

Attenuation of the p53 response to DNA damage by high cell density

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The p53 tumor suppressor is critical for preventing cancer progression. Numerous observations suggest that p53 function can be modulated by the cells' microenvironment. We addressed specifically the impact of cell crowding on the induction of p53 by DNA damage. We report that cell crowding attenuates markedly p53 upregulation, transcriptional activation and subsequent p53-dependent apoptosis following exposure to genotoxic stress. The p53 protein remains short-lived in confluent cultures regardless of the extent of DNA damage, even though it undergoes efficient phosphorylation on the mouse equivalent of human p53 serine 15. This inhibitory effect of cell crowding is not a secondary consequence of density-dependent cell cycle arrest (contact inhibition). Microscopic examination indicates that dense cultures display prominent cadherin-mediated cell–cell junctions, and only poor cell–matrix focal adhesions, whereas sparse cells possess conspicuous matrix adhesions and essentially no cell–cell contacts. High-density cell culture might recapitulate the microenvironment of cells in a living organism, where the response of p53 to DNA damage is reported to be low in some organs and ages. The impact of cell density on p53 activation may have important bearings on the involvement of p53 in tumor suppression and the cellular response to anticancer therapy.

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Introduction

p53 is a tumor suppressor, whose inactivation is a major facilitator of cancer progression (for recent reviews, see Wahl and Carr, 2001; Bargonetti and Manfredi, 2002; Michael and Oren, 2002). The importance of p53 is underscored by the high frequency of p53 mutations in sporadic human cancer (Hollstein *et al.*, 1999; El-Deiry, 2001; Hussain *et al.*, 2001). Inherited p53 germline mutations in humans, as well as genetic disruption of this gene in mice, confer a markedly increased suscept-

ibility to cancer (Malkin *et al.*, 1990; Srivastava *et al.*, 1990; Donehower *et al.*, 1992). p53 executes its tumor suppressor function by 'sensing' a wide array of potentially carcinogenic alterations. In the presence of such alterations, p53 orchestrates a cellular response that may entail apoptotic cell death, cell cycle arrest and replicative senescence, as well as accelerated repair of DNA damage. These changes in cell fate serve to ensure that cancer-prone cells, harboring constitutive carcinogenic alterations, are eliminated from the replicating pool before progressing into overt life-threatening tumors (for recent reviews, see Vousden, 2000; Pluquet and Hainaut, 2001; Michael and Oren, 2002; Vousden, 2002).

Although genetic alterations in tumor suppressor genes and oncogenes are critical for tumor initiation and progression, cell-autonomous changes are not the only determinant of this process. Tumor-induced angiogenesis, stromal reactions to the tumor, cancer cell invasion and metastasis, all involve complex interactions of the cancer cell with multiple cell types and extracellular components, which can modulate its cancerous potential. Premalignant cells will progress differently in different microenvironments, highlighting the role of the microenvironment in carcinogenesis (for recent reviews, see Arias, 2001; Bissell and Radisky, 2001; Elenbaas and Weinberg, 2001; Liotta and Kohn, 2001). The interplay between tumor-suppressor genes such as p53 and the microenvironment could be an important issue in carcinogenesis.

The ability of p53 to respond to genotoxic stress is crucial for its role as a tumor suppressor (Pluquet and Hainaut, 2001). There exists ample evidence that this ability is modulated differently in different cells, probably involving both cell-autonomous and cell nonautonomous factors. Of particular note, the extent of p53 activation following whole-body exposure of an organism to DNA damage differs widely among different organs and even different components of the same organ (Midgley *et al.*, 1995; MacCallum *et al.*, 1996; Gottlieb *et al.*, 1997; Komarova *et al.*, 1997; Wilson *et al.*, 1998). A possible explanation is that environmental factors can affect the activation of p53 in response to DNA damage, and thereby the ability of a cell to undergo p53-mediated fate changes. Numerous observations suggest an influence of the microenvironment on p53 activity. Excessive integrin signaling can lead to p53 activation and apoptosis (Bachelder *et al.*, 1999), while loss of cell anchorage, with consequent

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deprivation of integrin signaling, downregulates p53 (Nigro *et al.*, 1997; Lewis *et al.*, 2002). The levels and activity of p53 are also affected by growth factors and other secreted ligands to which the cell is exposed. Thus activation of the Ras/Raf and PI3K/AKT pathways, components of the response to growth factors, leads to induction of Mdm2 and thereby inactivation of p53 (Ries *et al.*, 2000; Mayo and Donner, 2001; Zhou *et al.*, 2001; Ashcroft *et al.*, 2002; Gottlieb *et al.*, 2002).

In vivo, cells usually exhibit close interactions with neighboring cells, unlike the situation in most *in vitro* models of cell growth. In the present study, we addressed specifically the possible contribution of cell density to the induction of p53 by DNA damage. Culture density affects cell–cell and cell–matrix interactions and adhesion-dependent signaling. In addition, cell density may affect diverse cellular processes including cytoskeletal dynamics, cell motility, growth factor availability and more. High cell density was also shown to have complex effects on neoplastic transformation of cells in culture (Rubin, 2001) and acquisition of p53 gene mutations (Rittling and Denhardt, 1992). We report that p53 activation by genotoxic stress is suppressed in densely plated cells, and the consequent biological effects are attenuated. This is due to the failure of p53 to become efficiently stabilized in confluent cells, despite undergoing proper phosphorylation on the mouse equivalent of human p53 serine 15, a hallmark of the DNA damage response. This inhibitory effect of cell crowding is not a secondary consequence of density-dependent cell cycle arrest (contact inhibition). High-density cell culture might recapitulate the micro-environment of cells in a living organism, where the response of p53 to DNA damage is reported to be low in some organs and ages (Midgley *et al.*, 1995; MacCallum *et al.*, 1996; Gottlieb *et al.*, 1997; Komarova *et al.*, 1997a; Wilson *et al.*, 1998). The impact of cell density on p53 activation, possibly through modulation of cell–cell and cell–matrix interactions, may have important bearings on the involvement of p53 in tumor suppression and on the cellular response to anticancer therapy.

