Original Research

p21 induction plays a dual role in anti-cancer activity of ursolic acid

Xudong Zhang[1,2,*], Xinhua Song[1,*], Shutao Yin[1], Chong Zhao[1], Lihong Fan[3] and Hongbo Hu[1]

[1]Department of Nutrition and Health, College of Food Science and Nutritional Engineering, Beijing Key Laboratory of Functional Food from Plant Resources, China Agricultural University, Beijing 100083, China; [2]Development Center of Science and Technology, Ministry of Agriculture, Beijing 100193, China; [3]College of Veterinary Medicine, China Agricultural University, Beijing 100193, China
*The first two authors contributed equally to this work.
Corresponding author: Hongbo Hu. Email: hongbo@cau.edu.cn

Abstract

Previous studies have shown that induction of G1 arrest and apoptosis by ursolic acid is associated with up-regulation of cyclin-dependent kinase inhibitor (CDKI) protein p21 in multiple types of cancer cells. However, the functional role of p21 induction in G1 cell cycle arrest and apoptosis, and the mechanisms of p21 induction by ursolic acid have not been critically addressed. In the current study, we demonstrated that p21 played a mediator role in G1 cell cycle arrest by ursolic acid, whereas p21-mediated up-regulation of Mcl-1 compromised apoptotic effect of ursolic acid. These results suggest that p21 induction plays a dual role in the anti-cancer activity of ursolic acid in terms of cell cycle and apoptosis regulation. p21 induction by ursolic acid was attributed to p53 transcriptional activation. Moreover, we found that ursolic acid was able to inhibit murine double minute-2 protein (MDM2) and T-LAK cell-originated protein kinase (TOPK), the two negative regulator of p53, which in turn contributed to ursolic acid-induced p53 activation. Our findings provided novel insights into understanding of the mechanisms involved in cell cycle arrest and apoptosis induction in response to ursolic acid exposure.

Keywords: Ursolic acid, cell cycle, apoptosis, p21, p53, MDM2, TOPK, Mcl-1

***Experimental Biology and Medicine* 2016; 241: 501–508. DOI: 10.1177/1535370215616195**

Introduction

p21, also known as cyclin-dependent kinase 1, plays an important role in a number of cellular processes including cell cycle regulation, apoptosis, and autophagy.[1–3] As a cyclin-dependent kinase, p21 can directly bind to and inhibit the activity of cyclin-CDK2, -CDK1, and -CDK4/6 complexes, leading to cycle arrest.[4] In addition, p21 can also regulate cell proliferation through interacting with proliferating cell nuclear antigen (PCNA), a DNA polymerase accessory factor.[5] Apart from its role in cell cycle regulation, p21 is also involved in the regulation of apoptosis.[2,6] Several studies have shown that p21 induction functions as pro-apoptotic signaling to facilitate apoptosis induction in response to certain apoptotic stimuli.[7,8] In contrast, several other studies argue that p21 induction may function as pro-survival signaling and antagonize apoptosis induction.[9,10]

Ursolic acid (UA) is a pentacyclic triterpenoid found in apple, rosemary, and holy basil. It has been shown that UA is effective against various types of cancer both *in vitro* and *in vivo*.[11,12] The induction of G1 cell cycle arrest and apoptosis by UA has been suggested to contribute to its anti-cancer activities.[13–16] Previous studies have shown that induction of G1 arrest and apoptosis by UA is associated with up-regulation of cyclin-dependent kinase inhibitor (CDKI) protein p21Cip1 in multiple types of cancer cells.[13–16] However, whether p21 induction plays a mediator role in G1 arrest and apoptosis in response to UA exposure has not been addressed. In the current study, we demonstrated for the first time that p21 induction plays a dual role in the anti-cancer activity in terms of cell cycle and apoptosis regulation. Our findings provide evidence for a better understanding of mechanisms underlying cell cycle arrest and apoptosis induction by UA.

Materials and methods

Chemicals and reagents

UA was purchased from Sigma-Aldrich (St. Louis, MO). Antibodies specific for p21, p53, phospho-p53, myeloid cell leukemia-1 (Mcl-1), survivin, Akt, phospho-Akt, TOPK, phospho-TOPK, cleaved poly (ADP-ribose) polymerase (PARP; p89), and β-actin were purchased from Cell Signaling Technology (Beverly, MA). Antibody for cellular Fas-associated death domain-like IL-1 beta-converting

enzyme inhibitory protein (c-FLIP) was purchased from Bioworld Technology (Minneapolis, MN). Antibody for MDM2 was purchased from Abcam (Cambridge, UK).

Cell culture and treatments

MCF-7 (Michigan Cancer Foundation-7) breast cancer cells were obtained from the American Type Culture Collection. Human colon cancer cells HCT-116 p53 (+/+) and p53 (−/−) colon cancer cells were kindly provided by Dr Feng Zhu (Huazhong University of Science and Technology, Wuhan, China). The cells were grown in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum without antibiotics. At 24–48 h after plating when cells were 50–60% confluence, the medium was changed before starting the treatment with UA and/or other agents.

