Original Article
Combination of B7-H3 vaccination and Id-1 silencing eliminates melanoma in the murine B16 melanoma model *in vivo*

Yiran Liu[1,2], Bin Guo[3], Yu Dou[1], Ting Wu[4], Meng Liu[5], Hiroyuki Hamakawa[6], Shaohua Liu[1]

[1]Department of Oral and Maxillofacial Surgery, Qilu Hospital, Institute of Stomatology, Shandong University, 107 Wenhuaxi Road, Jinan 250012, P. R. China; [2]Department of Nephrology, Peking University First Hospital, Beijing, P. R. China; [3]Department of Stomatology, Jinan Central Hospital, Shandong University, Jinan, Shandong, P. R. China; [4]Department of Endodontics, Jinan Stomatologic Hospital, Jinan, Shandong, P. R. China; [5]Department of Clinical Chemistry and Transfusion Medicine, Sahlgrenska University Hospital, Göteborg, Västra Götalands län, Sweden; [6]Department of Oral and Maxillofacial Surgery, Ehime University Graduate School of Medicine, Ehime 791-0295, Japan

Abstract: Traditional therapies of malignant melanoma, such as surgical excision, demonstrate limitations of therapeutic effects in the late-stage melanoma. The emerging therapies, including immune therapy, gene therapy and combination therapy, have exhibited the superiority of the therapeutic effects. Using B16 melanoma model in Kunming mice, we demonstrated that both B7-H3 vaccination and Id-1 silencing highly suppressed the tumor growth and improved the survival rate in B16 melanoma model. Significantly, the combination of B7-H3 vaccination and Id-1 silencing eventually eliminated the tumors, and demonstrated a remarkable survival rate (66.67%) 150 days post tumor implantation. The enhanced cytotoxic activities of the splenic lymphocytes and increased serum IFN-γ levels from B7-H3 vaccinated mice indicated the tumor suppression might be mediated by the immune response. Consistent with the expressions of Id-1, the expressions of VEGF of Id-1 silencing tumor tissue were significantly reduced. Interestingly, the apoptotic cells in the tumor tissue from the mice received B7-H3 vaccination and Id-1 silencing combination treatment were significantly increased compared to the mice only treated with Id-1 silencing, suggesting the importance of apoptosis in the suppression of tumor growth mediated by Id-1, and also the facilitation of B7-H3 in the process. Taken together, these findings indicate that therapeutic alliance of B7-H3 immunization and Id-1 silencing is a valid approach of the treatment of malignant melanoma. Immune responses, suppression of angiogenesis and induction of apoptosis might be implicated in the process.

Keywords: Immunotherapy, genetic therapy, melanoma, Id-1, B7-H3

Introduction

Malignant melanoma, a malignant tumor of the epidermal melanocytes, develops with a poor survival rate and a broad pattern of metastasis. The traditional treatments, such as wide-excision surgery, chemotherapy, radiotherapy, or immunotherapy, have achieved certain therapeutic effects on early-stage melanoma but limited effects on late-stage melanoma [1, 2]. It has been generally believed that melanoma is susceptible to immune destruction and immune surveillance [3]. Accordingly, IFN-α, IL-2 and ipilimumab (antibody of cytotoxic T-lymphocyte 4 antigen (CTLA-4)), which have been approved for melanoma therapy, assumedly limit melanoma by the regulation of immune response [4-6]. The emerging therapy, gene therapy, exhibits its advantages in the discrimination of tumor and normal cells and the protection of immune function, however, the therapeutic effect of gene therapy is limited due to the availabilities of target genes and their functions [7]. Thus, the combination of gene therapies and immune therapies gives the potential of legitimacy and superiority, and might be the inevitable development direction of cancer gene therapy.

B7-H3, also known as CD276, is a recently identified member of B7 co-stimulatory molecules, which is the second signal required in the activation of T lymphocytes in anti-tumor immunity [8, 9]. The B7 family molecules play critical roles in the regulation of anti-tumor immunity, which enhance T-cell proliferation, increase IFN-γ secretion and prevent cell apoptosis, thereby stimulate T-cell responses [10]. However, the role of B7-H3 in T-cell regulation and anti-tumor immunity remains controversial. It has been found associated with better postoperative prognosis when over expressed in pancreatic cancer [11] and gastric cancer [12], but not in some other studies [13, 14]. B7-H3 over-expressed oral squamous cancer cell vaccine significantly enhanced the tumor-specific immune response including the proliferation, IFN-γ induction and the cytotoxicity of T-cells [15]. Hence, the vaccination of B7-H3 might play an important role in the regulation of the immune response to melanoma cells and might even be a potential therapeutic target for late-stage melanoma.

Id-1, inhibitor of DNA binding 1, belongs to the Id protein family, which is involved in normal cell differentiation [16, 17]. Id-1 has also been found up-regulated in numerous human cancer cells including melanoma cells, and found to promote cell proliferation through inactivation of tumor suppressors [18]. Further more, it promotes tumor angiogenesis through the induction of the VEGF [19], and protects the cancer cells from apoptosis resulting from chemotherapeutic drugs [20, 21]. These evidences implicate a positive role of Id-1 in the tumourigenesis of a wide range of human cancers through multiple aspects, and also suggest that the therapeutic implications through inactivation of Id-1 in the treatment of melanoma should also be addressed.

In this study, genetic immunization of B7-H3, Id-1 silencing and their alliance were conducted to treat malignant melanoma *in vivo*. Tumor growth, survival rate and apoptosis rate were investigated. We also test the cytotoxic activities of the splenic lymphocytes and serum IFN-γ levels from B7-H3 vaccinated mice, and the VEGF expressions of Id-1 silencing tumor tissue.

Materials and methods

Reagents

Thiazolyl Blue Tetrazolium Bromide (MTT) was purchased from Amresco in USA. Primary antibodies against B7-H3, Id-1, VEGF, pan-actin and Horseradish peroxidase (HRP)-conjugated anti-rabbit antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). pcDNA3.1-B7-H3, was gifted from Professor Xueying Sun of the University of Auckland in New Zealand.