Results

Induction of p53 by Cis-Platinum is inhibited by high culture density

A major feature of p53 activation in response to DNA damage is the increase in the steady-state levels of the protein, owing to the inhibition of its proteolytic degradation. To evaluate the impact of cell density on the p53 response, we compared the accumulation of stabilized p53 in dense and sparse cultures, following exposure to DNA damage. DNA damage was elicited by incubation with Cis-diamminedichloroplatinum(II) (Cis-Platinum, CisPt), a DNA cross-linking agent widely used in cancer chemotherapy. In order to study cell density as an independent variable, an experimental system devoid of some possible confounding factors was used (see Figure 1a and Materials and methods).

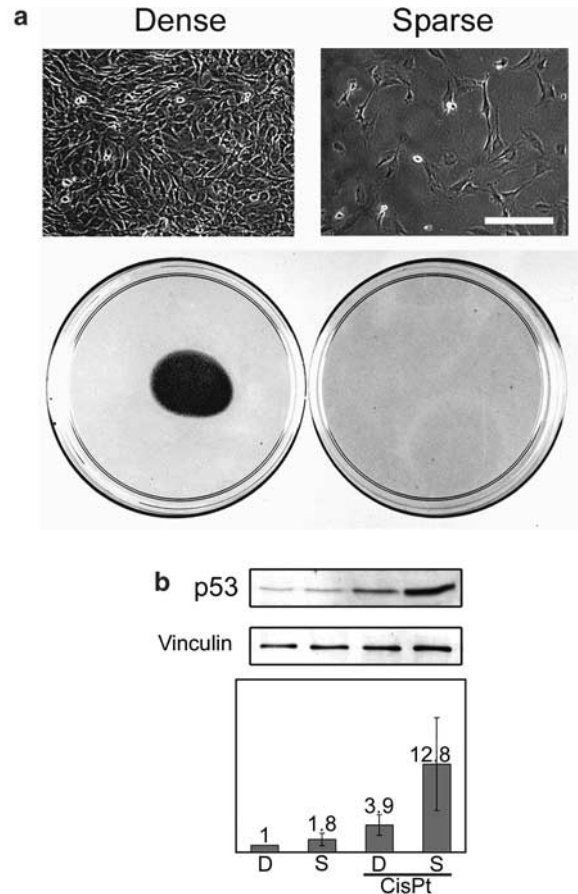


Figure 1 p53 induction by CisPt is attenuated in dense cultures. (a) MEFs were plated in a drop of medium, and allowed to adhere to the area covered by the drop for 1 h before further addition of culture medium (Dense). A similar number of cells were seeded in an identical dish by the standard protocol, allowing them to disperse evenly over the entire surface of the dish to create a sparse culture (Sparse). At 24 h after plating, cultures were photographed under an Axiovert microscope (ZEISS) using the DeltaVision acquisition system (top panel; scale bar = 50 μ m), and then fixed, stained with Giemsa stain and photographed (bottom panel; 90 mm dishes). (b) Wt MEFs were either drop plated (D) or seeded evenly at low density (S), as illustrated in (a). After 16 h of culture, CisPt was added to a final concentration of 4 μ g/ml, for a duration of 8 h. Cells were harvested, and proteins were analysed by SDS-PAGE followed by Western blot analysis. A typical result for p53 (CM5 antibody) is shown in the upper part, with vinculin as a loading control. Bands obtained in seven independent experiments were scanned and quantified by the NIH-Image software and p53 levels were corrected for the corresponding vinculin signals. Bar graphs (lower part) represent average results and standard deviations

Primary mouse embryonic fibroblasts (MEFs) were plated at either high or low culture density, and p53 protein levels were monitored 8 h after addition of CisPt. Strikingly, while CisPt led to prominent p53 accumulation in sparse (S) culture, its effect was far less pronounced in dense (D) culture (Figure 1b). This difference was reproducibly observed with the polyclonal anti-p53 antibody CM5 (Figures 1a and 2) as well as with a mixture of the monoclonal anti-p53 antibodies PAb421 and PAb248 (Figure 5). In contrast, only minor differences in basal p53 levels were found between sparse

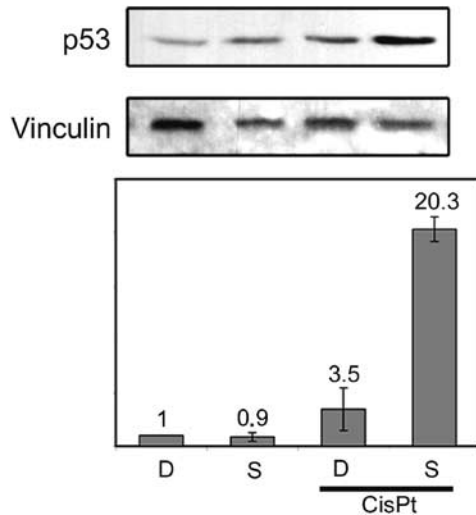


Figure 2 Density-dependent attenuation of p53 induction does not require ARF. ARF knockout (KO) MEFs were either drop plated (D) or seeded evenly at low density (S), treated and proteins were analysed as described for Figure 1b. A typical result for p53 is shown in the upper part, with vinculin as a loading control. Scanning of results and quantification of three different experiments were performed as described for Figure 1b

and dense untreated cultures (Figure 1b and see Figure 2). The failure to induce p53 accumulation also resulted in a failure to trigger p53-dependent transcriptional activity, as measured by evaluating the increase in p21^{WAF1}, product of a well-characterized transcriptional target of p53 (data not shown). Hence, high culture density inhibits specifically DNA damage-induced p53 accumulation and transactivating activity.

