The Polycomb Gene Product BMI1 Contributes to the Maintenance of Tumor-Initiating Side Population Cells in Hepatocellular Carcinoma

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Abstract

Side population (SP) cell analysis and sorting have been successfully applied to hepatocellular carcinoma (HCC) cell lines to identify a minor cell population with cancer stem cell properties. However, the molecular mechanisms operating in SP cells remain unclear. The polycomb gene product BMI1 plays a central role in the self-renewal of somatic stem cells in a variety of tissues and organs and seems to be implicated in tumor development. In this study, we determined the critical role of BMI1 in the maintenance of cancer stem cells with the SP phenotype in HCC cell lines. BMI1 was preferentially expressed in SP cells in Huh7 and PLC/PRF/5 HCC cells compared with the corresponding non-SP cells. Lentiviral knockdown of *BMI1* considerably decreased the number of SP cells in both Huh7 and PLC/PRF/5 cells. Long-term culture of purified SP cells resulted in a drastic reduction in the SP subpopulation upon the *BMI1* knockdown, indicating that BMI1 is required for the self-renewal of SP cells in culture. More importantly, the *BMI1* knockdown abolished the tumor-initiating ability of SP cells in nonobese diabetic/severe combined immunodeficiency mice. Derepression of the *INK4A* and *ARF* genes that are major targets for BMI1 was not necessarily associated with impaired self-renewal of SP cells caused by *BMI1* knockdown. In conclusion, our findings define an important role for BMI1 in the maintenance of tumor-initiating SP cells in HCC. BMI1 might be a novel therapeutic target for the eradication of cancer stem cells in HCC. [Cancer Res 2008;68(19):7742–9]

Introduction

According to the recent "cancer stem cell hypothesis," tumors consist of a minor component of tumorigenic cells and a major component of nontumorigenic cells (1, 2). The minor population, termed cancer stem cells or tumor-initiating cells, construct a hierarchical structure containing varied descendants in a similar fashion to the normal stem cell systems and possesses a prominent ability to initiate new tumors in xenograft transplantation (3, 4). In addition, these cells seem to be highly resistant to traditional forms of anticancer therapy such as chemotherapy and radiotherapy (5, 6), resulting in residual cancer stem cells which, in many instances, lead to the recurrence of the cancer (7). Therefore, an overall understanding of the various biological aspects of cancer stem cells is of paramount importance to both the elucidation of mechanisms underlying carcinogenesis and the establishment of novel therapeutic strategies.

Side population (SP) cell analysis and sorting were initially used for the isolation of hematopoietic stem cells in bone marrow cells (8). Currently, they are widely applied to the enrichment of putative normal stem cells in a variety of tissues and organs (9–11). The SP phenotype is determined by the ability to efflux the Hoechst 33342 dye through an ATP-binding cassette (ABC) membrane transporter. Of note, recent studies showed that SP cells isolated from diverse cancer cell lines harbor stem cell–like properties (12–14). Given that many different types of cancer cells frequently show overexpression of ABC transporters and exhibit drug resistance (15), it is quite reasonable to detect stem-like fractions in cancer cells using this approach. We previously applied SP analysis and sorting to established hepatocellular carcinoma (HCC) cell lines and found that in Huh7 and PLC/PRF/5 cells, SP fractions made up <1% of the total cell population (12). As expected, the SP subpopulations showed cancer stem cell–like properties both in culture and in an *in vivo* transplant model. These stem cell biology-based strategies enabled us to perform further analyses.

We and others previously reported that the polycomb-group (PcG) gene *Bmi1* plays a critical role in the self-renewal of a range of somatic stem cells, including hepatic stem cells, based on gain-of-function and loss-of-function analyses (16, 17). It seems likely that both normal and cancer stem cells share not only a number of surface marker phenotypes but also a list of molecular mechanisms for self-renewal and differentiation. This has been well shown in the leukemic stem cell system (18–20), although little is known in solid cancers.

