



Repositorio Institucional de la Universidad Autónoma de Madrid

<https://repositorio.uam.es>

Esta es la **versión de autor** del artículo publicado en:

This is an **author produced version** of a paper published in:

Carcinogenesis 39.3 (2018): 447-457

DOI: <http://doi.org/10.1093/carcin/bqx146>

Copyright: © 2017 The Author(s)

El acceso a la versión del editor puede requerir la suscripción del recurso

Access to the published version may require subscription

Title: In1-ghrelin splicing variant is associated with reduced disease-free survival of breast cancer patients and increases malignancy of breast cancer cells lines.

Authors: David Rincón-Fernández^{1,2,3,4}; Michael D. Culler⁵; Natia Tsomaia⁵; Gema Moreno-Bueno⁶; Raúl M. Luque^{1,2,3,4,*}; Manuel D. Gahete^{1,2,3,4,*}; Justo P. Castaño^{1,2,3,4,*}

Affiliations: ¹Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC); Córdoba, Spain.

²Department of Cell Biology, Physiology and Immunology, Universidad de Córdoba; Córdoba, Spain.

³Hospital Universitario Reina Sofía; Córdoba, Spain. ⁴CIBER Fisiopatología de la Obesidad y Nutrición

(CIBERObn); Córdoba, Spain. ⁵Ipsen Bioscience, Inc., Ipsen, Cambridge, MA, USA. ⁶Department of Biochemistry, Universidad Autónoma de Madrid (UAM), Instituto de Investigaciones Biomédicas "Alberto Sols" (CSIC-UAM), IdiPAZ & MD Anderson Internacional Foundation, Madrid, Spain.

* These authors co-directed the study and should be considered co-senior authors

Keywords: In1-ghrelin, splicing variant, breast cancer, malignancy, survival.

Funding: BIO-0139, CTS-1406, PI-639-2012, PI-0541-2013 (Junta de Andalucía), BFU2013-43282-R, BFU2016-80360-R (MINECO), PI13-00651, PI16/00264 (Proyectos de Investigación en Salud FIS, funded by Instituto de Salud Carlos III), GETNE Grant 2014, Merck Serono Grant 2013 and CIBERobn (to RML and JPC); PI13/00132, RETICC RD12/0036/0007, CIBERonc and S2010/BMD-2303 (to GMB). CIBER is an initiative of Instituto de Salud Carlos III, Ministerio de Sanidad, Servicios Sociales e Igualdad, Spain.

Address correspondence to: Justo P. Castaño, Manuel D. Gahete or Raúl M. Luque. Instituto Maimónides de Investigación Biomédica de Córdoba. Edificio IMIBIC. Avda. Menéndez Pidal s/n. 14004 Córdoba, Spain. E-mail: justo@uco.es, bc2gaorm@uco.es, raul.luque@uco.es

Conflict of interest disclosure: The authors declare no potential conflicts of interest.

© The Author(s) 2017. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com.

Abstract

Ghrelin gene generates several variants that regulate multiple pathophysiological functions, including tumor-related processes. In1-ghrelin is a splicing variant that was previously shown to be overexpressed in breast cancer (BCa), where it correlated with proliferation markers; however, its possible association with clinical outcome of BCa patients and underlying mechanisms are still unknown. To address this issue, expression levels and clinical associations of In1-ghrelin were analysed in a cohort of 117 BCa samples. Additionally, a battery of cellular and molecular assays were implemented using two BCa cell lines (MCF-7 and MDA-MB-231), wherein the role of In1-ghrelin on proliferation, migration, dedifferentiation and signaling pathways was explored. The results generated revealed that high expression of In1-ghrelin in BCa samples was associated with lymph node metastasis and reduced disease-free survival. Indeed, In1-ghrelin overexpression stimulated proliferation and migration in MCF-7 and MDA-MB-231 cells. Similar results were found by treating MDA-MB-231 and MCF-7 with In1-ghrelin-derived peptides. Conversely, In1-ghrelin silencing decreased proliferation and migration capacities of MDA-MB-231. Furthermore, In1-ghrelin (but not ghrelin) overexpression increased the capacity to form mammospheres in both cell lines. These effects could be associated with activation of MAPK-ERK, Jag1/Notch, Wnt/ β -catenin, and/or TGF- β 1 pathways. Altogether, our data indicate that In1-ghrelin could play relevant functional roles in the regulation of BCa development and progression, and may provide insights to identify novel biomarkers and new therapeutic approaches for this pathology.

Implication: In1-ghrelin could represent a novel biomarker and provide novel therapeutic opportunities in BCa as it was associated with lymph node metastasis and reduced disease-free survival.

Introduction

Breast cancer (BCa) is the most frequently diagnosed malignancy in women [1]. Although mortality has been diminishing over the last years, BCa is still the second leading-cause of cancer-related deaths in women [1]. The search for common molecular markers among the vast genetic heterogeneity in the different BCa types [2], which could facilitate the discovery of new drug targets and help to develop more efficient diagnostic, prognostic and therapeutic markers, has led to the identification of aberrant alternative splicing events as a common hallmark in cancer development and progression [3, 4]. Indeed, appearance and/or overexpression of certain splicing variants in tumor cells has been associated with increased drug resistance [5], escape from the immune system [6] or increased malignancy features and metastatic potential [3].

In this scenario, ghrelin gene has emerged as a complex and tightly regulated system, relevant in tumor pathologies, and with potential value as molecular marker and/or therapeutic target [7]. Thus, besides the known actions of native ghrelin as a truly pleiotropic hormone, the ghrelin gene itself comprises an intricate system [8], which includes the existence of several splicing variants [9], the proteolytic processing of pre-pro-peptides to yield different hormones [10], or specific post-transcriptional modification, such as acylation, that generates the best-known biologically-active peptide, acyl-ghrelin. Interestingly, the presence of acyl-ghrelin and its canonical receptor GHS-R1a has been reported in several endocrine and non-endocrine tumors [11], where their function varies from modulation of growth hormone expression and secretion in pituitary tumors, to proliferative effects on prostate [12], breast [13] or follicular thyroid carcinoma [14] cell lines. In spite of these observations, the actual significance of ghrelin system in tumor pathologies is poorly understood, likely due to controversial results from studies showing that ghrelin may be a good prognostic marker in BCa [15], but may inhibit proliferation [16] or display a biphasic effect in prostate cancer cell lines [17].

Additionally, elucidation of the precise biological significance of the ghrelin system has been hampered by the intrinsic complexity of the ghrelin peptide/receptor family [8]. Indeed, over the last years, several

splicing variants of the ghrelin and ghrelin receptor genes have been identified and associated with several human pathologies [8]. Among the ghrelin-gene splicing products, special attention has been focused on the In1-ghrelin variant [18], a recently discovered splicing-derived variant generated by the retention of the intron 1 [18]. The In1-ghrelin variant is overexpressed in different tumor pathologies, such as BCa [18], pituitary [19] and neuroendocrine tumors [20]. Moreover, its expression seems to be associated with tumor malignancy, since In1-ghrelin mRNA expression levels correlate with proliferation markers, like Ki-67 and cyclin D3 in BCa [18], and In1-ghrelin overexpression and/or treatment induces higher cell viability and inhibition of apoptotic processes [19, 20], and also increases hormone hypersecretion from pituitary and neuroendocrine tumors [19, 20]. Altogether, these data support a plausible relevance of the In1-ghrelin splicing variant in tumor processes, and suggest the necessity to implement further studies to clarify the specific functions and putative clinical implications of this splicing variant in relevant tumor pathologies, such as BCa. Accordingly, the aim of this study was to explore the putative clinical implications of the expression of this variant in samples from a well-characterized human BCa cohort as well as to determine the functional and mechanistic implications of In1-ghrelin on two representative BCa cell lines.

Material and methods

Human samples

A total of 117 infiltrating grade 3 ductal (IDC) BCa samples acquired from the archives of the Pathology Department of MD Anderson Cancer Center (Madrid, Spain) and previously described in [21, 22] were included. Patients underwent surgery between 2003 and 2004 with a mean age at surgery of 54.9years (range, 27-79years). According to the TNM Classification staging, 48 of the tumors were stage I, 34 were stage II, and 35 were stage III-IV. Relevant clinical data of the patients are provided in Supplemental Table 1. This study was performed following standard ethical procedures of the Spanish regulation (Ley de Investigación Orgánica Biomédica, 14 July 2007) and was approved by the ethic committee of MD Anderson Cancer Center, Madrid, Spain.

Cell lines

Invasive ER-negative (MDA-MB-231) and non-invasive ER-positive (MCF-7) BCa cell lines (ATCC, Barcelona, Spain) were validated by analysis of STRs (GenePrint® 10 System, Promega, Barcelona, Spain) and checked for mycoplasma contamination by PCR [23]. Cells were cultured in DMEM with 4,5g glucose (Lonza, Basel, Switzerland), 2mM L-Glutamine (Thermo Scientific, Wilmington, NC, USA), 10% FBS (Sigma, Barcelona, Spain) and 0,2% gentamicin/amphotericin (Thermo Scientific) and maintained at 37°C and 5% CO₂.

