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p53 mediates density-dependent growth arrest☆

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Abstract While the stress-response-associated importance of the p53 tumor suppressor is well established, recent studies have also linked p53 with several basic parameters in the normal behavior of cells. Here, we present evidence that basal p53 expression in WI38 human embryonic lung fibroblasts restricts growth rate and mediates density-dependent inhibition of growth and the associated G1 phase arrest of the cell cycle by affecting the density-dependent regulation of p16/INK4a. Additionally, we show that prolonged culturing of hTert-immortalized WI38 cells leads to a loss of density-dependent growth inhibition that correlates with p27/KIP deregulation as well as the previously shown INK4a locus silencing, and to an onset of contact-induced, p53-dependent cell death.

Key words: p53; p16/INK4a; Contact inhibition; Density-dependent

1. Introduction

The p53 tumor suppressor protein has been shown to constitute a major crossroads in the cellular response to a variety of stress stimuli, including radiation, genotoxic damage, hypoxia, etc. Under such stress conditions p53 is stabilized and accumulates in the nucleus in the form of an active tetramer, acting as a transcription factor in the regulation of a variety of genes, chiefly pro-apoptotic and DNA-repair-oriented. p53 is lost or modified in most tumors, indicating its vital role in limiting the growth of aberrant cells. The levels of p53 in the cell are tightly controlled, primarily by a negative feedback loop involving HdM2, which is transcriptionally induced by p53 and targets p53 to ubiquitination and subsequent proteasomal degradation (reviewed in[1,2]). Recent studies have shown a functional role for p53 in its basal level, present in non-stressed cells, particularly in cell motility and adhesion [3,4].

Density-dependent inhibition of growth is a characteristic of all types of normal somatic mammalian cells. In vitro, this property manifests itself in a saturation curve of cell growth, as the cells enter reversible growth arrest in a density-dependent manner. Density-dependent inhibition of growth is frequently absent in tumor cells and in tumor-derived cell lines [5]. In WI38 fibroblasts, in particular, this phenomenon has been thoroughly characterized by Pignolo et al. and Owen et al. [6,7].

Several cellular factors and pathways have been connected to density-dependent inhibition of growth (reviewed in [8]), among them p16/INK4a [9–11], p21/WAF [12,13], p27/KIP [14–17], Gadd45α [18], S100C [19], VHL [20], and the cadherin–β-catenin signaling module [21,22]. Cadherin-mediated cell–cell adhesion was recently shown to be regulated by Bcl-2 [23], which is itself repressed by p53 in some cell types [24]. β-Catenin is also known to interact with p53 via a negative feedback loop (reviewed in [2]), and p53 mutations have been shown to result in abnormal β-catenin accumulation [25]. Besides the mutual connection to β-catenin, density-dependent growth inhibition is a known factor in p53 regulation, suppressing the p53 response to stress signals [26]. However, the direct contribution of p53 to the density-dependent growth inhibition response has remained largely unknown.

2. Materials and methods

2.1. Cell culture

Primary human embryonic lung fibroblasts (WI38), amphotropic and ecotropic Phoenix retrovirus-producing cells were purchased from ATCC. WI38/hTert-immortalized cells from early and late passages were prepared and provided by M. Milyavsky. WI38 cells were grown in minimal essential medium supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 2 mM L-glutamine and antibiotics. Phoenix cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS and 2 mM L-glutamine and antibiotics. All cells were maintained in a humidified incubator at 37°C and 5% CO_2. Cells were split near confluence by incubation with trypsin and replated into a new 100 mm plate at a cell density of 1500 cells/cm^2. Cumulative population doublings (CPDLs) were calculated using the formula: CPDL = log (cell output/cell input)/log2.

Density-dependent growth inhibition was assessed by plating series of 10000 cells/35 mm culture dish, periodical harvesting by trypsinization and hemacytometer counting.

2.2. Retroviral constructs

Ecotropic receptor retroviral construct was kindly provided by Dr. David Ginsberg (Weizmann Institute of Science), PLXSN-GSE56-Neo was obtained by the subcloning of the GSE56 *Bam*HI fragment from pBabe-GSE56-puro [27,28] into PLXSN. pRetro-Super (Puro) containing small interfering RNA (siRNA) against p53 was kindly provided by Dr. Moshe Oren (Weizmann Institute of Science). pRetro-Super (Puro) containing siRNA against p16 was kindly provided by Dr. Reuven Agami (The Netherlands Cancer Institute).

