

## Original Article

# p53 is essential for embelin-induced apoptosis in p53-wildtype prostate cancer cells

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**Abstract:** *Background:* Embelin is a natural product that has shown anti-tumor activities in prostate cancer, however whether P53 is a possible target remains unknown. Here, the effects of embelin on prostate cancer cells with wildtype p53 were determined. *Materials and methods:* Cell viability was detected by MTT assay and apoptosis was detected by flow cytometry. Caspase 3 activity was detected by fluorescence assay, and proteins were examined by Western blot. Luciferase-based p53 reporter assay was performed to assess p53 activity. *Results:* Embelin suppressed cell viability and enhanced Annexin V positive and sub-G1 populations. Further, embelin promoted caspase 3 activation and PARP cleavage and p53/P21 expression, and inhibited androgen receptor (AR) and XIAP. Knocking down p53 made cells less sensitive to drug-induced apoptosis. Conversely, re-introducing p53 re-sensitized cells to the drug. *Conclusion:* Embelin efficiently inhibits proliferation and induces apoptosis in p53 wild type prostate cancer cells. Importantly, p53 is required for embelin-induced apoptosis. These novel findings suggest embelin as a potential natural product for prostate cancer with wild type p53.

**Keywords:** Embelin, apoptosis, p53, prostate cancer

## Introduction

Prostate cancer is one of the most common malignancies and a leading cause of cancer-related death worldwide in men [1]. Because chemotherapy usually yields a poor outcome with severe side effects, there is an intensive need for the development of agents with higher effectiveness and lower unwanted toxicity. Since plants have been widely used as an abundant resource for traditional medicine for the treatment of various diseases, many natural products have been developed and tested for their cancer-killing potential.

Naturally occurring compounds have been found to inhibit multiple malignant features in prostate cancer, such as growth, survival, angiogenesis, invasion and metastasis [2]. For example, curcumin is reported to effectively suppress prostate cancer proliferation and survival, regardless of hormone status [3]. In addition, genistein, a key soy isoflavone from soybean, has shown great inhibition on tumor growth, angiogenesis and metastasis in pros-

tate cancer models [4]. Many of these natural products have proven potential activities against human malignancies demonstrated *in vitro* and *in vivo*.

Embelin is an active natural product originally isolated from the Japanese Ardisia Herb (*Herba Ardisiae Japonicae*) and considered as a male contraceptive [5]. Recent studies have shown that embelin exhibits considerable anti-tumor activities in various preclinical models such as breast cancer [6, 7], gastric cancer [8] and lung cancer [9, 10]. A few studies have reported that embelin augments apoptosis and metastasis by activating varied pathways in breast cancer cells [6, 11, 12]. Other scientists find that embelin is able to reverse chemotherapy resistance and induce apoptosis via p38/JNK pathway in lung cancer cells [9, 10]. In prostate cancer, embelin is also recognized as a potential candidate for therapeutic interventions [13]. Embelin inhibits growth and induces apoptosis by impeding anti-apoptotic pathways in prostate cancer cells [14-16]. Moreover, pre-

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clinical studies have shown that embelin can enhance treatment effects of traditional therapies such as hormone therapy [17] and radiation therapy [18] for prostate cancer.

It is appreciated that many natural products are able to modulate multiple cellular processes, and apoptosis induction is perhaps one of the major phenomena. Apoptosis machinery can be regulated by proteins including oncoproteins and tumor suppressors. p53 is an essential tumor suppressor that plays a pleiotropic role in cancer controlling [19, 20]. A plethora of studies have revealed that p53 is a pivotal transcriptional factor that activates gene expression of various pro-apoptotic proteins such as BH-3 only proteins in Bcl-2 family [21-23]. Alternatively, p53 can also trigger apoptosis by repressing various anti-apoptotic gene products, for example, Bcl-2 [24], Mcl-1 [25] and XIAP [26], and subsequently, activate caspase cascades. Caspase 3 functions as a downstream effector of both extrinsic and intrinsic apoptosis pathways and is recognized as a typical marker of classic apoptosis. Caspase 3 further cleaves PARP that eventually induces DNA break and cell death [27].

Although a small group of cancer-related signaling molecules have been identified as targets for embelin, whether p53 is one of them remains unknown. In the current study, the anti-proliferative and pro-apoptotic effects of embelin on prostate cancer cells with wild type p53 were determined. Further, molecular pathways associated with p53 and apoptosis were examined. Finally, genetic modifications were pursued to explore the functional role of p53 in embelin-mediated apoptosis.