Cell cycle analysis

Cell cycle distribution was measured by flow cytometry analysis of DNA content following staining with propidium iodide (PI). Briefly, the cells were exposed to various concentrations of UA for the time indicated. Seventy-five percent ethanol was used to fix the cells at −20°C overnight. After fixation, the cells were washed with phosphate-buffered saline (PBS) and were resuspended in PBS containing RNAse A (1 mg/mL), and incubated at 37°C for 1 h. The fixed cells were labeled with PI (10 μg/mL) at room temperature in dark. After the labeling, the DNA content was examined by the FACS Calibur flow cytometry using CellQuest Software (Becton Dickinson, Franklin Lakes, NJ).

Apoptosis evaluation

Apoptosis was assessed by two methods. The first was sub-G1 analysis by flow cytometry. The second method was Western blotting analysis of cleaved PARP.

Western blot analysis

The cell lysate was prepared in ice-cold radioimmunoprecipitation assay buffer. Cell lysates were resolved by electrophoresis and transferred to a nitrocellulose membrane. The blot was then probed with primary antibody followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies. The signal was visualized by enhanced chemiluminescence (Pierce, Rockford, IL) and recorded on an X-ray film (Eastman Kodak Company, Rochester, NY).

RNA interference

siRNAs targeting p21 and non-targeting siRNA were purchased from Cell Signaling Technology. siRNAs targeting p53, TOPK, MDM2, and non-targeting siRNA were purchased from Life Technologies. siRNAs targeting Akt and non-targeting siRNA were purchased Santa Cruz Biotechnology. The cells were transfected with 50 or 5 nmol/L of specific or non-targeting siRNA using siPORT^{TM} NeoFX^{TM} Transfection Agent (Ambion, Austin, TX) for 24 h and then were used for subsequent experiments. The knockdown efficiency was validated by Western blotting analysis of the targeting gene expression in three independent experiments.

Statistical analysis

Data are presented as mean ± SD. These data were analyzed with the ANOVA with appropriate *post hoc* comparison among means. $P < 0.05$ was considered statistically significant.

Results

p21 induction makes a major contribution to UA-induced G1 cell cycle arrest in MCF-7 breast cancer cells

To determine the inhibitory effect of UA on cell growth in MCF-7 breast cancer cells, we measured the changes of cell cycle distribution in response to various concentrations of UA exposure by flow cytometry following staining with PI. As shown in Figure 1(a), treatment with UA in the concentration range of 10–17.5 μmol/L resulted in significant increase of cell number in G1 phase, whereas the number of cells in G1 phase gradually decreased starting at 20 μmol/L with onset of apoptosis (Figure 1b). To investigate the mechanisms of G1 cell cycle arrest by UA, we examined changes of p21, a key regulator of cell cycle, in response to UA by Western blotting. As shown in Figure 1(c), UA caused a concentration-dependent up-regulation of p21 that is well correlated with the changes of cell cycle distribution (Figure 1a). To critically determine the role of p21 induction in UA-induced G1 cell cycle arrest in MCF-7 breast cancer cells, we tested influences of p21 knockdown on the changes of cell cycle distribution by UA. As shown in Figure 1(d), under the condition that p21 was silenced by its siRNA, UA-induced G1 cell cycle arrest was nearly abolished, suggesting p21 functions as key mediator in cell cycle arrest induced by UA in MCF-7 breast cancer cells.

p21 induction counteracts apoptotic effect of UA in MCF-7 breast cancer cells

Having found the role of p21 induction in UA-induced G1 cell cycle arrest, we next asked whether p21 induction also played a role in apoptosis induction by UA in MCF-7 breast cancer cells. We measured PARP cleavage and apoptosis induction under the condition that p21 was inactivated by RNAi approach using Western blotting and sub-G1 analysis, respectively. As shown in Figure 2(a), when p21 was inhibited, treatment with UA for 36 h caused a significantly increased PARP cleavage. Consistent with the increase of PARP cleavage, knockdown of p21 led to a significant increase of apoptosis induction compared with the found in UA/con-si (Figure 2b). These results suggest that p21 induction functions as pro-survival signal counteracting apoptotic effect of UA. To decipher the mechanisms underlying the pro-survival function of p21 induction in response to UA, we investigated the role of anti-apoptotic Bcl-2 family protein Mcl-1 in this event since up-regulation of Mcl-1 by p21 has been reported in hyperoxia-induced cell death in H1299 human lung adenocarcinoma cells.[17] As shown in Figure 2(c), UA induced a concentration-dependent up-regulation of Mcl-1, which was well

