

utNgn1 locus is maintained in a poised (or inactive) state in the late stage of neocortical NPC development by a PcG protein-mediated mechanism, given that the locus is enriched with both H3K27me3 and H3K4me3 and that *utNgn1* expression was derepressed by ablation of Ring1B. These findings thus suggest that PcG proteins may directly suppress enhancers in addition to promoters for a class of developmental genes in a developmental context-dependent manner.

The mechanism of enhancers' regulation of gene expression and that of the regulation of enhancers themselves are fundamental to an understanding of gene control mechanisms. Given our finding that *utNgn1* is necessary for the efficient activation of *Neurog1* expression, a next key question will be how this transcript promotes gene activation, which is likely by recruitment of effector proteins, including histone modifiers or transcription factors, to the *Neurog1* promoter. Although recruitment or maintenance of mixed lineage leukemia (MLL) complex is implicated in the action of lincRNAs such as *HOTTIP* and *Mistral* (23, 25), other mechanisms likely account for the action of *utNgn1*, because the level of H3K4me3 (mediated by MLL complex) of the *Neurog1* promoter was not significantly reduced, despite a marked reduction of *utNgn1* expression, in the late developmental stages (Fig. 5). It will also be important to elucidate how PcG protein-mediated regulation of the *utNgn1* locus relates to that of the *Neurog1* promoter. It is possible that PcG proteins associated with the

enhancer might physically and functionally interact with those associated with the promoter. Further elucidation of *utNgn1*'s regulation and function should bring new insights into the role of enhancers and the regulation of transcription.

Materials and Methods

Full details of procedures are provided in *SI Materials and Methods*. Briefly, primary NPCs were isolated and cultured as described previously (18). The siRNA were transfected using Neon transfection system (Invitrogen). Antibodies used in ChIP assay were anti-H3K9K14Ac (Upstate; 06–599), anti-H3K27me3 (Upstate; 07–449), anti-H3K4me3 (Active Motif; 39916), anti-H3K36me3 (Abcam; 9050), and control rabbit IgG (Santa Cruz; sc-2027).

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Bmi1 Promotes Hepatic Stem Cell Expansion and Tumorigenicity in Both *Ink4a/Arf*-Dependent and -Independent Manners in Mice

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We previously reported that forced expression of *Bmi1* (B lymphoma Moloney murine leukemia virus insertion region 1 homolog) in murine hepatic stem/progenitor cells purified from fetal liver enhances their self-renewal and drives cancer initiation. In the present study, we examined the contribution of the *Ink4a/Arf* tumor suppressor gene locus, one of the major targets of *Bmi1*, to stem cell expansion and cancer initiation. *Bmi1*^{-/-} Delta-like protein (Dlk)⁺ hepatic stem/progenitor cells showed de-repression of the *Ink4a/Arf* locus and displayed impaired growth activity. In contrast, *Ink4a/Arf*^{-/-} Dlk⁺ cells gave rise to considerably larger colonies containing a greater number of bipotent cells than wild-type Dlk⁺ cells. Although *Ink4a/Arf*^{-/-} Dlk⁺ cells did not initiate tumors in recipient nonobese diabetic/severe combined immunodeficiency mice, enforced expression of *Bmi1* in *Ink4a/Arf*^{-/-} Dlk⁺ cells further augmented their self-renewal capacity and resulted in tumor formation *in vivo*. Microarray analyses successfully identified five down-regulated genes as candidate downstream targets for *Bmi1* in hepatic stem/progenitor cells. Of these genes, enforced expression of *sex determining region Y-box 17* (*Sox17*) in Dlk⁺ cells strongly suppressed colony propagation and tumor growth. **Conclusion:** These results indicate that repression of targets of *Bmi1* other than the *Ink4a/Arf* locus plays a crucial role in the oncogenic transformation of hepatic stem/progenitor cells. Functional analyses of *Bmi1* target genes would be of importance to elucidate the molecular machinery underlying hepatic stem cell system and explore therapeutic approaches for the eradication of liver cancer stem cells. (HEPATOLOGY 2010;52:1111-1123)

Abbreviations: Alb, albumin; *Bmi1*, B lymphoma Moloney murine leukemia virus insertion region 1 homolog; CDK, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; CK7, cytokeratin 7; DDC, 3,5-dihydroxycarbonyl-1,4-dihydrocollidine; Dlk, delta-like protein; EGFP, enhanced green fluorescent protein; ESC, embryonic stem cell; GO, Gene Ontology; H2Aub1, monoubiquitinated histone H2A; HCC, hepatocellular carcinoma; HSC, hematopoietic stem cell; KO, Kusabina-Orange; MACS, magnetic activated cell sorting; NOD/SCID, nonobese diabetic/severe combined immunodeficiency; NSC, neural stem cell; PcG, polycomb group; PRC, polycomb repressive complex; Rb, retinoblastoma protein; RT-PCR, reverse transcription polymerase chain reaction; *Sox17*, sex determining region Y-box 17.

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Polycomb group (PcG) proteins operate as the cellular memory machinery through epigenetic chromatin modifications and are indispensable to the maintenance of cellular identity.^{1,2} In particular, Bmi1, a core molecule of polycomb repressive complex 1 (PRC1), plays an important role in the self-renewal of various stem cell systems, including hepatic stem cells.³

Recent evidence obtained using stem cell biology-based approaches implies that a rare population of cells in tumors, termed cancer stem cells, possess extreme tumorigenic potential and share several distinctive molecular mechanisms concerning self-renewal, differentiation, and proliferation.^{2,4} Of note, it has been demonstrated that Bmi1 is necessary for the maintenance of not only leukemic stem cells but also cancer stem cells in solid tumors.^{5,6} Considering that high expression levels of Bmi1 are reported in a wide range of malignancies, Bmi1 could be a general regulator of cancer stem cells as in normal stem cells.

Disruption of the tightly regulated self-renewal process is considered a key early event in carcinogenesis.⁷ Enhancement or reacquisition of the self-renewal capability in hematopoietic stem or progenitor cells is essential for leukemogenesis.⁸ We also showed that forced expression of *Bmi1* accelerated the self-renewal of hepatic stem/progenitor cells and eventually induced their transformation in an *in vivo* transplant model.³ However, the molecular machinery underlying the Bmi1-mediated transformation of hepatic stem/progenitor cells remains unclear.

The *Ink4a/Arf* locus, which encodes a cyclin-dependent kinase (CDK) inhibitor, $p16^{Ink4a}$, and a tumor suppressor, $p19^{Arf}$, is a pivotal target of Bmi1.⁹ We showed that de-repressed $p16^{Ink4a}$ and $p19^{Arf}$ expression in *Bmi1*-deficient mice was tightly associated with a loss of self-renewing hematopoietic stem cells (HSCs). Deletion of both the *Ink4a* and *Arf* genes substantially restored the self-renewal capacity of *Bmi1*-deficient HSCs. Bmi1 thus regulates HSCs by acting as a critical failsafe against the $p16^{Ink4a}$ and $p19^{Arf}$ -dependent senescence pathway.^{10,11} Deletion of *Ink4a/Arf* similarly rescues neural stem cell (NSC) self-renewal and frequencies in *Bmi1*-deficient mice, although its effect is reportedly partial.¹² In the oncogenic setting, the *Ink4a*-retinoblastoma protein (Rb) and *Arf*-p53 cellular senescence pathways trigger oncogene-induced senescence to eliminate transforming cells that potentially develop into cancer stem cells.² Given that enhanced expression of *BMI1* and reduced expression of *INK4A/ARF* are frequently observed in human hepatocellular carcinoma (HCC) samples,^{13,14} it would be of importance to understand the contribu-

tion of the *Ink4a/Arf* locus to the oncogenic functions of Bmi1 in cancer and search for as-yet-unknown target genes of Bmi1 other than *Ink4a/Arf*.

In the present study, we prepared hepatic stem/progenitor cells from fetal livers of *Bmi1*-deficient and *Ink4a/Arf*-deficient mice and characterized their self-renewal capacity and effects of *Bmi1* overexpression on them. Through these analyses, we found that the *Ink4a/Arf*-independent function of Bmi1 is also essential for its full oncogenic activity in hepatic stem/progenitor cells. Our microarray screening successfully identified candidate downstream targets for Bmi1 in hepatic stem/progenitor cells.

Materials and Methods

Mice. *Bmi1*^{+/-} mice¹⁵ and *Ink4a-Arf*^{+/-} mice (Strain code 01XB1) obtained from Mouse Models of Human Cancers Consortium in the National Cancer Institute (NCI, Frederick, MD) in the C57BL/6 background were used. Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were purchased from Sankyo Laboratory (Tsukuba, Japan). All experiments using these mice were performed in accordance with our institutional guidelines for the use of laboratory animals.