Plasmids, siRNAs and transfection

The expression vector pCDNA3.1 (Thermo Scientific) was used for cloning and overexpression. Details regarding In1-ghrelin cloning have been previously reported [18]; while ghrelin sequence was purchased from PlasmidID (Harvard Medical School, Boston, MA) and subcloned into pCDNA3.1. Cells were transfected using Lipofectamine-2000 (Gibco, Barcelona, Spain) following manufacturer's instructions and stable-transfected cells were selected by addition of geneticin (1%; Gibco). To generate monoclonal cell lines, single clones were selected by limiting dilutions and validated by quantitative

real-time PCR (qPCR). Stably-transfected cells with empty pCDNA3.1 (control) were used as a negative control.

In1-ghrelin knock-down on MDA-MB-231 cells, which exhibited a basal, appreciable endogenous expression of In1-ghrelin, was carried out by using two custom-designed In1-ghrelin specific siRNAs [siRNA-1 (5'-GAGTCCTAAACAGACTGTT-3') already published [19] and siRNA-2 (5'-CACUGUUUCUGGAAGGACATT-3')] and compared with cells treated with a commercial control-siRNA (Silencer Select Negative Control No.1 siRNA, Thermo Scientific). Specifically, cells were transfected with these siRNAs (100nM) using Lipofectamine RNAiMAX (Gibco) and following manufacturer's instructions. siRNA effectiveness was validated by qPCR. All studies were developed two days after siRNA transfection, in identical condition to plasmid-transfected cells.

Peptides

Human acylated-ghrelin was commercially available (SC1357, PolyPeptide Laboratories, Limhamn, Sweden), while In1-ghrelin-derived acylated peptides were synthesized in collaboration with IPSEN Bioscience (Cambridge, MA, USA). Although the endogenous In1-ghrelin derived mature peptides have not yet been purified, pre-pro-In1-ghrelin precursor exhibits well-recognized target sites for protein-convertases, suggesting a proteolytic processing. As previously reported, In1-ghrelin precursor processing could generate 19-aa or 40-aa long peptides (named In1-19: GSSFLSPEHQVRVQVRPPHK and In1-40: GSSFLSPEHQVRVQVRPPHKAPHVVPALPLSNQLCDLEQQR), which share with native ghrelin the initial 13-aa, including the acylation site at Ser3 (for details, see Supplemental Figure 2). Thus, In1-19 and In1-40 peptides were synthesized as their acylated forms [19]. Paclitaxel and IGF1 were purchased from Sigma-Aldrich.

RNA isolation and qPCR

RNA extraction, quantification and reverse-transcription, as well as the development, validation and application of qPCR to measure the expression levels of different human transcripts, have been previously reported by our group [18, 24-27]. Briefly, total RNA was extracted from cell lines and

paraffin-embedded BCa samples with Trizol (Thermo Scientific) following manufacturer's protocol and subsequently treated with DNase (Promega). Total RNA concentration and purity was assessed using Nanodrop-2000 spectrophotometer (Thermo Scientific), and subsequently retro-transcribed using random hexamer primers and cDNA First Strand Synthesis kit (Thermo Scientific). Complementary DNA derived from cell lines and from paraffin-embedded BCa samples were amplified by qPCR using Brilliant III SYBR Green Master Mix in the Stratagene Mx3000p instrument (Agilent, La Jolla, CA, USA) [24-26]. Thermal profile consisted of an initial step at 95°C for 3 minutes, followed by 40 cycles of denaturation (95°C for 30 seconds) and annealing/extension (60°C for 30 seconds); and finally, a dissociation cycle to verify that only one product was amplified. Primer details are presented in Supplemental Table 2. The expression level (copy-number) of each transcript was adjusted by the most appropriate housekeeping gene(s) selected through Genorm software analysis. Specifically, BCa samples were normalized using ACTB expression levels, while cell lines data were normalized by a normalization factor (NF) calculated from HPRT and GAPDH expression levels.

In vitro cell proliferation

Cell proliferation was evaluated in stably transfected cells using Alamar-Blue reagent (Thermo Scientific) as previously reported [18, 28]. Briefly, 3000 cells/well were plated in 96-wells plates and the day of measurement, cells were incubated for 4 hours in 10% Alamar-Blue blue/serum free-DMEM and then, Alamar-Blue reduction was measured in a FlexStation system plate reader, exciting at 560 nm and reading at 590 nm. Alternatively, we implemented proliferation measurements by MTT assay (Sigma-Aldrich). Briefly, 3000 cells/well were plated in 96-wells plates and the day of measurement, 100µl of MTT diluted in d-PBS (Sigma-Aldrich) were added to the cells and then incubated 3 hours at 37°C. Subsequently, cells were detached with lysis buffer (10% SDS, 0,56% glacial acetic acid in DMSO) and absorbance measured using the FlexStation system plate reader, at 570nm. In all instances, cells were plated per quadruplicate and all assays were repeated a minimum of five times. Results are expressed as percentage vs. control cells.

Migration Assay

The ability of cells to migrate in response to In1-ghrelin or ghrelin overexpression or treatment was evaluated by wound healing technique as previously reported [28]. Briefly, cells were plated at sub-confluence in 12-wells plates. The wound was made using a 200µl sterile pipette tip on confluent cells. Then, cells were incubated for 24h in medium without FBS to prevent cell proliferation. Wound healing was calculated as the area recovered after 24hours. At least, four experiments were performed in triplicates on independent days, in which three-four random pictures along the wound were acquired. Results are expressed as percentage vs. control. In treatment assays, ghrelin, In1-ghrelin peptides, IGF and paclitaxel (positive and negative controls) were added with the culture medium at time 0.

Mammospheres formation assays

The ability to form mammospheres in response to In1-ghrelin or ghrelin overexpression or treatment was evaluated by mammospheres formation assay [29]. Briefly, 6.000-10.000 cells were seeded in pHEMA (Sigma-Aldrich) pretreated 6-wells plates using phenol red-free DMEM/F12 medium (Gibco) supplemented with rEGF (20ng/ml; Sigma-Aldrich) and B27 supplement without vitamin-A (Thermo Scientific). Then, cells were incubated for 7 days at 37°C and 5%CO₂ and, subsequently, mammospheres were counted. In treatment assays, peptide was added to the medium at day zero and refreshed each two days. To analyze mammospheres size, they were collected, disaggregated with trypsin/EDTA (Gibco), passed through a 25G needle and finally the number of individual cells was counted using a Neubauer chamber. Average number of cells was adjusted by the number of mammospheres as a representation of the size of each mammospheres.

Western Blot

Cells were cultured to subconfluence in 6-well plates and lysed in pre-warmed SDS-DTT buffer (62,5mM Tris-HCl, 2% SDS, 20% glicerol, 100mM DTT) followed by sonication for 10 sec and boiling for 5min at 95° C. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline/0.05% Tween

20 and incubated with the primary antibodies for ERK1/2 (Santa Cruz, Dallas, TX, USA), phospho-ERK1/2, phospho-Akt, or Akt (Cell Signaling, Beverly, MA) and the appropriate secondary antibody (Anti Rabbit IgG HRP-linked, Cell Signaling). Proteins were developed using ECL-2 (GE Healthcare, Madrid, Spain) following manufacturer's instructions. Densitometric analysis of the bands was carried out with ImageJ software. The relative phospho-ERK and phospho-Akt values were obtained from normalization of phospho-ERK1/2 or phospho-Akt values against the total ERK1/2 or Akt values, respectively.

Statistical analysis

For the analysis of human breast carcinoma data, samples were categorized as low, moderate and high In1-ghrelin levels according to quartile In1-ghrelin expression levels (0-50% as low, 50-75% as moderate and 75-100% as high). Significant correlations between categorized In1-ghrelin mRNA expression, presence of metastasis and disease-free survival were studied using Chi-square and Long-rank-p-value methods. For *in vitro* experiments, data are expressed as mean \pm SEM obtained. Data were evaluated for heterogeneity of variance using the Kolmogorov–Smirnov test. Statistical analysis was carried out using one-way ANOVA followed by Dunnett's test. Statistical analyses were carried out with GraphPad Prism 6 (La Jolla, CA, USA) and SPSS 17.0 (IBM). P-values smaller than 0.05 were considered statistically significant.

