A NOVEL ROLE OF ONCOSTATIN M IN INVASIVE BREAST CANCER: INDUCTION OF CATHEPSIN D AND LYSOSOMAL TRAFFICKING

by

Jordan Barrie Koncinsky

A thesis

submitted in partial fulfillment

of the requirements for the degree of

Master of Science in Biology

Boise State University

December 2013

BOISE STATE UNIVERSITY GRADUATE COLLEGE

DEFENSE COMMITTEE AND FINAL READING APPROVALS

of the thesis submitted by

Jordan Barrie Koncinsky

Thesis Title: A Novel Role of Oncostatin M in Invasive Breast Cancer: Induction of Cathepsin D and Lysosomal Trafficking

Date of Final Oral Examination: 31 July 2013

The following individuals read and discussed the thesis submitted by student Jordan Barrie Koncinsky, and they evaluated her presentation and response to questions during the final oral examination. They found that the student passed the final oral examination.

Cheryl L Jorcyk, Ph.D.	Chair, Supervisory Committee
Julia T. Oxford, Ph.D.	Member, Supervisory Committee
Ken Cornell, Ph.D.	Member, Supervisory Committee

The final reading approval of the thesis was granted by Cheryl L. Jorcyk, Ph.D., Chair of the Supervisory Committee. The thesis was approved for the Graduate College by John R. Pelton, Ph.D., Dean of the Graduate College.

DEDICATION

To my mother, Sandra. Your battle with breast cancer left me with a quest to gain knowledge and guided me to a path in science.

ACKNOWLEDGEMENTS

First of all, I would like to thank Dr. Cheryl Jorcyk for the opportunity to work in her lab. I still can't believe she accepted me before she even met me, but through it all, she never lost her trust in my abilities. The members of the Jorcyk lab also helped me so much during my three years at BSU. I would especially like to thank Dr. Randy Ryan for guidance on my project, Ken Tawara for always answering any question I asked, and Dr. Celeste Bolin for always being willing to help at the VA. The others lab members, Dollie, Jake, and Hunter, have become great friends, and I would like to thank them for helping me get through the tough times. I would like to thank my committee members, Dr. Julia Oxford and Dr. Ken Cornell, for all of their help and direction. Within the Biology Department, I would like to thank Raquel Brown for donating immunofluorescent antibodies and helping with Confocal imaging, and Laura Bond for helping retrieve Oncomine data. At the VA, my thanks go to Donna MacDonald and Dr. Craig Peterson. Finally, I would like to thank my family. You have supported me in every decision I have made; even my decision to be a perpetual student. Thanks again to all of you!

ABSTRACT

Oncostatin M (OSM) is an interleukin-6 (IL-6) family cytokine shown to be important in inflammation, hematopoiesis, development, and bone homeostasis. Despite its role as a growth suppressor for many cancers, including breast cancer, OSM is currently being studied for its ability to promote tumor invasion and metastasis. Cathepsin D (CTSD) is a lysosomal protease found to be overexpressed and hypersecreted in breast and other cancers. In this study, we found OSM to induce the expression of CTSD protein in human breast cancer cells via the STAT3 and JNK2 pathways. Next, we investigated mechanisms resulting in the increased secretion of CTSD from tumor cells. Previous reports have shown that acidic extracellular pH and cellular transformation stimulate lysosomal trafficking, increase secretion of lysosomal proteases, and increase invasion. In this study, we observed that OSM induced a change in cellular morphology and that CTSD-containing lysosomes traffic to the newly formed cellular protrusions. The trafficking and secretion of CTSD was dependent on Na^+/H^+ exchanger (NHE) activity and OSM activation of the PI3K and p38 MAPK pathways. OSM induced the secretion of physiologically active CTSD, which correlated with lysosomal location. Knockdown of CTSD also prevented an increase in invasive potential, even in the presence of OSM. Together, these results suggest that the expression of CTSD and location of CTSD-containing lysosomes are important aspects of the increase in invasive potential of tumor cells induced by OSM. This study provides

further evidence that OSM may be an important therapeutic target for in the early stages of breast cancer metastasis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
BSU	Boise State University
CTSB	Cathepsin B
CTSD	Cathepsin D
CM	Conditioned media
ECM	Extracellular matrix
EIPA	5-(*N*-ethyl-*N*-isopropyl)-amiloride, NHE inhibitor
EMT	Epithelial-mesenchymal transition
ER	Estrogen Receptor
FBS	Fetal bovine serum
gp130	Glycoprotein 130 IL-6 family cytokine receptor
HGF	Hepatocyte growth factor
hOSM	Human oncostatin M
IF	Immunofluorescence
IL-6	Interleukin-6
JAK-STAT	Janus Kinase-Signal Transducer and Activator of Transcription

JNK	c-jun NH2-Terminal Kinase
kDa	Kilodalton
LAMP1	Lysosomal-associated membrane protein 1
LIF	Leukemia inhibitory factor
LIFR	Leukemia inhibitory factor receptor
LIFRβ	Leukemia inhibitory factor receptor beta
MAPK	Mitogen-Activated Protein Kinase
MCF7	Weakly metastatic luminal human breast cancer cell line
MKK	Mitogen-Activated Protein kinase kinase
MMP	Matrix Metalloproteinase
NHE	Na^+/H^+ Exchanger
OSM	Oncostatin M
OSMR	Oncostatin M receptor
OSMRβ	Oncostatin M receptor beta
p38 MAPK	p38 mitogen-activated protein kinases
PBS	Phosphate-buffered saline
PBSAT	1X PBS/1% BSA/0.5%Triton X-100 buffer
RhoA	Ras homolog gene family, member A
ROCK1	Rho-associated, coiled-coil containing protein kinase 1

PI3K	Phosphatidylinositol 3-Kinase
siRNA	Small Interfering Ribonucleic Acid
SRC	Proto-oncogene tyrosine-protein kinase SRC
STAT	Signal transducers and activators of transcription
T47D	Weakly metastatic luminal human breast cancer cell line
VEGF	Vascular endothelial growth factor

INTRODUCTION

Over 200,000 new cases of breast cancer are reported each year in the United States. Moreover, breast cancer is the most commonly diagnosed cancer among women worldwide. The five-year survival rate of patients who present with distant metastases at the time of diagnosis is less than 25% [1], and breast cancer most commonly metastasizes to distant organs such as bone, liver, lung, and brain [2]. Metastasis is a complex, multi-step process involving primary tumor cell invasion, intravasation, survival in the circulation, extravasation, and colonization of a secondary organ [3]. In primary tumor cell invasion, proteases that are tightly controlled during mammary gland development and remodeling become deregulated and lead to excess extracellular matrix (ECM) degradation [4,5]. In addition, the ECM is remodeled via processes normally involved in inflammation and wound-healing [6]. This remodeling allows immune cells to infiltrate and provide additional ECM degrading proteases. The infiltrating immune cells also produce inflammatory cytokines that promote tumor progression such as those in the interleukin-6 (IL-6) family that have been related to increased malignancy in several cancer types [7–12].

The pleiotropic IL-6 family cytokine oncostatin M (OSM) has been shown to be important in inflammation, hematopoiesis, liver development and regeneration, neurogenesis, and bone homeostasis [13,14]. OSM was originally found to act as an anti-proliferative factor for A375 melanoma and other tumor cells *in vitro* [15], including multiple myeloma, lung cancer, glioblastoma, and breast cancer [16–18]. In contrast, OSM

has been shown to increase proliferation in prostate and ovarian cancer cells [19,20]. Despite its anti-proliferative effect, *in vivo* breast cancer research suggests a role for OSM in metastasis [8,21,22]. We and others have shown that *in vitro* OSM 1) suppresses estrogen receptor expression implying the progression to a more aggressive phenotype [23], 2) enhances the transition of cancer cells from an epithelial to mesenchymal phenotype (EMT), which has been linked to the acquisition of tumor stem cell-like properties [22,24,25], and 3) increases detachment, invasion, and migration [21,26–29] via the upregulation of proteases that degrade the extracellular matrix (ECM) [7,27,30–33] and induction of proangiogenic factors, such as vascular endothelial growth factor (VEGF) [7,33–35], which are all important in the initial stages of metastasis.

OSM and IL-6-family cytokines are known to activate several signal transduction pathways, including JAK/STAT3, PI3K/AKT, ERK1/2, JNK, and p38 MAPK [36–39], which have all been shown to play a role in breast cancer oncogenesis [40–43]. OSM activates these pathways via its binding and heterodimerization of gp130 with either OSMR or LIFR [36–39,44]. In ductal carcinoma *in situ* and invasive breast carcinoma, OSM[8,45] (our unpublished results) and OSMR[8] are expressed at higher levels than normal mammary epithelial cells, which express both at low levels. OSM can also be produced by both tumor-associated macrophages and neutrophils in response to cancer cells *in vitro* [46–48], suggesting autocrine and paracrine signaling by OSM in early stages of the metastatic cascade. How the OSM signaling pathway influences regulation of gene expression, angiogenesis, proteinase secretion, and cell motility and invasion are major areas of ongoing research.

Tumor cell invasion through the basement membrane and into the ECM involves local proteolysis and migration of tumor cells. Local proteolysis is facilitated by cell membrane bound and/or secreted proteases. Lysosomal proteases normally regulate many biological processes, such as tissue homeostasis, remodeling, and renewal by degrading and activating proteins within the lysosomes [49–51]. Alterations in the expression, activities, processing, and localization of lysosomal enzymes have been observed in several cancer types[52]. Therefore, many studies have looked at the role of lysosomal enzymes in ECM proteolysis, mainly the cysteine proteases cathepsins B and L and aspartic acid protease cathepsin D. All have been shown to degrade components of the ECM either intracellularly within phagolysosomes [53,54] or extracellularly due to altered localization to the plasma membrane [55,56]. Research also shows that anterograde trafficking of lysosomes (lysosomal movement away from the peri-nuclear region to the cell periphery) in malignant cells results in the secretion and exocytosis of lysosomal proteases [57–61]. These enzymes are synthesized as pro-enzymes and require activation at acidic pH found within endolysosomal vesicles [62–64]. However, extracellular pro-enzymes, such as cathepsin D (CTSD), can also undergo autoactivation [64,65] within the acidic extracellular environment hypothesized to occur in the tumor microenvironment [66]. In addition, active CTSD can be secreted from necrotic cells [67]. Once activated, CTSD can activate cathepsin B [68], promoting a cancer-associated proteolytic cascade further enhancing ECM degradation [69–72].

