Integrin β4 reduces DNA damage-induced p53 activation in colorectal cancer

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Abstract. Integrin contributes to the maintenance of cell adhesion. In turn, cell adhesion triggers certain integrin signaling cascades, and influences cell biological behavior. In the present study, we explored the role and mechanism of integrin β4 in the DNA damage response in colorectal cancer (CRC) using a three-dimensional (3D) cell culture model. Under 3D culture condition, dispersed CRC cells automatically formed multicellular spheroids, which consisted of layers of cells with cell junctions commonly distributed. The expression level of integrin β4 in HCT116 3D cultures was slightly higher compared with two-dimensional (2D) cultures, while the expression level in LoVo 3D cultures was similar to or slightly lower than that in 2D cultures. Knockdown of integrin β4 by lentiviral delivery of shRNA did not markedly change the architectural formation of 3D cultures under an inverted microscope or transmission electron microscope. Platinum increased p53 and p-p53 (ser15) in a time-dependent manner in 3D cultures. Knockdown of integrin β4 increased sensitivity to cisplatin (CDDP) in 3D cultures. Under 3D culture condition, knockdown of integrin β4 did not detectably change the basal p53 protein level but increased p53 and p-p53 (ser15) protein accumulation induced by platinum. Integrin β4 knockdown did not detectably change p53 protein level in HCT116 2D cultures with or without CDDP treatment. Knockdown of wild-type p53 decreased sensitivity to platinum in 3D cultures. Since it has been proven that platinum damages DNA to kill cells and p53 plays a key role in the DNA damage response, our results indicated that integrin β4 reduced DNA damage-induced p53 activation to decrease chemosensitivity in CRC. This may be due to integrin β4 activation in 3D cultures.

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Abbreviations: CRC, colorectal cancer; 3D, three-dimensional; ECM, extracellular matrix; 2D, two-dimensional; AOM, azoxymethane; DSS, dextran sodium sulfate; H&E, hematoxylin and eosin; PBS, phosphate-buffered saline; p-p53, phospho-p53 (Ser15); GAPDH, glyceraldehyde 3-phosphate dehydrogenase; sh, small hairpin; shcontrol, control shRNA; CDDP, cisplatin; DAPI, 4',6-diamidino-2-phenylindole; WST, water-soluble tetrazolium salt; L-OHP, oxaliplatin

Key words: integrin β4, p53, platinum, three-dimensional culture, colorectal cancer

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males, and the second most common in females worldwide (1). The highest incidence rates are mainly in developed countries and the incidence is increasing in developing countries, which is partly attributable to lipid metabolism (1-3). Despite an enormous amount of effort spent in the development of therapies to treat CRC, the 5-year survival rate has increased slowly in the USA, from 60% during the 1980s to 66% during 2005 to 2011 (4). To a large extent, this discouraging observation is largely due to the fact that patients with CRC demonstrate resistance to both new and older anticancer drugs (3,5).

Three-dimensional (3D) cell culture models provide an optimal experimental system to study the mechanisms of anticancer drug resistance, as they represent a suitable *in vivo* approximation of solid tumor tissue microenvironment, including cell adhesion (3,6,7). The formation and stability of cell adhesion, including cell-cell adhesion and cell-extracellular matrix (ECM) adhesion, rely on cell adhesion molecules, particularly the integrin family (6,8,9). Conversely, cell adhesion triggers certain integrin signaling cascades, and influences tumor cell biological behavior, including progression, proliferation, survival and chemosensitivity (6,9-11). Accumulating evidence has demonstrated that the expression of integrin is negatively correlated with prognosis in multiple cancer types (9,10,12). However, the underlying mechanism remains unclear (9,10,12).

In the present study, 3D cultures were used to explore the role of integrin β4 in the response of human CRC cells to platinum. Our data demonstrated that integrin β4 reduced DNA damage-induced p53 activation in CRC.

Materials and methods

Cell lines and cell culture. HCT116 and LoVo, two human CRC cell lines that contain stabilized wild-type p53 protein (3), and the cell line 293T were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

Two-dimensional (2D) cultures were grown and passaged routinely as previously described (3). In brief, HCT116 cells were grown in McCoy's 5A (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA, F12K (Gibco Thermo Fisher Scientific, Inc.) for LoVo cells or DMEM (Gibco) for 293T cells, respectively supplemented with 100 ml/l newborn calf serum (Gibco Thermo Fisher Scientific, Inc.), 100,000 IU/l penicillin and 100 μg/ml streptomycin (Gibco Thermo Fisher Scientific, Inc.) under a humidified atmosphere of 5% CO_2 at 37˚C.

Subsequently, 3D cultures were prepared without the use of any extracellular components as previously described (3,6,13). In brief, plates were coated with poly-2-hydroxyethylmethacrylate (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) as previously described (13). Exponentially growing CRC cells were inoculated into the plates. The plates were then gently horizontally swirled for 2 min every 4 h for the first 24 h, then 6 min every 8 h (3,6). The cells were incubated under a humidified atmosphere of 5% CO_2 at 37˚C. Half of the medium was replaced every day.

Preparation of slides for scanning electron microscopy. Sample slides were routinely prepared. In brief, 3D cultures were fixed in 2.5% glutaraldehyde. Samples were then sent to The Medical Research Center of The Third Military Medical University for preparation. The sections were imaged using a scanning electron microscope (S3400N II; Hitachi, Tokugawa, Japan).

Hematoxylin and eosin (H&E) staining. HCT116 3D cultures were fixed in 4% paraformaldehyde, and OCT-embedded samples were sectioned at a thickness of 10 μm. Sample slides were routinely stained with H&E (3).

Preparation of slides for transmission electron microscopy. Sample slides were routinely prepared as previously described (3). In brief, 3D cultures were fixed in 2.5% glutaraldehyde and then in 1% osmium tetroxide. Samples were dehydrated and ultrathin sections were generated. The sections were stained with uranium acetate and lead citrate and were observed using a transmission electron microscope (Tecnai 10; Philips, Amsterdam, The Netherlands).

Western blot analysis. Western blotting was performed as previously described (3,6). Cells were washed with PBS and lysed in 2X SDS loading buffer [0.1 M Tris-HCl (pH 6.8), 0.2 M DTT, 4% SDS, 20% glycerol and 0.2% bromophenol blue] with Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail 2 and Phosphatase Inhibitor Cocktail 3 (all from Sigma-Aldrich; Merck KGaA) for 5 min on ice, inverting the tube. Following sonication, protein was quantitated using the RC DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

The protein was resolved by SDS/PAGE and blotted on nitrocellulose membranes (Bio-Rad Laboratories). The nitrocellulose membranes were incubated with specific primary antibodies overnight at 4˚C. Following incubation with secondary antibodies for 90 min at 37˚C, immunoreactive proteins were visualized using the Enhanced Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.).

