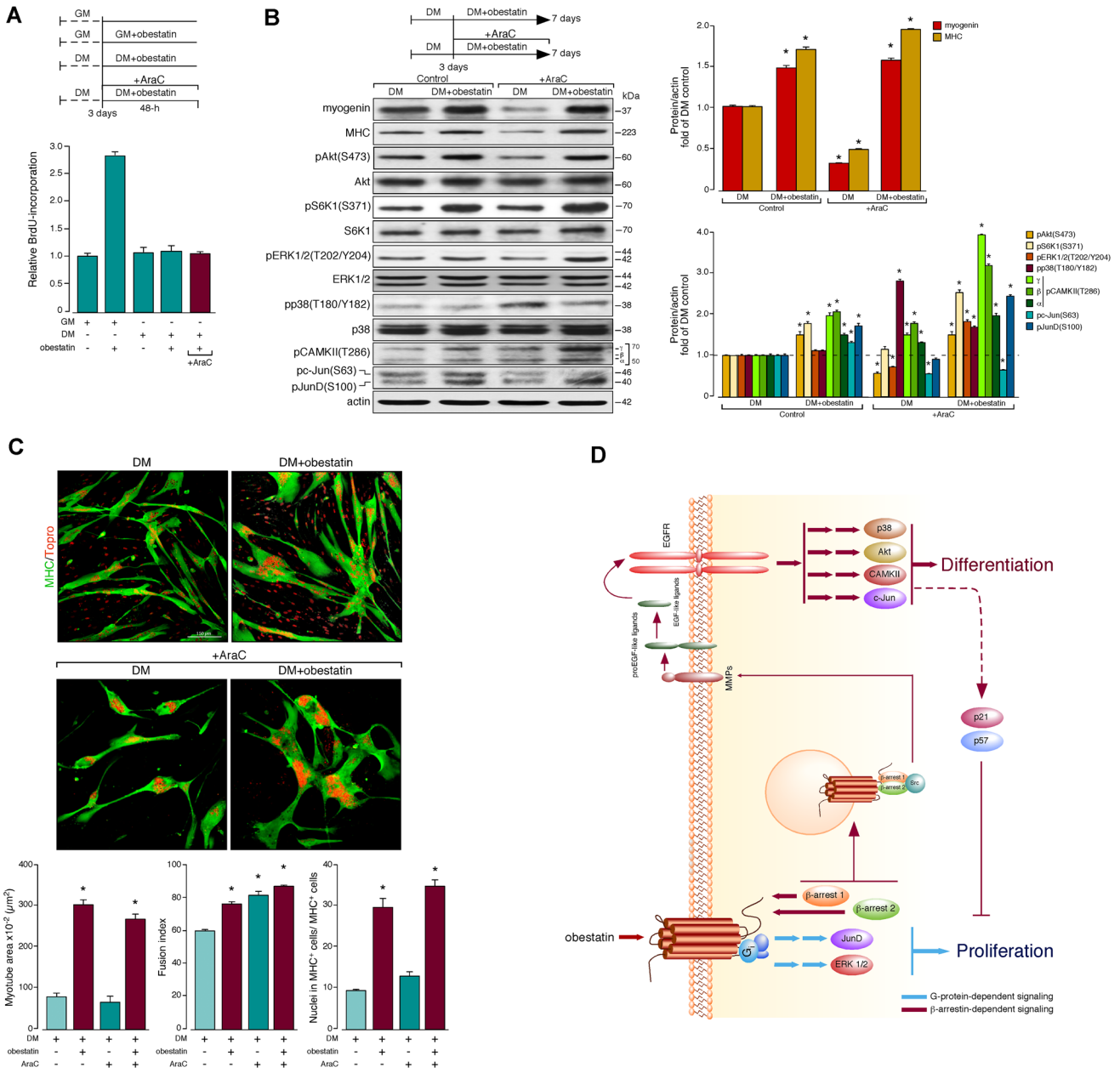


Fig. 7 The hypertrophic response to obestatin is associated with the recruitment and fusion of myoblast and further activation of protein synthesis. **a** Effect of obestatin (10 nM) on KM155C25 myoblast proliferation (48 h) applied at day three in GM, DM or DM + AraC (50 μM). Results were expressed as a fold of the BrdU incorporation relative to control cells maintained in GM. **b** Immunoblot analysis of pAkt(S473), pERK1/2(T202/Y204), pS6K1(S6371), pp38(T180/Y182), pc-Jun(S63), pJunD(S100), pCAMKII(T286), myogenin and MHC expression in KM155C25 myotubes at the 10-day point under DM (control) or DM + obestatin (10 nM) + AraC (50 μM) applied at day three of differentiation. Immunoblots are representative of mean values from each group. **c** Immunofluorescence detection of MHC and Topro in myotube cells at 10-day point after stimulation under DM (control) or DM + obestatin (5 nM) + AraC (50 μM) applied at day three of differentiation. Right panel, the myotube area (μm^2), fusion index and the mean number of nuclei within individual myotubes were evaluated ($n = 10$). Data were expressed as mean ± s.e.m. $^*P < 0.05$ versus control values. **d** Proposed model for the G-protein and β-arrestin-mediated activation of myogenesis through the obestatin/GPR39 system. Obestatin binding to GPR39 receptor results in the activation of $G_{i/o}$ protein-dependent pathway, leading to the activation of ERK1/2 and JunD signaling 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

Discussion

A detailed understanding of the molecular mechanisms that control human myogenesis is a crucial step in identifying new effective therapies. Here, we provide an overall molecular outline showing that obestatin and its receptor GPR39 is a relevant system in modulating the human myogenic program through a coordinate autocrine–paracrine loop. Our results have evidenced that: (1) based on the up-regulation of the obestatin/GPR39 system during myogenesis, and its role in promoting myogenic differentiation and fusion of human myoblasts, obestatin exerts an autocrine–paracrine effect on the skeletal myogenic process; (2) obestatin specifically regulates the different stages of myogenesis, i.e., proliferation, fusion and myotube growth, in a coordinated manner involving both G-protein-dependent and -independent mechanisms linking the activated receptor with distinct sets of accessory and effector proteins, thereby controlling the specificity, efficiency and capacity of the signals; (3) the obestatin-associated mitogenic action was determined by G-protein-dependent activation defining the intricate pathways