Previously, we have shown the OSM increases the secretion of the cathepsins D and L in T47D breast cancer cells [27]. In human breast cancer, increased CTSD expression is associated with a poor prognosis, worse overall survival, and poor relapse-free survival

[73,74]. Higher expression levels of CTSD have been seen in node positive versus node negative breast cancer tissue. In addition, higher CTSD levels are seen in cells involved in metastatic reoccurrence[75,76], while CTSD expression in the tumor stroma is associated with more aggressive breast cancer and an increase in microvessel density [77]. *In vitro*, CTSD is upregulated by estrogen, epidermal growth factor, insulin-like growth factor I, and basic fibroblast growth factor in human estrogen responsive breast cancer cells [78–80]. In human estrogen-independent breast cancer cell lines, CTSD was shown to be constitutively overexpressed [79,81], but recent reports show that thrombin [82] and the transcription factor c-myb [83] upregulate CTSD expression in these cell lines. Decreasing the expression, activity, and/or secretion of CTSD leads to a decrease in invasion and migration in human breast cancer and glioma cell lines [61,82–85], showing its importance in tumor cell metastasis.

In this study, we show that OSM has a double influence on CTSD in human breast cancer cells. OSM both increases the expression of CTSD via STAT3 and JNK2 activation and induces secretion of CTSD by promoting trafficking of lysosomes to the cell periphery in a manner dependent on PI3K, p38 MAPK, and NHE activity. In addition, we show that CTSD plays a role in the increased invasion seen by OSM-treated cancer cells. Overall, these findings indicate roles for lysosomal trafficking and lysosomal cathepsin D are key events in OSM-induced invasion of breast cancer cells.

MATERIALS AND METHODS

Cell Culture and Cytokine Stimulation

MCF7 and T47D human breast cancer cells are luminal epithelial-like estrogen receptor positive cells[86]. MCF7 and T47D cells were obtained directly from the American Type Culture Collection (Rockville, MD), maintained in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) and 100 U/mL each of penicillin and streptomycin. Cells were maintained at 37°C, 5% carbon dioxide, and 95% humidity. All media and supplements were obtained from Hyclone (Logan, UT). Human Oncostatin M (OSM) (Peprotech, Rocky Hill, NJ), interleukin-6 (IL-6), and leukemia inhibitory factor (LIF) (R & D Systems, Minneapolis, MN) were added at 25 ng/ml.

Western Blot Analysis

For whole cell lysates, 5 x 10^4 cells were seeded into 24-well plates (Thermo Fischer Scientific, Rockford, IL) unless otherwise stated. At the end of the experiment, plates were rinsed with ice-cold PBS, then placed on ice with 125 µl of RIPA buffer (Thermo Fischer Scientific) supplemented with 1:100 dilution of Sigma Protease Inhibitor Cocktail and 1 µl/ml phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, MO) for 15 minutes, mixing every 5 minutes. Plates were scraped and the contents transferred to an eppendorf tube. Tubes were then centrifuged at 14,000 rpm for 20 minutes.

For conditioned media, 2 x 10^5 cells were plated in a T25 flask containing RPMI media with 10% FBS. After 24 h, cells were treated with cytokines for 6 days. After 5 days of cytokine treatment, media was replaced with serum-free RPMI along with fresh cytokines. Conditioned media was collected after 24 h. Floating cells were removed by centrifugation and media was concentrated using an Amicon Ultra-4 Centrifugal Filter with a 10K membrane (Millipore, Billerica, MA).

Lysates were run on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Immunoblot analysis was performed using primary antibodies for human cathepsin D (1:500) (Calbiochem), JNK2 (1:500) (R & D Systems), β-actin (1:2000), phospho-STAT3 (Tyr705; 1:1000), STAT3 (1:500), or JNK1 (1: 500) (Cell Signaling, Beverly, MA). Secondary antibodies were HRP-conjugated donkey anti-rabbit or goat anti-mouse (1:5000-10,000) (Jackson Immuno Research Labs, West Grove, PA).

Conditioned media was run as described above except for as a loading control the membrane was incubated in Ponceau S stain (0.1% (w/v) in 5% (v/v) acetic acid) for 1 hour and then destained using distilled water before immunoblot analysis.

RNA Interference and Chemical Inhibitors

Gene knockdown of STAT3, JNK1, or JNK2-specific ON-TARGET plus siRNA or ON-TARGET plus non-targeting siRNA (Dharmacon, Lafayette, CO) was conducted using Hyperfect reagent (Qiagen, Valencia, CA) at a final concentration of 20 nM for 24 hours before OSM stimulation. Seventy-two hours after the addition of OSM, cells were harvested for immunoblotting analysis. Gene knockdown of CTSD was conducted using 2.5 x 10^5 cells in a 6-well plate (Thermo Fischer Scientific). A CTSD-specific Silencer Select siRNA (Applied Biosystems, Foster City, CA) was added using Hyperfect reagent

at a final concentration of 25 nM. After 24 hours, the media was replaced, and then 24 hours later, media was replaced with serum-free media containing OSM. 72 hours after the addition of OSM, cells were processed for a Matrigel invasion assay, as described below. At the same time, media was removed and condensed, and cells were harvested as described above for immunoblotting.

Inhibitors of the NA^+/H^+ exchanger (25 μM) (EIPA; Santa Cruz Biotechnology), MEK1/2 (5 μM) (U0126; Cell Signaling), PI3K (10 μM) (LY294002), p38 MAPK (10 μM) (SB203580), Stat3 (5 μM) (Stat3 peptide inhibitor) or JNK (10 μM) (JNK inhibitor II; all from Calbiochem) were added on day 4 of cytokine treatment for conditioned media and replaced with the serum-free media and cytokines on day 5. For immunofluorescence, inhibitors were added on day 3 of cytokine treatment.

Immunofluorescence Staining and Microscopy

2.5×10^5 cells were plated onto glass coverslips (Thermo Fisher Scientific). After 24 hours, cells were treated with or without OSM for 5 days. After experimental treatment, cells were rinsed once with PBS and then fixed in ice-cold methanol for 20 minutes. Cells were then rinsed 3x with PBS and permeabilized in 0.1% Triton X-100/1X PBS for 10 minutes. Cells were then blocked in PBSAT (1X PBS/1% BSA/0.5%Triton X-100) for 30 minutes and then incubated in PBSAT containing the primary antibodies specific for human cathepsin D (1:200), LAMP1 (1:200; Millipore, Temecula, CA), and/or actin (1:400; Cell Signaling) overnight. Cells were then washed 3× in PBS and incubated with PBSAT containing goat anti-mouse Alexa Flour 488 (1:1000) and donkey anti-rabbit Alexa Flour 546 (1:500; Molecular Probes, Invitrogen, Carlsbad, CA) secondary antibodies for 2 h. Cells were then washed 3× in PBS and mounted using

Prolong Gold DAPI (Molecular Probes). Stains were visualized and confocal microscopy images were taken using a Zeiss LSM 510 Meta system combined with the Zeiss Axiovert Observer Z1 inverted microscope and ZEN 2009 imaging software (Carl Zeiss, Inc., Thornwood, NY).

Invasion Assay

The invasion assay was conducted as described previously [46] with the following modifications. A total of 2.5 x 10^4 cells from CTSD or non-target siRNA treated cells were plated with or without OSM in the upper chamber of the Matrigel-coated invasion chambers (BD Biosciences, Bedford, MA) in serum-free media, while media containing 10% FBS was added to the bottom chamber as a chemoattractant and cells were allowed to invade for 22 hours. Invasion chambers were fixed in methanol and stained using crystal violet. Data are presented as previously described [21].

Activity Assay

Conditioned media was collected as described above. Active cathepsin D was then detected via a fluorescent cathepsin D (CTSD) activity assay (Anaspec, Fremont, CA). The assay was run according to the manufacturer's protocol. To summarize, 2 x 105 cells were plated in a T25 flask containing RPMI media with 10% FBS. After 24 h, cells were treated with cytokines for 6 days. After 5 days of cytokine treatment, media was replaced with serum-free RPMI along with fresh cytokines. Conditioned media was collected after 24 h. Floating cells were removed by centrifugation and media was concentrated using an Amicon Ultra-4 Centrifugal Filter with a 10K membrane (Millipore, Billerica, MA). CTSD activity was then measured using a Mca/FRET

peptide, in which the FRET peptide is a cleavage site for CTSD. The fluorescence of Mca is quenched until the peptide is cleaved and measured as at excitation/emission of 330nm/390nm. For data analysis, the relative fluorescent units (RFU) measured after 60 minutes versus concentration of a serially diluted Mca standard were plotted. The amount of Mca produced by active CTSD from each experimental sample was determined from the standard.

RESULTS

OSM Increases the Expression and Secretion of CTSD

Cathepsin D (CTSD) has been found to be overexpressed in several neoplastic tissues [87] and carcinoma cells[88] and can be induced by estrogen and other growth factors in breast cancer cells [78]. CTSD is synthesized as a prepro-enzyme and after removal of the signal peptide, the 52 kDa proCTSD is targeted to the lysosomes[89]. The cleavage of the N-terminus results in a 48 kDa intermediate enzyme and further cleavage yields the active form composed of heavy (34 kDa) and light (14 kDa) chains associated by non-covalent bonds [90–92].

We have previously shown that OSM induces the secretion of CTSD from T47D human breast cancer cells, but it was not known if OSM-induced the expression of CTSD. To determine if OSM regulates CTSD expression, we treated MCF7 and T47D luminal epithelial-like/ER^+ human breast cancer cells for 24, 48, and 72 hours with 25 ng/ml of OSM. OSM induced a time-dependent induction of protein expression of all cellular isoforms of CTSD (Fig. 1). OSM treatment in MCF7 human breast cancer cells induced a greater increase in CTSD protein levels as early as 24 hours (Fig. 1A; left panel). T47D human breast cancer cells also induced CTSD protein levels, but differences in induction by OSM were not identified until 48 hours (Fig. 1A; right panel). To see if other IL-6 inflammatory cytokines also regulate CTSD expression, we treated MCF7 and T47D cells with IL-6 and leukemia inhibitory factor (LIF) for 72 hours. In

MCF7 cells, we observed a dramatic increase in CTSD protein expression with OSM treatment compared to IL-6 and LIF treatment (Fig. 1B; left panel). This trend was also seen in T47D cells (Fig. 1B; right panel). This indicates that OSM strongly promotes protein expression of CTSD and that OSM is a more potent inducer than other IL-6 family cytokines.

