Low Cell Density Induced Epithelial-Mesenchymal Transition in MCF10A Cell is Regulated by Proteasome Activity

Jiejun Fu[1,2], Donghong Ju[1] and Youming Xie[1]*

[1]*Karmanos Cancer Institute and Department of Oncology, Wayne State University School of Medicine, Detroit, Michigan, USA.*
[2]*Center for Translational Medicine, Guangxi Medical University, Nanning, Guangxi, 530021, China*

Address for Correspondence
Youming Xie, Karmanos Cancer Institute and Department of Oncology, Wayne State University School of Medicine, 110 E Warren Avenue, Detroit, MI 48201, USA, Tel: 313-578-4319; Fax: 313-831-7518; E-mail: xiey@karmanos.org

Keywords: Proteasome; EMT; E-cadherin; Gene regulation; MCF10A cells

Abstract

Epithelial-mesenchymal transition (EMT) is a key process in tumor progression and metastasis. Previous studies have shown that MCF10A human mammary epithelial cells undergo EMT when cultured at low cell density, but the underlying mechanism remains poorly understood. Here we show that the expression of proteasome genes and the proteasome activity increase in response to low cell density. Moreover, the proteasome inhibitor MG132 blocks EMT in sparse MCF10A cells. The transcription of E-cadherin gene is repressed in sparse MCF10A cells, but is recovered upon the treatment with MG132. While the mRNA levels of Snail and Slug are markedly elevated at low cell density, the steady state protein levels are similar between sparse and confluent cells. These results suggest that the suppression of E-cadherin gene expression in sparse MCF10A cells is likely caused by downregulation of its transcription activator(s) rather than upregulation of its repressors such as Snail and Slug. This study reveals that cell density-dependent EMT in MCF10A cells is regulated by proteasome activity.

Introduction

Epithelial-mesenchymal transition (EMT) is a crucial event in embryonic development, tissue remodeling, wound repair, and tumor metastasis [1-3]. During EMT, epithelial cells lose polarity and epithelial characteristics, but acquire mesenchymal characteristics of fibroblast-like morphology with cytoskeleton reorganization and increase of cell motility. EMT induction is accompanied with downregulation of epithelial makers such as E-cadherin and cytokeratin, and on the other hand, with upregulation of mesenchymal markers including N-cadherin and vimentin [4,5]. E-cadherin represents one of the best characterized markers of EMT. Transcriptional repression is thought to be a predominant mechanism that controls the expression of E-cadherin in most carcinomas. Several transcriptional repressors of E-cadherin have been characterized, including Snail (Snail1), Slug (Snail2), SIP-1 (ZEB-2), δEF1 (ZEB-1) and Twist. These transcriptional repressors act as EMT inducers.

In cultured cells, EMT can be induced by cytokines and growth factors [1,6]. Induction of EMT appears to be highly tissue- and cell type-specific because EMT inducers have very different effects in different cells. MCF-10A is a spontaneously immortalized, but nontransformed human mammary epithelial cell line [7,8]. MCF10A cells exhibit numerous features of normal breast epithelia, including lack of tumorigenicity in nude mice and lack of anchorage-independent growth. MCF-10A cells are often used as a normal control in breast cancer studies and turn out to be a good model for understanding the process of EMT [9]. It has been shown that MCF10A cells undergo spontaneous morphologic and phenotypic EMT-like changes when cultured at low cell density [10,11]. However, the underlying mechanism remains unclear. In this study, we demonstrate that cell density-dependent EMT in MCF10A cells is regulated by proteasome activity.

Materials and Methods

Cell culture and reagents

MCF10A cells were obtained from the cell bank at Karmanos Cancer Institute and maintained in DMEM/F12 with 5% horse serum (Invitrogen), 10 μg/ml of insulin (Sigma-Aldrich), 20 ng/ml of epidermal growth factor (Invitrogen), 0.5 μg/ml of hydrocortisone (Sigma-Aldrich), and 100 ng/ml of cholera toxin (Calbiochem). MG132 (carbobenzoxyl-L-leucyl-L-leucyl-L-leucinal) was purchased from Boston Biochem and prepared as 10 mM stock in DMSO.

