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Paracrine Apoptotic Effect of p53 Mediated by Tumor Suppressor Par-4

Ravshan Burikhanov, Tripti Shrestha-Bhattarai, Nikhil Hebbar, Shirley Qiu, Yanming Zhao, Gerard P. Zambetti, and Vivek M. Rangnekar

INTRODUCTION

Lung cancer is the leading cause of cancer deaths in both men and women in the United States (Siegel et al., 2012). Activation of the Ras oncogene and loss of tumor suppressor p53 are the two most commonly occurring alterations in lung cancer. Given that p53 function is essential for growth arrest and cell death by diverse chemotherapeutic agents and ionizing radiation, loss of p53 function in the tumors may confer therapeutic resistance ultimately leading to death of the patients (Levine, 1997; Chen et al., 2010). P53 knockout or p53 mutant mice develop spontaneous as well as inducible tumors (Donehower et al., 1992; Jacks et al., 1994), whereas p53 transgenic mice exhibit tumor-free survival with an increased life span (García-Cao et al., 2002). Because up- or downregulation of transcriptional target genes involved in cell-cycle progression or apoptosis plays an important role in the tumor suppressor function of p53, it is not surprising that p53 is mutated within its DNA binding domain in a large number of human cancers (Petitjean et al., 2007).

Although a major part of p53 research has focused on its cell-autonomous functions, there is emerging evidence that tumor latency is reduced in p53-null mice relative to wild-type mice, indicating that p53 activity in the host may exert an inhibitory effect on cancer progression (Kiaris et al., 2005). Moreover, tumor progression imposes a strong selective pressure for loss of p53 function in cancer-associated fibroblasts (Hill et al., 2005). These observations support the view that p53 function in both normal and cancer compartments of the tumor is important for suppression of tumor progression. However, the precise mechanism by which p53 loss in normal or stromal cells may contribute to tumor progression is not well understood. Because normal cells in the body of the patient except in the tumor microenvironment are expected to express wild-type p53, we determined whether activation of p53 function in normal cells causes paracrine growth inhibitory effects in cancer cells. Specifically, we asked whether p53 activation in normal cells induces the secretion of proapoptotic proteins systemically and whether such proteins act to inhibit the growth of p53-deficient tumor cells in a paracrine manner. Our findings indicate that activation of p53 in normal cells promotes the secretion of Prostate apoptosis response-4 (Par-4), a proapoptotic tumor suppressor protein (Burikhanov et al., 2009). This action of p53 involves direct inhibition of UACA (uveal autoantigen with coiled-coil domains and ankyrin repeats), a gene first identified in patients suffering from inflammatory eye disease (Yamada et al., 2001) and recently reported to be upregulated by NF-κB (Burikhanov et al., 2013 and references cited therein). Importantly, secreted Par-4 mediates a paracrine growth-inhibitory effect by inducing apoptosis of p53-deficient cancer cells.

RESULTS

Normal Fibroblasts Secrete Par-4 Protein in a p53-Dependent Manner

To examine whether p53 activation in normal cells exhibits paracrine effects in cancer cells, we used cocultures of mouse
embryonic fibroblasts (MEFs) from p53 +/+ or p53−/− mice with p53-null H1299 and PC-3 cells and p53 mutant HOP92 cells. The cell cultures were treated with Nutlin-3a, a specific activator of p53 (Vassilev et al., 2004). Nutlin-3a induced apoptosis in p53-deficient cancer cells that were cocultured with p53 +/+ MEFs but not with p53−/− MEFs (Figure 1A, left). As expected, the p53-deficient cancer cells and the MEFs were resistant to apoptosis by Nutlin-3a when cultured individually (Figure 1A, right). Because p53 may function by partial inhibition of NF-κB activity (Dey et al., 2007), we combined Nutlin-3a with PS-1145, a small molecule that specifically inhibits IKKb (see Burikhanov et al., 2013). By itself, PS-1145 does not induce apoptosis of normal or lung cancer cells (Figure 1A, right). 

To determine whether extracellular factors secreted by the MEFs in response to Nutlin-3a and/or PS-1145 treatment contributed to apoptosis of the cancer cells, the MEFs were treated with these small molecules, and conditioned medium (CM) was transferred to p53-deficient lung cancer cells or normal lung cells. The CM from p53 +/+ MEFs but not from p53−/− MEFs induced apoptosis of H1299 cells (Figure 1B). Apoptosis of H1299 cells was scored after 24 hr by ICC for active caspase-3.

