Cancer Cells Cue the p53 Response of Cancer-Associated Fibroblasts to Cisplatin

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Abstract

Current understanding of the p53 response is based mainly upon in vitro studies of homogeneous cell populations. However, there is little information on whether the same principles operate within heterogeneous tumor tissues that are comprised of cancer cells and other cell types, including cancer-associated fibroblasts (CAF). Using ex-vivo tissue cultures, we investigated p53 status and responses to cisplatin in tumor cells and CAFs from tissue specimens isolated from 32 lung cancer patients. By comparing cultivated tissue slices with the corresponding tumor tissues fixed immediately after surgery, we found that morphology, proliferation, and p53 staining pattern were preserved during cultivation. Unexpectedly, when CAFs were analyzed, p53 accumulation and induction of p21 was observed only in tumors with constitutively low p53 protein and accumulation upon cisplatin treatment. In contrast, in tumors with no p53 accumulation in cancer cells there was also no p53 accumulation or p21 induction in adjacent CAFs. Furthermore, induction of cisplatin-induced apoptosis in CAFs was selectively observed in tumors characterized by a parallel induction of cancer cell death. Our findings reveal an interdependence of the p53 response in cancer cells and adjacent CAFs within tumor tissues, arguing that cancer cells control the response of their microenvironment to DNA damage. Cancer Res; 72(22); 109. ©2012 AACR.

Introduction

One of the major molecular players in cellular stress responses is the p53 tumor suppressor protein. Under non-stressed conditions, p53 is rapidly degraded and, therefore, not present at detectable levels in the cell. Upon various types of cellular stresses, p53 gets stabilized and rapidly accumulates within the nucleus (1–3). The tight regulation of the p53 system is impaired in most if not all cancers (4). More than 50% of all tumors carry TP53 mutations (5, 6). In many of the remaining cancers p53 is thought to be inactivated, for example, through loss of upstream regulators such as ARF (7) or overexpression of p53 inhibitors such as Mdm2 or MdmX (8).

The vast majority of research on p53 activities upon genotoxic stress has been conducted in homogeneous 2-dimensional (2D) cultures of tumor cell lines. However, in intact tissues a variety of different cell types interact with each other (9) and might thereby influence their respective reactions to stress. Several lines of evidence support the hypothesis that activation of p53 in 1 cell can influence the response of neighboring cells, particularly via transcriptional activation of genes that encode for secreted factors. For instance, in mouse xenograft models as well as in cell culture experiments using conditioned medium, it has been shown that the p53 activity of epithelial cancer cells (10, 11). Therefore, attenuation of the p53 response of carcinoma-associated fibroblasts (CAF) should support tumor growth. Moreover, facilitating the survival of CAFs by diminishing p53 function under stressful conditions might also help maintain a favorable environment for tumor progression.

Although mutations within the TP53 gene locus in stromal cells have been described (12, 13), their existence remains controversial (14–16). Alternatively, cancer cell-derived mechanisms might exist by which the malignant clone suppresses p53 function in the adjacent stromal cells. Indeed, experiments using conditioned medium from epithelial cancer cells indicated that such cells can antagonize this stromal activity by silencing p53 activation in their adjacent fibroblasts upon DNA damage (17). Yet, to date no studies have examined the influence of cancer cells on the p53 response of stromal fibroblasts within the intact tumor tissue.

In the present work, we analyzed p53 responses to cisplatin in 32 primary lung carcinomas using our previously described tissue slice technology (18). This culture system allowed us to analyze different cellular components (cancer cells and CAFs)

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for their sensitivity as well as for their p53 response within an intact tumor microenvironment. Remarkably, we found a close correlation between p53 accumulation and activation in cancer cells and in their adjacent CAFs. These results strongly suggest that, within the intact tumor microenvironment, the p53 response of cancer cells and CAFs is coordinately regulated via a cross-talk between these 2 cell types.