Oligonucleotide Array Analysis. Biotin-labeled complementary RNA was prepared with a two-cycle complementary DNA synthesis kit (Affymetrix, Santa Clara, CA) from purified total RNA equivalent to 10,000 cells, and was hybridized to an Affymetrix GeneChip Mouse Genome 430 2.0 array (Affymetrix). The array images were scanned using Affymetrix GeneChip Scanner 3000 7G. The expression value (Signal) for each probe set was calculated using GeneChip Operating Software version 1.4 (Affymetrix). The change value (Signal Log Ratio) and change call (Increase, Marginal Increase, No Change, Marginal Decrease, or Decrease) for each probe set were calculated. Data were obtained for quadrant samples from four independent experiments. To identify differentially expressed genes, we selected probe sets that presented a change call of Increase and a Signal Log Ratio value of ≥ 1 (\geq twofold up-regulation) or a change call of Decrease and a Signal Log Ratio value of ≤ -1 (\geq twofold down-regulation) in more than three experiments. Moreover, the Welch *t* test or paired *t* test was performed to determine significance. Gene Ontology (GO) annotations were performed using the GeneSpring annotation tool (Agilent Technologies, Santa Clara, CA). Microarray data are available at

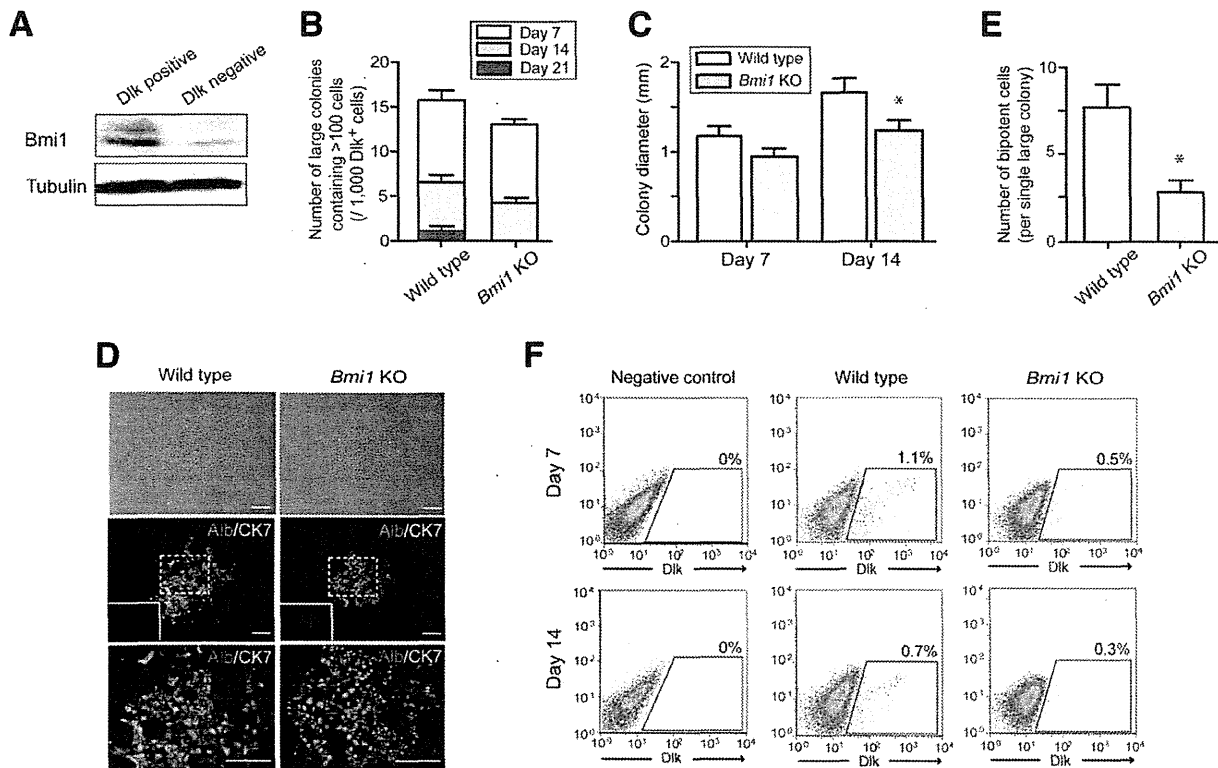


Fig. 1. Colony assays of *Bmi1*^{-/-} hepatic stem cells. (A) Western blot analysis of Bmi1 expression in Dlk⁺ and Dlk⁻ cells purified from wild-type fetal liver. Tubulin was used as a loading control. (B) The number of large colonies containing more than 100 cells at day 7 of culture was traced up to day 21. (C) Colony diameter at days 7 and 14 of culture. *Statistically significant ($P < 0.05$). (D) Bright-field images and immunocytochemical analyses of colonies at day 7 of culture. Alb (red) and CK7 (green) expression was merged. Nuclear DAPI staining (blue) is shown in the insets. (E) The absolute number of Alb⁺CK7⁺ bipotent cells in large colonies derived from wild-type or *Bmi1*^{-/-} Dlk⁺ cells at day 7 of culture. *Statistically significant ($P < 0.05$). (F) Flow cytometric profiles of colonies derived from wild-type or *Bmi1*^{-/-} Dlk⁺ cells at days 7 and 14 of culture. The percentages of Dlk⁺ cells are shown as mean values for three independent analyses.

<http://www.ncbi.nlm.nih.gov/geo/> (accession number: GSE17462).

Other methods are shown in Supporting Materials and Methods.

Results

Impaired Colony Propagation of *Bmi1*^{-/-} Hepatic Stem Cells. Similar to the hematopoietic components, the hepatic components developed normally in *Bmi1*^{-/-} fetal livers and we could recover a comparable number of delta-like protein (Dlk)⁺ hepatic stem/progenitor cells from them. Western blot analysis showed a higher level of Bmi1 expression in wild-type Dlk⁺ cells than Dlk⁻ cells (Fig. 1A). To gain an insight into the role of Bmi1 in hepatic stem cells, we conducted colony assays of wild-type and *Bmi1*^{-/-} Dlk⁺ cells purified by magnetic activated cell sorting (MACS). Flow cytometric analysis revealed that the purity of the sorted Dlk⁺ cells was greater than 90% (Supporting Fig. 1). Approximately 1.5% of wild-type

Dlk⁺ cells gave rise to large colonies (consisting of >100 cells) at day 7 of culture. Only a portion of the day 7 colonies kept growing and could be detected as large colonies at days 14 and 21, whereas the majority of colonies stopped expanding and disappeared by days 14 and 21 (Fig. 1B). Although the total number of large colonies did not differ significantly between wild-type and *Bmi1*^{-/-} Dlk⁺ cells at day 7 of culture (Fig. 1B), the diameter of colonies derived from *Bmi1*^{-/-} Dlk⁺ cells was slightly reduced (Fig. 1C). The impeded expansion of *Bmi1*^{-/-} Dlk⁺ cell-derived colonies was obvious at day 14 of culture (Fig. 1B,C). Approximately 10% of large colonies from wild-type Dlk⁺ cells continued to proliferate up to day 21 of culture, whereas no colonies derived from *Bmi1*^{-/-} Dlk⁺ cells expanded beyond day 21 (Fig. 1B).