related to the ERK1/2 and JunD axis; and, (4) the transactivation of EGFR through the β-arrestin signal complex determined the cell cycle exit, the development and the progression of obestatin-dependent differentiation through a kinase hierarchy determined by the Akt, CAMKII, c-Jun and p38 axes. It is well established that β-arrestins play an important role in GPCR desensitization, internalization, and trafficking [36]. Our data add a new component by which these scaffolding proteins arbitrate a crosstalk between the β-arrestin-dependent and G-protein-dependent signaling pathways to direct the progression of the myogenic program. This type of information, together with the validation of myogenic action of the obestatin/GPR39 system presented in this work, highlights the importance of the receptor capacity to regulate G-protein and β-arrestin signaling, and, in this way, to trigger intracellular activity patterns.

From the data presented so far, obestatin acts in an autocrine–paracrine manner in the myogenic program in human. Obestatin was preferentially expressed by myotubes, whereas GPR39 was equally expressed in both myoblast and myotube cells. Expression of both obestatin and GPR39 was coordinately up-regulated during the early stages of myogenesis, and their levels remained sustained throughout terminal differentiation. Furthermore, preproghrelin knockdown experiments uncovered obestatin contribution to human myogenesis, which reduced its myogenic potential through down-regulation of cyclin-dependent kinase inhibitors, p21 and p57, myogenic regulator factors (Myf5 and myogenin) and MHC. The effect on the activation and proliferation of myoblasts, with the resulting growth arrest and initiation of myogenesis reinforce our hypothesis. Further support is demonstrated by the effect GPR39 knockdown on the activation of obestatin action and myogenic differentiation. The preproghrelin up-regulation and, as a consequence, the increase of obestatin biosynthesis and secretion by myotubes, together with its weak expression in myoblasts, demonstrated that obestatin exerted a autocrine–paracrine role on myoblasts via a GPR39-dependent mechanism. At this point, it is possible to speculate about the role of obestatin as a limiting factor for the activation of the myogenic program. This hypothesis is supported by the temporal expression of obestatin linked to cell proliferation and differentiation. Thus, the loss of obestatin might limit the effective progression of muscle precursor cells along the myogenic lineage pathway. These findings emphasize the remarkable role of the obestatin/GPR39 system into the myogenic program.