ARF is dispensable for density-dependent inhibition of p53 induction

Deregulated growth signals, elicited by activated oncogenes, cause p53 stabilization and accumulation through induced expression of ARF, the alternative reading frame product of the *INK4A* tumor suppressor locus (reviewed in Sherr and Weber, 2000). ARF binds to Mdm2, and inhibits Mdm2-mediated p53 degradation. Although ARF has so far been implicated primarily in the activation of p53 by oncogenes, recent data indicate that ARF can also play a role in relaying integrin-dependent signals to p53 (Lewis *et al.*, 2002). Furthermore, ARF expression is stimulated by excess activity of nuclear β -catenin (Damalas *et al.*, 2001), leading to substantial p53 accumulation (Damalas *et al.*, 1999). Since the distribution of β -catenin between the membrane-anchored pool and the free nucleocytoplasmic pool can be modulated by cell–cell adhesion, it appeared plausible that cell crowding might affect the induction of ARF and thereby the stabilization of p53. However, the difference between sparse and dense cultures was also maintained in ARF-null MEFs (Figure 2). Hence, ARF is dispensable for the density-dependent differential regulation of p53 induction.

Interestingly, the variations in the extent of p53 induction in sparse cultures among individual experiments were substantially smaller in the ARF-null MEFs, as compared to the wild-type (wt) MEFs (note error bars in Figure 1b). This may be due to the ARF-null cells being spontaneously immortalized, whereas wt MEFs undergo rapid asynchronous senescence in culture, which may lead to differences in the extent of p53 induction (Seluanov *et al.*, 2001). To obtain greater uniformity, ARF-null MEFs were used in most subsequent experiments.

Attenuated p53 induction in dense cultures is not secondary to cell cycle alterations

The differential activation of p53 in sparse versus crowded cells may conceivably be a secondary consequence of differences in cell cycle distribution. Indeed, the subcellular localization of p53, which may impact on its stabilization by DNA damage, varies among different stages of the cell cycle (Shaulsky *et al.*, 1990; David-Pfeuty *et al.*, 1996). Moreover, following irradiation, p53 localizes to the nucleus mainly in the G1 and S phases of the cell cycle (Komarova *et al.*, 1997b), and the induction of p53 in response to UV radiation in NIH3T3 cells, and to hypoxia in melanoma cell lines, was most evident in the S phase (Haapajarvi *et al.*, 1995; Danielsen *et al.*, 1998).

The cell cycle distribution of cultures seeded at either low or high density was therefore compared at different time points after seeding. As shown in Figure 3a, up to 24 h after seeding the patterns appeared very similar between dense and sparse cultures. The effect of high culture density on cell cycle distribution, attributable to density-dependent contact inhibition, became apparent only 48 h after seeding (Figure 3a). The S-phase fraction of the cell cycle was further monitored by BrdU incorporation assays. Following 24 h of culture at either high or low density, a similar fraction of cells were found in the S phase under both conditions (57.5 ± 1.5 versus $56.7 \pm 2.5\%$ in dense and sparse cultures, respectively; data not shown). Representative fields are shown in Figure 3b. Furthermore, preferential induction of p53 in sparse cells was already observable as early as 10 h after seeding, and was as pronounced at that time as it was at 24 h (data not shown). The attenuated induction of p53 in dense cultures is thus not due to cell cycle alterations arising from density-dependent contact inhibition.

We also investigated whether the inhibitory effect of culture density on p53 induction is mediated by p27^{KIP1}, a cyclin-dependent kinase (CDK) inhibitory protein that is upregulated in cells that are growth arrested due to cadherin-mediated cell–cell interactions (St Croix *et al.*, 1998; Levenberg *et al.*, 1999). p27^{KIP1} mediates a G1 cell cycle arrest in confluent cells (Polyak *et al.*, 1994), although cells derived from p27^{KIP1} knockout mice and from their wt counterparts demonstrate a similar contact inhibition (Nakayama *et al.*, 1996). Consistent with the lack of evidence for a cell cycle arrest, no increase in p27^{KIP1} levels was detected in cells maintained