2.3. Retroviral infection

Amphotropic and ecotropic Phoenix packaging cells were transfected with 10 μg DNA of the appropriate retroviral construct by a standard calcium phosphate procedure. Culture supernatants were collected 36–48 h post transfection and filtered. WI38 cells were infected with the filtered viral supernatants in the presence of 4 μg/ml

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polybrene (Sigma) for 12 h, after which the medium was changed. Fresh viral suspensions were added after a 24 h interval for an additional 12 h. Infected cells were selected with 1 μg/ml puromycin (5 days), 400 μg/ml G418 (14 days) or 300 μg/ml hygromycin (5 days).

2.4. Western blotting analysis

Cells were lysed in TLB buffer [Tris–HCl (50 mM), NaCl (100 mM), Triton X-100 (1%), Na-deoxycholate (0.5%), sodium dodecyl sulfate (SDS, 0.1%)] supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail I and II (Sigma) for 30 min on ice. Extracts were analyzed for protein concentration by the Bradford assay. 50 μg of protein extract was dissolved in sample buffer (140 mM Tris pH 6.8, 22.4% glycerol, 6% SDS, 10% β-mercaptoethanol and 0.02% bromophenol blue), boiled and loaded on 10% polyacrylamide gels containing SDS. Proteins were transferred to nitrocellulose membranes.

The following primary antibodies were used: mouse monoclonal anti-p53 (1801, kindly provided by Dr. D. Lane), rabbit polyclonal anti-p53 (home-made), mouse monoclonal anti-HDM2 (4B2, 2A9, 2A10; kindly provided by Dr. M. Oren), rabbit polyclonal anti-p21 (C-19; Santa Cruz), rabbit polyclonal anti-p16 (C-20; Santa Cruz), mouse monoclonal anti-β-tubulin (Sigma), mouse monoclonal anti-p27 (kindly provided by Dr. M. Oren), mouse monoclonal anti-vinculin (kindly provided by Dr. Benny Geiger), rabbit polyclonal anti-β-catenin (kindly provided by Dr. A. Bershadsky).

The protein–antibody complexes were detected using horseradish peroxidase-conjugated secondary antibodies and the SuperSignal enhanced chemiluminescence system (Pierce).

2.5. Cell cycle analysis

Cells were detached with trypsin, fixed in 70% ethanol, and treated with 5 μg/ml propidium iodide plus 0.1 mg/ml RNase A. Samples were analyzed by flow cytometry using a FACS Sorter machine (Becton Dickinson). At least 10 000 cells were analyzed per sample.

2.6. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using the RNAeasy kit (Qiagen). 2 mg was reverse-transcribed using MMLV RT (Promega) and random hexamer primers. Real-time quantitative RT-PCR for total p16/INK4A mRNA was performed on an ABI 7000 (Applied Biosystems) machine using SYBR Green PCR mastermix (Applied Biosystems). The values for p16 were normalized to the GAPDH housekeeping control. Primer sequences for SYBR Green real-time PCR of GAPDH were 5′-ccaccaccctgttgctgta and 3′-accacagtccatgccatcac; p16/INK4a: 5′-gagcagcatggagccttcgg and 3′-catggttactgcctctggtg.

3. Results

3.1. Suppression of p53 in WI38 fibroblasts by GSE56 increases growth rate and delays the onset of replicative senescence

We chose to suppress p53 in WI38 human embryonic lung fibroblasts by means of a dominant-negative peptide, GSE56, described in [27,28]. Briefly, the peptide competitively binds the oligomerization domain of p53, thus preventing the formation of transcriptionally active p53 tetramers while stabilizing a large amount of inactive p53 (probably due to a lack of HdM2 up-regulation by p53, and perhaps also by avoiding nuclear export, as may be inferred from the supplementary figure). GSE56 was subcloned into a retroviral vector, PLXSN, with which the cells were infected. Expression of GSE56 and functional suppression of p53 in the infected cells were verified by Western blot of p53, GSE56 and two well-known transcriptional targets of p53, HdM2 and p21/WAF (Fig. 1). Introduction of GSE56 into primary WI38 fibroblasts prolonged the replicative life span in these cells (Fig. 2b), while in hTert-immortalized WI38 cells GSE56 enhanced the rate of growth (Fig. 2a).