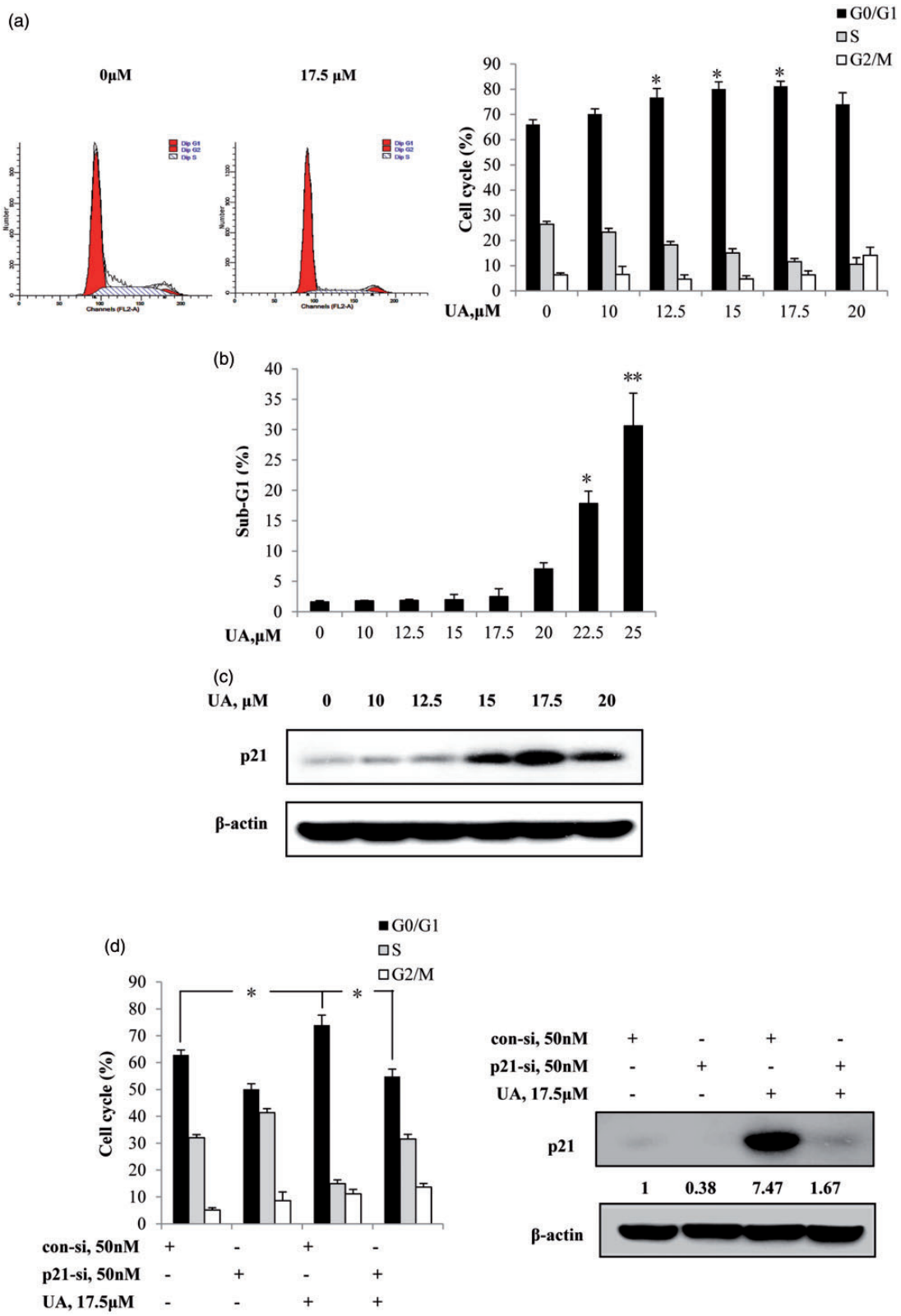

Figure 1 p21 induction makes a major contribution to ursolic acid-induced G0/G1 cell cycle arrest in MCF-7 breast cancer cells. (a) Ursolic acid-induced G0/G1 phase cell cycle arrest in MCF-7 cells. The cells were treated with various concentrations of ursolic acid for 24 h and cell cycle distribution was measured by flow cytometry following staining with propidium iodide. (b) Ursolic acid-induced apoptosis in MCF-7 cells. The cells were treated with various concentrations of ursolic acid for 24 h and apoptosis was analyzed by sub-G1 analysis. (c) Ursolic acid induced a concentration dependent up-regulation of p21 in MCF-7 cells. The cells were treated with various concentrations of ursolic acid for 24 h and p21 expression was measured by Western blotting. (d) p21 silenced nearly abolished G0/G1 phase cell cycle arrest induced by ursolic acid in MCF-7 cells. The cells were transfected with p21 or non-targeting siRNAs using siPORT^{TM} NeoFX^{TM} Transfection Agent. After 24 h transfection, the cells were treated with 17.5 μmol/L UA for 24 h. Cell cycle distribution was measured by flow cytometry (left) and knockdown efficiency was validated by Western blotting (right) ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$). (A color version of this figure is available in the online journal.)