The secretion of CTSD from the cell appears to play a role in the progression of many cancers. The secreted forms of CTSD include the pro-form (52 kDa) and the mature form (heavy chain 34 kDa; light chain 14 kDa). Pro-CTSD acts as a mitogen for cancer and stromal cells [93,94], while the mature, active CTSD can directly modulate the ECM [49] or activate a proteolytic cascade in which other proteases modulate the ECM [69,70]. Immunoblot analysis was performed using 10 µg of concentrated conditioned media collected from MCF7 and T47D human breast cancer cells treated with or without OSM, IL-6, or LIF (25 ng/ml) for 6 days. As seen in Fig. 1C, OSM-induced secretion of pro-CTSD (52 kDa) and mature CTSD (34 kDa) in both MCF7 and T47D human breast cancer cell lines. The secretion of CTSD by OSM was greater than that of the control, IL-6, or LIF treatments. Ponceau staining of the immunoblot confirmed equal loading of secreted protein (Fig. 1C; bottom panel). This indicates that OSM enhances the secretion of both the pro and active forms of CTSD to a greater extent than other IL-6 family cytokines.

STAT3 and JNK2 Pathways Are Required for OSM-Induced CTSD Expression

To identify the signaling pathway(s) necessary for the induction of CTSD expression by OSM in human breast cancer cells, we selectively inhibited the JAK/STAT pathway using a siRNA to STAT3 or the JNK pathway using JNK1 and JNK2 siRNAs

during cytokine treatment in MCF7 and T47D cells. In both the MCF7 and T47D cells, inhibition of STAT3 expression knocked down the induction of CTSD by OSM (Fig. 2A) while inhibition of JNK1expression did not affect OSM-induced CTSD expression (Fig. 2B). Inhibition of JNK2 also reduced the induction of CTSD by OSM in both cell lines (Fig. 2C). It is interesting to note that although STAT3 and JNK2 knockdown blocked OSM-induced CTSD expression, basal levels remained and active CTSD (34 kDa) was present at high levels even though proCTSD (52 kDa) expression was inhibited, especially in the T47D cells (Fig 2 A-C; right panels). This could imply that OSM regulates the transcription of new CTSD protein and not the processing of CTSD from the pro to active forms. In addition, inhibition using inhibitors of PI3K (LY294002), MEK1/2 (U0126), and p38 MAPK (SB203580) had no effect on OSM-induced CTSD expression (data not shown). Thus, activation of STAT3 and JNK2 appear to be necessary for the transcriptional regulation of CTSD by OSM.

OSM Induces Trafficking of CTSD-Containing Lysosomes to Cellular Protrusions

A hallmark of OSM-stimulated MCF7 or T47D human breast cancer cells *in vitro* is a loss in cobblestone epithelial morphology, leading to an increased migration and invasive potential [26–28]. To determine if the formation of cellular protrusions induced by OSM treatment was accompanied by a change in the intracellular distribution of lysosomes, T47D cells were treated for 5 days before staining for the lysosomal marker, lysosome-associated membrane protein-1 (LAMP-1) and the cytoskeletal filament, actin (Fig. 3A). Figure 3A (right panel) shows that with OSM treatment, LAMP-1 positive vesicles (red) undergo anterograde trafficking toward the cell periphery. The figure also shows that OSM induces cytoskeletal rearrangement of actin (green), resulting in

increased cellular protrusions, similar to pseudopodia observed by others [95,96]. Lysosomal trafficking to the cellular protrusions was observed. Thus, morphological changes induced by OSM occur with anterograde lysosomal trafficking.

To determine if the lysosomes contained CTSD, cells were co-stained to detect LAMP-1 (red) and CTSD (green) after 5 day treatment with OSM (Fig. 3B). Co-localization is seen by the yellow vesicles in both the absence and presence of OSM (Fig. 3B; right panels). The OSM treatment shows not only co-localization, but also anterograde trafficking of lysosomes containing CTSD to cellular protrusions with a high concentration of co-localized vesicles at the end of the protrusions (Fig. 3B; lower panels). Lysosomal cathepsins have been shown to be important in invasion when trafficking is altered; therefore, this could an important mechanism in the increased secretion of cathepsins and increased invasive potential seen with OSM treatment.

Moreover, increased activity of Na^+/H^+ exchangers (NHE) have been shown to play a role in lysosome trafficking [97,98] and NHE activity has been shown to be related to invadopodia-like structures [96,99] that degrade the ECM [100]. To determine if OSM-induced cellular protrusions and lysosomal trafficking require NHE activity, cells were treated with 25 μM of the NHE inhibitor, EIPA (5-[*N*-ethyl-*N*-isopropyl]-amiloride) with or without OSM. EIPA was able to prevent OSM-induced anterograde lysosomal trafficking and decreased the secretion of CTSD by OSM (Fig. 3C). These data suggest that the NHE activity drives OSM-induced lysosomal trafficking and CTSD secretion from human breast cancer cells.

PI3K and p38 MAPK Pathways Are Required for OSM-Induced Lysosomal Trafficking

To determine which pathway(s) are important for OSM-induced anterograde lysosomal trafficking, pathway specific inhibitors were used to block OSM signaling in T47D human breast cancer cells. PI3K inhibition with LY294002 and p38 MAPK inhibition with SB203580 almost completely reversed anterograde lysosomal trafficking containing CTSD to cellular protrusions (Fig. 4; panels 2-3). However, inhibition of MEK with U0126, STAT3 with STAT3 inhibitor peptide, or JNKs 1, 2 and 3 with JNK inhibitor II did not alter lysosomal trafficking (Fig. 4; panels 4-6). This suggests that both PI3K and p38 MAPK pathways play a major role in OSM-induced lysosomal trafficking. Furthermore, these results imply that the signaling pathway for OSM-induced lysosomal trafficking is different than the pathway for OSM-induced CTSD expression.

Enzymatically Active CTSD and Upregulation of CTSD Is Important for OSM-Induced Invasion

Altered localization and secretion of lysosomal cathepsins have been implicated in the localized proteolysis of the ECM and increased cell invasion [57,83,84,95]. PI3K and p38 MAPK inhibition not only block lysosomal trafficking, but also the secretion of CTSD in T47D human breast cancer cells. Figure 5A shows that the secretion of both the pro and active forms of CTSD also decreased with PI3K (LY294002) and p38 MAPK (SB203580) inhibition, suggesting a role of lysosomal trafficking in OSM-induced CTSD secretion.

To determine if the increased secretion of CTSD by OSM (Fig. 1C, Fig 5A) had a physiological consequence, the activity of the secreted CTSD was measured using a

fluorescent activity assay with a CTSD specific substrate. OSM treatment induced a two-fold increase in the amount of active CTSD in the media (Fig. 5B). We also found that PI3K (LY294002) and p38 MAPK (SB203580) inhibitors blocked the OSM induced CTSD activity (Fig. 5B). This shows a correlation between the trafficking of lysosomes and the amount of active CTSD secreted from the cell.

Next, to confirm the role of CTSD in OSM-induced invasion, RNAi-mediated silencing of CTSD in T47D cells was performed resulting in a decrease of OSM-induced CTSD expression (Fig. 5C; right panel). Cells were pretreated for 3 days with or without OSM, plated onto a Matrigel chamber, and allowed to invade for 22 hours. OSM treatment induced invasion of control siRNA T47D human breast cancer cells through a Matrigel chamber. OSM treated T47D cells with reduced CTSD induction had invasion levels similar to non-treated, control siRNA T47D cells (Fig. 5C; left panel). This data shows that knockdown of CTSD prevented the increased invasive potential, suggesting that CTSD plays an important role in OSM-induced invasion.

DISCUSSION

Tumor growth and invasion can be regulated by many factors in the tumor microenvironment. The presence of IL-6 family cytokines have been suggested to contribute to tumor progression since it has been shown that chronic inflammation promotes tumorigenesis [6,101,102]. While OSM has an anti-proliferative effect on many tumor types, it appears to play a role in tumor invasion and metastasis. Here we report a novel role for OSM signaling in breast cancer. When activated, OSM pathways induce the expression of CTSD and anterograde trafficking of lysosomes containing CTSD, resulting in an increase in CTSD secretion and invasion. OSM-induced CTSD expression depends on STAT3 and JNK2 pathways, while OSM-induced lysosomal and CTSD trafficking depends on NHE activity and the PI3K and p38 MAPK pathways. OSM induces the secretion of physiologically active CTSD, which also requires the PI3K and p38 pathways and may play a role in OSM-induced invasion since invasion was inhibited with CTSD knockdown. These data suggest that OSM signaling controls lysosomal distribution in tumor cells and that invasion may depend on lysosomal distribution and secretion of CTSD.

In tumor progression, proteolytic enzymes have important functions and are often abnormally expressed or secreted [69].These proteases mediate the degradation of the ECM, increasing tumor cell invasion to a secondary site. CTSD is a lysosomal protease found to be overexpressed and hypersecreted in breast cancer [78,80,103,104]. Studies have shown that

CTSD is important for invasion, migration, metastasis, and angiogenesis [61,82–85,95]. Here we show that OSM is a potent inducer of CTSD expression and secretion in MCF7 and T47D human breast cancer cell lines and this increase in expression and secretion are greater than IL-6 and LIF (Fig. 1A-C). Our data show that the protease CTSD may contribute to the increase in breast cancer cell invasion, migration, metastasis, and angiogenesis by OSM that we and others have seen.

Next, signaling pathways of OSM-induced CTSD expression were investigated. STAT3 is the classical signaling pathway for OSM and other IL-6 family cytokines. OSM has also been shown to be a strong activator of the JNK MAPK pathway specifically through the gp130/OSMRβ complex [44]. We found that JNK1 has no effect on CTSD induction, but that OSM signals through STAT3 and JNK2 induce CTSD expression (Fig. 2). The JNK protein kinases are activated by MKK4 or MKK7 in response to cytokines or cell stress, and studies have shown their importance in malignancy [105]. However, JNK2 may not directly promote CTSD expression. MKK4 [106], the upstream activator or JNK2, and JNK2 [107] both regulate the serine 727 phosphorylation of STAT3. Phosphorylation of this site is required for maximal STAT3 transcriptional activation [108,109]. Thus, JNK2 may be required for maximal transcription of cathepsin D induced by OSM via STAT3.

Several studies have focused on OSM-induced EMT changes in cell morphology [22,24,25], but our data shows another important and novel implication of the morphological changes induced by OSM. We show that OSM promotes cellular protrusions containing lysosomes and CTSD that have undergone anterograde trafficking (Fig. 4B). Others have shown that cellular protrusions containing lysosomes or CTSD were induced by cellular

transformation [57,84,110], HGF treatment [59], or acidic extracellular pH [95,98]. Additionally, Tu et al. have shown that lysosomal trafficking to podosomes in SRC-transformed fibroblasts resulted in ECM degradation due to protease secretion [111]. Since OSM induces the secretion of lysosomal proteases, OSM-induced lysosomal trafficking is likely to be critical for the increased invasive potential with OSM treatment. More work will need to be completed to identify whether lysosomal phagocytosis or exocytosis occurs in OSM-induced trafficking. We see large vesicles co-stained with LAMP-1 and CTSD that undergo anterograde trafficking with OSM (Fig. 3b). These could represent degradation of the ECM via phagocytosis of ECM[53,54], which would mean CTSD would be actively degrading the ECM within the acidic vesicles. However, we do see an increase in secretion of CTSD with OSM treatment. This suggests induction of lysosomal exocytosis [60,61], especially because our cells were not cultured on an artificial ECM, and thus it would be unlikely that the large vesicles contain phagocytic material.