### Materials and methods

#### Reagents

Embelin was purchased from Sigma (St. Louis, MO, USA). The powder was reconstituted in dimethyl sulfoxide (DMSO) and stored at -70°C. All cell culture medium and supplemental reagents including trypsin and EDTA were purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Sijiqing Biological Engineering Materials (Hangzhou, China). Antibodies against poly(ADP-ribose) polymerase (PARP), cleaved PARP, androgen receptor (AR), p53, P21, XIAP and

Actin were from Santa Cruz Biotechnology (Santa Cruz, CA). All secondary antibodies were purchased from Boster Biological Technology (Wuhan, China). Protease inhibitors and other chemicals were from Sigma unless otherwise indicated.

#### Cell culture

Human p53 wildtype prostate cancer cell line LNCaP and 22Rv1 were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin, and incubated in a 5% CO<sub>2</sub> humidified incubator at 37°C. The cells were passaged once every 3~4 days. All experiments were conducted using cells in 20 passages.

#### MTT assay

To determine drug-induced cytotoxicity, the MTT assay was used. Briefly, cells were cultured in 96-well plates with a seeding density 8000/well and treated with embelin for 72~96 h. At the end of the experiment, MTT (1 mg/ml) was added to each well (100 µl/well), and the cells were incubated for 4 h at 37°C. After incubation, DMSO (100 µl/well) was added to dissolve the precipitate. The absorbance was read with a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 570 nm.

#### Cell growth curve

The overall proliferation of cells was addressed by growth curve analysis. Briefly, cells were seeded in a 24-well plate with equal density ( $2 \times 10^4$ /well), followed by the desired treatment. Every 24 h, attached cells in triplicate wells were harvested by trypsinization. Viable cells determined by trypan blue exclusion were counted at 24-h intervals over 4 days in a culture using a Coulter cell counter (Beckman). Cell death were characterized by trypan blue positive staining. Percentage of cell death was defined by the ratio of trypan blue positive cells and total cells.

#### Flow cytometry

Flow cytometry was used to analyze cell apoptosis through 1) Annexin V/PI staining and 2) sub-G1 analysis. Briefly, the cells were harvest-

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ed and washed twice with ice-cold PBS. For 1), apoptotic cells that present phosphatidylserine on their outside surface were captured by Annexin V-FITC agent (Roche, Indianapolis, IN). Annexin V-FITC (1  $\mu$ l) was added to 500  $\mu$ l of Annexin V-FITC binding buffer, and the cells were incubated at room temperature for 20 min. After washing the cells with 1 $\times$  binding buffer, 10  $\mu$ l propidium iodide (PI) was added to the binding buffer, and the cells were analyzed with a flow cytometer (FACS Calibur; BD Biosciences). The percentages of early apoptotic (Annexin V+PI-) and total apoptotic cells (Annexin V+) were estimated by the defined quadrants. For 2), the harvested cells were fixed in 70% ethanol at 4°C overnight for sub-G1 analysis. Cells were incubated with PI (50  $\mu$ g/ml) and RNase A (1  $\mu$ g/ml) for 30 min. Samples were analyzed by flow cytometry (FACS Calibur, BD Biosciences). sub-G1 population was calculated from hypodiploid DNA fluorescent in the cell cycle histogram (Refs). Data were analyzed using WinMDI 2.8 software (Purdue University Cytometry Laboratory).

### *Western blot analysis*

Whole cell lysates were extracted using the radioimmunoprecipitation assay (RIPA) lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.25% Sodium deoxycholate, 1 mM EDTA, and protease inhibitors] and quantified with Bradford assay following the manufacturer's instructions (Bio-Rad, Hercules, CA). After addition of the sample loading buffer, protein samples were electrophoresed using 8%-12% SDS-PAGE and subsequently transferred to nitrocellulose membranes (Bio-Rad). Each membrane was blocked at room temperature for 30 min and probed with desired primary antibody at 4°C overnight. The membrane was then washed three times using PBST (PBS and 0.1% Tween 20) and incubated in the appropriate HRP-conjugated secondary antibody at room temperature for 2 h. The immunoreactive protein was visualized using the chemiluminescent reagent ECL (Pierce, Rockford, IL).

### *Caspase-3 activity assay*

Caspase-3 activation was determined following the instructions of a fluorescence Caspase-3 detection kit from Cayman Chemical (Ann Arbor, MI). Briefly, cells were dissolved in a lysis buffer (Cayman Chemical) and whole cell lysates (25

$\mu$ g) were incubated with fluorogenic substrate N-Ac-DEVD-N'-MC-R110 in a reaction buffer (containing 5 mM DTT) at 37°C for 2 h. Proteolytic release of the highly fluorescent product was monitored at  $\lambda_{ex}$  =485 nm and  $\lambda_{em}$  =535 nm using a microplate reader (Molecular Device, Sunnyvale, CA). Fold increase of the fluorescence signal was calculated by dividing the normalized signal activity for the treated sample with that of the untreated control.