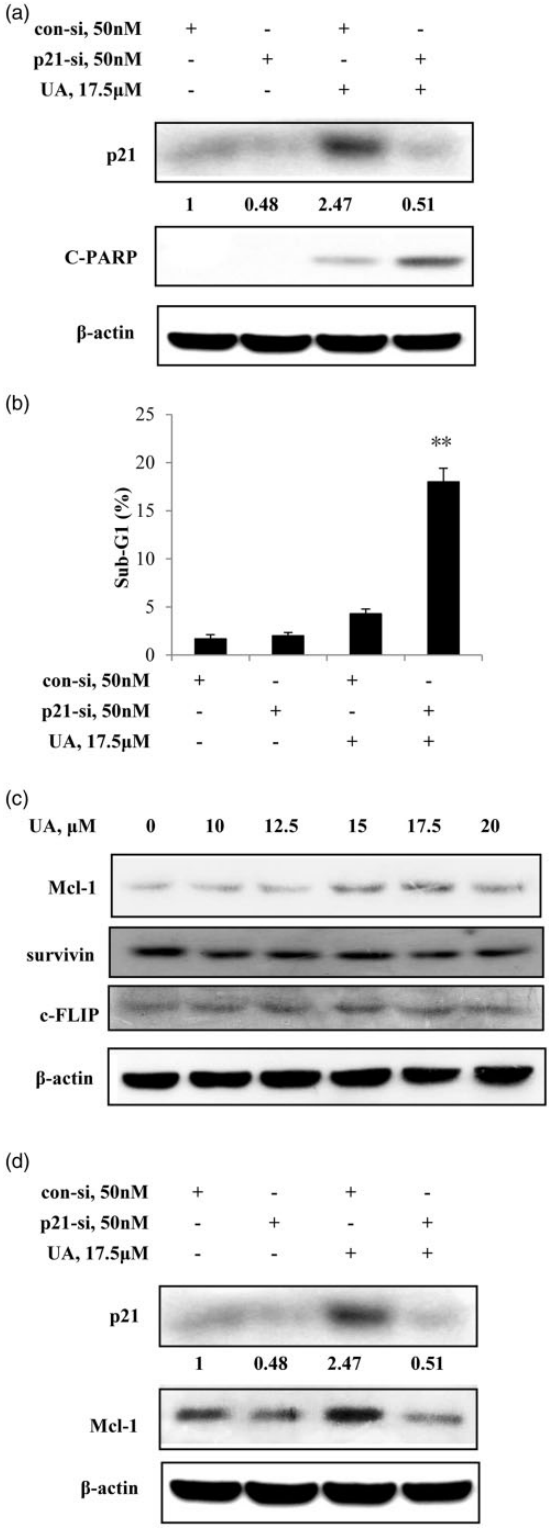

Figure 2 p21 induction counteracts apoptotic effect of ursolic acid in MCF-7 breast cancer cells. (a) p21 inactivation by RNAi increased PARP cleavage induced by ursolic acid in MCF-7 cells measured by Western blotting. (b) p21 inactivation by RNAi increased apoptosis induced by ursolic acid in MCF-7 cells assessed by flow cytometry. (c) Ursolic acid-induced dose-dependent up-regulation of anti-apoptotic protein Mcl-1 in MCF-7 cells analyzed by Western blotting. (d) p21 inactivation by RNAi abolished Mcl-1 induction by ursolic acid analyzed by Western blotting ($n = 3$, $^{**}P < 0.01$)

correlated with p21 induction (Figure 1c). We also measured the effects of UA on expression of survivin and c-FLIP, the two anti-apoptotic proteins. Unlike Mcl-1 induction, no obvious changes of these two proteins were detected in the experimental condition (Figure 2c). Furthermore, when p21 was silenced by its siRNA, Mcl-1 induction by UA was dramatically reduced (Figure 2d). Together, these results suggest that p21 induction compromises apoptotic effect of UA through up-regulation of anti-apoptotic protein Mcl-1 in MCF-7 breast cancer cells.

UA activates p21 through regulation of MDM2/TOPK-p53 axis

p21 is a well-known transcriptional target of p53.[18] To investigate the mechanisms of p21 induction by UA in MCF-7 breast cancer cells, the role of p53 was investigated. We first analyzed changes of p53 in response to UA by Western blotting. As shown in Figure 3(a), treatment with UA induced a concentration-dependent up-regulation of p53, which is generally consistent with p21 induction. To determine the role of p53 in UA-induced p21 in MCF-7 cells, we examined the effects of p53 knockdown on p21 expression. As shown in Figure 3(b), when p53 was inhibited by its siRNA, p21 induction by UA was nearly abolished. Consistent with the changes of p21 expression, p53 inactivation led to a significantly decreased G1 cell cycle arrest in response to UA (Figure 3c). These results support that p53 activation played a role in UA-induced p21 up-regulation in MCF-7 cells. We further investigated the mechanisms of p53 activation by UA. The expression of MDM2, a major negative regulator of p53,[19] was assessed by Western blotting and results are shown in Figure 3(d). Treatment with UA caused a concentration-dependent down-regulation of MDM2 expression. It has been shown that Akt can positively regulate MDM2.[20] We next asked whether inhibition of MDM2 by UA was due to suppression of Akt. The phosphorylation status of Akt was examined by Western blotting. As shown in Figure 3(d), UA induced a concentration-dependent inhibition of Akt phosphorylation which is paralleled with down-regulation of MDM2. Furthermore, inactivation of Akt by RNAi indeed resulted in down-regulation of MDM2 and activation of p53-p21 axis. In addition, we also measured effects of UA on TOPK because it has been shown to regulate p53 activity in certain cell types including MCF-7 breast cancer cells.[21] As shown in Figure 3(e). UA inhibited TOPK phosphorylation concentration-dependently. Moreover, inhibition of TOPK indeed triggered activation of p53–p21 pathway in MCF-7 cells. To determine the relationship between MDM2 and TOPK inhibition induced by UA, we tested the effect of MDM2 inhibition on TOPK/p-TOPK. As shown in Figure 3(f), no obvious changes of TOPK/p-TOPK were observed under the condition of MDM2 silencing, indicating suppression of TOPK by UA is not associated with MDM2 inhibition in the present condition. Taken together, these results suggest that p21 induction is attributed to activation of MDM2/TOPK-p53 axis in MCF-7 breast cancer cells.