Primary antibodies against integrin β4 (1:500; cat. no. 14803), p53 (1:1,000; cat. no. 2524), phospho-p53 (Ser15) (p-p53; 1:500; cat. no. 9286), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1,000; cat. no. 5174) and HRP-linked secondary antibodies (anti-mouse IgG; 1:5,000; cat. no. 7076; anti-rabbit IgG; 1:5,000; cat. no. 7074) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Lentiviral delivery of small hairpin (sh)RNA. Integrin β4 and p53 were knocked down by lentiviral vector-mediated shRNA interference using The RNAi Consortium system (Open Biosystems, Inc., Huntsville, AL, USA) according to the manufacturer's instructions (3). In brief, an integrin β4 or p53-targeting shRNA-pLKO.1 vector or a control shRNA-pLKO.1 vector, with the packaging plasmid pCMV-Dr8.91 and the enveloping plasmid pCMV-VSV-G, was co-transfected into 293T cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions (3). Virus-containing medium was collected at 48 and 72 h post-transfection and was filtered using a 0.22-μm filter (EMD Millipore, Billerica, MA, USA). Cells were infected with the lentivirus, and then were selected using puromycin (Sigma-Aldrich; Merck KGaA). Control shRNA (shcontrol) was targeted against green fluorescent protein, and the sense sequence of this shRNA is TACAACAGCCACAACGTCTAT (3). Sense sequences of shRNAs targeting specific genes were GAGGGTGTCATC ACCATTGAA (shβ4-1) (14) or ACGATGACAACCGACCTA TTG (shβ4-2) for integrin β4, and GACTCCAGTGGTAAT CTACT for p53 (3). Knockdown efficiency was confirmed by western blotting.

Immunohistochemical staining. HCT116 3D cultures were fixed in 4% paraformaldehyde, and OCT-embedded samples were sectioned at a thickness of 10 μm. Immunohistochemistry was performed according to the protocol of the SPlink Detection kits (ZSGB-Bio, Beijing, China), as previously described (3).

Immunofluorescence staining. Immunofluorescence staining was performed as previously described (7). HCT116 cells were grown as 2D cultures on cover slides, and then treated with 2.5 μg/ml cisplatin (CDDP; Sigma-Aldrich; Merck KGaA) for 24 h. Following fixation in 4% paraformaldehyde for 30 min, cells were incubated in 0.2% Triton X-100 in 2% BSA/PBS for 30 min. Subsequently, cells were incubated in p53 (Cell Signaling Technology, Inc.; 1:500) antibody solution and Alexa Fluor® 555 goat anti-mouse (Invitrogen; Thermo Fisher Scientific, Inc.; 1:1,000) solution for 2 h and 30 min, respectively. The nuclei were stained by incubating with 4',6-diamidino-2-phenylindole (DAPI, 1 μg/ml) (Sigma-Aldrich; Merck KGaA) for 30 min.

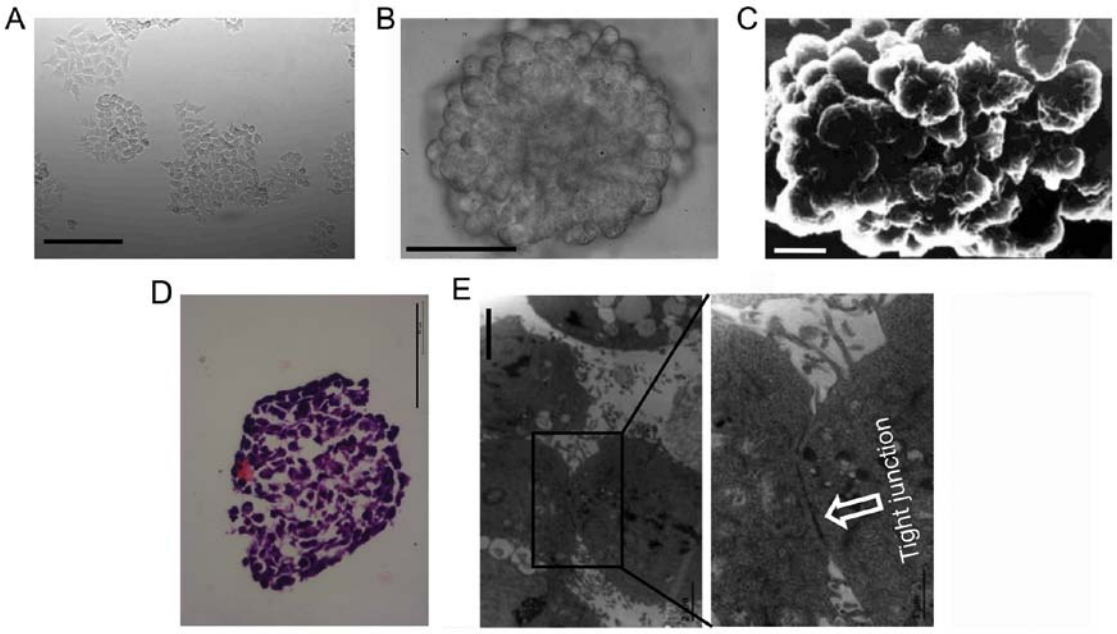

Figure 1. Histopathology of 3D cultures. (A) HCT116 cells were cultured routinely under 2D conditions for 4 days, or (B) under 3D conditions for 5 days. Cells were imaged using an inverted microscope. Scale bars represent 100 μm. (C) HCT116 3D cultures were imaged using a scanning electron microscope. The scale bar represents 10 μm. (D) HCT116 3D cultures were stained with H&E. The scale bar represents 100 μm. (E) HCT116 3D cultures were imaged using a transmission electron microscope. The scale bar represents 2 μm.

Water-soluble tetrazolium salt (WST) assay. 3D cultures were treated with 10 μg/ml CDDP or 5 μg/ml oxaliplatin (L-OHP) (Sigma-Aldrich; Merck KGaA) for 48 h. Control cultures received 10 μl PBS only. Cell viability was assayed using WST [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt], as previously described (3,7). The WST assay was performed using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions (3,7). In brief, 3D cultures detached using accutase (Non-enzyme Cell Detach Solution; Applygen Technologies, Beijing, China) were incubated with WST/media for 3-4 h, after which the absorbance at 450 nm was determined using a microplate reader with a reference wavelength of 650 nm. Cell viability was normalized to the control.