In the current study, we examined the crucial role of BMI1 in the maintenance of the tumor-initiating SP subpopulation in HCC cells. Taking advantage of lentivirus-mediated knockdown and retrovirus-mediated overexpression techniques, we examined whether BMI1 regulates the self-renewal and differentiation of SP cells in culture and their tumorigenicity in a nonobese diabetic/severe combined immunodeficient (NOD/SCID) xenograft transplant model.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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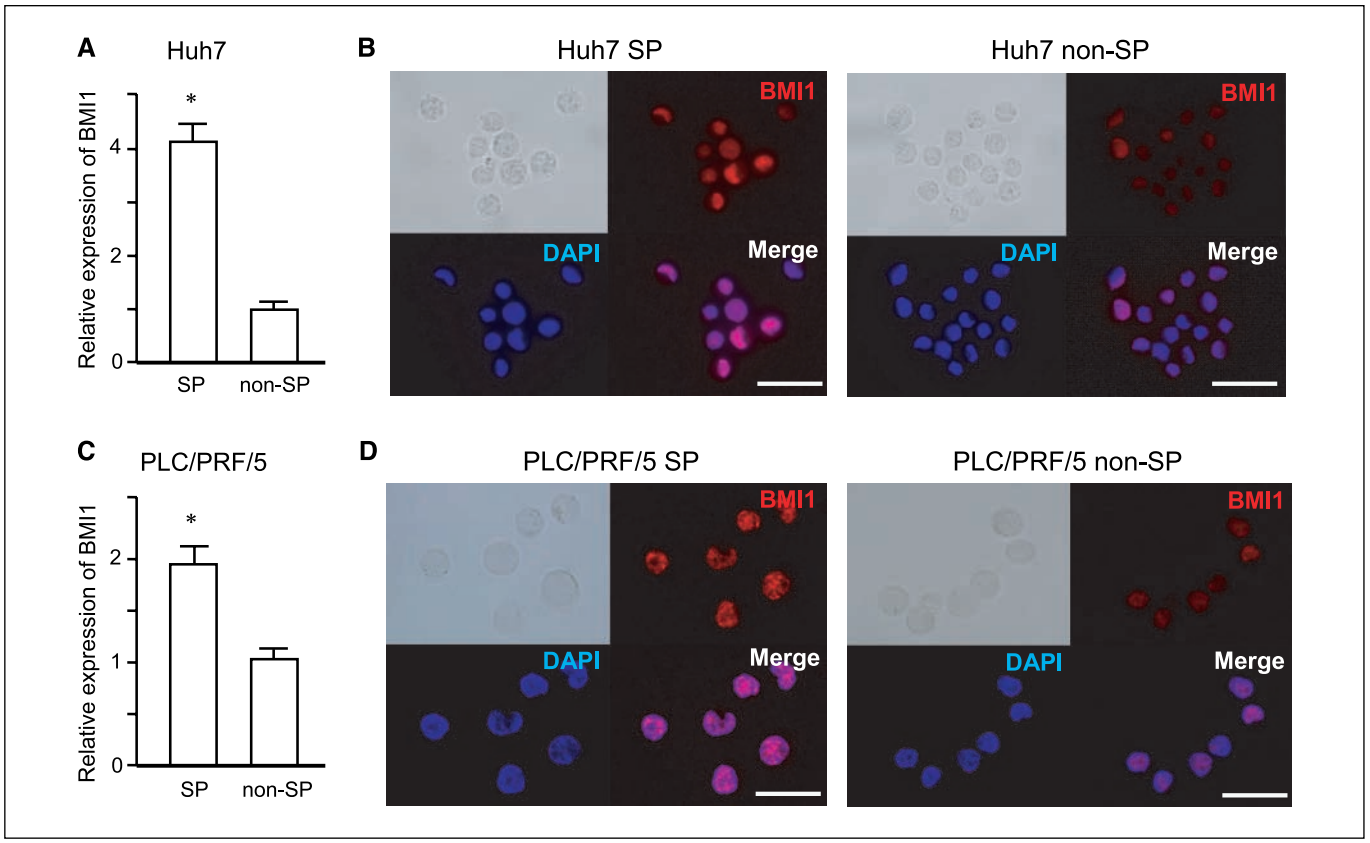

Figure 1. Expression of BMI1 in SP and non-SP cells. Real-time RT-PCR analyses of *BMI1* expression in SP and non-SP cells from Huh7 (*A*) and PLC/PRF/5 cells (*C*). Immunocytochemical analyses of BMI1 expression in SP and non-SP cells from Huh7 (*B*) and PLC/PRF/5 cells (*D*). Nuclear DAPI staining (*blue*) and immunofluorescent labeling of BMI1 (*red*) are merged. *, statistically significant ($P < 0.05$). *Scale bar,* 50 μm.

Materials and Methods

Mice. NOD/SCID mice were purchased from Sankyo Laboratory Co. Ltd. They were bred and maintained in accordance with our institutional guidelines for the use of laboratory animals.

Cell culture. The human liver cancer cell lines Huh7 and PLC/PRF/5 were cultured in DMEM (Invitrogen Life Technologies) containing 10% FCS and 1% penicillin/streptomycin (Invitrogen).

Flow cytometry. SP analysis and sorting were performed, as described previously (12). Briefly, the suspended cells were incubated at 37°C for 90 min with 20 μg/mL Hoechst 33342 (Sigma Chemical), either alone or in the presence of 50 μmol/L verapamil (Sigma). For the analysis of CD133 expression, cells were incubated with phycoerythrin-conjugated CD133/1 (Miltenyi Biotec). Propidium iodide (BD PharMingen) was added for the elimination of dead cells. Cell analysis and sorting were performed using MoFlo (DakoCytomation).

Immunocytochemistry. Freshly isolated SP cells and non-SP cells were placed on poly-L-lysine–coated slide glasses. After fixation with 2% paraformaldehyde and blocking in 10% goat serum, the cells were incubated with 0.5% Triton-X in PBS for 20 min at room temperature. After incubation, the cells were stained with a primary antibody, anti-Bmi1 (F6; Upstate Biotechnology), at a dilution of 1:200 for 12 h at 4°C. The cells then were washed and incubated with Alexa-555–conjugated goat anti-mouse IgG (Molecular Probes) at a dilution of 1:500 for 2 h at room temperature. After being washed in PBS, the cells were coverslipped with a mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories).

Viral production and transduction. Lentiviral vectors (CS-H1-shRNA-EF-1α-EGFP) expressing short hairpin RNA (shRNA) that targets human *BMI1* (target sequence: sh-*BMI1*-1, 5′-CAGATGAAGATAAGAGAAT-3′; sh-*BMI1*-2, 5′-GAGAAGGAATGGTCCACTT-3′) and *luciferase* were constructed. Human *BMI1* cDNA (a kind gift from Dr. Kazuhito Yamamoto) was cloned into a site upstream of IRES–enhanced green fluorescent protein (EGFP) in the pMCs-IG retroviral vector (21). Recombinant lentiviruses and retroviruses were produced as described before (17, 22).