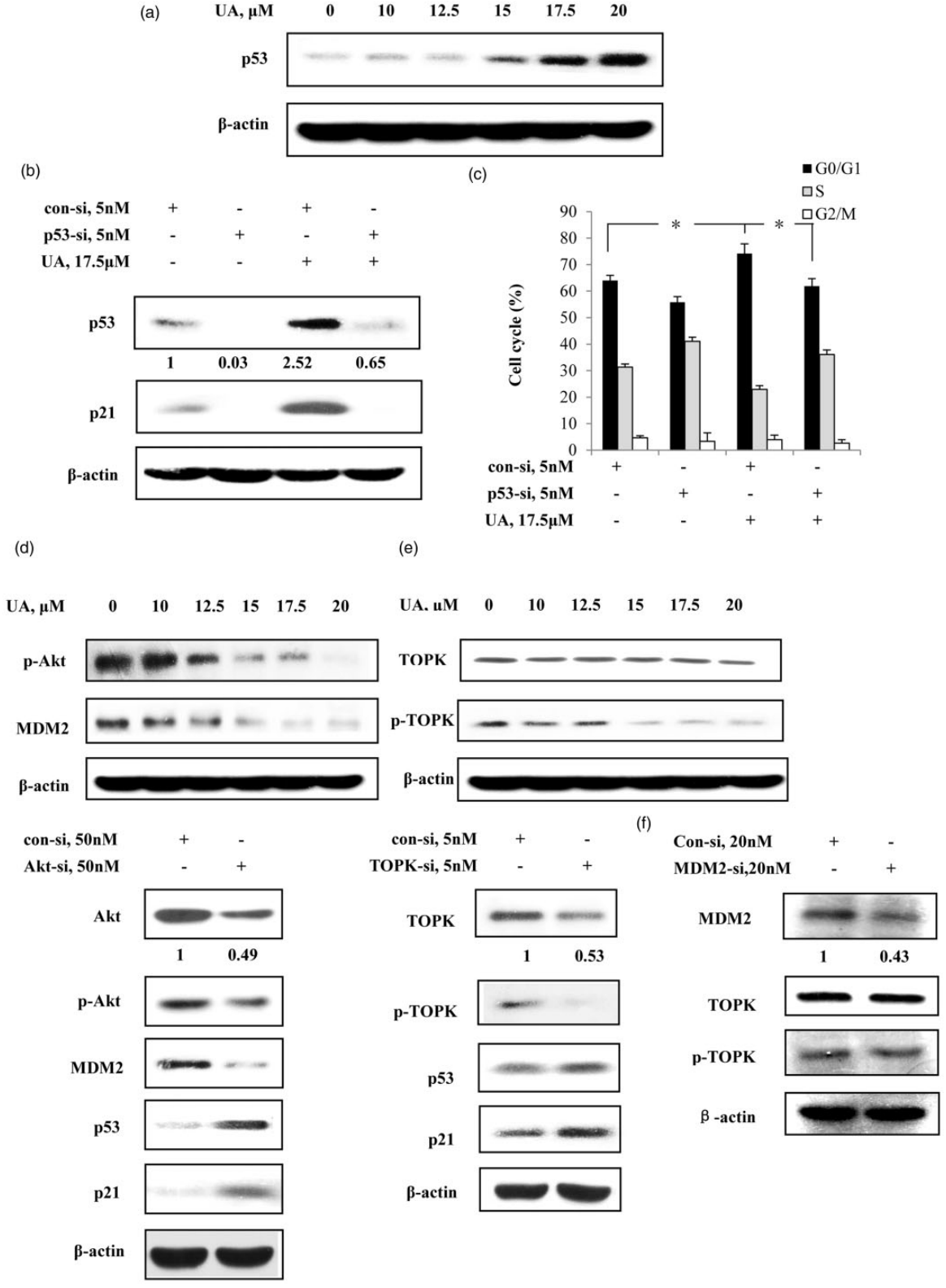

Figure 3 Ursolic acid activates p21 through regulation of MDM2/TOPK-p53 axis. (a) Ursolic acid induced a concentration-dependent up-regulation of p53 in MCF-7 cells. The cells were treated with various concentrations of ursolic acid for 24 h. p53 expression was measured by Western blotting. (b) p53 inactivation by RNAi decreased p21 induction by ursolic acid in MCF-7 cells measured by Western blotting. (c) p53 inactivation by RNAi decreased G0/G1 phase cell cycle arrest in response to ursolic acid in MCF-7 cells assessed by flow cytometry. (d) Ursolic acid activates p21 through regulation of MDM2-p53 axis. Ursolic acid inhibited phosphorylation of Akt and expression of MDM2 in MCF-7 cells (upper panel). The cells were treated with various concentrations of ursolic acid for 24 h, and phosphor-Akt and MDM2 expression were analyzed by Western blotting. Effects of Akt inhibition on expression of MDM2, p53, and p21 in MCF-7 cells (lower panel). The cells were transfected Akt or non-targeting siRNAs for 24 h, and Akt, p-Akt, MDM2, p53, and p21 expression were measured by Western blotting. (e) Ursolic acid activates p21 through regulation of TOPK-p53 axis. Ursolic acid inhibited phosphorylation of TOPK (upper panel). The cells were treated with various concentrations of ursolic acid for 24 h, and total and phosphor-TOPK were analyzed by Western blotting. Effects of TOPK inhibition by RNAi on p53 and p21 expression analyzed by Western blotting (lower panel). (f) Influences of MDM2 inhibition by RNAi on TOPK/p-TOPK assessed by Western blotting