It has been reported that Dlk⁺ cells are composed of albumin (Alb)⁺ cytokeratin 7 (CK7)⁺ cells and Alb⁺CK7⁻ cells, and Alb⁺CK7⁺ cells mainly contribute to the regeneration in retrorsine-treated liver.^{16,17} These findings suggest that Alb⁺CK7⁺ cells, which

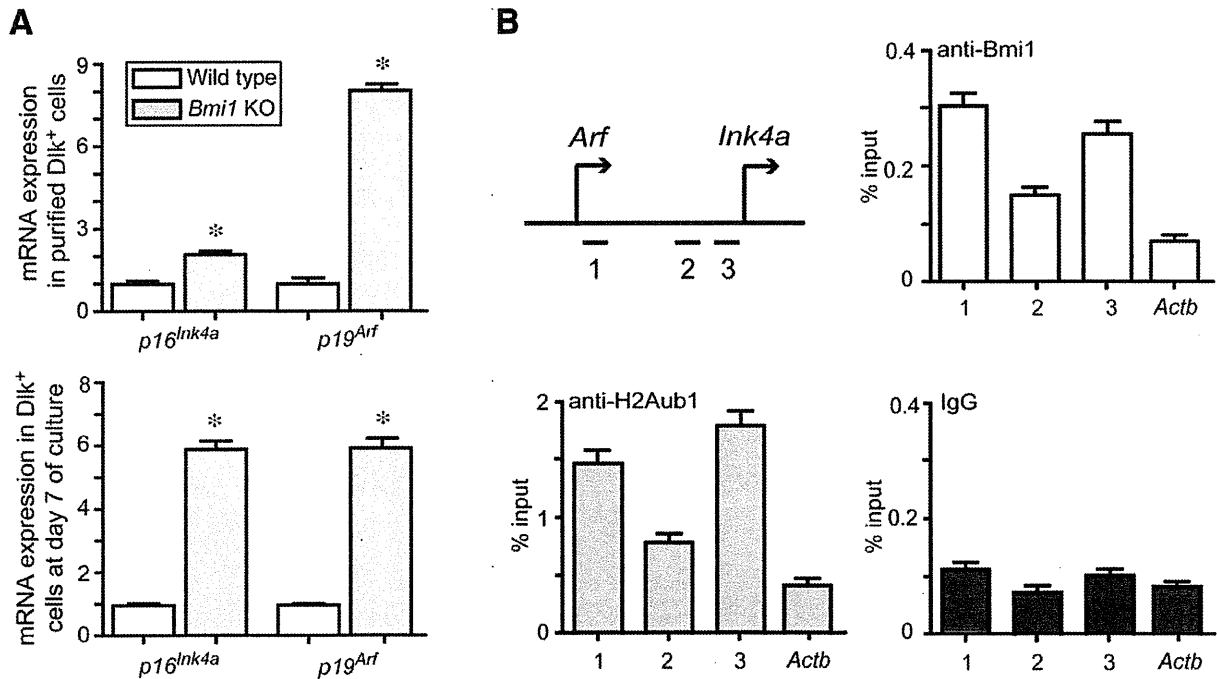


Fig. 2. Regulation of *Ink4a/Arf* expression by *Bmi1*. (A) Real-time RT-PCR analyses of the *p16^{Ink4a}* and *p19^{Arf}* genes in freshly purified *Dlk⁺* cells and *Dlk⁺* cells cultured for 7 days. *Statistically significant ($P < 0.05$). (B) ChIP analyses of *Dlk⁺* cells freshly purified from wild-type fetal livers were performed on the *Ink4a/Arf* locus (primer sets 1-3) and the β -actin (*Actb*) control promoter region using indicated antibodies. IgG, immunoglobulin G; mRNA, messenger RNA.

have the capacity to give rise to both $\text{Alb}^+\text{CK7}^-$ and $\text{Alb}^-\text{CK7}^+$ progenies, function as hepatic stem/progenitor cells. Therefore, the quantification of $\text{Alb}^+\text{CK7}^+$ impotent cells is one of the approaches to evaluate the content of hepatic stem/progenitor cells, although not all $\text{Alb}^+\text{CK7}^+$ cells necessarily have the capacity for bipotential differentiation. Immunocytochemical analyses revealed that the ability of *Bmi1*^{-/-} *Dlk*⁺ cells to differentiate into Alb^+ hepatocytes and CK7^+ cholangiocytes was preserved (Fig. 1D). However, the absolute number of $\text{Alb}^+\text{CK7}^+$ bipotent cells were significantly decreased in large colonies derived from *Bmi1*^{-/-} *Dlk*⁺ cells compared to those in wild-type large colonies (Fig. 1D,E). The absolute number of $\text{Alb}^+\text{CK7}^+$ cells per each large colony was 7.6 ± 1.5 and 2.8 ± 0.4 , respectively ($P < 0.05$) (Fig. 1E).

Consistent with these findings, flow cytometric analyses demonstrated that the *Dlk*⁺ population in *Bmi1*^{-/-} colonies decreased rapidly compared to that in wild-type colonies (Fig. 1F). The *Dlk*⁺ fraction in wild-type colonies was $1.1\% \pm 0.2\%$ at day 7 and $0.7\% \pm 0.1\%$ at day 14 of culture, whereas that in *Bmi1*^{-/-} colonies was $0.5\% \pm 0.1\%$ and $0.3\% \pm 0.1\%$, respectively. Conversely, forced expression of *Bmi1* in wild-type *Dlk*⁺ cells significantly promoted colony expansion (Supporting Fig. 2A-C) and

increased the *Dlk*⁺ fraction and number of bipotent cells (Supporting Fig. 2D,E).

Oval cells, although their origin is controversial, have been considered stem/progenitor cells in adult liver.¹⁸ Histological analyses demonstrated a drastic decrease in A6-positive oval cell numbers in 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-treated *Bmi1*^{-/-} adult liver (Supporting Fig. 3). Together, these findings suggest that *Bmi1* plays an important role in the maintenance and expansion of stem/progenitor cells in both fetal and adult livers.

Transcriptional Regulation of the *Ink4a/Arf* Gene by *Bmi1*. To examine whether deletion of *Bmi1* causes de-repression of the *Ink4a* and *Arf* genes as observed in HSCs, we conducted real-time reverse transcription polymerase chain reaction (RT-PCR) analyses (Fig. 2A). As expected, messenger RNA levels of *Ink4a* and *Arf* were 2.1-fold and 8.0-fold higher in freshly purified *Bmi1*^{-/-} *Dlk*⁺ cells than in wild-type *Dlk*⁺ cells, respectively. Colonies derived from *Bmi1*^{-/-} *Dlk*⁺ cells also showed increased (5.8-fold greater) expression compared to the wild-type colonies. To determine whether *Bmi1* is involved in transcriptional regulation of the *Ink4a/Arf* locus, we performed chromatin immunoprecipitation (ChIP) assays using wild-type *Dlk*⁺ cells. ChIP assays demonstrated the binding of