Clonogenic assay. Clonogenic assay was performed as previously described (3,7). In brief, 3D cultures with the same numbers of cells were treated with 10 μg/ml CDDP for 48 h. Then, the 3D cultures were detached as single-cell suspensions using accutase, and the same ratio was inoculated into 24-well plates. The cells were grown for 7 days and were subsequently stained with crystal violet and counted using a stereomicroscope and an automatic 'counting colony counter pen'.

Statistical analysis. The data shown represent the mean ± standard error. Statistical differences between groups were analyzed by Student's t-test or one-way ANOVA. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Histopathology of 3D cultures. HCT116 cells grew as a layer of cells under 2D condition (Fig. 1A). Under 3D condition, dispersed cells aggregated automatically and formed 3D cultures (multicellular spheroids). The surface of 3D cultures was observed using an inverted microscope (Fig. 1B) or a scanning electron microscope (Fig. 1C). The cells in the outermost layer of 3D cultures were uniformly spherical.

HCT116 3D cultures were stained with H&E to further analyze the inner structure. 3D cultures consisted of layers of cells packed tightly. The cells in the inner layers were heteromorphic, not uniformly spherical as in the outermost layer (Fig. 1D). These structures mimicked colorectal tumors at an avascular stage or avascular regions of colorectal tumors *in vivo* (2,7). The ultrastructure of 3D cultures was observed using a transmission electron microscope. Cells adhered with each other in 3D cultures, and cell junctions were commonly found (Fig. 1E).

Association between integrin β4 and 3D cultures. Western blot analysis revealed that the integrin β4 expression level in HCT116 3D cultures was slightly higher than in 2D cultures. The integrin β4 expression level in LoVo 3D cultures was similar to or mildly lower than in 2D cultures (Fig. 2A). These results indicated that the function of cell adhesion in integrin β4 expression may be cell-type specific.

To explore the role of integrin β4 in the architectural formation of 3D cultures, integrin β4 was knocked down by lentiviral delivery of shRNA. The efficiency was confirmed by western blot analysis (Fig. 2B). Cells with integrin β4-knockdown (shβ4) or cells transfected with control lentivirus (shcontrol) were cultured under 3D condition for 15 days and were observed under an inverted microscope (Fig. 3A). LoVo 3D cultures were smaller and more uniform than HCT116 3D cultures. Cells with integrin β4 knockdown grew as multicellular spheroids, similar to the respective shcontrol. No differences

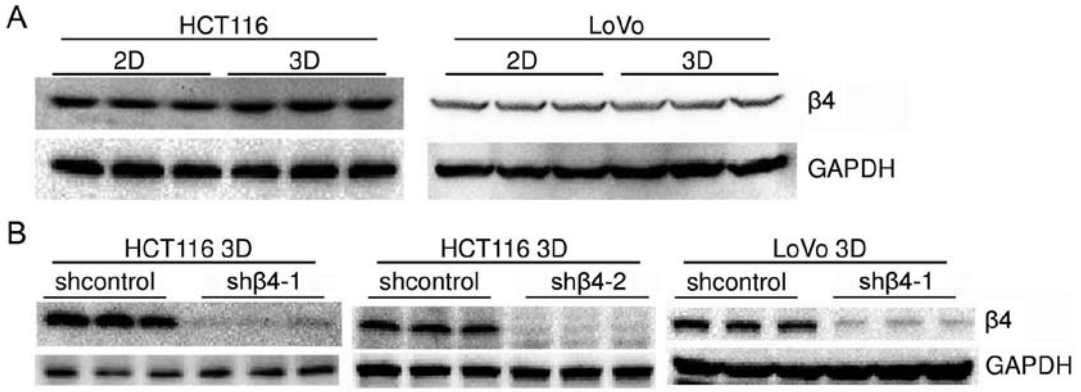

Figure 2. The function of 3D architecture in integrin β4 expression. (A) The integrin β4 protein level was assayed by western blotting. Protein was extracted from 2D cultures grown for 4 days or from 3D cultures grown for 5 days. (B) Integrin β4 was knocked down by lentiviral delivery of shRNA (shβ4-1 and shβ4-2), and the efficiency was assayed by western blotting.