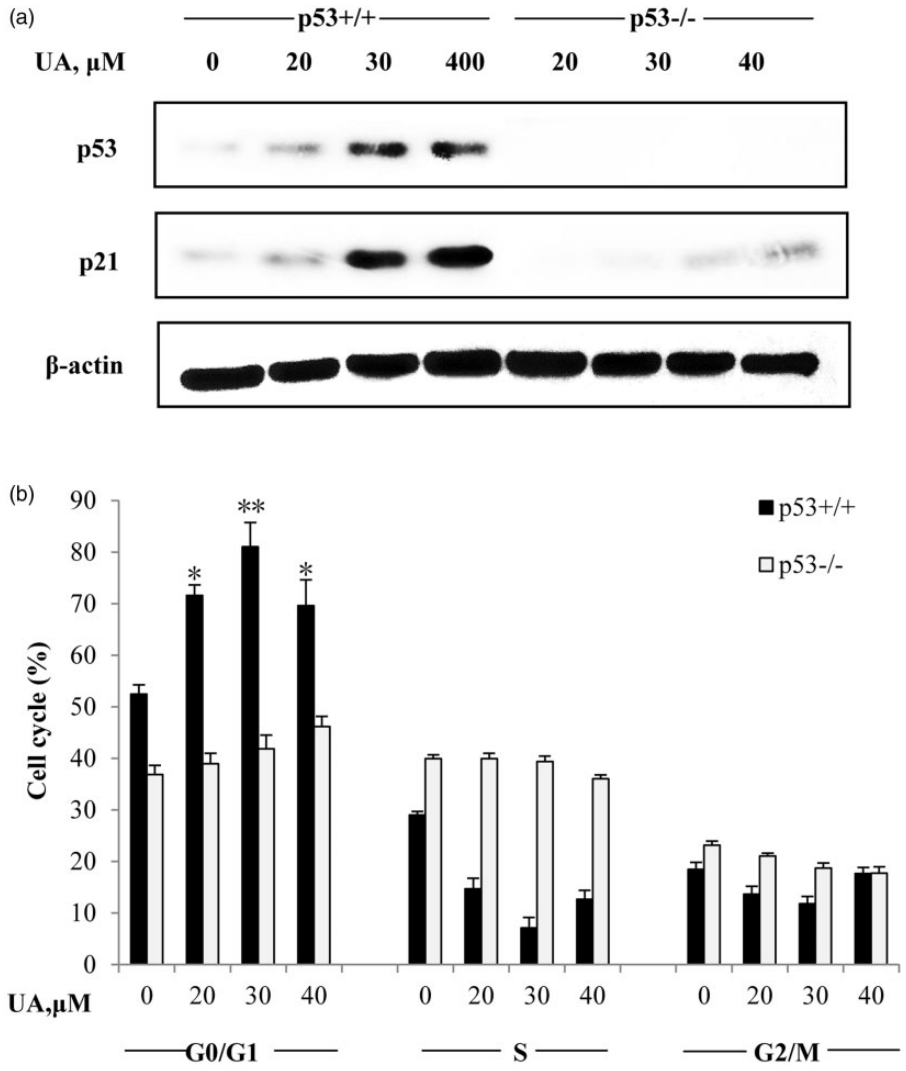

Figure 4 Ursolic acid induces G1 cell cycle arrest via p53-p21 pathway in HCT-116 colon cancer cells. (a) Ursolic acid induced a p53 dependent up-regulation of p21 in HCT-116 cells. The HCT-116 p53 (+/+) and p53 (−/−) cells were treated with various concentrations of ursolic acid for 24 h. p21 and p53 expression were measured by Western blotting. (b) Ursolic acid induces p53-p21-dependent G0/G1 phase cell cycle arrest in HCT-116 cells. The HCT-116 p53 (+/+) and p53 (−/−) cells were treated with various concentrations of ursolic acid for 24 h. Cell cycle distribution was measured by flow cytometry

UA induces G1 cell cycle arrest via p53–p21 pathway in HCT-116 colon cancer cells

To determine the general application of ursolic-induced p53/p21-mediated G1 cell cycle arrest, we tested the functional role of p53/p21 in cell cycle arrest by UA in HCT-116 p53 (+/+) and p53 (−/−) colon cancer cells. We first analyzed the changes of p21 expression in response to UA exposure in these cells. As shown in Figure 4(a), treatment with UA induced a concentration-dependent up-regulation of p21 in both the p53 wild-type and knockout HCT-116 cells. However, the magnitude of p21 induction in the cells with wild-type p53 is significant stronger than that found in the cells without p53. Consistent with the changes of p21 expression, UA caused a concentration-dependent up-regulation of p53 in p53 wild-type HCT-116 cells. In line with the activation of p53–p21 pathway, UA induced a significant G1 cell cycle arrest in p53 well-type HCT-116 cells, whereas only a slight G1 cell cycle arrest was detected in p53 knockout HCT-116 cells (Figure 4b). Together, these results suggest that UA induces p53–p21-dependent G1 cell cycle arrest in HCT-116 colon cancer cells which is consistent with that found in MCF-7 breast cancer cells.