### *Transfection*

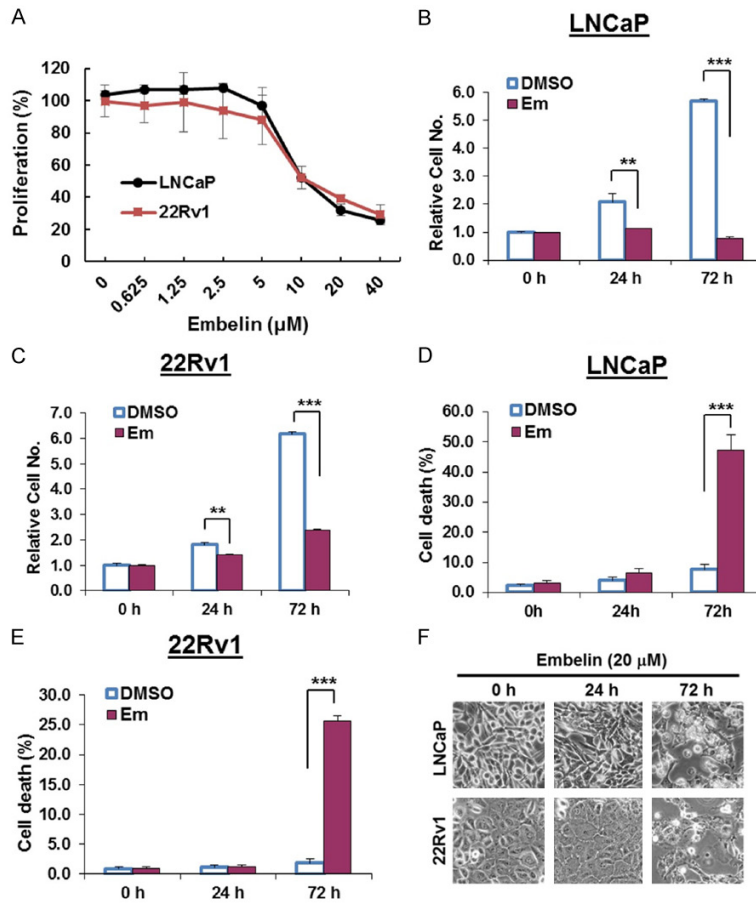
p53 shRNA (RC232563) and wildtype p53 cDNA (TR320558) constructs were purchased from Origene (Rockville, MD). Plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Transfected cells were maintained in kanamycin (25  $\mu$ g/ml) for future experiments.

### *p53 luciferase reporter assay*

To establish cells with an integrated p53 reporter plasmid, the p53luciferase reporter vector (Affymetrix, Santa Clara, CA), which codes for luciferase under the control of a p53 responsive element was introduced into cells using Lipofectamine 2000. pTL-Luc was used as the vector control. After transfection for 48 h, cells were exposed to embelin for 24 h and cell lysates were harvested by adding lysis buffer (10 mM Tris-HCl, 25 mM NaCl, 0.2 mM EDTA, 1 mM DTT and 0.1% Triton X-100) at 4°C for 20 min. The lysates were centrifuged and supernatant was incubated with Bright-Glo luciferase kit (Promega, Madison, WI) and  $\beta$ -galactosidase enzyme assay kit (Promega) respectively, according to the manufacturer's instructions. Samples were finally transferred to a luminometer plate and luciferase activity was measured by a microplate reader (Molecular Device). Fold increase of luciferase was calculated by dividing normalized luciferase activity of treated sample by that of the untreated control.

### *Statistical analysis*

All data were expressed as the mean  $\pm$  SD. The data were analyzed using two-tailed Student's *t*-test. GraphPad Prism 5.0 (San Diego, CA) was employed to perform the analysis. A threshold of  $P < 0.05$  was defined as statistically significant.



**Figure 1.** Embelin induces growth inhibition and cell death in p53-wildtype prostate cancer cells. (A) Cells were seeded into 96-well plates and treated with embelin at serially diluted concentrations for 4 days. Cell proliferation was determined by MTT assay. Bars, SD (n=3). (B-E) LNCaP (B, D) and 22Rv1 (C, E) cells were treated with DMSO or embelin at 20 μM for 24 and 72 h, respectively. Cells were trypsinized and mixed with trypan blue. Living cells that were exclusive from trypan blue were counted by hemocytometer. Relative cell number was calculated by dividing the counted number at each time point by the initial seeding number (B, C). Dead cells were featured as trypan blue positive. Cell death (%) was calculated by the ratio of dead cells with total cells (D, E). Columns, mean; bars, SD (n=3). \*\*\*P<0.001. (F) Cell morphology after embelin treatment. Original magnification, 200x.