Cells and cell culture

B16 (mouse malignant melanoma cell lines) and HEK293T cells were obtained from the Cell Bank of Chinese Academy of Sciences (CBCAS, Shanghai, China). The cells were maintained in RPMI-1640 (HyClone, USA) supplemented with 10% fetal bovine serum (HyClone, USA), 2 mM L-glutamine (HyClone, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (HyClone, USA) in the incubator with 5% CO_2 at 37°C.

Gene cloning

Murine B7-H3 amplified from pcDNA3.1-B7-H3 was inserted into an adenoviral shuttle vector, pAd-GFP. The recombinant, pAd-B7-H3-GFP, was selected with kanamycin and identified by sequencing. To knock down mouse *Id-1*, three hairpin RNA (shRNA), Id-1-164, Id-1-279 and Id-1-271, were designed. The amplified DNA was ligated to a lentivirus vector, Lv-GFP and then transformed into DH5α-competence *E. coli* cells. The selected colonies were amplified using PCR and the recombinants were identified with sequencing.

Adenovirus and lentivirus infection

Adenovirus infection: The pAd-B7-H3-GFP recombinants and pAd-GFP vectors were transfected into HEK293T cells by liposome to obtain the corresponding adenovirus. B16 cells were seeded at a density of 1×10^5 cells/ml in a 6-well plate, and were infected with titrated adenovirus of pAd-B7-H3-GFP or pAd-GFP at MOI 100:1 when the cells reached 80% confluence (Figure S1).

Lentivirus infection: The lentiviral vectors carrying Id-1 shRNA were packaged in HEK293T

cells. B16 cells were seeded at a density of 5×10^4 cells/ml in a 6-well plate and 24 hours later, were treated with 1×10^7 TU lentivirus for three days.

Cell proliferation and cytotoxicity assay

The cells were seeded in a 96-well plate at a density of 1×10^3/ml. At the desired time point, the cells were stained with 5 mg/ml sterile MTT and resolved in dimethyl sulfoxide (DMSO). The absorbance at 570 nm was measured via Microplate Reader (GloMax, Multi+, Promega).

Western blot

The protein of the cell lysates in RIPA buffer (Solarbio, China) was quantified using the BCA protein assay kit (Boster, China). Primary polyclonal antibodies against B7-H3, Id-1 and pan-actin (dilution 1:500) overnight at 4°C, HRP-conjugated anti-rabbit antibodies (dilution 1:1000) for 2 h, Immobilon Western Chemiluminescent HRP substrate (Millipore, USA), and FluorChem Q system (AlphaInnotech, USA) were used. The intensities of bands were quantified using AlphaInnotech-AlphaView. The desired protein quantification (B7-H3 or Id-1) was normalized to the internal control (pan-actin).

Enzyme-linked immunosorbent assay (ELISA)

IFN-γ in the serum of the mice was detected by ELISA kit purchased from R&D in USA. The procedures were performed according to the manufacture's instructions. The absorbance (at 450 nm) was measured using a Microplate Absorbance Reader (GloMax, Multi+, Promega). The amount of IFN-γ was determined by the interpolation from the standard curve.

Mice and in vivo study

The *in vivo* study including the anticipated tumor size and all the procedures were conducted in accordance with the protocols approved by the Animal Ethics Committee of Shandong University. Kunming (KM) mice (outbred mice derived from Swiss albino mice) were obtained from Experimental Animal Center (Shandong University, Jinan, China), and were maintained under specific pathogen free conditions. To investigate the effect of B7-H3 vaccination and Id-1 silencing on highly malignant melanoma, large tumor size (35 mm in diame-

ter) was approved and applied in this study. Euthanasia was applied when the maximum diameter of the tumor reached 35 mm. Butorphanol (0.05-0.1 mg/kg, i.m., qd) was used to reduce the stress of the mice when the diameter of the tumor reached 20 mm. Avertin (0.2 g/kg, i.p.) followed by cervical dislocation was used for sacrifice.

Tumor model: The *in vivo* melanoma model was developed by injecting 1×10^6 B16 cells subcutaneously at the left groin. The conditions of the mice were monitored every day since B16 cell inoculation. The maximum and minimum diameters (D_{max} and D_{min}) of the tumors were measured with a caliper and the tumor volumes were calculated using the formula: $V = D_{max} \times D_{min}^2/2$.

B7-H3 vaccination: B16 cells were infected with adenovirus of pAd-B7-H3-GFP, or pAd-GFP or mock at MOI 100:1 for 72 h. Alive cells identified with free stain of trypan blue were re-suspended in PBS at a density of 2×10^6/ml. 100 μl of the cell suspension (2×10^5) was injected into KM mice subcutaneously at the right anterior armpit for vaccination.

Id-1 silencing: To knock down Id-1 in KM mice, 1×10^8 TU of shId-1-164, shControl or mock lentivirus was intra-tumorally injected in the KM mice.

Effective T cells preparation: The spleens were grinded on a F200 mesh in sterile cold erythrocyte lysis buffer (Tris-NH$_4$Cl, 0.01 M Tris + 0.14 M NH$_4$Cl). The yeild splenocytes (more than 95% free stain with trypan blue), were cultured with RPMI1640 supplemented with 5 μg/ml phytohemagglutinin P (Solarbio, China) and 1 ng/ml interleukin-2 (IL-2, Peprotech, England) in the incubator at 37°C, 5% CO$_2$ for three days for effective T cell inductions.