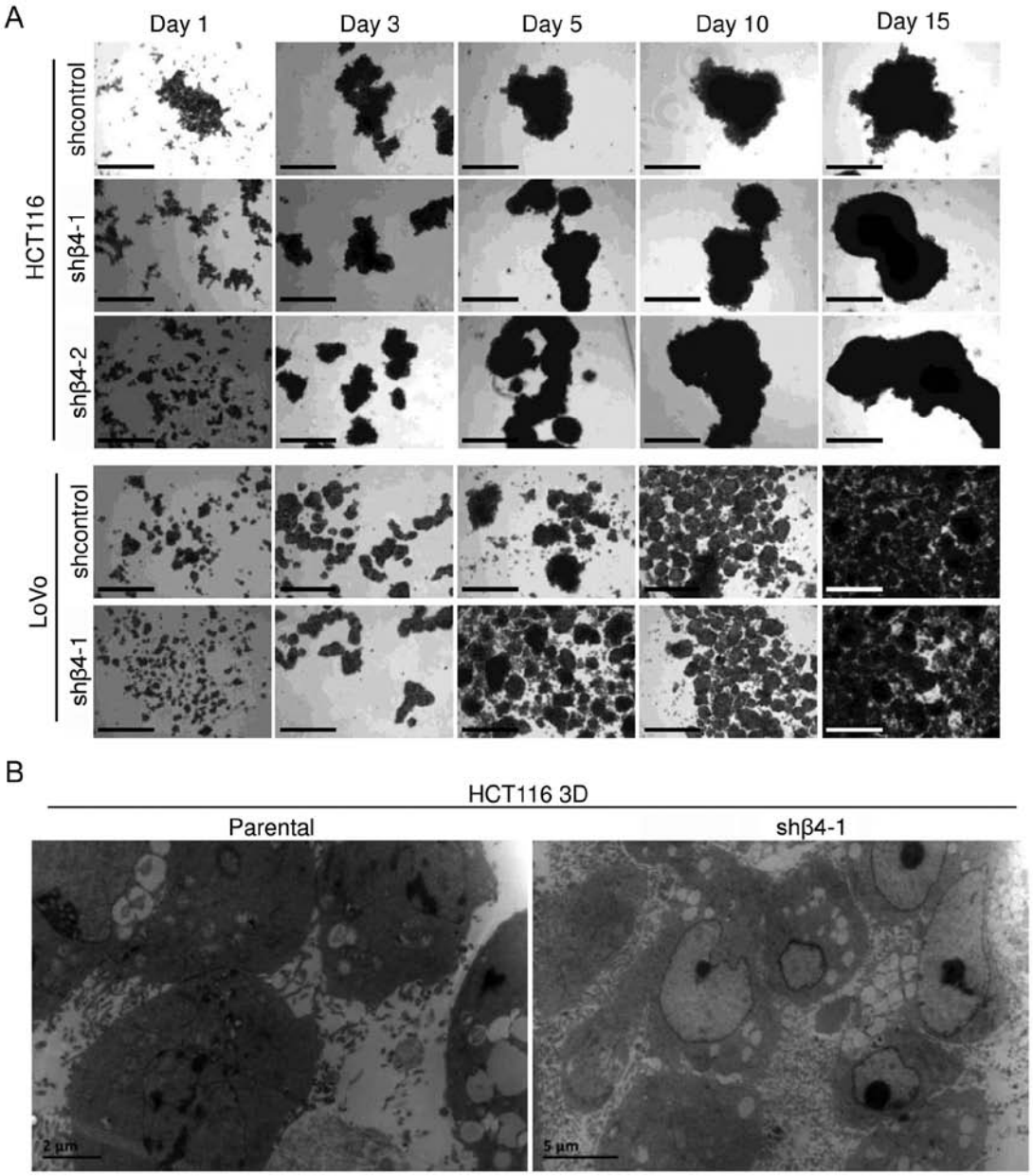

Figure 3. The function of integrin β4 in 3D architecture. (A) Cells were cultured under 3D condition and imaged at multiple time-points using an inverted microscope. Scale bars represent 500 μm. (B) Parental HCT116 cells and HCT116 cells with integrin β4 knockdown (shβ4-1) were cultured under 3D condition for 5 days and then imaged using a transmission electron microscope.

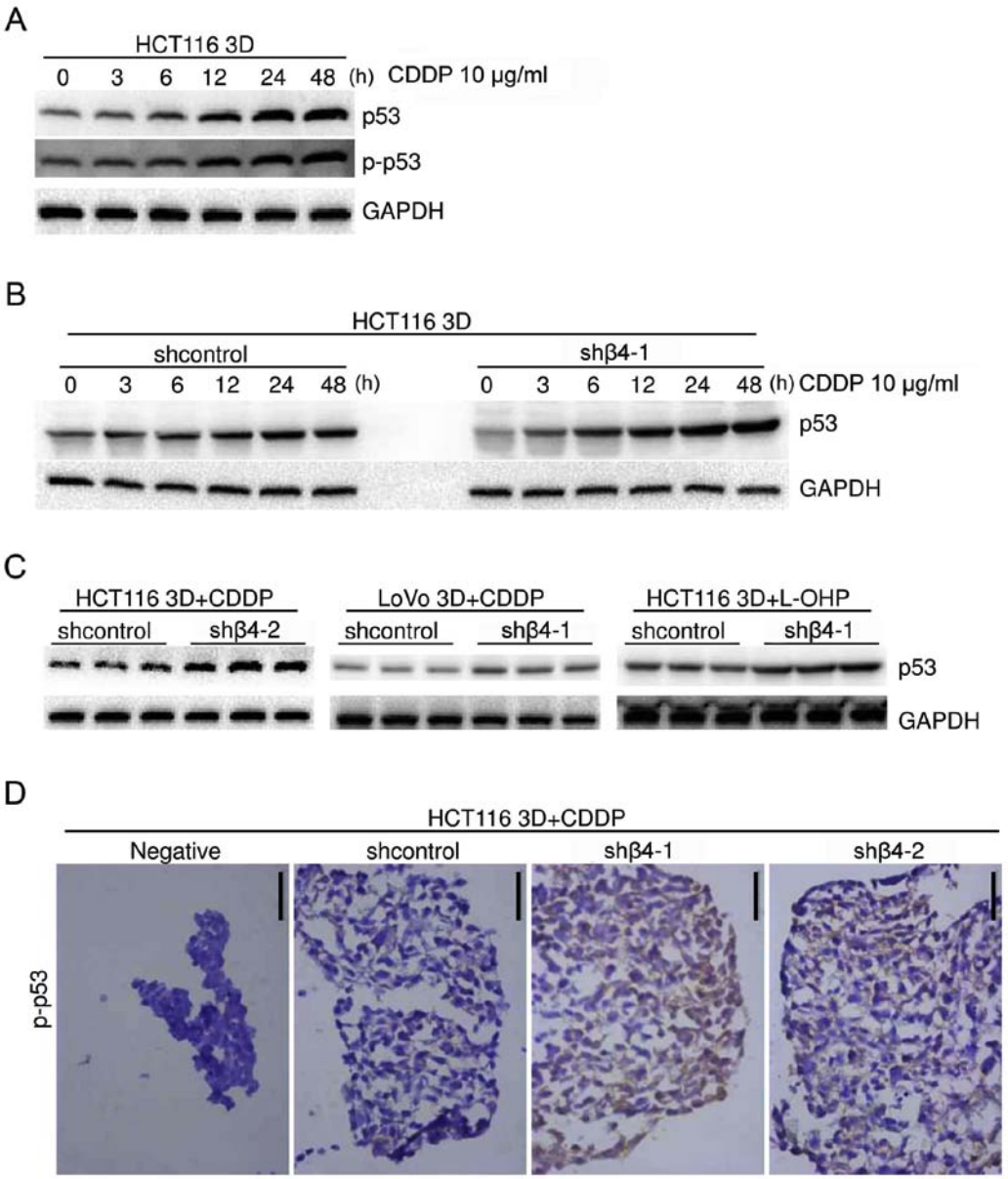

Figure 4. Integrin β4 reduces DNA damage-induced p53 activation in 3D cultures. (A) CDDP induced p53 and p-p53 (ser15) accumulation in a time-dependent manner. (western blotting). (B) Integrin β4 knockdown increased the p53 accumulation induced by CDDP, and did not detectably change the basal p53 protein level (western blotting). (C) Integrin β4 knockdown reduced the p53 accumulation induced by CDDP (10 μg/ml, 48 h) or L-OHP (5 μg/ml, 48 h) in HCT116 or LoVo cells, respectively (western blotting). (D) Immunohistochemical staining of p-p53 in HCT116 3D cultures treated with CDDP (10 μg/ml, 48 h). Scale bars represent 50 μm; (h, hours).

between shβ4 and the respective shcontrol were observed. The ultrastructures of parental HCT116 3D cultures and of shβ4 HCT116 3D cultures were observed using a transmission electron microscope (Fig. 3B). Integrin β4 knockdown did not detectably change cell adhesion, and no significant difference was observed. These results demonstrated that integrin β4 knockdown did not detectably change the architecture of 3D cultures.