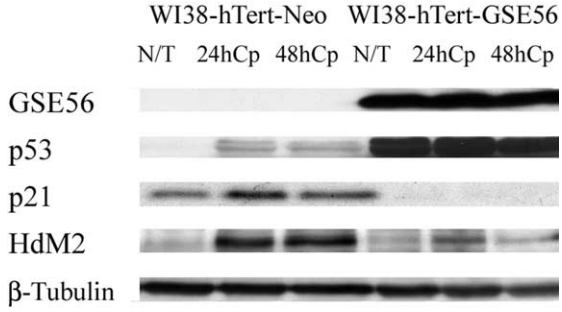

Fig. 1. GSE56 functionally suppresses p53 while stabilizing inactive p53 monomers. hTert-immortalized WI38 fibroblasts were infected with pLXSNeo retroviral plasmid containing GSE56 or the control vector. DNA damage was induced by treatment with 1 μg/ml cisplatin for periods of 24 and 48 h, and Western blot analysis was used to probe for GSE56, p53 and two well-known transcriptional targets of p53, p21/WAF and HdM-2. β-Tubulin was used as loading control. N/T: non-treated.

3.2. Suppression of p53 in WI38 fibroblasts reduces density-dependent inhibition of growth by abolishing G1 phase cell cycle arrest

To assess the effects of p53 suppression on density-dependent inhibition of growth, hTert-immortalized WI38 fibroblasts with and without GSE56 expression, and primary WI38 fibroblasts with and without anti-p53 siRNA, were seeded in culture dishes and allowed to grow beyond confluence, with periodical counting. As seen in Fig. 2c,d, GSE56-expressing and p53RNAi-expressing cells, in addition to having a higher growth rate in subconfluent culture, achieved a significantly higher final density in confluent culture. Thus, suppression of p53 by both GSE56 and p53RNAi results in a reduced density-dependent growth inhibition response.

To assess whether this change is mirrored by the cell cycle distribution of the cells, FACS cell cycle analysis was performed on dense cultures of hTert-immortalized WI38 cells with and without GSE56 expression. While control cells in dense culture showed an almost uniform G1 phase arrest (91% of cells were in G1, 4% in S and 5% in G2/M), GSE56-expressing cells show a cell cycle distribution similar to that of a proliferating culture, even after confluence (dense GSE56-expressing cells: 75% G1, 5% S, 20% G2/M; sparse control cells: 57% G1, 13% S, 30% G2/M; Fig. 3). Similar differences were observed between dense cultures of primary WI38 cells with and without GSE56 (data not shown). Thus, the suppression of density-dependent growth inhibition by inactivation of p53 is characterized by an abolition of G1 phase arrest in dense culture.

3.3. Suppression of p53 in late-passage, INK4a-locus-deficient immortalized WI38 fibroblasts by GSE56 overcomes contact-induced cell death

The cyclin-dependent kinase inhibitor, p16/INK4a, has been shown to mediate density-dependent inhibition of growth and to undergo significant up-regulation in dense culture [10]. To assess whether the observed effects of GSE56 on density-dependent growth inhibition correlate with changes in the status of the INK4a locus, the growth of late-passage immortalized WI38 fibroblasts with or without GSE56 expression was followed beyond confluence (Fig. 2e). These cells were characterized [29] as having undergone a silencing of the INK4a locus by means of promoter region methylation, associated with an increased growth rate and loss of contact inhibition. While all late-passage cells reached markedly high-