Results

In1-ghrelin expression correlated with lymph node metastasis and lower disease-free survival in BCa patients

To ascertain the putative clinical significance of In1-ghrelin in BCa patients, we evaluated the expression of In1-ghrelin in a cohort of 117 BCa samples, and explored its association with malignancy-associated features, such as lymph node metastasis and disease-free survival. To accomplish this, samples were categorized as low, moderate and high In1-ghrelin expression according to quartile In1-ghrelin expression levels (0-50% as low, 50-75% as moderate and 75-100% as high) (Figure 1A-B). This analysis showed a remarkable association ($p=0.001$) between In1-ghrelin expression and lymph node metastasis, wherein patients with high In1-ghrelin expression levels presented more lymph node metastasis (Figure 1C). Moreover, BCa patients with high expression of In1-ghrelin presented significantly ($p<0.001$) lower disease-free survival than those with low or moderate In1-ghrelin expression (Figure 1D), while no significant differences were observed between patients with low or moderate In1-ghrelin expression (Figure 1D). Remarkably, the same analysis implemented with the canonical variant ghrelin, or with the ratio between In1-ghrelin and ghrelin, did not show any significant association with lymph node metastasis or disease-free survival (data not shown).

In1-ghrelin increased cell proliferation and migration.

In1-ghrelin expression levels were low in non-invasive MCF-7 cell line but considerably higher in invasive MDA-MB-231; while ghrelin was almost undetectable in both cell lines (Supplemental Table 3). As expected, stably-transfected In1-ghrelin cells exhibited elevated levels of In1-ghrelin by qPCR, with no changes in ghrelin expression; whereas, stably-transfected ghrelin cells exhibited elevated levels of ghrelin without changes in In1-ghrelin expression (Figure 2A).

In1-ghrelin overexpressing MDA-MB-231 and MCF-7 cells exhibited a significantly increased cell proliferation rate compared to control cells by Alamar-Blue assay (MDA-MB-231: 113% and 117% at 24 and 72 hours respectively; MCF-7: 151%, 159% and 185% at 24, 48 and 72 hours respectively) (Figure 2B). Additionally, overexpression of ghrelin increased cell proliferation after 48-72h in MDA-

MB-231 (117% at both 24 and 72 hours), but not in MCF-7 (Figure 2B). These results were further confirmed using MTT assay, which generated similar results (Supplemental Figure 1), in that In1-19 (120% and 119% at 48 and 72 hours respectively), In1-40 (122%, 124% and 116% at 24, 48 and 72 hours respectively) and native ghrelin (124% at 72 hours) increased proliferation in MDA-MB-231 cells compared to vehicle, but only In1-ghrelin derived peptides stimulated proliferation in MCF-7 (113% and 115% at 72 hours for In1-19 and In1-40 respectively) (Figure 2C). As expected, Paclitaxel treatment (used as a control for cell proliferation and migration inhibition) induced a clear decrease in cell proliferation in both, MDA-MB-231 (72%, 50% and 37% at 24, 48 and 72 hours, respectively, compared with vehicle-treated cells) and MCF-7 (86%, 75% and 57% at 24, 48 and 72 hours, respectively, compared with vehicle-treated cells) cells lines; while IGF-I (a classical stimulator of proliferation and migration) significantly increased proliferation rate at 48 and 72 hours in MDA-MB-231 (119% and 120% respectively) and at 72 hours in MCF-7 cells (112%), which suggest a similar stimulatory effect of In1-ghrelin peptides than IGF-I in these cell lines.

In general, these results robustly and consistently demonstrate that the presence of In1-ghrelin, or its derived peptides, is able to increase the proliferation rate of breast cancer cells, although the extent of the induction is not exactly the same in all the experimental conditions. However, the particular differences observed between MDA-MB-231 and MCF-7 cells could be related to the actual difference in basal expression of In1-ghrelin variant between both cell lines; indeed, MDA-MB-231 cells present a higher basal expression of In1-ghrelin than MCF-7, which may suggest that endogenous In1-ghrelin in MDA-MB-231 cells could be exerting a role by increasing basal proliferation so that additional expression of this splicing variant presents a lower effect than in cells with a lower basal expression, as is the case of MCF-7 cells. Alternatively, it is also possible that the higher basal proliferation rate of MDA-MB-231 compared with MCF-7 could have an effect in the observed differences. On the other hand, the differences between overexpression and peptide treatment effects could be related to the different dynamics of both types of experiments in that treatment with peptides commonly evoke more acute, short-lived effect; whereas, overexpression experiments seem to maintain higher levels over time,

and therefore, likely provides a better model to observe an effect on cell proliferation in response to an overexposure.

In1-ghrelin overexpressing cells exhibited increased migration ability (Figure 3A), whereas ghrelin-overexpressing cells did not show any difference in migration capacity compared with control cells (Figure 3A). Similarly, non-transfected cells exhibited higher migration capacity when treated with In1-ghrelin peptides compared with vehicle-treated cells (Figure 3A). Interestingly, acyl-ghrelin treatment only increased migration capacity significantly in MDA-MB-231 cells (Figure 3A). It should be noted that both, stimulatory and inhibitory, controls (IGF-I and paclitaxel) exerted the expected results in the migration ability of both cell lines. Moreover, In1-ghrelin, but not ghrelin, overexpression induced an increase in basal ERK phosphorylation with no changes in AKT phosphorylation (Figure 4).

In1-ghrelin down-regulation decreased cell proliferation and migration

Two different In1-ghrelin specific siRNAs that successfully reduced In1-ghrelin mRNA expression (Figure 3B) were deployed to analyze proliferation rate and migration capacity. In1-ghrelin inhibition induced a clear decrease in migration and proliferation rates compared with control siRNA transfected cells (Figures 3C-D). Similar results were obtained with both independent siRNAs.

In1-ghrelin overexpression increased mammosphere formation

In1-ghrelin but not ghrelin overexpression induced a marked increase in the number of mammospheres in both cell lines (Figure 5A), and, in the case of MDA-MB-231 cell line, in mammospheres size (Figure 5B), as compared with control and ghrelin-transfected cells. Consistently, cells treated during 7 days with In1-ghrelin derived peptides exhibited increased capacity to form mammospheres compared with vehicle- and ghrelin-treated cells (Figure 5A). Although it is not clear the reason why In1-ghrelin overexpression only increases mammosphere size (cell content) in MDA-MB-231 cells (which could be due to intrinsic cell line-specific factors, including the own endogenous In1-ghrelin expression), these results demonstrate that In1-ghrelin overexposure has a profound effect on mammosphere formation.

To explore the molecular basis of these In1-ghrelin-induced changes, the expression pattern of key genes of three signaling pathways related to dedifferentiation (TGF- β 1 [30], Notch [31] and Wnt/ β -catenin [32]) was explored. Overexpression of ghrelin and In1-ghrelin increased TGF- β 1 expression. We also measured JAG1, a Notch pathway ligand, and β -catenin (CTNNB1), a Wnt pathway effector protein, which has been clearly correlated with dedifferentiation processes [33, 34]. JAG1 showed elevated mRNA levels in In1-ghrelin and ghrelin transfected cells (Figure 6). Interestingly, β -catenin showed increased mRNA levels in cell lines overexpressing In1-ghrelin, but not ghrelin (Figure 6). Finally, we also explored the expression levels of the canonical receptor for native ghrelin, GHS-R1a, as well as its truncated form GHS-R1b, and found that both were absent or close to the detection limit (GHS-R1a: Ct = 37,0 – 37,1 in MDA-MB-231 and MCF-7 cell lines and GHS-R1b: Ct = 35,2 – 33,4 in MDA-MB-231 and MCF-7 cell lines, respectively).

Discussion

Aberrant alternative splicing events have been observed in several cancer types and are currently considered as an emerging cancer hallmark [4, 35]. These processes promote the aberrant appearance of novel mRNA and protein variants, distinct from those present in healthy tissues [36], and whose expression is often associated with malignant features and poor prognosis. Indeed, the pathophysiological role of these aberrant splicing variants span from the promotion of apoptosis [37] or immune system escape [38], to increased malignancy features, such as proliferation, migration, invasion or metastatic ability [39, 40]. In this scenario, previous studies have demonstrated that expression of the splicing variant In1-ghrelin is tightly linked to enhanced malignancy and/or aggressiveness features in human BCa [18], and in pituitary [19] and neuroendocrine [20] tumors; however, the exact functions of In1-ghrelin splicing variant in BCa pathophysiology is yet to be determined.