Integrin β4 reduces DNA damage-induced p53 activation in 3D cultures. Platinum and irradiation kills cells by damaging DNA, and p53 plays a key role in the DNA damage response (3,15,16). CDDP caused p53 protein accumulation in HCT116 3D cultures in a time-dependent manner (Fig. 4A). DNA damage induces the phosphorylation of p53 at ser15 (16,17). Western blot analysis revealed that CDDP induced p-p53 (ser15) protein accumulation in a time-dependent manner (Fig. 4A). These results are consistent with those of previous studies (3,15,17,18) and, collectively, these results demonstrated that platinum caused DNA damage to induce p53 activation in a time-dependent manner.

HCT116 3D cultures were treated with 10 μg/ml CDDP in a time-gradient manner and the p53 protein level was assayed using western blot analysis. Knockdown of integrin β4 did not detectably change the basal p53 protein level but increased the CDDP-induced p53 protein accumulation (Fig. 4B). HCT116 and LoVo 3D cultures were treated with 10 μg/ml CDDP or 5 μg/ml L-OHP for 48 h, respectively. Western blot analysis revealed that integrin β4 knockdown increased platinum-induced p53 protein accumulation (Fig. 4C), but did

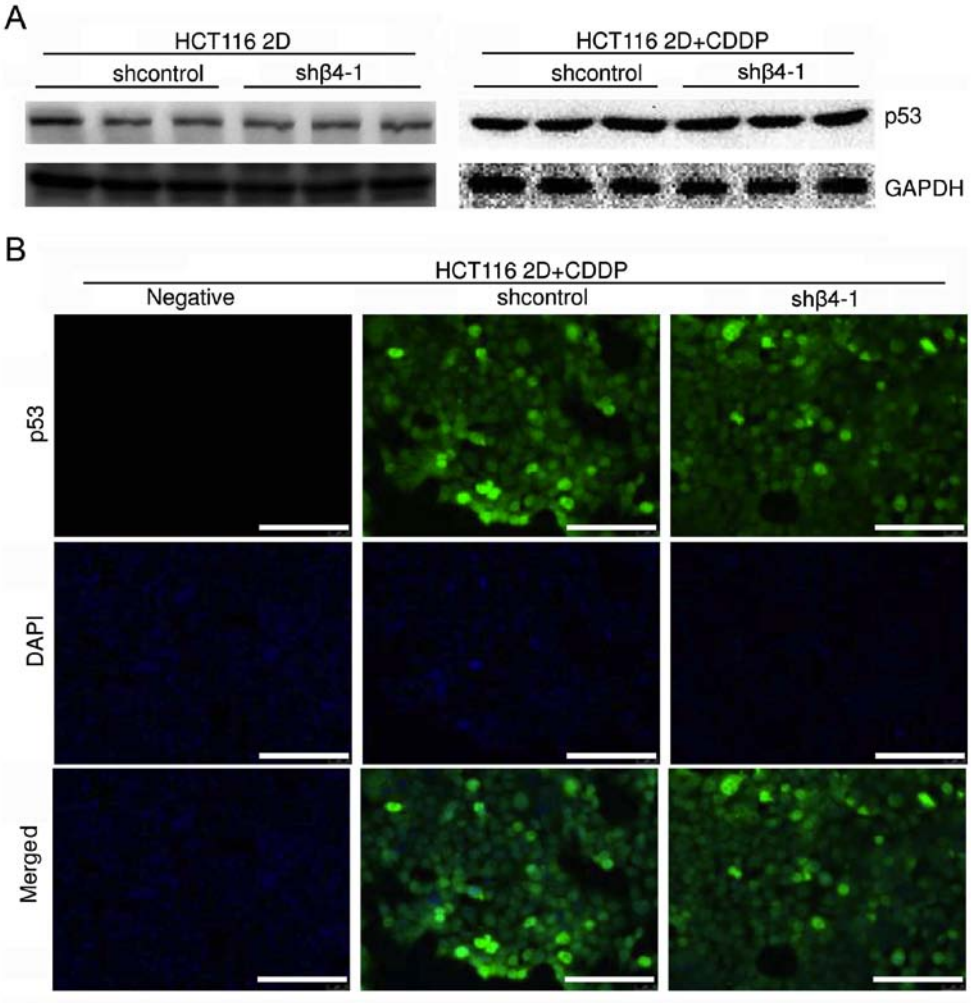

Figure 5. Integrin β4 does not detectably alter DNA damage-induced p53 accumulation in HCT116 2D cultures. (A) Integrin β4 knockdown did not detectably change the basal p53 protein level, or CDDP-induced (2.5 μg/ml, 48 h) p53 accumulation (western blotting). (B) Results of immunofluorescence revealed that integrin β4 knockdown did not markedly change CDDP-induced (2.5 μg/ml, 24 h) p53 accumulation. Scale bars represent 100 μm. (h, hours).

not detectably change the basal p53 level (data not shown). The effect of integrin β4 knockdown on p-p53 was explored. HCT116 3D cultures were treated with 10 μg/ml CDDP for 48 h, and p-p53 was evaluated using immunohistochemical staining. Integrin β4 knockdown increased the CDDP-induced p-p53 accumulation that arose from DNA damage (16,17) (Fig. 4D). These results unanimously supported the conclusion that integrin β4 reduced p53 activation from platinum-induced DNA damage in 3D cultures.

The effect of integrin β4 on p53 in HCT116 2D cultures was studied. Since 2D cultures are more sensitive to chemotherapy and radiotherapy than 3D cultures (3,8,19), 2D cultures were treated with a lower concentration of CDDP. Results of western blot analysis revealed that integrin β4 knockdown (shβ4-1) did not markedly change the basal p53 protein level, or the CDDP-induced (2.5 μg/ml, 48 h) p53 protein level (Fig. 5A). Results of immunofluorescence staining were consistent with those of western blot analysis. Integrin β4 knockdown did not markedly change the p53 protein level in HCT116 2D cultures treated with CDDP (2.5 μg/ml, 24 h) (Fig. 5B). In summary, these results indicated that integrin β4 reduced DNA damage-induced p53 activation in 3D cultures, but not in HCT116 2D cultures.