Asterisks in (A), (B), and (D) indicate statistical significance (p < 0.001) by the Student’s t test; **N+P is significantly (p < 0.001) more effective than individual treatments based on two-way ANOVA. Error bars indicate SD.

Figure 1. P53 Activation in Normal Cells Produces Paracrine Apoptosis in p53-Deficient Cancer Cells

(A) Activation of p53 in MEFs induces apoptosis of cocultured p53-deficient cancer cells. Cocultures of p53+/+ or p53−/− MEFs with the indicated cancer cells were treated with vehicle (v), Nutlin-3a (N, 10 μM), and/or PS-1145 (P, 10 μM) for 24 hr. The cells were subjected to ICC for cytokeratins to detect epithelial cancer cells and for active caspase-3 to determine apoptotic cells.

(B) Apoptosis of cancer cells by CM from p53-activated MEFs. P53+/+ or p53−/− MEFs were treated with vehicle (v), Nutlin-3a (N, 10 μM), and/or PS-1145 (P, 10 μM) for 24 hr, and their CM was transferred to normal (HEL) or p53-deficient cancer (H1299) cells. Apoptotic cells were scored after 24 hr by ICC for active caspase-3.

(C) Coparallel activation of p53 and inhibition of NF-κB activity additively increases Par-4 secretion in fibroblasts. MEFs were treated with vehicle (v), Nutlin-3a (N), and/or PS-1145 (P) for 24 hr, and their CM, as well as whole-cell lysates, was subjected to western blot analysis for Par-4. β-actin was used to normalize loading of lysate. Coo massie blue-stained albumin in the serum was used to normalize loading of CM. Par-4 secretion, but not Col1A1 secretion, was dependent on the p53 status of the cells. Fold change in secreted Par-4 is shown.

(D) Par-4 secretion is critical for the paracrine effect resulting from simultaneous p53-activation and NF-κB-inhibition. The CM from p53+/+ cells treated with Nutlin-3a + PS-1145 was incubated with polyclonal antibody for Par-4 or PTEN control and then added to H1299 cells. Apoptosis of H1299 cells was scored after 24 hr by ICC for active caspase 3 (left panel). Par-4+/+ and Par-4−/− MEFs were treated with Nutlin-3a + PS-1145 (N+P) or vehicle, and then the CM was applied to the indicated cell lines, and apoptotic cells were scored by ICC for active caspase-3 (middle panel). Expression of Par-4 in CM was verified on western blots (right panel).
We then examined the CM for secreted proteins, especially TRAIL, maspin, IGFBP3, and Par-4, which are known to act extracellularly and induce cancer-specific apoptosis. The CM from p53+/+ MEFs treated with Nutlin-3a or PS-1145 showed elevated levels of Par-4 protein, and combination of Nutlin-3a plus PS-1145 additively increased the secretion of Par-4 (Figure 1 C). None of the other proteins showed elevated secretion with Nutlin-3a plus PS-1145 (R.B. and T.S.-B., unpublished data). By contrast, p53+/−/−/− MEFs accumulated Par-4 protein in the lysate but failed to secrete it in response to these treatments (Figure 1 C). Moreover, doxorubicin, which is known to activate p53, induced Par-4 secretion from p53+/+ but not p53−/−/− MEFs, and the CM collected from p53+/+ but not p53−/−/− MEFs induced apoptosis of p53-deficient cancer cells (Figure S1 D). Together, these findings suggest that p53 regulates the secretion of Par-4 protein, which selectively induces apoptosis in cancer cells.

P53 Induces Systemic Expression of Par-4 in Normal Mice

We interrogated whether p53 regulates the secretion of Par-4 in vivo. The serum of untreated C57BL/6 p53+/+ mice contained higher levels of Par-4 relative to p53−/− mice (Figure 2A, left). When p53+/+, p53−/−, and p53−/−/− mice were injected with Nutlin-3a plus PS-1145 or vehicle control, and serum samples were collected for analysis of Par-4, we noted that, relative to vehicle control, Nutlin-3a plus PS-1145 (N+P) were preincubated with Par-4 antibody, control PTEN antibody or no antibody (no antibody [Ab]), and then applied on lung cancer cells. After 24 hr of treatment, the cells were scored for apoptosis. Asterisks in (A) and (B) indicate statistical significance (p < 0.001) by the Student’s t test. Error bars indicate SD.

Figure 2. Activation of p53 in Mice Induces Systemic Expression of Par-4 Proapoptotic Activity

(A) p53 function is essential for induction of systemic Par-4 in mice. Serum samples from p53+/+ mice (4 μl per lane) and p53−/− mice (8 μl per lane) (left panel) and also from p53+/+ , p53−/− , and Par-4−/− mice (right panel) injected i.p. route with either vehicle (v) or with Nutlin-3a + PS-1145 (N+P) were examined for Par-4 expression by western blot analysis using serum albumin to normalize loading. Data are representative of four mice per treatment.