In the present study, we have demonstrated for first time a relevant association between high In1-ghrelin expression levels and lymph node metastasis. Indeed, patients with BCa samples categorized as bearing low levels or no expression of In1-ghrelin presented with less metastasis than those whose samples showed a high level of In1-ghrelin expression. Moreover, 10-year follow-up studies on these patients revealed that high expression of In1-ghrelin strongly correlated with increased recurrence or *de novo* tumor appearance, demonstrating a relevant implication of this splicing variant on the clinical outcome of BCa patients and suggesting its putative value as prognostic marker and/or therapeutic target in BCa. Mechanistically, proliferation and migration rates were increased in In1-ghrelin overexpressing MDA-MB-231 and MCF-7 cells and in response to In1-ghrelin derived peptides, which is in line with previous results [18]. Indeed, In-ghrelin overexpression and treatment increases malignancy features in cultured pituitary and neuroendocrine tumor cells [19, 20], thereby providing a plausible rationale to suggest that In1-ghrelin may play a common, malignancy-linked role among different cancer types. Herein, In1-ghrelin overexpression was accompanied by changes in basal phosphorylation of the crucial, tumor-associated signaling pathway MAPK/ERK [41], which may explain the observed changes in cellular proliferation and migration. Importantly, these results were further corroborated by In1-ghrelin

downregulation using two independent siRNAs in MDA-MB-231 cells, reinforcing the notion that counteracting In1-ghrelin may confer a therapeutic benefit.

The precise role of the ghrelin system in cancer is still a matter of controversy [7, 15-17], likely due to the diversity of components of this complex system. In this context, in the present study, side-by-side comparison of ghrelin and In1-ghrelin peptides revealed that although both induced changes in proliferation and migration, the fact that only In1-ghrelin altered the ERK signaling pathway, suggests that the two splicing variants of the ghrelin gene could be exerting their functions by different receptors and/or signaling cascades. Surprisingly, MDA-MB-231 and MCF-7 cells lines did not show relevant expression levels of the canonical ghrelin receptors GHS-R1a, or its spliced variant GHS-R1b, which would imply that the observed changes may be mediated by as of yet unknown, and likely distinct receptors.

Finally, the clinical findings found herein compare well with the higher number of CSCs induced by In1-ghrelin overexposure in both tumor cell lines, since CSCs have been associated with tumor relapse and metastatic potential [42]. Particularly, the *in vitro* results shown herein indicate a clear increase in mammosphere number and, in MDA-MB-231 cells, mammosphere size, when the cells are transfected with In1-ghrelin or treated with In1-ghrelin peptides, but not with native ghrelin. These results further demonstrate a clear capacity of the In1-ghrelin variant to increase the ability of MDA-MB-231 and MCF-7 cells to dedifferentiate, and to stimulate the mammosphere proliferation rate of MDA-MB-231 cells. Therefore, with the aim of further elucidating the molecular mechanisms underlying the association between In1-ghrelin and the dedifferentiation processes, we determined the expression levels of several candidate genes that have been shown to be involved in signaling pathways associated with cell plasticity and CSCs in previous studies [33, 43]. In particular, these tumor processes are tightly associated with activation of certain signaling pathways such as TGF- β 1 [30], Notch [31] and Wnt/ β -catenin [32], which have been shown to crosstalk in order to modulate EMT and CSCs biology [44]. Interestingly, we discovered that In1-ghrelin overexpression induced a clear up-regulation of TGF- β 1

expression, which was particularly marked in MDA-MB-231 cells. In addition, In1-ghrelin overexpression increased mRNA levels of JAG1, one of the main activators of Notch signaling [45], and those of β -catenin, which was especially evident in MCF-7 cells, and which would explain the clear changes induced by In1-ghrelin in forming mammospheres. It is interesting to note that In1-ghrelin overexpression induced a similar, albeit not completely identical change in the expression levels of differentiation-related genes in both cell lines. Specifically, In1-ghrelin overexpression in MDA-MB-231 mainly altered the expression levels of TGF- β 1, while it only moderately changed β -catenin and JAG1 expression levels. Contrarily, In1-ghrelin overexpression in MCF-7 cells alters moderately TGF- β 1 mRNA expression but strongly increase the expression levels of JAG1. These data, together with the fact that ghrelin overexpression induced distinct, though less pronounced, changes in the expression of the mentioned signaling pathways, suggests that In1-ghrelin triggers specific changes in TGF- β 1, Notch and Wnt/ β -catenin signaling pathways to modulate cell plasticity and CSCs biology, which could be BCa cell line-dependent.

The present results demonstrate that the In1-ghrelin splicing variant may have relevant clinical implications, in that In1-ghrelin may contribute to human tumor progression, metastasis, and relapse, as indicated by the data collected from patient samples. The cellular and molecular approaches demonstrated that In1-ghrelin enhances the malignant features of two BCa derived cell lines, MDA-MB-231 and MCF-7. Moreover, we provide plausible key mechanisms of action underlying the In1-ghrelin effects; specifically, activation of the MAPK-ERK, Jag1/Notch, Wnt/ β -catenin and/or TGF- β 1 signaling pathways. The fact that the effects of In1-ghrelin are different from those exerted by the canonical native variant of the ghrelin gene, ghrelin, underscore the promising value of In1-ghrelin as a biomarker and therapeutic target in BCa treatment and diagnosis.

Acknowledgments: We appreciate the generous contribution of Virginia Ruiz-Murillo, Maria E. Prados, Ramón Peiteado-Santamaria, Marta Hergueta and Alejandro Rojas-Sebastian in the initial phase of these studies.

References

- [1] Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. *CA Cancer J Clin.* 2017;67:7-30.
- [2] Baird RD, Caldas C. Genetic heterogeneity in breast cancer: the road to personalized medicine? *BMC Med.* 2013;11:151.
- [3] Chen J, Weiss WA. Alternative splicing in cancer: implications for biology and therapy. *Oncogene.* 2015;34:1-14.
- [4] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011;144:646-74.
- [5] Dehm SM. mRNA splicing variants: exploiting modularity to outwit cancer therapy. *Cancer Res.* 2013;73:5309-14.
- [6] Moreau P, Flajollet S, Carosella ED. Non-classical transcriptional regulation of HLA-G: an update. *J Cell Mol Med.* 2009;13:2973-89.
- [7] Chopin LK, Seim I, Walpole CM, Herington AC. The ghrelin axis--does it have an appetite for cancer progression? *Endocr Rev.* 2012;33:849-91.
- [8] Gahete MD, Rincon-Fernandez D, Villa-Osaba A, Hormaechea-Agulla D, Ibanez-Costa A, Martinez-Fuentes AJ, et al. Ghrelin gene products, receptors, and GOAT enzyme: biological and pathophysiological insight. *J Endocrinol.* 2014;220:R1-24.
- [9] Seim I, Herington AC, Chopin LK. New insights into the molecular complexity of the ghrelin gene locus. *Cytokine Growth Factor Rev.* 2009;20:297-304.
- [10] Garg A. The ongoing saga of obestatin: is it a hormone? *J Clin Endocrinol Metab.* 2007;92:3396-8.
- [11] Papotti M, Duregon E, Volante M. Ghrelin and tumors. *Endocr Dev.* 2013;25:122-34.
- [12] Jeffery PL, Herington AC, Chopin LK. Expression and action of the growth hormone releasing peptide ghrelin and its receptor in prostate cancer cell lines. *J Endocrinol.* 2002;172:R7-11.
- [13] Jeffery PL, Murray RE, Yeh AH, McNamara JF, Duncan RP, Francis GD, et al. Expression and function of the ghrelin axis, including a novel preproghrelin isoform, in human breast cancer tissues and cell lines. *Endocr Relat Cancer.* 2005;12:839-50.
- [14] Jeffery PL, Herington AC, Chopin LK. The potential autocrine/paracrine roles of ghrelin and its receptor in hormone-dependent cancer. *Cytokine Growth Factor Rev.* 2003;14:113-22.

- [15] Gronberg M, Fjallskog ML, Jirstrom K, Janson ET. Expression of ghrelin is correlated to a favorable outcome in invasive breast cancer. *Acta Oncol.* 2012;51:386-93.
- [16] Cassoni P, Papotti M, Ghe C, Catapano F, Sapino A, Graziani A, et al. Identification, characterization, and biological activity of specific receptors for natural (ghrelin) and synthetic growth hormone secretagogues and analogs in human breast carcinomas and cell lines. *J Clin Endocrinol Metab.* 2001;86:1738-45.
- [17] Cassoni P, Ghe C, Marrocco T, Tarabra E, Allia E, Catapano F, et al. Expression of ghrelin and biological activity of specific receptors for ghrelin and des-acyl ghrelin in human prostate neoplasms and related cell lines. *Eur J Endocrinol.* 2004;150:173-84.
- [18] Gahete MD, Cordoba-Chacon J, Hergueta-Redondo M, Martinez-Fuentes AJ, Kineman RD, Moreno-Bueno G, et al. A novel human ghrelin variant (In1-ghrelin) and ghrelin-O-acyltransferase are overexpressed in breast cancer: potential pathophysiological relevance. *PLoS One.* 2011;6:e23302.
- [19] Ibanez-Costa A, Gahete MD, Rivero-Cortes E, Rincon-Fernandez D, Nelson R, Beltran M, et al. In1-ghrelin splicing variant is overexpressed in pituitary adenomas and increases their aggressive features. *Sci Rep.* 2015;5:8714.
- [20] Luque RM, Sampedro-Nuñez M, Gahete MD, Ramos-Levi A, Ibañez-Costa A, Rivero-Cortes E, et al. In1-ghrelin, a splice variant of ghrelin gene, is associated with the evolution and aggressiveness of human neuroendocrine tumors: Evidence from clinical, cellular and molecular parameters. *Oncotarget.* 2015.
- [21] Gahete MD, Rincon-Fernandez D, Duran-Prado M, Hergueta-Redondo M, Ibanez-Costa A, Rojo-Sebastian A, et al. The truncated somatostatin receptor sst5TMD4 stimulates the angiogenic process and is associated to lymphatic metastasis and disease-free survival in breast cancer patients. *Oncotarget.* 2016.
- [22] Moreno-Bueno G, Salvador F, Martin A, Floristan A, Cuevas EP, Santos V, et al. Lysyl oxidase-like 2 (LOXL2), a new regulator of cell polarity required for metastatic dissemination of basal-like breast carcinomas. *EMBO Mol Med.* 2011;3:528-44.

