1-9-2014

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

Ravshan Burikhanov
University of Kentucky, rburi2@uky.edu

Tripti Shrestha-Bhattarai
University of Kentucky, tripti.shrestha@uky.edu

Nikhil Hebbar
University of Kentucky, nikhilhebbar@uky.edu

Shirley Qiu
University of Kentucky

Yanming Zhao
University of Kentucky, yzhao@uky.edu

See next page for additional authors

Click here to let us know how access to this document benefits you.

Follow this and additional works at: https://uknowledge.uky.edu/radmed_facpub

Part of the Medical Immunology Commons, Medical Microbiology Commons, Medical Toxicology Commons, Oncology Commons, and the Radiology Commons

Repository Citation
Burikhanov, Ravshan; Shrestha-Bhattarai, Tripti; Hebbar, Nikhil; Qiu, Shirley; Zhao, Yanming; Zambetti, Gerard P.; and Rangnekar, Vivek M., "Paracrine Apoptotic Effect of p53 Mediated by Tumor Suppressor Par-4" (2014). Radiation Medicine Faculty Publications. 1.
https://uknowledge.uky.edu/radmed_facpub/1

This Article is brought to you for free and open access by the Radiation Medicine at UKnowledge. It has been accepted for inclusion in Radiation Medicine Faculty Publications by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.
Authors
Ravshan Burikhanov, Tripti Shrestha-Bhattarai, Nikhil Hebbar, Shirley Qiu, Yanming Zhao, Gerard P. Zambetti, and Vivek M. Rangnekar

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

Notes/Citation Information
Published in Cell Reports.

© 2014 The Authors All rights reserved.

This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial-No Derivative Works License, which permits non-commercial use, distribution, and reproduction in any medium, provided the original author and source are credited.

Digital Object Identifier (DOI)
http://dx.doi.org/10.1016/j.celrep.2013.12.020

This article is available at UKnowledge: https://uknowledge.uky.edu/radmed_facpub/1
Paracrine Apoptotic Effect of p53 Mediated by Tumor Suppressor Par-4

Ravshan Burikhanov,1,6 Tripti Shrestha-Bhattarai,2,6 Nikhil Hebbar,2 Shirley Qiu,3 Yanming Zhao,2 Gerard P. Zambetti,4 and Vivek M. Rangnekar1,2,3,5,*

1Department of Radiation Medicine, University of Kentucky, Lexington, KY 40536, USA
2Graduate Center for Toxicology, University of Kentucky, Lexington, KY 40536, USA
3Department of Microbiology, Immunology, and Molecular Genetics, University of Kentucky, Lexington, KY 40536, USA
4Department of Biochemistry, St. Jude Children’s Research Hospital, Memphis, TN 38105, USA
5L.P. Markey Cancer Center, University of Kentucky, Lexington, KY 40536, USA
6These authors contributed equally to this work

*Correspondence: vmrang01@email.uky.edu

This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial-No Derivative Works License, which permits non-commercial use, distribution, and reproduction in any medium, provided the original author and source are credited.

SUMMARY

The guardian of the genome, p53, is often mutated in cancer and may contribute to therapeutic resistance. Given that p53 is intact and functional in normal tissues, we harnessed its potential to inhibit the growth of p53-deficient cancer cells. Specific activation of p53 in normal fibroblasts selectively induced apoptosis in p53-deficient cancer cells. This paracrine effect was mediated by p53-dependent secretion of the tumor suppressor Par-4. Accordingly, the activation of p53 in normal mice, but not p53+/- or Par-4-/- mice, caused systemic elevation of Par-4, which induced apoptosis of p53-deficient tumor cells. Mechanistically, p53 induced Par-4 secretion by suppressing the expression of its binding partner, UACA, which sequesters Par-4. Thus, normal cells can be empowered by p53 activation to induce Par-4 secretion for the inhibition of therapy-resistant tumors.