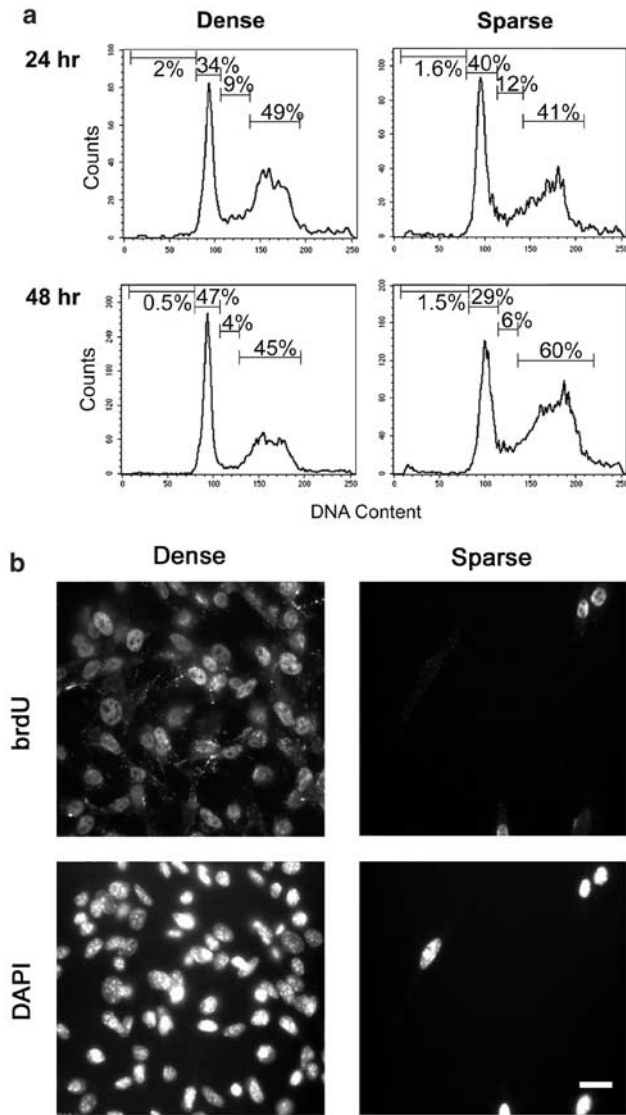


Figure 3 Attenuated p53 induction in dense cultures is not secondary to alterations in cell cycle. **(a)** ARF-null MEFs were either drop plated (Dense) or seeded evenly (Sparse). Cells were harvested either 24 or 48 h after plating, fixed, stained with propidium iodide and subjected to FACS analysis for DNA content. **(b)** ARF-null MEFs were plated on coverslips as either sparse or dense. After 24 h, cells were labeled by a 2 h exposure to 10 μ M BrdU. Following fixation and staining for BrdU and DAPI, representative fields were photographed for BrdU labeling (upper panels), indicating active DNA replication, and for DAPI (lower panels), to visualize all nuclei in the field. The right panels represent sparsely plated cells, while the left panels represent dense cells. Bar = 35 μ M

at high density for 24 h, although p27^{KIP1} accumulated to high levels within 72 h of high culture density (data not shown). Moreover, MEFs derived from p27^{KIP1} knock-out mice retained the ability to respond to high culture density by inhibition of p53 activation upon DNA damage (data not shown). Hence, p27^{KIP1} is not involved in the density-dependent attenuation of the p53 response to DNA damage.

Attenuated p53 induction in dense cultures is not due to different extents of DNA damage in sparse and dense cultures

A trivial explanation for the attenuated induction of p53 in dense cultures could be that high culture density blocks cellular access of CisPt, resulting in insufficient DNA damage. To address this possibility, densely plated cells were treated with CisPt, and then the drug was removed and the cells replated either densely or sparsely. If indeed high culture density interferes with efficient acquisition of DNA damage, one might expect that p53 will fail to accumulate even if the cells are subsequently replated sparsely. However, as seen in Figure 4, this was not the case. Although 5 h after replating p53 levels were still low in both dense and sparse cultures, pronounced p53 induction became evident later on (22 h postreplating) in the sparse, but not in the dense culture. This argues that crowded cells do acquire sufficient DNA damage, but the potential of this damage to lead to p53 accumulation is somehow repressed as long as cells are kept at high culture density.

Density-dependent attenuation of p53 induction is observed following different genotoxic treatments

We next examined the induction of p53 in crowded versus sparse cells by different genotoxic agents. As seen in Figure 5, camptothecin (a topoisomerase I inhibitor, lanes 5 and 6), and 4-NQO (a generator of bulky DNA adducts, lanes 7 and 8), induced much greater p53 accumulation in sparse cells as compared to crowded cells, similar to CisPt (lanes 3 and 4). Hence, inhibition of p53 induction by DNA damage in crowded cells is a general phenomenon.

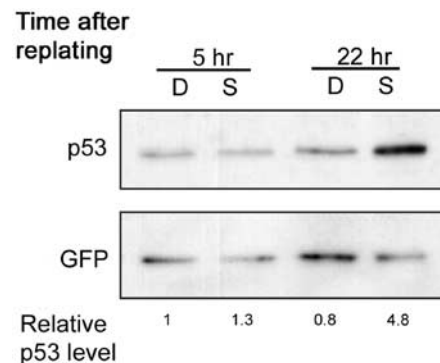


Figure 4 Efficient p53 induction is restored upon low-density replating of CisPt-treated dense cultures. ARF-null MEFs, previously infected with GFP retrovirus, were seeded at high culture density. At 12 h after plating, CisPt was added to a final concentration of 4 μ g/ml, for a duration of 16 h. The culture was then trypsinized, and cells were replated either densely (D) or sparsely (S), with no further exposure to CisPt. Cultures were harvested at the indicated time points after replating in the absence of CisPt, and subjected to protein analysis as in Figure 1b. p53 (CM5 antibody) and GFP bands were scanned and quantified by the NIH-Image software and p53 levels were corrected for the corresponding GFP signals

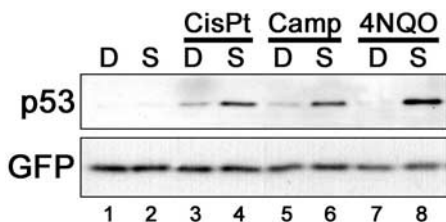


Figure 5 Attenuated induction of p53 in crowded cells in response to different genotoxic agents. ARF-null MEFs, previously infected with GFP retrovirus, were plated as dense (D) or sparse (S). At 22 h after plating, cells were treated for 4 h with either CisPt (lanes 3 and 4, 16 μ g/ml), camptothecin (Camp, lanes 5 and 6, 2 μ g/ml) or 4-Nitroquinoline 1-Oxide (4NQO, lanes 7 and 8, 0.2 μ g/ml). Cells were then harvested, extracted and subjected to SDS-PAGE. Western blot analysis was carried out to detect p53 (PAb421 and PAb248), and GFP as a loading control

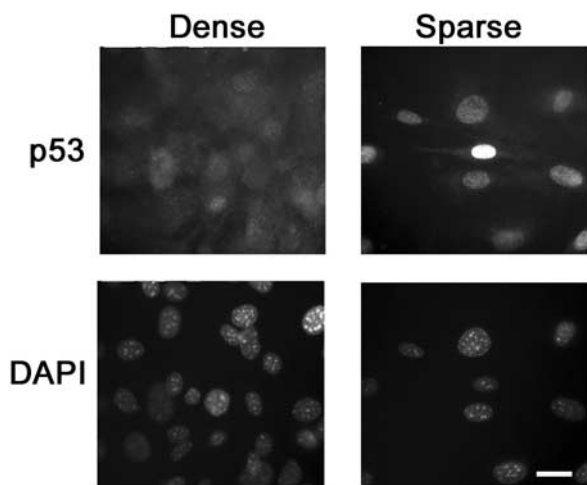


Figure 6 Densely cultured cells do not prevent p53 induction in adjacent sparse cells. ARF-null MEFs were drop plated on a glass coverslip. At 5 h after plating, CisPt (4 μ g/ml) was added for another 14 h, at which time the cells were fixed and stained with DAPI to visualize nuclei (lower panels) and with the PAb421 antibody to visualize p53 (upper panels). While the cells were highly crowded throughout most of the area covered by the drop (left panels), sparser cells could be observed in the periphery (right panels). As noted in the upper right panel, almost all these cells displayed intense nuclear p53 staining, unlike the bulk of the dense culture shown at the upper left. Bar = 25 μ m