Western blotting. Cells transduced with the indicated viruses were selected by cell sorting for EGFP expression and subjected to Western blot analysis using anti-Bmi1 (F6) and anti–α-tubulin (Ab-1; Oncogene Science) antibodies.

Reverse transcription–PCR. Total RNA extraction and cDNA synthesis were conducted, as described previously (12). Real-time PCR was performed using TaqMan technology and the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). TaqMan probe and primers for *BMI1* (assay ID Hs00180411_m1) and *β-actin* (assay ID Hs99999903_m1) were obtained from TaqMan gene expression assays (Applied Biosystems). To examine the mRNA expression of *INK4A/ARF* genes in SP cells following *BMI1* knockdown, multiplex reverse transcription–PCR (RT-PCR) was performed as described previously (23). PCR for *BMI1* and *β-actin* was conducted using the following primers: *BMI1* (forward 5′-AGC AGC AAT GAC TGT GAT GC-3′, reverse 5′-CAG TCT CAG GTA TCA ACC AG-3′), *β-actin* (forward 5′-ATC CTG CGT CTG GAC CTG GCT GG-3′, reverse 5′-ACA TGC CGG AGC CGT TGT CGA CGA-3′).

Xenograft transplantation. Various numbers of SP and non-SP cells stably expressing shRNA against *BMI1* or *luciferase* were suspended in DMEM and Matrigel (Becton Dickinson; 1:1) and transplanted to NOD/SCID mice (male, 6–10 wk) under anesthesia. *BMI1* knockdown cells and control cells were implanted into the s.c. space on the right and left sides of the back of recipient mice, respectively. To examine whether enforced expression of *BMI1* in SP cells promotes tumorigenesis, 1×10^4 Huh7 SP cells transduced with *BMI1* and *EGFP* retroviruses were also transplanted.

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Tumor formation was observed weekly for 14 wk. The transplantation assays were performed in accordance with institutional guidelines for the use of laboratory animals.

Immunohistochemical analysis. The subcutaneous tumors formed in NOD/SCID mice were fixed in formalin and embedded in paraffin. The sections were subjected to H&E staining. For dual immunohistochemical analyses, the sections were stained with anti-EGFP (BD Biosciences Clontech) and anti-BMI1 (F6), followed by incubation with Alexa-488–conjugated goat anti-rabbit IgG and Alexa-555–conjugated goat anti-mouse IgG (Molecular Probes), respectively.

Statistical analysis. Data are presented as the means ± SE. Statistical differences between two groups were analyzed using the Mann-Whitney units test. P values <0.05 were considered significant.

Results

Preferential expression of BMI1 in SP cells. To gain insight into the crucial role of the polycomb gene product BMI1, we first examined the basal expression of BMI1 in the SP population in Huh7 and PLC/PRF/5 cells. Real-time RT-PCR analyses showed that the mRNA expression of *BMI1* was 4.10 ± 0.36-fold and 1.92 ± 0.25-fold higher in Huh7 and PLC/PRF/5 SP cells than in the corresponding non-SP cells, respectively (Fig. 1*A* and *C*). Immunocytochemical analyses confirmed that BMI1 is highly expressed in the nuclei of SP cells rather than the corresponding non-SP cells in both cell lines (Fig. 1*B* and *D*).

Stable knockdown of *BMI1* by shRNA. We next performed loss-of-function analyses of BMI1 *in vitro*. Stable knockdown of *BMI1* in Huh7 and PLC/PRF/5 cells was achieved by lentivirus-mediated delivery of shRNA against *BMI1*. A lentiviral vector expressing shRNA against *luciferase* was used as a control. We obtained stable cell lines expressing shRNA against *BMI1* or *luciferase* by cell sorting using EGFP as a marker for infection. Western blot analysis showed that both sh-*BMI1*-1 and sh-*BMI1*-2 markedly repressed BMI1 expression in both cell lines, although sh-*BMI1*-1 was less effective than sh-*BMI1*-2 (Fig. 2*A*). Both shRNA inhibited the growth of HCC cell lines. In good agreement with the Western blot data, sh-*BMI1*-2 was more effective in growth suppression than sh-*BMI1*-1 (Fig. 2*B* and *C*). The viability of cells expressing shRNA against *BMI1* was comparable with that of control cells (data not shown).

Detection and isolation of SP cells. SP cell analysis and sorting were performed in Huh7 and PLC/PRF/5 cells stably expressing shRNA against *BMI1* (Fig. 3 and Supplementary Fig. S1). *BMI1* knockdown using sh-*BMI1*-2 considerably decreased the size of the SP population from 0.67 ± 0.09% to 0.19 ± 0.03% in Huh7 cells and from 0.87 ± 0.10% to 0.40 ± 0.04% in PLC/PRF/5 cells (Fig. 3). On the other hand, *BMI1* knockdown using sh-*BMI1*-1 slightly decreased the percentage of SP cells from 0.59 ± 0.04% to 0.32 ± 0.03% in Huh7 cells and from 0.82 ± 0.06% to 0.47 ± 0.03% in PLC/PRF/5 cells (Supplementary Fig. S1). The SP population showed a drastic reduction in number on treatment with the calcium channel blocker verapamil.