- [23] Uphoff CC, Drexler HG. Detection of mycoplasma contaminations. *Methods Mol Biol.* 2005;290:13-23.
- [24] Martinez-Fuentes AJ, Moreno-Fernandez J, Vazquez-Martinez R, Duran-Prado M, de la Riva A, Tena-Sempere M, et al. Ghrelin is produced by and directly activates corticotrope cells from adrenocorticotropin-secreting adenomas. *J Clin Endocrinol Metab.* 2006;91:2225-31.
- [25] Neto LV, Machado Ede O, Luque RM, Taboada GF, Marcondes JB, Chimelli LM, et al. Expression analysis of dopamine receptor subtypes in normal human pituitaries, nonfunctioning pituitary adenomas and somatotropinomas, and the association between dopamine and somatostatin receptors with clinical response to octreotide-LAR in acromegaly. *J Clin Endocrinol Metab.* 2009;94:1931-7.
- [26] Taboada GF, Luque RM, Bastos W, Guimaraes RF, Marcondes JB, Chimelli LM, et al. Quantitative analysis of somatostatin receptor subtype (SSTR1-5) gene expression levels in somatotropinomas and non-functioning pituitary adenomas. *Eur J Endocrinol.* 2007;156:65-74.
- [27] Gahete MD, Luque RM, Yubero-Serrano EM, Cruz-Teno C, Ibanez-Costa A, Delgado-Lista J, et al. Dietary fat alters the expression of cortistatin and ghrelin systems in the PBMCs of elderly subjects: putative implications in the postprandial inflammatory response. *Mol Nutr Food Res.* 2014;58:1897-906.
- [28] Duran-Prado M, Gahete MD, Hergueta-Redondo M, Martinez-Fuentes AJ, Cordoba-Chacon J, Palacios J, et al. The new truncated somatostatin receptor variant sst5TMD4 is associated to poor prognosis in breast cancer and increases malignancy in MCF-7 cells. *Oncogene.* 2012;31:2049-61.
- [29] Shaw FL, Harrison H, Spence K, Ablett MP, Simoes BM, Farnie G, et al. A detailed mammosphere assay protocol for the quantification of breast stem cell activity. *J Mammary Gland Biol Neoplasia.* 2012;17:111-7.
- [30] Watabe T, Miyazono K. Roles of TGF-beta family signaling in stem cell renewal and differentiation. *Cell Res.* 2009;19:103-15.
- [31] Pannuti A, Foreman K, Rizzo P, Osipo C, Golde T, Osborne B, et al. Targeting Notch to target cancer stem cells. *Clin Cancer Res.* 2010;16:3141-52.

- [32] Bandapalli OR, Dihlmann S, Helwa R, Macher-Goeppinger S, Weitz J, Schirmacher P, et al. Transcriptional activation of the beta-catenin gene at the invasion front of colorectal liver metastases. *J Pathol.* 2009;218:370-9.
- [33] Nosedà M, McLean G, Niessen K, Chang L, Pollet I, Montpetit R, et al. Notch activation results in phenotypic and functional changes consistent with endothelial-to-mesenchymal transformation. *Circ Res.* 2004;94:910-7.
- [34] Czerwinska P, Kaminska B. Regulation of breast cancer stem cell features. *Contemp Oncol (Pozn).* 2015;19:A7-A15.
- [35] Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell.* 2000;100:57-70.
- [36] Ghigna C, Valacca C, Biamonti G. Alternative splicing and tumor progression. *Curr Genomics.* 2008;9:556-70.
- [37] Lin JC, Lin CY, Tarn WY, Li FY. Elevated SRPK1 lessens apoptosis in breast cancer cells through RBM4-regulated splicing events. *RNA.* 2014;20:1621-31.
- [38] Prendergast GC. Immune escape as a fundamental trait of cancer: focus on IDO. *Oncogene.* 2008;27:3889-900.
- [39] Okumura N, Yoshida H, Kitagishi Y, Nishimura Y, Matsuda S. Alternative splicings on p53, BRCA1 and PTEN genes involved in breast cancer. *Biochem Biophys Res Commun.* 2011;413:395-9.
- [40] Klingbeil P, Isacke CM. The 'alternative' EMT switch. *Breast Cancer Res.* 2011;13:313.
- [41] Ciuffreda L, Incani UC, Steelman LS, Abrams SL, Falcone I, Curatolo AD, et al. Signaling intermediates (MAPK and PI3K) as therapeutic targets in NSCLC. *Curr Pharm Des.* 2014;20:3944-57.
- [42] Velasco-Velazquez MA, Popov VM, Lisanti MP, Pestell RG. The role of breast cancer stem cells in metastasis and therapeutic implications. *Am J Pathol.* 2011;179:2-11.
- [43] Kotiyal S, Bhattacharya S. Breast cancer stem cells, EMT and therapeutic targets. *Biochem Biophys Res Commun.* 2014;453:112-6.
- [44] Guo X, Wang XF. Signaling cross-talk between TGF-beta/BMP and other pathways. *Cell Res.* 2009;19:71-88.

[45] Li D, Masiero M, Banham AH, Harris AL. The notch ligand JAGGED1 as a target for anti-tumor therapy. *Front Oncol.* 2014;4:254.

Figure Legends

Figure 1. In1-ghrelin expression levels are associated with lymph node metastasis and lower disease-free survival in breast cancer patients. **A)** Expression levels of In1-ghrelin (adjusted by ACTB) in the 117 grade 3 breast carcinomas and their classification as low, medium or high In1-ghrelin mRNA levels. **B)** Average expression levels of In1-ghrelin (adjusted by ACTB) in the breast carcinoma samples within each group (low, medium or high In1-ghrelin mRNA levels). Samples were categorized as low, moderate and high In1-ghrelin expression according to quartile In1-ghrelin expression levels (0-50% as low, 50-75% as moderate and 75-100% as high). **C)** Association between the presence of In1-ghrelin and lymph node metastasis in breast carcinoma samples. Data, obtained from a frequency table, showed an association between In1-ghrelin expression and lymph node metastasis. **D)** Kaplan-Meier plots showing the association of increased In1-ghrelin mRNA expression and disease-free survival in breast carcinoma series. Significant correlation was studied using a Chi-square and Long-rank-p-value methods. Asterisks (***, $p < 0.001$) indicate significant differences between groups by One-way ANOVA followed by Dunnett's test.

Figure 2. In1-ghrelin stimulated cell proliferation in breast cancer cell lines. **A)** Validation of In1-ghrelin and ghrelin overexpression by qPCR in MDA-MB-231 and MCF-7 stably transfected cells. Copy number are normalized by a normalization factor (NF) and data are showed as percentage of control cells ($n=10$ different cell preparations). **B)** Proliferation rates determined by Alamar blue technique ($n=6$ for each cell line). **C)** Proliferation rate assayed by Alamar blue after treatment with In1-ghrelin and ghrelin peptides ($n=8-7$ in MDA-MB-231 and MCF-7 cell lines, respectively). Values represent means \pm SEM. Asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) indicate significant differences between groups by One-way ANOVA followed by Dunnett's test.

Figure 3. In1-ghrelin stimulated migration rate in breast cancer cell lines and its downregulation decreased proliferation rate and migration in MDA-MB-231 cells. **A)** Migration ability was

determined by wound healing technique in overexpressing (n=4-6 in MDA-MB-231 and MCF-7 cell lines, respectively) and treated (n=4-5 in MDA-MB-231 and MCF-7 cell lines, respectively) cells. **B)** Validation of In1-ghrelin downregulation by siRNAs transfections expressed as percentage of control siRNA expression levels (n=6-4 in siRNA-1 and siRNA-2, respectively). **C)** Migration ability analysis by wound healing technique (n=3-4 in siRNA-1 and siRNA-2, respectively) represented as percentage of control siRNA-transfected cell migration, and a representative picture of each transfected cells at 0 and 24 hours. **D)** Proliferation rates in cells transfected with specific siRNAs compared with control siRNA-transfected cells (n=5-3 in siRNA-1 and siRNA-2, respectively). Values represent means \pm SEM. Asterisks above each point (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) indicate significant differences between groups by One-way ANOVA followed by Dunnett's test

Figure 4. In1-ghrelin stimulated ERK phosphorylation in MDA-MB-231 cells. Basal ERK (n=5) and AKT (n=4) phosphorylation in In1-ghrelin and ghrelin transfected MDA-MB-231 cell lines compared with Control. Values represent means \pm SEM. Asterisks above each point (*, $p<0.05$) indicate significant differences between groups by One-way ANOVA followed by Dunnett's test. pERK states for phospho-ERK while pAkt states for phospho-Akt.

