Adenovirus-mediated truncated Bid overexpression induced by the Cre/LoxP system promotes the cell apoptosis of CD133+ ovarian cancer stem cells

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Abstract. Cancer stem cells are a small subset of cancer cells that contribute to cancer progression, metastasis, chemoresistance and recurrence. CD133-positive (CD133+) ovarian cancer cells have been identified as ovarian cancer stem cells. Adenovirus-mediated gene therapy is an innovative therapeutic method for cancer treatment. In the present study, we aimed to develop a new gene therapy to specifically eliminate CD133+ ovarian cancer stem cells by targeting CD133. We used the Cre/LoxP system to augment the selective expression of the truncated Bid (tBid) gene as suicide gene therapy in CD133+ ovarian cancer stem cells. The adenovirus (Ad)-CD133-Cre expressing Cre recombinase under the control of the CD133 promoter and Ad-CMV-LoxP-Neo-LoxP-tBid expressing tBid under the control of the CMV promoter were successfully constructed using the Cre/LoxP switching system. The co-infection of Ad-CMV-LoxP-Neo-LoxP-tBid and Ad-CD133-Cre selectively induced tBid overexpression, which inhibited cell growth and triggered the cell apoptosis of CD133+ ovarian cancer stem cells. The Cre/LoxP system-mediated tBid overexpression activated the pro-apoptotic signaling pathway and augmented the cytotoxic effect of cisplatin in CD133+ ovarian cancer stem cells. Furthermore, in xenograft experiments, co-infection with the two recombinant adenoviruses markedly suppressed tumor growth *in vivo* and promoted cell apoptosis in tumor tissues. Taken together, the present study provides evidence that the adenovirus-mediated tBid overexpression induced by the Cre/LoxP system can effectively eliminate CD133+ ovarian cancer stem cells, representing a novel therapeutic strategy for the treatment of ovarian cancer.

Introduction

Ovarian cancer is one of the most lethal gynecologic cancers with high morbidity in women worldwide (1). Surgical resection followed by chemotherapy has been considered the most effective treatment for ovarian cancer; however, most patients are diagnosed at advanced stages and undergo recurrence after treatment, which leads to a poor survival rate (2,3). To date, there is still lack of an effective therapeutic method for ovarian cancer. It is of great importance to develop promising strategies to improve the treatment of ovarian cancer.

Cancer stem cells, a small subset of cancer cells, are capable of self-renewal and multi-lineage differentiation into cells that can form tumors (4). These cells have been identified in various types of cancer and contribute to cancer progression, metastasis, chemoresistance and recurrence (5-8). Therefore, the elimination of cancer stem cells may be a potential and promising therapy for the treatment of cancer. Cancer stem cells express distinct cell surface markers such as CD24, CD44, CD119 and CD133 (6,9,10). Among these, CD133+ cancer stem cells have been widely studied in ovarian cancers as CD133+ cells isolated from ovarian cancer cells or primary ovarian tumor tissues show self-renewal, multi-lineage differentiation and tumor initiation capabilities (10-12). Given these findings, the elimination of CD133+ ovarian cancer stem cells could be a novel and effective therapeutic strategy for preventing ovarian cancer.

Truncated Bid (tBid), the C-terminal of full-length Bid (a BH3-only subgroup of the Bcl-2 family member) cleaved by caspase-8, with a molecular weight of 15 kDa, is a potent inducer of cell apoptosis (13,14). tBid translocates to mitochondria and activates the release of cytochrome *c* from mitochondria to

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the cytosol wherein cytochrome *c* activates caspase-9/-3/-7 to trigger cell apoptosis (14-16). Thus, tBid has been proposed as a promising molecular target for killing cancer cells (17,18). Recombinant adenoviruses have been suggested as an effective gene delivery tool for cancer treatment. However, adenovirus-mediated tBid expression initiated by the CMV promoter may kill packaging cells during the production of the recombinant adenovirus, making it difficult to obtain high titers of recombinant adenovirus. Therefore, a conditional expression system is needed.

Cre ('Causes recombination') is a site-specific recombinase that can mediate the specific recombinant process between two LoxP (locus of crossover P1) sequences (19,20). In the present study, we designed a recombinant adenovirus carrying tBid (with CMV promoter) and a null gene expression cassette (Neo) flanked by LoxP which was inserted upstream of tBid to block tBid expression (Ad-CMV-LoxP-Neo-LoxP-tBid). To conditionally initiate tBid expression, we introduced another recombinant adenovirus carrying Cre (without CMV promoter), with a CD133 promoter inserted upstream of Cre. Thus, Cre may only be expressed when the CD133 promoter was activated (Ad-CD133-Cre). We hypothesized that infection of Ad-CD133-Cre into CD133⁺ ovarian cancer stem cells may trigger the expression of Cre which may then mediate the LoxP recombinant and cut-off the null gene expression cassette between the two LoxP, ultimately leading to tBid overexpression. The activity of the CD133 promoter is weaker than that of the CMV promoter, but it has been reported that a very small amount of Cre is able to cut-off LoxP sequences effectively (19,21). Therefore, CD133 promoter-mediated Cre expression in CD133⁺ ovarian cancer stem cells is sufficient to trigger tBid overexpression. Together, this Cre/LoxP system mediated-tBid overexpression could specifically function in and kill CD133⁺ ovarian cancer stem cells, representing a potential and promising adjuvant therapy for the prevention of ovarian cancer metastasis and recurrence.

Materials and methods

Animals. Six-week-old female severe combined immune deficient (SCID) mice (weighing 18-25 g) were purchased from the Guangdong Medical Laboratory Animal Center (Guangzhou, China) and housed under specific pathogen-free conditions (21±2˚C and 12/12 h light/dark) with free access to food and water. The animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Nanjing Medical University Affiliated Suzhou Hospital.