Stable overexpression of *BMI1* by retroviral vector. Next, we tested the overexpression of *BMI1* in HCC cells (Supplementary Fig. S2*A*). In clear contrast with the knockdown experiment, the SP subpopulation increased nearly 8-fold with the overexpression of *BMI1* in Huh7 cells (Supplementary Fig. S2*B*). Next, we examined the tumorigenicity of Huh7 SP cells transduced with *BMI1* in NOD/SCID xenograft transplantation. The implantation of 1×10^4 SP cells transduced with *BMI1* resulted in early onset and aggressive

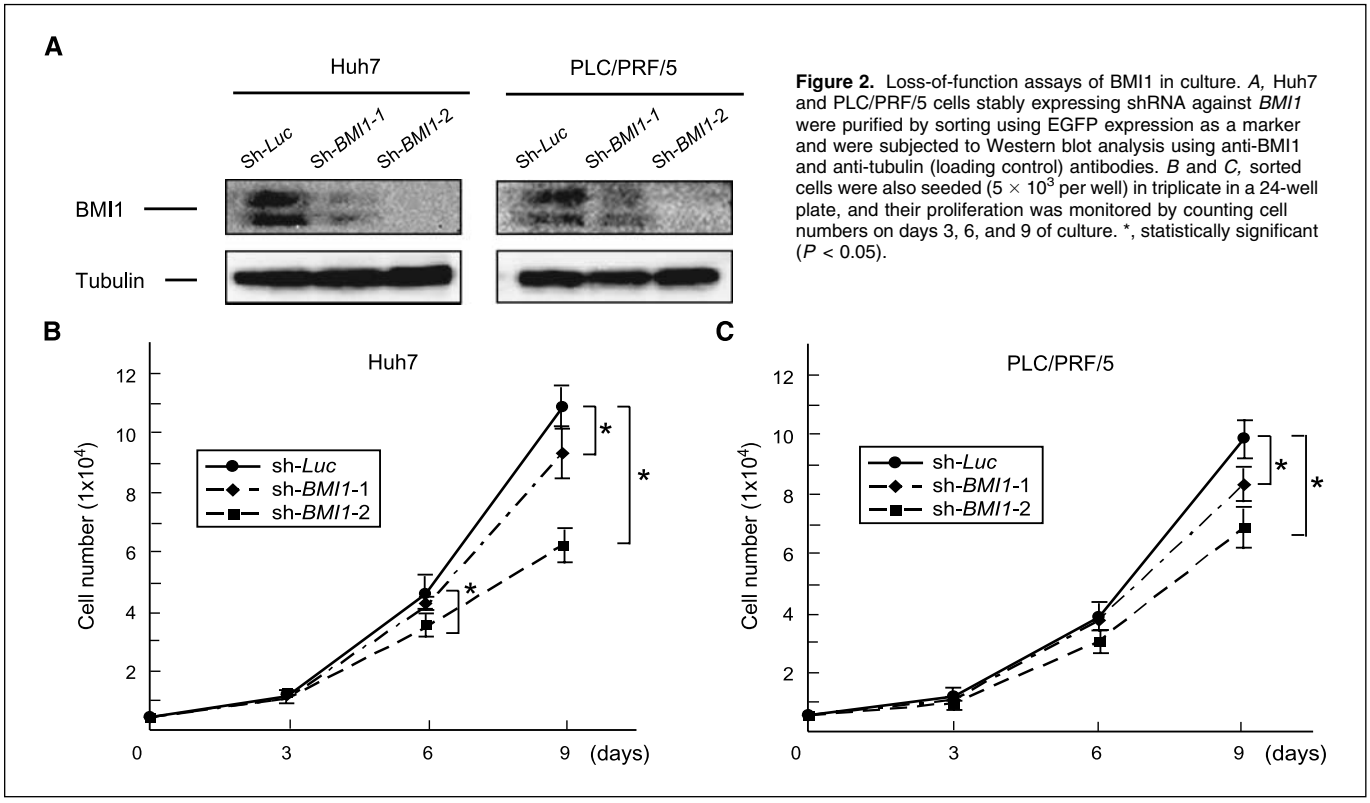

Figure 2. Loss-of-function assays of BMI1 in culture. *A,* Huh7 and PLC/PRF/5 cells stably expressing shRNA against *BMI1* were purified by sorting using EGFP expression as a marker and were subjected to Western blot analysis using anti-BMI1 and anti-tubulin (loading control) antibodies. *B* and *C,* sorted cells were also seeded (5×10^3 per well) in triplicate in a 24-well plate, and their proliferation was monitored by counting cell numbers on days 3, 6, and 9 of culture. *, statistically significant ($P < 0.05$).