(B) Serum from Nutlin-3a plus PS-1145-treated p53+/+ mice induces ex vivo apoptosis of cancer cells. Serum samples collected from p53+/+, p53−/−, and Par-4−/− mice injected i.p. with either vehicle (lower panel) or with Nutlin-3a + PS-1145 (upper panel) was applied at a final concentration of 10% to the indicated normal or cancer cell lines. The cells were scored for apoptosis after 24 hr. Fetal bovine serum (FBS, 10%) was used as an additional control.

(C) Secreted Par-4 is essential for the paracrine effect of p53 activation. Serum samples collected from p53+/+ mice treated with vehicle or Nutlin-3a + PS-1145 (N+P) were preincubated with Par-4 antibody, control PTEN antibody or no antibody (no antibody [Ab]), and then applied on lung cancer cells. After 24 hr of treatment, the cells were scored for apoptosis.

We then examined the CM for secreted proteins, especially TRAIL, maspin, IGFBP3, and Par-4, which are known to act extracellularly and induce cancer-specific apoptosis. The CM from p53+/+ MEFs treated with Nutlin-3a or PS-1145 showed elevated levels of Par-4 protein, and combination of Nutlin-3a plus PS-1145 additively increased the secretion of Par-4 (Figure 1 C). None of the other proteins showed elevated secretion with Nutlin-3a plus PS-1145 (R.B. and T.S.-B., unpublished data). By contrast, p53+/−/−/− MEFs accumulated Par-4 protein in the lysate but failed to secrete it in response to these treatments (Figure 1 C). Moreover, doxorubicin, which is known to activate p53, induced Par-4 secretion from p53+/+ but not p53−/−/− MEFs, and the CM collected from p53+/+ but not p53−/−/− MEFs induced apoptosis of p53-deficient cancer cells (Figure S1 D). Together, these findings suggest that p53 regulates the secretion of Par-4 protein, which selectively induces apoptosis in cancer cells.
for upregulation of Par-4 secretion in mice. Importantly, the serum from Nutlin-3a plus PS-1145-treated p53+/+ mice, but not p53−/− mice or Par-4−/− mice, induced ex vivo apoptosis of cancer cell cultures but not normal cell cultures (Figure 2B, top). By contrast, the serum from vehicle-treated mice failed to induce apoptosis over background levels in normal or cancer cells (Figure 2B, bottom). Moreover, preincubation of the serum samples collected from Nutlin-3a plus PS-1145-treated p53+/+ mice with the Par-4 antibody but not the PTEN control antibody significantly inhibited the ability of the serum to induce apoptosis of cancer cell cultures (Figure 2C). Altogether, these findings suggest that p53 activation in normal mice induces adequate levels of systemic Par-4 protein that is functionally effective in inducing apoptosis of cancer cells.

**P53 Downregulates the Expression of UACA to Induce Par-4 Secretion**

Because Par-4 does not contain a p53 consensus binding site in its DNA, Par-4 secretion is likely regulated by another downstream target of p53. To identify the primary gene target of p53, we used three criteria: (1) it should encode a protein that binds to Par-4, (2) it should be regulated by p53, and (3) it should be induced by NF-κB activity. We recently identified UACA as a strong binding partner of Par-4 (Burikhanov et al., 2013). UACA was coimmunoprecipitated from normal MEF and HEL fibroblast with Par-4 antibody, and Par-4 was reciprocally coimmunoprecipitated with UACA antibody (Figure S2A). A comparison of p53+/+ and p53−/− MEFs indicated that UACA was lower in p53+/+ normal cells and mouse tissues relative to p53−/− cells and mouse tissues (Figure 3A). UACA levels in p53+/+ and p53−/− MEFs correlated inversely with the levels of Par-4 secreted in the CM (Figure S2B). Nutlin-3a suppressed the expression of UACA in p53+/+ but not p53−/− MEFs.
classical endoplasmic reticulum (ER)-Golgi pathway (Burikhanov et al., 2013). Previous studies have indicated that Par-4 is secreted via the Classical Pathway (Yu et al., 2009), as well as via the exosomal pathway (Wang et al., 2012). On the other hand, p53 has been shown to promote secretion of proteins via the exosomal pathway (Yu et al., 2009). To determine whether p53 activation leads to Par-4 secretion via the classical pathway, we used Brefeldin A (BFA), which blocks the ER-Golgi pathway. BFA inhibited the secretion of Par-4 by p53 restoration at 32°C in (10)1/Val5 fibroblasts (Figure 3D, left panel). As both Nutlin-3a and PS-1145 induced the secretion of Par-4 in a p53-dependent manner, we investigated whether Par-4 secretion following Nutlin-3a plus PS-1145-treatment occurred via the classical pathway. Nutlin-3a plus PS-1145-inducible secretion of Par-4 from p53+/+ MEFs was about 60% inhibited by BFA (Figure 3D, middle panel). These findings imply that p53 regulates the secretion of Par-4 largely by the classical pathway. Moreover, Par-4 secretion following UACA knockdown in p53+/+ MEFs was inhibited by BFA (Figure 3D, right). Collectively, these findings indicate that UACA inhibition by siRNA, Nutlin-3a plus PS-1145, or p53 activation induces Par-4 secretion via the classical pathway.