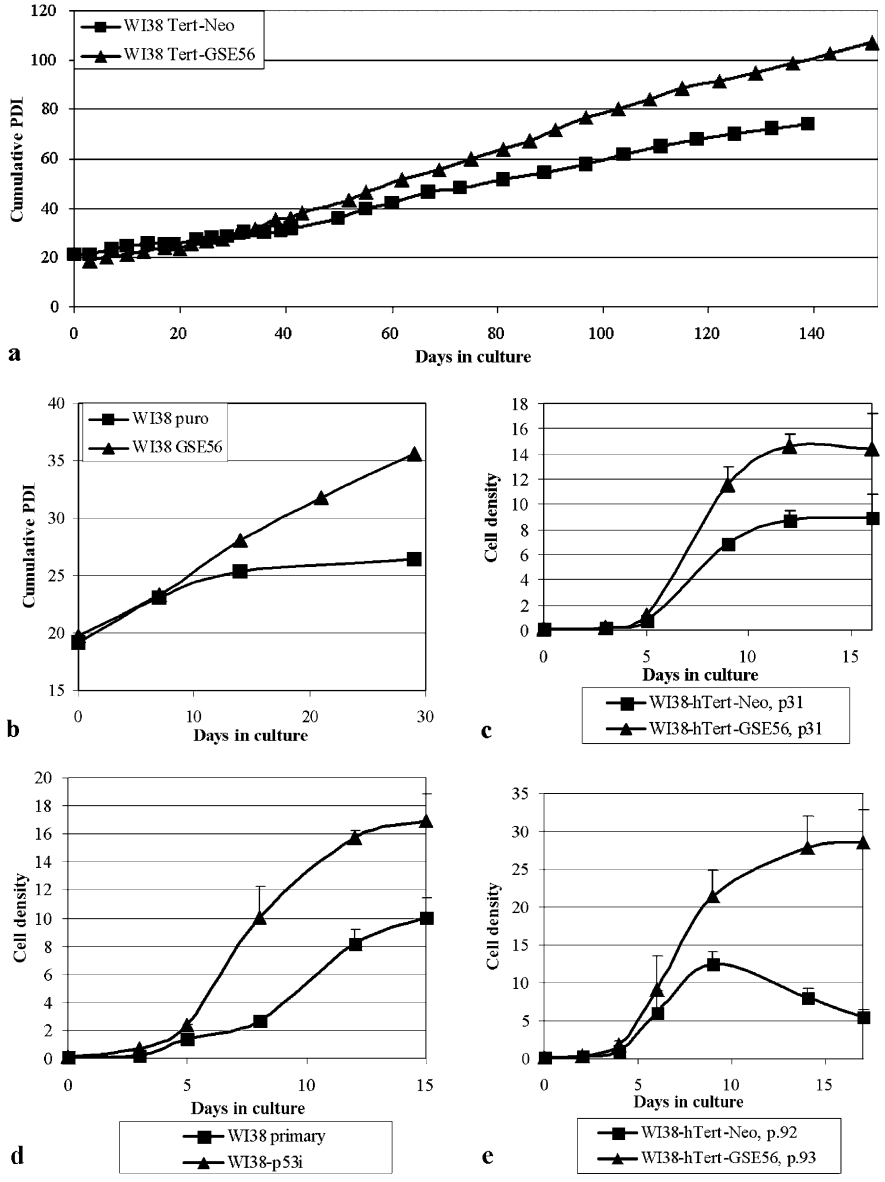

Fig. 2. Suppression of p53 in WI38 fibroblasts enhances growth rate, postpones replicative senescence, reduces contact inhibition of growth and prevents contact-induced cell death. a: Cumulative growth of hTert-immortalized WI38 fibroblasts with and without GSE56 expression over a period of ~140 days. b: Cumulative growth of primary WI38 fibroblasts with and without GSE56 expression over a period of ~30 days. In panels a and b cells were cultured in 100 mm dishes, detached prior to confluence by trypsin, counted with a hemocytometer and re-seeded to an initial density of 10^5 cells/plate. c: Contact inhibition of growth in early-passage (~p.30) hTert-immortalized WI38 fibroblasts with and without GSE56 expression. d: Contact inhibition of growth in primary WI38 fibroblasts (~p.18) with and without p53-inhibitory RNA expression. e: Contact inhibition of growth and contact-induced cell death in late-passage (~p.90) hTert-immortalized WI38 fibroblasts with and without GSE56 expression. In panels c–e cells were seeded to an initial density of 10^4 cells/plate in 35 mm dishes, periodically detached by trypsin and counted with a hemocytometer. The data was averaged from four to six samples per time point. Cell density is in units of 10^4 cells/cm^2. PDL: population doublings. Error bars: S.D.

er densities than early-passage immortalized cells described above (in agreement with [29]), late-passage cells without GSE56 showed the peculiar property of undergoing massive cell death after reaching confluence, while some cells in the same culture continued proliferating. As no such phenomenon was observed before the culture reached confluence (data not shown), it seems likely that the observed cell death was cell-contact-induced. This assumption is also supported by the predominantly perpendicular orientation of the surviving cells that suggests a selective advantage for minimizing cell–cell contacts in this culture, as opposed to all other confluent cultures examined, in which most cells had a locally parallel orientation that allows for a better filling of the available space (Fig. 4). In contrast, GSE56-expressing cells of the same passage after immortalization showed no density-induced death, while maintaining a similar and high growth rate and high final density. Thus, p53 mediates contact-induced cell death in late-passage, pre-malignant, INK4a-deficient WI38 fibroblasts. Notably, this function cannot be described as a stress response in a traditional sense, as the cells were maintained in normal culture conditions, at which no up-regulation of p53 was noticeable (Fig. 7).