Lysosomal proteases that undergo exocytosis still need an acidic environment for maximal enzymatic activity. Increased Na^+/H^+ exchanger isoform 1 (NHE1) activity decreases the pericellular pH. NHE1 activity results in cytoskeletal reorganization and formation of cellular protrusions at the leading edge [97] thought to represent invadopodia and lamellipodia [97,99] in which NHE1 becomes localized [96]. NHE1 activation may be a key event in oncogenesis[112] as the decrease in pericellular pH creates a favorable environment for many proteases, such as lysosomal cathepsins, that play a role in tumor cell migration and invasion due to degradation of the ECM at the invadopodia-like structures [100]. Since the NHE inhibitor, EIPA, reversed the anterograde lysosomal trafficking in cells treated with OSM (Fig. 3C), we show that lysosomal trafficking is

mediated through the activity of NHEs. Our 2D model supports a link between OSM and NHE activity, which to our knowledge has never been shown. Based on these data, we hypothesize that OSM increases NHE1 activity to promote cellular protrusions and decrease pericellular pH, which result in lysosomal trafficking, the subsequent secretion and activation of lysosomal proteases, and cancer cell invasion. This would support the role of lysosomal exocytosis instead of phagocytosis. EIPA inhibits several NHE isoforms; thus, more work will need to be done to verify that the NHE1 is the most important isoform in OSM-induced lysosomal trafficking as seen by others. In addition, future studies will need to be done to determine if using EIPA to block NHE activity not only blocks the movement of lysosomes but also decreases OSM-induced invasive potential. The use of 3D models will help to show whether the increase in NHE activity and the movement of lysosomes to the periphery induced by OSM participate in the direct degradation of the ECM.

We also saw that inhibitors of the PI3K (Fig. 4; panel 2) and p38 MAPK (Fig. 4; panel 3) pathways reversed the lysosomal trafficking induced by OSM. Others have shown that trafficking of lysosomes induced by HGF and acidic extraceullular pH is mediated through the PI3K pathway [59,98] and can induce cytoskeletal changes [24,43]. While others have shown the p38 MAPK to be important in cytoskeletal changes [113–115]. Cardone et al. [97] summarize the role of both PI3K and p38 in regulating NHE1 activity. At the pseudopodia distal tip, p38 regulates NHE1 via the PKA–RHOA–ROCK1–p38 signaling cascade. This process is also mediated by PI3K [116]. In normal cells, p38 has also been shown to directly phosphorylate NHE1, which could indicate a disregulated mechanism in cancer cells [117]. Our data indicate that PI3K and p38 pathways activated by

OSM might drive NHE1 activity, leading to anterograde lysosomal trafficking. However, more work needs to be done to elucidate the roles of PKA, RHOA and ROCK1 in the signaling cascade.

While reports have been conflicting about the roles of precursor versus active CTSD in the extracellular milieu, previous studies have shown that an increase in active CTSD is a useful indicator of poor outcome in node positive breast cancer patients [118]and that mature CTSD in patient serum was a valuable marker since it was only found in breast cancer patients with metastatic disease [74]. We saw a two-fold increase in active CTSD secretion induced by OSM (Fig. 5B) in breast cancer cell lines supporting the role of enzymatically active CTSD in the direct degradation of the ECM [119–123] and its role in the proteolytic cascade [69–72] leading to an increased rate of metastases. Moreover, we showed that OSM-induced CTSD plays a role in the increased invasion seen with OSM (Fig. 5C). These data support the role of lysosomal exocytosis since OSM induces the secretion of active CTSD due to lysosomal trafficking that depends on NHE activity.

A proposed model summarizing OSM signaling in breast cancer is illustrated in Figure 6. The first label shows that OSM induces CTSD expression through activation of the STAT3 and JNK2 pathways. Second, OSM signaling through PI3K and p38 MAPK induces lysosomal trafficking to cellular protrusions that exhibit altered actin organization. Third, OSM regulates NHE activity, which induces lysosomal trafficking. PI3K and p38MAPK activation by OSM may mediate the increase in NHE signaling by OSM.

In conclusion, this study demonstrates for the first time that OSM promotes CTSD expression and secretion via lysosomal trafficking. We show a novel role for OSM

in lysosomal trafficking that is mediated through OSM-induced NHE1 activity and possible link to invadopodia-like structures. Based on this study, we suggest OSM could be a viable target in the early stages of breast cancer cell metastasis.

Figure 1

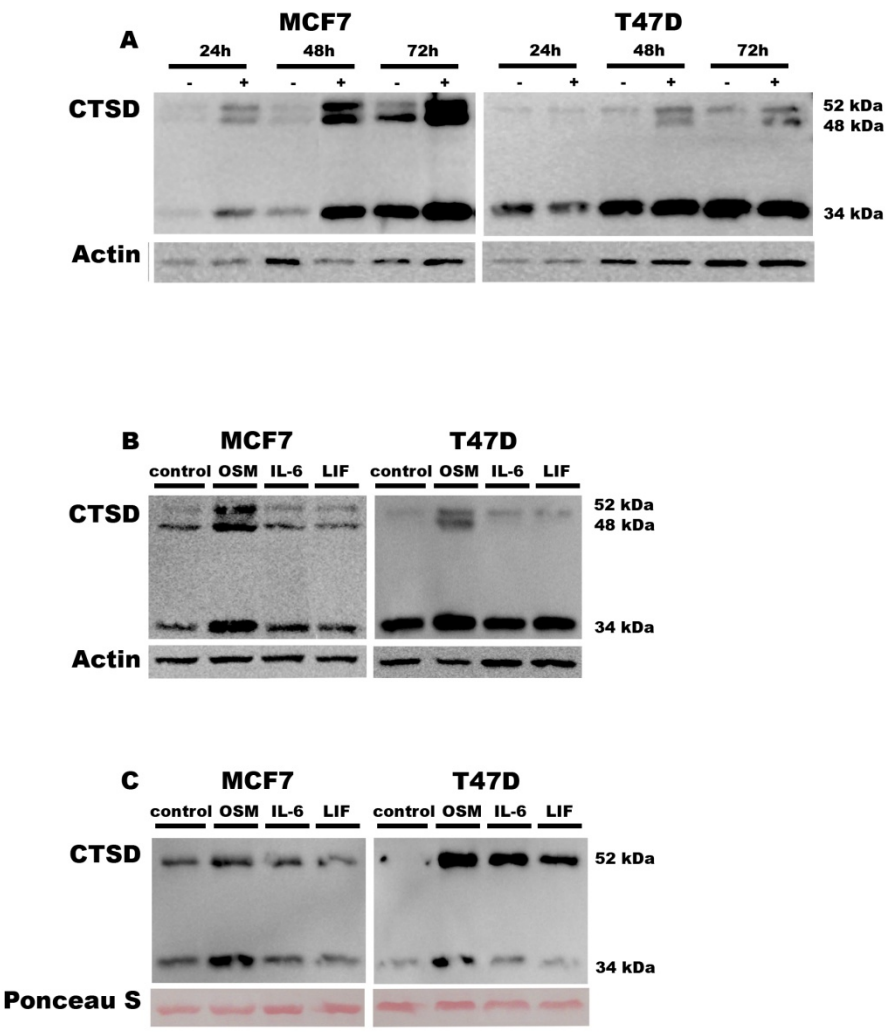

Figure 1. OSM induces expression and secretion of cathepsin D from human breast cancer cells *in vitro*. A) Time dependent induction of intracellular CTSD protein by OSM, and B) induction of CTSD protein by IL-6 family cytokines in MCF7 and T47D cells. Cells were treated for 72 hours with 25 ng/ml of the indicated cytokine. C) Secreted CTSD levels (as measured by Western blot analysis) were higher in OSM treated MCF7 and T47D than other IL-6 family cytokines. Cells were treated for 6 days with 25 ng/ml of the indicated cytokine.

Figure 2

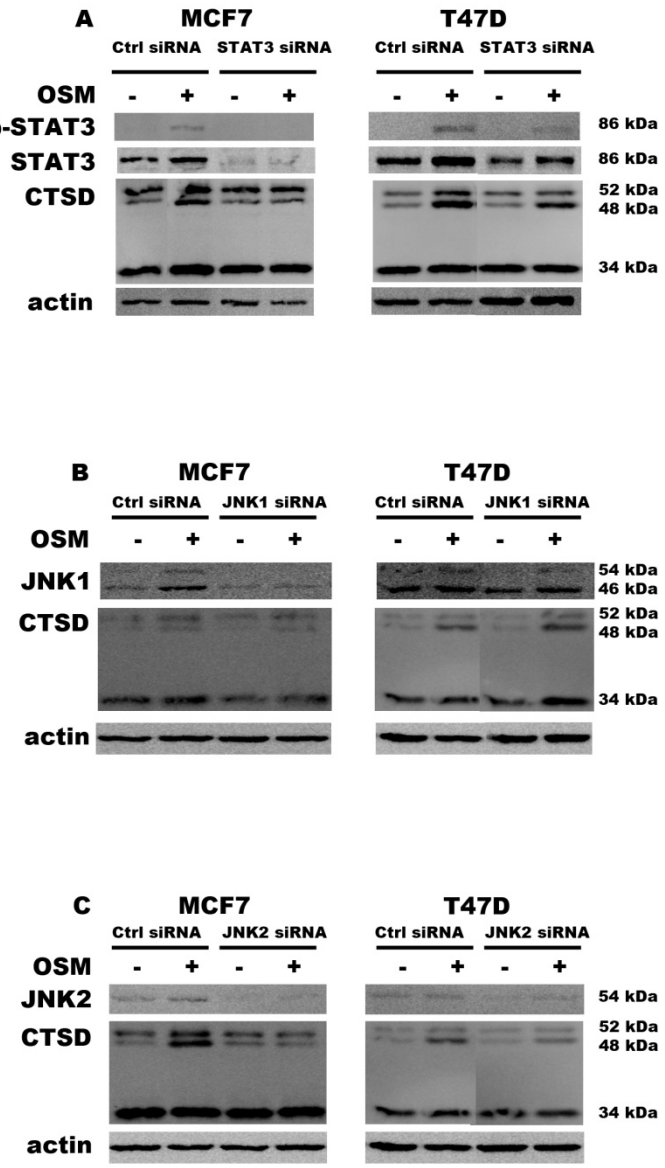

Figure 2. STAT3 and JNK2 signaling regulate cathepsin D expression from human breast cancer cells *in vitro*. CTSD protein levels after A) STAT3 inhibition by transfection of STAT3 siRNA, B) JNK1 siRNA, and C) JNK2 siRNA 24h before OSM treatment in MCF7 and T47D cells. Cells were harvested after 72h treatment with or without 25 ng/ml of OSM and analyzed by Western blot analysis.