UACA Is a Direct Target of p53

A potential p53-binding site (el-Deiry et al., 1992) is present in the DNA corresponding to exon 1 within the coding region of UACA (Figure S3A). P53 suppresses the expression of UACA RNA, as judged by quantitative RT-PCR (qRT-PCR) (Figure S3B). To determine direct binding of p53 to its consensus binding site in UACA, we performed chromatin immunoprecipitation studies. HEL cells treated with Nutlin-3a, but not with vehicle, showed chromatin immunoprecipitation of endogenous p53 bound to the p53-consensus motif in UACA (Figure 4A). These findings were corroborated by chromatin immunoprecipitation studies in p53-deficient cells that were transfected with p53 expression construct (Figure S3C). Consistently, Nutlin-3a treatment, which activates endogenous p53, suppressed the expression of UACA in HEL cells (Figure 4B).

To determine whether p53 regulated UACA expression via its binding motif in an NF-kB activity-independent manner, we performed experiments in IKKβ−/− MEFs, which lack NF-kB activity (Burikhanov et al., 2013; Li et al., 1999). Nutlin-3a treatment of IKKβ−/− MEFs resulted in suppression of UACA expression (Figure 4B). Moreover, ectopic p53 suppressed the expression of UACA containing the p53-binding motif but did not suppress the expression of mutant-UACA, which contained mutations in the p53-binding sequence, in IKKβ−/− MEFs (Figure S4). Together, these findings suggest that p53 suppressed the expression of UACA via its binding motif in an NF-kB-independent manner. Thus, UACA is a relevant target of p53, and p53 may regulate Par-4 secretion by downregulation of UACA.

**DISCUSSION**

The tumor suppressor p53 is known to suppress tumor growth by intracellular activation of growth arrest and apoptotic cell death pathways. However, p53 is mutated in over 50% of cancers, and mutant forms of p53 may render cancer cells resistant to both chemotherapy and radiation therapy. Lung cancer, the leading cause of cancer-related deaths in the US, is often associated with inactivating mutations in p53. We determined

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**Figure 4. P53 Directly Binds to UACA and Inhibits Its Expression**

(A) P53 binds to its consensus binding motif in UACA. HEL cells were treated with Nutlin-3a (N) or vehicle (v) for 24 hr and subjected to chromatin immunoprecipitation analysis with p53 antibody (Ab) or control rabbit immunoglobulin (Ig) G Ab, and immunoprecipitated DNA fragments were analyzed by PCR with primers flanking the p53-binding site in UACA. Primers flanking the p53-binding motif #1 in p21 (see Figure S3A) or for GAPDH (which does not contain a p53-binding site) were used as positive or negative control, respectively. Input samples for each set of primers are shown. (B) Nutlin-3a causes inhibition of endogenous UACA expression in an NF-kB-independent manner. IKKβ−/− MEFs or HEL cells were treated with Nutlin-3a or vehicle for 24 hr, and whole-cell lysates were subjected to western blot analysis for UACA, p53, or actin.
whether wild-type p53 function in normal cells could be efficiently propelled to target lung cancer cells. The present study revealed that p53 activation in normal cells induces paracrine apoptosis of p53-deficient lung cancer and prostate cancer cells. This action of p53 is mediated by the tumor suppressor protein Par-4. The paracrine effect of p53 activation on Par-4 secretion was bolstered by coparallel inhibition of NF-κB activity in normal cells. We noted that UACA binds to Par-4 and prevents it from being secreted, and inhibition of UACA by p53 activation and/or inhibition of NF-κB activity, or by several different siRNAs, results in elevated secretion of Par-4. Importantly, Par-4 secreted by cells following coparallel activation of p53 and inhibition of NF-κB activity caused apoptosis in p53-deficient lung cancer cells but not normal cells. The physiological relevance of the findings was confirmed by studies indicating that normal cells in mice can be triggered to secrete proapoptotic Par-4 activity in circulation in a p53-dependent manner. The elevated levels of Par-4 in serum induced ex vivo apoptosis in tumor cells but not normal cells. Because our previous studies indicated a good correlation between inhibition of lung tumors in immunocompetent mice and induction of ex vivo apoptosis by Par-4 in the serum of these mice (Zhao et al., 2011), the activation of p53 to trigger proapoptotic Par-4 protein secretion and elevate its systemic levels may be an effective strategy to induce apoptosis of cancer cells that metastasize through the circulatory route. Collectively, our findings suggest that secreted Par-4 mediates the paracrine apoptotic effects of p53, and that secretagogues of Par-4 may therefore empower normal cells to execute paracrine tumor growth inhibition.

