

Cell density-dependent regulation of p73 in breast cancer cells

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Abstract. Molecular regulation of p73, a p53 family member, remains unclear. Here we report that p73 expression is significantly regulated by cell densities. In particular, we found that p73 α and p73 β are differentially regulated. While p73 β protein levels were inversely correlated with cell densities, p73 α protein levels behaved oppositely. We further showed that density-dependent changes of p73 α follow the same patterns as E2F-1 and TAp73 mRNA levels, suggesting transcriptional regulation. Our data also suggest that high levels of p73 β at lower densities may be due to increased protein stability. However, AIP-4/Itch appeared not to be involved in downregulation of p73 β at high densities. Moreover, we also found that subcellular location of p73 isoforms changes with the culture density increases. While high level of p73 β at low density was mainly presented in the nucleus, low levels of this protein at high densities were mainly in the cytosol. Taken together, these findings reveal a novel mechanism that differentially regulates p73 isoforms and underscores the role of cell-cell interaction in p73 regulation, which may advance our understanding of p73 expression and function in human cancers.

Introduction

p73 is a member of the p53 family of transcription factors, which is implicated in cell differentiation, development, apoptosis and tumor suppression (1). The p73 gene encodes two groups of isoforms: TAp73s and Δ Np73s. TAp73s contain a transactivation (TA) domain in their N-termini, while Δ Np73s have the TA domain missing. Alternative splicing at the carboxyl terminus of both TAp73 and Δ Np73 yields further p73 isoforms. For example, TAp73s include p73 α , β , χ , δ , ϵ , φ , γ , η (2). The major isoforms of p73 are TAp73 α and TAp73 β (3). In general, TAp73 and Δ Np73 are functional antagonists to each other. TAp73s are able to activate the p53 pathway; Δ Np73s function as dominant negative mutants that inactivate p53 and TAp73 (2).

Expression and regulation of p73 in human cancer remain unclear. In contrast to frequent p53 mutation, p73 mutation in human cancer is rare (2). However, overexpression of wild-type p73 is frequently detected in different cancers, such as breast, prostate, bladder and esophageal cancers (4). Previous studies demonstrate that p73 can be regulated at both mRNA and protein levels by a number of factors, including DNA damage, interactions with oncoproteins and viral proteins (5,6). The TAp73 promoter is known to contain binding sites for the transcription factors E2F-1, c-Myc, and c-Myb (7,8). The Δ Np73 promoter contains binding sites for p53 and TAp73 (9,10). Activation of TAp73 transcription contributes to E2F-1-induced apoptosis (11). p73 activity is also regulated through its physical interaction with a number of binding partners, including c-Abl, p300, WT1, c-Myc, MM1, MDM-2, E1A and others (12-14). In particular, when c-Abl is activated in response to DNA damage, it phosphorylates TAp73 and increases its protein stability (5,6,15). Recently, it has been shown that p73 degradation is regulated by AIP4, a ubiquitin ligase (16).

Despite significant advances, more factors that regulate p73 remain to be identified. Here we show that the expression of p73 is closely regulated by cell density. Interestingly, p73 α and p73 β are differentially regulated at different cell densities. This novel finding suggests that cell-cell interaction and signaling from the microenvironment might contribute to the regulation of p73 expression and functional modulation.

Materials and methods

Cell culture and transfection. MCF-7, MDA-MB-231 and HS578T breast cancer cell lines were purchased from the American Type Culture Collection (ATCC). The cells were cultured in DMEM/F12 medium (Sigma Co., St. Louis, MO) containing 10% fetal bovine serum (FBS). MCF-7 cell line expressing p53 siRNA was established by transfecting MCF-7 cells with plasmid encoding p53-specific siRNA (Imgenex), followed by G418 selection. G418 resistant clones were pooled for further characterization. For determining p73 levels at different culture densities, cells were inoculated into 60-mm dishes with cell number as low as 1×10^4 cells/dish to as high as 3×10^6 cells/dish and incubated for 48 h. The cells were then collected to examine p73 RNA and protein levels. For AIP-4 siRNA (Dharmacon RNA Technologies, Lafayette, CO) transfection, 1×10^6 cells were inoculated into a 60-mm dish 24 h before transfection. The transfections were performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol.