Figure 3

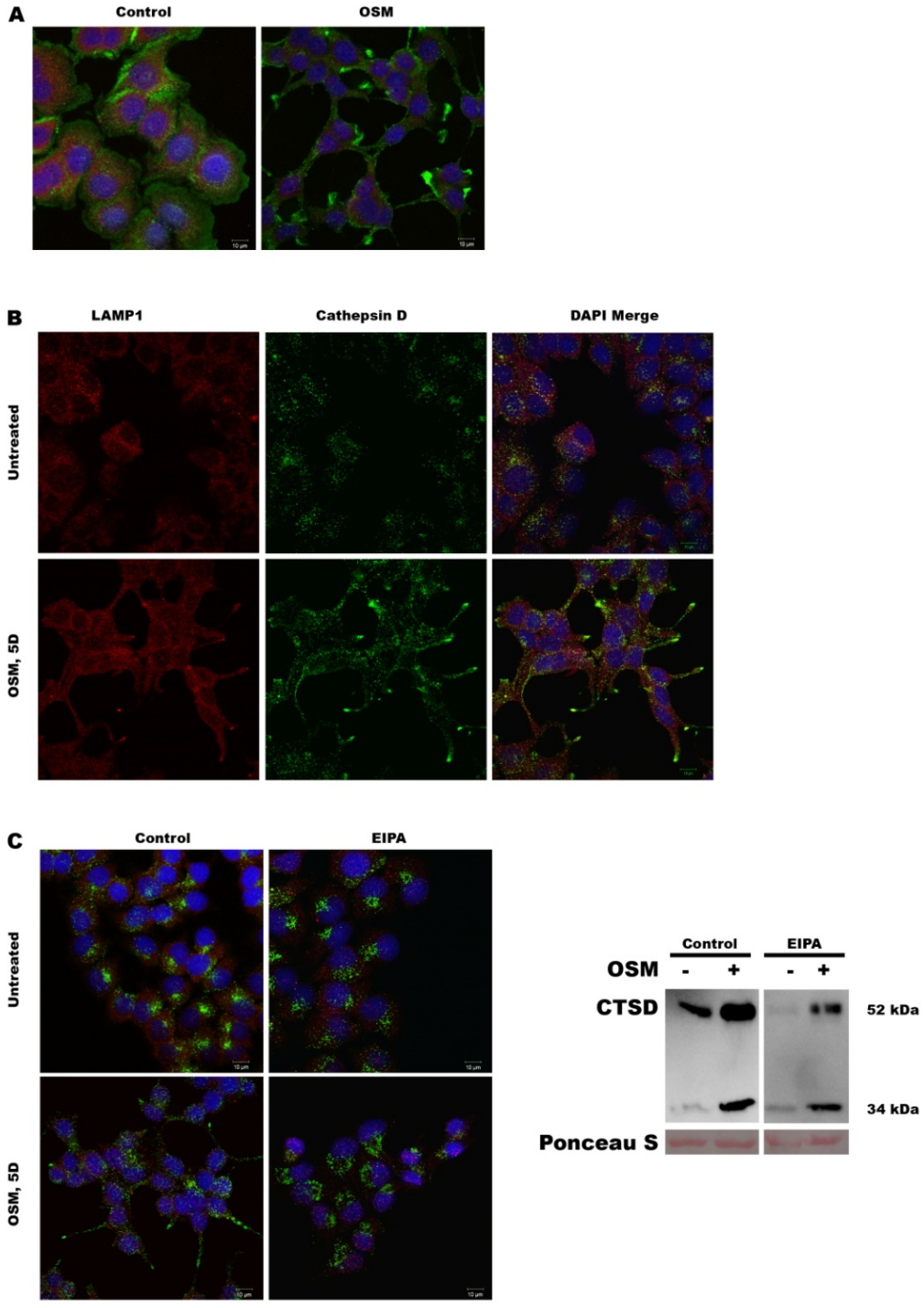

Figure 3. OSM induces changes in cell morphology and anterograde lysosomal trafficking requires NHE activity. A) Representative merged IF images depicting the

morphological changes of actin (green) and trafficking of lysosomes (red) before and after 5d treatment with OSM in T47D cells. DAPI stained nuclei appear blue. B) LAMP1 (red; left panels) and cathepsin D (green; middle panels) colocalize (yellow; right panels) in the absence and presence of OSM. Shown also are nuclei (blue). Vesicles traffic to the cell periphery in OSM treated cells (bottom panels). C) Representative merged IF images of EIPA, a sodium-proton exchangers (NHE) inhibitor, treatment on day 3 of 5d OSM treatment; lysosomes (red), cathepsin D (green), and nuclei (blue). Secreted CTSD levels (Western blot) also decreased with EIPA treatment even in the presence of OSM.

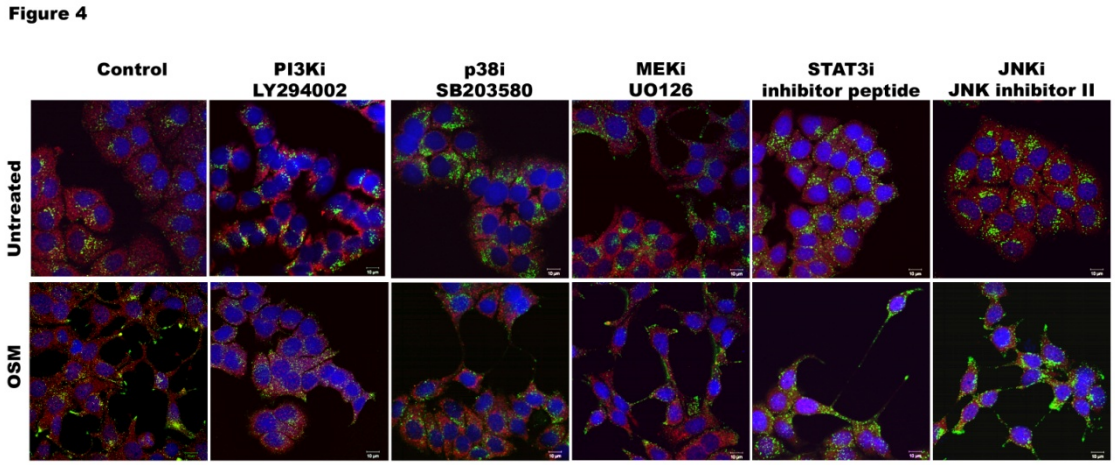

Figure 4. Trafficking of CTSD within the lysosomes is dependent on PI3K and p38 MAPK pathways. Representative merged IF images of signaling inhibitor treatment added on day 3 of 5d OSM treatment. LAMP1 (red) and cathepsin D (green) colocalize (yellow) in the absence and presence of OSM. Shown also are nuclei (blue).Vesicle trafficking to the cell periphery is blocked with the PI3K (LY294002) and the p38 MAPK (SB203580) inhibitors.

Figure 5

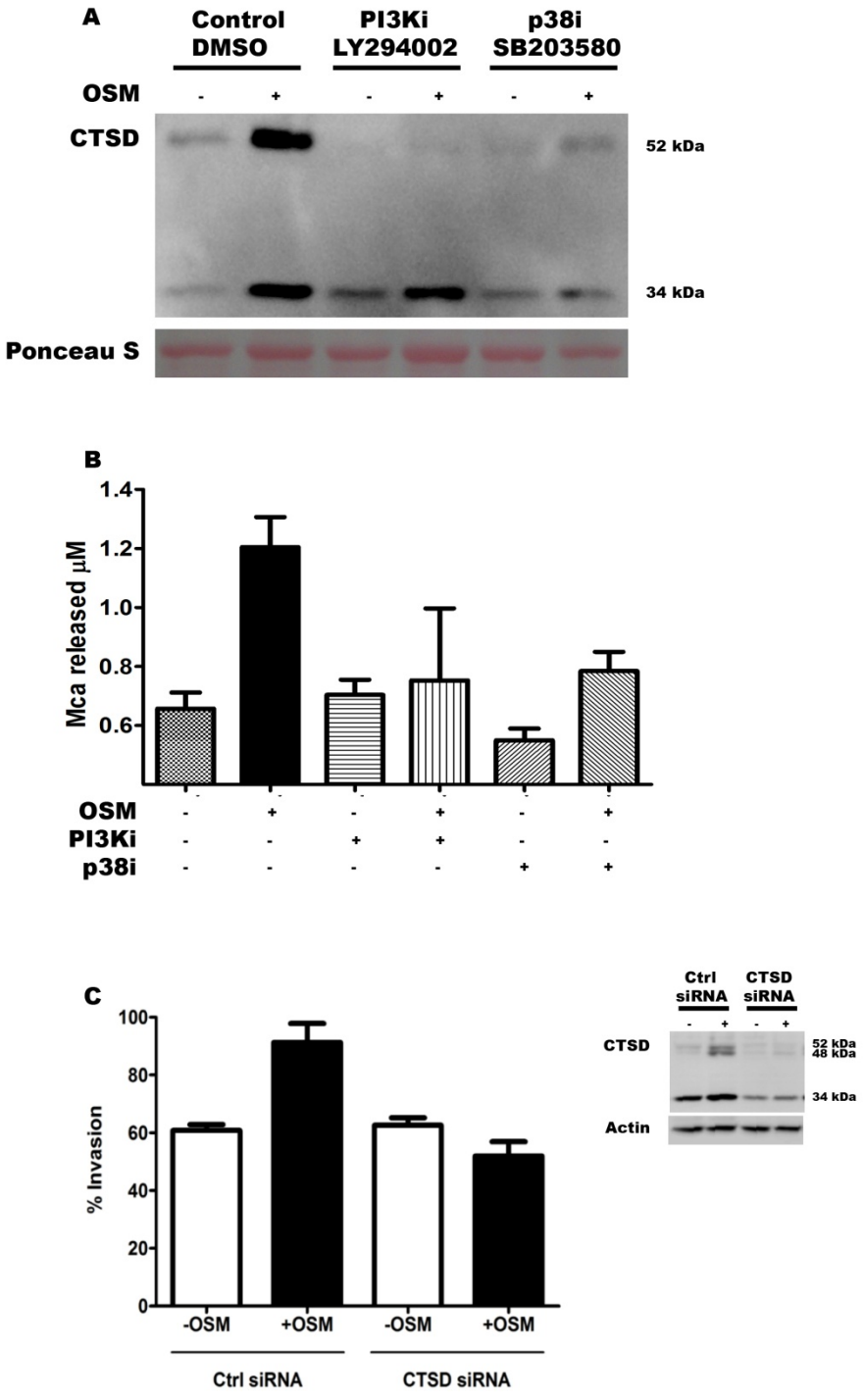

Figure 5. OSM-induced CTSD is active and important for OSM-promoted invasive potential. T47D cells were treated for 6 days with OSM. PI3K (LY294002) and

p38 MAPK (SB203580) inhibitors were added on day 4 of cytokine treatment. A) Secreted levels of both proCTSD (52 kDa) and active CTSD (34 kDa) protein levels (as measured by Western blot analysis) decrease with inhibitor treatment B) The amount of active CTSD secreted into the culture media was determined using an activity assay. C) T47D cells were pre-treated with control or CTSD siRNA, in the absence or presence of OSM. Cells were then seeded onto Matrigel chambers and allowed to invade for 22h in the presence or absence of OSM.