Immunohistochemistry (IHC)

The slides of paraffin-embedded sections (4 μm) were treated with 3% hydrogen peroxide, subjected to antigen restoration in a microwave, blocked with 1.5% goat serum, and then incubated with the rabbit polyclonal antibodies against Id-1 and VEGF overnight at 4°C and streptavidin-HRP reagent (DAKO, Denmark) at 37°C for 30 min. After exposition to DAB, the
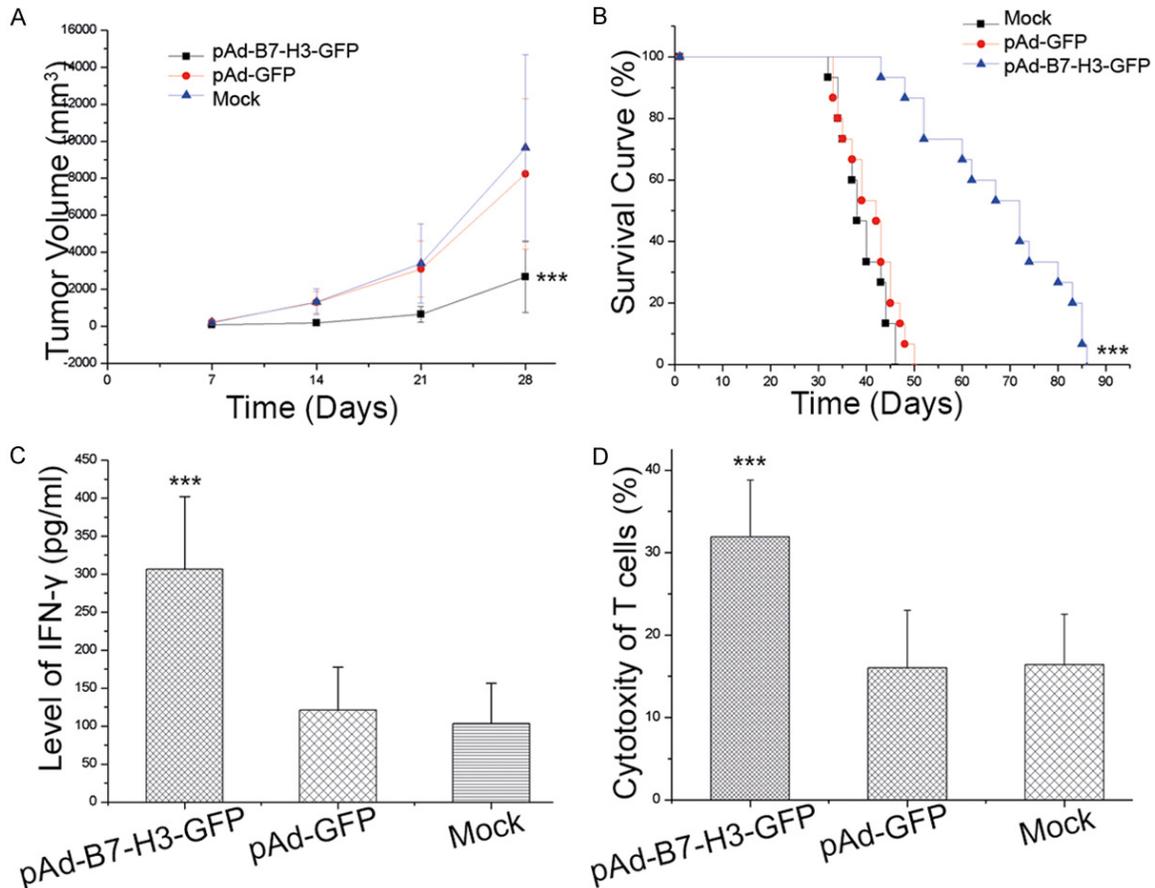
Figure 1. Tumor growth, survival rate, cyototoxicity and IFN-γ induction of B7-H3 vaccinated mice. The KM mice were vaccinated with pAd-B7-H3-GFP, pAd-GFP or mock adenovirus infected B16 cells before subject to B16 cell inoculation. (A, B) The tumor volumes (A) and the survival rates (B) post tumor implantation were analyzed, n = 15, ***P ≤ 0.001. (C) Serum IFN-γ levels of the KM mice seven days post tumor implantation were detected by ELISA, n = 6, ***P ≤ 0.001. (D) The splenic T cells from the KM mice two weeks post vaccination were applied to MTT assay. n = 6, ***P ≤ 0.001.

slides were counterstained with hematoxylin before dehydration and mount.

TUNEL assay

The procedures were followed by the instructions of TUNEL kit purchased from Beyotime Institute of Biotecnology in China. The counterstained slides were observed at high magnification (× 400). The apoptotic cells (N_{apop}) and normal cells (N_{norm}) in five representative fields were counted. The apoptosis index (AI) was calculated according to the formula: AI = N_{apop}/(N_{apop} + N_{norm}) × 100.

Statistical analysis

The survival rates were analyzed using Cox regression model and the other statistical sig-

nificances were analyzed using ANOVA. The analyses were performed using SPSS software (SPSS 17.0, USA). Data was presented as mean ± SD. *P* ≤ 0.05 was considered as statistical significance.

Results

B7-H3 vaccination inhibits tumor growth

B7-H3 vaccination suppressed the melanoma development: The KM mice were inoculated with pAd-B7-H3-GFP, pAd-GFP or mock-infected B16 cells (15 mice each group). Two weeks post the vaccination, the KM mice were injected with 1 × 10⁶ wild-type B16 cells to develop melanoma (Day 0). The volume of the tumors was recorded, and the survival time of KM mice were plotted (**Figure 1A** and **1B**). 28 days post