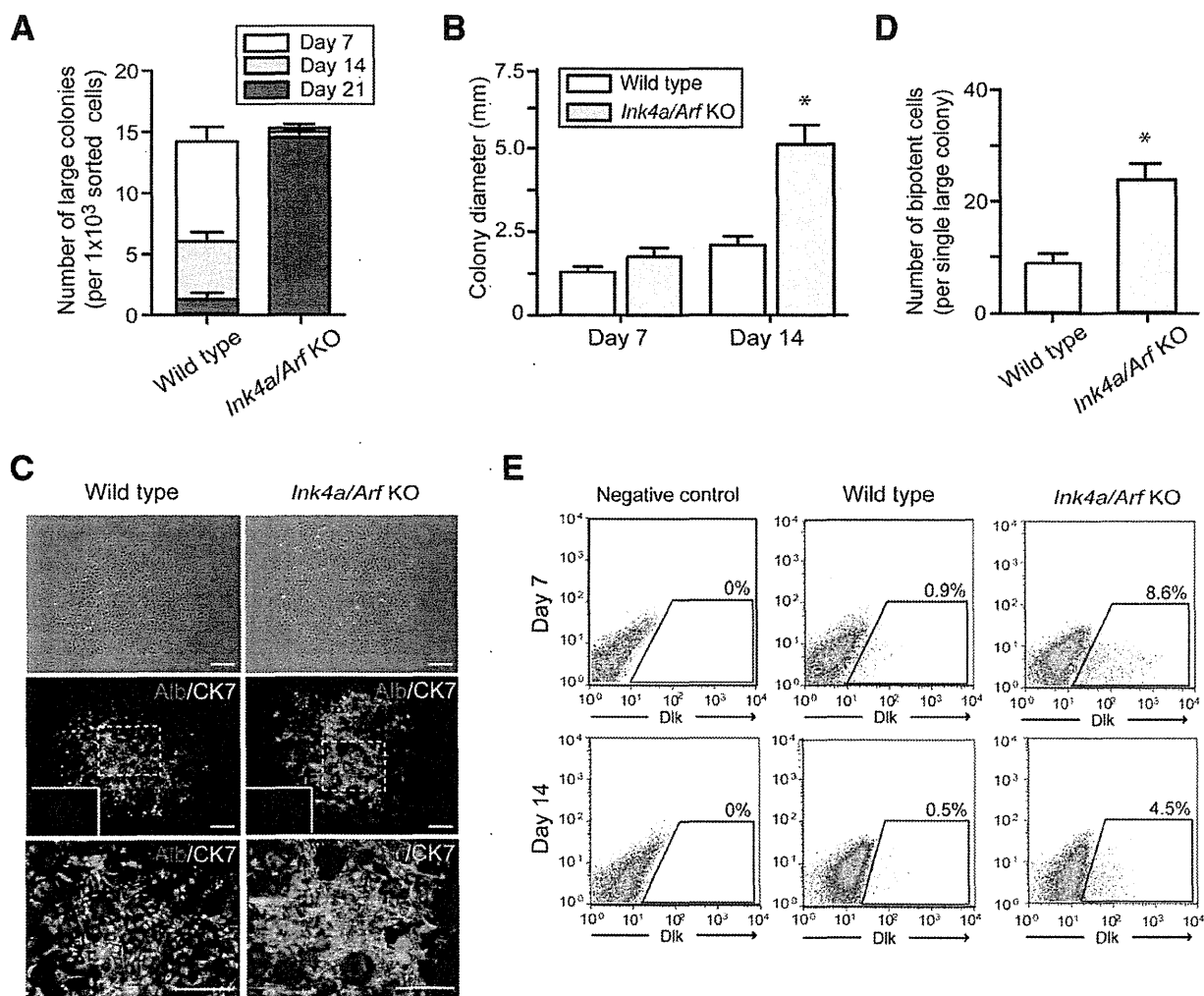


Fig. 3. Colony assays of *Ink4a/Arf*^{-/-} hepatic stem cells. (A) The number of large colonies containing more than 100 cells at day 7 of culture was traced up to day 21. (B) Colony diameter at days 7 and 14 of culture. *Statistically significant ($P < 0.05$). (C) Bright-field images and immunocytochemical analyses of colonies at day 7 of culture. Alb (red) and CK7 (green) expression was merged. Nuclear DAPI staining (blue) is shown in the insets. Scale bar = 200 μ m. (D) The absolute number of Alb⁺CK7⁺ bipotent cells in large colonies derived from wild-type or *Ink4a/Arf*^{-/-} Dlk⁺ cells at day 7 of culture. *Statistically significant ($P < 0.05$). (E) Flow cytometric profiles of colonies derived from wild-type or *Ink4a/Arf*^{-/-} Dlk⁺ cells at days 7 and 14 of culture. The percentages of Dlk⁺ cells are shown as mean values for three independent analyses.

Bmi1 to the *Ink4a/Arf* locus and increased levels of monoubiquitinated histone H2A (H2Aub1) (Fig. 2B).

Augmented Colony-Forming Activity of *Ink4a/Arf*^{-/-} Hepatic Stem Cells. To understand the role of the *Ink4a* and *Arf* genes in hepatic stem cells, we next analyzed *Ink4a/Arf*^{-/-} Dlk⁺ cells in culture. In clear contrast with *Bmi1*^{-/-} Dlk⁺ cells, *Ink4a/Arf*^{-/-} Dlk⁺ cells showed pronounced growth activity in culture. The number of large colonies (consisting of more than 100 cells) derived from *Ink4a/Arf*^{-/-} Dlk⁺ cells was significantly increased compared to that derived from wild-type Dlk⁺ cells (Fig. 3A,B). By day 14 of culture,

Ink4a/Arf^{-/-} Dlk⁺ cells gave rise to distinctly abnormal and large colonies compared to wild-type Dlk⁺ cells (Fig. 3B,C). More than 95% of large colonies from *Ink4a/Arf*^{-/-} Dlk⁺ cells further expanded beyond day 21 of culture, although wild-type colonies barely maintained their growth activity (Fig. 3A). Immunocytochemical analyses showed an increase in the proportion and number of Alb⁺CK7⁺ bipotent cells in colonies derived from *Ink4a/Arf*^{-/-} Dlk⁺ cells, particularly in their central area (Fig. 3C). The absolute number of bipotent cells in large colonies derived from wild-type and *Ink4a/Arf*^{-/-} Dlk⁺ cells at day 7 of culture was 8.2 ± 2.3 versus 22.7 ± 4.6 ($P < 0.05$) (Fig.

3D). Flow cytometric analyses revealed that the percentage of Dlk⁺ cells in wild-type colonies was 0.9% ± 0.2% at day 7 and 0.5% ± 0.1% at day 14 of culture, although that in *Ink4a/Arf*^{-/-} colonies was 8.6% ± 0.7% and 4.5% ± 0.3%, respectively (Fig. 3E). These findings indicate the enhanced self-renewal capability of hepatic stem cells on the loss of *Ink4a/Arf* expression. Of note, messenger RNA expression of *Bmi1* was comparable between wild-type and *Ink4a/Arf*^{-/-} Dlk⁺ cells (data not shown).

As expected, but importantly, the ability of wild-type Dlk⁺ cells to propagate colonies was extremely compromised by cotransduction with *Ink4a* and *Arf* retroviruses. Immunocytochemical analyses and flow cytometric analyses showed that the Dlk⁺ fraction and bipotent cells were significantly reduced in culture (Supporting Fig. 4).

Enhanced Self-Renewal of *Ink4a/Arf*^{-/-} Hepatic Stem Cells by *Bmi1* Overexpression. We previously reported that forced expression of *Bmi1* enhances the self-renewal capacity of hepatic stem/progenitor cells and eventually induces their transformation.³ To elucidate whether the functional significance of *Bmi1* is attributable to the repression of *Ink4a/Arf*, we performed gain-of-function assays of *Bmi1* in *Ink4a/Arf*^{-/-} cells. *Ink4a/Arf*^{-/-} Dlk⁺ cells were transduced with either control enhanced green fluorescent protein (EGFP) or *Bmi1* 12-18 hours after purification. Enforced expression of *Bmi1* was verified by western blot analysis (Fig. 4A). Exogenous *Bmi1* in *Ink4a/Arf*^{-/-} Dlk⁺ cells did not significantly increase colony number (Fig. 4B). Of note, however, the diameter of *Bmi1*-overexpressing colonies was significantly larger than that of the control colonies (Fig. 4C). Furthermore, flow cytometric analyses showed that the percentage of *Ink4a/Arf*^{-/-} Dlk⁺ cells labeled with EGFP was higher in *Bmi1* cultures than in control cultures (22.6% ± 2.3%, 14.0% ± 1.2%, and 8.8% ± 0.7% versus 8.4% ± 1.1%, 3.4% ± 0.5%, and 2.1% ± 0.2% at days 7, 14, and 28 of culture, respectively) (Fig. 4D).

We next carried out single-cell sorting of Dlk⁺ cells contained in primary colonies at days 14 and 28 of culture in order to evaluate their self-renewal capacity in terms of replating activity. Dlk⁺ cells overexpressing *Bmi1* gave rise to 3.1-fold to 4.0-fold more secondary colonies than the control (Fig. 5A). Secondary colonies were generated in a similar fashion to the original colonies. Immunocytochemical analyses demonstrated that the frequency of Alb⁺CK7⁺ bipotent cells was significantly higher in secondary colonies derived from Dlk⁺ cells collected from the primary *Bmi1*-transduced *Ink4a/Arf*^{-/-} colonies at days 14 and 28 of culture (Fig. 5B,C).

In contrast, *Bmi1*^{-/-}*Ink4a/Arf*^{-/-} Dlk⁺ cells behaved like *Ink4a/Arf*^{-/-} Dlk⁺ cells (Supporting Fig. 5). Although loss of *Bmi1* still affected the function of *Ink4a/Arf*^{-/-} hepatic stem/progenitor cells to some extent, these findings indicate that *Ink4a/Arf* is the major target of *Bmi1* in hepatic stem cells as in HSCs and NSCs.

Acquisition of Tumorigenic Capacity by *Bmi1*-Transduced *Ink4a/Arf*^{-/-} Hepatic Stem Cells. We then tested whether the loss of both *Ink4a* and *Arf* is enough for the transformation of hepatic stem cells. Considering that a large number of cells were necessary for transplantations assays, these cells were allowed to form colonies in culture for 28 days. Immunocytochemical analyses showed that more than 90% of cells transduced with *Bmi1* expressed both EGFP, a marker antigen for retrovirus integration, and Flag-tagged *Bmi1* (Supporting Fig. 6). Subsequently, a total of 2 × 10⁶ transduced cells were transplanted into the subcutaneous space of NOD/SCID mice (Fig. 5D). Although all the mice transplanted with *Bmi1*-transduced *Ink4a/Arf*^{-/-} Dlk⁺ cells developed tumors, none of those transplanted with control *Ink4a/Arf*^{-/-} Dlk⁺ cells did. Histological analyses revealed that the subcutaneous tumors consisted of both Alb⁺ parenchymal cells and a CK7⁺ glandular structure (Fig. 5D). The histological finding is consistent with our previous observation in tumors derived from *Bmi1*-transduced wild-type hepatic stem cells.³ These findings clearly indicate that repression of the *Ink4a* and *Arf* genes is not enough for *Bmi1* to achieve its tumorigenic potential in hepatic stem cells.