Discussion

In the current, we investigated the functional role of p21 induction in cell cycle arrest and apoptosis induction by UA. Our results show that p21 induction plays a paradoxical role in the anti-cancer activity of UA. On the one hand, p21 induction made a major contribution to UA-induced G1 cell cycle arrest. On the other hand, p21 induction compromised UA-apoptotic effect through up-regulation of Bcl-2 anti-apoptotic protein Mcl-1. Our findings provide a novel insight into understanding of the mechanisms of anti-cancer property of UA.

The implication of p21 in apoptosis regulation still remains controversial. Induction of p21 functions either as pro-apoptotic or pro-survival signaling.[2] Our results show that inhibition of p21 induction by RNAi approach led to enhanced PARP cleavage and apoptosis induction by UA, suggesting p21 induction conferring cancer cells resistant to ursolic acid-induced apoptosis, which could compromise anti-cancer activity of UA in terms of apoptosis induction. It has been shown that p21 can exert anti-apoptotic effect through multiple mechanisms including up-regulation of Bcl-2 anti-apoptotic proteins.[17] Our data show that p21 induction was well correlated with up-regulation of anti-apoptotic Bcl-2 family protein Mcl-1. Moreover, inactivation of p21 by RNAi approach led to a significant reduction of Mcl-1 expression induced by UA. These results therefore indicate that Mcl-1 is a downstream target of p21 induction to mediate anti-apoptotic signaling against UA-induced apoptotic effect.

p21 can be activated by p53-dependent or -independent pathway.[2] To decipher the mechanisms of p21 induction by UA, we examined the effects of p53 knockdown or p53 knockout on p21 expression in MCF-7 and HCT cells, respectively. The results show that knockdown of p53 led to a significant reduced p21 induction by UA in MCF-7 cells. Consistent with the finding in MCF-7 cells, a significant reduced p21 was also observed in p53 knockout HCT-116 cells. In line with the effect of p53 on p21 induction, G1 cell cycle arrest induced by UA was nearly abolished when p53 was inactivated by either knockdown or knockout approaches. These results clearly support involvement of p53-dependent pathway in UA-induced p21 induction.

MDM2 functions as an endogenous inhibitor of p53 to be involved in metabolic regulation of p53 activity through promotion of p53 degradation or p53 nuclear exportation. T-LAK cell-originated protein kinase (TOPK/PBK), a member of serine-threonine mitogen-activated protein kinase kinase family, is found to inhibit p53 activation through directly physical interaction.[21] In the present study, we found that both MDM2 and TOPK were highly expressed in MCF-7 breast cancer cells, which are consistent with previous literatures.[22–25] Moreover, our results showed that UA induced a dose-dependent p53 activation which was paralleled with inhibition of MDM2 and TOPK. Suppression of either MDM2 or TOPK indeed caused activation of p53/p21 axis in MCF-7 breast cancer cells. Regarding the relationship between MDM2 and TOPK inhibition in our current system, our data demonstrated that inhibition of MDM2 did not affect TOPK/p-TOPK, suggesting they functioned separately rather than connected. These data for the first time demonstrated MDM2 and TOPK as the targets for UA to trigger p53 activation.

In summary, p21 induction plays a pivotal role in UA-induced G1 cell cycle arrest. However, p21 activation by UA counteracts its apoptotic effect through up-regulation of Mcl-1. p21 is activated through p53-dependent pathway, whereas p53 activation is associated inhibition of MDM2 and TOPK, two negative regulator of p53. Our findings provide evidence for a better understanding of mechanisms underlying cell cycle arrest and apoptosis induction by UA.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; XZ, XS, SY, and CZ conducted the experiments, LF participated in design of the study and helped to draft the manuscript. HH conceived of the study, designed the experiments, and drafted and edited the manuscript.

ACKNOWLEDGMENTS

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Chinese Universities Scientific Fund and by grant from the Ministry of Science and Technology of China (2012BAD33B09).