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Sulforhodamine B (SRB) assay. Cells were inoculated in a 24-well plate at low (500/well) or higher (25,000/well) densities. Cells from each group were then fixed with 10% trichloroacetic acid (TCA) at 24 and 48 h after inoculation. TCA fixed cells were then stained with 0.4% SRB for 30 min followed by four washes. Protein-bound dye was dissolved in 10 mM Tris base and plates were read at 565 nm using an ELISA reader. Cell proliferation rate was calculated based on the increase in SRB absorbance between 24 and 48 h of the same density group (3 parallel samples in each group). The data were analyzed with Student's t test.

Western blotting. The cells lysate was collected at indicated time after inoculation. Protein concentration was determined using a BCA kit. Protein lysate (50 μ g) was separated with 12 or 15% SDS-PAGE gel and transferred to nitrocellulose membrane. The membrane was probed with a specific primary antibody at a dilution of 1:300 to 2000, followed by washing, probing with a corresponding secondary antibody and autoradiography using an ECL kit (Amersham/GE Health Care). Antibodies against p53, p73 α (H79), E2F-1 AIP-4/Itch and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against p73 β (AB3) was from Calbiochem. Anti-p73 α (C24) antibody for immunofluorescence was purchased from Vector laboratories (Burlingame, CA).

RNA extraction and RT-PCR. Total RNA was isolated from control and treated cells using RNeasy Mini Kit (Qiagen, CA). First strand cDNA synthesis was performed using 3 μ g total RNA and SuperscriptIII™ First Strand synthesis system (Invitrogen). mRNA levels of p73 and actin were detected with PCR using the following primers: TAp73 forward: 5'-CGG GAC GGA CGC CGA TG-3'; Δ Np73 forward: 5'-CGC CTA CCA TGC TGT ACG TCG GTG-3; p73 reverse: 5'-CTT GGC GAT CTG GCA GTA G-3'. Actin forward: 5'-GCA CCA CAC CTT CTA CAA TGA GC-3'; actin reverse: 5'-GAC GTA GCA CAG CTT CTC CTT AAT G-3'.

Results

Cell density-dependent regulation of p73 β and its correlation with proliferation rate. During our study of p73 regulation by other factors, we observed that p73 β protein levels varied with culture conditions. This was based on the results detected with a well established antibody (AB3) that specifically recognizes p73 β (17). To test the correlation between cell density and p73 β expression, we examined p73 β protein levels in MCF-7 cells grown at different densities, which varied from 1x10⁴ to 3x10⁶/60 mm dish. We found that p73 β levels were inversely correlated with cell density. MCF-7 cells cultured at very low density expressed very high protein levels of p73 β , which decreased with increasing cell density (Fig. 1A). To exclude the possibility that density regulated p73 β in MCF-7 cells is a cell line-specific phenomenon, we examined the effect of cell density on p73 β expression in MDA-MB-231 and HS578T breast cancer cell lines. As shown in Fig. 1B and C, each of the cell lines exhibited similar density-dependent regulation of p73 β . These results indicate that cell density-dependent regulation of p73 β is

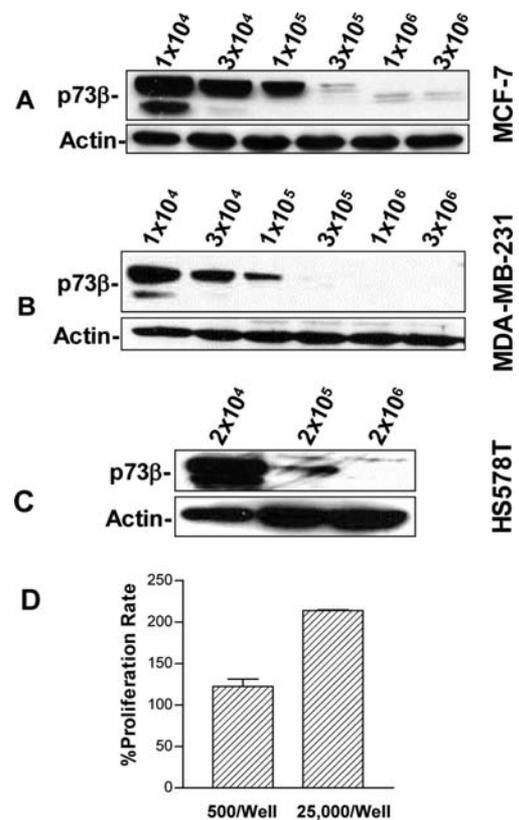


Figure 1. Cell density regulates p73 expression in breast cancer cells (A-C). MCF-7 (A), MDA-MB-231 (B) and HS578T (C) cells were seeded at different densities and cultured for 48 h. Protein levels of p73 β were analyzed with Western blot (the p73 antibody was AB3). (D) Proliferation rate of MCF-7 cells at different densities. The cells were inoculated in a 24-well plate at 500/well or 25,000/well. Cell proliferation rate was calculated based on SRB absorbance of 48 h over 24 h. *p<0.01.