Ovarian cancer cell culture. The ovarian cancer cell line A2780 purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from Gibco, Rockville, MD, USA), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma, St. Louis, MO, USA). Cells were routinely cultured in a humidified incubator containing 5% CO_2 at 37˚C.

Generation of CD133⁺ ovarian cancer stem cells. The CD133⁺ ovarian cancer stem cells were isolated by magnetic bead sorting as previously described (22). Briefly, cells were dissociated with 0.25% trypsin (Sigma) and suspended in ice-cold phosphate-buffered saline (PBS). Then, single cells were incubated with microbeads labeled with anti-CD133/1 monoclonal antibodies (Miltenyi Biotech, Auburn, CA, USA) for 20 min at 4˚C in the dark. The samples were then washed with ice-cold PBS and sorted on a BD fluorescence-activated cell sorting (FACS)Aria system (BD Biosciences, San Jose, CA, USA). The CD133⁺ cells were cultured in serum-free medium containing 10 ng/ml human recombinant basic fibroblast growth factor and 20 ng/ml human recombinant epidermal growth factor (Invitrogen, Carlsbad, CA, USA).

Construction, production and purification of recombinant adenovirus. The cDNA fragments of the CD133 promoter and Cre were amplified and inserted into adenovirus pShuttle plasmid (Stratagene, Santa Clara, CA, USA) to construct the Ad-CD133-Cre recombinant adenovirus. The cDNA fragments of LoxP-Neo-LoxP and tBid were inserted into pShuttle-CMV plasmid (Stratagene) to construct the Ad-CMV-LoxP-Neo-LoxP-tBid recombinant adenovirus. The recombinant pShuttle and pAdEasy-1 plasmids (Stratagene) were homologously recombined in BJ5183 bacteria. The recombined plasmids were linearized and transfected into 293T cells (ATCC) to produce the recombinant virus. After 14 days, the cells were harvested and lyzed by freeze-thawing. The supernatants were collected and concentrated by CsCl gradient centrifugation. The titers were determined using the 50% tissue culture infectious dose method (23). Cells were infected with Ad-CD133-Cre at a multiplicity of infection (MOI) of 20 and Ad-CMV-LoxP-Neo-LoxP-tBid at MOI 30.

Western blot analysis. Mitochondrial and cytosolic proteins were extracted using a mitochondria isolation kit (Pierce, Rockford, IL, USA). The proteins were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was then blocked in 2.5% non-fat milk at 37˚C for 1 h. The membrane was blotted with primary antibodies at 4˚C overnight. After washing three times with Tris-buffered saline (TBS)-Tween (TBST) and once with TBS, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000; Bioss Antibodies, Beijing, China) for 1 h at 37˚C. After washing with TBST, the protein bands were visualized using a chemiluminescence detection procedure (Amersham Biosciences, Beijing, China). The band intensity was quantified using Image Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). The primary antibodies used in this experiment were as follows: anti-Bid, anti-Cre, anti-cytochrome *c*, anti-cleaved caspase-9 and anti-β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-cleaved caspase-3 and anti-COX IV (Abcam, Cambridge, MA, USA).

Cell viability assay. Cell growth and viability were detected by MTT assay. Briefly, cells were plated onto a 96-well plate at a density of 1x10⁴ cells/well and cultured overnight. Thereafter, the cells were infected with Ad-CD133-Cre or/and Ad-CMV-LoxP-Neo-LoxP-tBid and incubated for 48 h. The medium was replaced with fresh medium and 20 µl of MTT
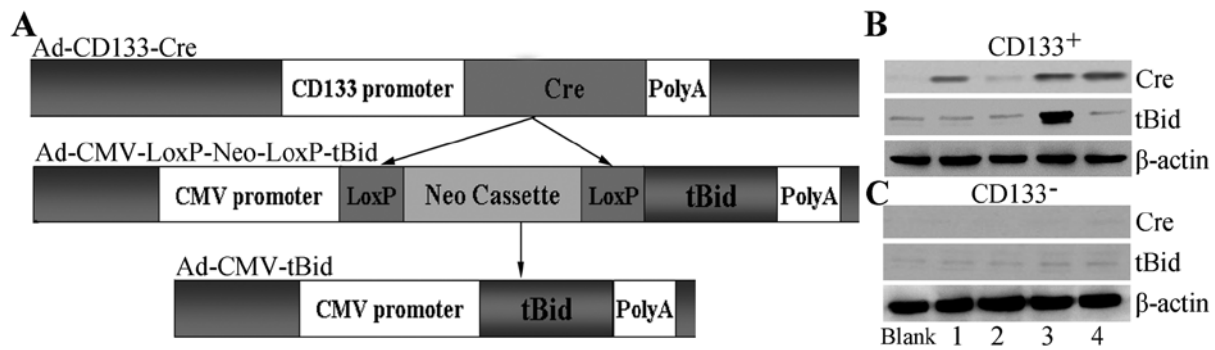
Figure 1. Cre/LoxP system-mediated tBid overexpression in CD133+ ovarian cancer stem cells. (A) Diagram of Cre/LoxP system-mediated tBid overexpression. Western blot analysis of Cre and tBid protein expression in (B) CD133+ ovarian cancer stem cells or (C) CD133- ovarian cancer cells. Blank, cells without treatment; lane 1, cells infected with Ad-CD133-Cre; lane 2, cells infected with Ad-CMV-LoxP-Neo-LoxP-tBid; lane 3, cells co-infected with Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-tBid; and lane 4, cells co-infected with Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-GFP.

stock solution (5 mg/ml; Sigma) was added to each well. After culturing for 4 h, 200 μl of dimethyl sulfoxide was added to each well to dissolve the formazan crystals. Then, absorbance at a wavelength of 490 nm was detected using a microplate reader (BioTek Instruments, Winooski, VT, USA).