**Results**

*Embelin induces growth inhibition and cell death in p53-wildtype prostate cancer cells*

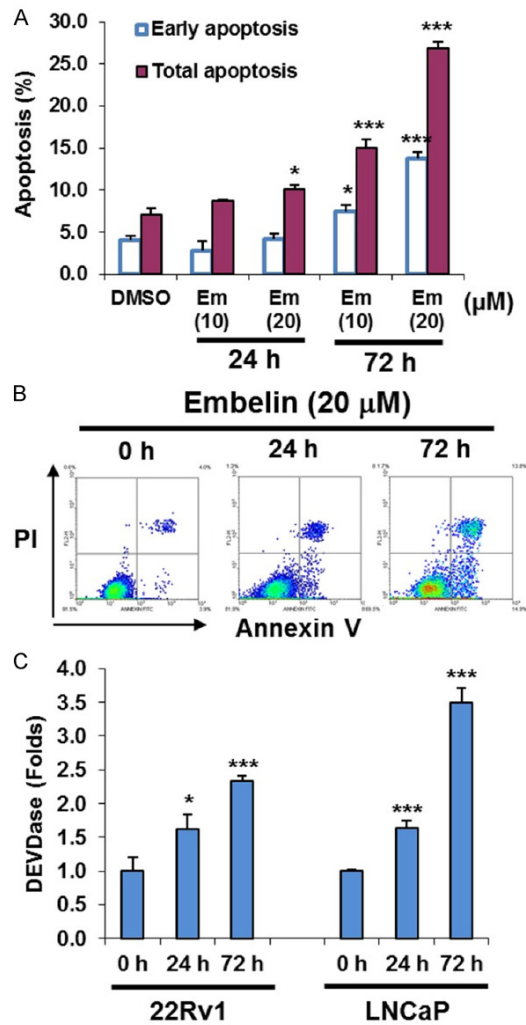
p53-wild type LNCaP and 22Rv1 cells were treated with embelin with serially diluted concentrations for 96 h and cell proliferation was detected by MTT assay. Embelin inhibited proliferation in a dose-dependent manner for both cells (Figure 1A). Non-linear regression of the dose-effect curve indicated that the IC<sub>50</sub> for LNCaP and 22Rv1 cells were 14.6 μM and 18.3 μM, respectively. To test whether such drug

effects were also dependent on exposure period, cells were treated with embelin at 20 μM and living cells were counted at 24 h and 72 h post-treatment. As expected, embelin significantly inhibited cell proliferation in a time-dependent manner in both LNCaP (Figure 1B) and 22Rv1 (Figure 1C). To further examine whether reduced cell number was due to cell-killing effects by embelin, dead cells were counted by trypan blue staining. Data showed that drug treatment for 72 h significantly induced cell death as reflected by trypan blue positive cells in LNCaP (Figure 1D) and 22Rv1 (Figure 1E). These data were supported by morphological images that 72 h drug treatment resulted in clearly less attached cells and more floating cells (Figure 1F).

*Embelin induces apoptosis in p53-wildtype prostate cancer cells*

To further detect whether embelin-induced cell death is apoptosis, drug-treated cells were subjected to several tests related with apoptotic cell death. Annexin V/PI double staining followed by flow cytometry analysis indicated that drug treatment for 72 h with both concentrations (10, 20 μM) significantly enhanced early and total apoptotic cell populations in LNCaP (Figure 2A). At 24 h treatment, only higher concentration (20 μM) showed meaningful increase in total apoptosis (Figure 2A). Representative flow cytometry illustrations of LNCaP were shown in Figure 2B. Further, cellular caspase 3 activity was detected since caspase 3 activation is a hallmark of classic apoptosis. In both cells drug treatment resulted in significant elevation of released fluorescent substrate DEVD, suggesting the strong activation of caspase 3 (Figure 2C).

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**Figure 2.** Embelin induces apoptosis in p53-wildtype prostate cancer cells. (A) LNCaP cells were treated with embelin at 10 or 20  $\mu\text{M}$  for 24 and 72 h, respectively. Cells were processed to Annexin V/PI double staining followed by flow cytometry. Early apoptosis (Annexin V+ PI+) and total apoptosis (Annexin V+) were gated by defined quadrants. Columns, mean; bars, SD ( $n=3$ ). \* $P<0.05$ ; \*\*\* $P<0.001$  compared to DMSO. (B) Typical illustrations in (A) were shown. (C) 22Rv1 and LNCaP cells were treated with embelin (20  $\mu\text{M}$ ) for 24 and 72 h, respectively. Caspase 3 function was determined by incubating whole cell lysates with fluorogenic substrate N-Ac-DEVD-N'-MC-R110 followed by monitoring emitted fluorescence. Fold of enzymatic activity was calculated by dividing the fluorescent signal in the treated sample by that in the untreated control. Columns, mean; bars, SD ( $n=3$ ). \* $P<0.05$ ; \*\*\* $P<0.001$  compared to 0 h control.