Real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using Trizol Reagent (Invitrogen) and treated with DNase I to eliminate contaminated genomic DNA. cDNA was prepared using the Superscript III first strand synthesis kit (Invitrogen) according to the manufacturer's recommendations. Real-time RT-PCR was performed using the SYBR Green PCR reagents (Applied Biosystems) according to the manufacturer's instruction. PCR amplification was carried out in a GeneAmp 5700 Sequence Detection System (Applied Biosystems) under thermal cycling conditions (50°C for 2 min, an initial denaturation step at 95°C for 10 min, followed by 15s at 95°C and 1min at 60°C for 40 cycles). Relative quantification using GAPDH as an internal control was defined using the comparative Ct (threshold cycle) method. Oligo primers used in real-time PCRs are listed in Table 1.

Immunoblotting analysis

Cells were harvested and lysed with RIPA buffer (50

Table 1: Primers used for real-time PCR experiments

Gene	NCBI accession no.	Primer sequence (5'-3')	
GAPDH	NM_002046	Forward	CAGCCTCAAGATCATCAGCA
		Reverse	TGTGGTCATGAGTCCTTCCA
E-cadherin	NM_004360	Forward	CTGAGAACGAGGCTAACG
		Reverse	TTCACATCCAGCACATCC
N-cadherin	NM_001792	Forward	GAAAGACCCATCCACG
		Reverse	CCTGCTCACCACCACTA
PSMA1	NM_148976	Forward	TTATGCGTCAGGAGTGTT
		Reverse	GTGTTGGTATCTGGGTCT
PSMA5	NM_002790	Forward	AGCAATTGGCTCTGCTTCAG
		Reverse	GCATTCAGCTTCTCCTCCAT
PSMB6	NM_002798	Forward	CTGGGAAAGCCGAGAAGT
		Reverse	ATGCGGTCGTGAATAGGT
PSMC2	NM_002803	Forward	ATGTTGGTGGCTGTAAGG
		Reverse	CACGCATCAGTCCGATTA
PSMD3	NM_002809	Forward	ACGCTATCTCGCGCTCGTGTG
		Reverse	ACGCGTGATGTGGAAGGCAGCAT
SNAIL	NM_005985	Forward	AATCGGAAGCCTAACTACAGCG
		Reverse	GTCCCAGATGAGCATTGGCA
SLUG	NM_003068	Forward	AGCATTTCAACGCCTCCA
		Reverse	GGATCTCTGGTTGTGGTATGAC

mM Tris-HCI, pH 7.5, 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing protease inhibitor cocktail for 20 min at 4°C. The lysates were centrifuged at 14,000 rpm at 4°C for 15 min to remove debris. Protein concentrations were determined using the Bio-Rad Protein Assay kit. For immunoblotting analysis, 50 μg of cell lysates were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore). Membranes were blocked in 0.2% PBS containing 0.1% casein for 1 h, and incubated respectively with anti-Snail and anti-Slug antibodies (Santa Cruz Biotechnology) for 2 h. After 3 washes, membranes were incubated with a secondary antibody conjugated with Alexa Fluor 680 (Invitrogen) for 40 min, and visualized using an Odyssey Infrared Imaging system (LI-COR Biosciences). Comparable input of cell extracts was confirmed by reprobing the blot with an anti-GAPDH antibody.

Proteasome activity assay

Cells were washed 3 times with ice-cold PBS and scraped for centrifugation. Cell pellets were resuspended in lysis buffer (50 mM Tris-Cl, PH 7.5, 10 mM $MgCl_2$, 100 mM KCl, 1% Triton X-100, 10% glycerol, 1 mM DTT) and mixed with rotation at 4°C for 2 h. Extracts were centrifuged at 13,200 rpm for 10 min at 4°C. Protein concentrations were determined using Bradford Assay Kit (Bio-Rad). The chymotrypsin-like activity of proteasome was measured by using the fluoregenic Suc-Leu-Leu-Val-Tyr-AMC (Suc-LLVY-AMC) peptide substrate (Boston BioChem). Specifically, 20 μg of cell extract was incubated with 40 μM Suc-LLVY-AMC in 20 μl of assay buffer (50 mM Tris-Cl, pH 7.5) at 37°C for 1 h. The release of fluorescence signal (AMC group) was monitored with an excitation filter of 355 nm and an emission filter of 460 nm using a Spectramax Gemini XS

microplate fluorometer (Molecular Devices).

Statistical analysis

All experiments were performed in triplicate. Results were analyzed by Student's t-test and presented as mean ± SD.