Our findings indicate that Par-4 secretion following p53 activation occurs via the classical BFA-sensitive pathway. Secretion of Par-4 via this pathway is dependent on downmodulation of UACA, a functional target of p53. Chromatin immunoprecipitation experiments confirmed that p53 directly binds to its consensus motif in UACA. Importantly, p53 suppressed the expression of UACA in an NF-κB-independent manner. Given that UACA is a principal binding partner that sequesters Par-4 in normal cells, UACA inhibition by p53 is necessary to release Par-4 for secretion via the classical pathway. However, normal cells lacking p53 are deficient in Par-4 secretion despite UACA downregulation, implying that p53 function is critical for Par-4 secretion and is suggestive of an additional role for p53 in triggering Par-4 secretion. Several integral components of the classical ER–Golgi-transport vesicle/membrane fusion pathway are currently being investigated as targets for p53-dependent Par-4 secretion. Given that previous studies have indicated that p53 regulates protein secretion via the exosomal pathway, we tested whether Par-4 secretion is also regulated via the exosomes. Our findings indicate that, of the total Par-4 secreted by cells, <5% is secreted via the exosomal pathway, and the large majority is secreted by the classical pathway (T.S.-B., unpublished data).

In summary, our findings suggest that the tumor suppressor p53 regulates the secretion of the proapoptotic, tumor suppressor Par-4 via the classical pathway by suppressing UACA, and that Par-4 executes the paracrine apoptotic effects of p53. As systemic Par-4 inhibits the growth of lung tumors (Zhao et al., 2011), Par-4 secretagogues can be exploited to activate p53 and unleash the power of normal cells in the tumor micro- and macroenvironment to elevate systemic Par-4 and suppress tumor cell survival.

**EXPERIMENTAL PROCEDURES**

**Cells and Chemical Reagents**

Lung cancer cells H1299, HOP92, LLC1, prostate cancer cells PC-3, and primary lung fibroblast cells HEL were from ATCC. IKKα+/+ and IKKβ−/− MEFs were previously described (Burikhanov et al., 2013). Par-4+/+ and Par-4−/− MEFs were derived from wild-type and Par-4-null C57BL/6 mice, respectively (Figure S1C). KP lung tumor cells, and P53+/+ and P53−/− MEFs in the third passage were from Tyler Jackson (Massachusetts Institute of Technology). Nutlin-3a was from Cayman Chemicals. Immortalized Balc/c fibroblasts (101), with no endogenous p53, and (10.1)Va15 cell line derived from (10)1 cells by stable transfection with the temperature-sensitive p53 allele encoding valine at 135 aa, were from Arnold Levine (Institute for Advanced Study, NJ). The p53 mutant in (10.1)Va15 cells exhibits wild-type conformation at 32°C, judged by p21/WAF1 induction, and mutant conformation at 37°C–39°C (Wu and Levine, 1994).

**Animal Experiments**

Whole-blood samples and various tissues were collected from mice 24 hr after injection via the intraperitoneal (i.p.) route with Nutlin-3a plus PS-1145 (10 and 5 mg/kg body weight, respectively) or corn oil (100 μl) as vehicle control. Serum was separated from the blood samples and used for testing. All animal procedures were performed with IACUC approval.

**Statistical Analysis**

All experiments were performed in triplicate to verify the reproducibility of the findings. Statistical analyses were carried out with Statistical Analysis System software (SAS Institute). Mean ± SD bars are shown. p values were calculated using the Student’s t test.

All other reagents and experimental procedures are presented in the Supplemental Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.12.020.

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