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 1993;**75**:805–16
2. Gartel AL, Tyner AL. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. *Mol Cancer Ther* 2002;**1**:639–49
3. Fujiwara K, Daido S, Yamamoto A, Kobayashi R, Yokoyama T, Aoki H, Iwado E, Shinojima N, Kondo Y, Kondo S. Pivotal role of the cyclin-dependent kinase inhibitor p21WAF1/CIP1 in apoptosis and autophagy. *J Biol Chem* 2008;**283**:388–97
4. Harper JW, Elledge SJ, Keyomarsi K, Dynlacht B, Tsai L, Zhang P, Dobtowolski S, Bai C, Connell-Crowley L, Swindell E, Fox MP, Wei N. Inhibition of cyclin dependent kinases by p21. *Mol Biol Cell* 1995;**6**:387–400
5. Flores-Rozas H, Kelman Z, Dean FB, Pan ZQ, Harper JW, Elledge SJ, O'Donnell M, Hurwitz J. Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase delta holoenzyme. *Proc Natl Acad Sci USA* 1994;**91**:8655–59
6. Coqueret O. New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? *Trends Cell Biol* 2003;**13**:65–70
7. Kondo S, Barna BP, Kondo Y, Tanaka Y, Casey G, Liu J, Morimura T, Kaakaji R, Peterson JW, Werbel B, Barnett GH. WAF1/CIP1 increases the susceptibility of p53 non-functional malignant glioma cells to cisplatin-induced apoptosis. *Oncogene* 1996;**13**:1279–85
8. Lincet H, Poulain L, Remy JS, Deslandes E, Duigou F, Gauduchon P, Staedel C. The p21(cip1/waf1) cyclin-dependent kinase inhibitor enhances the cytotoxic effect of cisplatin in human ovarian carcinoma cells. *Cancer Lett* 2000;**161**:17–26
9. Helt CE, Rancourt RC, Staversky RJ, O'Reilly MA. p53-dependent induction of p21(Cip1/WAF1/Sdi1) protects against oxygen-induced toxicity. *Toxicol Sci* 2001;**63**:214–22
10. Gorospe M, Cirielli C, Wang X, Seth P, Capogrossi M, Holbrook N. P21 (WAF1/CIP1) protects against p53-mediated apoptosis of human melanoma cells. *Oncogene* 1997;**14**: 929–35
11. Bishayee A, Ahmed S, Brankov N, Perloff M. Triterpenoids as potential agents for the chemoprevention and therapy of breast cancer. *Front Biosci* 2011;**16**:980–96
12. Kowalczyk MC, Walaszek Z, Kowalczyk P, Kinjo T, Hanausek M, Slaga TJ. Differential effects of several phytochemicals and their derivatives on murine keratinocytes *in vitro* and *in vivo*: implications for skin cancer prevention. *Carcinogenesis* 2009;**30**:1008–15

13. Kim DK, Baek JH, Kang CM, Yoo MA, Sung JW, Chung HY, Kim ND, Choi YH, Lee SH, Kim KW. Apoptotic activity of ursolic acid may correlate with the inhibition of initiation of DNA replication. *Int J Cancer* 2000;**87**:629–36
14. Hsu YL, Kuo PL, Lin CC. Proliferative inhibition, cell-cycle dysregulation, and induction of apoptosis by ursolic acid in human non-small cell lung cancer A549 cells. *Life Sci* 2004;**75**:2303–16
15. Nam H, Kim MM. Ursolic acid induces apoptosis of SW480 cells via p53 activation. *Food Chem Toxicol* 2013;**62**:579–83
16. Prasad S, Yadav VR, Sung B, Reuter S, Kannappan R, Deorukhkar A, Diagaradjane P, Wei C, Baladandayuthapani V, Krishnan S, Guha S, Aggarwal BB. Ursolic acid inhibits growth and metastasis of human colorectal cancer in an orthotopic nude mouse model by targeting multiple cell signaling pathways: chemosensitization with capecitabine. *Clin Cancer Res* 2012;**18**:4942–53
17. Wu YC, O'Reilly MA. Bcl-X(L) is the primary mediator of p21 protection against hyperoxia-induced cell death. *Exp Lung Res* 2011;**37**:82–91
18. Chen X, Bargonetti J, Prives C. p53, through p21 (WAF1/CIP1), induces cyclin D1 synthesis. *Cancer Res* 1995;**55**:4257–63
19. Momand J, Zambetti GP, Olson DC, George D, Levine AJ. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* 1992;**69**:1237–45
20. Feng J, Tamaskovic R, Yang Z, Brazil DP, Merlo A, Hess D, Hemmings BA. Stabilization of Mdm2 via decreased ubiquitination is mediated by protein kinase B/Akt-dependent phosphorylation. *J Biol Chem* 2004;**279**:35510–7
21. Hu F, Gartenhaus RB, Eichberg D, Liu Z, Fang HB, Rapoport AP. PBK/TOPK interacts with the DBD domain of tumor suppressor p53 and modulates expression of transcriptional targets including p21. *Oncogene* 2010;**29**:5464–74
22. Agrawal A1, Yang J, Murphy RF, Agrawal DK. Regulation of the p14ARF-Mdm2-p53 pathway: an overview in breast cancer. *Exp Mol Pathol* 2006;**81**:115–22
23. Xia L, Paik A, Li JJ. p53 activation in chronic radiation-treated breast cancer cells: regulation of MDM2/p14ARF. *Cancer Res* 2004;**64**:221–8
24. Park JH, Lin ML, Nishidate T, Nakamura Y, Katagiri T. PDZ-binding kinase/T-LAK cell-originated protein kinase, a putative cancer/testis antigen with an oncogenic activity in breast cancer. *Cancer Res* 2006;**66**:9186–95
25. Zhu F, Zykova TA, Kang BS, Wang Z, Ebeling MC, Abe Y, Ma WY, Bode AM, Dong Z. Bidirectional signals transduced by TOPK-ERK interaction increase tumorigenesis of HCT116 colorectal cancer cells. *Gastroenterology* 2007;**133**:219–31

(Received May 14, 2015, Accepted October 15, 2015)