### Embelin activates p53

To determine the molecular alterations by drug treatment, LNCaP cells were treated with

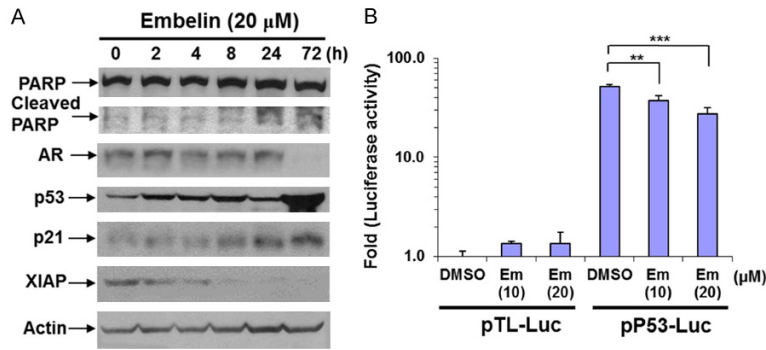
embelin (20  $\mu\text{M}$ ) for varied time points, and proteins related with apoptotic pathways were detected by Western blot. Embelin induced cleavage of PARP, a typical substrate of caspase 3 after 24 h (Figure 3A), which is in line with caspase 3 activation (Figure 2C). Interestingly, embelin quickly and markedly enhanced p53 expression, starting at as early as 2 h, and lasted for over 24 h, with the strongest activation at 72 h (Figure 3A). Such findings were supported by increased expression of P21, the most classic downstream signaling molecule of p53, and decreased AR (Figure 3A), a typical prostate cancer marker that has been shown to have inverse correlation with p53 [28]. In addition, embelin rapidly reduced anti-apoptotic protein XIAP (Figure 3A), consistent with previous studies that embelin is a natural IAP inhibitor [16].

To further evaluate whether elevated p53 expression leads to enhanced functional activity, p53 reporter assay was employed to monitor p53's function as a transcriptional factor that is able to drive luciferase reporter expression. After treatment in LNCaP cells containing p53 luciferase reporter constructs (p53-Luc), embelin significantly reduced p53 function by decreasing luciferase activity, with 28% and 42% inhibition by 10 and 20  $\mu\text{M}$ , respectively (Figure 3B). By contrast, the drug showed no effect in cells with vector control reporter (pTL-Luc) (Figure 3B).

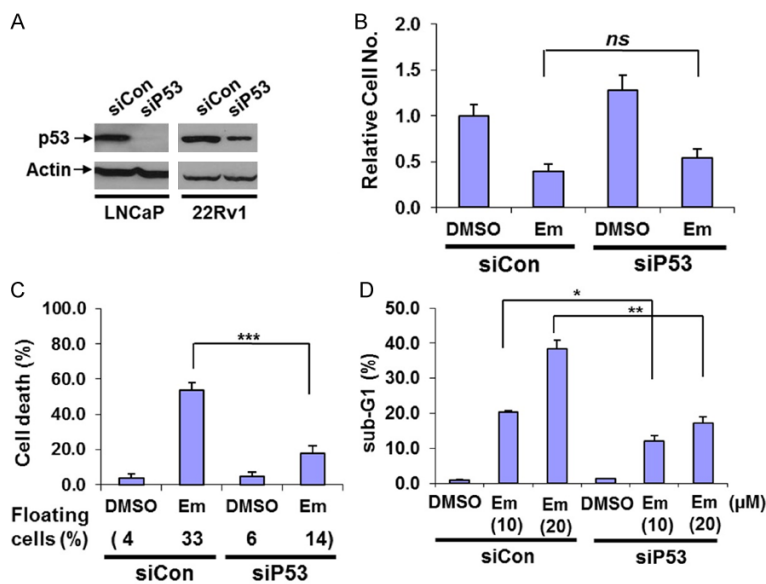
### Knockdown p53 attenuates embelin-induced cell death in p53 wildtype cells