universal. To correlate p73 β expression with cell proliferation, the proliferation rate of MCF-7 cells at low or higher densities was measured using SRB assays. As shown in Fig. 1D, cell proliferation rate at lower density (500/well of 24-well plate) was significantly lower than the higher density group (25,000/well), suggesting a correlation between high p73 β levels and slower cell growth at low density.

Cell density-dependent regulation of p73 α , p53 and E2F-1. Based on cell density-dependent changes in p73 β levels, it is reasonable to question whether p73/p53 family members and their regulatory factors are modulated concomitantly. We therefore examined the protein levels of p73 α , p53 and E2F-1 in MCF-7 cells at different densities. Of note, p73 α protein was identified based on a 73 Kd band recognized by a well-established p73 antibody, H79 (18). As shown in Fig. 2A, we found that the expression of all these factors was regulated by cell density, although to a lesser extent. In contrast to p73 β , protein levels of p73 α , p53 and E2F-1 were lower at low density but higher in high density. These results indicate that the pattern of cell density-dependent regulation of p73 α , p53 and E2F-1 was opposite to that of p73 β .

Previous studies suggest that expression of both TAp73 and Δ Np73 expression can be regulated by p53 (19,20). Our results show that, in cell density-related regulation, p53 levels

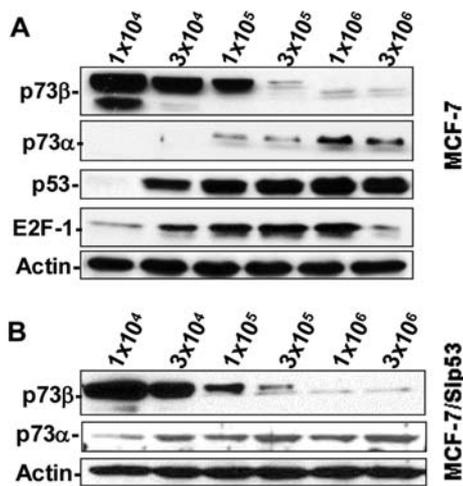


Figure 2. (A) Regulation of p53, p73 family of proteins by cell density. MCF-7 cells were inoculated at increasing cell densities and cultured for 48 h. Protein levels of individual markers were detected using Western blot with anti-p73 α (H79), anti-p53 (DO-1), anti-E2F-1 (KH-95) and AIP4/Itch (G-11) antibodies. (B) Density-dependent regulation of p73 is independent of cellular p53 status. Control and MCF-7/p53siRNA cells were inoculated at indicated densities and p73 protein levels were determined by Western blotting with anti-p73 β antibody (AB3).

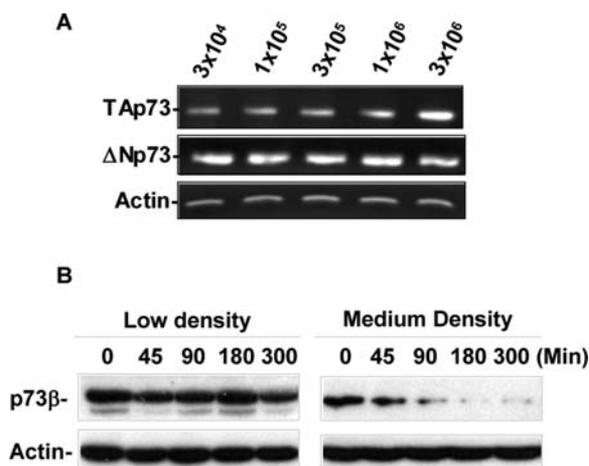


Figure 3. (A) Cell density-dependent regulation of p73 transcription. MCF-7 cells were cultured at indicated densities for 48 h, followed by RNA extraction. TAp73 mRNA levels were detected by RT-PCR using gene-specific primers. (B) p73 β protein stability is increased in low density cultures. MCF-7 cells were cultured at low (2×10^4) and medium (2×10^5) densities overnight and treated with 50 μ M CHX for indicated time points. Whole cell lysates were collected and subjected to Western blot analysis using anti-p73 β antibody (AB3).