Results

Aberrant overexpression of proteasome genes is implicated in the formation of cancer malignance [12-18]. We wanted to examine if alteration of proteasome activity plays a role in EMT using MCF10A cells a model system. We first confirmed the previous observation that MCF10A cells commit EMT at low cell density. As shown in Figure 1A, the morphology of MCF10A cells became more fibroblast-like at low cell density. Moreover, the expression level of E-cadherin was much lower in sparse than in confluent cells (Figure 1B, left panel). In contrast, the N-cadherin expression level was substantially higher in sparse cells compared to their confluent counterparts (Figure 1B, right panel).

We next compared the expression levels of proteasome genes between sparse and confluent MCF10A cells using real time RT-PCR. Five proteasome genes including three encoding 20S core subunits (PSMA1, PSMA5 and PSMB6) and two encoding 19S regulatory
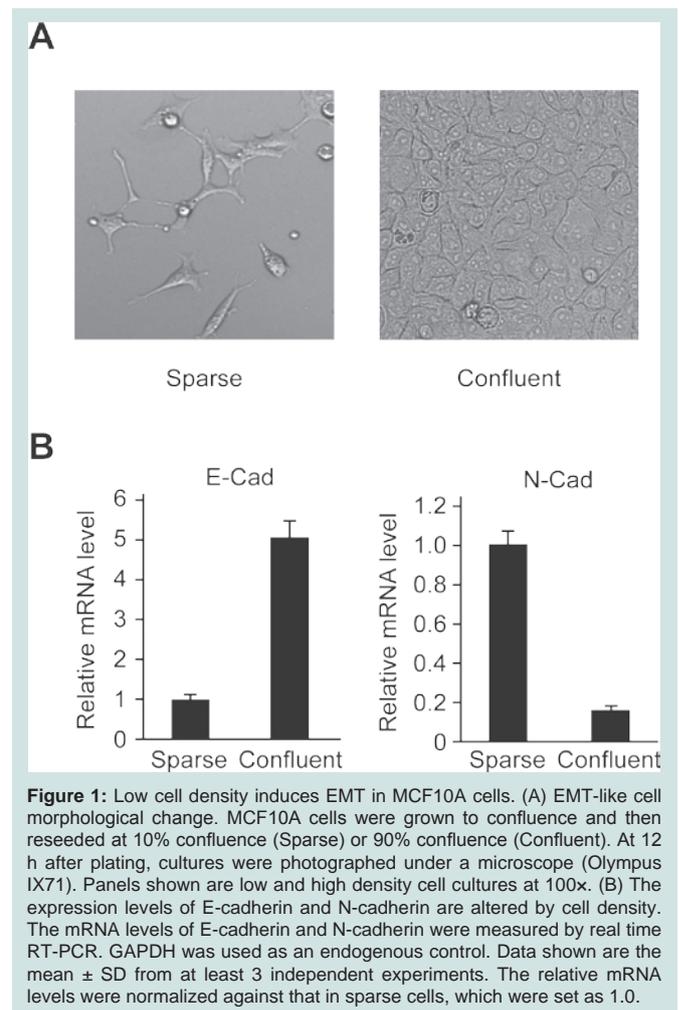

Figure 1: Low cell density induces EMT in MCF10A cells. (A) EMT-like cell morphological change. MCF10A cells were grown to confluence and then reseeded at 10% confluence (Sparse) or 90% confluence (Confluent). At 12 h after plating, cultures were photographed under a microscope (Olympus IX71). Panels shown are low and high density cell cultures at 100×. (B) The expression levels of E-cadherin and N-cadherin are altered by cell density. The mRNA levels of E-cadherin and N-cadherin were measured by real time RT-PCR. GAPDH was used as an endogenous control. Data shown are the mean ± SD from at least 3 independent experiments. The relative mRNA levels were normalized against that in sparse cells, which were set as 1.0.