Based on the findings in Figure 3, subsequent experiments are designed to assess the role of p53 in embelin-induced apoptosis by stably knocking down p53 using shRNA. Western blot confirmed efficient p53 knockdown effects in both cells (Figure 4A). Modified LNCaP cells were then exposed to embelin with indicated concentrations and time periods. Embelin did not alter proliferating profile in cells with p53 knockdown (LNCaP-sip53) (Figure 4B). However, the drug did cause significantly less cell death in LNCaP-sip53 compared to cells with vector control (LNCaP-siCon), which was consistent with the proportion of floating cell populations (14% vs. 33%, Figure 4C). Flow cytometry analysis suggested that embelin induced significantly less apoptosis in LNCaP-sip53 than in LNCaP-siCon cells, as indicated by

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**Figure 3.** Embelin activates p53 in LNCaP cells. A. LNCaP cells were treated with embelin for varied time points and whole cell lysates were made for Western blot. Proteins associated with apoptosis and p53 pathway were probed. Actin is a loading control. B. LNCaP cells were transfected with p53 reporter plasmid (pP53-Luc) or control plasmid (pTL-Luc) for 48 h and then treated with embelin (10 or 20  $\mu$ M) for 24 h. Cell lysates were processed for luciferase assay. Fold of luciferase activity was calculated by dividing the luciferase signal in the sample by that in the control (pTL-Luc transfection and DMSO treatment). Columns, mean; bars, SD ( $n=3$ ). \*\* $P<0.01$ ; \*\*\* $P<0.001$ .



**Figure 4.** Knockdown p53 attenuates embelin-induced cell death in p53 wild-type cells. (A) Both LNCaP and 22Rv1 cells were stably transfected with p53 shRNA or control vector. Whole cell lysates were detected for p53 expression. Actin is a loading control. (B-D) LNCaP cells with stable p53 knockdown (LNCaP-siP53) or control (LNCaP-siCon) plasmid were treated with embelin (10, 20  $\mu$ M) or DMSO for 72 h. Attached cells were trypsinized and mixed with trypan blue, and floating cells were also harvested (B, C). Living cells that were exclusive from trypan blue were counted by hemocytometer (B). Relative cell number was calculated by dividing the counted number at each time point by the initial seeding number (B). Trypan blue positive cells were defined as dead cells and cell death (%) was calculated by the ratio of dead cells with total cells (C). In addition, floating cells were counted by hemocytometer and divided by total cell number to yield percentage of floating cells (C). (D) Cells were processed to flow cytometry to acquire sub-G1 populations. Data are from one of two independent experiments (D). Columns, mean; bars, SD ( $n=3$ ). \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$ . ns, not significant.

reduced sub-G1 population in LNCaP-sip53 (Figure 4D).

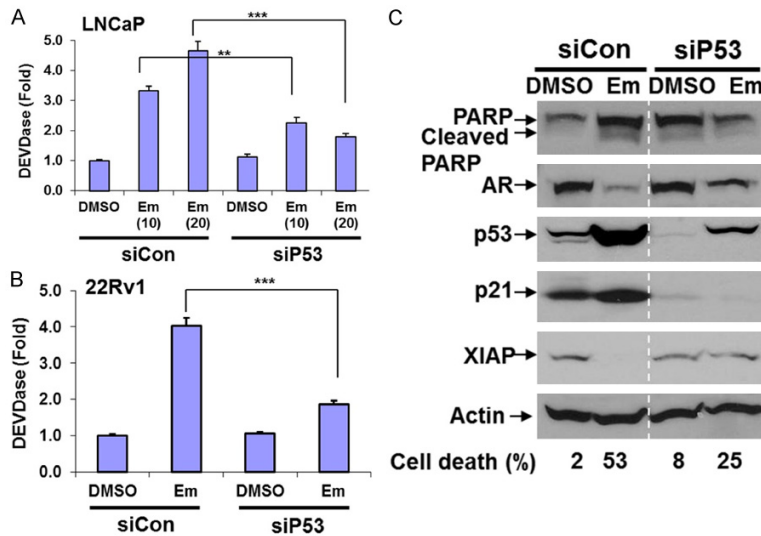
*Knockdown p53 impairs embelin-induced apoptosis in p53 wildtype cells*

Apoptosis-associated molecular features, such as caspase 3 activation, PARP cleavage, as well as p53 pathway were also determined to follow up previous studies. In line with the flow cytometry data (Figure 4D), embelin induced strong caspase 3 activation in LNCaP-siCon cells, while in LNCaP-sip53, such effects were significantly weakened at both drug concentrations tested (Figure 5A). Similar outcomes were observed in 22Rv1 that embelin enhanced much less caspase 3 activity in p53 knockdown cells (Figure 5B). Consistent with caspase 3 status (Figure 5A), PARP cleavage was clearly reduced in LNCaP-sip53 by embelin treatment, compared to that in LNCaP-siCon (Figure 5C). Such observation tended to explain less cell death in p53 knockdown cells by drug treatment (25%) than vector control cells (53%) (Figure 5C). In addition, while embelin induced strong expression of p53 and P21 in vector control cells, these two proteins were dramatically less expressed by drug treatment in p53 knockdown cells (Figure 5C). Interestingly, compared to control cells, embelin showed less inhibition on AR and no inhibition on XIAP in p53 knockdown cells (Figure 5C).