TUNEL assay. Cell apoptosis was assessed using the terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) kit (Roche, Indianapolis, IN, USA). Briefly, the infected cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Then, the cells were incubated with TUNEL reaction mixtures for 1 h at 37˚C. Then, the cells were observed using a fluorescence microscope (Olympus, Tokyo, Japan), and apoptotic cells were counted in five random fields/slide.

Flow cytometric analysis. Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) stained cells were detected by flow cytometric analysis according to the manufacturer's instructions (Jiancheng Bioengineering Institute, Nanjing, China). Briefly, cells were dissociated with trypsin, washed with ice-cold PBS and resuspended in binding buffer at 1×10^6 cells/ml. Then, Annexin V and PI stock solution were added and incubated at 4˚C in the dark. Thereafter, the samples were detected by FACS and the data were analyzed by FlowJo software (BD Biosciences).

In vivo xenograft experiments. CD133+ ovarian cancer stem cells (1×10^5 cells) were suspended in 200 μl of PBS and subcutaneously injected into the right flank of SCID mice (n=6 mice/group). Approximately 1×10^{10} plaque-forming units of recombinant adenovirus diluted in 50 μl of PBS were intratumorally injected every 3 days. Tumor volume (V) was measured using an external caliper and calculated according to the formula: V = length x width^2 x π/6. The mice were sacrificed by subcutaneous injection with sodium pentobarbital (100 mg/kg) 21 days after inoculation and the tumors were excised for western blot analysis and TUNEL assay.

Data analysis. Data are presented as mean ± standard deviation (SD). Statistical analyses were performed by one-way analysis of variance followed by Bonferroni post hoc test using

SPSS version 11.5 (SPSS, Inc., Chicago, IL, USA). Differences were considered statistically significant at p<0.05.

Results

Cre/LoxP system-mediated tBid overexpression in CD133+ ovarian cancer stem cells. To achieve conditional tBid overexpression, we introduced a Cre/LoxP-mediated expression system (Fig. 1A). Briefly, we cloned the CD133 promoter into an adenovirus and inserted the open reading frame of Cre downstream from the CD133 promoter. Therefore, Cre was expressed only when the CD133 promoter was activated. Meanwhile, we constructed another recombinant adenovirus containing a flanked LoxP cassette upstream of tBid. Thus, tBid was expressed only when the LoxP cassette was deleted. To test whether this Cre/LoxP system was successful, we infected these recombinant adenoviruses into CD133+ ovarian cancer stem cells. The results showed that Cre was expressed in the Ad-CD133-Cre-infected CD133+ ovarian cancer stem cells (Fig. 1B). The single infection of Ad-CMV-LoxP-Neo-LoxP-tBid did not result in tBid overexpression whereas co-infection of Ad-CD133-Cre with Ad-CMV-LoxP-Neo-LoxP-tBid into CD133+ ovarian cancer stem cells resulted in tBid overexpression (Fig. 2B). In contrast, in CD133- cells, infection of Ad-CD133-Cre or/and Ad-CMV-LoxP-Neo-LoxP-tBid did not produce Cre or tBid (Fig. 1C). These results indicated that the Cre/LoxP-mediated tBid overexpression system was successfully established in the CD133+ ovarian cancer stem cells.

Cre/LoxP system-mediated tBid overexpression promotes cell apoptosis of CD133+ ovarian cancer stem cells. To determine the efficiency of Cre/LoxP system-mediated tBid overexpression in gene therapy against ovarian cancer, we assessed its biological effect on cell growth and apoptosis in CD133+ ovarian cancer stem cells *in vitro.* The results showed that the single infection of Ad-CD133-Cre or Ad-CMV-LoxP-Neo-LoxP-tBid had no obvious effect on cell growth or viability, as detected by MTT assay (Fig. 2A). However, the co-infection of Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-tBid significantly inhibited the growth of CD133+ ovarian cancer stem cells (Fig. 2A). Furthermore, Annexin V-FITC/PI assay
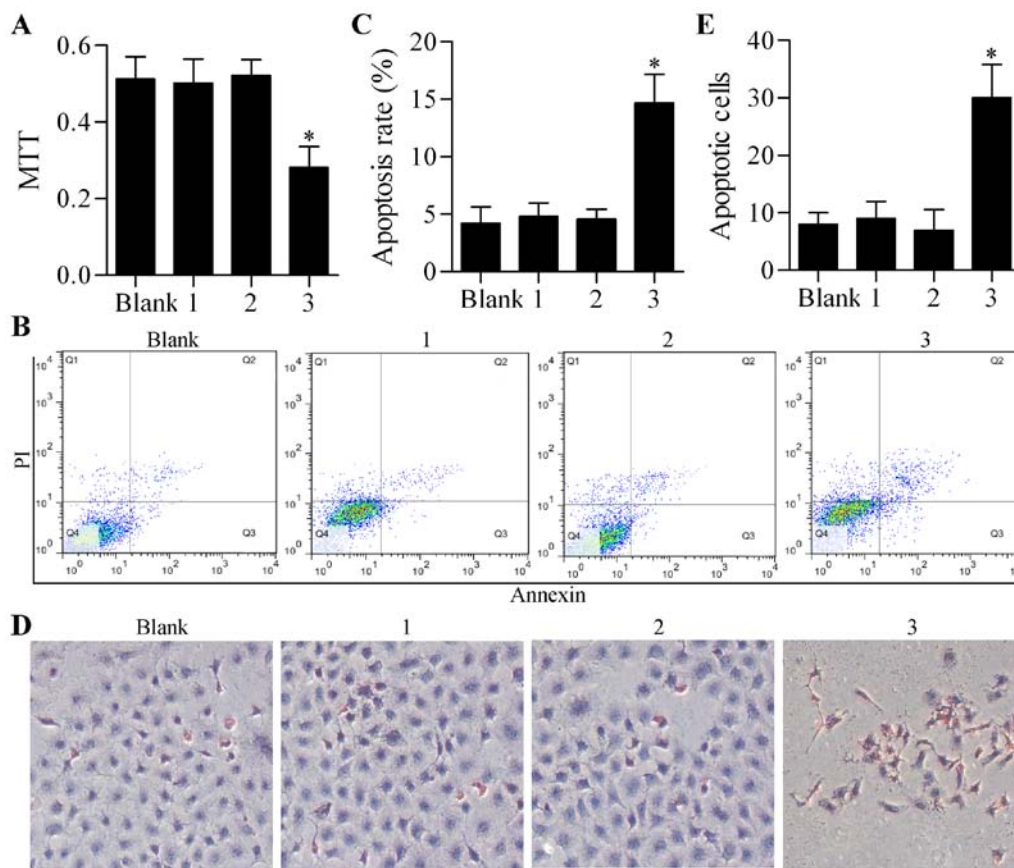
Figure 2. Cre/LoxP system-mediated tBid overexpression promotes the apoptosis of CD133+ ovarian cancer stem cells. CD133+ ovarian cancer stem cells were infected with recombinant adenovirus (blank, cells without treatment; 1, cells infected with Ad-CD133-Cre; 2, cells infected with Ad-CMV-LoxP-Neo-LoxP-tBid; and 3, cells co-infected with Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-tBid) and incubated for 48 h, and subjected to the following procedures: (A) cell growth was detected by MTT assay, (B) cell apoptosis was detected by Annexin V-FITC/PI or (D) TUNEL assays. (C) Quantitative statistics of apoptosis rate in B. (E) Quantitative statistics of apoptotic cells in D; *p<0.05 vs. blank, group 1 and 2.