Crowded cells do not inhibit p53 induction in adjacent sparse cells

We considered the possibility that high culture density leads to secretion of a diffusible factor that represses the p53 response. p53 induction was therefore monitored in sparse cells growing in close proximity to a larger number of highly crowded cells. As seen in Figure 6, p53 was efficiently induced in these sparse cells, although it failed to be induced to a comparable extent in the adjacent crowded cells. Secretion of a diffusible factor by the near-by dense islands might have been expected to inhibit p53 induction also in neighboring sparse cells. Hence, the data do not support the involvement of such a factor.

Crowded cells are protected against CisPt-induced apoptosis in a p53-dependent manner

To explore the functional consequences of density-dependent differential p53 induction, we compared the ability of CisPt to elicit apoptotic cell death in sparse and crowded MEFs. FACS analysis revealed a pronounced accumulation of cells with sub-G1 DNA content, indicative of apoptosis, in the CisPt-treated sparse cultures (Figure 7a, upper right panel), but significantly less so in confluent cultures (upper left panel; see also Figure 7b). To probe the role of p53 in this differential apoptotic response to DNA damage, a similar analysis was performed with p53-null MEFs. As shown in Figure 7a (lower panels) and Figure 7b,

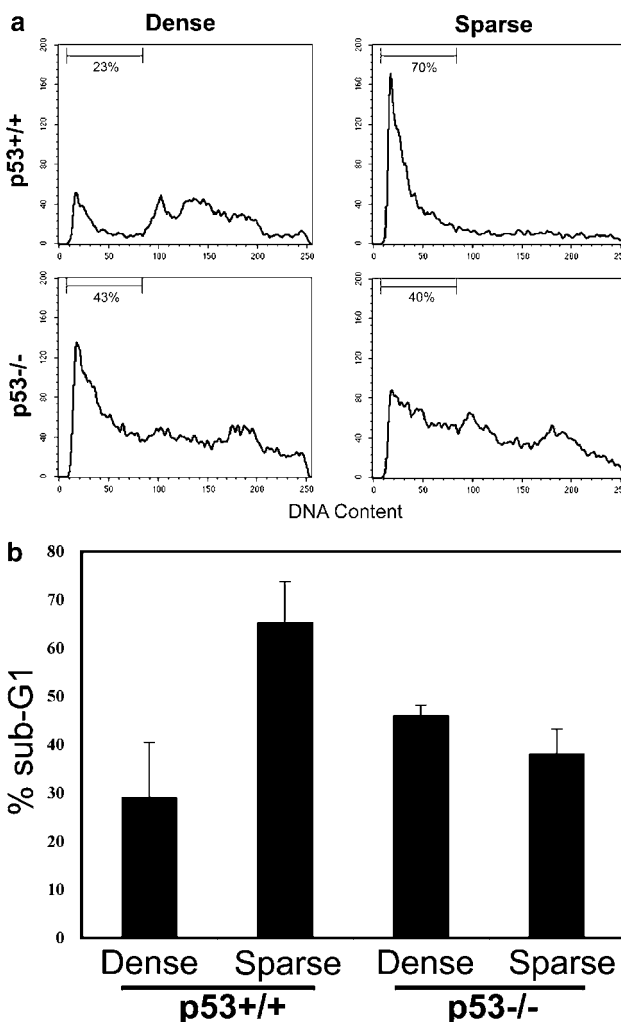


Figure 7 Crowded cells are protected against CisPt-induced apoptosis in a p53-dependent manner. (a) p53 +/+ (ARF-null) and p53 -/- MEFs were plated at either high (left panels) or low (right panels) culture density. After 24 h, CisPt was added to a final concentration of 4 μ g/ml. After 48 h of treatment, suspended and adherent cells were collected and combined, and subjected to cell cycle analysis as in Figure 3a. The fraction of cells with sub-G1 DNA content, indicative of apoptosis, is denoted in each panel. (b) Data from three separate experiments performed as in (a) were pooled and averaged. Standard deviations are indicated by error bars

ablation of p53 eliminated the difference between sparse and dense cultures. This implies that the preferential induction of p53 in sparse, but not dense cells, is responsible for their differential ability to be killed by extended exposure to CisPt.

Surprisingly, whereas sparse p53^{+/+} MEFs were more prone to apoptosis than their p53^{-/-} counterparts, as expected, the opposite appeared to hold for densely cultured cells, where more apoptosis was actually seen in the absence of p53 than in its presence (43 versus 23% of sub-G1 cells in Figure 7a; see also Figure 7b). This raises the interesting possibility that, in dense cultures, p53 may even confer increased resistance to apoptosis.

p53 is short-lived in dense cultures exposed to DNA damage

The increase in p53 steady-state levels upon exposure to DNA damage is believed to occur primarily through inhibition of the proteasomal degradation of p53 (Alarcon-Vargas and Ronai, 2002; Michael and Oren, 2002; Vousden, 2002). Consistent with this notion, incubation of MEFs with the proteasomal inhibitor MG132 led to a marked increase in p53 protein in both sparse and dense untreated cultures (Figure 8a, compare lanes 5, 6 to 1, 2). Remarkably, p53 levels were practically identical in all MG132-treated cultures, irrespective of DNA damage or culture density (lanes 5–8). This implies that all differences in p53 levels seen in the absence of MG132 are due to different protein stability. This conclusion was corroborated more

directly by pulse-chase analysis. Following inhibition of protein synthesis by cycloheximide in CisPt-treated cells, p53 disappeared much faster in densely cultured MEFs than in their sparse counterparts (Figure 8b). Densitometric analysis revealed that the calculated half-lives of p53 were 12 and 66 min in dense and sparse cells, respectively (data not shown). These data argue that, despite acquisition of sufficient DNA damage, p53 remains extensively short-lived in confluent cells. Taken together, these observations support the conclusion that high culture density interferes with the ability of DNA damaging stress to protect p53 against proteasomal degradation.