Material and Methods

Tissue slice preparation and culture

Fresh tumor tissue was obtained from Klinik Schillerhöhe immediately after surgery of lung cancer patients. The local ethics committee approved the investigation (project number 396/2005V) and informed consent was obtained from the patients. After arrival, the fresh material was incubated in Oncostore (Oncoscience AG) containing 100 μg/mL Colistin until the start of the experiment to avoid bacterial contamination of tissue slices. Cores with a diameter of 5 mm were punched out of the tissue by hand and slices of 200 μm thickness were prepared using a Krumdieck tissue slicer (Krumdieck, Alabama Research and Development Corp., Munsford). The slices were then put in 24-well plates containing Airway Epithelial Cell Medium + Supplements (PromoCell) and incubation was conducted for 96 hours in a constant atmosphere of 5% CO2 and 37°C. Treatment with cisplatin (13 μmol/L) started 24 hours after preparation of slices for additional 72 hours. Cisplatin was obtained from the pharmacy of Robert Bosch Hospital at a concentration of 1 mg/mL. Fixation was done using buffered formalin before slices were embedded in paraffin for further investigation.

Isolation, cultivation, and characterization of carcinoma-associated fibroblasts

Tumor tissues from 6 patients were enzymatically digested using a tissue disaggregation buffer (120 mmol/L NaCl, 5.6 mmol/L glucose, 2.5 mmol/L MgCl2 × 6H2O, 5.4 mmol/L KCl, 1 mmol/L NaH2PO4, 20 mmol/L HEPES, pH 7.2) supplemented with collagenase (167 U/mL), DNase (250 U/mL), and protease (0.25 mg/mL) for 90 minutes at 37°C. The disaggregated material was filtered through a 70-μm cell strainer (BD Falcon) and the flow-through was seeded in cell culture flasks. The outgrowing fibroblasts were cultivated with RPMI1640 medium supplemented with 10% FCS.

Immunohistochemistry

For immunohistochemical analysis, the embedded slices were cut into 3 μm sections. Staining for Ki67 (anti-human KI67 Antigen, Clone MIB-1, 1:50) was done using the DAKO Envision Kit on a DakoCytomation Autostainer (DAKO). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was done according to the manufacturer’s instructions (ApopTag Kit S7100, Chemicon). Staining for p21 (anti-p21 WAF1 Ab-3, clone DSC-602, Thermo Fisher Scientific) and p53 (anti-p53 DM082, clone DO-7, ACRIS) was done using the DAKO Envision Kit. Before staining epitope, retrieval was achieved by incubating at pH6 (DAKO target retrieval solution pH6) for 15 minutes in a steam heater. The p21 and p53 antibodies were diluted 1:50 and 1:40 respectively using DAKO antibody diluent. Visualization was done with the Leica DM 4000 B microscope (Leica Microsystems). Immunohistochemical assessment was conducted blinded independently by at least 2 observers. For all stainings, the percentage of positive cells was determined using the ×20 and ×40 objectives. Discrepancies were resolved by simultaneous examination using a double-headed microscope.

Protein expression

For total lysates the cellular pellet was resuspended in lysis buffer (50 mmol/L Tris-HCl pH7.6, 250 mmol/L NaCl, 0.1% Triton x-100, 5 mmol/L EDTA) supplemented with complete protease inhibitor cocktail (Roche). Western blot was conducted using following antibodies: anti-p53 clones DO1 and 1801 (Santa Cruz) and anti-β-actin (Sigma).

DNA isolation

DNA isolation from paraffin embedded material was done using the Qiagen DNeasy Blood & Tissue Kit (Qiagen). Paraffin blocks of tumor tissues from patients were obtained from the pathology of the Robert Bosch Hospital and cut into several 10 μm-thick sections. Areas of high tumor content were chosen for DNA isolation as determined by hematoxylin and eosin staining of an additional sequential section.

Sequencing

Before sequencing DNA, the exons of interest had to be amplified via PCR. The same primers were used for the PCR and sequencing reaction. The current primers of International Agency for Research on Cancer were used: Exon 4: P-329 and P330, Exon 5: P-312 and P-271, Exon 6: P-239 and P-240, Exon 7: P-333 and P-313, Exon 8: P-316 and P-319, Exon 8–9: P-314 and P-315, and Exon 10: P-El0Li and P-562. Afterward PCR products were sent to GENterprise (GENterprise) for sequencing.

Software

Statistics were conducted using GraphPad Prism 5.0 Software (Graph Pad Prism Software Incorp). Pictures of immunohistochemical stainings were taken using the Leica Application Suite and the DFC 245 camera (both Leica Microsystems). Sequences were analyzed using Chromas Lite 1.62 (Technelysium Pty. Ltd) and Geneious (Biomatters Ltd). X54156 dna from p53-knowledgebase (http://p53.bii.a-star.edu.sg/index.php) was used as reference sequence for p53.