also showed a significantly high apoptosis rate in the CD133+ ovarian cancer stem cells co-infected with Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-tBid (Fig. 2B and C). Similarly, TUNEL assay showed that the co-infection of Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-tBid markedly increased cell apoptosis of the CD133+ ovarian cancer stem cells compared with single infection of Ad-CD133-Cre or Ad-CMV-LoxP-Neo-LoxP-tBid (Fig. 2D and E). Taken together, these results suggested that Cre/LoxP system-mediated tBid overexpression effectively promoted the apoptosis of CD133+ ovarian cancer stem cells.

Cre/LoxP system-mediated tBid overexpression activates the pro-apoptotic signaling pathway. To further verify the effect of Cre/LoxP system-mediated tBid overexpression on induction of cell apoptosis, we assessed its effect on the pro-apoptotic signaling pathway. The results showed that the co-infection of Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-tBid significantly upregulated the release of cytochrome *c* from mitochondria to the cytosol compared with the other groups (Fig. 3A and B). Furthermore, the activated forms of caspase-9 and caspase-3 (Fig. 3C) were also significantly upregulated by co-infection of Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-tBid in the CD133+ ovarian cancer stem cells. Taken together, these results indicated that the Cre/LoxP system-

mediated tBid overexpression significantly activated the pro-apoptotic signaling pathway in the CD133+ ovarian cancer stem cells.