CisPt induces efficient phosphorylation of mouse p53 on serine 18 in dense cultures

Stabilization of p53 in response to DNA damage is associated with multiple phosphorylation events, occurring mainly within its N-terminal domain (reviewed in Alarcon-Vargas and Ronai, 2002; Michael and Oren, 2002; Vousden, 2002). The best studied of these phosphorylation sites is serine 15 of human p53, corresponding to serine 18 (Ser18) of mouse p53, which is targeted directly by the ATM and ATR protein kinases. As seen in Figure 9, the relative extent of Ser18 phosphorylation following CisPt treatment, when corrected for total p53 protein levels, appeared as high in confluent as in sparse cells. Thus the signal transduction pathway leading to Ser18 phosphorylation is triggered efficiently by CisPt irrespective of cell density, but is not followed by p53 stabilization.

Comparison of adhesive interactions in dense and sparse cultures

To gain an insight into the differences between dense and sparse cultures that may underlie the differential

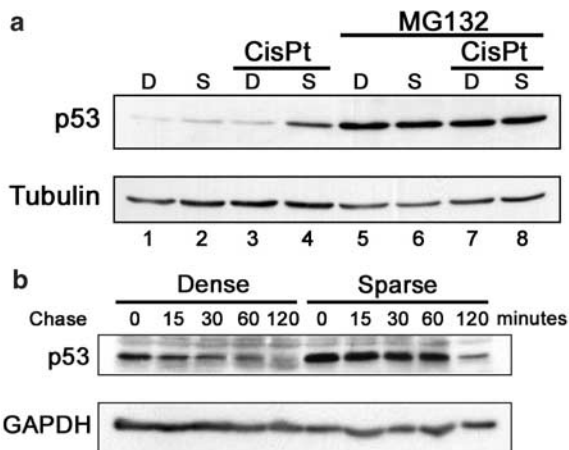


Figure 8 p53 is not efficiently stabilized by DNA damage in dense cells. (a) Wt MEFs were plated either densely (D) or sparsely (S). After 16 h, CisPt (4 μ g/ml) and the proteasomal inhibitor MG132 (25 μ g/ml) were added where indicated. Cells were harvested after an additional 8 h, and processed as in Figure 1b. p53 was detected with a mixture of PAb421 and PAb248. α -tubulin was used as a loading control. (b) ARF-null MEFs were plated as dense or sparse. After 6 h, CisPt (4 μ g/ml) was added for 20 h. At the end of the treatment, the growth medium was replaced with serum-free DMEM containing cycloheximide (50 μ g/ml). Cells were harvested at the indicated time points after addition of cycloheximide, and proteins were extracted and resolved by SDS-PAGE. Western blot analysis was carried out for p53 (CM5) and for GAPDH as a loading control

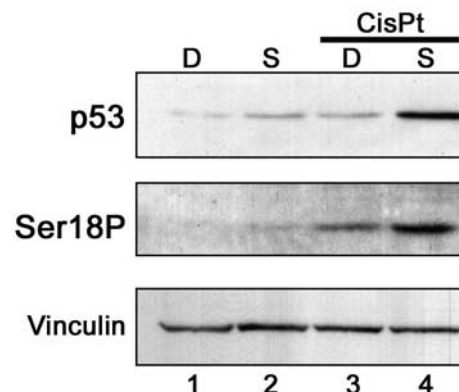


Figure 9 Efficient induction of mouse p53 Serine 18 phosphorylation in CisPt-treated dense culture. ARF-null MEFs were plated at different culture densities as indicated. At 16 h after plating CisPt (4 μ g/ml) was added, where indicated, for an additional 8 h. Cell extracts were subjected to Western blot analysis with a mixture of PAb421 and PAb248 (p53) or with an antibody specific for phosphorylated Serine 15 of human p53, recognizing phosphoserine 18 of mouse p53 (Ser18P). Membranes were reprobbed for vinculin as a loading control

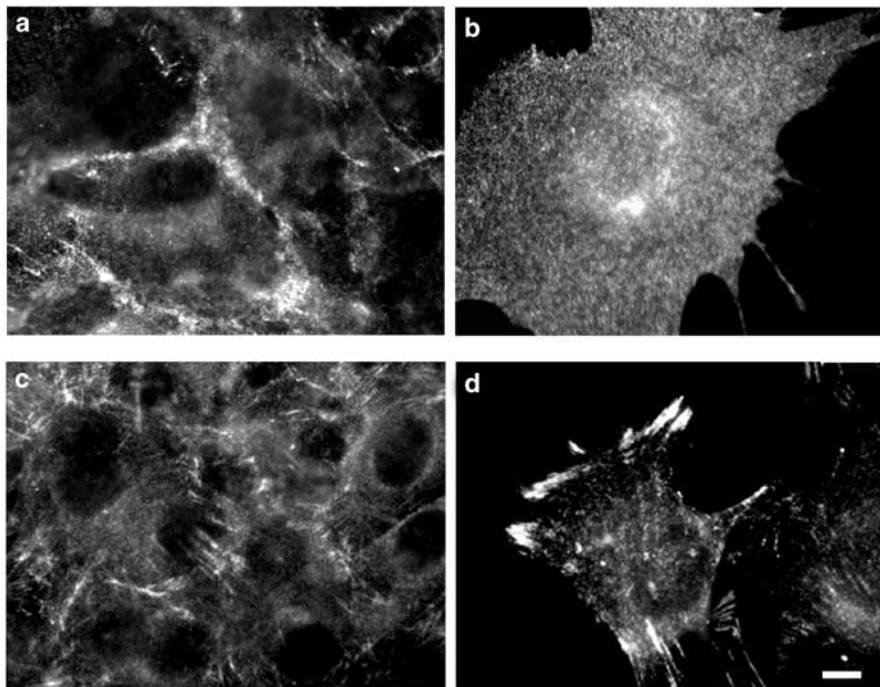


Figure 10 Different distribution of cadherins and paxillin in sparse and dense cells. ARF-null MEFs were seeded on coverslips at either high (panels a and c) or low (b and d) cell density. Following 24 h of culture, cells were fixed and stained with either the CH-19 anti-pan-cadherin antibody (a and b), or with antipaxillin antibody (c and d). Scale bar = 10 μ M