B7-H3 vaccination plus Id-1 silencing eliminates melanoma
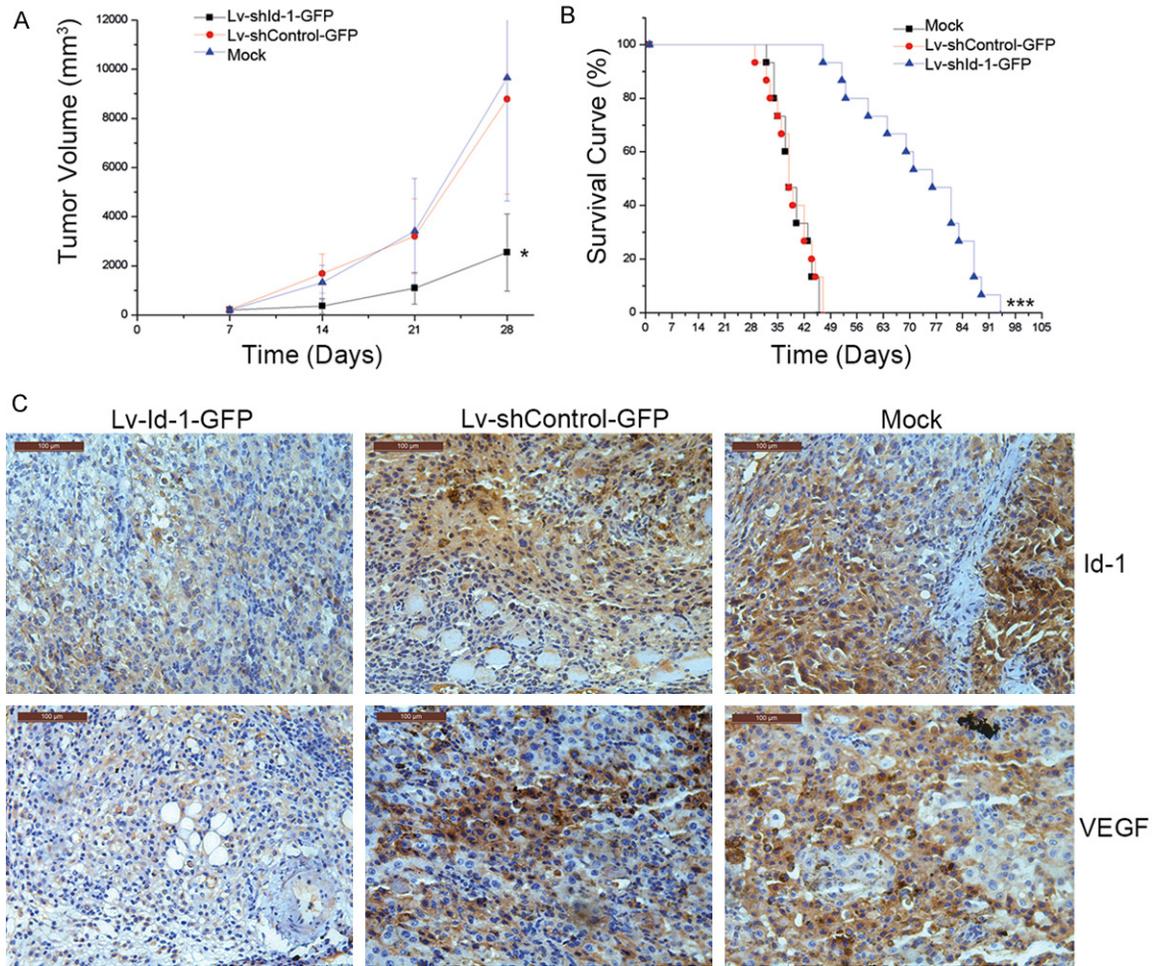
Figure 2. Tumor growth, survival rate and the epressions of Id-1 and VEGF of Id-1 silenced mice. The KM mice were subject to Lv-shId-1-164-GFP, Lv-shControl-GFP or mock lentivirus seven days post B16 cell inoculation. (A, B) The tumor volume (A) and the survival rates (B) post the inoculaton was plotted, n = 15, *P ≤ 0.05, ***P ≤ 0.001. (C) The expressions of Id-1 and VEGF in the mice seven days post lentiviral infection were evaluated by IHC.

tumor implantation (Day 28), the tumor volumes of B7-H3 vaccinated KM mice were significantly larger than pAd-GFP and mock vaccinated KM mice. Consistently, B7-H3 vaccinated KM mice exhibited longer survival time compared to pAd-GFP and mock-vaccinated KM mice. This suggested that B7-H3 vaccination suppressed the tumor growth of B16 melanoma in KM mice.

B7-H3 increased the cytotoxicity of T cells and the levels of IFN-γ: Effective T cells were induced by infecting splenic lymphocytes of the KM mice with pAd-B7-H3-GFP, pAd-GFP or mock adenovirus (6 mice each group) for two weeks. The B16 cells (2 × 10^4/ml) were co-cultivated with the effective T cells of three groups (1 × 10^5/ml) for 48 h. MTT assay was per-

