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RESEARCH ARTICLE

Bmi-1 is essential for the oncogenic potential in CD133$^+$ human laryngeal cancer cells

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Abstract It has been hypothesized that cancer stem cells (CSCs) are a principal culprit of tumor initiation, invasion, metastasis, and treatment resistance. Previous studies have confirmed that cancer stem cells can be detected in laryngeal carcinoma. This study aimed to evaluate whether population of CD133$^+$ cells that existed in primary human laryngeal carcinoma have characteristic of CSCs with enhanced capacity of proliferation and invasion, and to understand whether and how *Bmi-1* implicated in self-renewal and tumorigenesis. We clarified the tumorigenic potential of CD133 sorted populations of cancer cells derived from primary human laryngeal tumor sample. After fluorescence activated cell sorting, real-time polymerase chain reaction (PCR) and western blot confirmed *Bmi-1* was differentially expressed in CD133 sorted laryngeal tumor cells. *Bmi-1* was knocked down, and proliferation, colony formation, invasion, cell cycle assay, and apoptosis assays were performed, and the impact on Bmi-1 pathway was evaluated. It was found that CD133$^+$ cells existed in primary human laryngeal tumor with enhanced capacity of proliferation and invasion. *Bmi-1*, implicated in self-renewal and tumorigenesis, was coexpressed with the CD133. Furthermore, knockdown of *Bmi-1* expression in CD133$^+$ cells led to inhibition of cell growth, colony formation, cell invasion in vitro, and tumorigenesis in vivo, through up-regulation of p16^{INK4A} and p14^{ARF}. Our data indicate that *Bmi-1* expression is central to the tumorigenicity of CD133$^+$ cells, which functions as a pleiotropic regulator that maintains the viability and proliferative capacity of human laryngeal tumor. It negatively regulates the transcription of the downstream INK4a/ARF gene and inhibits expression of P16^{ink4a}/P14^{ARF}, so as to maintain the high ability of proliferation and differentiation in laryngeal cancer stem cells.

Keywords Laryngeal carcinoma · Cancer stem cells · *Bmi-1* gene

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Introduction

Laryngeal tumor occurs mainly in adult males and represents the second most common malignancy of the head and neck worldwide. The incidence rate comprises 1 to 5 % of all cancer with an estimated incidence rate of 5.1/100,000 cases in males worldwide [1]. The major pathology (95 to 98 %) of laryngeal cancer is squamous cell carcinomas [2]. Although the definite cause of laryngeal cancer is not yet determined, a variety of epidemiological studies demonstrated that smoking and alcohol consumption play a critical role in the development of the disease [3]. Although progress has occurred in the diagnosis and treatment of this disease, the locoregional recurrence characterized by increased invasiveness and high rate of metastasis is the most important factor determining the survival of laryngeal cancer patients in the advanced stage [2]. Identification of tumor markers associated with metastasis plays a vital role in characterizing these cells at the molecular level and developing new therapeutic strategies.

Cancer stem cells (CSCs) are defined as a subset of cells in tumors with properties of self-renewal and multilineage

differentiation; thus, they can either initiate or maintain a tumor and are also thought to be responsible for tumor metastasis and resistance to conventional chemo- and radiotherapies [4]. Human CD133 (prominin-1) is a glycosylated protein with five transmembrane domains and two large extracellular loops [5, 6]. It was initially characterized as a marker for hematopoietic stem cells [7]. In brain tumors, $CD133^+$ cells revealed properties of cancer stem cells [8]. CD133 has been identified as a marker of cancer stem cells in the human laryngeal tumor Hep-2 cell line [9]. In an in vivo study, $CD133^+$ cells sorted from the Hep-2 cell line showed higher tumorigenic potential than $CD133^-$ or unsorted cells [10]. In laryngeal squamous cell carcinomas, Bmi-1 is highly enriched in $CD133^+$ cells, induces the proliferation of these cells, and prevents apoptosis. The analysis of these studies reveals that CD133 is a useful cancer stem cell marker in (Head and Neck Squamous Cell Carcinoma (HNSCC) and might serve as a putative biomarker to identify head and neck cancer patients who are resistant to conventional chemotherapy [11].

The Polycomb group (PcG) gene Bmi-1 (B lymphoma Mo-MLV insertion region 1 homolog) functions as a transcriptional repressor, which partly attributed to negative regulation of the INK4A/ARF locus [12]. *Bmi-1*, as a critical regulator of cell cycle, is essential for maintaining the self-renewal of normal and cancer stem cells [13], and overexpression of Bmi-1 is also correlated with tumor invasion, metastasis, cancer therapy failure, and poor prognosis [14].

In laryngeal tumor, subpopulation of $CD133^+$ cells or SP cells sorted from the laryngeal cancer cell line Hep-2 possesses distinct properties of cancer stem-like cells, such as increased expression of specific stem cell markers, higher potential for clonogenicity, invasion and tumorigenicity in vivo as well as increased chemoresistance [15, 16]. However, it is unknown how the $CD133^+$ cells of primary human laryngeal tumor maintain self-renewal ability; the roles of Bmi-1 still remain largely elusive. Therefore, research on $CD133^+$ cells to develop effective therapeutic strategies for solid laryngeal tumor is urgently needed.

We describe here that CD133 is critical to sustain the ability of cell proliferation and self-renewal in primary human laryngeal tumor and confer laryngeal tumor cells an enhanced invasive potential. We further provide evidence that *Bmi-1* is significantly upregulated in $CD133^+$ laryngeal tumor cells and confirmed *Bmi-1* is a key gene of self-renewal and multilineal differentiation in laryngeal cancer stem cells. It negatively regulates the transcription of the downstream INK4a/ARF gene and inhibits expression of protein $P16^{ink4a}$/$P14^{ARF}$, so as to maintain the high ability of proliferation and differentiation in laryngeal cancer stem cells.

Materials and methods

Clinical specimens

Six-week-old male BALB/c nude mice were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai. All animal experiments were done in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the institutional biomedical research ethics committee of Fudan University (Shanghai, China). Fresh human laryngeal tumor with patient written consent was obtained in accordance with the ethical standards of the Ethics Committee for Human Research at Fudan University. The patient was an untreated 45-year-old Chinese male who underwent laryngectomy for squamous cell carcinoma deriving from the epiglottis, Stage IVa, T4aN2M0, based on the sixth edition Union for International Cancer Control (UICC) TNM classification system. Notably, he had no family history of head and neck cancer, but had a history of smoking and long year history of alcohol use.

Laryngeal tumor xenograft establishment

Primary specimens obtained from patients undergoing surgical resection were implanted under the skin of immunocompromised mice. Surgical laryngeal tumor specimens were obtained at the time of resection from the patient. The sample was received in the laboratory within 20 min. Tumor specimens were immersed in cold triple antibiotic phosphate buffered saline (PBS) containing 1 % penicillin/streptomycin and scissored into pieces of about 1×2×2 mm in dimension, which were implanted subcutaneously into the armpit of each nude mouse. Tumors were pinched into their final position, and then the incision was sealed with surgical staple.