subuits (PSMC2 and PSMD3) were chosen for this study. We found that all proteasome genes tested were expressed at a higher level (> 2 fold) in sparse than in confluent cells (Figures 2A-2E). Consistently, the proteasome activity was higher in sparse than in confluent cells (Figure 2F). These results suggest that increase in proteasome activity may be important for EMT in sparse MCF10A cells. To test this hypothesis, we used proteasome inhibitor MG132 to treat MCF10A cells at low or high cell density. Indeed, MG132 was able to inhibit EMT in sparse MCF10A cells (compare Figure 3A and Figure 1A). In line with this observation, MG132 averted the downregulation of E-cadherin in sparse MCF10A cells (Figure 3B). Thus, EMT in sparse MCF10A cells is regulated by proteasome activity, probably through
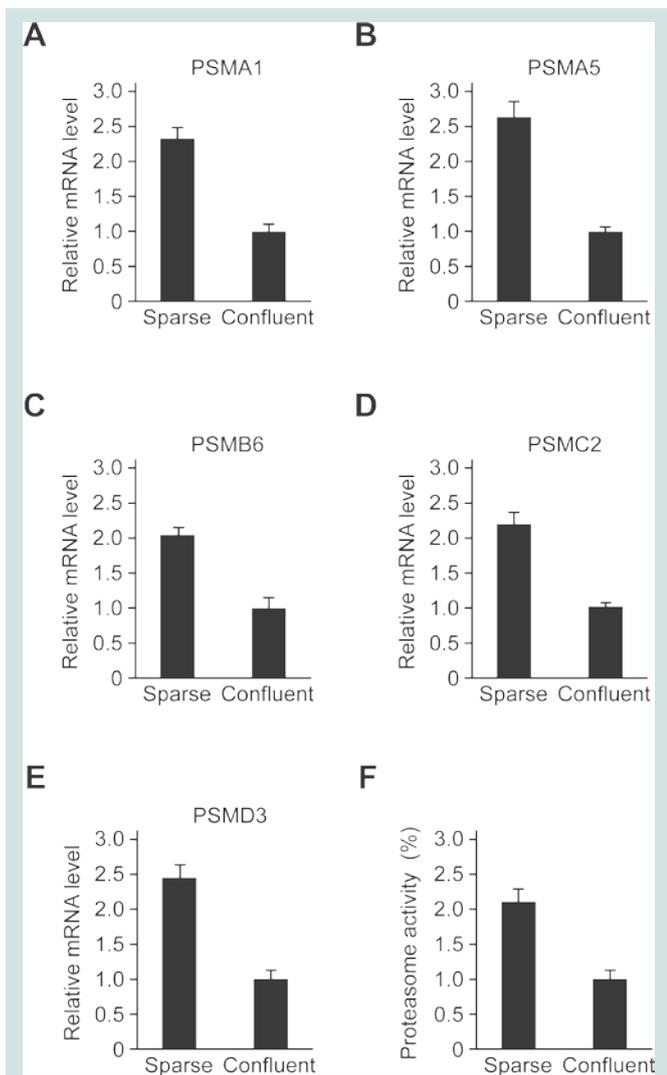

Figure 3: Inhibition of proteasome blocks EMT in sparse MCF10A cells. (A) MG132 stops EMT in sparse MCF10A cells. (B) Downregulation of E-cadherin in sparse MCF10A cells is averted by MG132. The mRNA levels of E-cadherin in sparse and confluent MCF10A cells treated with MG132 or DMSO were measured by real time RT-PCR as described in Figure 1.

modulating the expression of E-cadherin.

The transcription repressor Snail has been shown to play an important role in EMT through downregulation of E-cadherin expression by interacting with the proximal E-boxes of the E-cadherin promoter [4,5]. We found that the mRNA level of Snail markedly increased in sparse MCF10A cells (Figure 4A). However, the steady state level of Snail protein was only slightly higher in sparse than in confluent MCF10A cells (Figure 4B). We also compared the expression of Slug, another member of the conserved Snail family of transcription repressors, in low and high cell density cultures. Like Snail, the mRNA level of Slug is higher but the steady state protein level is similar in sparse MCF10A cells in comparison to their confluent counterparts (Figures 4A and 4B). The "discrepancy" between mRNA and protein levels may result from faster degradation of Snail and Slug in sparse cells due to their higher proteasome activity. It has been shown that Snail and Slug are unstable proteins and are degraded by the proteasome [19,20]. These results indicate that the suppression of E-cadherin expression in sparse MCF10A cells is unlikely caused by upregulation of Snail and Slug genes.

Discussion

It is well known that MCF10A cells undergo spontaneous EMT-like phenotype changes depending on cell density. However, the signals and mechanisms triggering EMT are not completely understood. Previous studies have shown that the expression of E-cadherin and N-cadherin in MCF10A cells was dependent on cell