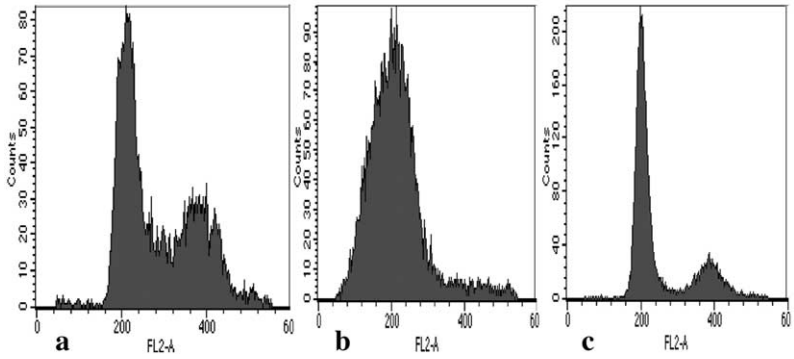

Fig. 3. Cell cycle analysis of a sparse culture of WI38-hTert-Neo (a) and dense cultures of WI38-hTert-Neo (b) and WI38-hTert-GSE56 (c). Cells were cultured in 100 mm plates until 40% confluence (a) or until 7 days after confluence (b,c), FACS analysis was performed as described in Section 2. Results shown are representative from three samples per category.

3.4. *The reduction of density-dependent inhibition of growth in cells with suppressed p53 correlates with a smaller up-regulation of p16/INK4a by cell density than in control cells*

p16/INK4a is an established mediator and marker of density-dependent growth inhibition [10]. To assess whether the diminished density-dependent growth inhibition response in cells with suppressed p53 activity correlates with a change in the regulation of p16 in these cells, p16 protein levels were determined by Western blot in WI38 fibroblasts from sparse and dense cultures. The analysis was performed on the following cell lines: primary WI38 (p.19), WI38 expressing p53i (p.19), hTert-immortalized WI38 (p.30–40 and p.90+), hTert-immortalized WI38 expressing GSE56 (p.30–40 and p.90+). As shown in Figs. 5 and 6, suppression of p53 activity by both mechanisms (GSE56 and p53-inhibitory RNA) resulted in a weaker up-regulation of p16 by culture density than in the appropriate control cells, and in some cases, in no up-regulation at all (Fig. 5). This result was obtained in three independent experiments.

3.5. *The effect of p53 suppression on the regulation of p16 by culture density is not transcriptional*

p16/INK4a is not an established transcriptional target of p53, neither was it present in a microarray analysis results list of genes whose expression was significantly altered in GSE56-expressing cells (data not shown). To check whether the diminished density-dependent up-regulation of p16 in cells with suppressed p53 activity is mirrored at the mRNA level, cDNA was obtained from dense cultures of the different WI38

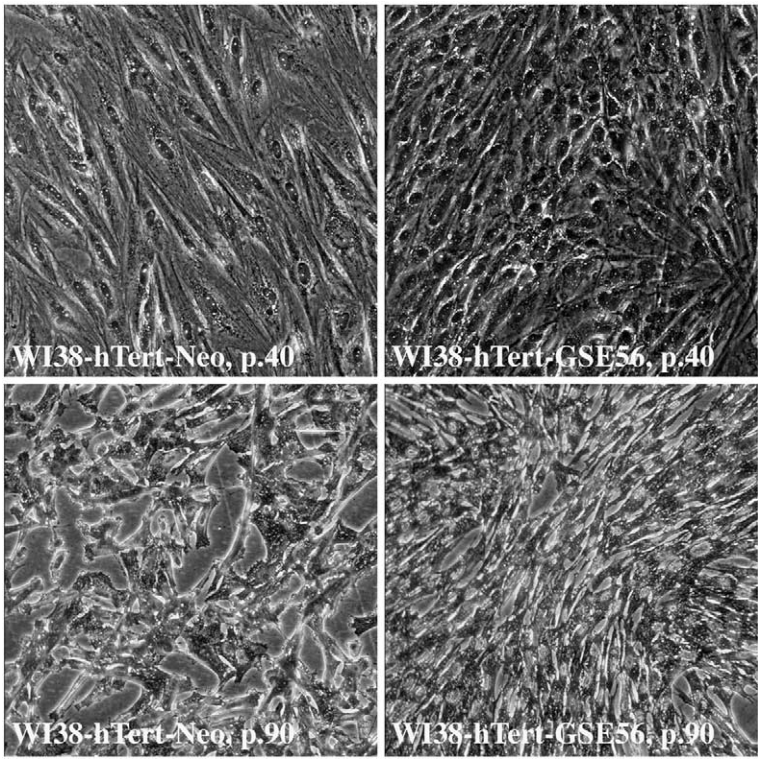

Fig. 4. Spatial organization of different WI38 strains in confluent culture is indicative of defects in cell–cell contact signaling pathways. hTert-immortalized WI38 fibroblasts of early (p.30–p.40, top) and late (p.90–p.95, bottom) passages were infected with GSE56 (right) or the control vector (left), grown to confluence in 10 cm plates, fixed and stained with crystal violet and photographed at ×200 magnification.