Knockdown of wild-type p53 decreases sensitivity to platinum in CRC. The role of p53 in the sensitivity of HCT116 (containing stabilized wild-type p53 protein (3)) to platinum was explored. p53 was knocked down by lentiviral delivery of shRNA. The efficiency was confirmed by western blot analysis (Fig. 6A). The specificity was previously verified (3,20). Immunohistochemistry revealed that knockdown of p53 significantly decreased p-p53 level in HCT116 3D cultures treated with 10 μg/ml CDDP for 48 h (Fig. 6B). Results of the WST assay revealed that knockdown of p53 significantly decreased chemosensitivity of HCT116 3D cultures to platinum (Fig. 6C). The viability of shcontrol HCT116 cells treated with CDDP (10 μg/ml, 48 h) was 44.1±2.6%, whereas that of HCT116 cells with p53 knockdown was 64.6±3.2% ($P<0.01$). The viability of shcontrol HCT116 cells treated with L-OHP (5 μg/ml, 48 h) was 51.4±2.4%, whereas that of HCT116 cells with p53 knockdown was 67.7±4.2% ($P<0.05$). There was no significant difference in viability between the parental HCT116 3D cell cultures and the shcontrol ($P\geq0.05$) (data not shown). The results of the clonogenic assay were consistent with these of the WST assay. The clonogenicity of the shp53 HCT116 cells was higher than that of the shcontrol (Fig. 6D).

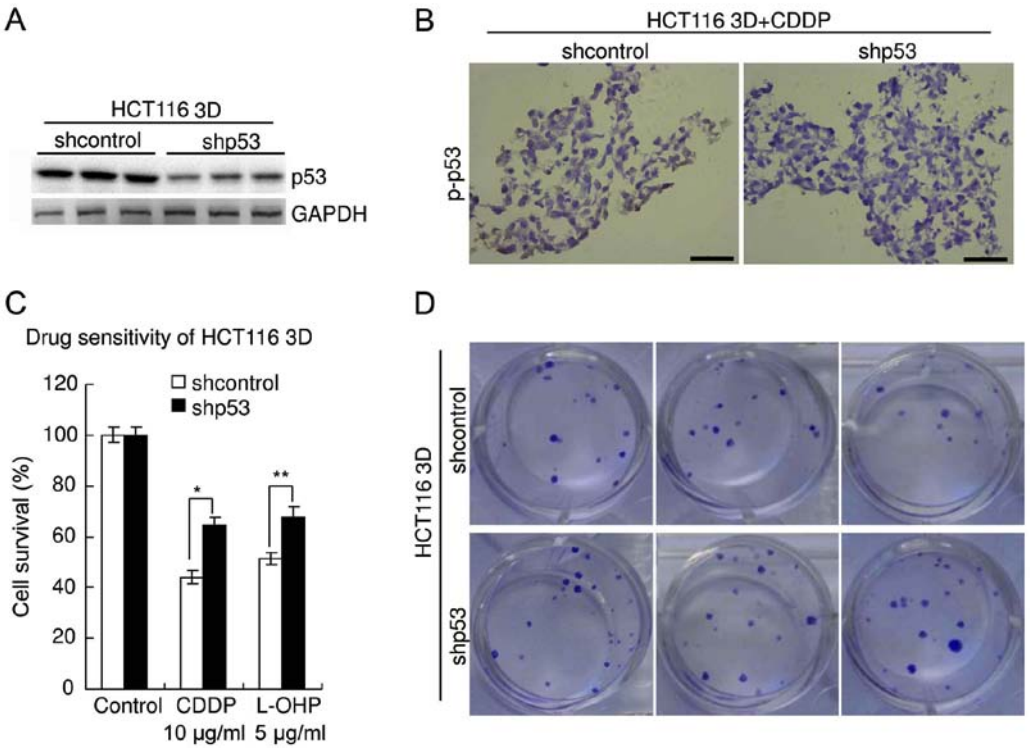

Figure 6. Knockdown of p53 decreases sensitivity to platinum in HCT116 3D cultures. (A) p53 was knocked down by lentiviral delivery of shRNA (shp53), and the efficiency was assayed by western blot. (B) Immunohistochemical staining of p-p53 in HCT116 3D cultures treated with CDDP (10 μg/ml, 48 h). Scale bars represent 50 μm. (C) Cell viability was assayed by WST. *P<0.01; **P<0.05. (D) Clonogenic assay. The clonogenicity of the shcontrol HCT116 cells was lower than that of the shp53 cells. (h, hours).

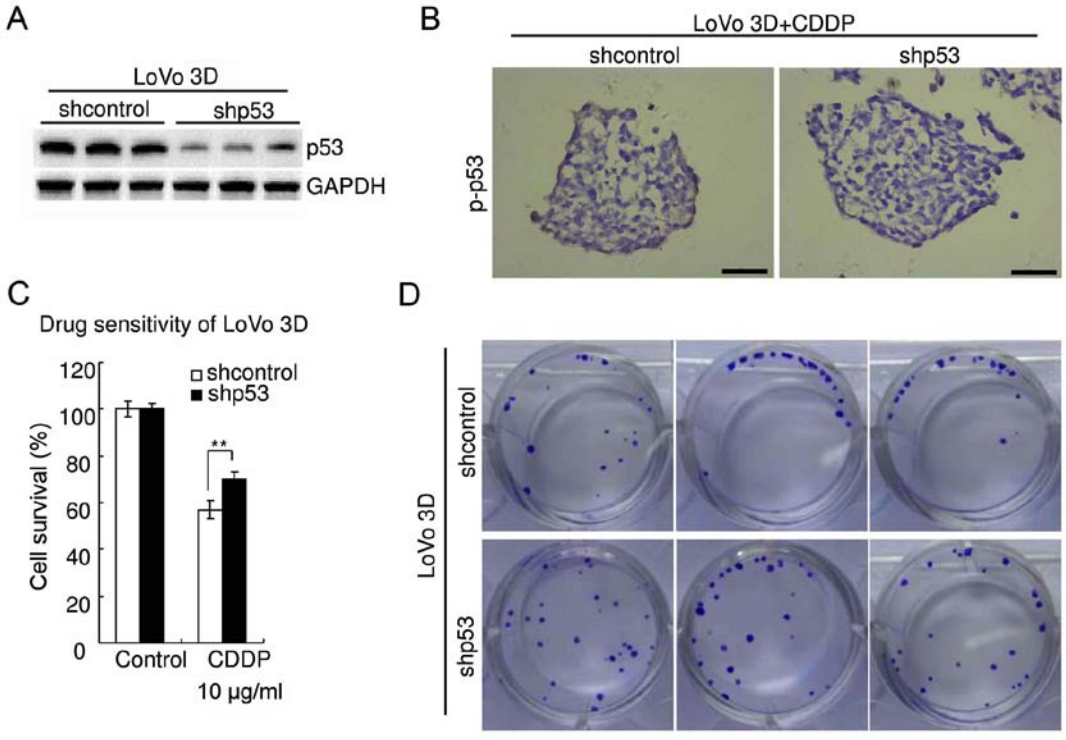

Figure 7. Knockdown of p53 decreases sensitivity to CDDP in LoVo 3D cultures. (A) Western blot assay. (B) Immunohistochemical staining of p-p53 in LoVo 3D cultures treated with CDDP (10 μg/ml, 48 h). Scale bars represent 50 μm. (C) Cell viabilities. **P<0.05. (D) Clonogenic assay.