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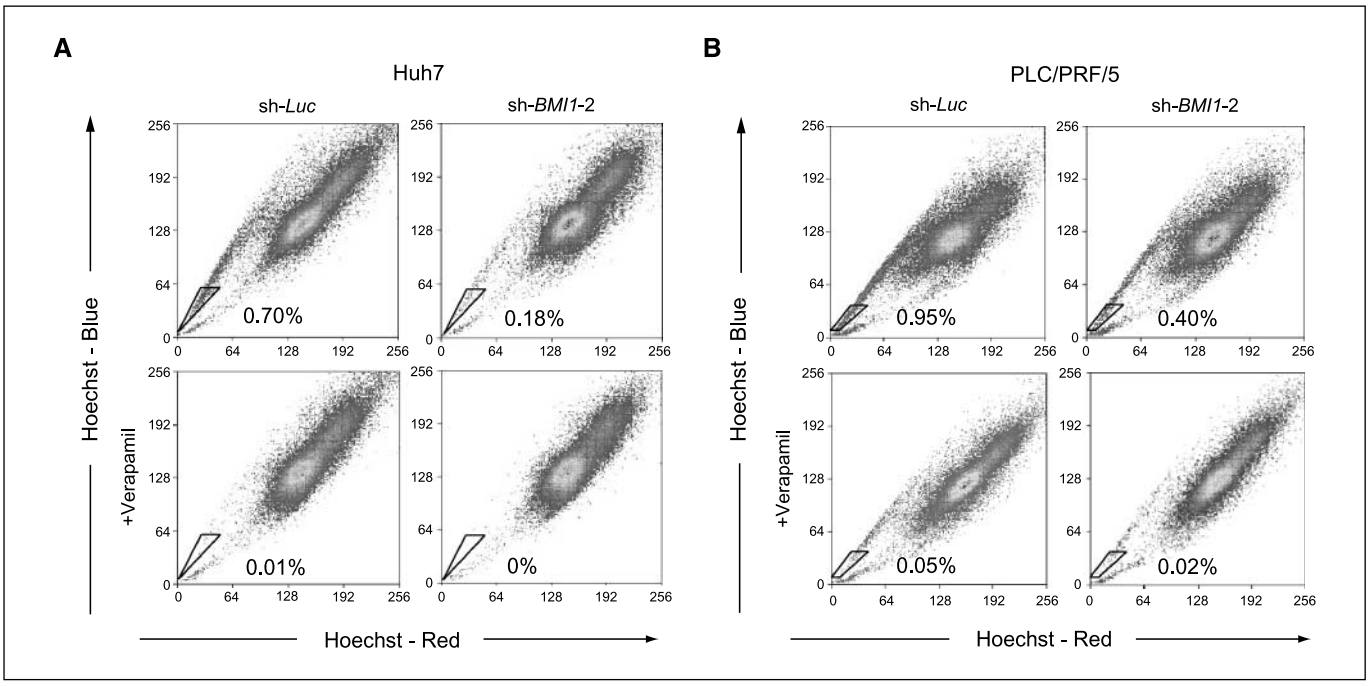

Figure 3. SP cell analysis in *BMI1* knockdown HCC cells by sh-*BMI1*-2. Flow cytometric profiles of SP cells among Huh7 (*A*) and PLC/PRF/5 cells (*B*) after stable knockdown of *BMI1* by sh-*BMI1*-2. SP cell profiles in the presence of verapamil are depicted at the bottom. The percentages of SP cells are indicated.

tumor growth compared with that of control SP cells expressing EGFP (Supplementary Fig. S2*C* and *D*). These results indicated that forced expression of *BMI1* leads to not only enhanced self-renewal but also increased tumorigenicity of SP cells.

Expression of the *INK4A/ARF* gene in *BMI1* knockdown SP cells. To address whether the knockdown of *BMI1* causes derepression of the *INK4A* and *ARF* genes as observed in *Bmi1*-deficient hematopoietic stem cells (HSC; ref. 17), we examined their mRNA expression in SP cells freshly isolated from *BMI1* knockdown and control cells (Fig. 4*A*). In Huh7 SP cells, in which *INK4A* expression is repressed by aberrant DNA methylation in its promoter region (23) and *ARF* is constitutively expressed, *BMI1* knockdown did not affect their expression at all. Conversely, *BMI1* knockdown in PLC/PRF/5 SP cells, in which both *INK4A* and *ARF* are moderately expressed, greatly augmented their expression.

Reanalysis of isolated SP cells. We have previously reported that purified SP cells self-renew and generate nontumorigenic non-SP cells through asymmetrical cell division *in vivo* (12). Purified SP cells repopulate the same hierarchical structure as the original tumor cells consisting of a minor component of SP cells and a major component of non-SP cells (12, 24). This process occurs both *in vitro* and *in vivo*. The SP cells in repopulated tumor retain the same tumor-initiating capacity as the original SP cells. We purified SP cells from both *BMI1* knockdown and control cells and cultured them for 4 weeks to examine the role of BMI1 in this process in culture. The SP subpopulation in *BMI1* knockdown Huh7 cells profoundly decreased (5.5%) compared with that in the control cells (18.8%; Fig. 4*B*). Likewise, the percentage of PLC/PRF/5 SP cells among *BMI1* knockdown and control cells was 6.1% and 22.0%, respectively (Fig. 4*B*). These results imply that BMI1 regulates the self-renewal capability of tumor-initiating SP cells and loss of BMI1 accelerates differentiation toward nontumorigenic non-SP cells.

The role for BMI1 in the maintenance of tumorigenic CD133-positive Huh7 cells. It has been reported that CD133-positive cells possessed greater tumorigenicity than CD133-negative cells in HCC cells, including Huh7 cells (25). Although the majority of Huh7 cells are CD133-positive (Fig. 4*C*), it has been shown that CD133 expression is stronger in SP cells than in non-SP cells (24). We then evaluated the expression of CD133 in the context of BMI1 expression using flow cytometry. *BMI1* knockdown decreased the CD133-positive fraction from 74.2% to 60.9%, whereas *BMI1* overexpression increased it from 71.6% to 84.4% (Fig. 4*C*). These findings indicate that the expression level of BMI1 is tightly correlated with the cancer stem cell phenotype represented not only by SP cells but also by CD133-positive cells.