Laryngeal tumor cell isolation

Once the orthotopic tumor developed into a gross tumor bulk, it was removed and minced with a sterile scalpel and digested for 30 min at with 0.25 % trypsin-EDTA (Invitrogen) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen). Every 10 min, the solution was mixed through a 10-ml pipette to allow complete digestion. After this enzymatic digestion, the single-cell suspensions were filtered through a 40-μm cell strainer and washed twice with D-Hank's Balanced Salt Solution (HBSS; Gibco/Invitrogen)/fetal bovine serum (FBS; Hyclone), then seeded in 60-mm Petri dishes in a humidified 5 % CO_2 incubator at 37 °C. Cancer cells near confluence were detached with 0.25 % trypsin-EDTA, and primary cells were serially passaged. Cells were stored in liquid nitrogen from passage 1. Then cells were labeled for flow cytometry and fluorescence activated cell sorting.

Fluorescence activated cell sorting (FACS)

The single-cell suspensions were washed with HBSS containing 2 % FBS. Phycoerythrin (PE)-conjugated anti-human CD133 antibody or compared isotype control antibody was added and the cells were incubated for 30 min on ice, and the samples were washed twice with HBSS/2 % FBS. Samples were analyzed and sorted on BD FACSAria (BD Biosciences). The purity of sorted $CD133^+$ and $CD133^-$ cells was evaluated by standard flow cytometric analysis.

Gene promoter cloning and luciferase assays

The CDKN2A gene encodes two alternatively spliced transcripts, $p16^{INK4A}$ and $p14^{ARF}$, which function as inhibitors of cell cycle progression [17]. From the $CD133^+$ laryngeal carcinoma cell DNA genomic, *INK4a*, *ARF* (*INK4a* gene sequence number of NM_000077, *ARF* gene sequence number is NM_078487) gene promoter region was subcloned into the pGL3-Basic Vector luciferase reporter plasmid (Promega). PCR was carried out with primers containing the restriction sites for the Nhe I and Xho I enzymes using Pfu-based DNA polymerase (TOYOB) according to the manufacturers' instructions. PCR products and plasmids were validated by sequencing. Luciferase activity was determined in dual-luciferase reporter assay using the phRL-TK control according to the manufacturers' instructions (Promega). Briefly, cells plated on 24-well plates were transiently transfected with both of $p16^{INK4A}$ or $p14^{ARF}$ promoter-firefly luciferase plasmids and pRL-TK plasmid (Promega) as internal control for monitoring transfection efficiency. After 24 h, luciferase activity was measured, and the ratio of firefly activity normalized with the Renilla activity was calculated for each transfection. Transfections were performed in triplicate and repeated three times to ensure reproducibility.

Construction of shRNA targeted BMI-I

The siRNA sequence for *Bmi-1* (5′-ACCTAATACTTTCCAG ATTGAT-3′) was selected after screening to validate potential siRNAs. Non-silencing siRNA (*5′-TTCTCCGAACGTGTCA CGT-3′*) was used as control. ShRNAs, corresponding to siRNA sequences for Bmi-1 and non-silencing, were then generated, respectively. The annealed oligonucleotide fragments were subcloned into pLVTHM vector (Clontech). The control or shRNA vectors were co-transfected with the psPAX2 and pMD2.G packaging plasmid (Clontech) into HEK-293T cells by using Fugene HD (Roche). The CD133 sorted cells were infected by the lentiviral particles at a multiplicity of infection (MOI) of 30 plus polybrene. Real-time PCR and Western blot were used respectively to detect the expression of *Bmi-1*.

In vivo tumorigenicity assay

For detecting tumor initiation ability, 1×10^5 sorted $CD133^+$, $CD133^-$ cells and RNA-interference cells suspended in serum-free medium were mixed with Matrigel (2:1, volume/volume) injected into the subcutaneous space of the axillary fossa of nude mice. Tumors were measured in two dimensions every 3 or 4 days with calipers, and the volumes were calculated in accord with Chinnaiyan's formula. Mice were euthanized 4 weeks later, and all tumor nodules were photographed and weighed.

Cell culture and clonogenicity

Cells were cultured before assays in DMEM supplemented with 10 % FBS in an incubator at 37 °C with 5 % CO_2. Freshly isolated $CD133^+$, $CD133^-$ cells and RNA-interference cells were plated at a density of 1500 cells/well in six-well plates and cultured for 1–2 weeks. Finally, the cells were fixed and Giemsa stained, and colonies were counted using Quantity One software (Bio-Rad). The three generations of clone forming rate were detected. Each experiment was performed three times.

Analysis of apoptosis

Apoptosis assay was performed by flow cytometry using FITC-Annexin V and propidium iodide (PI; Sigma) according to the manufacturer's protocol. The Annexin V positive cells (early apoptosis) and Annexin V/PI double positive cells (late apoptosis) served as the apoptotic cell population. The samples were analyzed on BD FACSAria. Each analysis was performed utilizing at least 10,000 events.

Cell cycle assay

After cell seeding for 24 h, cells were synchronized in a medium containing 8 mM glutamine and 0.04 % FBS for another 24 h. After synchronization, the cells were harvested, washed with PBS, fixed in 70 % ethanol for overnight at −20 °C, and stained with 50 μg/ml PI and 100 μg/ml RNase A in PBS for 30 min at 37 °C in the dark. The DNA content of the labeled cells was measured by flow cytometry (BD FACSAria).

Cell invasion assay

After the Matrigel (BD) polymerized, 1×10^5 cells/ml of cell suspension in 100 μl DMEM was added to the upper chamber of a Transwell chamber (Corning) and 500 μl of DMEM containing 20 % FBS was added to the lower chamber. The Transwell chamber was placed in a 37 °C incubator. After 48 h of incubation, the Transwell chamber was taken out and washed twice with PBS. The cells adhesive to the

Table 1 Primer sequences of target genes

Target genes	Primer sequences
Human *Bmi-1* F	TCAGTCACCAGAGAGATGGACT
Human *Bmi-1* R	GAAGAGGTGGAGGGAACTCCT
Human *INK4a* F	CCCGAGAGGTCTTTTTCCGAG
Human *INK4a* R	CCAGCCCATGATGGTTCTGAT
Human *ARF* F	TTGGATGCACAACATGAATCAGG
Human *ARF* R	TCTTCTGACTGAGAGCTATGGTC
Human *GAPDH* F	CACCATCTTCCAGGAGCGAG
Human *GAPDH* R	AAATGAGCCCCAGCCTTCTC

chamber surface were fixed and stained with crystal violet (Sigma). Cells migrating through the Transwell polycarbonate membrane were considered as invading cells and examined by microscope (Leica); eight randomly selected fields were counted.