induction of p53 by DNA damage, we compared the interactions of such cells with each other and with the matrix. Cell–cell interactions between fibroblasts are largely mediated by homophilic cadherin–cadherin interactions (Juliano, 2002). Immunostaining with an anti-pan-cadherin antibody revealed the presence of prominent adherens junctions along the cell periphery in dense cells (Figure 10a). Such adhesions were not observed in the sparse cells (Figure 10b). On the other hand, staining for paxillin, a focal adhesion-associated molecule involved in integrin-mediated cell–matrix interactions, revealed prominent focal adhesions along the ventral aspects of the sparse cells (Figure 10d). In the dense cultures, large focal adhesions were not formed and only small streak-like paxillin-rich contacts were noted at the cell–matrix optical focal plane (Figure 10c) (Geiger *et al.*, 2001). These differences may implicate both cell–cell and cell–matrix adhesive interactions in the differential induction of p53 by DNA damage.

Discussion

The ability of p53 to become stabilized and activated in response to DNA damage is an important aspect of its action as a potent tumor suppressor. Here, we report that this ability is markedly repressed in cells maintained at high culture density. p53 continues to undergo a relatively rapid turnover in DNA-damaged crowded cells, and its accumulation is severely impaired, even though the cells acquire sufficient DNA damage. The

attenuated stabilization of p53 in crowded cells is not secondary to density-dependent cell cycle arrest, nor does it rely on p27^{KIP1}, a CDK inhibitor upregulated by cadherin-mediated cell–cell contact.

The molecular mechanisms underlying the inhibition of p53 accumulation by high culture density remain to be elucidated. One attractive possibility is that the differential effects of cell crowding rely on intercellular adhesive interactions (reviewed in Juliano, 2002), which are enhanced in crowded cells (Figure 10a, b). It is noteworthy that β -catenin, a pivotal signaling molecule (reviewed in Fodde *et al.*, 2001), is efficiently sequestered to cell–cell junctions in the dense central areas of tumors, but is liberated from such junctions in the sparser cells constituting the invasive front of the tumor, allowing it to reach the nucleus and presumably modify the transcriptional program of these cells (Brabletz *et al.*, 2001). Deregulated β -catenin, operating as a transcription factor, can indeed promote p53 accumulation even without DNA damage, in an ARF-dependent manner (Damalas *et al.*, 1999, 2001). However, we find that the differential effects of culture density on the p53 response to DNA damage are independent of ARF. Thus, if nuclear translocation of β -catenin is necessary for the response of p53 to DNA damage, this effect must be exerted through an ARF-independent mechanism; this possibility is presently under investigation.

There often exists an inverse correlation between the organization status of cadherin-mediated cell–cell interactions and integrin-mediated cell–matrix interactions (Levenberg *et al.*, 1998; von Schlippe *et al.*, 2000).

Indeed, sparse MEFs, which exhibit only minimal cadherin-mediated cell–cell junctions, possess prominent integrin-mediated focal adhesions (Figure 10). Integrin signaling may be required for efficient induction of p53 following DNA damage (Lewis *et al.*, 2002), although other reports indicate that integrin signaling actually downregulates p53 (Ilic *et al.*, 1998; Wang *et al.*, 2002). It is also of note that whereas the positive effect of integrins on p53 activation was shown to correlate with an increase in ARF protein levels (Lewis *et al.*, 2002), ARF is dispensable for the effects described in our study, implying the involvement of a different molecular mechanism.

ATM-dependent phosphorylation of p53 on serine 15 (Nakagawa *et al.*, 1999) has been proposed to entail reduced association with Mdm2 (Shieh *et al.*, 1997), and may be required for the induction of p53 by some types of DNA damage (Bean and Stark, 2001). However, other studies do not support a direct role for serine 15 phosphorylation in p53 stabilization (Ashcroft *et al.*, 1999; Dumaz *et al.*, 2001), which is in line with our finding that p53 is efficiently degraded in dense cells despite undergoing phosphorylation on the mouse equivalent of human serine 15. It remains possible that other DNA damage-induced post-translational modifications of p53, and possibly also Mdm2, may be inhibited in dense cells in a manner that interferes with p53 stabilization.

We have shown that while genotoxic treatment leads to effective protection of p53 against proteasomal degradation in sparse cells, it fails to do so to a similar extent in confluent cells. In accordance, a recent study demonstrated that transiently transfected p53 is degraded faster in lung adenocarcinoma cells (H1299), cultured at high density (Yin *et al.*, 2002). H1299 are p53-null tumor-derived cells that may experience constitutive intrinsic stress probably associated with increased genomic instability. Therefore, when p53 is reintroduced into such cells, it may be immediately subjected to the biochemical signals that impinge on endogenous p53 in nontransformed cells only after DNA damage.

What are the implications of our findings? High cell density culture might mimic the physiological situation in tissues, where cells are in close association with their neighbors. Most studies addressing p53 induction by DNA damage are traditionally performed in cultured, often subconfluent cells. An important aspect of regulation of p53 levels and activity might be overlooked in these studies. Indeed, while upregulation of p53 by genotoxic stress is a robust phenomenon in culture, this is not always the case in the context of a whole organism (Midgley *et al.*, 1995; MacCallum *et al.*, 1996; Gottlieb *et al.*, 1997; Komarova *et al.*, 1997a; Wilson *et al.*, 1998).