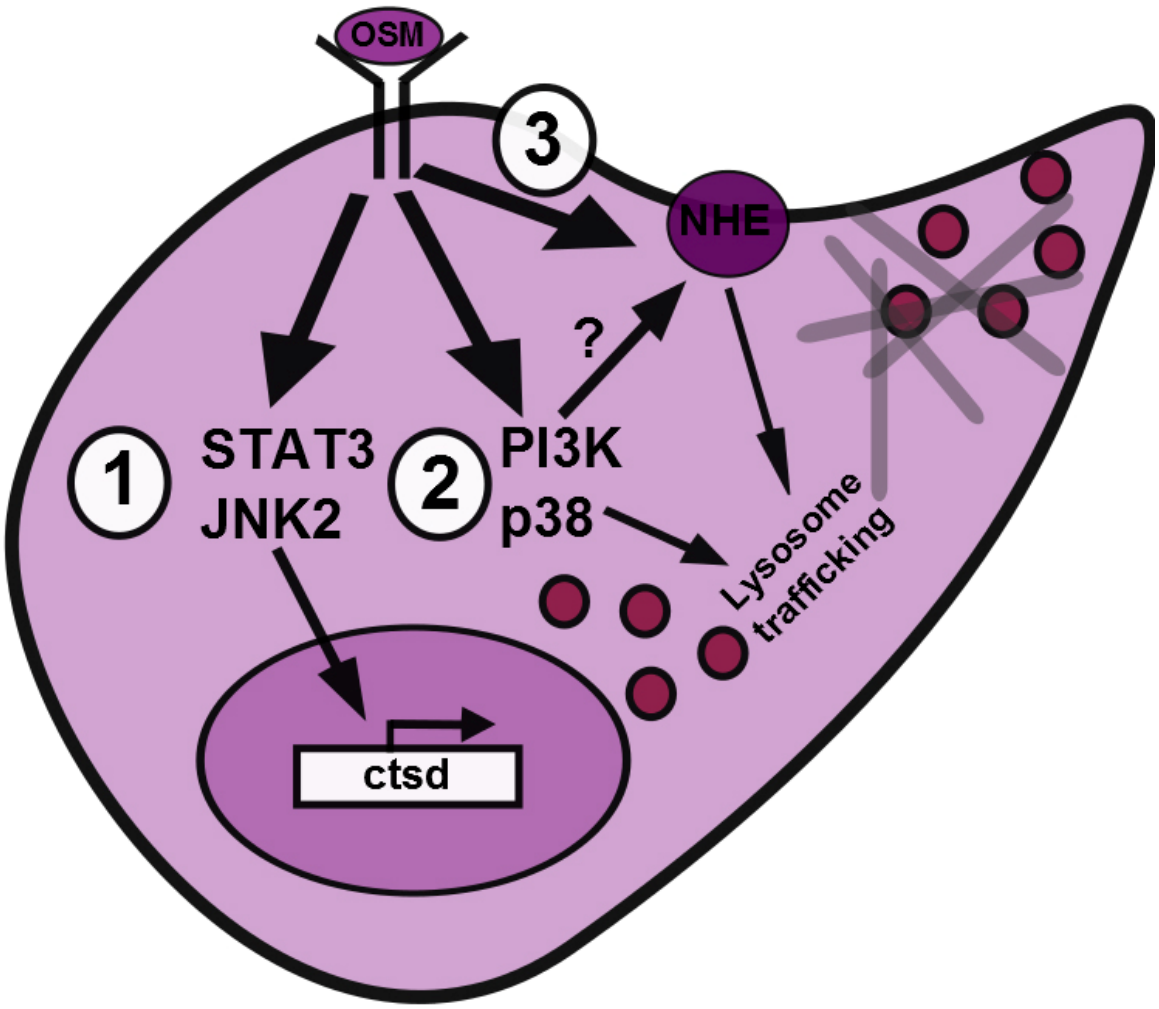

Figure 6 Overview of OSM signaling regulating CTSD expression and lysosome trafficking. 1) OSM activates STAT3 and JNK2 to induce the expression of CTSD after 24-48 hours. 2) OSM signaling through PI3K and p38 MAPK induces lysosomal trafficking to cellular protrusions with altered actin organization. 3) NHE activity induced by OSM induces lysosomal trafficking and may be mediated via PI3K and p38.

REFERENCES

1. Siegel, R. Cancer statistics, 2013. *CA. Cancer J. Clin.* **63,** 11–30 (2013).

2. Foulkes, W. Triple-negative breast cancer. *N. Engl. J. Med.* **363,** 1938–1948 (2010).

3. Nguyen, D. X. & Massagué, J. Genetic determinants of cancer metastasis. *Nat. Rev. Genet.* **8,** 341–52 (2007).

4. Wiseman, B. & Werb, Z. Stromal effects on mammary gland development and breast cancer. *Science (80-.).* **296,** 1046–1049 (2002).

5. Green, K. a & Lund, L. R. ECM degrading proteases and tissue remodelling in the mammary gland. *BioEssays* **27,** 894–903 (2005).

6. Balkwill, F., Charles, K. a & Mantovani, A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* **7,** 211–7 (2005).

7. Weiss, T., Simak, R. & Kaun, C. Oncostatin M and IL-6 induce u-PA and VEGF in prostate cancer cells and correlate in vivo. *Anticancer Res.* **31,** 3273–3278 (2011).

8. García-Tuñón, I. *et al.* OSM, LIF, its receptors, and its relationship with the malignance in human breast carcinoma (in situ and in infiltrative). *Cancer Invest.* **26,** 222–9 (2008).

9. Savarese, T. M. *et al.* Coexpression of oncostatin M and its receptors and evidence for STAT3 activation in human ovarian carcinomas. *Cytokine* **17,** 324–34 (2002).

10. Tumminello, F. M. *et al.* Serum interleukin-6 in patients with metastatic bone disease: correlation with cystatin C. *Med. Oncol.* **26,** 10–5 (2009).

11. Chavey, C. *et al.* Oestrogen receptor negative breast cancers exhibit high cytokine content. *Breast Cancer Res.* **9,** R15 (2007).

12. Knüpfer, H. & Preiss, R. Significance of interleukin-6 (IL-6) in breast cancer (review). *Breast Cancer Res. Treat.* **102,** 129–35 (2007).

13. Tanaka, M. & Miyajima, a. Oncostatin M, a multifunctional cytokine. *Rev. Physiol. Biochem. Pharmacol.* **149,** 39–52 (2003).

14. Heinrich, P. C. *et al.* Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem. J.* **374,** 1–20 (2003).

15. Zarling, J. M. *et al.* Oncostatin M: a growth regulator produced by differentiated histiocytic lymphoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **83,** 9739–43 (1986).

16. Liu, J., Spence, M. & Wallace, P. Oncostatin M-specific receptor mediates inhibition of breast cancer cell growth and down-regulation of the c-myc proto-oncogene. *Cell growth Differ.* **8,** 667–676 (1997).

17. Horn, D. *et al.* Regulation of cell growth by recombinant oncostatin M. *Growth Factors* **2,** 157–165 (1990).

18. Friedrich, M. *et al.* Complete inhibition of in vivo glioma growth by oncostatin M. *J. Neurochem.* **76,** 1589–92 (2001).

19. Li, Q. *et al.* Oncostatin M promotes proliferation of ovarian cancer cells through signal transducer and activator of transcription 3. *Int. J. Mol. Med.* **28,** 101–8 (2011).

20. Mori, S., Murakami-Mori, K. & Bonavida, B. Oncostatin M (OM) promotes the growth of DU 145 human prostate cancer cells, but not PC-3 or LNCaP, through the signaling of the OM specific receptor. *Anticancer Res.* **19,** 1011–1015 (1999).

21. Bolin, C. *et al.* Oncostatin m promotes mammary tumor metastasis to bone and osteolytic bone degradation. *Genes Cancer* **3,** 117–30 (2012).

22. Guo, L. *et al.* Stat3-coordinated Lin-28-let-7-HMGA2 and miR-200-ZEB1 circuits initiate and maintain oncostatin M-driven epithelial-mesenchymal transition. *Oncogene* **32,** 5272–82 (2013).

23. West, N. R., Murphy, L. C. & Watson, P. H. Oncostatin M suppresses oestrogen receptor-α expression and is associated with poor outcome in human breast cancer. *Endocr. Relat. Cancer* **19,** 181–195 (2012).

24. Argast, G. M. *et al.* Cooperative signaling between oncostatin M, hepatocyte growth factor and transforming growth factor-β enhances epithelial to mesenchymal transition in lung and pancreatic tumor models. *Cells. Tissues. Organs* **193,** 114–32 (2011).

25. West, N. R., Murray, J. I. & Watson, P. H. Oncostatin-M promotes phenotypic changes associated with mesenchymal and stem cell-like differentiation in breast cancer. *Oncogene* 1–10 (2013). doi:10.1038/onc.2013.105

26. Holzer, R. G., Ryan, R. E., Tommack, M., Schlekeway, E. & Jorcyk, C. L. Oncostatin M stimulates the detachment of a reservoir of invasive mammary carcinoma cells: role of cyclooxygenase-2. *Clin. Exp. Metastasis* **21,** 167–76 (2004).

27. Jorcyk, C. L., Holzer, R. G. & Ryan, R. E. Oncostatin M induces cell detachment and enhances the metastatic capacity of T-47D human breast carcinoma cells. *Cytokine* **33,** 323–36 (2006).