Results

Morphology, proliferation, and p53 staining in tissue slices from primary lung cancer specimens are preserved during cultivation

To study the p53 response within an experimental setting that is as similar as possible to the intact tumor tissue, we used lung cancer tissue slices cultured ex vivo. Immediately after surgery, tissue samples were sliced on a Krumdieck tissue slicer at 200-μm-thick slices. Tissue slices from 35 primary lung carcinomas were cultured for 96 hours, then fixed in formalin, and embedded in paraffin as described previously (16, 18).
Three cases were excluded because of distinct necrotic areas in control tissues (more than 50%).

As a quality control we compared morphology, Ki67, and p53 immunostaining in cultivated tissue slices with the corresponding tumor tissue material fixed immediately after surgery (pathological routine material). As shown in Fig. 1 (representative cases in left), morphology was highly preserved during cultivation. In addition, the range of Ki67 scores obtained from cultivated tissue samples significantly correlated to that of the corresponding tissues fixed immediately after surgery (Fig. 1A, right) and was also comparable to Ki67 scores described in non–small cell lung carcinoma (NSCLC) patient studies (19). As shown in the representative examples in Fig. 1B, p53 staining pattern was also preserved during cultivation. Furthermore, there was a significant correlation between the percentage of p53-positive cancer cells in cultivated tissues and in the corresponding pathological routine sections (Spearman correlation = 0.82; \( P < 0.0001 \)).

TP53 mutations were detected in 18 of the 32 cases (56.2%) with 12 missense substitutions and 6 deletions. The overall distribution of missense mutations was comparable to the dataset for NSCLC published in the International Agency for Research on Cancer TP53 database (http://www-p53.iarc.fr). TP53 status and patient characteristics as well as medication are summarized in the Supplementary Table S1. We found a significant but slightly higher number of Ki67-positive cancer cells in tumors harboring mutated p53 compared with wtp53 tumors (Fig. 1C).

### Characterization of tumors by p53 status and Ki67 expression

We next examined the p53 response of cancer cells to cisplatin in their intact tissue microenvironment. For this, we incubated tissue slices from 32 individual lung cancer patients with or without cisplatin and analyzed them for p53 protein expression by immunohistochemistry (IHC; Fig. 2A). According to their p53 immunostaining characteristics, the tumor samples could be divided into 3 categories. In 14 of 32 cases (43.7%), we found constitutively low (less than 20% of cancer cells) or undetectable p53 levels in cancer cell nuclei in untreated samples and an accumulation of p53 in the cancer cells upon cisplatin treatment (Fig. 2A, category I). Cases with constitutively high p53 levels in the cancer cell compartment (more than 20% of cancer cells), with no further p53 accumulation after cisplatin treatment (7 of 32; 21.9%) were defined as category II tumors (Fig. 2A). In a third subgroup (11 of 32; 34.4%), p53 protein was completely undetectable in cancer cell nuclei both without and with cisplatin treatment (Fig. 2A, category III).

The Ki67 score of category I and II tumors was comparable. A trend toward lower proliferation rate was detected in samples with undetectable p33 (category III tumors) regardless of the presence of cisplatin (Fig. 2B).

In all tissues (7 of 7) from category II tumors, TP53 mutations could be detected (Fig. 2B; mutant p53 indicated by red squares). This result was expected since, within established tumors, p53 proteins produced by missense mutations are often constitutively high in the cancer cell compartment. Cases with undetectable p53 (category III tumors) regardless of the presence of cisplatin (Fig. 2B).

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Response of cancer cells to cisplatin treatment

Response to cisplatin treatment in the cancer cells was evaluated by reduction of Ki67 score by at least 10% (Fig. 3A) and by an increase of at least 20% in TUNEL-positive cells (Fig. 3B). Five of 13 tumors harboring wtTP53 (38%) responded to cisplatin by reduced proliferation. Of note, 4 of those 5 responders were category I tumors (Fig. 3A, top). Of the 17 tumors harboring mutant p53, 4 responded to cisplatin by reduced proliferation (23.5%). Interestingly, only 1 of those 4 tumors was of category I, whereas the remaining 3 were of category II, characterized by constitutively high p53 protein staining (Fig. 3A, bottom).