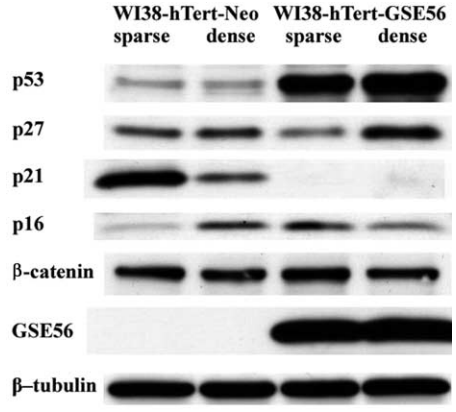

Fig. 5. Expression of several known contact inhibition markers (p27/KIP, p21/WAF, p16/INK4a, β-catenin) in sparse and dense cultures of early-passage (p.30–p.40) hTert-immortalized WI38 fibroblasts expressing GSE56 (two right lanes) or the control vector (two left lanes). β-Tubulin was used as loading control.

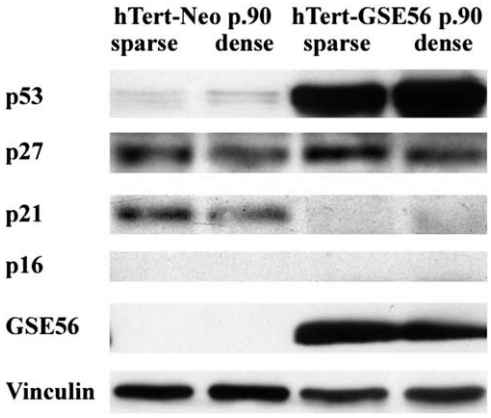

Fig. 7. Expression of several known contact inhibition markers (p27/KIP, p21/WAF, p16/INK4a) in sparse and dense cultures of late-passage (p.90–95) hTert-immortalized WI38 fibroblasts expressing GSE56 (two right lanes) or the control vector (two left lanes). A positive control for p16 was used on the same membrane (not shown). Vinculin was used as loading control.

cell lines described, and real-time quantitative PCR was performed. As seen in Fig. 8, both p53-inhibitory RNA and GSE56 expression actually caused an increase in p16 mRNA expression in both sparse and dense cultures, in contrast with the Western blot results. Furthermore, cells with suppressed p53 exhibited a density-dependent up-regulation of p16 mRNA similar to control cells. Thus, basal expression of p53 is necessary for a non-transcriptional regulatory pathway acting on p16/INK4a and mediating density-dependent inhibition of growth.

3.6. *Effect of prolonged culturing, but not of p53 suppression, on density-dependent growth inhibition correlates with inability to up-regulate p27*

To assess whether the observed effects of GSE56 on density-dependent growth inhibition correlate with changes in the status of p27, an established marker for density-dependent growth inhibition [14,16,17], the levels of p27 protein in sparse and dense cells with and without GSE56 expression were probed by Western blotting analysis. As seen in Fig. 5, expression of GSE56 did not result in a decrease of the expression levels of p27, or in a lack of its up-regulation in dense culture. However, as seen in Fig. 7, in late-passage, INK4a-deficient cells, p27 failed to accumulate as a function of culture density, again regardless of p53 status.

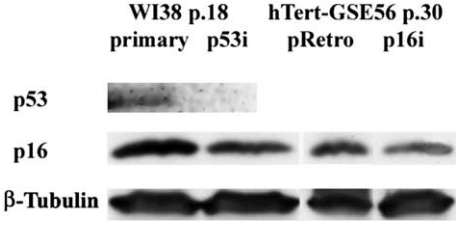

Fig. 6. Expression of p16/INK4a in dense cultures of primary WI38 fibroblasts with and without p53-inhibitory RNA expression (two left lanes) and early-passage hTert-immortalized WI38 fibroblasts expressing GSE56, with p16-inhibitory RNA or the control vector (two right lanes). β-Tubulin was used as loading control. Levels of p53 expression are shown for the two left lanes only.

4. Discussion

While density-dependent inhibition of growth is a phenomenon of considerable complexity, it is among the easiest to measure and observe in vitro, pointing on the cells' interaction with their immediate environment, their growth control status, and sometimes indicative of their tumorigenic potential. The contributions of particular pathways and factors to density-dependent growth arrest are only now being elucidated.