Western blot

Cells were lysed in RIPA buffer for 30 min on ice and the lysates were centrifuged at 10,000×*g* for 15 min at 4 °C. Protein concentration was quantified using the Bradford reagent (Sigma, St Louis, MO) according to the manufacturer's instructions. Equal amounts of total cellular protein

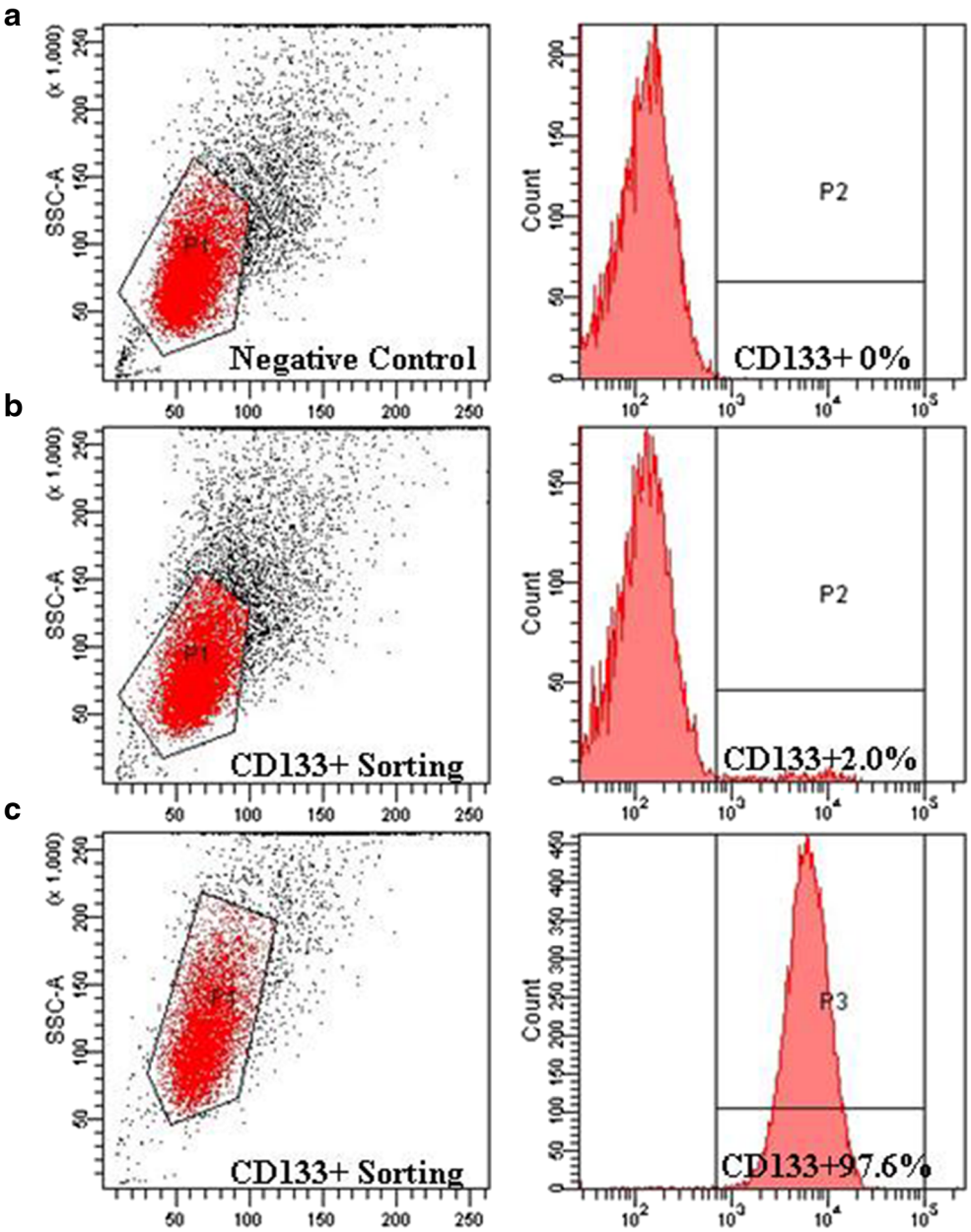

Fig. 1 Representative diagrams of flow cytometry analysis of $CD133^+$ cells in freshly resected human laryngeal tumor. Human laryngeal tumor cells were immunostained with isotype (**a**) or PE-CD133/1 (**b**) antibody, and the fraction of $CD133^+$ cells was sorted using FACS. The purity of sorted $CD133^+$ cells was confirmed by flow cytometry after staining with PE-CD133/1 antibody (**c**)

were mixed with loading buffer and subjected to 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and immunoblotted with specific antibodies. All immunoblots were visualized using an enhanced chemiluminescence kit (Pierce Rockford, IL).

Proliferation assay

The in vitro tumor cell growth was measured by MTT assay. Cells were plated in 96-well plates with a concentration of 1000 cells per well. To measure cell growth, 20 μl 5 mg/ml MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) was added into the medium and cultured at 37 °C.

