Cell density-dependent regulation of p73 in breast cancer cells

CHAITALI TOPKHANE, SHIHE YANG, ZHIZHUANG JOE ZHAO and XIAOHE YANG

Department of Pathology, University of Oklahoma Health Sciences Center; Oklahoma City, OK 73104, USA

Received July 30, 2009; Accepted August 27, 2009

DOI: 10.3892/ijo_00000461

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 p73α and p73ß are differentially regulated. While p73ß protein levels were inversely correlated with cell densities, p73α protein levels behaved oppositely. We further showed that density-dependent changes of p73α follow the same patterns as E2F-1 and TAp73 mRNA levels, suggesting transcriptional regulation. Our data also suggest that high levels of p73ß at lower densities may be due to increased protein stability. However, AIP-4/Itch appeared not to be involved in downregulation of p73ß at high densities. Moreover, we also found that subcellular location of p73 isoforms changes with the culture density increases. While high level of p73ß 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 ΔNp73s. TAp73s contain a transactivation (TA) domain in their N-termini, while ΔNp73s have the TA domain missing. Alternative splicing at the carboxyl terminus of both TAp73 and ΔNp73 yields further p73 isoforms. For example, TAp73s include p73α, β, δ, ε, γ, η (2). The major isoforms of p73 are TAp73α and TAp73β (3). In general, TAp73 and ΔNp73 are functional antagonists to each other. TAp73s are able to activate the p53 pathway; ΔNp73s 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 ΔNp73 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, p73α and p73ß 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 1x10^4 cells/dish to as high as 3x10^6 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, 1x10^6 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.

Correspondence to: Dr Xiaohe Yang, Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA
E-mail: xiaohe-yang@ouhsc.edu

Key words: p73, cell density, p53, E2F-1, ITCH/AIP4, breast cancer
Sulforhodamine B (SRB) assay. Cells were incubated 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 incubation.
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 μg) was separated with 12 or 15% SDS-PAGE gel and transferred to nitricellulose
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, p73α (H79), E2F-1 AIP-4/Itch
and actin were purchased from Santa Cruz Biotechnology
(Santa Cruz, CA). Antibody against p73β (AB3) was from
Calbiochem. Anti-p73α (C24) antibody for immunofluores-
cence 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 μg
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'; ΔNp73 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 p73β and its correlation
with proliferation rate. During our study of p73 regulation by
other factors, we observed that p73β protein levels varied with
culture conditions. This was based on the results detected
with a well established antibody (AB3) that specifically
recognizes p73β (17). To test the correlation between cell
density and p73β expression, we examined p73β protein
levels in MCF-7 cells grown at different densities, which
density and p73β expression, we examined p73β protein
levels were inversely correlated with cell density. MCF-7
cells cultured at very low density expressed very high protein
levels of p73β, which decreased with increasing cell density
(Fig. 1A). To exclude the possibility that density regulated
p73β in MCF-7 cells is a cell line-specific phenomenon, we
examined the effect of cell density on p73β 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 p73β. These results
indicate that cell density-dependent regulation of p73β is
universal. To correlate p73β 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 p73β
levels and slower cell growth at low density.

Cell density-dependent regulation of p73α, p53 and E2F-1.
Based on cell density-dependent changes in p73β 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 p73α, p53 and E2F-1 in
MCF-7 cells at different densities. Of note, p73α 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
p73β, protein levels of p73α, 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 p73α,
p53 and E2F-1 was opposite to that of p73β.

Previous studies suggest that expression of both TAp73 and
ΔNp73 expression can be regulated by p53 (19,20). Our
results show that, in cell density-related regulation, p53 levels
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 TAIP73 and ANP73. As shown in Fig. 3A, TAIP73 RNA levels increased with increasing density, which is parallel to the protein pattern of p73α (Fig. 2A). Since it was reported that ANP73 mRNA and protein levels were higher in confluent cells than at low density (20), we also examined the mRNA levels of ANP73. However, our data demonstrated that ANP73 levels were basically unchanged at different cell densities.

To verify the PCR results and evaluate TAIP73 promoter activity at different densities, we established a stable MCF-7 subline transfected with a reporter plasmid carrying TAIP73 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 TAIP73 mRNA levels (Fig. 3). These results suggest that high levels of p73β at low density are independent of p73α.

p73β protein stability is cell density-dependent. To test whether modulation of p73β stability was involved in cell density-dependent regulation of p73β, MCF-7 cells at low (2x10^4) and higher density (2x10^5) 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 p73β protein levels were analyzed by Western blotting. The results show that p73β 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 p73β at higher densities.

Cell density-dependent regulation of p73β 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 p73β, 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 p73β expression. We next examined whether knocking down AIP4 using AIP4-specific siRNA modulate p73β 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 p73α levels but little change in p73β levels. Although the experiments in Fig. 3B suggest a correlation between lower

were inversely correlated with p73β but parallel to the changes in p73α. To test whether cell density-dependent regulation of p73 is p53-dependent, we examined the p73β levels in MCF-7/p53siRNA cell line, which is a stable MCF-7 subline transfected with p53-specific siRNA. As shown in Fig. 2B, p73β 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 p73β is p53-independent. This is supported by similar density-dependent expression of p73β in MDA-MB-231 cells (Fig. 1B), which are known to express mutant p53.
levels of p73ß and decreased protein stability at higher density, results from AIP4 knockdown do not support the role of AIP4 in p73ß degradation at higher density.

Subcellular localization of p73 in MCF-7 cells at different densities. To verify the differential expression of p73ß at different cell densities, we examined p73ß expression by immunocytochemistry. To this end, MCF-7 cells were inoculated in chamber slide at different density followed by immunostaining. The results show that p73ß 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 p73ß in cells at lower density were primarily located in the nucleus. In contrast, substantial p73ß in the cells at higher density appeared to be cytosolic. To further investigate this issue, we examined p73ß 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 p73ß 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 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 p73α, whose levels increase with cell densities, p73ß 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 p73ß and p73ß 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 discern 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
p73α and p73β 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 p73α levels behaved concomitantly with E2F-1 levels and TAp73 mRNA levels in response to density variation, cell density-dependent regulation of p73α 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, p73β behaved oppositely with p73α, E2F-1 and TAp73 mRNA, suggesting a different mechanism. Indeed, results from CHX blocking experiments suggest that p73β 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 p73α but not p73β (Fig. 4). As p73 stability could be regulated by other factors, such as promyelocytic leukemia (PML) (23), how p73β 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 p73β was more evident than p73α (Fig. 2). Previous reports suggest that p73β was more potent than p73α in regulating p53-dependent genes (3,24). Based on our data of cell density-dependent proliferation (Fig. 1D), high levels of p73β 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 p73α and p73β 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 p73α or β. We acknowledge the limit of this approach in explana-tion 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 p73α and p73β levels are differentially regulated in a cell density-dependent manner. In particular, expression of p73β was more sensitive to cell density. Mechanistic studies suggest that cell density-dependent regulation of p73β mainly occurs at the protein level. In contrast, the regulation of 73α appears to be at the transcriptional level. Moreover, regulation of p73β 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.

Acknowledgements

This work was supported by a Seed Grant to X.Y. from the University of Oklahoma Cancer Center Institutional Research Grant from the American Cancer Society ASC-IRG #IRG-05-066-01; X.Y. is also supported by a Health Research Grant from Oklahoma Center for Advancement of Science and Technology (HR07-108) and a Research Scholar Grant from the American Cancer Society (RSG-08-138-01-CNE).

References