were inversely correlated with p73 β but parallel to the changes in p73 α . To test whether cell density-dependent regulation of p73 is p53-dependent, we examined the p73 β levels in MCF-7/p53siRNA cell line, which is a stable MCF-7 subline transfected with p53-specific siRNA. As shown in Fig. 2B, p73 β protein levels in MCF-7/p53siRNA cells at different densities show a similar pattern to MCF-7 cells. The results suggest that cell density-dependent regulation of p73 β is p53-independent. This is supported by similar density-dependent expression of p73 β in MDA-MB-231 cells (Fig. 1B), which are known to express mutant p53.

Cell density-dependent regulation of p73 transcription. To study the underlying mechanism of cell density-dependent regulation of p73, we next examined mRNA levels of p73 in cells at different densities using RT-PCR. Isoform-specific primers were used to detect TAp73 and Δ Np73. As shown in Fig. 3A, TAp73 RNA levels increased with increasing density, which is parallel to the protein pattern of p73 α (Fig. 2A). Since it was reported that Δ Np73 mRNA and protein levels were higher in confluent cells than at low density (20), we also examined the mRNA levels of Δ Np73. However, our data demonstrated that Δ Np73 levels were basically unchanged at different cell densities.

To verify the PCR results and evaluate TAp73 promoter activity at different densities, we established a stable MCF-7 subline transfected with a reporter plasmid carrying TAp73 promoter from -2713 to +20, which was named MCF-7/p73PF/Luc. It has been confirmed that the reporter gene in the clone used for the following study is not affected by the positional effect (data not shown). Results from luciferase assays on p73 promoter activity in MCF-7/p73PF/Luc at different density indicate that p73 promoter activation increases with cell density, which is consistent with the variation of TAp73 mRNA levels (Fig. 3). These results suggest that high levels of p73 β at low density are independent of p73 transcription. In contrast, concomitant changes in p73 α protein levels, E2F-1, TAp73 mRNA levels suggest that transcriptional regulation plays a critical role in cell density-dependent regulation of p73 α .

p73 β protein stability is cell density-dependent. To test whether modulation of p73 β stability was involved in cell density-dependent regulation of p73 β , MCF-7 cells at low (2×10^4) and higher density (2×10^5) were treated with the protein synthesis inhibitor cycloheximide (CHX) in a time-course experiment (for 0 min, 45 min, 1.5, 3 and 5 h) and p73 β protein levels were analyzed by Western blotting. The results show that p73 β was relatively stable in cells at low density, whereas its degradation in cells at higher density was faster (Fig. 3B). This suggests that modified protein stability may contribute to lower protein levels of p73 β at higher densities.

Cell density-dependent regulation of p73 β is independent of ubiquitin ligase AIP4/Itch. AIP4/Itch is a human Hect-ubiquitin protein ligase (E3) that binds to p73 and targets it to proteasome-dependent degradation (21). AIP4/Itch expression is downregulated upon DNA damage, thereby allowing stabilization of the p73 protein (16). To test whether AIP4/Itch-mediated regulation of p73 contributes to cell density-dependent modulation of p73 β , we first tested whether the expression of AIP4/Itch is cell density-dependent. As shown in Fig. 4A, AIP4/Itch protein levels increased in a density-dependent manner, which are inversely correlated with p73 β expression. We next examined whether knocking down AIP4 using AIP4-specific siRNA modulate p73 β protein levels at a relatively low density. As shown in Fig. 4B, AIP4 siRNA effectively inhibited the expression of AIP4 in the transfected cells. Interestingly, AIP4 knockdown results in increase in p73 α levels but little change in p73 β levels. Although the experiments in Fig. 3B suggest a correlation between lower