At least in certain types of cancer, p53 mutations occur at late stages of tumor development. An apparent paradox is the continued growth of precancerous cells while bearing a not-yet-mutated p53 (Fearon and Vogelstein, 1990). Genomic instability, oncogene activation and hypoxia are probably present in these cells, but

nevertheless proliferation goes on, suggesting that the antiproliferative activities of p53 do not come into full play in the early stages of carcinogenesis. Early-stage epithelial neoplastic lesions retain an epithelial phenotype, including close cell–cell contacts. In advanced stages, however, epithelial to mesenchymal transition (EMT) may take place, disrupting adherens junctions and desmosomes (Savagner, 2001). One interesting prediction of our data is that EMT might serve as a driving force for acquisition of p53 mutations, due to unleashing of p53's activity when cells lose contact with their neighbors.

In accordance with the preferential upregulation of p53 in response to DNA damage, p53 sensitizes sparse cells to killing by CisPt. Surprisingly, p53 actually seems to confer increased resistance to apoptosis when cells are maintained at high culture density (Figure 7). The molecular basis of this apparent cytoprotection is presently unknown. However, it is noteworthy that antiapoptotic effects of wt p53 have indeed been documented (Lassus *et al.*, 1996; McKay *et al.*, 2001). Moreover, in addition to its large arsenal of proapoptotic genes, p53 can upregulate the transcription of a number of antiapoptotic genes, such as HB-EGF (Fang *et al.*, 2001) and several decoy receptors (Sheikh *et al.*, 1999; Meng *et al.*, 2000), and induction of p21^{WAF1} by p53 can also confer increased resistance to cytotoxic therapy (Bunz *et al.*, 1999). One attractive possibility is that high culture density alters the target gene specificity of p53, perhaps favoring the expression of antiapoptotic genes and thereby conferring increased cytoprotection. Such antiapoptotic effects of wt p53 may account for the surprising observation that loss of wt p53 function can sometimes restrict, rather than accelerate, early stages of tumorigenesis (Kemp *et al.*, 1993), where cells typically still maintain quasinormal tissue architecture involving extensive cell–cell interactions.

Materials and methods

Materials

CisPt was purchased from ABIC (Israel). 4-Nitroquinoline 1-oxide (4-NQO), Camptothecin and Cycloheximide were purchased from Sigma (Israel). MG132 was purchased from Calbiochem (San Diego, CA, USA).

Cells and cell analysis

Primary MEFs were prepared from day 13.5 embryos of C57/BL mice according to standard protocols (Zindy *et al.*, 1997). p53-null MEFs were similarly prepared from C57/BL p53 knockout mice ((Jacks *et al.*, 1994); obtained from Jackson Labs, Maine). ARF-null MEFs, and p27^{KIP1} knockout, ARF-null MEFs (Kamijo *et al.*, 1997) were kindly provided by Drs M Roussel and CJ Sherr (Memphis). All mouse fibroblasts were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma), nonessential amino acids (Beit Haemek, Israel), beta mercapto-ethanol (60 μ M, Sigma) and penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA). CisPt treatment was carried out in the presence of 20% FCS.

Dense cells were plated at a density in the range of 600–800 cells/mm², about 10 times denser than the control sparse cultures. Within 24 h of culture, cell number and cell density increased about twofold.

Cell cycle analysis was performed as described before (Haupt *et al.*, 1995). Cells were analysed by FACSORT (Becton Dickinson), using the CellQuest software.

Retroviral infection

LZRSpBMN-IRES-EGFP, a retroviral plasmid encoding green fluorescent protein (GFP), was kindly provided by G Nolan (Stanford). Infectious virus stocks were prepared and used as described (Gottlieb and Oren, 1998).

Antibodies

Mouse p53 was detected with either the CM5 polyclonal antibody (Novocastra, UK) (Midgley *et al.*, 1995), or with a mixture of the monoclonal antibodies PAb421 and PAb248 (generous gift from Dr D Lane). Antiphosphorylated serine 15 of human p53, and anti-p21^{WAF1} polyclonal serum were generous gifts from Drs Y Taya (Tokyo) and C Schneider (Trieste), respectively. Anti-p27^{KIP1} and antipaxillin were from Signal Transduction Labs. Anti-GFP was purchased from Roche (Switzerland). Anti-BrdU (5-bromo-2'-deoxy-uridine) was purchased from Becton Dickinson (NJ, USA). Antivinculin, anti- α -tubulin (clone DM 1A) and CH-19 anti-pan cadherin antibodies were from Sigma (Israel).

Immunoblotting and immunofluorescence staining

For immunoblotting, equivalent amounts of extracts of each sample were subjected to SDS–polyacrylamide gel

electrophoresis (PAGE) and transferred onto nitrocellulose (Schleicher and Schuell, NH, USA) or PVDF membranes (Amersham Pharmacia, NJ, USA). Western blots were developed using the ECL method (Amersham Pharmacia, NJ, USA). In some experiments, the expression of retrovirally transduced GFP was employed to monitor equal protein loading. To this end, cells were infected with a GFP retrovirus to yield a 5–10% infection rate, prior to plating for the experiment. At the end of the experiments, GFP was visualized by Western blotting with appropriate antibodies.

Immunostaining was performed as previously described (Sadot *et al.*, 2002). Secondary antibody (goat anti-mouse or goat anti-rabbit, Cy3 or Cy2 conjugated, Jackson Labs, Maine) was used along with DAPI. Staining for BrdU was carried out in triplicate, as previously described (Shaulian *et al.*, 2000). Following fixation and staining, cells were scored for BrdU incorporation by fluorescent microscopy. Fields were randomly selected and at least 300 cells were counted on each plate.

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