Here, we have demonstrated that basal expression of p53 in non-stressed WI38 fibroblasts is crucial for keeping cell proliferation in check, both by restricting cellular growth rate and by establishing a density-induced growth arrest. It is apparent that at least two distinct pathways mediate these effects downstream of p53. On the one hand, expression of the p21/WAF/CIP1 cyclin-dependent kinase inhibitor, a known transcriptional target of p53, declines dramatically in the absence of the basal expression of p53, presumably affecting cell cycle progression and growth rate. On the other hand, p16/INK4a, another cyclin-dependent kinase inhibitor, fails to accumulate in dense cultures, in correlation with a reduced density-dependent growth inhibition response (which it was shown to mediate by [10]) and a lack of G1 arrest in these cultures (which is also its known role, reviewed in [30]). Thus, the observed changes in the regulation of p16 appear to be highly relevant to the observed phenotypic effects, following p53 suppression. However, it is impossible to exclude the involvement of additional pathways in the mediation of density-dependent growth inhibition by p53.

p16/INK4a is not a known transcriptional target of p53, and our results indicate that the induction of p16 mRNA upon density-dependent growth inhibition is not p53-dependent. However, we have shown that the p16 protein failed to accumulate in dense WI38 cells with suppressed p53 to an extent similar to that observed in control cells.

Assuming (from the suppression mechanism of GSE56) that transcriptional activity of p53 is necessary for its contribution to the density-dependent growth inhibition response, it is natural to expect that the involvement of p53 in density-dependent inhibition of growth is achieved through an additional signaling module, that is transcriptionally activated by p53

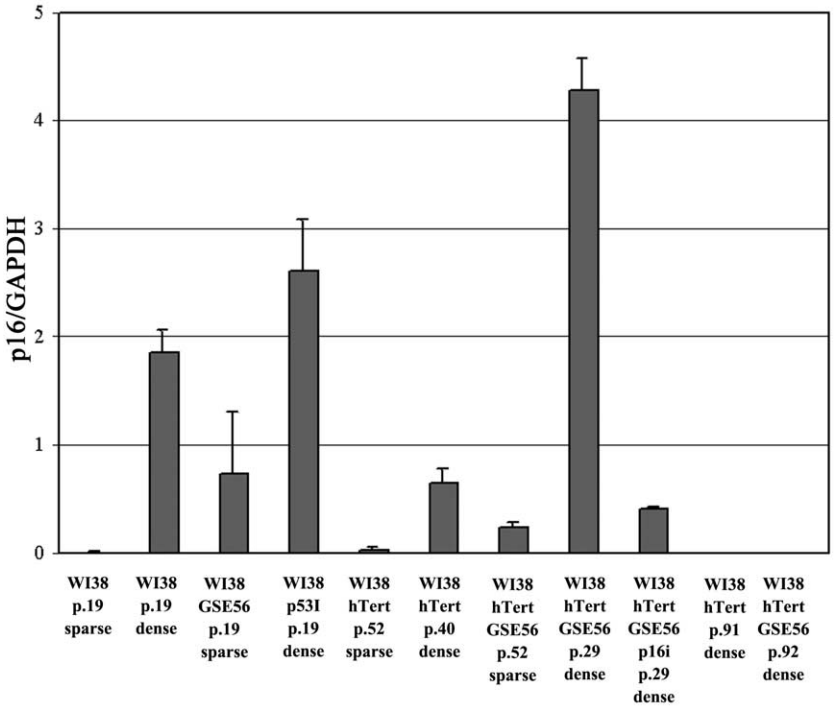

Fig. 8. p16/INK4a mRNA expression levels in different WI38-derived cell types, normalized by GAPDH levels. The RT-PCR procedure is described in Section 2. Error bars: S.D. from duplicates.

and modulates the levels of p16 in a non-transcriptional mechanism, possibly by affecting the stability of the protein itself.

Additionally, we have shown that the regulation of p27/KIP, another known mediator of density-dependent growth arrest, is not affected by the p53 status, while following prolonged culturing of immortalized cells its density-dependent regulation is disrupted, in correlation with higher cell growth rate and final culture density (also described in [29]). Prolonged culturing also causes the unique phenomenon of contact-induced cell death, which can be abolished by p53 inactivation and is, therefore, p53-dependent, in compliance with the traditional role of p53 as a pro-apoptotic tumor suppressor. Notably, this cell-death-promoting activity of p53 in late-passage immortalized WI38 cells was also mediated by basal amounts of p53, as no significant up-regulation of p53 was observed in these cells.