Cre/LoxP system-mediated tBid overexpression augments the cytotoxic effect of cisplatin in CD133+ ovarian cancer stem cells. To further determine the efficiency of Cre/LoxP system-mediated tBid overexpression in gene therapy against ovarian cancer, we investigated the effect of Cre/LoxP system-mediated tBid overexpression on cisplatin-induced cytotoxicity. The results showed that cisplatin treatment inhibited the growth and viability of CD133+ ovarian cancer stem cells, and that the single infection of Ad-CD133-Cre or Ad-CMV-LoxP-Neo-LoxP-tBid had no obvious effect on cisplatin-induced cytotoxicity (Fig. 4A). However, co-infection with Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-tBid significantly augmented the cytotoxic effect of cisplatin in the CD133+ ovarian cancer stem cells (Fig. 4A). Furthermore, cisplatin-induced cell apoptosis was also enhanced by co-infection with Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-tBid in comparison with single infection of Ad-CD133-Cre or Ad-CMV-LoxP-Neo-LoxP-tBid (Fig. 4B-E). Taken together, these results suggested that Cre/LoxP system-mediated tBid overexpression increased the sensitivity of CD133+ ovarian cancer stem cells to cisplatin.
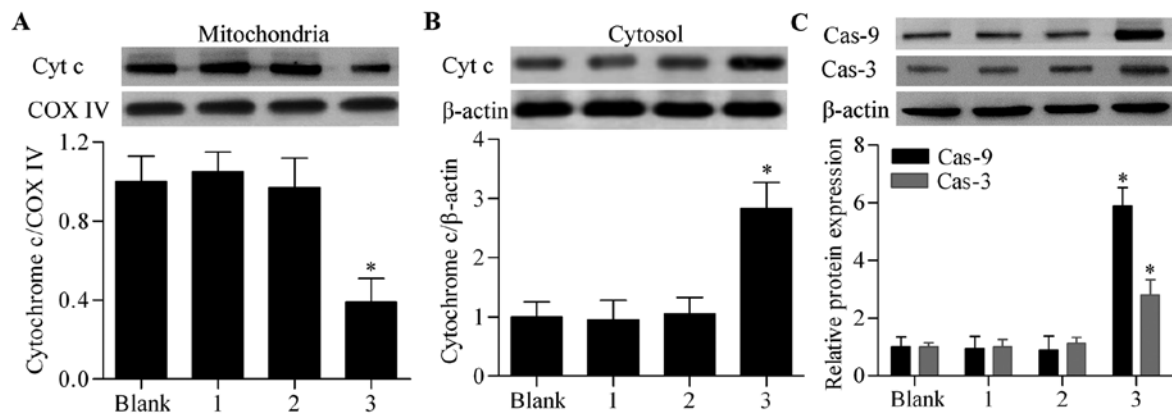
Figure 3. Cre/LoxP system-mediated tBid overexpression activates the pro-apoptotic signaling pathway. CD133+ ovarian cancer stem cells were infected with recombinant adenovirus (blank, cells without treatment; 1, cells infected with Ad-CD133-Cre; 2, cells infected with Ad-CMV-LoxP-Neo-LoxP-tBid; and 3, cells co-infected with Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-tBid) and incubated for 48 h, and subjected to western blot analysis. Western blot analysis of cytochrome c in (A) mitochondria or (B) cytosol. (C) Western blot analysis of cleaved caspase-9 and caspase-3 in the different cell groups; *p<0.05 vs. blank, group 1 and 2.
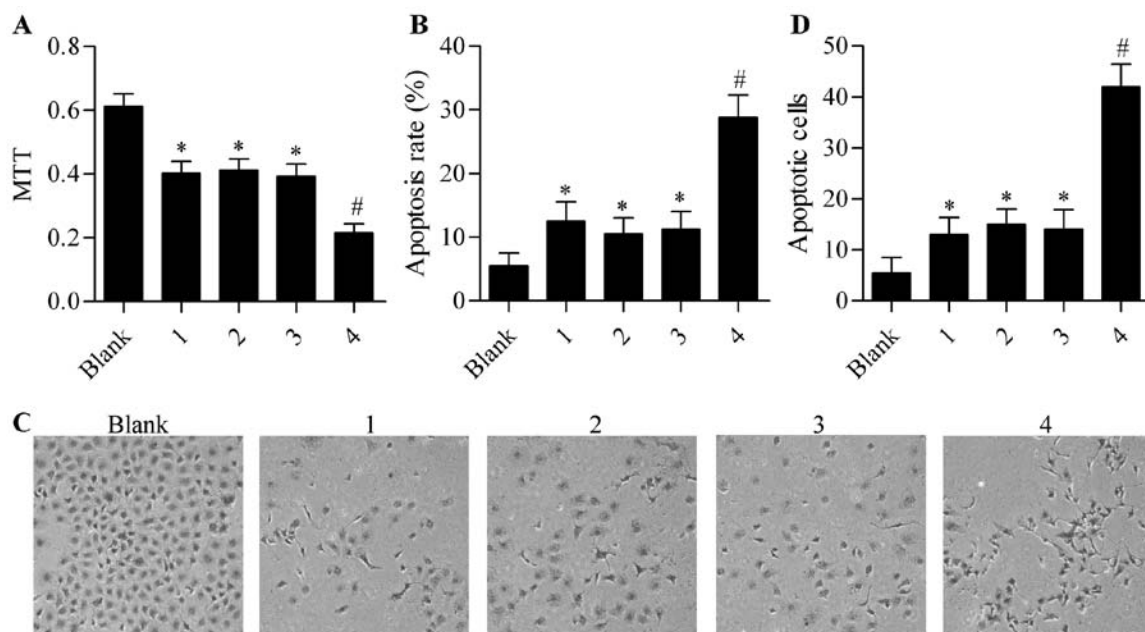

Figure 4. Cre/LoxP system-mediated tBid overexpression enhances the effect of cisplatin on apoptosis in the CD133+ ovarian cancer stem cells. CD133+ ovarian cancer stem cells were treated with cisplatin (10 µg/ml) and infected with recombinant adenovirus. Blank, cells without treatment; 1, cells treated with cisplatin; 2, cells treated with cisplatin and Ad-CD133-Cre; 3, cells treated with cisplatin and Ad-CMV-LoxP-Neo-LoxP-tBid; 4, cells treated with cisplatin and Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-tBid. (A) Cell growth was detected by MTT assay. (B) Cell apoptosis rate was detected by Annexin V-FITC/PI staining method. (C) TUNEL assay of cell apoptosis, and (D) quantitative statistics of apoptotic cells; *p<0.05 vs. blank; #p<0.05 vs. group 1, 2 and 3.

Cre/LoxP system-mediated tBid overexpression inhibits tumor growth in vivo. To further confirm the antitumor effect of Cre/LoxP system-mediated tBid overexpression against CD133+ ovarian cancer stem cells, we subcutaneously inoculated the tumor cells into SCID mice. The recombinant adenoviruses were intratumorally injected into the mice and the tumor volume was monitored. The results showed that the single infection of Ad-CD133-Cre or Ad-CMV-LoxP-Neo-LoxP-tBid had no obvious effect on tumor growth compared with the non-infected control group (Fig. 5A). As expected, the co-infection of Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-tBid