Figure 5. In1-ghrelin overexposure increased mammospheres formation and size. **A)** Percentage of mammospheres in transfected (n=5-3 in MDA-MB-231 and MCF-7 cells, respectively) and treated (n=4) cells compared with control and vehicle treated cells, respectively. **B)** Average number of cells by mammosphere in each transfected cell lines (n=3). Values represent means \pm SEM. Asterisks (*, $p<0.05$) indicate significant differences between groups by One-way ANOVA followed by Dunnett's test.

Figure 6. In1-ghrelin and ghrelin altered mRNA expression levels of dedifferentiation related genes. mRNA expression levels of b-Catenin (CTNNB1), Jagged1 (JAG1), and Tumor Growth Factor-b1 (TGF-b1) are showed as a percentage of mock cells expression (n=10 independent cell preparations).

Values represent means \pm SEM. Asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) indicate significant differences between groups by One-way ANOVA.

FIGURE 1

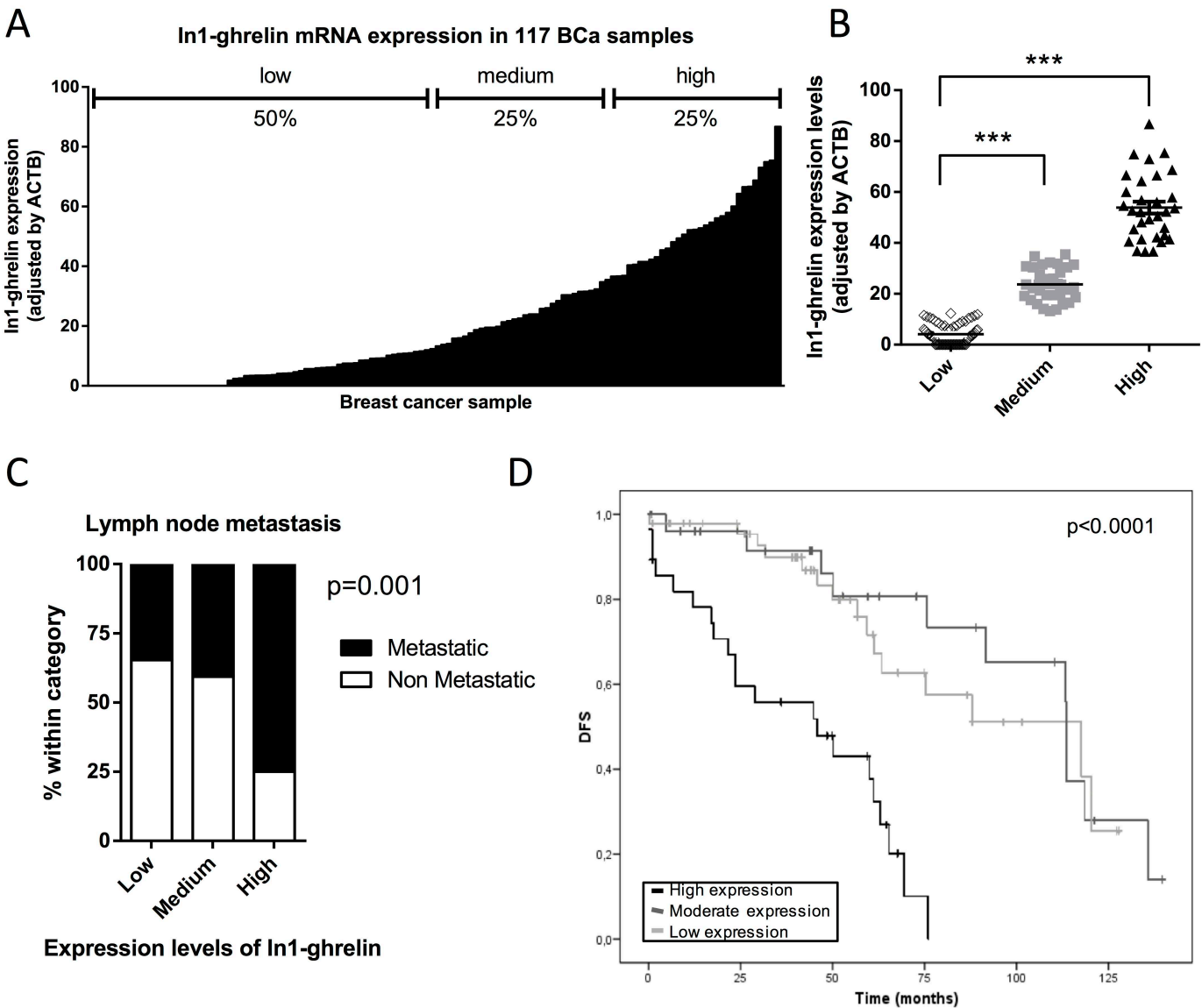


Figure 1. In1-ghrelin expression levels are associated with lymph node metastasis and lower disease-free survival in breast cancer patients. **A)** Expression levels of In1-ghrelin (adjusted by ACTB) in the 117 grade 3 breast carcinomas and their classification as low, medium or high In1-ghrelin mRNA levels. **B)** Average expression levels of In1-ghrelin (adjusted by ACTB) in the breast carcinoma samples within each group (low, medium or high In1-ghrelin mRNA levels). **Samples were categorized as low, moderate and high In1-ghrelin expression according to quartile In1-ghrelin expression levels (0-50% as low, 50-75% as moderate and 75-100% as high).** **C)** Association between the presence of In1-ghrelin and lymph node metastasis in breast carcinoma samples. Data, obtained from a frequency table, showed an association between In1-ghrelin expression and lymph node metastasis. **D)** Kaplan-Meier plots showing the association of increased In1-ghrelin mRNA expression and disease-free survival in breast carcinoma series. Significant correlation was studied using a Chi-square and Long-rank-p-value methods. Asterisks (***, $p < 0.001$) indicate significant differences between groups by One-way ANOVA followed by Dunnett's test.