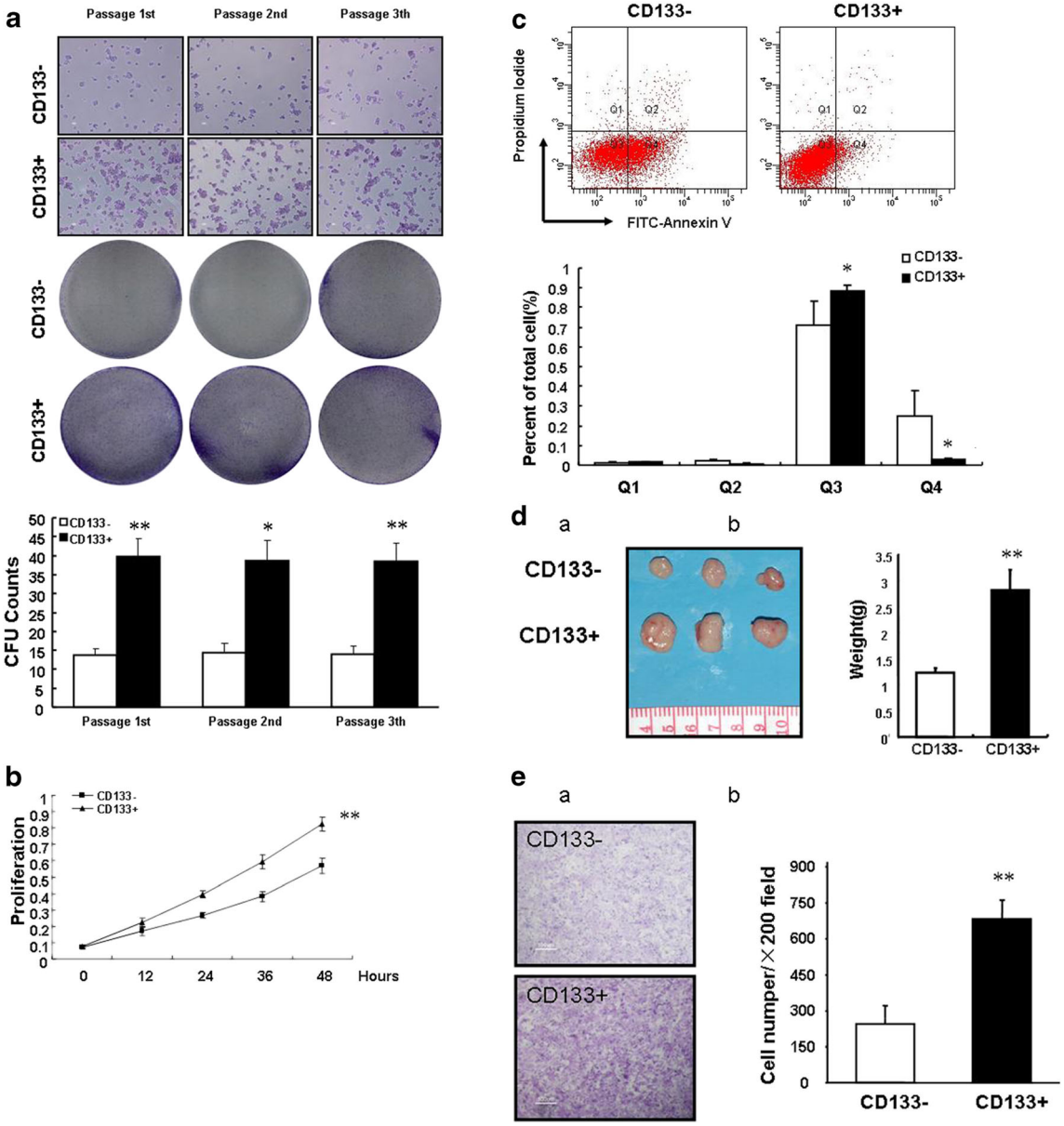

Fig. 2 Characterization of $CD133^+$ and $CD133^-$ cells. **a** Significant difference in the number of colonies between sorted $CD133^+$ and $CD133^-$ cells. Representative photographs of the plates containing colonies derived from either sorted $CD133^+$ or $CD133^-$ cells at 21 days after seeding of cells are shown, and total number of all clones was enumerated. **b** The cell viability was determined using MTT assay. $CD133^+$ cells possess much higher proliferation than $CD133^-$ cells. **c** Apoptotic index determined by the percentage of cells positive for both propidium iodine and annexin V. $CD133^+$ subpopulation showed a decreased proportion of apoptotic cells. **d** $CD133^+$ subpopulation was more tumorigenic than $CD133^-$ cells. *a* Representative subcutaneous tumors derived from $CD133^+$ or $CD133^-$ cells. *b* Data are generated from five mice in each group. **e** Examination of invasiveness ability of sorted $CD133^+$ and $CD133^-$ cells utilizing Transwell assay. *a* Representative images of invasion in $CD133^+$ and $CD133^-$ cells isolated from human laryngeal tumor. *b* Average number of cells per field was calculated. The results are representative of three independent experiments. *$p<0.05$; **$p<0.01$ versus $CD133^-$ cells group

After 4 h, the medium were removed, 200 μl DMSO was added to dissolve the generated formazan right after removing the medium, and the OD490 value of the solvent was measured by an automatic microplate reader (Bio-Tek). The measurement process was performed for 12, 24, 36, and 48 h to generate a cell growth curve. Ten replicates were used for each group, and the procedure was repeated three times.

Analysis of gene expression

Total RNA was extracted from cells using an RNeasy kit (QIAGEN) according to the manufacturer's instructions. 2 μg RNA from each sample was reverse transcribed into cDNA via Oligo (dT) with SuperScript III Reverse Transcriptase (Invitrogen). Quantitative RT-PCR in triplicate for each sample and each gene was performed by SYBR Green PCR Master Mix (Qiagen) using primers listed in Table 1. The transcript levels were determined by using the 7500 Real-Time PCR System (Applied Biosystems). The gene expression was normalized to GAPDH as an endogenous control.

Statistical analysis

All data are presented as means±standard deviation (SD) unless otherwise indicated. Statistical significance of the differences was assessed by two-tailed Student's *t* test for paired data or by one-way analysis of variance using SPSS 11.0 software (SPSS, Chicago, IL). Differences between groups were considered significant at $p < 0.05$.

Results

CD133 expression in primary laryngeal tumor cells and sorted CD133$^+$ cells display higher clonogenicity ability

We initially analyzed the expression of CD133 in the primary laryngeal tumor cells by flow cytometry. Our results showed that about 2.0±0.3 % of CD133$^+$ cells existed in the freshly clinical laryngeal tumor sample (Fig. 1a). CD133$^+$ cells significantly enriched up to about 97.6±1.6 % as confirmed by flow cytometry (Fig. 1b). CD133 has been identified as the CSCs marker in laryngeal tumor; thus, we tested the colony formation of initial passage CD133$^+$ (passage 1) and CD133$^-$ sorted cells. The colony formation efficiency for CD133$^+$ laryngeal tumor cells was significantly higher than that for CD133$^-$ cells. CD133$^+$ cells in the secondary (passage 2) and third (passage 3) passage also grew better than CD133$^-$ counterparts. Evident by colony formation assay (Fig. 2a), data shows, in the same view, that the clone formation rate of three generations in CD133$^+$ cells were 39.67±8.08, 38.67±9.29, and 38.33