Myogenesis is a multistep process by which myoblasts first proliferate, then withdraw from the cell cycle, and finally differentiate and fuse to form the multinucleated mature myotubes. This dynamic process is controlled by intracellular signaling pathways in response to extracellular cues that determine the association of the myogenic effector transcription factors to E boxes on muscle loci and their transcriptional activities [1, 2]. This study delineated MAPKs [ERK1/2, c-Jun N-terminal kinases (JNK1-3), and different p38 isoforms (α/β/γ)], Akt, p53, and CAMKII signaling pathways activated downstream of the obestatin/GPR39 system. These kinase hierarchies are decoded by the myogenic effectors that execute different phases of the differentiation process in response to the obestatin/GPR39. During the proliferation stage, an enhanced expression of Ki67, a proliferating cell marker, was observed. A signaling pathway that plays a fundamental role in cellular proliferation, migration, and fusion involves ERK1/2 [37, 38]. In the context of obestatin-induced activity, ERK activity initially increased, consistent with the initial proliferation of myoblasts, and then is followed by a decrease, which is necessary for differentiating myoblasts to overcome its inhibitory effect. Indeed, ERK was shown to inhibit differentiation by preventing the nuclear accumulation of members of the myocyte enhancer factor-2 [39], and the expression of certain myogenic factors, including MyoD [40] and the CDK inhibitor p21 [41]. Our results from phospho-protein array showed increased levels of pRSK1, which lies downstream of the Ras/MAPK/ERK signaling cascade exerting a feedback loop that limits ERK1/2 activation. A number of RSK functions can be deduced from the nature of the RSK substrates, ranging from the regulation of transcription, translation and protein stability to the control of cell survival, cell motility, cell

growth and proliferation [42, 43]. The withdrawal from the cell cycle was evidenced by the expression of p57 and p21 in the present work, which trigger and stabilize the cell cycle exit necessary for the early steps of differentiation [44, 45]. Exposure of myoblasts to obestatin triggered the activation of JNK (JNK1, JNK2 and JNK3), which can bind directly to and translate its activity into the phosphorylation of its downstream substrate c-Jun on S63 and S73 [46, 47]. Analogous to the ERK activity, JNK/c-Jun pathway increased in the early step of the differentiation consistent with a role in the cell cycle progression [47–49]. Indeed, the growth-promoting activity of c-Jun is mediated by the up-regulation of positive cell cycle regulators, the induction of cyclin D1 transcription and the repression of p21 [50]. In contrast to c-Jun activity, JunD phosphorylation showed a progressive increase during differentiation in response to obestatin stimulation. JunD is a constitutively expressed member of the activating protein-1 transcription complex (AP-1) family in contrast to what is generally observed for c-Jun and c-fos genes, which are considered immediate early genes. Phosphorylation of JunD at S100 results in an increase of its transcriptional activity, and this activation requires ERK1/2 [51]. Although it is not easy to establish a specific role for JunD in myogenesis, it was clearly described as a negative regulator of the cell growth in other systems [52]. In this sense, it is important to note that as part of the AP-1 transcription complex, c-Jun and JunD determine differential gene regulation based on its composition [53–55]. Thus, the differential regulation of the Jun proteins coordinates distinct responses determining the cell fate [50]. This denotes that AP-1 complex in differentiating muscle cells is distinct from that in proliferating myoblasts [54, 56]. p38 pathway is a crucial regulator that orchestrates sequential events in the myogenic pathway, including cell cycle arrest and differentiation of myoblasts [57]. p38 kinase activity increased over the course of differentiation under obestatin treatment, a fact that fits well with the established role of this kinase in the cell cycle arrest by antagonizing the JNK/c-Jun pathway [47] and in differentiation through the control of muscle-specific gene expression [57]. From phospho-kinase analysis, MKK3, but not MKK6, appears as candidate to activate p38 through phosphorylation at T180 and Y182 [58, 59]. In addition, heat-shock protein 27 (Hsp27), a p38 substrate via MK2 [59], was phosphorylated in response to obestatin. pHsp27 regulates the actin filaments dynamics in cytoskeleton organization [60, 61]. This protein was showed to inhibit the MEK/ERK signaling pathway by a mechanism involving both c-Raf activity attenuation and stimulation of MAPK phosphatase-1 (MKP1) through p38 MAPK leading to significant reduction of cyclin D1 levels and subsequent cell cycle arrest [62]. Moreover, Hsp27 is known to interact with p53, regulating its transcriptional activity [63], therefore having an effect in cell cycle regulation. Last but not least, pHsp27 negatively regulates apoptosis during myogenic differentiation by inhibiting apoptosis effectors [64].