Tumorigenic ability in xenograft transplantation. To determine whether loss of BMI1 affects the tumorigenicity *in vivo*, various numbers of SP and non-SP cells sorted from the *BMI1* knockdown or control HCC cells were transplanted into NOD/SCID mice (Fig. 5; Table 1). As few as 1×10^3 control SP cells were enough to initiate tumors for both cell lines. In contrast, 1×10^3 *BMI1* knockdown SP cells transduced with sh-*BMI1*-1 and 1×10^4 *BMI1* knockdown SP cells transduced with sh-*BMI1*-2 from Huh7 and PLC/PRF/5 cells failed to initiate subcutaneous tumors in any recipient mice. Tumors derived from control SP cells showed similar histologic features to those formed by the injection of unsorted cells and exhibited the nuclear expression of BMI1 (Fig. 5). Unexpectedly, 1×10^5 *BMI1* knockdown Huh7 and PLC/PRF/5 SP cells with sh-*BMI1*-2 gave rise to tumors in one of three and one of two mice, respectively. However, the tumor size was less than half that of control SP cells. Furthermore,

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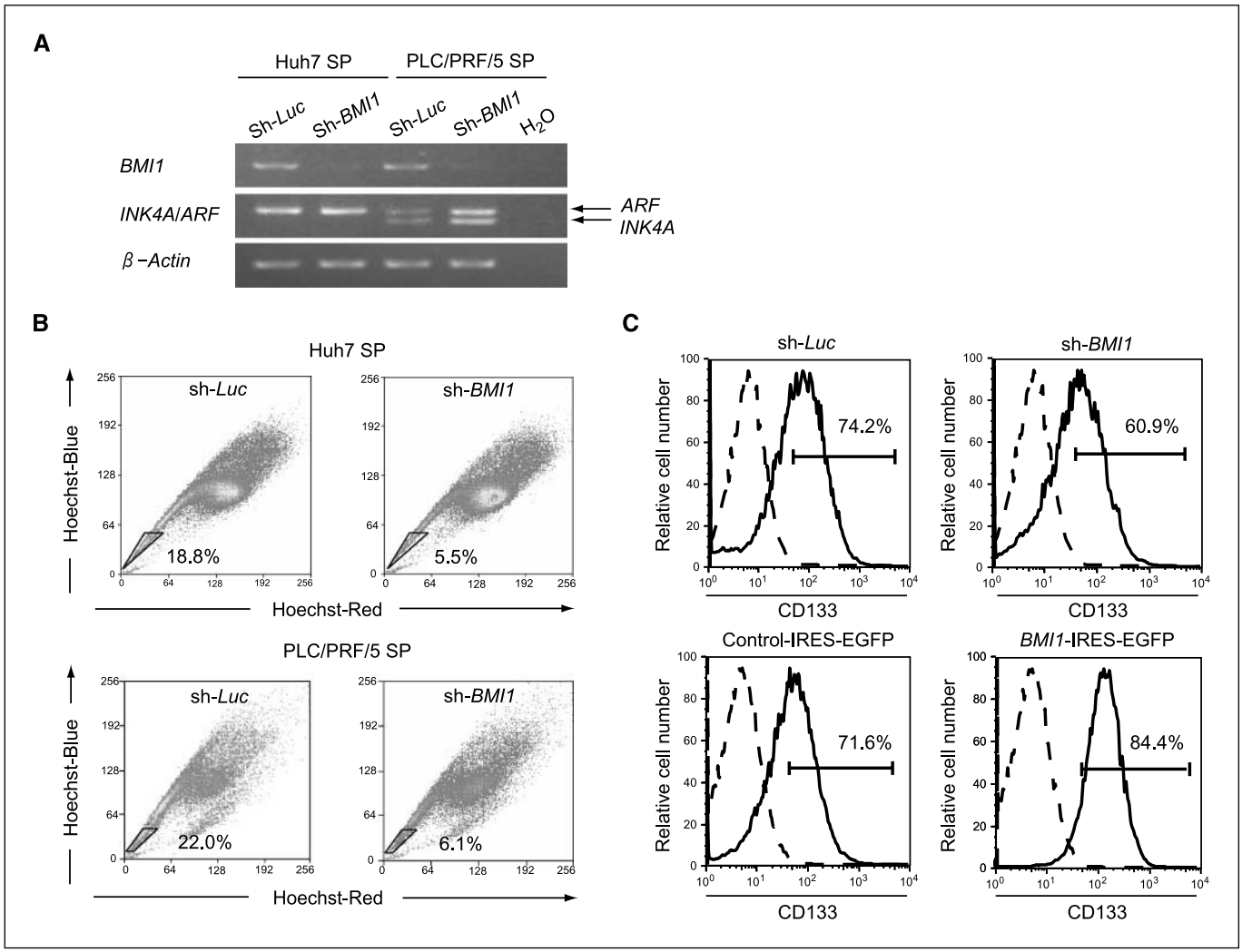

Figure 4. Role of BMI1 in *INK4A/ARF* expression and cancer stem cell phenotype. *A,* RT-PCR analysis of *INK4A/ARF* in *BMI1* knockdown SP cells from Huh7 and PLC/PRF/5 cells. Lane H_2O represents the negative control without the template. *B,* flow cytometric profiles of purified SP cells after culture. SP cells were purified from *BMI1* knockdown cells, cultured for 4 wk, and then subjected to the flow cytometric analysis. *C,* expression of CD133 in *BMI1* knockdown (*top*) and *BMI1* overexpressing cells (*bottom*) detected by flow cytometric analysis. Dotted line represents negative controls. The percentages of CD133-positive cells are indicated.

immunohistochemical analyses revealed that tumors predominantly consisted of contaminating EGFP-negative cells or EGFP-positive cells showing no obvious effects of *BMI1* knockdown (Supplementary Fig. S3). By contrast, tumors derived from control SP cells did not contain EGFP-negative cells at all (Fig. 5). These results further support that the tumor-initiating capacity is profoundly impaired by *BMI1* knockdown. In contrast, the injection of 1×10^6 non-SP cells from either cell line failed to generate tumors in any mice.