significantly suppressed tumor growth compared with the other groups (Fig. 5A). TUNEL assay showed that the apoptotic cells were not significantly altered in the Ad-CD133-Cre or Ad-CMV-LoxP-Neo-LoxP-tBid single-infected group, but markedly increased in the Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-tBid co-infected group (Fig. 5B). By analysis of xenograft tumor tissues, we found that tBid was overexpressed in the Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-tBid co-infected group (Fig. 5C and D). Moreover, caspase-3 activity was also markedly upregulated by co-infection with Ad-CD133-Cre and Ad-CMV-LoxP-
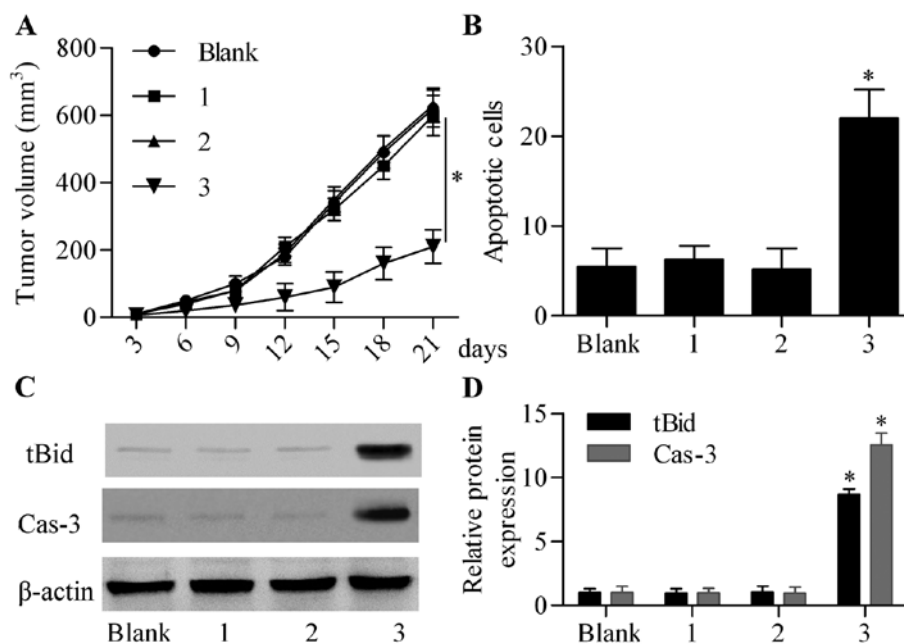
Figure 5. Cre/LoxP system-mediated tBid overexpression inhibits tumor growth *in vivo*. (A) The tumor growth curve of mice implanted with CD133⁺ ovarian cancer stem cells. The recombinant adenoviruses were intratumorally injected into the mice. Control, mice without infection; 1, mice infected with Ad-CD133-Cre; 2, mice infected with Ad-CMV-LoxP-Neo-LoxP-tBid; and 3, mice infected with Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-tBid. (B) Cell apoptosis was detected by TUNEL assay in tumor tissues of the different groups. (C) Western blot analysis of tBid and cleaved caspase-3 protein expression in tumor tissues of the different groups. (D) The relative quantitative protein expression of tBid and cleaved caspase-3 in tumor tissues of the different groups; *p<0.05 vs. blank, group 1 and 2.

Neo-LoxP-tBid (Fig. 5C and D). Taken together, these results revealed that Cre/LoxP system-mediated tBid overexpression triggered cell apoptosis and suppressed tumor growth *in vivo*.

Discussion

In the present study, we successfully established conditional truncated Bid (tBid) overexpression induced by the Cre/LoxP system. tBid overexpression mediated by the Cre/LoxP system was triggered in CD133⁺ ovarian cancer stem cells, significantly inducing cell apoptosis and inhibiting CD133⁺ ovarian cancer stem cell growth *in vitro* and *in vivo*. The present study suggests that adenovirus-mediated tBid overexpression induced by the Cre/LoxP system may represent a potential therapeutic tool for anti-ovarian cancer therapy.

CD133 has been suggested as a stem cell marker in many types of stem cells including hematopoietic, prostate epithelial and renal stem cells, and endothelial progenitor cells (24-27), implying that CD133 is associated with the original cell differentiation state. In malignant tumors, the promoter region of CD133 is in a hypomethylated state and CD133 is highly expressed in various types of cancers, including ovarian (6), breast (28), brain (5), prostate (29), colon (30) and pancreatic cancer (31). Increasing evidence suggests that CD133⁺ cells isolated from ovarian cancer cells or primary ovarian tumor tissues have self-renewal, multi-lineage differentiation and tumor initiation capabilities and are thus defined as ovarian cancer stem cells (10-12). A very small amount of CD133⁺ ovarian cancer stem cells can form tumors in SCID (12). Therefore, CD133 serves as a potential molecular target for eliminating CD133⁺ cancer stem cells. One approach for molecular-targeted therapy is monoclonal antibody therapy,

for example using monoclonal antibodies targeting CD44 and CD123 in the removal of human acute myeloid leukemic stem cells (32,33). Smith *et al* reported that the CD133 single-chain antibody conjugated with cytotoxic drugs inhibited hepatocellular and gastric cancer cell growth (34). However, the effect of CD133 antibody targeted therapy is still limited due to the complex molecular structure of CD133 (35,36).

Utilizing specific transcriptional regulation is another strategy. The carcinoembryonic antigen (CEA) promoter-mediated Cre/LoxP system was found to induce the expression of the cytosine deaminase and specifically inhibit CEA-producing gastric cancer cells *in vitro* and *in vivo* (37). Similarly, in CD133⁺ cells, the CD133 promoter can be used to control selective gene expression. In the present study, we wanted to introduce tBid, a potent suicide gene, into CD133⁺ ovarian cancer stem cells in order to kill the cells. However, the recombinant adenovirus carrying tBid driven by the CMV potent promoter is difficult to obtain since the tBid expression may kill packaging cells during the production of the recombinant adenovirus. Therefore, a conditional expression system was needed. Miao *et al* constructed a recombinant adenovirus containing a tBid gene driven by an α-fetoprotein (AFP) promoter and tBid was overexpressed in AFP-producing hepatocellular carcinoma cells (38). Similarly, a recent study demonstrated that a selected fragment of the AFP promoter designated as EA4D exhibited the highest reporter activity and that the tBid driven by EA4D was highly and specifically expressed in AFP-producing hepatocellular carcinoma cells (17). In CD133⁺ cancer stem cells, we used the CD133 promoter to drive tBid expression. However, the expression level of tumor-specific promoters is generally low and the activity of the CD133 promoter is weaker than that of

the CMV promoter (39). The tBid expression driven directly by the CD133 promoter is so low that it cannot effectively kill CD133$^+$ ovarian cancer stem cells. Therefore, a way of augmenting the selective expression of the tBid gene was needed.

