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Decreased Expression of Bmi1 Is Closely Associated with Cellular Senescence in Small Bile Ducts in Primary Biliary Cirrhosis

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Cellular senescence of biliary epithelial cells with p16^{INK4a} and p21^{WAF1/Cip} expression in damaged small bile ducts may be critical for progressive bile duct loss in primary biliary cirrhosis. We investigated the involvement of *bmi1*, a polycomb group gene repressing p16^{INK4a} expression, in the pathogenesis of biliary cellular senescence. Bmi1 expression was examined immunohistochemically in livers taken from the patients with primary biliary cirrhosis (*n* = 18) and other diseased (*n* = 19) and normal livers (*n* = 16). We examined the effect of oxidative stress and a short interference RNA (siRNA) targeting *bmi1* on cellular senescence in cultured mouse biliary epithelial cells. Bmi1 was widely expressed in the nuclei of biliary epithelial cells in the control livers. In contrast, *bmi1* expression was significantly decreased in damaged small bile ducts in 43% of livers with primary biliary cirrhosis of stage 1/2, coordinating with the increased p16^{INK4a} expression. In cultured biliary epithelial cells, oxidative stress by H₂O₂ treatment significantly decreased *bmi1* expression, followed by increased P16^{INK4a} expression. A knockdown of *bmi1* induced increased p16^{INK4a} expression, decreased cell proliferation, and increased cellular senescence. In conclusion, the decreased *bmi1* expression caused by oxidative stress may be involved in the pathogenesis of cellular senescence of biliary epithelial cells in primary biliary cirrhosis. (Am J Pathol 2006; 169:831–845; DOI: 10.2353/ajpath.2006.051237)

Primary biliary cirrhosis (PBC) is an organ-specific autoimmune disease and presents with chronic, progressive cholestasis and liver failure.^{1–3} PBC is characterized histologically as a cholangitis of small bile ducts (chronic nonsuppurative destructive cholangitis; CNSDC) eventu-

ally followed by the extensive loss of small bile ducts.^{2–4} Although there have been many studies on the immunopathological features,^{5–7} there have been few studies on the pathogenesis of bile duct loss in PBC. We have recently reported cellular senescence of biliary epithelial cells (BECs) with the augmented expression of p16^{INK4a} and p21^{WAF1/Cip} in damaged small bile ducts in PBC and suggested that the cellular senescence may be involved in the pathogenesis of progressive bile duct loss in PBC.⁸

Cellular senescence is defined as a condition in which a cell no longer has the ability to proliferate. Senescent cells are irreversibly arrested at the G₁ phase of the cell cycle and do not respond to various external stimuli but remain metabolically active. Senescent cells display several characteristics, including histological changes *in vitro* and *in vivo*,^{9,10} shortened telomeres, increased expression of p16^{INK4a} and p21^{WAF1/CIP}, and increased activity of senescence-associated β -galactosidase (SA- β -gal).¹¹ Although we have also shown a possible association of oxidative stress with cellular senescence in PBC,⁸ the mechanism inducing cellular senescence in PBC has not so far been fully clarified.

Recently, *bmi1*, a transcriptional receptor belonging to the polycomb group gene family,¹² has been noted as a senescence regulator.¹³ A critical target of *bmi1* is the *INK4a* locus, which encodes the p16^{INK4a} and p19^{ARF} (p14^{ARF} in humans) tumor suppressor proteins.¹⁴ Overexpression of *bmi1* extends the replicative life spans of mouse and human fibroblasts,^{14,15} possibly by repressing p16^{INK4a} and p19/p14^{ARF}. In the absence of *bmi1*, both the p16^{INK4a} and p19^{ARF} genes from the *Ink4a* locus are expressed.¹⁶ Mouse embryonic fibroblasts (MEFs) from *bmi1* knockout mice show a premature senescence feature with the increased expression of p16^{INK4a}.¹⁴

Given the critical role of *bmi1* in the regulation of cellular senescence and p16^{INK4a} expression, we hypothe-

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sized that the decreased expression of *bmi1* may be closely associated with the cellular senescence of BECs in PBC. Therefore, we examined *bmi1* expression and its correlated expression with senescence-associated $p16^{\text{INK4a}}$ and $p21^{\text{WAF1/CIP}}$ in BECs in PBC and control human livers. Furthermore, we took advantage of cultured mouse BECs to address the issues whether or not oxidative stress could influence *bmi1* expression and whether or not a knockdown of *bmi1* expression could induce the up-regulation of $p16^{\text{INK4a}}$ and cellular senescence. We also examined the involvement of the other polycomb group protein EZH2 that is required for the recruitment of *bmi1* to polycomb group bodies.¹⁷

Materials and Methods

Human Study

Classification of Intrahepatic Biliary Tree

The intrahepatic biliary tree is classified into the intrahepatic large and small bile ducts by their size and distribution in the portal tracts.^{4,18} In this study, septal and interlobular bile ducts are termed small bile ducts. Bile ductules were evaluated separately and not included in the small bile ducts.