Significant induction of cell death in response to cisplatin was observed in both wt and mutant p53 tumors. However, the proportion of responders was higher in wtTP53 tumors than in those harboring mutant p53. The responders in tumors harboring wtTP53 were distributed among category I and III (Fig. 3B, top). Among the 4 responder tumors with mutant p53, 3 were from category I tumors and only 1 from category II (Fig. 3B, bottom). Irrespective of the p53 mutation status, we found a trend toward higher sensitivity of category I tumors to cisplatin when compared with the other 2 tumor groups indicating that the observed differences in p53 response might also have functional consequences (Supplementary Figure).

The response of CAFs to cisplatin mirrors the response of their adjacent cancer cells

We next investigated p53 protein expression in both CAFs and their adjacent cancer cells within the intact tumor tissue samples, using p53 IHC and hematoxylin counterstaining (representative examples in Fig. 4A). In the absence of cisplatin, p53 was undetectable or low (<2%) in CAFs irrespective of the different p53 levels in cancer cells of the 3 tumor categories (Supplementary Table S2). Remarkably, upon cisplatin treatment we observed a strong correlation between p53 accumulation in the cancer cells and p53 accumulation in their adjacent CAFs (Spearman correlation = 0.88; P < 0.0001). Strikingly, in category I tumors this correlation was seen regardless of the p53 mutation status of the tumor (Fig. 4B, left), and was mirrored by parallel induction of the p53 target p21 in CAFs (Fig. 4B, right). In sharp contrast, tumor tissues with no detectable induction of p53 accumulation in the cancer cells was excessively stable, owing to escape for the posttranslational mechanisms that otherwise promote rapid p53 degradation (20–22). However, TP53 mutations could also be detected in many tumors characterized by low or undetectable p53 staining in untreated control samples from categories I (7 of 14 with TP53 mutation; indicated by red squares in Fig. 2B) and III (4 of 11 with TP53 mutation; indicated by red squares in Fig. 2B).

**Figure 2.** p53 response in cultivated tissue slices upon cisplatin treatment. Tumor tissue slices were incubated with or without cisplatin for 72 hours and evaluated for p53 (with and without cisplatin; A) and Ki67 (constitutive; B). A, representative examples of control samples (left) and cisplatin-treated samples (right). According to their p53 positivity in cancer cells before and after cisplatin treatment, the samples were subdivided into categories. The 3 categories were defined as follows: category I (top), no detectable or low constitutive expression of p53 (<20% of positive cancer cells) with accumulation after 72 hours of cisplatin treatment; category II (middle), constitutively high p53 levels with no further accumulation upon cisplatin treatment; and category III (bottom), undetectable p53 protein independent of cisplatin treatment. B, percentage of Ki67-positive cancer cells in the tumor categories I to III. Thirty-one of 32 tumor samples were available for Ki67 staining. TP53 mutation status is indicated by black (wild-type) and red squares (mutant). Horizontal lines indicate the mean percentage of Ki67-positive cancer cells within the 3 groups. Group III shows the lowest mean expression of Ki67, whereas there is only a slight difference between the groups I and II.
cells (categories II and III) showed no p53 accumulation (Fig. 4B, left) or p21 induction (Fig. 4B, right) also in their adjacent CAFs. To analyze if this phenotype is preserved in CAFs after disruption of the tumor tissue context, we used CAFs isolated from 2 tumors of each category and cultivated them in presence or absence of cisplatin. Importantly, induction of p53 accumulation was detected in all monocultivated CAF strains to a comparable degree irrespective of the tumor category (Fig. 4C), suggesting that the microenvironment predominantly determines the observed differences in the p53 response of CAFs to cisplatin. These results show for the first time a close correlation in p53 response between cancer cells and adjacent CAFs in the intact microenvironment of lung tumor tissues.