FIGURE 2

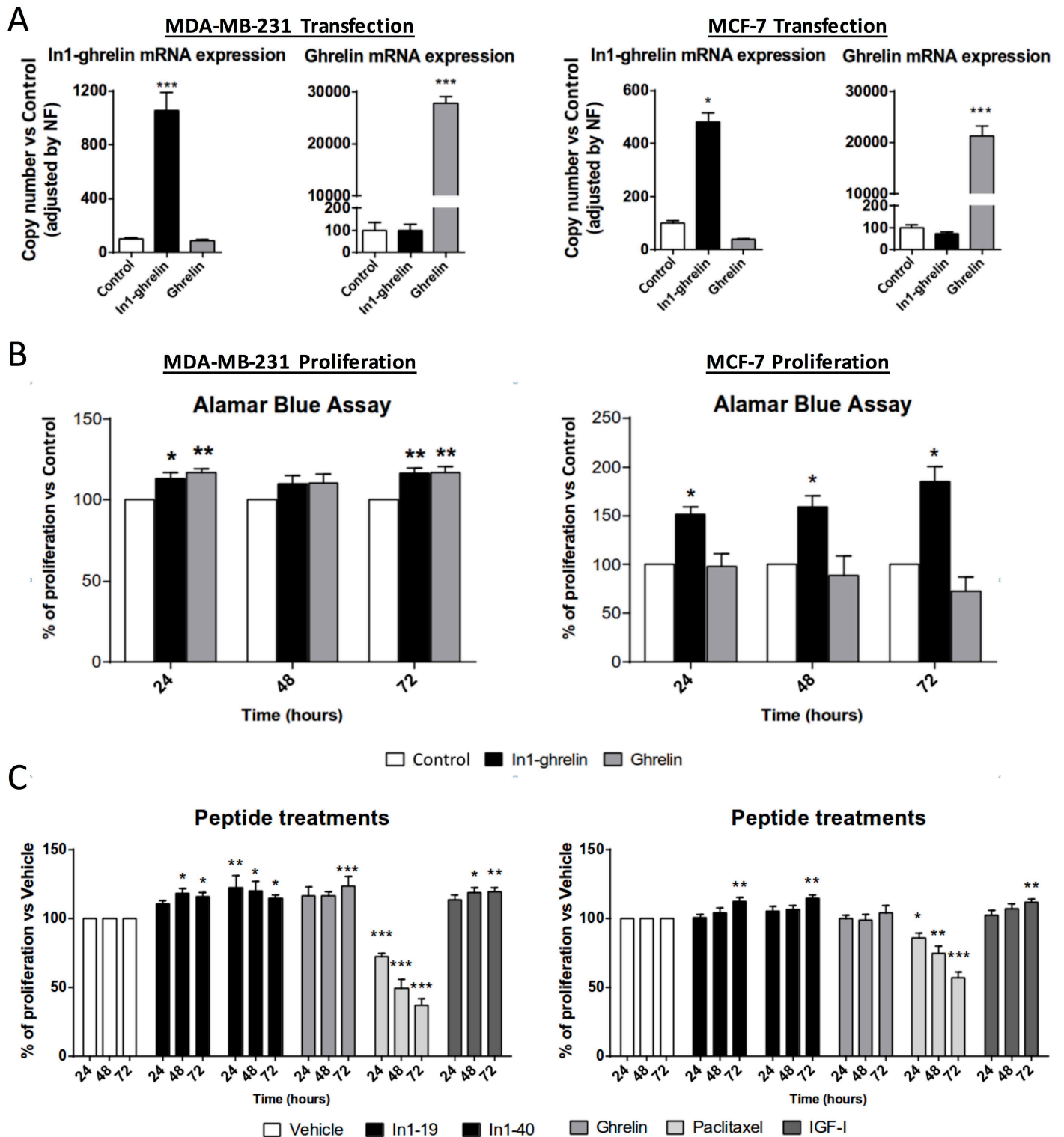


Figure 2. In1-ghrelin stimulated cell proliferation in breast cancer cell lines. A) Validation of In1-ghrelin and ghrelin overexpression by qPCR in MDA-MB-231 and MCF-7 stably transfected cells. Copy number are normalized by a normalization factor (NF) and data are showed as percentage of control cells (n=10 different cell preparations). **B)** Proliferation rates determined by Alamar blue technique (n=6 for each cell line). **C)** Proliferation rate assayed by Alamar blue after treatment with In1-ghrelin and ghrelin peptides (n=8-7 in MDA-MB-231 and MCF-7 cell lines, respectively). Values represent means \pm SEM. Asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) indicate significant differences between groups by One-way ANOVA followed by Dunnett's test.

FIGURE 3

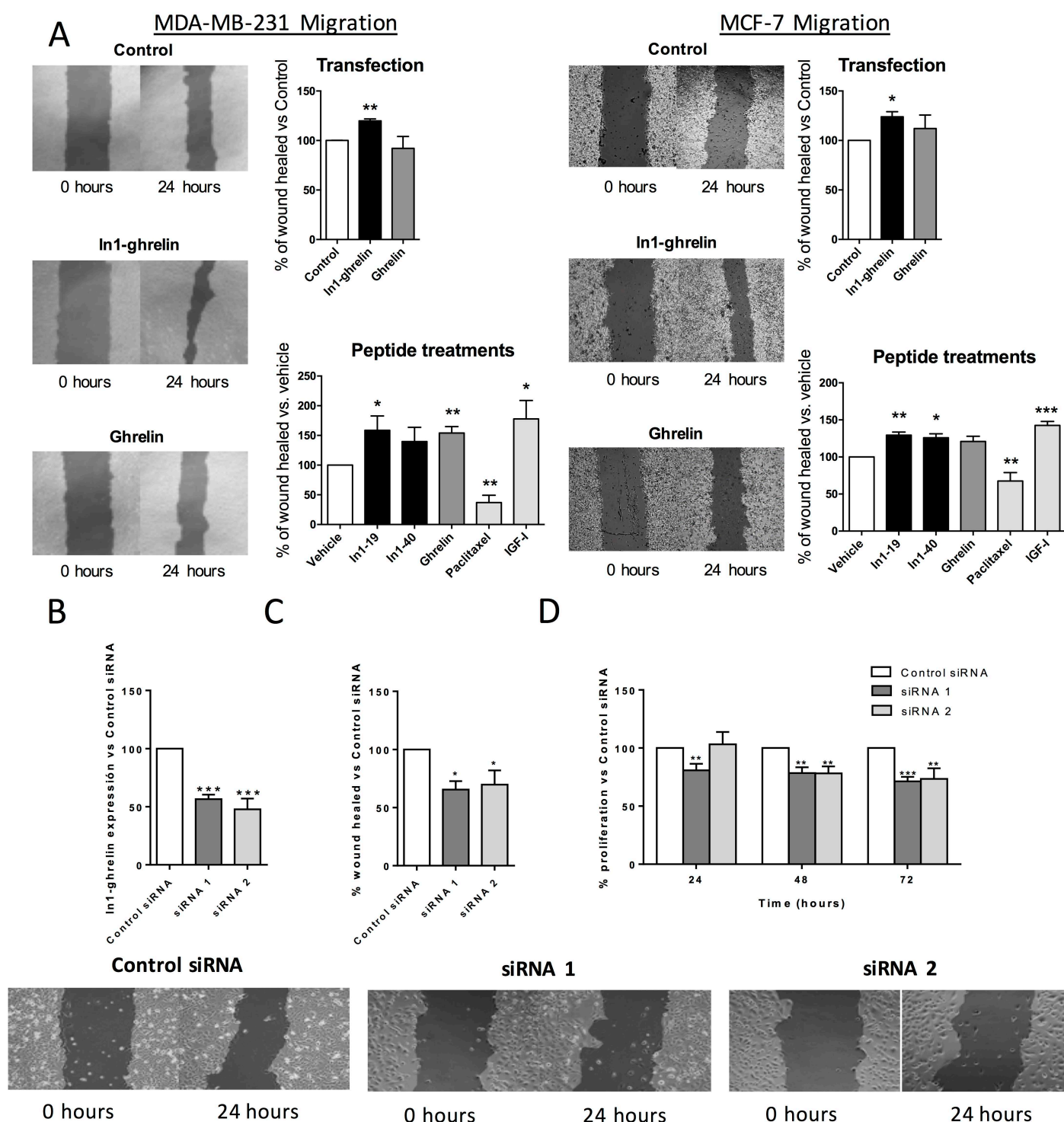


Figure 3. In1-ghrelin stimulated migration rate in breast cancer cell lines and its downregulation decreased proliferation rate and migration in MDA-MB-231 cells. **A)** Migration ability was determined by wound healing technique in overexpressing (n=4-6 in MDA-MB-231 and MCF-7 cell lines, respectively) and treated (n=4-5 in MDA-MB-231 and MCF-7 cell lines, respectively) cells. **B)** Validation of In1-ghrelin downregulation by siRNAs transfections expressed as percentage of control siRNA expression levels (n=6-4 in siRNA-1 and siRNA-2, respectively). **C)** Migration ability analysis by wound healing technique (n=3-4 in siRNA-1 and siRNA-2, respectively) represented as percentage of control siRNA-transfected cell migration, and a representative picture of each transfected cells at 0 and 24 hours. **D)** Proliferation rates in cells transfected with specific siRNAs compared with control siRNA-transfected cells (n=5-3 in siRNA-1 and siRNA-2, respectively). Values represent means \pm SEM. Asterisks above each point (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) indicate significant differences between groups by One-way ANOVA followed by Dunnett's test