Figure 2: Increase of proteasome gene expression and proteasome activity in sparse MCF10A cells. (A-E) Comparison of the mRNA levels of various proteasome subunits in sparse and confluent cell. Real time RT-PCR was used to measure the transcription levels of PSMA1 (A), PSMA5 (B), PSMB6 (C), PSMC2 (D), and PSMD3 (E) as described in Figure 1. The relative mRNA levels in confluent cells were set as 1.0. (F) Measurement of the proteasome activity in sparse and confluent MCF10A cells. The chymotrypsin-like proteolytic activity of proteasome was assayed by incubation of cell extracts of sparse and confluent cells with the Suc-LLVY-AMC peptide substrate. The released fluorescent signal (AMC group) was recorded. The proteasome activity of confluent cells was set as 1.0. Data shown are the mean ± SD of 3 independent experiments.
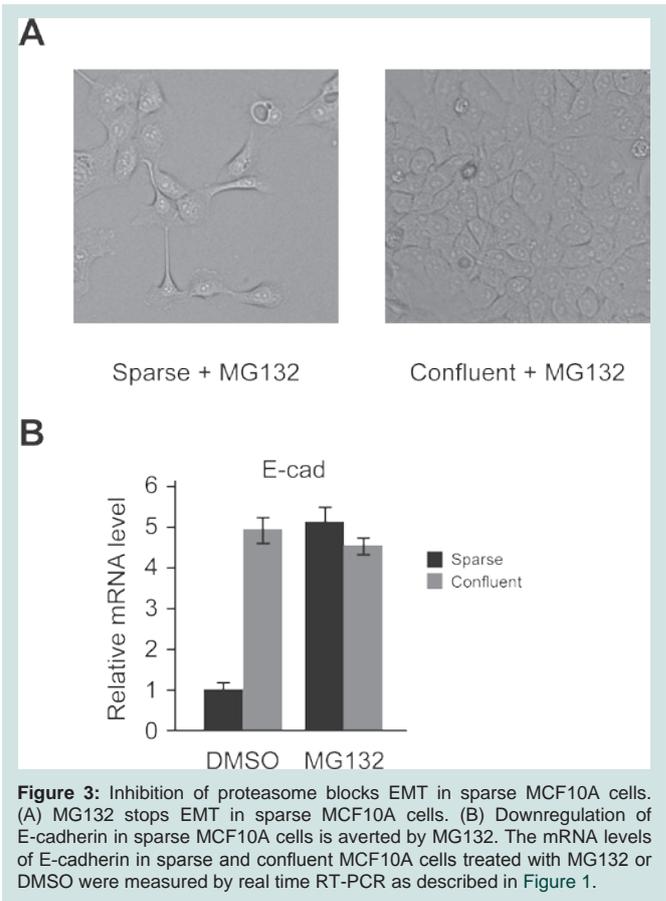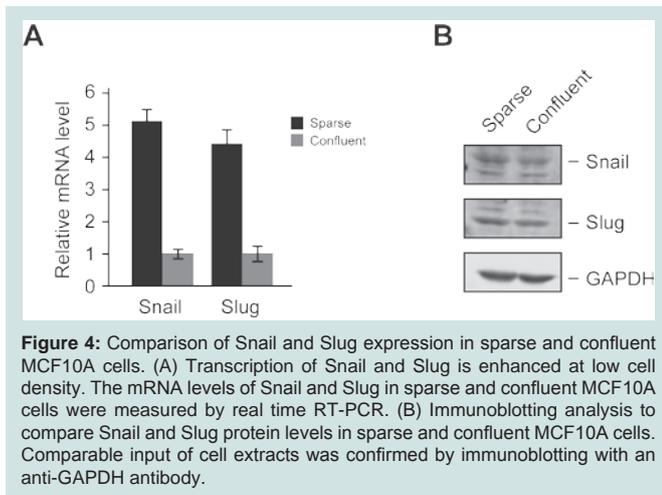
Figure 4: Comparison of Snail and Slug expression in sparse and confluent MCF10A cells. (A) Transcription of Snail and Slug is enhanced at low cell density. The mRNA levels of Snail and Slug in sparse and confluent MCF10A cells were measured by real time RT-PCR. (B) Immunoblotting analysis to compare Snail and Slug protein levels in sparse and confluent MCF10A cells. Comparable input of cell extracts was confirmed by immunoblotting with an anti-GAPDH antibody.

confluence [10,11]. In this study, we confirm the early observations that the expression level of E-cadherin is lower and that of N-cadherin is higher in MCF10A cells at low density. More importantly, we reveal that the expression levels of proteasome genes and proteasome activity increase in sparse MCF10A cells. Interestingly, treatment of sparse MCF10A cells with proteasome inhibitor MG132 blocks EMT. These results indicate that proteasome gene expression is tightly regulated by cell density and that the increase of proteasome activity in response to low cell density plays a critical role in EMT of sparse MCF10A cells.