Discussion

PcG proteins form chromatin-associated multiprotein complexes, polycomb repressive complex 1 (PRC1) and PRC2 and function as a cellular memory system through epigenetic chromatin modifications (26, 27). Bmi1, a component of PRC1, has been implicated in the regulation of self-renewal in a range of different stem cell systems (28, 29). Of note, Bmi1 is also required for the maintenance of self-renewing leukemic stem cells in a mouse model using *Bmi1*$^{-/-}$ HSCs (30). Recent reports described that BMI1 is preferentially expressed in the tumorigenic subpopulation in breast cancer and head and neck tumors (31, 32). Consistent with these reports, we previously showed that forced expression of *BMI1* promotes the self-renewal of hepatic stem/progenitor cells and contributes to malignant transformation (16). In addition, immunohistochemical analyses showed BMI1 to be overexpressed in >60% of human HCC cases examined.[6] Together, all these findings highlight the importance of BMI1 in hepatocarcinogenesis and implicate BMI1 in the self-renewal of cancer stem cells in HCC.

In the present study, we first examined the basal expression of BMI1 in Huh7 and PLC/PRF/5 SP cells. As expected, both the real-

[6] Unpublished data.

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time RT-PCR and immunocytochemical analyses showed BMI1 expression to be stronger in SP cells than non-SP cells in each cell line. We thus directly evaluated the role of BMI1 in cancer stem cell–like SP cells. Lentiviral shRNA-mediated knockdown of *BMI1* allowed a highly efficient loss-of-function assay of the SP subpopulation in culture and in an *in vivo* transplant model. The analysis showed a significant decrease in the frequency of SP cells among the *BMI1* knockdown cells compared with the corresponding control cells. Furthermore, analysis of the growth and differentiation of purified SP cells revealed that loss of BMI1 causes a considerable decrease in the SP subpopulation and facilitates differentiation toward non-SP cells. These results indicated that BMI1 contributes to the self-renewal of SP cells in culture.

Notably, when as few as 1×10^3 control SP cells were sufficient to initiate tumors in NOD/SCID mice, even 10 times more *BMI1* knockdown SP cells (1×10^4) failed to develop tumors. 1×10^5 *BMI1* knockdown SP cells gave increase to tumors in some of the recipient mice, but the tumor-initiating capacity was profoundly reduced. Collectively, the tumorigenic activity in the SP subpopulation seemed to be attenuated nearly 100-fold by the *BMI1* knockdown. Although the important role of Bmi1 in the maintenance of cancer stem cells has already been shown in a mouse leukemia model, this is the first direct evidence that the loss of BMI1 in established cancer stem cells can affect their ability to self-renew and tumorigenicity. The role of BMI1 in the regulation of tumor-initiating SP cells was further supported by the findings of the gain-of-function assay. Although stable knockdown of *BMI1* decreased SP cell numbers *in vitro*, it did not completely abolish SP cells and its effect varied among HCC cell lines. These results strongly indicate that the SP subpopulation is quite heterogeneous, and the contribution of BMI1 to the SP phenotype differs among the cell lines. Considering that BMI1 is just one of multiple self-renewal regulators, the different contributions of molecular machinery, including the Notch, Wnt, and Shh signaling pathways, might also influence the SP phenotype (33). Further analyses would be necessary to clarify the mechanisms underlying the regulation of cancer stem cells in HCC.

Bmi1 regulates the cell cycle, apoptosis and senescence by repressing the *Ink4a/Arf* locus (26, 34). In *Bmi1*-deficient mice, the expression of *Ink4a* and *Arf* is markedly increased in HSCs (17). Conversely, deletion of both *Ink4a* and *Arf* substantially restores the impaired capacity of *Bmi1*$^{-/-}$ HSCs to self-renew (22). These findings suggest that Bmi1 regulates HSCs by acting as a critical failsafe against the premature loss of HSCs induced by Ink4a and Arf-dependent senescence pathways. On the other hand, the Ink4a-Rb and Arf-p53–dependent cellular senescence pathways play a critical role in the triggering of oncogene-induced senescence, which is of substantial importance to the elimination of transforming cells that potentially develop into cancer stem cells (26, 35). In the present study, the expression of the *INK4A* and *ARF* genes was augmented by *BMI1* knockdown in PLC/PRF/5 cells. In this case, derepression of *INK4A* and *ARF* could account for the impaired self-renewal of PLC/PRF/5 SP cells with *BMI1* knockdown. On the other hand, knockdown of *BMI1* in Huh7 cells resulted in no remarkable changes in *INK4A* and *ARF* gene expression compared with the control. Given that the function of p53 is impeded by mutations in Huh7 cells (36), additional targets for BMI1 other than the *INK4A/ARF* locus might be responsible for