Obestatin activated Akt pathway during myogenesis differentiation. Akt has emerged as a critical signaling node within the myogenic program that control a broad array of responses, ranging from primarily metabolic functions such as glucose transport, glycolysis, glycogen synthesis, and the suppression of gluconeogenesis to cell cycle progression, increase in protein synthesis, as well as myotube hypertrophy, and apoptosis suppression [65]. These functions are related to different Akt isoforms. Akt1 was described to be essential in early differentiation leading to cell cycle progression [66]. By contrast, Akt2 triggers myoblast cell cycle exit and drives differentiation determining myotube maturation [66]. Two major downstream branches of the Akt pathway were activated by obestatin: the S6K1 and GSK3 pathways. Both of them control protein synthesis with relevant role in muscle hypertrophy [67, 68]. In the case of CAMKII, its signaling prevents the formation of MEF2–HDAC complexes by phosphorylation of HDAC4/HDAC5, promoting a nuclear export of these transcriptional repressors and a subsequent increase in MEF2 activity on muscle-specific genes such as MyoD and myogenin [69]. This pathway thus regulates gene expression, leading to hypertrophic growth and promotion of the slow versus fast fiber program [70, 71]. Phospho-protein array showed increased pp53 levels after obestatin stimulation. p53 activation acts on the differentiation program by activating both cell cycle exit via p21 and the muscle-specific genes [72]. In addition, CREB acts downstream of obestatin signaling as revealed by the phospho-protein array. CREB stimulates muscle differentiation by increasing the expression of the myogenic program [73, 74]. These findings emphasize the importance of a tight and intricate regulation of cellular processes that are essential for myogenesis by more than one pathway. Thus, the signaling nodes associated with the obestatin/GPR39 system act at the chromatin level defining the mechanism for regulation between mitogenic and myogenic signals to direct the transition from quiescence to terminal differentiation. In this regard, obestatin shows functional similarity with the insulin and insulin-like growth factors (IGF-I and IGF-II) pathways since such factors have the capacity to promote both proliferation and differentiation of muscle cells [75, 76].

A somewhat surprising, but also one of the most interesting finding in the obestatin/GPR39-mediated signaling was the observation that the diverse biological processes of myogenesis are specifically orchestrated by G-protein- and β-arrestin-dependent mechanisms. In this study, we demonstrated that obestatin activates the first