TUNEL IHC was next used to analyze induction of cell death upon cisplatin treatment of tissue slices (Fig. 4D). Cisplatin-induced increase of TUNEL positivity was observed in both the cancer cell and stromal cell compartments (Spearman correlation = 0.65; \(P = 0.0003\)). Notably, in 7 of 26 cases, a parallel increase of TUNEL positivity (above 20%) was observed in both cancer and stromal cells. In contrast, in only 4 of 26 cases, we found an apoptotic response to cisplatin in the cancer cell compartment but not in their adjacent CAFs. All cases with a response in CAFs showed also a response in the

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**Figure 3.** Reduction of proliferation and induction of cell death upon cisplatin treatment. Tumor tissue slices were incubated with or without cisplatin for 72 hours and analyzed for proliferation (Ki67 positivity of cancer cells; A) and cell death (TUNEL positivity of cancer cells; B). A, reduction of Ki67 score as a consequence of cisplatin treatment in tumor samples harboring wtp53 (top) and mutant p53 (mtp53; bottom). The left panel illustrates the Ki67 score in all samples. The right panels show the responses in the 3 categories. Wilcoxon matched-pairs signed rank test was used to test for differences (*, \(P < 0.05\)). B, induction of cell death in cancer cells upon cisplatin treatment in tumor samples with wtp53 (top) and mutant p53 (bottom). Again, left panel shows all samples, whereas the right panels illustrate cell death induction in the 3 categories. Wilcoxon matched-pairs signed rank test was used to test for differences (**, \(P < 0.01\)) and (***, \(P < 0.001\)). Tumor cases with a decrease of more than 10% Ki67-positive cancer cells and an increase of more than 20% for TUNEL positivity upon cisplatin treatment are labeled in red.
cancer cell compartment. Together, these findings argue that not only p53 induction but also induction of cell death upon cisplatin was markedly correlated between cancer and stromal cells.

**Discussion**

Understanding the response of complex tumor tissues to DNA damaging agents might facilitate the rational design of therapeutic regimens to treat cancer. Serial biopsies to study such stress responses in vivo are not feasible in cancer patients. Both 2D and 3-dimensional cell culture systems aim to mimic the tumor, but it is likely that the heterogeneity of primary tumor samples surpasses by far the complexity of even advanced in vitro culture systems. Cultivation of thin tissue slices prepared from freshly resected primary tumors is probably the only suitable ex vivo model system that adequately represents the complexity and heterogeneity of the individual in situ situation of carcinomas in patients (16, 18, 23). Using this ex vivo model, we were able to show a close correlation of the stress responses of cancer cells and the nonmalignant CAFs in intact lung tumor tissues: both accumulation of the major stress regulator p53 and induction of cell death were found to be coordinately regulated in the cancer and stromal compartments.

Our data show that constitutive expression of p53 protein as well as its accumulation upon cisplatin in cancer cells show a high degree of variability among individual tumors, which could not be completely predicted on the basis of the mutational status of TP53. In most clinical studies of NCSLC patient specimens, positive staining for p53 tended to be associated with the presence of TP53 mutations (for review see ref. 24). This is consistent with our findings, as we detected TP53 mutations in all tumors with constitutively high p53 levels in the nuclei of the cancer cells (category II tumors). However, we also found TP53 mutations in approximately 50% of tumors with constitutively low or undetectable p53 levels (category I and III tumors). Interestingly, in...
the majority of those latter cases we observed a significant induction of nuclear p53 staining in the cancer cell compartment in response to cisplatin treatment (category I tumors). The imperfect correlation of constitutive p53 IHC positivity and TP53 mutation status in our relatively small patient cohort has also been observed in other studies (25–27). Furthermore, analysis of a p53R172H knock-in mouse model confirmed that mutant p53 is inherently unstable in normal mouse tissues (28), and additional tumor specific alterations, such as altered Mdm2 or ARF expression, are required for stabilization of mutant p53 (22). Importantly, it could be shown in vivo that, similar to wtp53, mutant p53 protein can also be posttranslationally modified and stabilized in response to cellular stress signals (29); this is in agreement with our study where 7 of 10 mutant p53 tumor samples with constitutively low p53 levels responded to cisplatin by p53 accumulation.

