in embryonic development, tissue remodeling, wound repair, and mesenchymal markers including N-cadherin and vimentin [4,5]. With downregulation of epithelial makers such as E-cadherin and increase of cell motility. EMT induction is accompanied epithelial characteristics, but acquire mesenchymal characteristics independent growth. MCF-10A cells are often used as a normal including lack of tumorigenicity in nude mice and lack of anchorage-MCF10A cells exhibit numerous features of normal breast epithelia, but nontransformed human mammary epithelial cell line [7,8]. In different cells. MCF-10A is a spontaneously immortalized, cell type-specific because EMT inducers have very different effects factors [1,6]. Induction of EMT appears to be highly tissue- and 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), ZEB1 (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 (carbobenzoxy-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 demonstrated that cell density-dependent EMT in MCF10A cells is regulated by proteasome activity.
mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium deoxycholate) 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 MgCl₂, 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 was monitored with an excitation filter of 355 nm and an emission filter of 460 nm using a Spectramax Gemini X 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 subunits (PSMD3 and PSMD6) were chosen for this analysis because they have been implicated in cancer development. The mRNA levels of these genes were measured by real time RT-PCR and normalized against that in sparse cells, which were set as 1.0.

Table 1: Primers used for real-time PCR experiments

<table>
<thead>
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<th>Gene</th>
<th>NCBI accession no.</th>
<th>Primer sequence (5’-3’)</th>
<th>Gene</th>
<th>NCBI accession no.</th>
<th>Primer sequence (5’-3’)</th>
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<td></td>
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<td>PSMA1</td>
<td>NM_148976</td>
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<tr>
<td></td>
<td></td>
<td>Reverse: CTCGTCACCAACACTA</td>
<td>PSMA5</td>
<td>NM_002790</td>
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<td>Reverse: GGATCTCTGGTTGTATGAC</td>
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</table>

![Figure 1](image.png)

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 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.
subunits (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 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...
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 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 TM45F5 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


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