formed and the absorbance (OD values) of the co-cultivated B16 cells and effective T cells (OD_{B16+T}), effective T cells only (OD_T) and B16 cells only (OD_{B16}) were measured. The percentage of the cytotoxicity of the effective T cells was calculated according to the formula: Cytotoxicity (%) = [1 - (OD_{B16+T} - OD_T)/OD_{B16}] × 100. **Figure 1D** showed that the cytotoxic T-lymphocyte CTL activity was significantly enhanced in the B7-H3 vaccinated KM mice compared to pAd-GFP and mock-vaccinated mice. The IFN-γ levels of the serum from the orbital sinus blood were detected using ELISA as described above. IFN-γ levels in the serum of B7-H3 vaccinated mice increased significantly compared to pAd-GFP and mock vaccinated mice (**Figure 1C**). These indicated that the
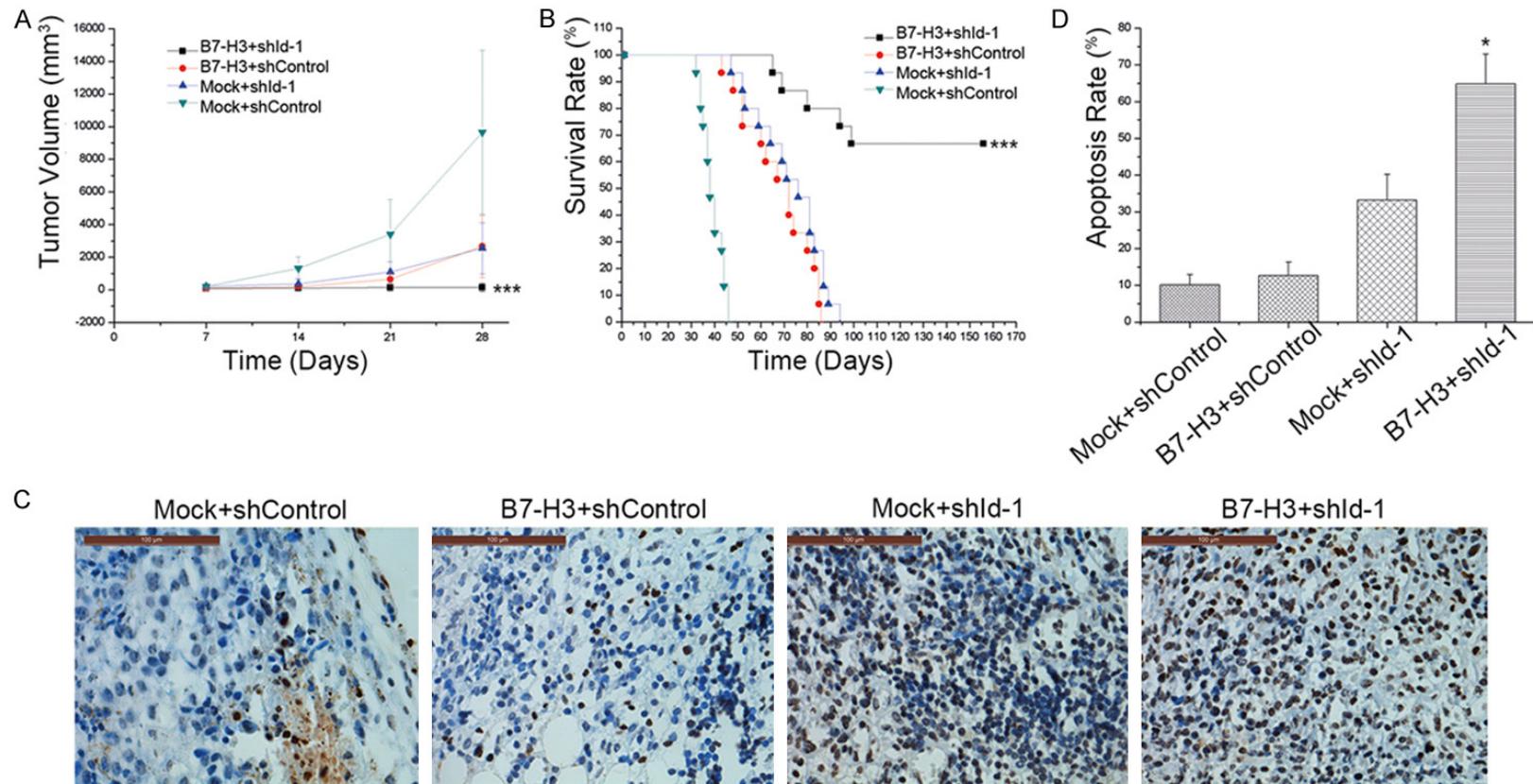
Figure 3. Tumor growth, survival rate, and apoptosis of B7-H3 vaccinated and/or Id-1 silenced KM mice. The KM mice were B7-H3 (or mock) vaccinated and Id-1 (or control shRNA) silenced seven days post B16 cell implantation. (A, B) The tumor volume (A) and the survival rates (B) post the implantation was plotted, n = 15, ***P ≤ 0.001. (C, D) The apoptosis cells in the tumor tissue seven days post tumor implantation were evaluated by TUNEL, n = 15, *P ≤ 0.05.

tumor suppression resulting from B7-H3 vaccination might be mediated or facilitated by CTL and IFN-γ.

Id-1 silencing suppresses the tumor growth

Id-1 silencing inhibited tumor growth and prolongs survival time: The lentivirus carrying Id-1 shRNA (Lv-shId-1-164-GFP, Lv-shId-1-279-GFP, Lv-shId-1-271-GFP or Lv-shControl-GFP) was produced as shown in Figure S2A. Lv-shId-1-164-GFP showing the most significant knockdown effects was used for Id-1 silencing in the *in vivo* study (Figure S2B and S2C). To knock down Id-1 in KM mice, lentiviral Id-1 shRNA was applied to KM mice. Seven days post tumor implantation (Day 7), shId-1-164, shControl or mock lentivirus was intra-tumorally injected in the KM mice (15 mice each group). The plots of tumor volume (**Figure 2A**) indicated that the tumor growth of Id-1 silenced mice was significantly inhibited compared to shControl and mock treated mice 28 days post implantation. The survival time of Id-1 silenced KM mice was also prolonged compared to shControl and mock treated KM mice (**Figure 2B**). This suggested that Id-1 silencing suppressed the tumor growth, and improved the survival time in murine B16 melanoma model.

VEGF expressions were reduced in Id-1 knocked-down tumor cells: KM mice were intra-tumorally injected with shId-1-164, shControl or mock lentivirus (15 mice each group) 7 days post B16 cell inoculation (Day 7). Seven days post the treatments (Day 14), the tumor masses of the mice were dissected and sectioned for immunohistochemistry as shown in **Figure 2C**. The expressions of Id-1 and VEGF of Lv-shId-1-164-GFP treated mice were significantly reduced, but not in Lv-shControl-GFP treated or mock treated mice. This indicated that the suppression of tumor growth by Id-1 silencing might result from the retaining of angiogenesis.

Combination of B7-H3 vaccination and Id-1 silencing

Combination of B7-H3 vaccination and Id-1 silencing eliminated melanoma: The combination of B7-H3 (or mock) vaccination and Id-1 (or control shRNA) silencing treatment was introduced to KM mice (15 mice each group). The volume of the tumors indicated that the

tumors of B7-H3 vaccination, Id-1 silencing and mock treated mice were enlarged during progression, but not for the mice received B7-H3 vaccination combined Id-1 silencing treatment (**Figure 3A**). Notably, the tumors of the mice received combined treatment eventually disappeared in the experiment. The survival rate of KM mice received combined treatment of B7-H3 vaccination and Id-1 silencing was significantly improved (66.67% 150 days post tumor implantation) (**Figure 3B**).