*Re-introducing p53 into p53-knockdown cells sensitizes embelin-induced apoptosis*

Previous studies have shown that p53 silencing is able to

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**Figure 5.** Knockdown p53 impairs embelin-induced apoptosis in p53 wild-type cells. Cells were stably transfected with p53 shRNA or control vector as described in **Figure 4**. (A and B) transfected cells were treated with embelin [10 & 20  $\mu$ M for LNCaP (A); 20  $\mu$ M for 22Rv1 (B)] or DMSO for 72 h. Caspase 3 function was determined by incubating whole cell lysates with fluorogenic substrate N-Ac-DEVD-N'-MC-R110 followed by monitoring emitted fluorescence. Fold of enzymatic activity was calculated by dividing the fluorescent signal in the treated sample by that in the untreated control. Columns, mean; bars, SD ( $n=3$ ). \*\* $P<0.01$ ; \*\*\* $P<0.001$ . (C) Transfected LNCaP cells were treated with embelin (20  $\mu$ M) for 72 h. Whole cell lysates were made for Western blot. Proteins associated with apoptosis and p53 pathway were probed. Actin is a loading control. Cell death was examined by trypan blue exclusion.

attenuate embelin-induced apoptosis, indicating that p53 is critical for drug mechanism. To confirm such conclusion, p53 is re-introduced into p53 knockdown cells to see whether the engineered cells re-gain sensitization to embelin-mediated apoptosis. LNCaP-sip53 cells stably transfected with p53 cDNA plasmids were successfully re-expressing p53 (**Figure 6A**). Annexin V/PI double staining revealed that embelin induced greater apoptosis in p53 re-expressing cells (p53) than pure p53 knock-down cells with vector control (pVec) (**Figure 6B**). Caspase 3 activity showed similar trend that matched the observation in **Figure 6B, 6C**. Finally, when treated with embelin (20  $\mu$ M), p53 restoring cells (p53) yielded stronger cleaved PARP and p53 expression than vector control (pVec) (**Figure 6D**).

### Discussion

In the current study, we have shown that embelin is able to effectively suppress cell proliferation and induces apoptosis in p53 wildtype

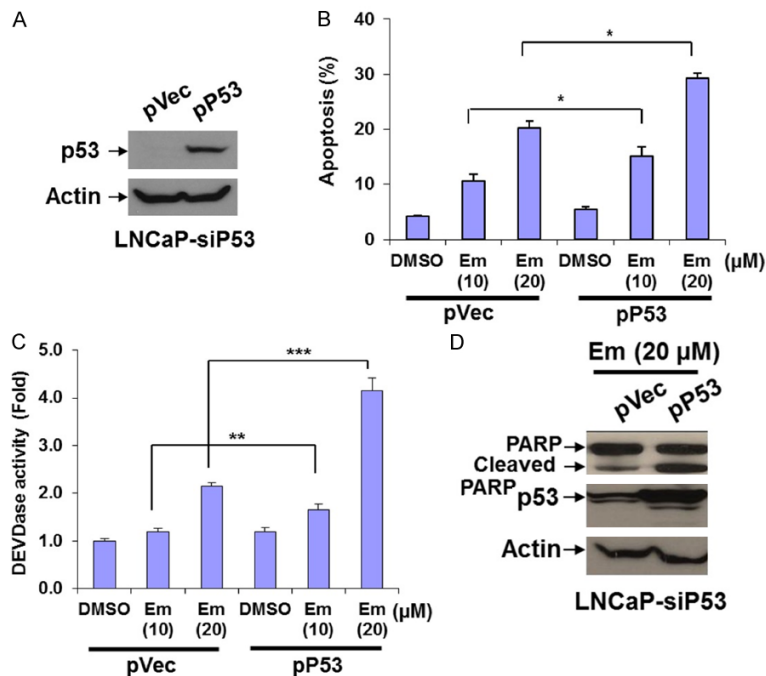
prostate cancer cells (**Figures 1 and 2**). Such cellular features are supported by molecular events that embelin induces caspase 3 activation and PARP cleavage (**Figures 2C and 3A**), and inhibits AR and XIAP expression (**Figure 3A**). Most importantly, embelin activates p53 at both expression level (**Figure 3A**) and function level (**Figure 3B**), suggesting p53 may be a potential target for embelin-induced apoptosis. Further, silencing p53 by shRNA weakens embelin-induced apoptosis (**Figures 4 and 5**). Finally, ectopically expressing p53 in p53-knockdown cells restores cell response to embelin (**Figure 6**). Taken together, these data suggest the important role of p53 in embelin-mediated phenotypes.