proliferation step of the myogenic process by G-protein-dependent mechanisms. The lack of effect of β-arrestin knockdown on myoblast proliferation rules out these scaffold proteins as upstream signals. This is further supported by the fact the suppression of β-arrestins by siRNAs did not blunt the obestatin-stimulated ERK1/2 phosphorylation, a critical node for myoblast proliferation. This is further in agreement with the lack of effect of β-arrestin knockdown on JunD activation that requires ERK1/2 activity for its phosphorylation [51]. On the other hand, we identified a GPR39-driven myogenic signal pathway, which is dependent on the scaffold protein β-arrestins as a crucial point in the activation of EGFR signaling and in the induction of a myogenic phenotype. We showed that the GPR39-dependent EGFR transactivation required activation of Src in a β-arrestin-dependent fashion. Thus, upon obestatin stimulus, β-arrestin 1 and 2 are recruited to the membrane, where they functionally interact with GPR39 and the downstream effector Src leading to Src activation and signalplex formation to EGFR transactivation through the activation of MMPs. MMPs, such as the ADAM (disintegrin and metalloprotease) family members, are able to cleave EGF-like ligands promoting ligand shedding into the extracellular space to bind to the EGFR and other EGFR family members, thereby encouraging EGFR dimerization and activation [77–79]. Indeed, obestatin induced autophosphorylation of the cytoplasmic Y992, Y1045 and Y1068 at COOH-terminal tail, residues that serve as docking sites for intracellular signaling proteins [80], ruling out a direct Src-dependent phosphorylation through β-arrestin/Src complex described in other systems [81] since Y845 phosphorylation of EGFR did not occur under obestatin stimulation. It is important to note that the human genome possesses about 40 ADAMs, more than 12 EGF ligands and four main isoforms of EGFR, and so it is expected that various combinations and permutations of these proteins are used in a cell-specific manner [80, 82, 83]. The obestatin-dependent β-arrestin recruitment acts as switch in the GPR39/EGFR crosstalk signaling to activate JNK/c-Jun, CAMKII, Akt and p38 pathways to endorse the mid- to late stages of differentiation. In fact, the activation of β-arrestin-dependent signalosome promoted the expression of myogenic regulator factors (i.e., myogenin) and MHC. Thus, GPR39/β-arrestins/Src complex might function specifically to control EGFR from being a mitogenic to myogenic stimulus, by conferring the EGFR the ability to activate signaling pathways that conform to the unique myogenic signature. This mechanism would allow the triggered EGFR to deliver a cell cycle exit signal to promote the differentiation to form multinucleated mature myotubes (Fig. 7d). Indeed, p21 and p57 expression was determined through the β-arrestin-dependent pathway. Thus, a clear specificity is associated with G-protein-, and β-arrestin-dependent mechanisms: pro-mitogenic and pro-myogenic, respectively. Such mode of action thus uncovers the interesting possibility to direct cellular signaling with specificity through agonists, i.e., biased agonists that selectively stabilize only a subset of GPR39 conformations, therefore activating particular signaling mechanisms. The finding presented so far not only suggests a bidirectional crosstalk between both receptors but also provides the first functional activity for β-arrestins in myogenesis leading to new avenues for therapeutic approach by targeting specific signaling arms of GPCRs involved in myogenesis. In recent years, the concept that ligands can differentially modulate the activity of the various signaling pathways engaged by a single receptor has gained wide acceptance and is now known as functional selectivity or ligand-biased signaling [84]. A common fact observed in biased agonism is that between G-protein-dependent and arrestin-dependent signaling, although there are likely to be many other variants, such as bias in GPCR coupling to different G-protein subtypes [85]. A potentially important consequence of functional selectivity at GPCR is the different physiological responses that could be triggered by biased ligands. Indeed, the recent ongoing clinical development of several biased agonists for a variety of indications proves to be a suitable tool to develop safer and more effective medications, thus opening new avenues for drug design.

Recruitment and fusion of myoblasts into multinucleated myotubes, and myotube hypertrophy, are required for developmental myogenesis and all contribute to postnatal maintenance of muscle mass in both physiological and pathological conditions. We report here that obestatin favors the recruitment and fusion of myoblasts into myotubes, as indicated by the increased fusion index and the number of myonuclei in MHC^{+} cells, with increased cell size or hypertrophic effect. To eliminate the possibility that the hypertrophic effect of obestatin could be due to an enhanced proliferation of myoblasts at early stages of differentiation, thus providing more differentiating cells at later stages, AraC was added to the medium of 3-day-old myotubes for 4 days. Under these conditions, obestatin induced both myotube hypertrophy and myoblast fusion, demonstrating that the formation of hypernucleated myotubes with increased size was not only a consequence of an increased number of undifferentiated myoblasts available for the fusion process. Functional screening of intracellular targets showed significant increase of obestatin-induced Akt and CAMKII activity parallel to myogenin and MHC expression, signaling nodes relevant to muscle hypertrophy [68]. Thus, the obestatin/GPR39 system exerts a role on protein synthesis regulating myotube hypertrophy, concomitant to the recruitment and fusion of myoblasts, with

each other or with existing myotubes, resulting in myonuclei addition. Protein synthesis may thus be a feature of more advanced hypertrophy.

In conclusion, these findings not only demonstrate the action of the obestatin/GPR39 system as an autocrine/paracrine signal to activate human myoblast expansion in combination with myotube growth but also unveil a molecular mechanism whereby the β-arrestins function as a link between GPR39 and EGFR to determine specific steps of myogenic process. The key observation that the receptor-mediated response can differ across the multiple signal transduction pathways, e.g., G-protein and β-arrestins, might have significant implications for the design of a novel class of drugs with enhanced efficacy on specific therapeutic pathways. Thus, our study exposes the potential therapeutic target of the obestatin/GPR39 system for a variety of conditions where muscle regeneration or hypertrophy may be desirable.

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