B7-H3 enhanced the apoptosis induced by Id-1: Seven days post B7-H3 (or mock) vaccination and Id-1 (or control shRNA) silencing treatment was introduced to KM mice (15 mice each group), the tumors of the mice were dissected, embedded and sectioned for TUNEL assay as shown in **Figure 3C** and **3D**. The apoptosis index (AI) of B7-H3 vaccination plus Id-1 silencing was significantly higher than the AI of B7-H3 vaccination only, Id-1 silencing only and mock vaccination and control shRNA treatment. And the differences of AI between Id-1 treatment and control shRNA group were significant. However, the differences of AI between B7-H3 vaccination and mock group were not significant (P = 0.47). This data indicated that Id-1, not B7-H3, promoted the apoptosis of tumor tissue, while B7-H3 facilitated the apoptosis induced by Id-1.

Discussion

Both B7-H3 vaccination and Id-1 silencing effectively reduced the tumor growth in KM mice and prolonged the survival time, however, neither of them completely eradicated the subcutaneous melanoma induced by B16 cells in KM mice. Remarkably, the alliance of two therapies intensively suppressed the tumor growth and eventually eliminated the tumor mass in KM mice. Moreover, it significantly improved the survival rate to 66.7% 150 days post tumor implantation. Although the mechanism underlining the intense suppression has not been well investigated or understood, the plots and the statistics indicate that the synergized effects of the combination therapy is not simply the effect of B7-H3 plus the effect of Id-1. To be addressed, the apoptotic cells of combined treatment and of Id-1 silencing treatment, but not of B7-H3 vaccination, were significantly increased. This indicated that Id-1, not B7-H3,

might play a predominant role in the apoptosis of melanoma induced by B16 cells, whilst B7-H3 might facilitate and enhance the apoptosis resulting from Id-1.

Although the role of B7-H3 in the immune response is controversial [8, 17], we found that the splenic T lymphocytes from B7-H3 vaccinated mice exhibited enhanced cytotoxic activities to B16 cells and increased serum IFN-γ levels. These suggested that the immune response in our study was enhanced to fight against the tumourigenesis and the tumor development. The tumor growth inhibition of Id-1 silencing has been demonstrated in adenoid cystic carcinoma and oral squamous cell carcinoma in mice in our previous studies [22]. The associations of Id-1 overexpression with tumor cell proliferation, tumor invasion, tumor angiogenesis, anti-apoptosis, and chemotherapeutic drug resistance have been shown in several cancers [19-21]. Consistent with these studies, we found that Id-1 knockdown by RNA interference inhibited the proliferation and promoted the apoptosis of B16 cell. Ling *et al*. found that ectopic expression of Id-1 in prostate cancer cells increased the expression of VEGF [19]. Moreover, the VEGF expressions of Id-1 silencing mice were found significantly reduced. Id-1 has also been found to activate NF-κB and inhibit TNF-α signaling to prevent the apoptosis in breast cancer cells [23]. This might explain the enhanced apoptosis of B7-H3 vaccinated cells together with Id-1 silencing. Based on the phenomena we observed, the effects of B7-H3 and Id-1 were not merely additional but possibly synergistic, however, not enough mechanism studies were undertaken, such as T-cell and cytokine analyses. Presumably, B7-H3 promotes IFN-α and IFN-γ induction, which might cross-talk with the NF-κB and TNF-α signaling to facilitate the apoptosis induced by deprivation of Id-1.

In summary, the therapeutic alliance of B7-H3 vaccine and Id-1 silencing exhibits a synergistic effect to shrink or even to eradicate the tumor mass, and provides a theoretical basis for the clinical treatment of malignant melanoma.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Shaohua Liu, Department of Oral and Maxillofacial Surgery, Qilu Hospital, Institute of Stomatology, Shandong University, 107 Wenhuaxi Road, Jinan 250012, P. R. China. Tel: +86 531 82166775; Fax: +86 531 82169075; E-mail: lshabccba@sdu.edu.cn

References

[1] Bhatia S, Tykodi SS, Lee SM and Thompson JA. Systemic therapy of metastatic melanoma: on the road to cure. Oncology (Williston Park) 2015; 29: 126-35.

[2] Aris M and Barrio MM. Combining immunotherapy with oncogene-targeted therapy: a new road for melanoma treatment. Front Immunol 2015; 6: 46.

[3] Morton D, Eilber FR, Malmgren RA and Wood WC. Immunological factors which influence response to immunotherapy in malignant melanoma. Surgery 1970; 68: 158-163; discussion 163-164.

[4] Schwartzentruber DJ, Lawson DH, Richards JM, Conry RM, Miller DM, Treisman J, Gailani F, Riley L, Conlon K, Pockaj B, Kendra KL, White RL, Gonzalez R, Kuzel TM, Curti B, Leming PD, Whitman ED, Balkissoon J, Reintgen DS, Kaufman H, Marincola FM, Merino MJ, Rosenberg SA, Choyke P, Vena D and Hwu P. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. N Engl J Med 2011; 364: 2119-2127.

[5] Phan GQ, Yang JC, Sherry RM, Hwu P, Topalian SL, Schwartzentruber DJ, Restifo NP, Haworth LR, Seipp CA, Freezer LJ, Morton KE, Mavroukakis SA, Duray PH, Steinberg SM, Allison JP, Davis TA and Rosenberg SA. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc Natl Acad Sci U S A 2003; 100: 8372-8377.

[6] Kirkwood JM and Ernstoff MS. Interferons in the treatment of human cancer. J Clin Oncol 1984; 2: 336-352.

[7] Viola JR, Rafael DF, Wagner E, Besch R and Ogris M. Gene therapy for advanced melanoma: selective targeting and therapeutic nucleic acids. J Drug Deliv 2013; 2013: 897348.

[8] Chapoval AI, Ni J, Lau JS, Wilcox RA, Flies DB, Liu D, Dong H, Sica GL, Zhu G, Tamada K and Chen L. B7-H3: a costimulatory molecule for

T cell activation and IFN-γ production. Nat Immunol 2001; 2: 269-274.

[9] Dong C, Temann UA and Flavell RA. Cutting edge: critical role of inducible costimulator in germinal center reactions. J Immunol 2001; 166: 3659-3662.