In the present study, we used the Cre/LoxP regulation system to selectively augment tBid expression. We constructed two recombinant adenoviruses, Ad-CMV-LoxP-Neo-LoxP-tBid and Ad-CD133-Cre. The tBid cannot be expressed in Ad-CMV-LoxP-Neo-LoxP-tBid, and thus we were able to obtain a high titer of recombinant adenovirus. With regards to Ad-CD133-Cre, the Cre is expressed in CD133$^+$ cancer stem cells. Even though the CD133 promoter was a weaker promoter, a very small amount of Cre was able to cut-off LoxP sequences effectively (19,21). After LoxP was cut-off in Ad-CMV-LoxP-Neo-LoxP-tBid, the tBid was highly overexpressed as driven by the CMV promoter in CD133$^+$ cancer stem cells. Therefore, a selective and augmented tBid expression system was established. Co-infection with Ad-CMV-LoxP-Neo-LoxP-tBid and Ad-CD133-Cre effectively inhibited cell growth and induced cell apoptosis in the CD133$^+$ ovarian cancer stem cells. tBid is a potent apoptosis inducer and sensitizes apoptosis induced by chemotherapeutic drugs in cancer cells (40). The present study also revealed that Cre/LoxP system-mediated tBid overexpression augmented the cytotoxic effect of cisplatin in CD133$^+$ ovarian cancer stem cells. The data indicate that Cre/LoxP system mediated-tBid overexpression represents a potential and promising adjuvant therapy to prevent ovarian cancer metastasis and recurrence.

In conclusion, the present study applied the Cre/loxP system to augment the selective expression of the tBid gene in CD133$^+$ ovarian cancer stem cells. The Cre/LoxP system mediated-tBid overexpression significantly induced the apoptosis of CD133$^+$ ovarian cancer stem cells *in vitro* and *in vivo*, representing a future clinical approach for preventing ovarian cancer metastasis and recurrence. Furthermore, the adenovirus-mediated tBid overexpression induced by the Cre/LoxP system could be applicable to other CD133$^+$ cancer stem cells.

Acknowledgements

References

1. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2016. CA Cancer J Clin 66: 7-30, 2016.
2. Chekerov R, Braicu I, Castillo-Tong DC, Richter R, Cadron I, Mahner S, Woelber L, Marth C, Van Gorp T, Speiser P, *et al*: Outcome and clinical management of 275 patients with advanced ovarian cancer International Federation of Obstetrics and Gynecology II to IV inside the European Ovarian Cancer Translational Research Consortium-OVCAD. Int J Gynecol Cancer 23: 268-275, 2013.
3. Peiretti M, Bristow RE, Zapardiel I, Gerardi M, Zanagnolo V, Biffi R, Landoni F, Bocciolone L, Aletti GD and Maggioni A: Rectosigmoid resection at the time of primary cytoreduction for advanced ovarian cancer. A multi-center analysis of surgical and oncological outcomes. Gynecol Oncol 126: 220-223, 2012.
4. Bonnet D and Dick JE: Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3: 730-737, 1997.
5. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J and Dirks PB: Identification of a cancer stem cell in human brain tumors. Cancer Res 63: 5821-5828, 2003.
6. Zhang S, Balch C, Chan MW, Lai HC, Matei D, Schilder JM, Yan PS, Huang TH and Nephew KP: Identification and characterization of ovarian cancer-initiating cells from primary human tumors. Cancer Res 68: 4311-4320, 2008.
7. Iwasaki H and Suda T: Cancer stem cells and their niche. Cancer Sci 100: 1166-1172, 2009.
8. Zhao J: Cancer stem cells and chemoresistance: The smartest survives the raid. Pharmacol Ther 160: 145-158, 2016.
9. Gao MQ, Choi YP, Kang S, Youn JH and Cho NH: CD24$^+$ cells from hierarchically organized ovarian cancer are enriched in cancer stem cells. Oncogene 29: 2672-2680, 2010.
10. Curley MD, Therrien VA, Cummings CL, Sergent PA, Koulouris CR, Friel AM, Roberts DJ, Seiden MV, Scadden DT, Rueda BR, *et al*: CD133 expression defines a tumor initiating cell population in primary human ovarian cancer. Stem Cells 27: 2875-2883, 2009.
11. Moserle L, Indraccolo S, Ghisi M, Frasson C, Fortunato E, Canevari S, Miotti S, Tosello V, Zamarchi R, Corradin A, *et al*: The side population of ovarian cancer cells is a primary target of IFN-alpha antitumor effects. Cancer Res 68: 5658-5668, 2008.
12. Baba T, Convery PA, Matsumura N, Whitaker RS, Kondoh E, Perry T, Huang Z, Bentley RC, Mori S, Fujii S, *et al*: Epigenetic regulation of *CD133* and tumorigenicity of CD133$^+$ ovarian cancer cells. Oncogene 28: 209-218, 2009.
13. Esposti MD: The roles of Bid. Apoptosis 7: 433-440, 2002.
14. Luo X, Budihardjo I, Zou H, Slaughter C and Wang X: Bid, a Bcl2 interacting protein, mediates cytochrome *c* release from mitochondria in response to activation of cell surface death receptors. Cell 94: 481-490, 1998.
15. Liu X, Kim CN, Yang J, Jemmerson R and Wang X: Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome *c*. Cell 86: 147-157, 1996.
16. Li H, Zhu H, Xu CJ and Yuan J: Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94: 491-501, 1998.
17. Hu BG, Liu LP, Chen GG, Ye CG, Leung KK, Ho RL, Lin MC and Lai PB: Therapeutic efficacy of improved α-fetoprotein promoter-mediated tBid delivered by folate-PEI600-cyclodextrin nanopolymer vector in hepatocellular carcinoma. Exp Cell Res 324: 183-191, 2014.
18. Cristofanon S and Fulda S: ABT-737 promotes tBid mitochondrial accumulation to enhance TRAIL-induced apoptosis in glioblastoma cells. Cell Death Dis 3: e432, 2012.
19. Stricklett PK, Nelson RD and Kohan DE: Site-specific recombination using an epitope tagged bacteriophage P1 Cre recombinase. Gene 215: 415-423, 1998.
20. Kanegae Y, Lee G, Sato Y, Tanaka M, Nakai M, Sakaki T, Sugano S and Saito I: Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. Nucleic Acids Res 23: 3816-3821, 1995.
21. Zhou L, Wei X, Cheng L, Tian J and Jiang JJ: CD133, one of the markers of cancer stem cells in Hep-2 cell line. Laryngoscope 117: 455-460, 2007.
22. Long H, Xie R, Xiang T, Zhao Z, Lin S, Liang Z, Chen Z and Zhu B: Autocrine CCL5 signaling promotes invasion and migration of CD133$^+$ ovarian cancer stem-like cells via NF-κB-mediated MMP-9 upregulation. Stem Cells 30: 2309-2319, 2012.
23. Kanegae Y, Makimura M and Saito I: A simple and efficient method for purification of infectious recombinant adenovirus. Jpn J Med Sci Biol 47: 157-166, 1994.
24. Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MA, *et al*: Expression of VEGFR-2 and AC133 by circulating human CD34$^+$ cells identifies a population of functional endothelial precursors. Blood 95: 952-958, 2000.
25. Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV, Tsukamoto AS, Gage FH and Weissman IL: Direct isolation of human central nervous system stem cells. Proc Natl Acad Sci USA 97: 14720-14725, 2000.
26. Corbeil D, Röper K, Hellwig A, Tavian M, Miraglia S, Watt SM, Simmons PJ, Peault B, Buck DW and Huttner WB: The human AC133 hematopoietic stem cell antigen is also expressed in epithelial cells and targeted to plasma membrane protrusions. J Biol Chem 275: 5512-5520, 2000.