28. Zhang, F., Li, C., Halfter, H. & Liu, J. Delineating an oncostatin M-activated STAT3 signaling pathway that coordinates the expression of genes involved in cell cycle regulation and extracellular matrix deposition of MCF-7 cells. *Oncogene* **22,** 894–905 (2003).

29. West, N. R. & Watson, P. H. S100A7 (psoriasin) is induced by the proinflammatory cytokines oncostatin-M and interleukin-6 in human breast cancer. *Oncogene* **29,** 2083–92 (2010).

30. Hui, W., Rowan, A. D., Richards, C. D. & Cawston, T. E. Oncostatin M in Combination With Tumor Necrosis Factor Alpha Induces Cartilage Damage and Matrix Metalloproteinase Expression In Vitro and In Vivo. *Arthritis Rheum.* **48,** 3404–3418 (2003).

31. Rowan, A. D., Hui, W., Cawston, T. E. & Richards, C. D. Adenoviral gene transfer of interleukin-1 in combination with oncostatin M induces significant joint damage in a murine model. *Am. J. Pathol.* **162,** 1975–84 (2003).

32. Tiffen, P. G. *et al.* A dual role for oncostatin M signaling in the differentiation and death of mammary epithelial cells in vivo. *Mol. Endocrinol.* **22,** 2677–88 (2008).

33. Fearon, U. *et al.* Oncostatin M induces angiogenesis and cartilage degradation in rheumatoid arthritis synovial tissue and human cartilage cocultures. *Arthritis Rheum.* **54,** 3152–62 (2006).

34. Rega, G. *et al.* Vascular Endothelial Growth Factor Is Induced by the Inflammatory Cytokines Interleukin-6 and Oncostatin M in Human Adipose Tissue In Vitro and in Murine Adipose Tissue In Vivo. *Arterioscler. Thromb. Vasc. Biol.* **27,** 1587–1595 (2007).

35. Vasse, M. *et al.* Oncostatin M induces angiogenesis in vitro and in vivo. *Arterioscler. Thromb. Vasc. Biol.* **19,** 1835–42 (1999).

36. Korzus, E., Nagase, H., Rydell, R. & Travis, J. The mitogen-activated protein kinase and JAK-STAT signaling pathways are required for an oncostatin M-responsive element-mediated activation of matrix metalloproteinase 1 gene expression. *J. Biol. Chem.* **272,** 1188–96 (1997).

37. Liu, J. *et al.* Oncostatin M-specific receptor expression and function in regulating cell proliferation of normal and malignant mammary epithelial cells. *Cytokine* **10,** 295–302 (1998).

38. Badache, A. & Hynes, N. Interleukin 6 inhibits proliferation and, in cooperation with an epidermal growth factor receptor autocrine loop, increases migration of T47D breast cancer cells. *Cancer Res.* **61,** 383–391 (2001).

39. Hurst, S. M. *et al.* Secretion of oncostatin M by infiltrating neutrophils: regulation of IL-6 and chemokine expression in human mesothelial cells. *J. Immunol.* **169,** 5244–51 (2002).

40. Dillon, R. L., White, D. E. & Muller, W. J. The phosphatidyl inositol 3-kinase signaling network: implications for human breast cancer. *Oncogene* **26,** 1338–45 (2007).

41. Clevenger, C. V. Roles and regulation of stat family transcription factors in human breast cancer. *Am. J. Pathol.* **165,** 1449–60 (2004).

42. Whyte, J., Bergin, O., Bianchi, A., McNally, S. & Martin, F. Key signalling nodes in mammary gland development and cancer. Mitogen-activated protein kinase signalling in experimental models of breast cancer progression and in mammary gland development. *Breast Cancer Res.* **11,** (2009).

43. Wallin, J. J. *et al.* Active PI3K pathway causes an invasive phenotype which can be reversed or promoted by blocking the pathway at divergent nodes. *PLoS One* **7,** e36402 (2012).

44. Underhill-Day, N. & Heath, J. K. Oncostatin M (OSM) cytostasis of breast tumor cells: characterization of an OSM receptor beta-specific kernel. *Cancer Res.* **66,** 10891–901 (2006).

45. Crichton, M. B., Nichols, J. E., Zhao, Y., Bulun, S. E. & Simpson, E. R. Expression of transcripts of interleukin-6 and related cytokines by human breast tumors, breast cancer cells, and adipose stromal cells. *Mol. Cell. Endocrinol.* **118,** 215–20 (1996).

46. Queen, M. M., Ryan, R. E., Holzer, R. G., Keller-Peck, C. R. & Jorcyk, C. L. Breast cancer cells stimulate neutrophils to produce oncostatin M: potential implications for tumor progression. *Cancer Res.* **65,** 8896–904 (2005).

47. Celis, J. E. *et al.* Proteomic characterization of the interstitial fluid perfusing the breast tumor microenvironment: a novel resource for biomarker and therapeutic target discovery. *Mol. Cell. Proteomics* **3,** 327–44 (2004).

48. Levano, K. S., Jung, E. H. & Kenny, P. a. Breast cancer subtypes express distinct receptor repertoires for tumor-associated macrophage derived cytokines. *Biochem. Biophys. Res. Commun.* **411,** 107–10 (2011).

49. Benes, P., Vetvicka, V. & Fusek, M. Cathepsin D--many functions of one aspartic protease. *Crit. Rev. Oncol. Hematol.* **68,** 12–28 (2008).

50. Mohamed, M. M. & Sloane, B. F. Cysteine cathepsins: multifunctional enzymes in cancer. *Nat. Rev. Cancer* **6,** 764–75 (2006).

51. Gocheva, V. & Joyce, J. Cysteine cathepsins and the cutting edge of cancer invasion. *Cell Cycle* **6,** 60–64 (2007).

52. Journet, A. & Ferro, M. The potentials of MS-based subproteomic approaches in medical science: the case of lysosomes and breast cancer. *Mass Spectrom. Rev.* **23,** 393–442 (2004).

53. Montcourrier, P. *et al.* Cathepsin D in breast cancer cells can digest extracellular matrix in large acidic vesicles. *Cancer Res.* **50,** 6045–6054 (1990).

54. Montcourrier, P. *et al.* Characterization of very acidic phagosomes in breast cancer cells and their association with invasion. *J. Cell Sci.* **107,** 2381–91 (1994).

55. Sloane, B. F. *et al.* Membrane association of cathepsin B can be induced by transfection of human breast epithelial cells with c-Ha-ras oncogene. *J. Cell Sci.* **107,** 373–84 (1994).

56. Sameni, M. *et al.* Cathepsin B and D are localized at the surface of human Breast Cancer cells. *Pathol. Oncol. Res.* **1,** 43–53 (1995).

57. Nishimura, Y., Sameni, M. & Sloane, B. F. Malignant transformation alters intracellular trafficking of lysosomal cathepsin D in human breast epithelial cells. *Pathol. Oncol. Res.* **4,** 283–96 (1998).

58. Kokkonen, N. *et al.* Defective acidification of intracellular organelles results in aberrant secretion of cathepsin D in cancer cells. *J. Biol. Chem.* **279,** 39982–8 (2004).

59. Steffan, J. J., Williams, B. C., Welbourne, T. & Cardelli, J. a. HGF-induced invasion by prostate tumor cells requires anterograde lysosome trafficking and activity of Na+-H+ exchangers. *J. Cell Sci.* **123,** 1151–1159 (2010).

60. Bastow, E. R. *et al.* Evidence for lysosomal exocytosis and release of aggrecan-degrading hydrolases from hypertrophic chondrocytes, in vitro and in vivo. *Biol. Open* **1,** 318–328 (2012).

61. Liu, Y., Zhou, Y. & Zhu, K. Inhibition of Glioma Cell Lysosome Exocytosis Inhibits Glioma Invasion. *PLoS One* **7,** (2012).

62. Gonzalez-Noriega, a, Grubb, J. H., Talkad, V. & Sly, W. S. Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. *J. Cell Biol.* **85,** 839–52 (1980).

63. Gieselmann, V., Pohlmann, R., Hasilik, a & Von Figura, K. Biosynthesis and transport of cathepsin D in cultured human fibroblasts. *J. Cell Biol.* **97,** 1–5 (1983).

64. Hasilik, A., von Figura, K., Conzelmann, E., Nehrkorn, H. & Sandohoff, K. Lysosomal enzyme precursors in human fibroblasts. *Eur. J. Biochem.* **125,** 317–321 (1982).

65. Conner, G. E. Isolation of procathepsin D from mature cathepsin D by pepstatin affinity chromatography. Autocatalytic proteolysis of the zymogen form of the enzyme. *Biochem. J.* **263,** 601–4 (1989).

66. Gatenby, R. a, Gawlinski, E. T., Gmitro, A. F., Kaylor, B. & Gillies, R. J. Acid-mediated tumor invasion: a multidisciplinary study. *Cancer Res.* **66,** 5216–23 (2006).

67. Nicotra, G. *et al.* The dilemma□: Does tissue expression of cathepsin D reflect tumor malignancy? The question□: Does the assay truly mirror cathepsin D mis-function in the tumor? *Cancer Biomarkers* **7,** 47–64 (2010).

68. Van der Stappen, J. W., Williams, a C., Maciewicz, R. a & Paraskeva, C. Activation of cathepsin B, secreted by a colorectal cancer cell line requires low pH and is mediated by cathepsin D. *Int. J. Cancer* **67,** 547–54 (1996).

69. Koblinski, J. E., Ahram, M. & Sloane, B. F. Unraveling the role of proteases in cancer. *Clin. Chim. Acta.* **291,** 113–35 (2000).

70. Leto, G., Tumminello, F. M., Crescimanno, M., Flandina, C. & Gebbia, N. Cathepsin D expression levels in nongynecological solid tumors: clinical and therapeutic implications. *Clin. Exp. Metastasis* **21,** 91–106 (2004).

71. Skrzydlewska, E., Sulkowska, M., Koda, M. & Sulkowski, S. Proteolytic-antiproteolytic balance and its regulation in carcinogenesis. *World J. Gastroenterol.* **11,** 1251–66 (2005).

72. Mason, S. D. & Joyce, J. a. Proteolytic networks in cancer. *Trends Cell Biol.* **21,** 228–37 (2011).

73. Foekens, J. a *et al.* Cathepsin-D in primary breast cancer: prognostic evaluation involving 2810 patients. *Br. J. Cancer* **79,** 300–7 (1999).

74. Brouillet, J. P. *et al.* Immunoradiometric Assay of Pro-cathepsin D in Breast Cancer Cytosol: Relative Prognostic Value Versus Total Cathepsin D. *Eur. J. Cancer* **29A,** 1248–1251 (1993).