Gene Expression Analyses of *Bmi1*-Transduced *Ink4a/Arf*^{-/-} Hepatic Stem Cells. In order to explore novel targets for *Bmi1*, *Ink4a/Arf*^{-/-} Dlk⁺ cells were infected with either the control EGFP or *Bmi1*-expressing retrovirus and allowed to form colonies. Dlk⁺ cells were purified from colonies at day 28 of culture by cell sorting and subjected to gene expression profiling using oligonucleotide microarrays. We selected genes exhibiting a twofold or greater change with statistical significance in *Bmi1*-transduced *Ink4a/Arf*^{-/-} Dlk⁺ cells compared to control *Ink4a/Arf*^{-/-} Dlk⁺ cells. As a result, we identified 75 down-regulated genes and 97 up-regulated genes in total (Supporting Table 1). Functional annotation based on GO showed significant enrichment for down-regulated genes which fell into the category "metabolism" and "transport", which included many hepatocyte maturation genes (Fig. 6A). This indicates that *Bmi1* strongly suppresses the differentiation and maturation of hepatocytes.

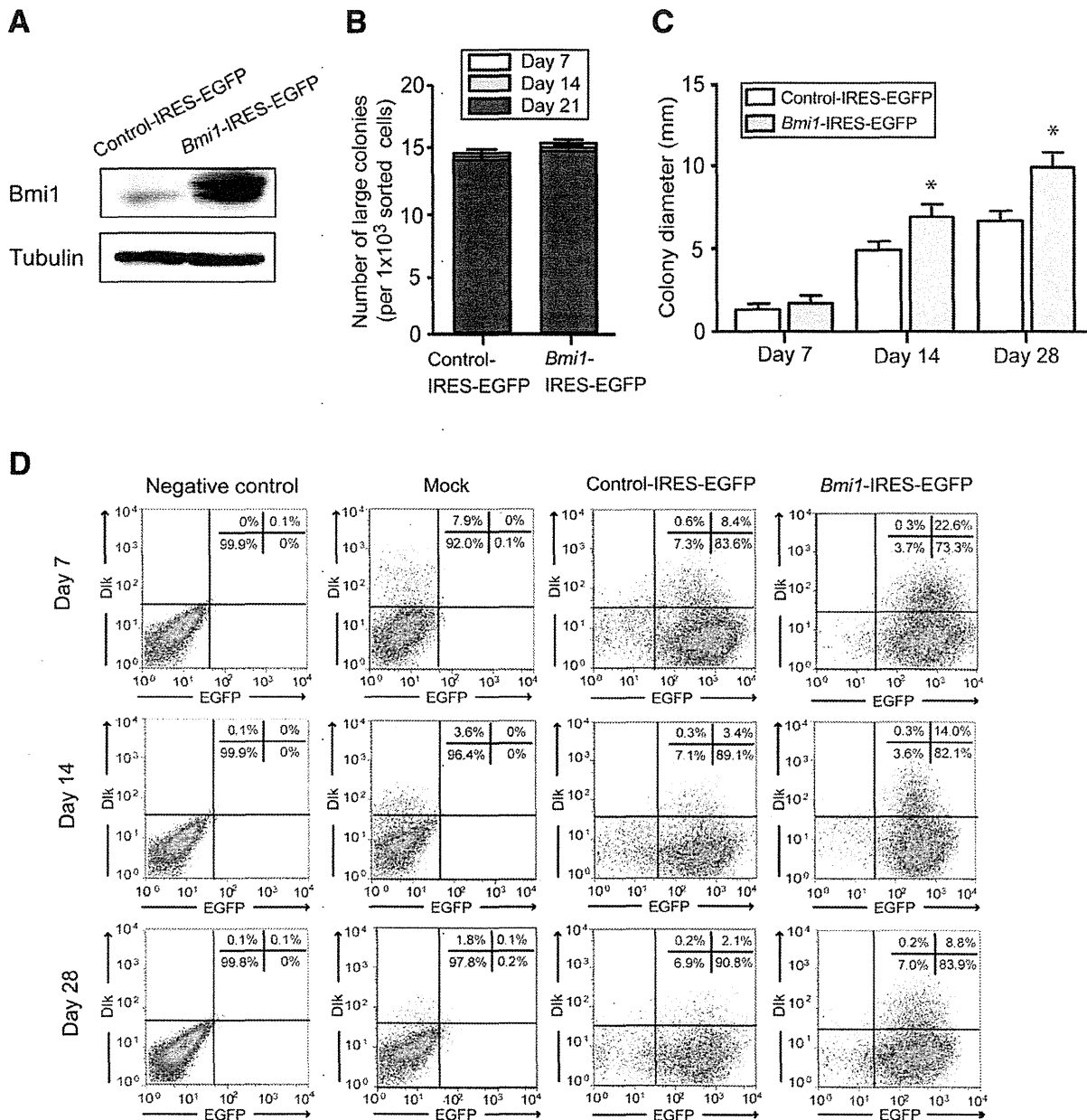


Fig. 4. Gain-of-function assays of *Bmi1* in *Ink4a/Arf*^{-/-} Dlk⁺ cells. (A) Cells transduced with indicated retroviruses were subjected to western blot analysis using anti-Bmi1 and anti-tubulin (loading control) antibodies. (B) The number of large colonies containing more than 100 cells at day 7 of culture was traced up to day 21. (C) The diameter of colonies at days 7, 14, and 28 after transduction of indicated retroviruses. *Statistically significant ($P < 0.05$). (D) Flow cytometric profiles of colonies derived from nontransduced (mock) and EGFP or *Bmi1*-transduced *Ink4a/Arf*^{-/-} Dlk⁺ cells at days 7, 14, and 28 in culture. The percentages of each fraction are shown as mean values for three independent analyses.

Recent whole-genome ChIP-on-chip analyses successfully identified genes that are bound by PRC1 and PRC2 complexes in embryonic stem cells (ESCs).¹⁹⁻²¹ Boyer et al. reported the genes occupied by PRC1 (Phc1 and Rnf2) and PRC2 (Suz12 and Eed) in murine ESCs.¹⁹ To explore a novel target of *Bmi1* in hepatic stem/progenitor cells, we compared the list of down-regulated genes with the ChIP-on-chip data

documented by Boyer et al.¹⁹ As a result, five genes namely, *Sox17*, *Irx5*, *Gjb2*, *Shox2*, and *Bhmt2* in the present study appeared to be regulated by both PRC1 and/or PRC2 in ESCs (Fig. 6B). We therefore considered these genes as candidates for direct targets of *Bmi1* in hepatic stem cells and performed further analyses on them. In order to confirm the altered expression of these 5 candidate genes, *Ink4a/Arf*^{-/-} Dlk⁺