27. Weigmann A, Corbeil D, Hellwig A and Huttner WB: Prominin, a novel microvilli-specific polytopic membrane protein of the apical surface of epithelial cells, is targeted to plasmalemmal protrusions of non-epithelial cells. Proc Natl Acad Sci USA 94: 12425-12430, 1997.
28. Marsden CG, Wright MJ, Pochampally R and Rowan BG: Breast tumor-initiating cells isolated from patient core biopsies for study of hormone action. Methods Mol Biol 590: 363-375, 2009.
29. Maitland NJ and Collins AT: Prostate cancer stem cells: A new target for therapy. J Clin Oncol 26: 2862-2870, 2008.
30. O'Brien CA, Pollett A, Gallinger S and Dick JE: A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 445: 106-110, 2007.
31. Li C, Lee CJ and Simeone DM: Identification of human pancreatic cancer stem cells. Methods Mol Biol 568: 161-173, 2009.
32. Jin L, Hope KJ, Zhai Q, Smadja-Joffe F and Dick JE: Targeting of CD44 eradicates human acute myeloid leukemic stem cells. Nat Med 12: 1167-1174, 2006.
33. Jin L, Lee EM, Ramshaw HS, Busfield SJ, Peoppl AG, Wilkinson L, Guthridge MA, Thomas D, Barry EF, Boyd A, *et al*: Monoclonal antibody-mediated targeting of CD123, IL-3 receptor alpha chain, eliminates human acute myeloid leukemic stem cells. Cell Stem Cell 5: 31-42, 2009.
34. Smith LM, Nesterova A, Ryan MC, Duniho S, Jonas M, Anderson M, Zabinski RF, Sutherland MK, Gerber HP, Van Orden KL, *et al*: CD133/prominin-1 is a potential therapeutic target for antibody-drug conjugates in hepatocellular and gastric cancers. Br J Cancer 99: 100-109, 2008.
35. Deonarain MP, Kousparou CA and Epenetos AA: Antibodies targeting cancer stem cells: A new paradigm in immunotherapy? MAbs 1: 12-25, 2009.
36. Bidlingmaier S, Zhu X and Liu B: The utility and limitations of glycosylated human CD133 epitopes in defining cancer stem cells. J Mol Med 86: 1025-1032, 2008.
37. Ueda K, Iwahashi M, Nakamori M, Nakamura M, Matsuura I, Yamaue H and Tanimura H: Carcinoembryonic antigen-specific suicide gene therapy of cytosine deaminase/5-fluorocytosine enhanced by the Cre/loxP system in the orthotopic gastric carcinoma model. Cancer Res 61: 6158-6162, 2001.
38. Miao J, Chen GG, Chun SY, Yun JP, Chak EC, Ho RL and Lai PB: Adenovirus-mediated tBid overexpression results in therapeutic effects on p53-resistant hepatocellular carcinoma. Int J Cancer 119: 1985-1993, 2006.
39. Sato Y, Tanaka K, Lee G, Kanegae Y, Sakai Y, Kaneko S, Nakabayashi H, Tamaoki T and Saito I: Enhanced and specific gene expression via tissue-specific production of Cre recombinase using adenovirus vector. Biochem Biophys Res Commun 244: 455-462, 1998.
40. Miao J, Chen GG, Chun SY, Chak EC and Lai PB: Bid sensitizes apoptosis induced by chemotherapeutic drugs in hepatocellular carcinoma. Int J Oncol 25: 651-659, 2004.