The aforementioned experiments were also performed in LoVo cells [containing stabilized wild-type p53 protein (3)]. The results were consistent with those observed in HCT116 cells. Western blot analysis confirmed the efficiency of p53-knockdown in LoVo 3D cultures (Fig. 7A). Immunohistochemistry revealed that knockdown of p53 significantly decreased

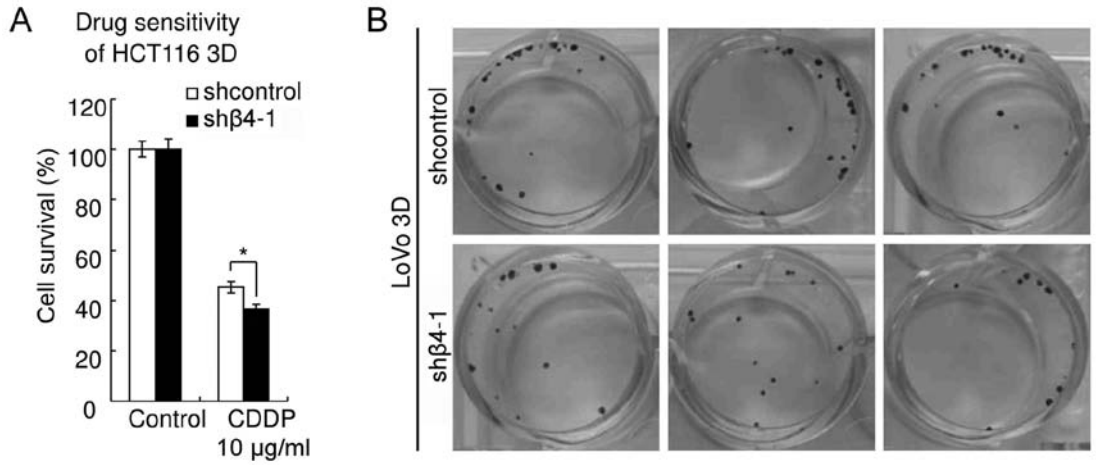

Figure 8. Knockdown of integrin β4 increases CRC sensitivity to CDDP. (A) Viabilities of HCT116 3D cultures. *P<0.01. (B) Clonogenic assay with LoVo cells. The clonogenicity of the shcontrol LoVo cells was lower than that of the shp53 cells.

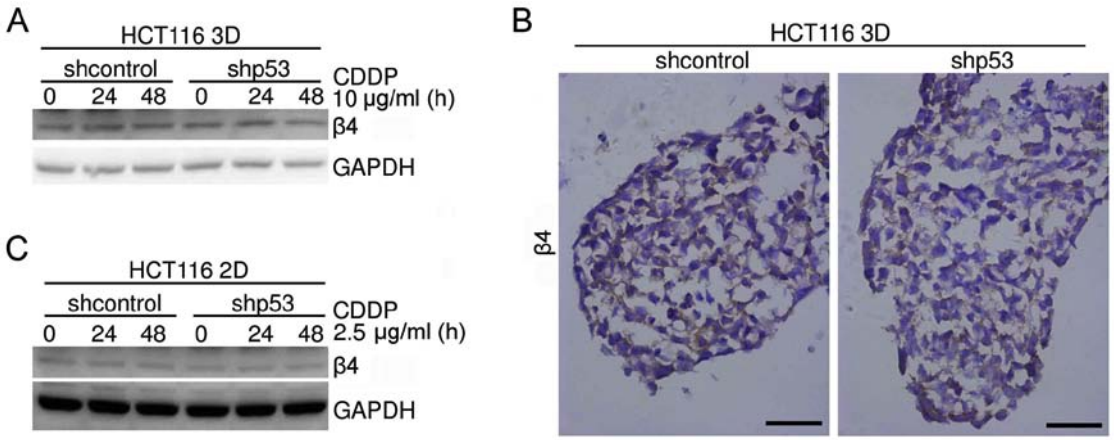

Figure 9. Knockdown of p53 does not detectably alter integrin β4 in HCT116 cells. (A) Knockdown of p53 did not detectably change the expression of integrin β4 in 3D cultures (western blotting). (B) Immunohistochemical staining of integrin β4 in 3D cultures. Scale bars represent 50 μm. (C) Knockdown of p53 did not detectably change integrin β4 expression in 2D cultures.

p-p53 in LoVo 3D cultures treated with 10 μg/ml CDDP for 48 h (Fig. 7B). WST assay revealed that knockdown of p53 significantly decreased chemosensitivity of LoVo 3D cultures to CDDP (P<0.05) (Fig. 7C). The cell viability of shcontrol LoVo treated with CDDP (10 μg/ml, 48 h) was 57.0±3.7%, whereas that of LoVo with p53 knockdown was 69.9±3.1% (P<0.05). The clonogenicity of the shp53 LoVo 3D cultures was higher than that of the shcontrol (Fig. 7D). There was no significant difference in viability between parental LoVo 3D cultures and shcontrol (P≥0.05) (data not shown).

Knockdown of integrin β4 increases sensitivity to CDDP in CRC. As aforementioned, integrin β4 reduced DNA damage-induced p53 activation (Fig. 4) and knockdown of wild-type p53 decreased sensitivity to platinum (Figs. 6 and 7). These results led to the hypothesis that knockdown of integrin β4 may increase sensitivity to platinum of CRC. Results of WST assay revealed that knockdown of integrin β4 significantly increased the chemosensitivity of HCT116 3D cultures to CDDP (Fig. 8A). The viability of shcontrol HCT116 cells treated with CDDP (10 μg/ml, 48 h) was 45.3±2.4%, whereas that of HCT116 cells with integrin β4 knockdown (shβ4-1) was 36.5±2.1% (P<0.01). The results of the clonogenic assay revealed that the clonogenicity of the shβ4-1 LoVo 3D cultures was lower than that of the shcontrol (Fig. 8B). In summary, these results indicated that integrin β4 reduces DNA damage-induced p53 activation to decrease CRC chemosensitivity to platinum.