These findings, combined with the comparison of the quantitative effects of prolonged culturing and of p53 suppression on density-dependent growth inhibition, suggest that the deregulation of density-dependent growth inhibition in these two cases is achieved by differentially acting on two distinct and complementary pathways that mediate density-dependent inhibition of growth. In agreement with this conclusion, the described effects appear to be additive: late-passage immortalized WI38 cells with an additional inactivation of p53 exhibit the highest final density in culture.

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References

[1] Oren, M. (1999) J. Biol. Chem. 274, 36031–36034.
[2] Oren, M. et al. (2002) Ann. NY Acad. Sci. 973, 374–383.
[3] Alexandrova, A., Ivanov, A., Chumakov, P., Kopnin, B. and Vasiliev, J. (2000) Oncogene 19, 5826–5830.
[4] Sablina, A.A., Chumakov, P.M. and Kopnin, B.P. (2003) J. Biol. Chem. (in press).
[5] Paul, D., Henahan, M. and Walter, S. (1974) J. Natl. Cancer Inst. 53, 1499–1503.
[6] Pignolo, R.J., Rotenberg, M.O. and Cristofalo, V.J. (1994) In Vitro Cell Dev. Biol. Anim. 30A, 471–476.
[7] Owen, T.A., Carter, R., Whitman, M.M., Soprano, D.R. and Soprano, K.J. (1990) J. Cell Physiol. 142, 137–148.
[8] Nelson, P.J. and Daniel, T.O. (2002) Kidney Int. 61 (Suppl. 1), 99–105.
[9] Higashi, H., Suzuki-Takahashi, I., Yoshida, E., Nishimura, S. and Kitagawa, M. (1997) Biochem. Biophys. Res. Commun. 231, 743–750.
[10] Wieser, R.J., Faust, D., Dietrich, C. and Oesch, F. (1999) Oncogene 18, 277–281.
[11] Yanagisawa, K., Kosaka, A., Iwahana, H., Nakanishi, M. and Tominaga, S. (1999) J. Biochem. (Tokyo) 125, 36–40.
[12] Huang, Z.Y., Wu, Y., Hedrick, N. and Gutmann, D.H. (2003) Mol. Cell. Biol. 23, 566–578.
[13] Ritt, M.G., Mayor, J., Wojcieszyn, J., Smith, R., Barton, C.L. and Modiano, J.F. (2000) Cancer Lett. 158, 73–84.
[14] Deleu, L., Fuks, F., Spitkovsky, D., Horlein, R., Faisst, S. and Rommelaere, J. (1998) Mol. Cell. Biol. 18, 409–419.
[15] Dietrich, C., Wallenfang, K., Oesch, F. and Wieser, R. (1997) Oncogene 15, 2743–2747.
[16] Fuse, T. et al. (2000) J. Neurochem. 74, 1393–1399.
[17] Levenberg, S., Yarden, A., Kam, Z. and Geiger, B. (1999) Oncogene 18, 869–876.
[18] Zhang, X., Ma, L., Enkemann, S.A. and Pledger, W.J. (2003) Oncogene 22, 4166–4174.

[19] Sakaguchi, M., Miyazaki, M., Inoue, Y., Tsuji, T., Kouchi, H., Tanaka, T., Yamada, H. and Namba, M. (2000) J. Cell Biol. 149, 1193–1206.
[20] Baba, M. et al. (2001) Oncogene 20, 2727–2736.
[21] Dietrich, C., Scherwat, J., Faust, D. and Oesch, F. (2002) Biochem. Biophys. Res. Commun. 292, 195–199.
[22] Stockinger, A., Eger, A., Wolf, J., Beug, H. and Foisner, R. (2001) J. Cell Biol. 154, 1185–1196.
[23] Li, L., Backer, J., Wong, A.S., Schwanke, E.L., Stewart, B.G. and Pasdar, M. (2003) J. Cell Sci. (in press).
[24] Wu, Y., Mehew, J.W., Heckman, C.A., Arcinas, M. and Boxer, L.M. (2001) Oncogene 20, 240–251.
[25] Cagatay, T. and Ozturk, M. (2002) Oncogene 21, 7971–7980.
[26] Deffie, A., Hao, M., Montes de Oca Luna, R., Hulboy, D.L. and Lozano, G. (1995) Mol. Cell. Biol. 15, 3926–3933.
[27] Ossovskaya, V.S. et al. (1996) Proc. Natl. Acad. Sci. USA 93, 10309–10314.
[28] Mittelman, J.M. and Gudkov, A.V. (1999) Somat. Cell Mol. Genet. 25, 115–128.
[29] Milyavsky, M. et al. (2003) Cancer Res. 63, 7147–7157.
[30] Rocco, J.W. and Sidransky, D. (2001) Exp. Cell Res. 264, 42–55.