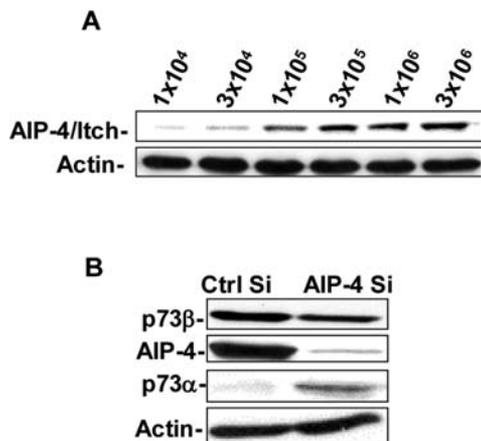


Figure 4. (A) AIP-4/Itch levels increased with increasing culture density. MCF-7 were inoculated at different densities as indicated. AIP-4 protein levels were determined by Western blotting. (B) Knockdown of AIP-4 in high density cells did not increase p73 β protein levels. AIP-4 was knocked down in MCF-7 cells using AIP-4-specific siRNA. AIP-4 and p73 levels in control siRNA and AIP-4 siRNA expressing MCF-7 cells were determined by Western blot using anti-p73 α (H79) and anti-p73 β (AB3) antibodies.

levels of p73 β and decreased protein stability at higher density, results from AIP4 knockdown do not support the role of AIP4 in p73 β degradation at higher density.

Subcellular localization of p73 in MCF-7 cells at different densities. To verify the differential expression of p73 β at different cell densities, we examined p73 β expression by immunocytochemistry. To this end, MCF-7 cells were inoculated in chamber slide at different density followed by immunostaining. The results show that p73 β signal was very strong in cells at low density but weak in cells at higher density (Fig. 5A), which is consistent with the Western blot data. While examining p73 expression, we noticed that high levels of p73 β in cells at lower density were primarily located in the nucleus. In contrast, substantial p73 β in the cells at higher density appeared to be cytosolic. To further investigate this issue, we examined p73 β localization in MCF-7 cells at different density by cell fractionation. As shown in Fig. 5B, preparation of nuclear and cytosolic fractions was successful, as demonstrated by distinctive signals of lamin B and actin, which are enriched in nucleus and cytosol, respectively. Clearly, as shown in Fig. 5B, p73 protein levels decreased with increasing cell density. Interestingly, however, a different pattern of p73 expression was observed in low versus high density cultures. In low density cultures, p73 was found mainly in nucleus whereas in dense cultures, p73 was predominantly cytosolic.

Discussion

In this study, we investigated the effect of variation in cell densities on p73 expression in breast cancer cells. We demonstrate that p73 levels, in particular p73 β protein levels, are sensitive to changes in cell density. This suggests that cell-cell interaction and extra cellular matrix-mediated signaling, which are involved in cell density-dependent regulation and in tumor microenvironment modulation, may affect

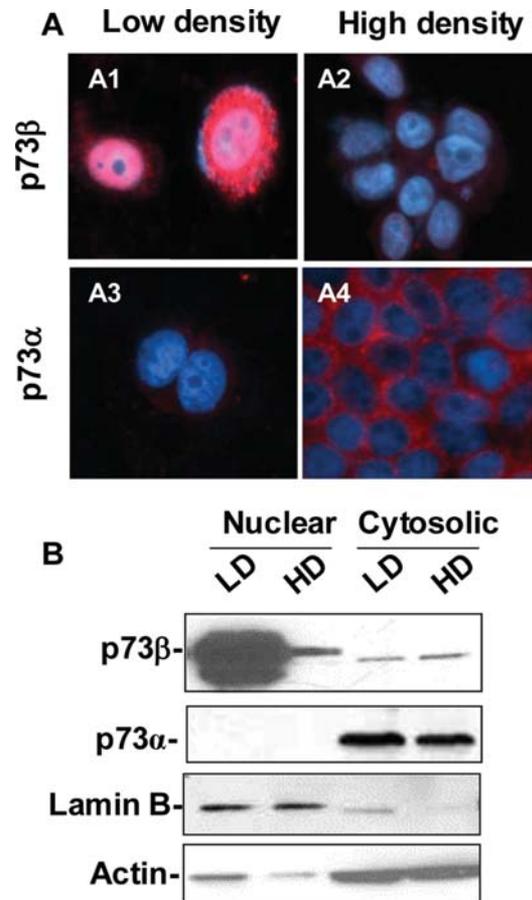


Figure 5. Subcellular localization of p73 changes in MCF-7 cells in a density-dependent manner. MCF-7 cells were cultured at indicated densities in 8-well coverglass chamber slides for 48 h. After 48 h, cells were fixed and immunostained for p73 using anti-p73 α (C24) and anti-p73 β (AB3) antibodies and FITC-labeled secondary antibody. Images were taken using (Carl-Zeiss) confocal microscope. (B) MCF-7 cells were cultured at low (2×10^4) and high (2×10^6) densities in 60-mm dishes. After 48 h of incubation, cells were collected. Nuclear and cytosolic fractions were prepared, and subjected to Western blot analysis using anti-p73 (AB3 and C24), anti-lamin B, and anti-actin antibodies.

p73 expression and function. This finding identified a novel mechanism that regulates p73 expression and may advance our understanding of p73 expression in human cancer tissues.