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 (Garcia-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 surpris-
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 IKK (see Burikhanov et al., 2013). By itself, PS-1145 does not induce apoptosis of normal or lung cancer cells (Figure 1A, right). Par-4 secretion, but not Col1A1 secretion, was dependent on the p53 status of the cells. Fold change in secreted Par-4 is shown.

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 the CM from p53 −/− MEFs treated with Nutlin-3a or PS-1145 produced apoptosis of H1299 cells (Figure 1B). Apoptosis of H1299 cells was scored after 24 hr by ICC for active caspase-3. By contrast, the CM from MEFs treated with Nutlin-3a and/or PS-1145 did not induce apoptosis in wild-type TP53 primary human lung fibroblasts HEL cells (Figure 1B). Similarly, CM from HEL cells treated with Nutlin-3a plus PS-1145 induced apoptosis of H1299 and HOP92 cells, but not in HEL cells (Figure S1A). These findings indicated that Nutlin-3a and PS-1145 may regulate the secretion of cancer-selective proapoptotic factor(s) in a p53-dependent manner.

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.
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 Par-4+/+ but not Par-4−/−/− 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−/− MEFs 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 induced apoptosis of p53-deficient cancer cells (Figure S1D). 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.

Figure 3. P53 Stimulates Par-4 Secretion by Suppressing the Expression of UACA

(A) P53 downregulates UACA. Whole-cell lysates from p53+/+ and p53−/− MEFs that were either untreated or treated with vehicle (v), Nutlin-3a (N, 10 μM), or PS-1145 (P, 10 μM) for 24 hr (left three panels), or whole-tissue lysates of highly vascular organs obtained from p53+/+ and p53−/− mice (right three panels) were examined for UACA by western blot analysis.

(B) Restoration of p53 activity inhibits UACA expression and promotes Par-4 secretion. P53−/− MEFs were infected with GFP-tagged p53- or GFP-producing adenoviral constructs (left panel). Also, the mouse fibroblasts (101), which do not express any p53, and (101)-derived Val5 cells, which are engineered to stably overexpress wild-type p53 at 32°C or mutant p53 at 37°C, were grown at 37°C or shifted to 32°C to activate p53 (right panel). Expression of the indicated proteins in the CM or whole-cell lysate was examined by western blot analysis.

(C) UACA inhibits Par-4 secretion. UACA expression was knocked down in mouse (p53+/+ or p53−/−) MEF and human (HEL) cells with distinct siRNA pools from two different sources, Dharmacon (D) and Santa Cruz Biotechnology (SC), and the CM, as well as the whole-cell lysates, was subjected to western blot analysis. C, control (scrambled) siRNA.

(D) P53 activation and UACA inhibition promotes secretion by a BFA-sensitive pathway. (101)/Val5 fibroblasts grown at 32°C were treated with BFA (1 μg/ml) or vehicle (v) for 3 hr (left panel). UACA expression was inhibited in MEFs (p53+/+) with Nutlin-3a plus PS-1145 (N+P; 10 μM each) (middle panel), or with an siRNA pool (from Dharmacon) (right panel), and then the cells were further placed in the presence of BFA or vehicle (v) for 3 hr. The CM, as well as the whole-cell lysates, was subjected to western blot analysis.

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.
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

**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 chromatim 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-κB activity-independent manner, we performed experiments in IKKβ−/− MEFs, which lack NF-κB 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-κB-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
whether wild-type p53 function in normal cells could be effectively 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 microenvironment 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 Jacks (Massachusetts Institute of Technology). Nutlin-3a was from Cayman Chemicals. Immortalized Bab/c fibroblasts (10), with no endogenous p53, and (10.1) Val5 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)Val5 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.

**ACKNOWLEDGMENTS**

This study was supported by KLCR and NIH/NCI grant CA060872 (to V.M.R.).

Received: July 31, 2013

Revised: November 25, 2013

Accepted: December 12, 2013

Published: January 9, 2014

**REFERENCES**