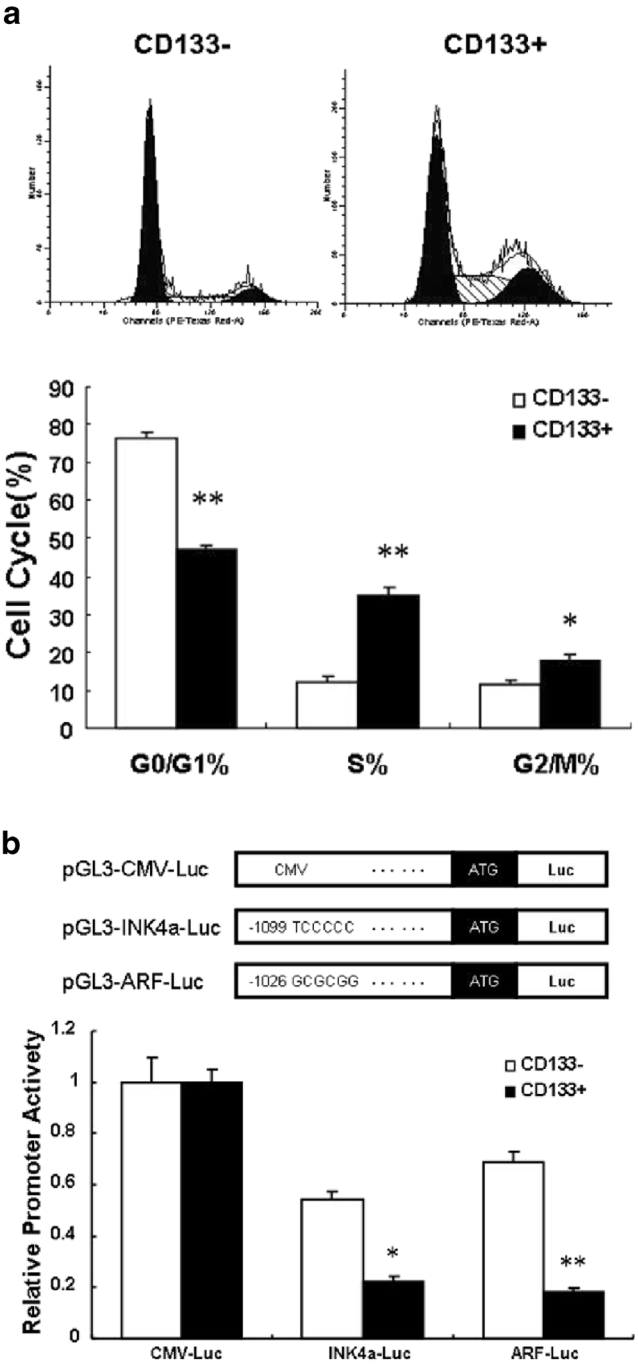

Fig. 3 Analysis of the cell cycle by flow cytometric and *INK4a*/*ARF* gene promoter activity. **a** Cell cycle analysis upon propidium iodide staining. Shown are representative histograms (*upper*) and quantification of cell cycle distribution (*lower*) in CD133$^+$ and CD133$^-$ cells. **b** Promoter activity of the *INK4a*/*ARF* luciferase constructs in sorted CD133$^+$ and CD133$^-$ cells. The promoter activity (in fold change) is calculated. pGL3-Myc was used as an internal control for the promoter activity. Histograms represented average of three independent experiments with duplicates. *$p<0.05$; **$p<0.01$ versus CD133$^-$ cells group

±8.33 %, while CD133$^-$ cells were 13.67±3.21, 14.33±4.51, and 14±3.61 %. The two groups have a statistically significant difference ($p<0.05$).

CD133$^+$ cells show greater tumorigenicity and metastatic potential

The significant increase in the proliferative capacity was observed in CD133$^+$ cells as compared to CD133$^-$ cells, as confirmed by the MTT assay (Fig. 2b), which suggested that CD133 is associated with increased viability in laryngeal tumor cells. CD133$^+$ cells were found to be more viable than corresponding CD133$^-$ counterparts, as evident by the lower apoptotic rates (Fig. 2c).

We isolated CD133$^+$ and CD133$^-$ laryngeal tumor cells and compared their tumorigenicity in nude mice. Moreover, 1.0×10^4 CD133$^+$ cells produced larger tumors (2.71±0.20 g) than CD133$^-$ cells (1.81±0.46 g) 30 days after injection (Fig. 2d), which verified the greater tumor initiating capability of CD133$^+$ cells.

CD133$^+$ laryngeal tumor cells displayed enhanced invasion and migratory properties in comparison with CD133$^-$ cells (Fig. 2e). Taken together, these findings suggest that CD133 plays a critical role in the invasive phenotype and metastatic capacity of laryngeal tumor cells.

CD133 regulates cell viability and cell cycles

The cell cycle is a crucial regulator of cell proliferation. Abnormal cell cycle regulation is an important mechanism of tumorigenesis [18]. Cell cycle analysis revealed a significant decrease in the number of cells at G0/G1 presented in CD133$^+$ subpopulation, accompanied by higher percentage of cells that were arrested in S and G_2/M phase. Meanwhile, CD133$^-$ subpopulation displayed accumulation in the G1 phase and fewer cells in the S phase (Fig. 3a).

The CDKN2A gene plays an important role in cell cycle regulation by decelerating cell progression from G1 phase to S phase, and its deletion leads to inactivation of the p16^{INK4A} and p14^{ARF} pathway and loss of cell cycle control [17]. CDKN2A is the most frequently deleted cancer locus in humans [19]. Thus, we examined the activity of p16^{INK4A} and p14^{ARF} pathway with luciferase reporter assays. The reporter analysis revealed that the p16^{INK4A} and p14^{ARF} promoter exhibits higher activity in CD133$^+$ cells than in CD133$^-$ cells (Fig. 3b). These findings suggest that CD133

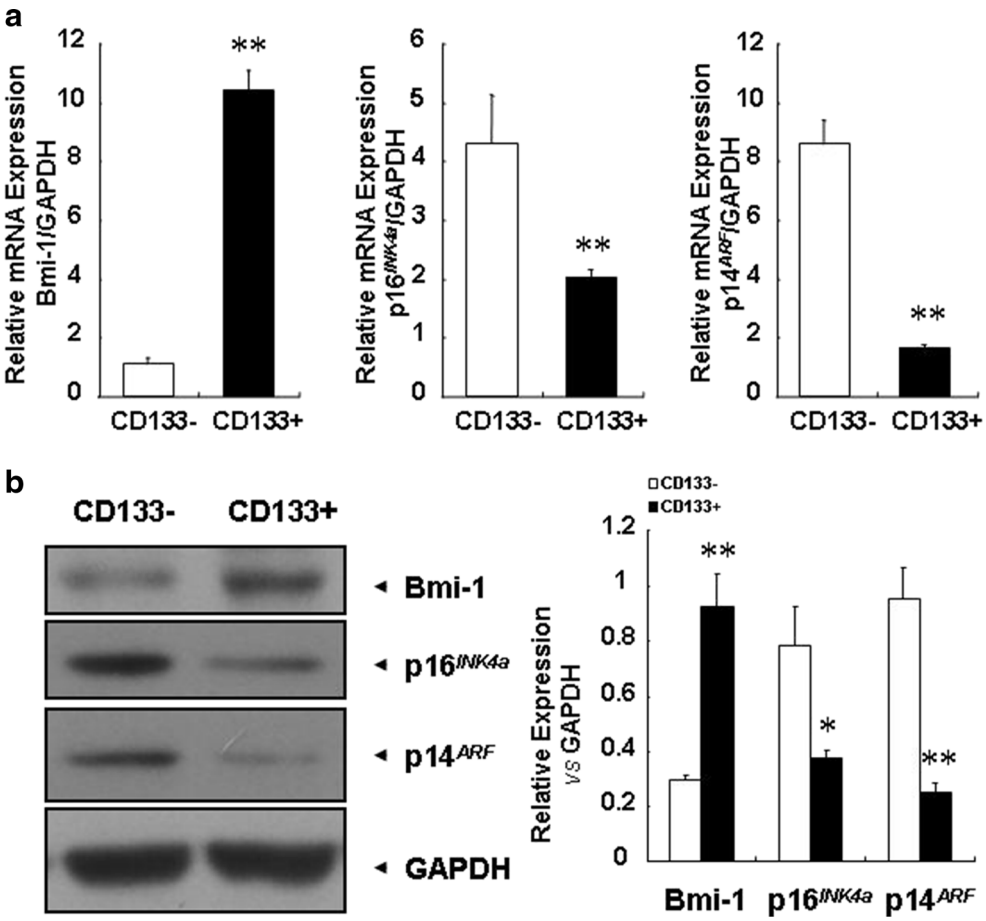

Fig. 4 Expression profile of *Bmi-1* and targeted genes in CD133 sorted cells by real-time PCR (**a**) and western blot (**b**) analysis. *Bmi-1* in CD133$^+$ cells were significantly higher than in CD133$^-$ cells; meanwhile, CD133$^+$ cells showed lower levels of expression of *INK4a/ARF* and p16^{INK4A}, p14^{ARF}. *$p<0.05$; **$p<0.01$ versus CD133$^-$ cells group

contributes to the increased cell viability of laryngeal tumor through deregulated $p16^{INK4A}$ and $p14^{ARF}$ pathways.