In the characterization of cell density-dependent regulation of p73, we found that p73 isoforms are differentially regulated by cell density. In contrast to p73 α , whose levels increase with cell densities, p73 β protein levels are high at low density and decrease with density increase. This suggests that in addition to alternate promoter sets and splicing (2), cell density-related factors may play a role in the differential regulation of p73 isoforms. In this study, identification of p73 α and p73 β was mainly based on established antibodies primarily recognizing specific isoforms. Although this may have certain limits in explaining other isoforms, evidence supporting the notion that cell density differentially regulates p73 isoforms is concrete. The effect of density-dependence of other p73 isoforms and more specific approaches that discerns individual forms will be tested in future studies.

The mechanisms of cell density-dependent regulation of p73 appear to be complicated. Cell density may regulate

p73 α and p73 β levels by different mechanisms. Our data showed that density-dependent regulation of p73 was p53-independent, although p53 levels also changed with cell densities. Since p73 α levels behaved concomitantly with E2F-1 levels and TAp73 mRNA levels in response to density variation, cell density-dependent regulation of p73 α is mainly regulated at the transcriptional level. This is consistent with a recent report that p73 may be induced in confluent cells with pRB pathway alterations (22). In contrast, p73 β behaved oppositely with p73 α , E2F-1 and TAp73 mRNA, suggesting a different mechanism. Indeed, results from CHX blocking experiments suggest that p73 β protein at low density is more stable than at high density. However, knockdown of AIP4/Itch, a ubiquitin ligase that regulates p73 degradation (16), in MCF-7 cells only resulted in increased protein levels of p73 α but not p73 β (Fig. 4). As p73 stability could be regulated by other factors, such as promyelocytic leukemia (PML) (23), how p73 β is regulated in density-dependent manner requires further investigation.

Given the differential regulation of p73 isoforms associated with cell density, the functional relevance of a specific isoform appears to be complicated. Relatively, cell density-dependent regulation of p73 β was more evident than p73 α (Fig. 2). Previous reports suggest that p73 β was more potent than p73 α in regulating p53-dependent genes (3,24). Based on our data of cell density-dependent proliferation (Fig. 1D), high levels of p73 β at very low density were associated with slower proliferation. This is consistent with the common observations that cells do not grow well under too sporadic conditions. More specific designs correlating p73 isoforms and cellular functions at different densities will be tested in future studies.

Immunostaining experiments indicate that subcellular localization of p73 isoforms changes with changes in cell density. Many signaling proteins are regulated by alterations in their subcellular location. MDM2 is known to alter subcellular localization of p73 (25). Differential regulation of p73 α and p73 β cellular localization at different culture densities adds an important aspect to be considered while manipulating p73-dependent gene expression for tumor cell growth inhibition and apoptosis. As in all other p73-related studies, differentiation among p73 isoforms is a complicated issue. In this study the differential regulation of p73 isoforms was mainly based on antibodies primarily recognizing p73 α or β . We acknowledge the limit of this approach in explanation of other p73 isoforms. Nevertheless, findings in this report are intriguing and may lead to more studies on p73 regulation by cell density and extra cellular matrix.

In summary, our data demonstrate an interesting finding that p73 α and p73 β levels are differentially regulated in a cell density-dependent manner. In particular, expression of p73 β was more sensitive to cell density. Mechanistic studies suggest that cell density-dependent regulation of p73 β mainly occurs at the protein level. In contrast, the regulation of p73 α appears to be at the transcriptional level. Moreover, regulation of p73 β was independent of p53 and AIP4/Itch, although involvement of other factors requires further exploration. Our results suggest that cell-cell, cell-matrix interactions, which are critical for tumor initiation and invasion, may play a role in regulating p73 expression. More detailed studies are required

to identify specific molecules involved in intercellular interactions that are responsible for density-mediated regulation of p73 expression in breast cancer.

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