Numerous studies have assessed a possible relationship between p53 protein expression and therapy outcome, and the findings have been inconsistent across those studies (for review see ref. 24). However, most studies do support a trend toward enhanced resistance to platinum-based therapy in NSCLCs with p53 IHC positivity, even if this association did not reach significance in a meta-analysis involving more than 1,000 NSCLC patients (30). In accordance with these clinical studies, we found that the majority of tumors with a constitutively high expression of p53 did not respond to cisplatin by induction of cell death (category II tumors; Fig. 3). This is in contrast to cases with p53 accumulation induced by cisplatin treatment (category I tumors), where a significant proportion showed an induction of cell death independent of the TP53 mutation status of the tumor. There are 2 possible explanations for this phenomenon. First, the mutational status of the cancer cells in cultured tissues might differ from that in the paraffin embedded material used for sequencing in our study. However, a previously published report shows that despite a high degree of variability in p53 IHC, the genetic TP53 alterations were homogeneously detectable in tissue sections of lung cancer (31). Therefore, a discrepancy between the cancer cells in cultured tissues and the corresponding primary tumor material used for sequencing seems unlikely. An alternative explanation might be that induction of cell death by cisplatin is independent of p53 in TP53 mutated tumors with low constitutive p53 levels, despite the observed increase of p53 protein (category I tumors; Fig. 3). The enhanced resistance of p53-mutated tumors harboring constitutively high levels of p53 protein might be because of the same mechanisms that cause increased stability of the mutant p53 protein, namely cancer-associated alterations in intracellular signaling pathways that enable those cancer cells to adapt to constitutive high stress.

Despite the high degree of variability in the reaction to cisplatin, both induction of p53 accumulation as well as induction of cell death was coordinately regulated in the cancer cells and in their neighboring fibroblasts. These data further show the central role of the microenvironment for the response of the whole tumor, including neoplastic cells as well as noncancerous stromal cells, to cytotoxic drugs. Furthermore, despite a presumably normal p53 genotype (16), CAF-specific induction of p53 and p21 occurred only in tumor tissues characterized by induction of p53 accumulation in the cancer cell compartment (category I tumors) whereas in tumors with no p53 accumulation in cancer cells (categories II and III) there was also no p53 accumulation or p21 induction in adjacent CAFs. Importantly, this phenotype seems to be dependent on the intact tissue environment, as isolated CAFs in 2D monoculture showed cisplatin-induced p53 accumulation independent of the tumor category they were originated from. So far, mutual cross-talk of the p53 responses of cancer cells and their microenvironment has been studied only in artificial cocultures. In such models, p53 activity of normal fibroblasts was shown to negatively influence cancer cell proliferation via paracrine mechanisms (32, 33). Furthermore, cancer cell proliferation was repressed in normal but not in p53 null mice (10). These observations suggest that tumors harboring a p53-deficient microenvironment may have a selective advantage. Indeed, using a prostate mouse model, Hill and colleagues showed that stromal fibroblasts tend to lose their wild type p53 during tumor progression (34). Moreover, TP53 mutations resulting in amino acid substitutions have been detected in microdissected stromal compartments from paraffin-embedded carcinoma samples (12, 13), even though the latter results were challenged by subsequent studies, using frozen tissues or fresh material (14–16). Therefore, if CAFs indeed typically maintain a wild type p53 genotype, a functional interaction between cancer cells and the p53 response of CAFs seems to be more likely. Indeed, such interactions could be shown in simple 2D cell line models (17, 35), where it was shown that epithelial cancer cells can suppress CAF-specific p53 accumulation. Our data provide for the first time evidence that this inhibitory effect of cancer cells on CAF p53 response may depend on the functionality of the cancer cell p53 response: complete abrogation of cisplatin-induced p53 accumulation in CAFs was selectively seen in tumor tissue samples characterized by lack of p53 accumulation in the cancer cell compartment. This implies that, independent of the mutational status of TP53, loss of the DNA damage-induced p53 response in cancer cells is sufficient to inhibit stromal p53 induction. We also observed a trend toward enhanced resistance to cisplatin-induced cell death in such tumors (Supplementary Figure), indicating that this inhibitory effect might also have functional consequences. However, because of the very heterogeneous treatment regimens (summarized in the Supplementary Table S1) and the relatively small sample size, statistically significant correlation between p53 functionally or mutational status and clinical outcome could not be obtained.

In sum, our findings highlight a hitherto undescribed level of cross-talk between the p53 response in cancer cells and in their immediate microenvironment, which is only partly accountable by p53 mutation status. Furthermore, they highlight the great potential of tissue slices for studying cancer-stroma interactions in a setting that is more representative of the
authentic tumor microenvironment and yet amenable to interrogation by a variety of experimental manipulations.

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