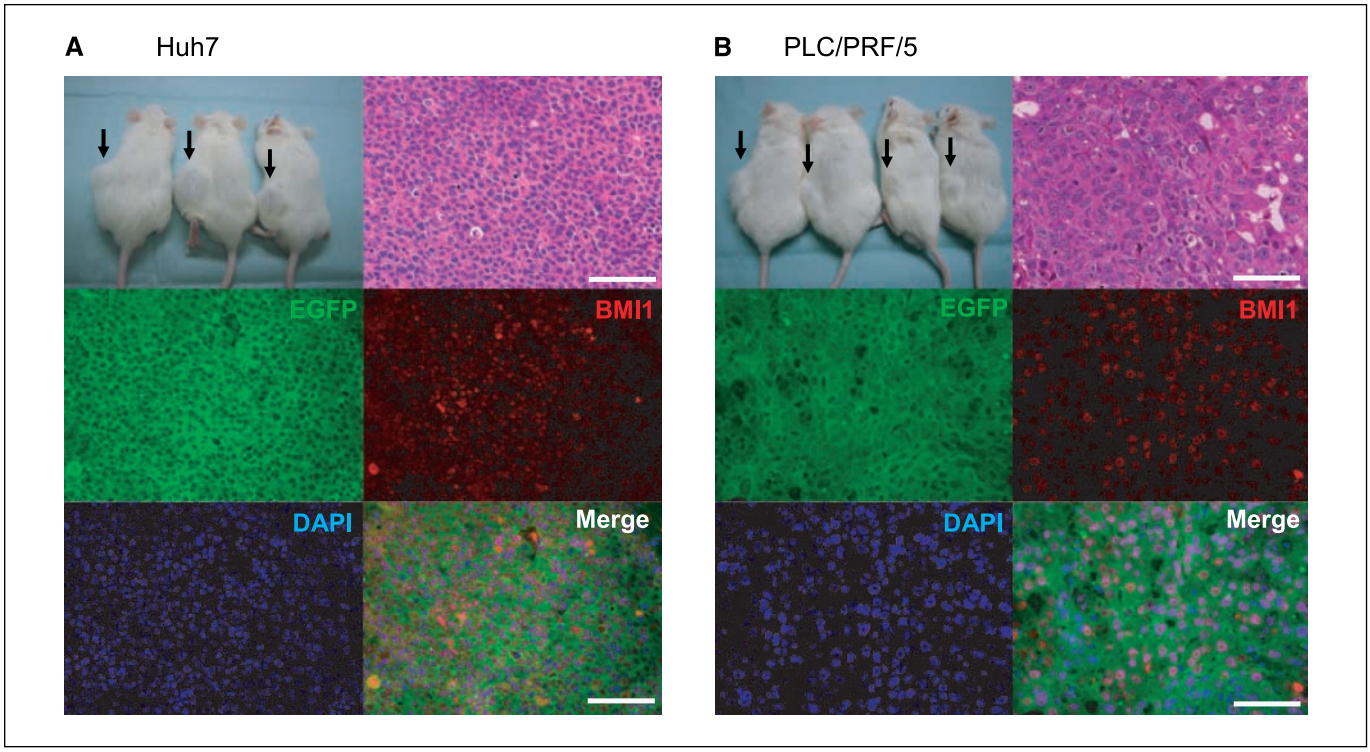

Figure 5. Loss of tumorigenicity in SP cells by *BMI1* knockdown. 1×10^3 control SP cells from Huh7 (*A*) and PLC/PRF/5 cells (*B*) generated tumors in the left subcutaneous space of recipient mice (*arrows*), whereas *BMI1* knockdown SP cells failed to initiate tumors in the right space. Immunohistochemical analyses revealed the nuclear localization of BMI1 in tumor cells generated by control SP cells. *Scale bar,* 100 μm.

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Table 1. Tumor-initiating ability of SP cells in the NOD/SCID xenograft transplant model

	No. implanted cells				
	100	1×10^3	1×10^4	1×10^5	1×10^6
Huh7					
sh-*Luc*					
SP cells	0/5	14/15	13/13		
Non-SP cells		0/15	0/14	0/10	0/5
sh-*BMI1*-1					
SP cells	0/5	0/6	3/6*		
Non-SP cells		0/6	0/6	0/5	0/5
sh-*BMI1*-2					
SP cells	0/5	0/9	0/8	1/3 †	
Non-SP cells		0/9	0/8	0/5	0/5
PLC/PRF/5					
Sh-*Luc*					
SP cells	0/5	14/14	14/14		
Non-SP cells		0/14	0/14	0/10	0/5
sh-*BMI1*-1					
SP cells	0/5	0/5	2/5*		
Non-SP cells		0/4	0/5	0/5	0/5
sh-*BMI1*-2					
SP cells	0/5	0/9	0/9	1/2 †	
Non-SP cells		0/9	0/9	0/5	0/5

NOTE: Tumor initiation was monitored for 14 wk after implantation.
*Delayed tumor formation and a decrease in tumor size were observed compared with tumors derived from control SP cells.
†Immunohistochemical data for tumors are displayed in Supplementary Fig. S3.

the impaired ability of Huh7 SP cells to self-renew. Thus, BMI1 might function in the maintenance of HCC cancer stem cells in both an *INK4A/ARF*-dependent and an *INK4A/ARF*-independent manner.

Finally, the present loss-of-function and gain-of-function assays revealed that BMI1 determines the self-renewal capability of SP cells, which directly contributes to the tumorigenic potential. However, further analysis will definitely be necessary to determine the role of BMI1 in primary HCC cancer stem cells (37). Our findings also indicate BMI1 to be a novel therapeutic target for the eradication of cancer stem cells in HCC. It would be of paramount importance to understand differential functions and targets of BMI1 in normal and cancer stem cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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