[10] Seliger B, Marincola FM, Ferrone S and Abken H. The complex role of B7 molecules in tumor immunology. Trends Mol Med 2008; 14: 550-559.

[11] Loos M, Hedderich DM, Ottenhausen M, Giese NA, Laschinger M, Esposito I, Kleeff J and Friess H. Expression of the costimulatory molecule B7-H3 is associated with prolonged survival in human pancreatic cancer. BMC Cancer 2009; 9: 463.

[12] Wu CP, Jiang JT, Tan M, Zhu YB, Ji M, Xu KF, Zhao JM, Zhang GB and Zhang XG. Relationship between co-stimulatory molecule B7-H3 expression and gastric carcinoma histology and prognosis. World J Gastroenterol 2006; 12: 457-459.

[13] Zang X, Sullivan PS, Soslow RA, Waitz R, Reuter VE, Wilton A, Thaler HT, Arul M, Slovin SF, Wei J, Spriggs DR, Dupont J and Allison JP. Tumor associated endothelial expression of B7-H3 predicts survival in ovarian carcinomas. Mod Pathol 2010; 23: 1104-1112.

[14] Parker AS, Heckman MG, Sheinin Y, Wu KJ, Hilton TW, Diehl NN, Pisansky TM, Schild SE, Kwon ED and Buskirk SJ. Evaluation of B7-H3 expression as a biomarker of biochemical recurrence after salvage radiation therapy for recurrent prostate cancer. Int J Radiat Oncol Biol Phys 2011; 79: 1343-1349.

[15] Yang HY, Chu M, Zheng LW, Zwahlen RA, Luo J, Zou DH and Sun ST. Transgenic B7-H3 therapy induces tumor-specific immune response in human oral squamous cell cancer: an in vitro study. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008; 106: 721-728.

[16] Hasskarl J and Münger K. Id proteins--tumor markers or oncogenes? Cancer Biol Ther 2002; 1: 91-96.

[17] Benezra R, Davis RL, Lockshon D, Turner DL and Weintraub H. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell 1990; 61: 49-59.

[18] Wong YC, Wang X and Ling MT. Id-1 expression and cell survival. Apoptosis 2004; 9: 279-289.

[19] Ling MT, Lau TC, Zhou C, Chua CW, Kwok WK, Wang Q, Wang X and Wong YC. Overexpression of Id-1 in prostate cancer cells promotes angiogenesis through the activation of vascular endothelial growth factor (VEGF). Carcinogenesis 2005; 26: 1668-1676.

[20] Cheung HW, Ling MT, Tsao SW, Wong YC and Wang X. Id-1-induced Raf/MEK pathway activation is essential for its protective role against taxol-induced apoptosis in nasopharyngeal carcinoma cells. Carcinogenesis 2004; 25: 881-887.

[21] Zhang X, Ling MT, Wang X and Wong YC. Inactivation of Id-1 in prostate cancer cells: a potential therapeutic target in inducing chemosensitization to taxol through activation of JNK pathway. Int J Cancer 2006; 118: 2072-2081.

[22] Liu P, Liu S, Qi H, Li Y, He H and Wei F. Effects of silencing Id-1 in cell culture of human adenoid cystic carcinoma. Oral Oncol 2009; 45: 783-788.