FIGURE 4

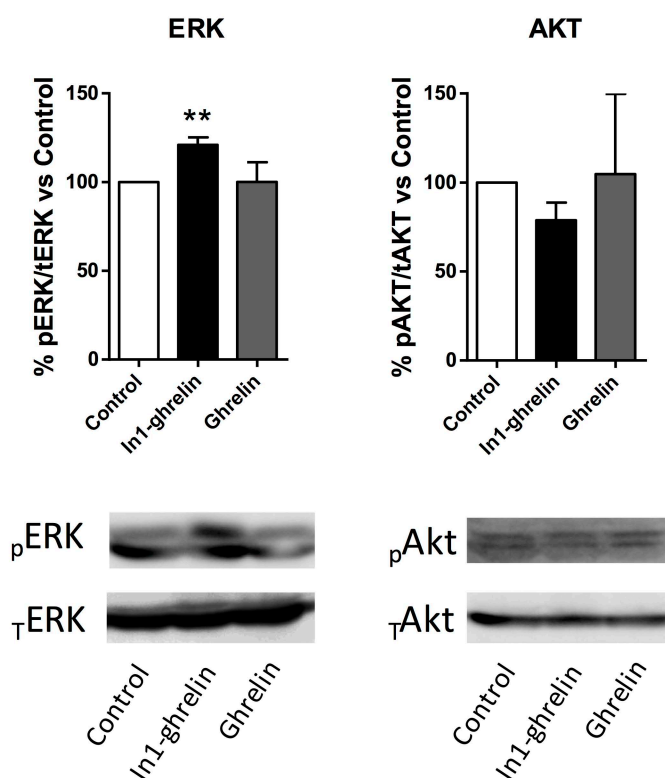
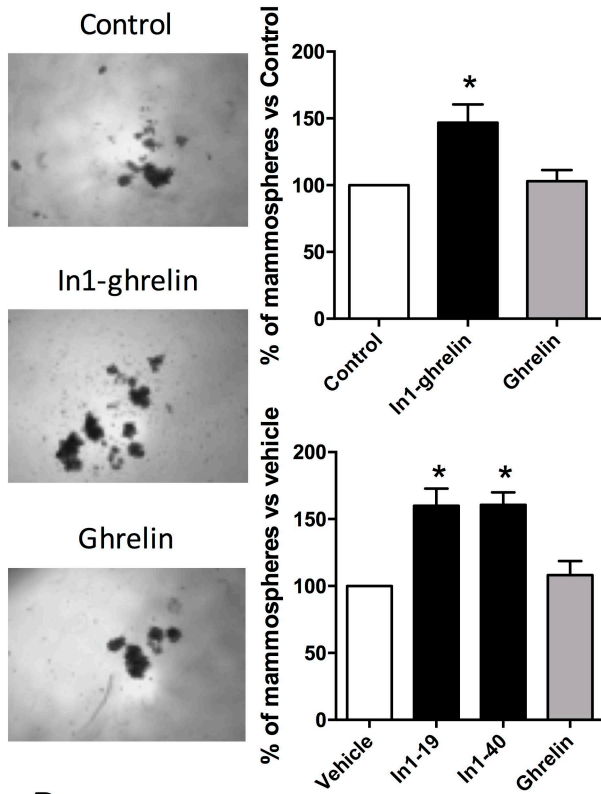


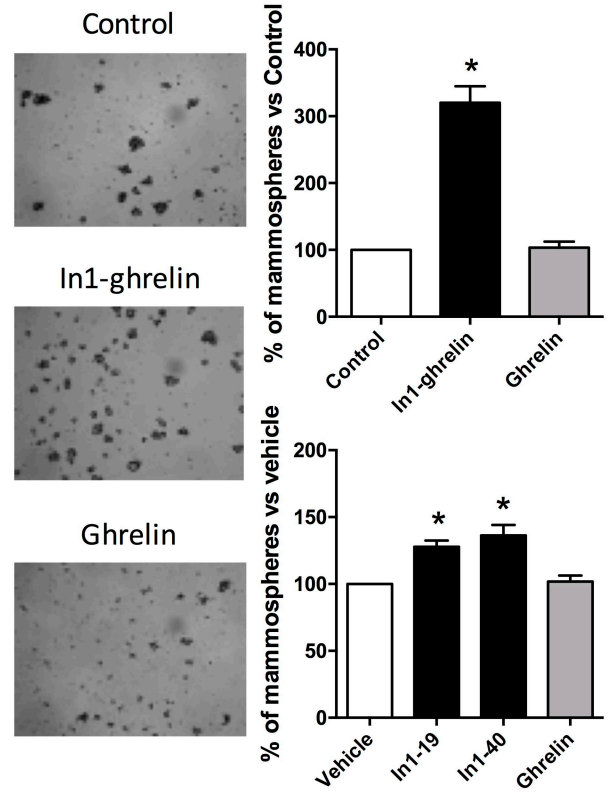
Figure 4. In1-ghrelin stimulated ERK phosphorylation in MDA-MB-231 cells. Basal ERK (n=5) and AKT (n=4) phosphorylation in In1-ghrelin and ghrelin transfected MDA-MB-231 cell lines compared with Control. Values represent means \pm SEM. Asterisks above each point (*, $p < 0.05$) indicate significant differences between groups by One-way ANOVA followed by Dunnett's test. pERK states for phospho-ERK while pAkt states for phospho-Akt.

FIGURE 5

A MDA-MB-231 Mammospheres



MCF-7 Mammospheres



B

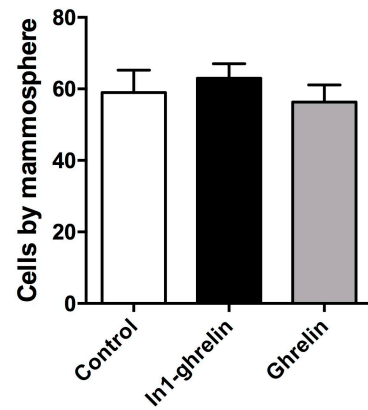
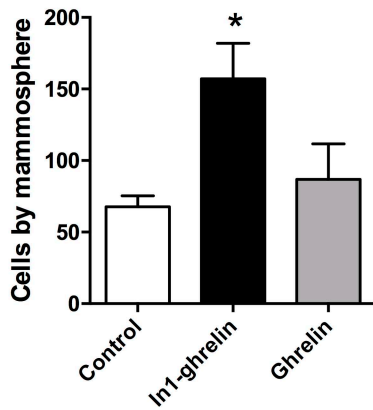


Figure 5. In1-ghrelin overexposure increased mammospheres formation and size. **A)** Percentage of mammospheres in transfected (n=5-3 in MDA-MB-231 and MCF-7 cells, respectively) and treated (n=4) cells compared with control and vehicle treated cells, respectively. **B)** Average number of cells by mammosphere in each transfected cell lines (n=3). Values represent means \pm SEM. Asterisks (*, $p < 0.05$) indicate significant differences between groups by One-way ANOVA followed by Dunnett's test.

FIGURE 6

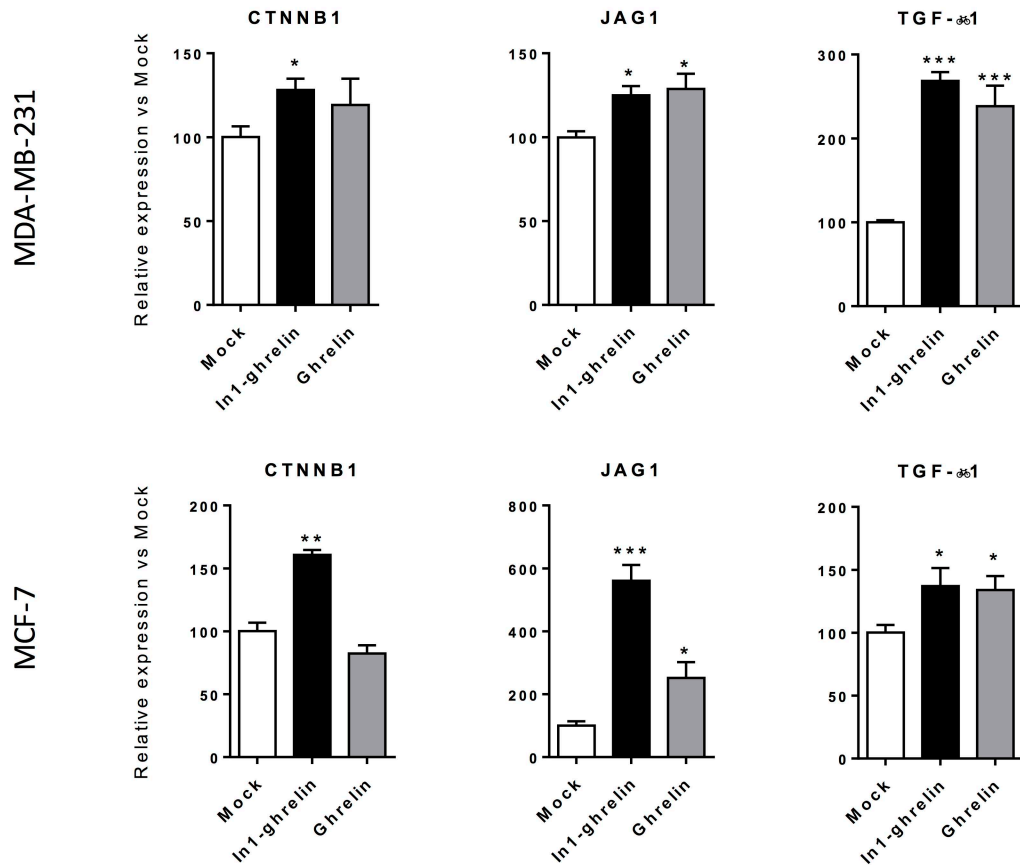


Figure 6. In1-ghrelin and ghrelin altered mRNA expression levels of dedifferentiation related genes. mRNA expression levels of β -Catenin (CTNNB1), Jagged1 (JAG1), and Tumor Growth Factor- β 1 (TGF- β 1) are showed as a percentage of mock cells expression (n=10 independent cell preparations). Values represent means \pm SEM. Asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) indicate significant differences between groups by One-way ANOVA.