Enhanced activity of *Bmi-1* in primary $CD133^+$ laryngeal tumor cells

We examined the expression of *Bmi-1* in CD133 sorted cell by Q-PCR and western blot analysis. *Bmi-1* is significantly higher in the $CD133^+$ cells, which was confirmed at the protein level. *Bmi-1* is known to epigenetically repress $p16^{INK4A}$ and $p14^{ARF}$; hence, decreased expression of $p16^{INK4A}$ and $p14^{ARF}$ was observed in $CD133^+$ cells (Fig. 4). A lentivirus-mediated shRNA knockdown strategy was used to study the functional effect of Bmi-1 loss on $CD133^+$ laryngeal tumor cells. Knockdown of Bmi-1 was confirmed by western blot (Fig. 5a). Results of the real-time PCR assay showed that the Bmi-1 mRNA expression level significantly decreased (Fig. 5b). The expression of Bmi-1-targeted genes, *INK4A* and *ARF*, was correspondingly enhanced in $CD133^+$/*Bmi-1* shRNA cells but no significant difference in $CD133^-$/*Bmi-1* shRNA (Fig. 5a, b). In corroboration with these findings, we also observed the upregulation of $p16^{INK4A}$ and $p14^{ARF}$ promoter reporter activity in $CD133^+$/Bmi-1 shRNA cells (Fig. 5c). These results indicated that BMI-1 pathway was efficiently downregulated.

BMI-1 knockdown suppresses oncogenic effects of CD133

Bmi-1 has an essential role in the self-renewal and differentiation of normal and cancer stem cells, and is implicated in the pathogenesis of various human malignancies. To determine if *Bmi-1* affects viability and proliferation of $CD133^+$ cells, we assessed the effects of altered Bmi-1 expression on the proliferation of CD133-sorted cells. In contrast to $CD133^-$ cells, in which *Bmi-1* knockdown had no effect on the cell survival, *Bmi-1* knockdown revealed a significantly inhibitory effect on both cell proliferation in vitro and tumor growth in vivo in $CD133^+$ cells (Fig. 6a, b). In addition, $CD133^+$ cells expressing sh*Bmi-1* showed an increased proportion of apoptotic cells, although there were no significant differences between $CD133^-$ cells and $CD133^-$/shBmi-1 (Fig. 6c). Moreover, Bmi-1-dependent reduction in clonogenicity was observed in $CD133^+$ cells but not in the $CD133^-$ cells by colony formation assays (Fig. 6d).

Cell cycle control is one of the major regulatory mechanisms of cell growth [20]. Therefore, cell cycle distribution was measured by flow cytometry to determine whether inhibition of cell growth was associated with cell cycle arrest. $CD133^+$ cells with downregulation of Bmi-1 displayed accumulation in G0/G1 and reduction in G2/M phase. The effect of inhibition on cell cycle by downregulation of *Bmi-1* was significantly stronger in $CD133^+$ cells when compared to $CD133^-$ cells (Fig. 6e). *Bmi-1* has been reported to exert its effects through suppression of $p16^{INK4A}$ and $p14^{ARF}$. Using a combination of real-time PCR, western blot, and reporter assay, we found that expression and activity of $p16^{INK4A}$ and

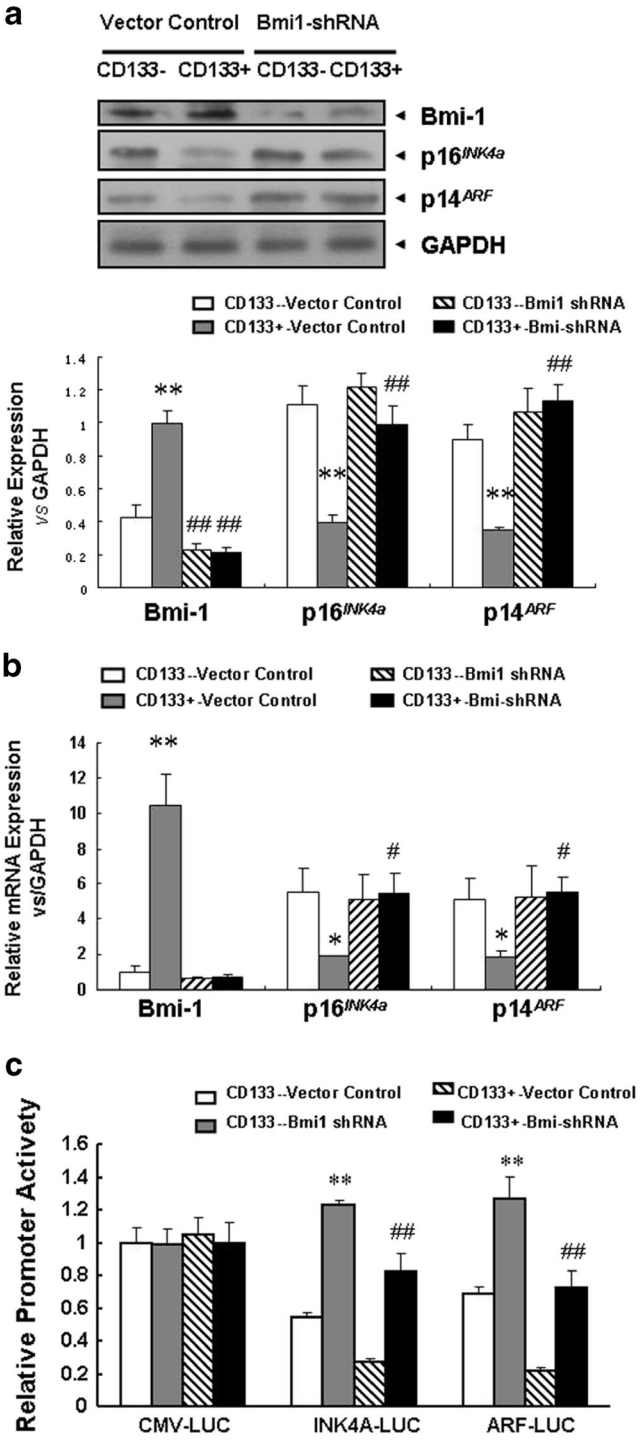

Fig. 5 *Bmi-1* specific shRNAs resulted in the reduction of *Bmi-1* mRNA and protein levels in CD133-sorted cells. **a** A drastic reduction of *Bmi-1* gene expression and upregulation of *INK4a*/*ARF* by real-time PCR analysis. **b** Western blot confirmed that *Bmi-1* knockdown released transcriptional repression of $p16^{INK4A}$ and $p14^{ARF}$. **c** The effects of silencing of *Bmi-1* on *INK4a*/*ARF* promoter activity were investigated using luciferase reporter gene. *Histograms* represented the average of three independent experiments with duplicates. $*p<0.05$; $**p<0.01$ versus $CD133^-$ cells group; $^{\#}p<0.05$ versus corresponding control