75. Barthell, E. *et al.* Immunohistochemical visualisation of cathepsin-D expression in breast cancer. *Anticancer Res.* **27,** 2035–9 (2007).

76. Dian, D., Vrekoussis, T. & Shabani, N. Expression of Cathepsin-D in Primary Breast Cancer and Corresponding Local Recurrence or Metastasis: An Immunohistochemical Study. *Anticancer Res.* **32,** 901–905 (2012).

77. González-Vela, M. C., Garijo, M. F., Fernández, F., Buelta, L. & Val-Bernal, J. F. Cathepsin D in host stromal cells is associated with more highly vascular and aggressive invasive breast carcinoma. *Histopathology* **34,** 35–42 (1999).

78. Cavailles, V., Garcia, M. & Rochefor, H. Regulation of Cathepsin D and pS2 Gene Expression by Growth Factors in MCF7 Human Breast Cancer Cells. *Mol. Endocrinol.* **3,** 552–8 (1989).

79. Westley, B. R. & May, F. E. B. Oestrogen regulates cathepsin D mRNA levels in oestrogen responsive human breast cancer cells. *Nucleic Acids Res.* **15,** 3773–3786 (1987).

80. Cavailles, V., Augereau, P. & Rochefort, H. Cathepsin D gene is controlled by a mixed promoter, and estrogens stimulate only TATA-dependent transciption in breast cancer cells. *Biochemistry* **90,** 203–207 (1993).

81. Augereau, P. *et al.* Cloning and sequencing of the 52K cathepsin D complementary deoxyribonucleic acid of MCF7 breast cancer cells and mapping on chromosome 11. *Mol. Endocrinol.* **2,** 186–192 (1988).

82. Hu, L., Roth, J. M., Brooks, P., Luty, J. & Karpatkin, S. Thrombin up-regulates cathepsin D which enhances angiogenesis, growth, and metastasis. *Cancer Res.* **68,** 4666–73 (2008).

83. Knopfová, L. *et al.* c-Myb regulates matrix metalloproteinases 1/9, and cathepsin D: implications for matrix-dependent breast cancer cell invasion and metastasis. *Mol. Cancer* **11,** 209 (2012).

84. Premzl, A. *et al.* Invasion of ras-transformed breast epithelial cells depends on the proteolytic activity of cysteine and aspartic proteinases. *Biol. Chem.* **382,** 853–857 (2001).

85. Glondu, M. *et al.* Down-regulation of cathepsin-D expression by antisense gene transfer inhibits tumor growth and experimental lung metastasis of human breast cancer cells. *Oncogene* **21,** 5127–34 (2002).

86. Lacroix, M. & Leclercq, G. Relevance of breast cancer cell lines as models for breast tumours: an update. *Breast Cancer Res. Treat.* **83,** 249–89 (2004).

87. Reid, W. A., Valler, M. J. & Kay, J. Immunolocalisation of cathepsin D in normal and neoplastic human tissues. *J. Clin. Pathol.* **39,** 1323–1330 (1986).

88. Rochefort, H. & Capony, F. Estrogen-Induced lysosomal proteases secreted by breast cancer cells: A role in carcinogenesis? *J. Cell. Biochem.* **35,** 17–29 (1987).

89. Hasilik, A. & Neufeldg, E. F. Biosynthesis of Lysosomal Enzymes in Fibroblasts. *J. Biol. Chem.* **25,** 4937–4945 (1980).

90. Erickson, A. H., Conner, G. E. & Blobel, G. Biosynthesis of a Lysosomal Enzyme. *J. Biol. Chem.* **256,** 11224–31 (1981).

91. Conner, G. E. & Richo, G. Isolation and characterization of a stable activation intermediate of the lysosomal aspartyl protease cathepsin D. *Biochemistry* **31,** 1142–7 (1992).

92. Gieselmann, V., Hasilik, A & von Figura, K. Processing of human cathepsin D in lysosomes in vitro. *J. Biol. Chem.* **260,** 3215–20 (1985).

93. Berchem, G. *et al.* Cathepsin-D affects multiple tumor progression steps in vivo: proliferation, angiogenesis and apoptosis. *Oncogene* **21,** 5951–5 (2002).

94. Laurent-Matha, V. *et al.* Catalytically inactive human cathepsin D triggers fibroblast invasive growth. *J. Cell Biol.* **168,** 489–99 (2005).

95. Glunde, K. *et al.* Extracellular acidification alters lysosomal trafficking in human breast cancer cells. *Neoplasia* **5,** 533–45 (2003).

96. Lagana, A. *et al.* Regulation of the formation of tumor cell pseudopodia by the Na+/H+ exchanger NHE1. *J. Cell Sci.* **113,** 3649–3662 (2000).

97. Cardone, R. A, Casavola, V. & Reshkin, S. J. The role of disturbed pH dynamics and the Na+/H+ exchanger in metastasis. *Nat. Rev. Cancer* **5,** 786–95 (2005).

98. Steffan, J. J., Snider, J. L., Skalli, O., Welbourne, T. & Cardelli, J. A. Na+/H+ exchangers and RhoA regulate acidic extracellular pH-induced lysosome trafficking in prostate cancer cells. *Traffic* **10,** 737–53 (2009).

99. Putney, L., Denker, S. & Barber, D. The changing face of the Na+/H+ exchanger, NHE1: structure, regulation, and cellular actions. *Annu. Rev. Pharmacol. Toxicol.* **42,** 527–552 (2002).

100. Busco, G. *et al.* NHE1 promotes invadopodial ECM proteolysis through acidification of the peri-invadopodial space. *FASEB J.* **24,** 3903–15 (2010).

101. De Visser, K. E., Eichten, A. & Coussens, L. M. Paradoxical roles of the immune system during cancer development. *Nat. Rev. Cancer* **6,** 24–37 (2006).

102. Zitvogel, L., Tesniere, A. & Kroemer, G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat. Rev. Immunol.* **6,** 715–27 (2006).

103. Rochefort, H. *et al.* Overexpression and hormonal regulation of pro-cathepsin D in mammary and endometrial cancer. *J. Steroid Biochem.* **34,** 177–182 (1989).

104. Augereau, P. *et al.* Characterization of the proximal estrogen-responsive element of human cathepsin D gene. *Mol. Endocrinol.* **8,** 693–703 (1994).

105. Davis, R. J. Signal transduction by the JNK group of MAP kinases. *Cell* **103,** 239–52 (2000).

106. Turkson, J. *et al.* Requirement for Ras/Rac1-mediated p38 and c-Jun N-terminal kinase signaling in Stat3 transcriptional activity induced by the Src oncoprotein. *Mol. Cell. Biol.* **19,** 7519–7528 (1999).

107. Nitta, R. T. *et al.* The role of the c-Jun N-terminal kinase 2-α-isoform in non-small cell lung carcinoma tumorigenesis. *Oncogene* **30,** 234–44 (2011).

108. Aznar, S. *et al.* Simultaneous tyrosine and serine phosphorylation of STAT3 transcription factor is involved in Rho A GTPase oncogenic transformation. *Mol. Biol. Cell* **12,** 3282–94 (2001).

109. Wen, Z., Zhong, Z. & Darnell, J. E. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell* **82,** 241–50 (1995).

110. Nishimura, Y. *et al.* Small guanosine triphosphatase Rho/Rho-associated kinase as a novel regulator of intracellular redistribution of lysosomes in invasive tumor cells. *Cell Tissue Res.* **301,** 341–351 (2000).

111. Tu, C. *et al.* Lysosomal Cathepsin B Participates in the Podosome-Mediated Extracellular Matrix Degradation and Invasion via Secreted Lysosomes in v-Src Fibroblasts. *Cancer Res.* **68,** 9147–56 (2008).

112. Reshkin, S. J. *et al.* Na+/H+ exchanger-dependent intracellular alkalinization is an early event in malignant transformation and plays an essential role in the development of subsequent transformation-associated phenotypes. *FASEB J.* **14,** 2185–2197 (2000).

113. Wei, J. *et al.* AEG-1 participates in TGF-beta1-induced EMT through p38 MAPK activation. *Cell Biol. Int.* **9999,** 1–6 (2013).

114. Antoon, J. W. *et al.* Inhibition of p38 mitogen-activated protein kinase alters microRNA expression and reverses epithelial-to-mesenchymal transition. *Int. J. Oncol.* **42,** 1139–50 (2013).

115. Das, S., Becker, B. N., Hoffmann, F. M. & Mertz, J. E. Complete reversal of epithelial to mesenchymal transition requires inhibition of both ZEB expression and the Rho pathway. *BMC Cell Biol.* **10,** 94 (2009).

116. Reshkin, S. J. *et al.* Phosphoinositide 3-Kinase Is Involved in the Tumor-specific Activation of Human Breast Cancer Cell Na+/H+ Exchange, Motility, and Invasion Induced by Serum Deprivation. *J. Biol. Chem.* **275,** 5361–5369 (2000).

117. Shrode, L. D., Rubie, E. A., Woodgett, J. R. & Grinstein, S. Cytosolic Alkalinization Increases Stress-activated Protein Kinase/c-Jun NH2-terminal Kinase (SAPK/JNK) Activity and p38 Mitogen-activated Protein Kinase Activity by a Calcium-independent Mechanism. *J. Biol. Chem.* **272,** 13653–13659 (1997).

118. Riley, L. *et al.* Analysis of cathepsin D in human breast cancer: usefulness of the processed 31 kDa active form of the enzyme as a prognostic indicator in node-negative and node-positive patients. *Breast Cancer Res Treat* **60,** 173–179 (2000).

119. Roughley, P. J. The Degradation of Cartilage Proteoglycans by Tissue Proteinases. Proteoglycan Heterogeneity and the Pathway of Proteolytic Degradation. *Biochem. J.* **167,** 639–646 (1977).

120. Handley, C. J. *et al.* Cathepsin D cleaves aggrecan at unique sites within the interglobular domain and chondroitin sulfate attachment regions that are also cleaved when cartilage is maintained at acid pH. *Matrix Biol.* **20,** 543–53 (2001).

121. Scott, P. G. & Pearson, C. H. Cathepsin D: cleavage of soluble collagen and crosslinked peptides. *FEBS Lett.* **88,** 41–45 (1978).

122. Scott, P. G. & Pearson, H. Cathepsin D: specificity of peptide-bond cleavage in type-I collagen and effects on type-III collagen and procollagen. *Eur. J. Biochem.* **114,** 59–62 (1981).

123. Tressel, T., Shively, J. E. & Pande, H. Human placental fibronectin: demonstration of structural differences between the A and B chains in the extra domain-A region. *Arch. Biochem. Biophys.* **266,** 639–43 (1988).