Knockdown of p53 does not markedly change integrin β4 protein levels in HCT116 cells. It was reported that p53 regulated integrin β4 expression in several cells types, including tumor cells (21-23). The effect of p53 on integrin β4 expression in HCT116 3D cultures was investigated. HCT116 3D cultures were treated with or without 10 μg/ml CDDP for 24 or 48 h, respectively. Integrin β4 expression levels were assayed using western blot analysis. No detectable difference in integrin β4 expression levels was observed between shcontrol HCT116 cells and the respective shp53 HCT116 cells (Fig. 9A). Immunohistochemical staining was employed to evaluate the integrin β4 protein level of HCT116 3D cultures without any treatment. Knockdown of p53 (shp53) did not detectably change the integrin β4 protein level (Fig. 9B). The effect of p53 on integrin β4 in HCT116 2D cultures was studied. HCT116 2D cultures were treated

with or without 2.5 μg/ml CDDP for 24 or 48 h, respectively. Integrin β4 expression levels were assayed using western blot analysis. No detectable difference in integrin β4 expression levels was observed (Fig. 9C). These results indicated that knockdown of p53 may not detectably change the expression of integrin β4 under these specific conditions.

Discussion

Solid tumor cells, including CRC cells, proliferate, survive and response to stimuli in a specific tissue microenvironment *in vivo* (3,7,8). In addition, 3D cell culture models provide an optimal experimental system for mimicking solid tumor tissue microenvironments, particularly cell adhesion *in vivo* (Fig. 1) (3,7,8). In 3D cultures, cells adhered to each other within layers of cells, and cell junctions were commonly found (Fig. 1B-E) (7). Their structures were more similar to these of tumors at avascular stage and avascular tumor regions (3,7,8).

The formation and stability of cell adhesion rely on cell adhesion molecules, particularly E-cadherin and the integrin family (6-11). Integrins, consisting of α and β subunits, are a group of transmembrane heterodimeric cell surface receptors that enhance cell anchorage to the ECM and cell-cell interaction (6,9-11). It has been reported that 24 definite integrin heterodimers are established by the amalgamation of 18 α subunits and 8 β subunits (9,10). In the present study, integrin β4 knockdown did not prevent suspended CRC cells from forming 3D cultures (Fig. 3A). This indicated that integrin β4 may not be essential for architectural formation of 3D cultures or very low expression of integrin β4 may be sufficient. Integrin β4 is different from other integrin β subunits for its exceptionally large cytoplasmic domain and its upregulation correlates with changes in cell biology (10,12,23,24). High expression of integrin β4 has been reported in human CRC, and it was also reported that cell adhesion affects cell adhesion molecules expression levels (8,14). In the present study, the integrin β4 expression level in HCT116 3D cultures was higher than in 2D cultures, while in LoVo 3D cultures, the integrin β4 expression level was similar to or slightly lower than in 2D cultures (Fig. 2A). These results indicated that the function of cell adhesion in integrin β4 expression may be cell-type specific in CRC. Another possible explanation is that the difference may be caused by the difference in the microenvironment between HCT116 3D cultures and LoVo 3D cultures since the two cell lines form different shapes of multicellular spheroids (Fig. 3B).

Integrin contributes to the maintenance of cell adhesion (6,9,10,23,25). On the other hand, mounting evidence has indicated that integrin couples intra- and extra-cellular signals, since it rapidly undergoes conformational switches transduced via cytoplasmic changes ('inside-out' signaling) and simultaneous ligand-induced rearrangements ('outside-in' signaling) (6,9,25). Inactive integrins are compact and bent, with their genu folded and the headpiece ~5 nm from the membrane. Separation of the α and β subunit legs destabilizes their interface with the headpiece, converting the bent structure to an overall extended conformation and relieving constraints on headpiece activation (6,9,10,21,25). Several factors, such as ECM-cell adhesion, cell-cell adhesion and force, were reported to activate integrins (6,9-11,25). Furthermore, 3D cultures represent the solid tumor tissue microenvironment, particularly cell adhesion *in vivo* (Fig. 1B-E) (3,6-8). Under 3D condition, integrin β4 exhibited the ability to reduce DNA damage-induced p53 acivation (Fig. 4B-D). This effect was not observed in HCT116 2D cultures (Fig. 5A and B). These results indicated that integrin β4 reduced DNA damage-induced p53 activation in 3D cultures and this may be due to integrin β4 activation. Due to a myriad of difference in the microenvironment between 3D cultures and 2D cultures (3,6,8), it is unclear what causes integrin β4 activation under the specific condition.

Platinum and irradiation damages DNA by binding to and causing crosslink of DNA to kill cells (3,15,16,26). Mounting evidence has demonstrated that the activation of p53 plays a key role in the DNA damage response, and loss of wild-type p53 leads to resistance to DNA damage-induced cell death (3,15,18,20,27,28). In the present study, integrin β4 reduced DNA damage-induced p53 activation, and knockdown of integrin β4 increased sensitivity to CDDP (Figs. 4, 5 and 8). Knockdown of wild-type p53 [both HCT116 and LoVo contain a stabilized wild-type p53 protein (3)] decreased sensitivity to platinum (Figs. 6 and 7). In summary, these findings indicated that integrin β4 reduced DNA damage-induced p53 activation. This may contribute to explain the phenomenon that the integrin expression level negatively correlated with prognosis in multiple cancer types (10,12,14,23). Furthermore, mutated *p53* or loss of p53 is frequently observed in cancer (17,21,27) and *p53* mutations can also inactivate the protein normal function (18,22,28). These results may also contribute to explain that loss of p53 or mutated *p53* often acquired drug resistance and that loss of p53 or mutated *p53* negatively correlated with survival of cancer patients, including CRC patients (3,15,20,23,26-28).

In the present study, knockdown of integrin β4 appeared to increase chemosensitivity to a lesser degree than that caused by knockdown of p53 (Figs. 6-8). Integrin β4 triggers numerous signaling cascades, and p53 can be regulated by a variety of factors (3,10,12,14,17,20-25,27). It is reasonable to hypothesize that other signaling cascades, besides integrin β4-p53 pathway, may be involved in the mechanism underlying the development of CRC resistance to DNA damage.

In summary, 3D cultures consist of layers of cells that preserve cell adhesive systems. These present a good model to deciphering the function of cell adhesive systems in cancer. Cell adhesion triggers certain integrin signaling cascades and influences tumor cell biological behavior, including chemosensitivity (6,9-11). Data in the present study indicated that integrin β4 reduced DNA damage-induced p53 activation to decrease DNA damage-induced cell death; this may be due to integrin β4 activation in 3D cultures. However, the mechanism of adhesion-associated CRC cell resistance to DNA damage *in vivo* is complex and deserves further investigation.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

JW, RZ and JL performed the experiments; JW and BL analyzed the data, designed the research and wrote the paper. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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