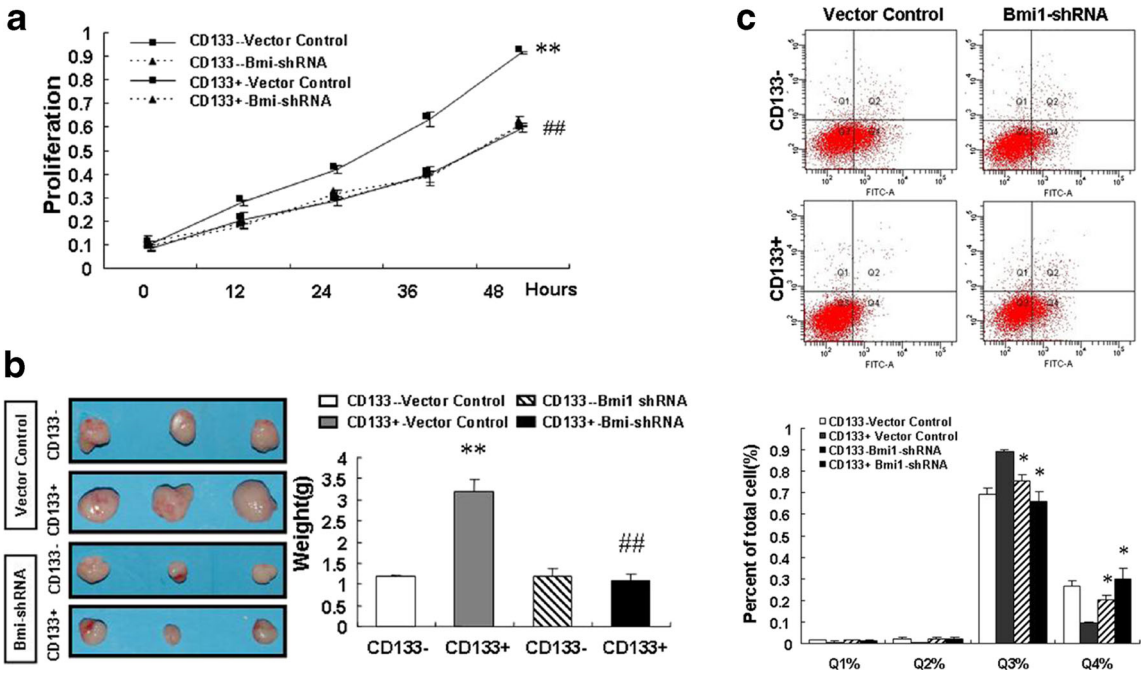

Fig. 6 Silencing of *Bmi-1* suppresses oncogenic properties of CD133$^+$ cells. **a** Effect of *Bmi-1* knockdown on cell growth of CD133-sorted cells by MTT assay. The cell proliferation ability was significantly decreased by sh*Bmi-1* in the CD133$^+$ group. **b** Effect of *Bmi-1* knockdown on the tumorigenesis of CD133-sorted cells in vivo. Silencing of sh*Bmi-1* showed a smaller tumor size in the CD133$^+$ cells group. Twenty-eight days after cell inoculation in nude mice, tumors were excised and weighed. Tumor weights of mice inoculated with CD133$^+$/CD133$^-$ vector or CD133$^+$/CD133$^-$ shRNA-*Bmi-1* cells are shown. CD133$^+$shRNA-*Bmi-1* cells formed smaller tumors than CD133$^+$/CD133$^-$ vector and CD133$^-$ shRNA-*Bmi-1* cells. **c** *Bmi-1* knockdown led to significantly higher levels of apoptosis in CD133$^+$ cells. The percentages of apoptotic cells were presented. **d** Effect of *Bmi-1*-shRNA on colony formation of CD133-sorted cells. Dramatically decreased colony number and size were observed in the CD133$^+$/sh*Bmi-1* group. Representative photographs of colony-forming assay of cells are shown and the number of colonies formed were counted and averaged. **e** The effect of *Bmi-1* downregulation on cell cycle was examined by flow cytometry. G2/M-phase cells were significantly reduced in the CD133$^+$/sh*Bmi-1* group. **f** *Bmi-1*-shRNA repressed the capacity of cell invasion. Representative image of a Transwell assay using *Bmi-1* shRNA knockdown in CD133-sorted cells for further elucidating the role of *Bmi-1* in tumor cell invasion. The experiments were repeated three times in triplicate. *$p<0.05$; **$p<0.01$ versus CD133$^-$ vector; $^{\#\#}p<0.01$ versus CD133$^+$/sh*Bmi-1*

p14^{ARF} were upregulated by *Bmi-1* knockdown in CD133$^+$ cells, which were unaffected in CD133$^-$ cells (Fig. 5c). Then, we tested the effect of *Bmi-1* inhibition on CD133-sorted cell metastasis. The effects of CD133 on invasion were reversed after knockdown of *Bmi-1* expression (Fig. 6f). Together, these data indicate that Bmi-1 expression is central to the oncogenic function of CD133$^+$ cells. It negatively regulates the transcription of the downstream INK4a/ARF gene and inhibits protein P16^{ink4a}/P14^{ARF} expression, so as to maintain the high proliferation of laryngeal cancer stem cells.

Discussion

It has been recognized that tumor tissues are composed of heterogeneous populations of cancer cells defined as CSC subset which are endowed with the tumorigenic capacity to self-renew, differentiate, and extensively form new tumors [21]. It was previously reported that CD133 was a marker of laryngeal CSCs using well-established cell lines [10]. In the present study, we have provided evidence that a rare subpopulation (CD133$^+$) of cancer cells (1–2 %) exist in primary human laryngeal tumor tissue. The migration and invasion of cancer cells is an important part of tumor metastasis, and only rare subpopulations of cancer cells are capable of metastasis. Our study demonstrates that the isolated CD133$^+$ laryngeal tumor cells were significantly more invasive than the CD133$^-$ cells. This minority subset possesses distinct properties, including high clonogenicity, increased invasiveness, and highly in vivo tumorigenic capacity, which support the widely held notion of the existence of a developmental hierarchy within human cancer [22]. Our study is the first to demonstrate the existence of CD133$^+$ cells with highly tumorigenic capacity in primary human laryngeal tumor cells.