It is currently unclear how exactly the proteasome participates in EMT. Our data show that enhanced proteasome activity suppresses the expression of E-cadherin. Although the mRNA levels of Snail and Slug are markedly elevated at low cell density, the protein levels are similar between sparse and confluent cells. These results suggest that the suppression of E-cadherin gene expression in sparse MCF10A cells is likely caused by downregulation of its transcription activator(s) through proteasomal degradation instead of upregulation of its repressors such as Snail and Slug. It will be of great interest to identify the relevant transcription activators. Of note, there have been discrepant reports on the involvement of proteasome in EMT. For example, a recent study showed that inhibition of proteasome gene expression by TM4SF5 or treatment with MG132 caused EMT-like changes in SNU449 cells [6]. Perhaps, the discrepancy regarding the role of proteasome in EMT is attributed to the use of different cell lines in different studies. In fact, we found that the expression of proteasome genes is not affected by cell density in the MDA-MB-231 breast cancer cell line (data not shown). Nevertheless, our study provides a plausible mechanism for EMT in sparse MCF10A cells.

References

1. Thiery JP (2002) Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2: 442-454.

2. Arias AM (2001) Epithelial mesenchymal interactions in cancer and development. Cell 105: 425-431.

3. Yang J, Weinberg RA (2008) Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev Cell 14: 818-829.

4. Zheng H, Kang Y (2013) Multilayer control of the EMT master regulators. Oncogene.

5. Thiery JP, Sleeman JP (2006) Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol 7: 131-142.

6. Kim JY, Nam JK, Lee SA, Lee MS, Cho SK, et al. (2011) Proteasome inhibition causes epithelial-mesenchymal transition upon TM4SF5 expression. J Cell Biochem 112: 782-792.

7. Soule HD, Maloney TM, Wolman SR, Peterson WD Jr, Brenz R, et al. (1990) Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res 50: 6075-6086.

8. Pauley RJ, Soule HD, Tait L, Miller FR, Wolman SR, et al. (1993) The MCF10 family of spontaneously immortalized human breast epithelial cell lines: models of neoplastic progression. Eur J Cancer Prev 3: 67-76.

9. Debnath J, Muthuswamy SK, Brugge JS (2003) Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods 30: 256-268.

10. Sarrio D, Rodriguez-Pinilla SM, Hardisson D, Cano A, Moreno-Bueno G, et al. (2008) Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. Cancer Res 68: 989-997.

11. Maeda M, Johnson KR, Wheelock MJ (2005) Cadherin switching: essential for behavioral but not morphological changes during an epithelium-to-mesenchyme transition. J Cell Sci 118: 873-887.

12. Xie Y (2010) Feedback regulation of proteasome gene expression and its implications in cancer therapy. Cancer Metastasis Rev 29: 687-693.

13. Xu H, Ju D, Jarois T, Xie Y (2008) Diminished feedback regulation of proteasome expression and resistance to proteasome inhibitors in breast cancer cells. Breast Cancer Res Treat 107: 267-274.

14. Schwartz AL, Ciechanover A (1999) The ubiquitin-proteasome pathway and pathogenesis of human diseases. Annu Rev Med 50: 57-74.

15. Kumatori A, Tanaka K, Inamura N, Sone S, Ogura T, et al. (1990) Abnormally high expression of proteasomes in human leukemic cells. Proc Natl Acad Sci USA 87: 7071-7075.

16. Chen L, Madura K (2005) Increased proteasome activity, ubiquitin-conjugating enzymes, and eEF1A translation factor detected in breast cancer tissue. Cancer Res 65: 5599-5606.

17. Bazzaro M, Lee MK, Zoso A, Stirling WL, Santillan A, et al. (2006) Ubiquitin-proteasome system stress sensitizes ovarian cancer to proteasome inhibitor-induced apoptosis. Cancer Res 66: 3754-3763.

18. Pilarsky C, Wenzig M, Specht T, Saeger HD, Grützmann R (2004) Identification and validation of commonly overexpressed genes in solid tumors by comparison of microarray data. Neoplasia 6: 744-750.

19. Wu Y, Deng J, Rychahou, PG, Qiu S, Evers BM, Zhou BP (2009) Stabilization of Snail by NF-κB is required for inflammation-induced cell migration and invasion. Cancer Cell 15: 416-428.

20. Wang SP, Wang WL, Chang YL, Wu CT, Chao YC, et al. (2009) p53 controls cancer cell invasion by inducing the MDM2-mediated degradation of Slug. Nat Cell Biol 11: 691-704.