Growing evidence has suggested embelin as a potential anti-tumor natural product for prostate cancer, and several

signaling pathways have been identified in the prostate cancer setting such as XIAP [16], Akt/mTOR [15], beta-catenin [14]. Here, we report for the first time that embelin is able to activate p53 that leads to significant pro-apoptotic effects in prostate cancer cells. Genetic approaches demonstrate that p53 is required for embelin-induced apoptosis. These findings provide an alternative pharmacological mechanism for embelin.

Interestingly, although we have shown that embelin effectively augments p53 at protein level, the reason behind it is not known. It could be possible that embelin may enhance p53 gene expression, or block p53 degradation, or affect other proteins that indirectly result in p53 increase. In addition, impact of embelin on p53 post-translational modifications (such as phosphorylation) and p53-related signaling pathways are also remained unclear. It is necessary to elucidate these molecular underpinnings in order to support the therapeutic potential of embelin.

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**Figure 6.** Re-introducing p53 into p53-knockdown cells sensitizes embelin-induced apoptosis. Stably p53-knockdown LNCaP cells (LNCaP-siP53) were transfected with plasmids encoding wildtype p53 (pP53) or vector control (pVec). (A) Whole cell lysates were probed for p53 expression, with Actin a loading control. (B) Cells were treated with embelin at 10 or 20  $\mu\text{M}$  for 72 h and processed to Annexin V/PI double staining followed by flow cytometry. Apoptosis (Annexin V+) were gated by defined quadrants. Columns, mean; bars, SD (n=3). \*P<0.05 compared to DMSO. (C) Cells were treated as in (B) and caspase 3 function was determined. Fold of enzymatic activity was calculated by dividing the fluorescent signal in the treated sample by that in the untreated control. Columns, mean; bars, SD (n=3). \*\*P<0.01; \*\*\*P<0.001. (D) Cells were treated with embelin (20  $\mu\text{M}$ ) for 72 h. Whole cell lysates were probed for PARP cleavage and p53 expression. Actin is a loading control.

In prostate cancer abnormally increased expression of AR is associated with transition to androgen independence and disease progression, an advanced stage often associated with the loss of p53 function. Clinical observations have revealed their correlation in patient samples [28-30]. At the molecular level, expression of AR is negatively regulated by p53, as demonstrated by the fact that p53 is able to directly bind to the promoter region of the AR gene, which contains a potential p53 DNA-binding consensus sequence [31]. In another study, Cronauer et al. reported that overexpression of wild-type p53 decreases AR function, suggesting a balance of AR and p53 during the androgen-dependent growth of prostate cancer [28]. We have shown that while embelin facilitates p53 expression at as early as 2 h, AR is reduced 24 h after drug treatment (Figure 3A), suggest-

ing a late event after p53 activation. However it is still unclear whether in our scenario p53 accumulation is required for AR loss. Future studies will be designed to explore the possible relationship between these two key players by embelin treatment.

Our data also show that embelin-induced rapid p53 activation is associated with XIAP suppression (Figure 3A) and p53 depletion restores XIAP loss by embelin (Figure 5C), suggesting the inverse relationship between these two proteins. Such finding is consistent with previous studies indicating their relationships. Carter et al. explain that since p53 induces apoptosis largely by modulating Bcl-2 family proteins and permeabilizing mitochondrial membrane, it is reasonable that p53 promotes release of Smac, the negative regulator of XIAP, therefore inhibits XIAP expression [32]. In ovarian cancer cells with wildtype p53, p53 is a determinant of XIAP-mediated chemoresistance, and inhibition of

XIAP sensitizes cell response to chemodrugs, suggesting that p53 function is required for XIAP interference [33]. In our study however, whether p53 activation is a cause or a consequence of XIAP inhibition by embelin still remains unknown. Delineating the clear regulation between these two key apoptotic signaling molecules undoubtedly assists to interpret detailed mechanisms of the drug.

In summary, our study shows that embelin efficiently inhibits proliferation and induces apoptosis in p53 wildtype prostate cancer cells. Interestingly, p53 is required for embelin-induced apoptosis, suggesting p53 may be a potential target for embelin-mediated efficacies in p53 wildtype prostate cancer. These novel findings suggest that embelin may represent a promising lead natural medicine for the



treatment of prostate cancer with wildtype functional p53, and therefore warrants future preclinical investigations.

**Disclosure of conflict of interest**

None.

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