Bmi-1 was initially known as an oncogene, which cooperates with c-myc in the development of murine lymphoma [12]. Several of studies suggested that *Bmi-1* has been associated with CSCs and involved in the metastasis of many cancers [23–26]. In corroboration with these findings, we also have found that *Bmi-1* is highly expressed in CD133$^+$ cells with increased tumorigenic capacity. Meanwhile, shRNA-mediated knockdown of *Bmi-1* effectively inhibited the

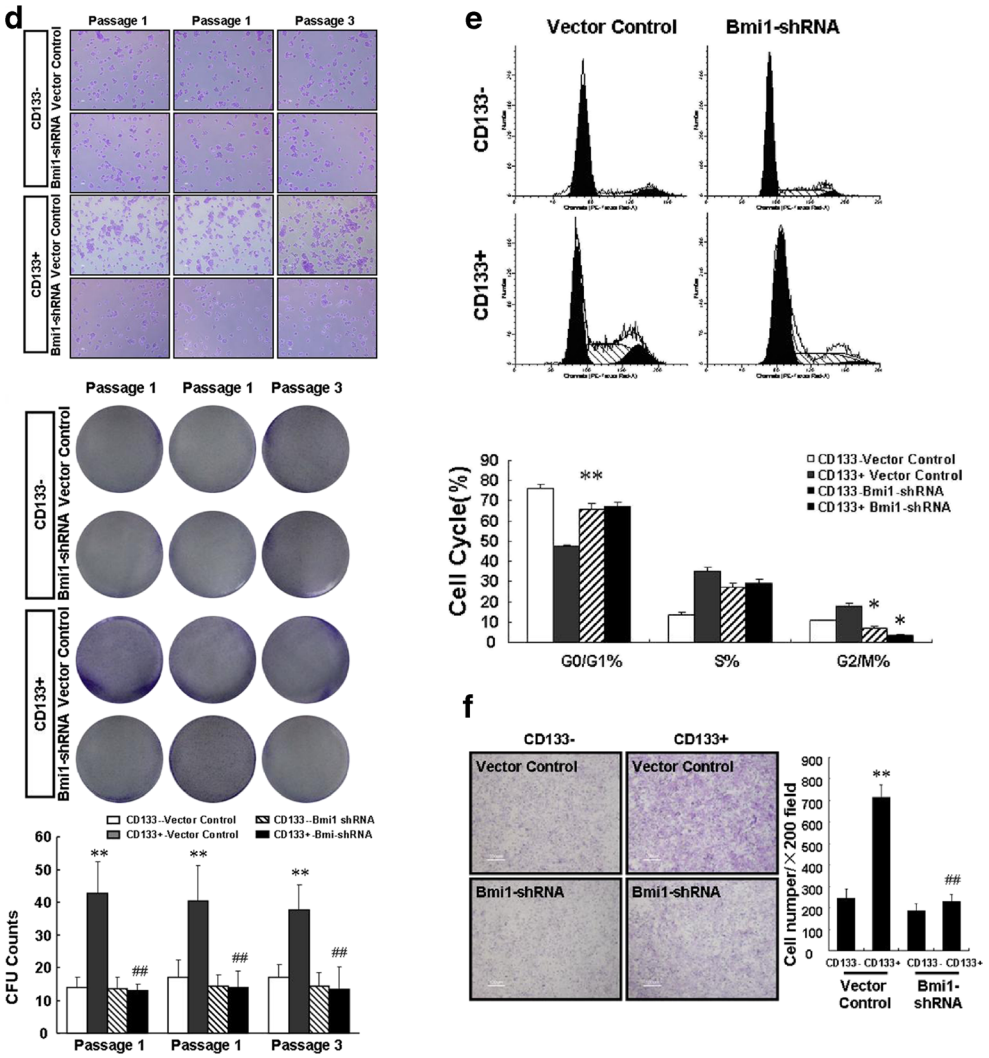

Fig. 6 (continued)

proliferation of $CD133^+$ laryngeal tumor cells in vitro and in vivo. Analysis of cell cycle distribution showed an accumulation of cells in the S and G2/M phase in $CD133^+$ cells compared with $CD133^-$ cells, with a concomitant decrease in the G0/G1 phase. It is well known that oncogenes or suppressor genes are linked to dysregulation of the cell cycle machinery in various types of human tumors, and changes in the G1- to S-phase transition genes are the most critical [18, 27]. We found *Bmi-1*-shRNA causes cell cycle arrest of $CD133^+$ cells in the G0/G1 phase, but not $CD133^-$ cells. The progression of the cell cycle is regulated by many factors, including cyclins, CDKs, and cyclin-dependent kinase inhibitors [20, 28]. We further clarified *Bmi-1*-mediated cell cycle arrest through the release of transcriptional repression of *INK4a*/*ARF* gene and $P16^{ink4a}$/$P14^{ARF}$ protein, which is consistent with previous reports [12, 29]. A clonogenic assay is an in vitro cell survival assay for studying the survival and proliferation of tumor cells, based on the ability of a single cell to grow into a colony. The assay essentially tests every cell in the population for its ability to undergo "unlimited" division [30]. We provide evidence that the proliferative potential $CD133^+$ cells lacking *Bmi-1* are compromised by clonogenic assay, which is likely due to a combination of increased apoptosis and blocked proliferation caused by upregulation of *INK4a*/*ARF* activity. These data indicate that the reduced tumor mass of $CD133^+$ cells is partially due to the combination of decreased proportion of cells going through S phase and increased fraction of cells undergoing cell apoptosis. Meanwhile, the invasiveness of $CD133^+$ cells was also inhibited by the downregulation of *Bmi-1*. These results provide substantial evidence that knockdown of *Bmi-1* gene expression contributes to diminishing $CD133^+$ cells metastasis and induced the $CD133^+$ cell to eventually undergo proliferation arrest and show signs of apoptosis. Taken together, our studies demonstrated that $CD133^+$ cells is involved in the aggressiveness of human laryngeal tumor, and that downregulation of *Bmi-1* activity blunted $CD133^+$ cell proliferation and invasion. It is also confirmed that the tumorigenic properties of $CD133^+$ cells are modulated

by *Bmi-1* through CDKN2A-dependent pathways to regulate cell proliferation and survival during cancer progression in primary human laryngeal tumor cells.

Just as Julie Lessard et al. [31, 32] have founded, retroviral introduction of Bmi-1 into the leukemic cell clones deficient in $p19^{ARF}$ and $p16^{INK4a}$ readily rescued their tumorigenic properties, suggesting that *Bmi-1* has one or more additional functions in leukemic stem cell besides repression of these cyclin-dependent kinase inhibitors such as $p19^{ARF}$ and $p16^{INK4a}$. Stem cell associated genes, cell survival gene, transcription factors, and other genes modulating proliferation may be altered respectively in *Bmi-1* knockout $CD133^+$ cells. It needs further investigation. Our results indicate that *Bmi-1* is essential for oncogenic potential in $CD133^+$ human laryngeal cancer cells.

Conclusion

The $CD133^+$ cells sorted from primary human laryngeal tumor are a tumor stem cell-like subpopulation that displays characteristics of high clone formation, strong tumorigenic ability, and enhanced capacity of proliferation and invasion. *Bmi-1*, implicated in self-renewal and tumorigenesis, was coexpressed with the CD133 and is central to the tumorigenicity of $CD133^+$ cells. It is indicated that *Bmi-1* is a pleiotropic regulator that maintains the viability and proliferative capacity of human laryngeal tumor. It negatively regulates the transcription of the downstream *INK4a*/*ARF* gene and inhibits expression of $P16^{ink4a}$/$P14^{ARF}$ protein, so as to maintain renewal ability of laryngeal cancer stem cells. *Bmi-1* may be a potential targeted gene for killing the laryngeal cancer stem cells.

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Conflicts of interests None

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