[23] Kim H, Chung H, Kim HJ, Lee JY, Oh MY, Kim Y and Kong G. Id-1 regulates Bcl-2 and Bax expression through p53 and NF-κB in MCF-7 breast cancer cells. Breast Cancer Res Treat 2008; 112: 287-296.
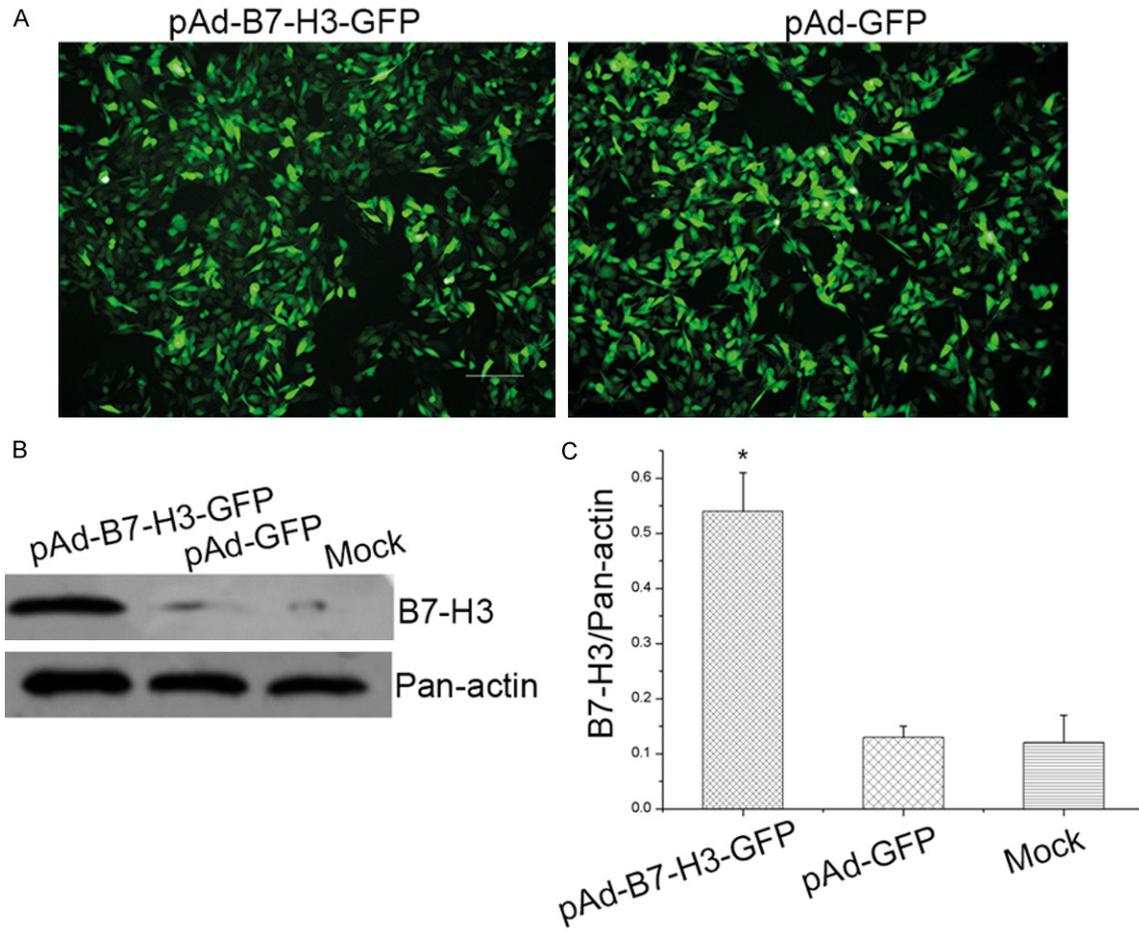
Figure S1. The infection of B7-H3 adenovirus in B16 cell. B16 cells were infected with pAd-B7-H3-GFP, pAd-GFP and mock adenovirus for 72 h. A. B7-H3 fused GFP or GFP only was indicated under fluorescent microscopy. B and C. B7-H3 expressions 72 h post infection was detected by western blot, n = 3, *P < 0.05.
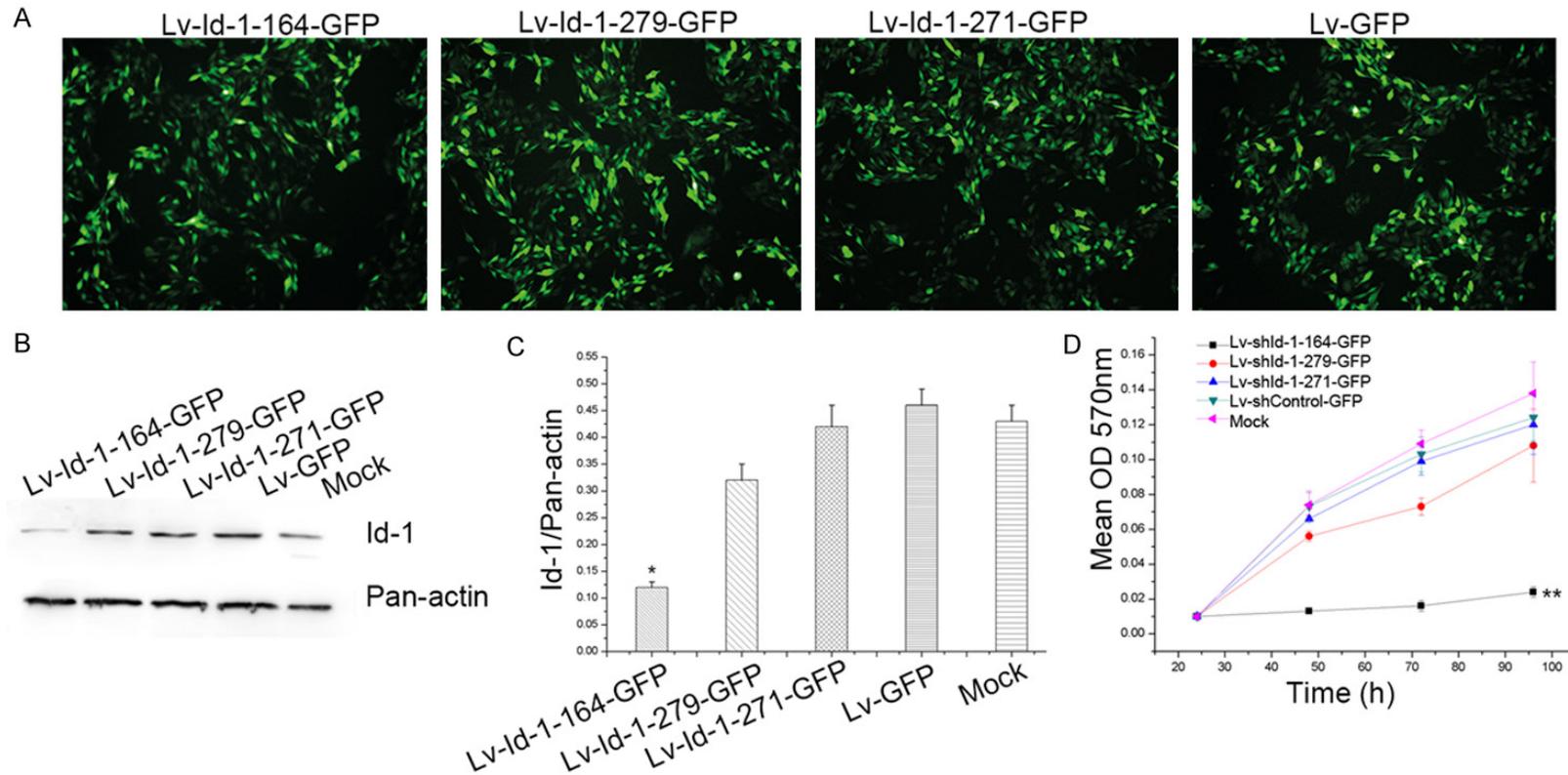
Figure S2. Id-1 silencing and the proliferation of Id-1 silenced B16 cells. B16 cells were infected with Lv-shId-1-164-GFP, Lv-shId-1-279-GFP, Lv-shId-1-271-GFP, Lv-shControl-GFP or mock lentivirus to knock down Id-1. A. GFP expressions of B16 cells infected with Lv-shId-1-164-GFP, Lv-shId-1-279-GFP, Lv-shId-1-271-GFP and Lv-shControl-GFP 72 h post infection were indicated under fluorescent microscopy. B and C. Id-1 expressions of infected B16 cells 72 h post infection were detected by western blot, n = 3, *P < 0.05. D. The proliferation of B16 cells 48 h, 72 h and 96 h post infection was assessed by MTT assay. shId-1-164 knocked down B16 cells exhibited inhibited proliferation, n = 6, ***P < 0.001.