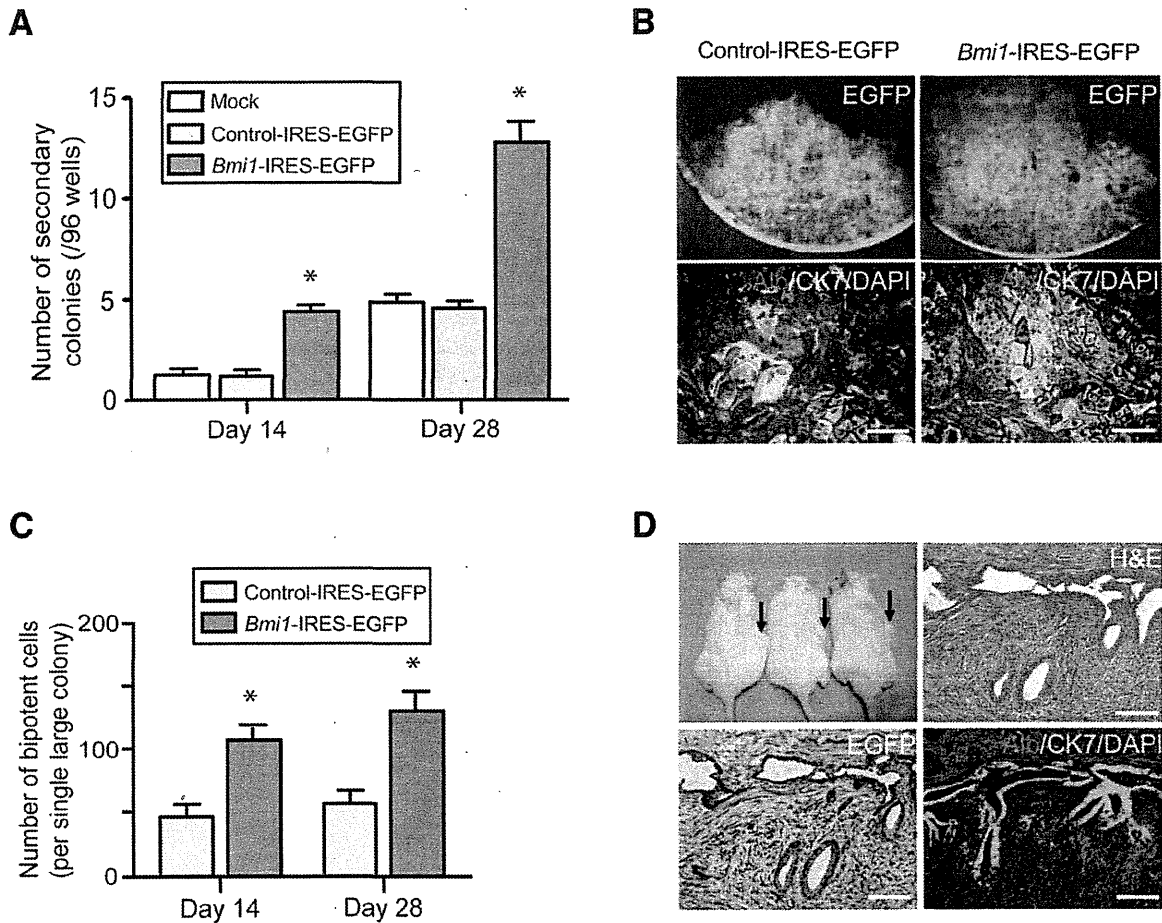


Fig. 5. Replating assays and implantation of *Bmi1*-transduced *Ink4a/Arf*^{-/-} Dlk⁺ cells. (A) Dlk⁺ cells in primary colonies generated from nontransduced (mock) and EGFP or *Bmi1*-transduced *Ink4a/Arf*^{-/-} Dlk⁺ cells were clone-sorted at days 14 and 28 of culture and allowed to form colonies. The replating efficiency of Dlk⁺ cells was evaluated by counting the number of secondary colonies containing more than 100 cells 14 days after replating by clone-sorting. *Statistically significant ($P < 0.05$). (B) Fluorescence images (upper panels) and dual immunostaining (lower panels) of secondary clonal colonies derived from EGFP or *Bmi1*-transduced *Ink4a/Arf*^{-/-} Dlk⁺ cells at day 28 of culture. Alb (red) and CK7 (green) expression in secondary colonies was merged with nuclear DAPI staining (blue). Scale bar = 100 μ m. (C) The absolute number of Alb⁺CK7⁺ bipotent cells in secondary large colonies at day 14 of subculture. *Statistically significant ($P < 0.05$). (D) *Ink4a/Arf*^{-/-} Dlk⁺ cells were transduced with the control EGFP or *Bmi1*-expressing retrovirus and a total of 2×10^6 transduced cells were transplanted into the subcutaneous space of NOD/SCID mice. *Bmi1*-transduced *Ink4a/Arf*^{-/-} cells formed tumors in the right subcutaneous space of recipient mice (arrows), whereas the same number of control EGFP-transduced *Ink4a/Arf*^{-/-} cells did not generate tumors in the left space. Hematoxylin and eosin (H&E) staining of tumors demonstrated histological features compatible with combined hepatocellular and cholangiocellular carcinoma. Immunohistochemical analysis revealed that the tumors were positive for EGFP and consisted of Alb⁺ parenchymal cells (red) and CK7⁺ glandular structures (green). Scale bar = 200 μ m.

cells transduced with either control EGFP or *Bmi1* were purified from colonies at day 28 of culture and subjected to real-time RT-PCR analyses. The selected five genes exhibited similar expression profiles as in the microarray analysis in *Ink4a/Arf*^{-/-} Dlk⁺ cells (Fig. 6C). Forced expression of *Bmi1* in wild-type Dlk⁺ cells significantly repressed the expression of these genes in a similar fashion to that in *Ink4a/Arf*^{-/-} Dlk⁺ cells (Fig. 6C).

Gain-of-Function Assays of Sox17 in Hepatic Stem Cells. Among candidates for *Bmi1* targets, *sex*

determining region Y-box 17 (Sox17) was most severely down-regulated following *Bmi1*-overexpression in hepatic stem cells (Fig. 6C). It has been reported that *Sox17* is highly expressed in the very early definitive endoderm²² and in hepatocyte-like cells derived from ESCs.²³ These findings prompted us to further examine the role of *Sox17* in hepatic stem cell self-renewal and tumorigenesis. ChIP assays in wild-type Dlk⁺ cells demonstrated specific binding of *Bmi1* and an increased level of H2Aub1 at the *Sox17* promoter only in cells transduced with the *Bmi1* retrovirus (Fig. 7A).

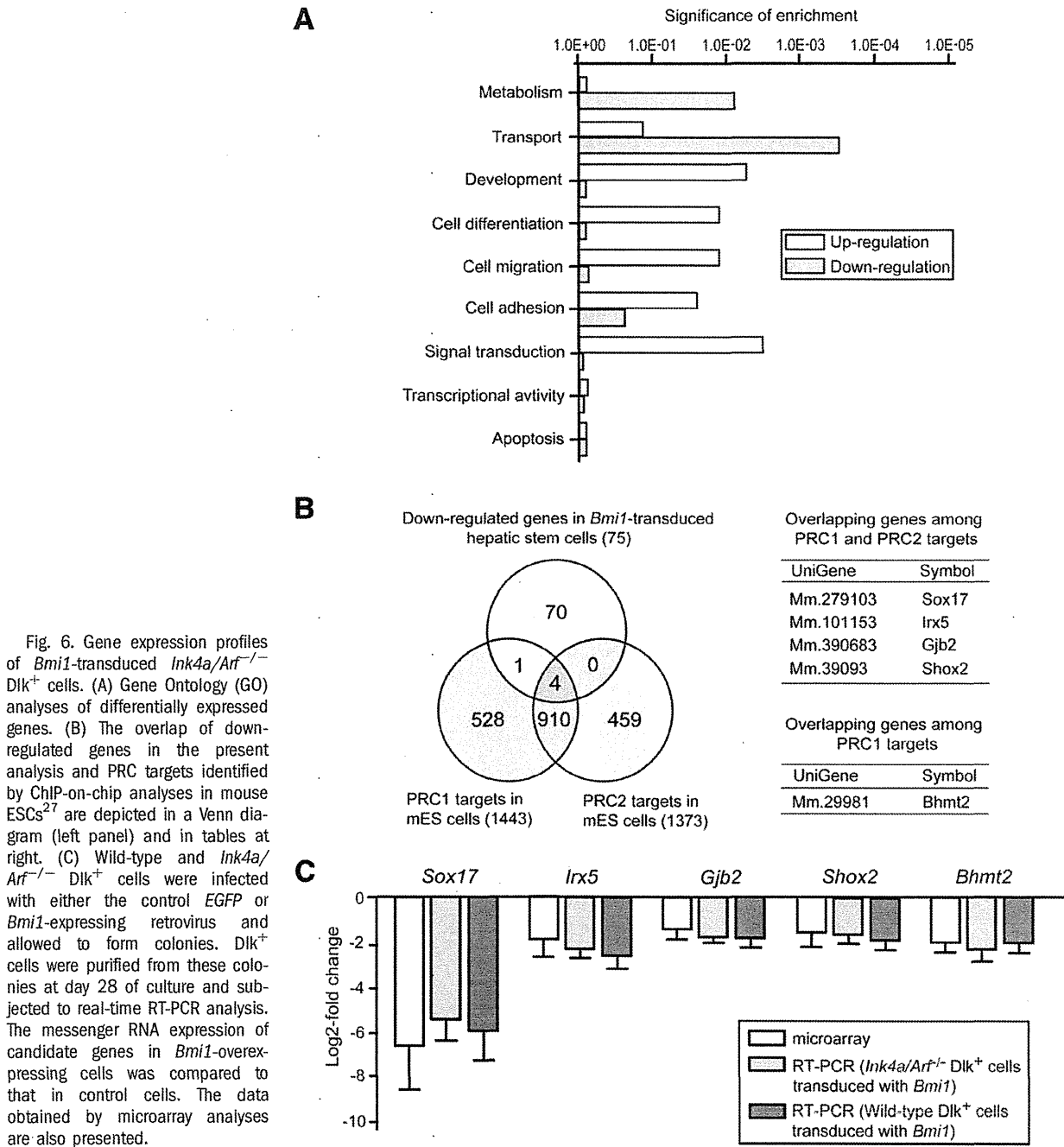


Fig. 6. Gene expression profiles of *Bmi1*-transduced *Ink4a/Arf*^{-/-} Dlk⁺ cells. (A) Gene Ontology (GO) analyses of differentially expressed genes. (B) The overlap of down-regulated genes in the present analysis and PRC targets identified by ChIP-on-chip analyses in mouse ESCs²⁷ are depicted in a Venn diagram (left panel) and in tables at right. (C) Wild-type and *Ink4a/Arf*^{-/-} Dlk⁺ cells were infected with either the control *EGFP* or *Bmi1*-expressing retrovirus and allowed to form colonies. Dlk⁺ cells were purified from these colonies at day 28 of culture and subjected to real-time RT-PCR analysis. The messenger RNA expression of candidate genes in *Bmi1*-overexpressing cells was compared to that in control cells. The data obtained by microarray analyses are also presented.

All these findings indicate that *Bmi1* could directly regulate the expression of *Sox17*.

We next tested the effect of *Sox17* in a gain-of-function assay. Overexpression of *Sox17* was confirmed by western blotting (Fig. 7B). Enforced expression of *Sox17* in wild-type Dlk⁺ cells severely impaired the formation of colonies and reduced the number as well as size of colonies (Fig. 7C,D). Dlk⁺ cells transduced with *Sox17* did not form any large colonies containing more than 100 cells at day 7 of

culture (Fig. 7C) and no colonies expanded beyond day 14 of culture (data not shown). Immunocytochemical analyses showed a decrease in number of Alb⁺CK7⁺ bipotent cells in colonies derived from Dlk⁺ cells transduced with *Sox17* compared to the control colonies (Fig. 7D,E). Concordant with this, flow cytometric analyses demonstrated that the Dlk⁺ fraction in *Sox17*-transduced colonies was 0.3% ± 0.1%, much lower than that in wild-type colonies (0.9% ± 0.2%) (Fig. 7F).

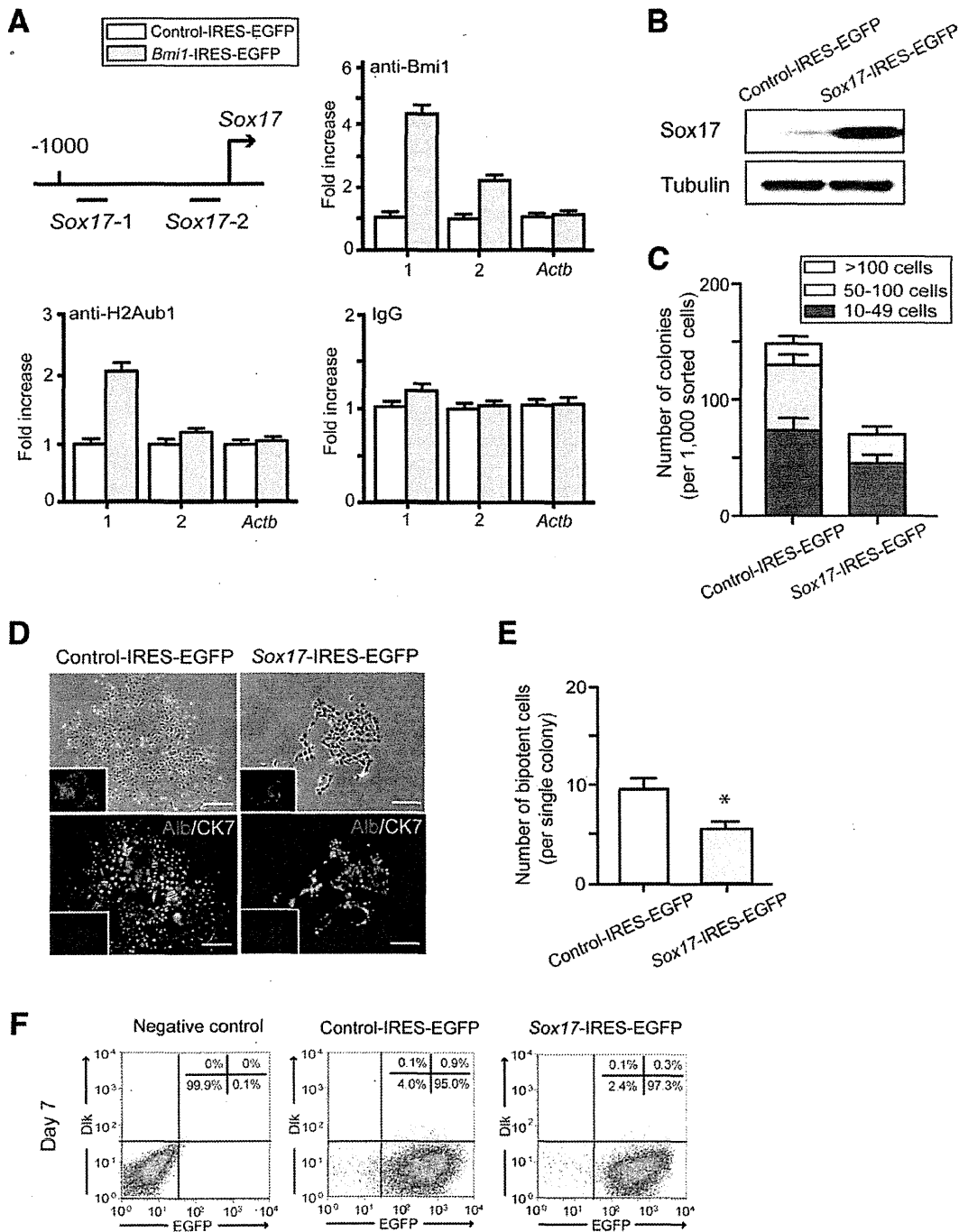


Fig. 7. Gain-of-function assay of *Sox17* in wild-type *Dlk*⁺ cells. (A) ChIP analyses of wild-type *Dlk*⁺ cells transduced with *EGFP* or *Bmi1* on the *Sox17* locus and *Actb* control promoter region using anti-*Bmi1* and anti-H2Aub1 antibodies. *Statistically significant ($P < 0.05$). (B) Western blot analysis in *Sox17*-transduced wild-type *Dlk*⁺ cells using anti-*Sox17* and anti-tubulin (loading control) antibodies. (C) Enforced expression of *Sox17* in wild-type *Dlk*⁺ cells markedly decreased both the total number of colonies and the number of large colonies containing more than 100 cells at day 7 of culture. *Statistically significant ($P < 0.05$). (D) Bright-field images and immunocytochemical analyses of colonies derived from wild-type *Dlk*⁺ cells transduced with *Sox17* at day 7 of culture. Alb (red) and CK7 (green) expression was merged. Nuclear DAPI staining (blue) is shown in the insets. Scale bar = 200 μ m. (E) The absolute number of Alb⁺CK7⁺ bipotent cells in colonies derived from wild-type *Dlk*⁺ cells transduced with *Sox17* at day 7 of culture. *Statistically significant ($P < 0.05$). (F) Flow cytometric profiles of colonies derived from *EGFP* or *Sox17*-transduced wild-type *Dlk*⁺ cells at day 7 of culture. The percentages of each fraction are shown as mean values for three independent analyses.

To elucidate the impact of *Sox17* on the tumorigenic process driven by *Bmi1*-overexpressing hepatic stem cells, we cotransduced *Ink4a/Arf*^{-/-} Dlk⁺ cells with *Bmi1* and *Sox17*. *Ink4a/Arf*^{-/-} Dlk⁺ cells were simultaneously transduced with *Sox17*-IRES-EGFP and *Bmi1*-IRES-Kusabira-Orange (KO)-expressing retroviral vectors (Supporting Fig. 7A). Flow cytometric profiles demonstrated that more than 90% of cells were successfully cotransduced (Supporting Fig. 7B). A total of 2×10^6 *Ink4a/Arf*^{-/-} cells cotransduced with *Bmi1* and *Sox17* or control *EGFP* were transplanted into the subcutaneous space of NOD/SCID mice. Cotransduction of *Bmi1* and *Sox17* resulted in a significant reduction in tumor volume compared to the cotransduction of *Bmi1* and control *EGFP* (Supporting Fig. 6C). This result indicates that *Sox17* suppresses the tumorigenic activity of *Bmi1*-overexpressing hepatic stem cells.

We then further tested the effect of *Sox17* knockdown in wild-type Dlk⁺ cells (Supporting Fig. 8). *Sox17* knockdown mildly promoted colony expansion and increased the Dlk⁺ fraction and the number of bipotent cells, although its effect was not statistically significant. Transplantation of 2×10^6 *Sox17*-knockdown Dlk⁺ cells did not develop subcutaneous tumors in NOD/SCID mice at all (data not shown).

Discussion

Bmi1, a component of PRC1, regulates the cell cycle, apoptosis and senescence by repressing the *Ink4a/Arf* locus.^{5,10} p19^{Arf} suppresses MDM2, which mediates ubiquitin-dependent degradation of p53, and subsequently activates p53 target genes involved in cell cycle arrest and apoptosis, including *p21*.²⁴ Direct binding of p16^{Ink4a} to CDK4 and CDK6 keeps Rb hypophosphorylated. Hypophosphorylated Rb represses E2F-dependent transcription leading to cell cycle arrest and senescence.²⁴ Thus, the repression of the *Ink4a/Arf* locus by *Bmi1* has a great impact on the maintenance of self-renewing stem cells.

In the present study, *Bmi1*^{-/-} hepatic stem cells showed high levels of *Ink4a* and *Arf* expression and significantly but modestly impaired colony expansion and self-renewal in culture. Although *Bmi1*^{-/-} liver is functionally and histologically normal,¹⁵ oval cell induction following DDC treatment was apparently impaired in *Bmi1*^{-/-} mice (Supporting Fig. 3). Considering the results of gain-of-function (Supporting Fig. 2) and loss-of-function assays of *Bmi1* (Fig. 1), the possibility exists that redundancy among other PcG molecules such as Me118 weakens the phenotype

of *Bmi1*^{-/-} hepatic stem cells in developing and adult liver.²⁵ In clear contrast, *Ink4a/Arf*^{-/-} hepatic stem cells exhibited enhanced colony formation and retained a large Dlk⁺ population in culture compared to the wild type. Furthermore, deletion of both *Ink4a* and *Arf* largely restored the impaired self-renewal capacity of *Bmi1*^{-/-} hepatic stem cells (Supporting Fig. 5). These findings indicate that *Ink4a/Arf* is the major target of *Bmi1* in hepatic stem cells as in HSCs and NSCs.^{11,12}

Bmi1 is also essential for cancer stem cells as demonstrated in a mouse leukemia model as well as in a mouse lung tumor model generated by the expression of a mutant K-ras gene in bronchioalveolar stem cells.^{5,26} In addition, we previously demonstrated that forced expression of *Bmi1* promotes the self-renewal of hepatic stem/progenitor cells and contributes to malignant transformation.³ All these findings highlight the important role of *Bmi1* in both the development and maintenance of cancer stem cell systems. Of interest, an *Ink4a/Arf*-independent contribution of *Bmi1* to not only self-renewal in neural stem cells but also tumorigenesis in a mouse model for glioma has been reported.^{27,28} The current *in vivo* transplant assays ascertained that *Bmi1*-transduced *Ink4a/Arf*^{-/-} Dlk⁺ cells but not control *Ink4a/Arf*^{-/-} Dlk⁺ cells acquire tumorigenic potential. *Bmi1*-transduced *Ink4a/Arf*^{-/-} Dlk⁺ cells showed an augmented self-renewal capability as evident from the higher replating efficiency in the single cell-sorting analysis compared to *Ink4a/Arf*^{-/-} Dlk⁺ cells. These results clearly demonstrated that repression of the *Ink4a/Arf* locus only does not directly drive tumor initiation in hepatic stem cells. Considering that *Ink4a/Arf*^{-/-} mice barely developed primary liver tumors in their lifetime,²⁹ repression of additional targets of *Bmi1* may be needed in cancer initiation.

To evaluate the impact of *Bmi1* on gene expression in hepatic stem cells and to explore the additional targets of *Bmi1* related to tumorigenesis, we conducted an oligonucleotide array analysis using *Bmi1*-transduced *Ink4a/Arf*^{-/-} Dlk⁺ cells and the control *Ink4a/Arf*^{-/-} Dlk⁺ cells. The screening of more than 39,000 transcripts successfully identified 75 down-regulated and 97 up-regulated genes (Supporting Table 1). As expected, enforced expression of *Bmi1* contributed to the maintenance of stemness features and suppression of differentiation-related genes. The present analysis revealed gene expression to be up-regulated for the hepatic stem cell markers *Prom1* (*CD133*) ($P = 0.041$) and *EpCAM* ($P = 0.017$) and down-regulated for the hepatocyte differentiation markers *Cps1* ($P = 0.010$), *Mat1a* ($P = 0.011$), and *Gjb2* (*Cx26*) ($P = 0.010$).

Among these, *Mat1a* knockout mice have been reported to be hypersensitive to oxidative stress and developed steatosis and HCC.³⁰ Furthermore, reduced expression of *Gjb2* (*Cx26*) is known to contribute to the promotion and progression of hepatocarcinogenesis in rats.³¹

Of interest, our microarray analysis unveiled the altered expression of genes involved in Wnt/ β -catenin signaling; down-regulation of the Wnt antagonist *Sox17* ($P = 0.009$), up-regulation of a Wnt downstream effector *Cyclin D1* ($P = 0.001$), and modestly increased expression of the Wnt receptor *Fzd7* ($P = 0.098$). Wnt/ β -catenin signaling is integrally associated with the regulation of stem cells and development of cancer³² and activated Wnt/ β -catenin signaling promotes the proliferation and transformation of hepatic stem/progenitor cells.³ Together, these results imply that enforced expression of *Bmi1* results in an enhancement of stemness features and the acquisition of malignant potential in normal hepatic stem/progenitor cells, at least in part, through the activation of Wnt signaling. However, further analysis would be necessary to elucidate the relationship between *Bmi1* and Wnt signaling.

Surprisingly but importantly, none of the 75 down-regulated genes following *Bmi1*-overexpression was included among the 305 up-regulated genes in neural progenitor cells after *Bmi1* knockdown.²⁷ Likewise, there existed no overlapping genes between the current expression profile and the 101 commonly regulated genes following *BMI1* knockdown between medulloblastoma and Ewing sarcoma cells.^{33,34} In contrast, we detected several genes down-regulated following *Bmi1*-overexpression in hepatic stem/progenitor cells which are also regulated by *Bmi1* in hematopoietic stem/progenitor cells (data not shown). These findings support the fact that PcG proteins function in a cell type-specific manner and the composition of PcG complexes is highly dynamic and differs in different cell-types and even at different gene loci.³⁵

A comparison of the down-regulated genes with the ChIP-on-chip data for PcG complexes in ESCs revealed five genes that are regulated by PRC1 in ESCs as potential direct targets of *Bmi1* in hepatic stem/progenitor cells (Fig. 6B). One of these genes, *Sox17*, is an endodermal marker gene and *Sox17*^{-/-} mice die in the embryonic stage because the endoderm fails to form properly.²² Therefore, its role in hepatic stem cells remained obscure. In the present study, self-renewal capacity of hepatic stem cells was inversely correlated with the *Sox17* expression levels. Furthermore, cotransduction of *Sox17* with *Bmi1* repressed

tumorigenic capacity of *Bmi1* in NOD/SCID mice. These findings suggest that *Sox17* acts as a tumor suppressor in a specific type of tumor originating from hepatic stem cells. The finding that it is transcriptionally silenced by DNA methylation in human colon cancer cells further supports its role as a tumor suppressor gene.³⁶ On the other hand, *Sox17*-knockdown in *Dlk*⁺ cells alone did not promote tumor initiation in immunodeficient mice. Tumor initiation usually requires multiple steps including activation of oncogenes and repression of tumor suppressor genes. As a number of candidate genes of *Bmi1* were identified in this study, coordinated regulation of multiple *Bmi1* targets might be needed to recapitulate *Bmi1*-mediated tumorigenesis *in vivo*. In this regard, knockdown of *Sox17* or other candidate target genes in *Ink4a/Arf*^{-/-} *Dlk*⁺ cells would be intriguing to assess for their tumorigenic activity *in vivo*.

Finally, our findings demonstrated that *Bmi1* regulates the self-renewal of hepatic stem/progenitor cells to a large extent through the suppression of *Ink4a/Arf*. However, it is evident that targets of *Bmi1* other than the *Ink4a/Arf* locus are also responsible for the development of cancer. Further analyses are necessary to determine the roles of the genes listed here in liver development, regeneration, and cancer.

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