Bile ducts were largely classified into inflamed and noninflamed bile ducts. Inflamed bile ducts included the bile ducts surrounded and occasionally infiltrated by inflammatory cells such as bile ducts involved in CNSDC in PBC and bile ducts embedded in infiltrating lymphoid cells in chronic viral hepatitis (CVH).

Liver Tissue Preparation

A total of 53 liver tissue specimens (all were wedge biopsied or surgically resected) were collected from the liver disease files of our laboratory and affiliated hospitals. The liver specimens consisted of 18 PBC, 14 CVH livers, five livers from extrahepatic biliary obstruction (EBO), and 16 histologically normal livers. All PBCs were from the patients fulfilling the clinical, serological, and histological characteristics consistent with the diagnosis of PBC.³ PBC livers were staged histologically.^{3,19} Twelve and six PBC were of stages 1/2 and of stages 3/4, respectively. Seven CVH were regarded as F0-2 and seven as F3/4, respectively.²⁰ Two and twelve CVH cases were serologically positive for hepatitis B surface B antigen (HBsAg) and anti-hepatitis C viral antibody (HCVAb), respectively. Causes of EBO were obstruction or stenosis of the bile duct at the hepatic hilum or the extrahepatic bile duct, because of carcinoma or stone, and the duration of jaundice was less than 1 month. Histologically normal livers were obtained from surgically resected livers for traumatic hepatic rupture or metastatic liver tumor. The liver tissues used were taken from the part sufficiently away from the trauma and tumor. There are no significant differences between the liver samples we used as histologically normal livers, liver tissue from organ donor, and histologically normal wedge biopsied

livers in the expression of *bmi1* and Ki67 antigen in preliminary study.

Liver tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin. More than 20 serial sections, 4 μm thick, were cut from each block. Several were processed routinely for histopathological study, and the remainder was processed for the following immunohistochemistry.

Immunohistochemistry

Bmi1 was detected by using mouse monoclonal anti-*bmi1* antibody (clone 229F6, dilution 1:400; Upstate Technology, Lake Placid, NY). This monoclonal antibody is characterized^{14,21} and has been reported to work on paraffin sections.²² As positive controls for immunohistochemistry, paraffin-embedded material from tonsil and lymph node was used. In addition, we used another monoclonal antibody recognizing *bmi1* (clone 1.T.21, dilution 1:100; Abcam, Cambridge, UK) to confirm the immunostaining. The staining pattern by both antibodies was similar in preliminary study and the former antibody was mainly used. In selected serial sections, $P16^{\text{INK4a}}$ was also detected using mouse monoclonal anti- $p16^{\text{INK4a}}$ protein (clone JC2, dilution 1:100; Neomarkers, Fremont, CA). Dual immunostaining of *bmi1* and $p16^{\text{INK4a}}$ was also performed in the selected sections as described previously.²³ In brief, deparaffinized sections were pretreated in ethylenediaminetetraacetic acid buffer (pH 9) in a pressure-cooker at 100°C, 5 minutes for *bmi1*, or in a microwave oven in citrate buffer (pH 6) at 95°C, 20 minutes for $P16^{\text{INK4a}}$. The sections were immersed in 0.3% H_2O_2 in methanol for 20 minutes to abolish endogenous peroxidase activities. The sections were then immersed in 5% normal goat serum for 60 minutes and incubated with the primary antibody at 4°C overnight. The Envision+ solution (DAKO, Glostrup, Denmark) was then applied for 30 minutes at room temperature. The reaction products were visualized using 3–3'-diaminobenzidine tetra hydrochloride (Sigma Chemical Co., St. Louis, MO) and H_2O_2 . The sections were then lightly counterstained with methyl green or hematoxylin. A similar dilution of the control mouse or control rabbit IgG (DAKO) was applied instead of the primary antibody as negative control. Positive and negative controls were routinely included. At least five portal tracts per liver section were evaluated.

In Vitro Study

Cell Culture and Treatments of Mouse Intrahepatic BECs

Mouse intrahepatic BECs (mBECs) were isolated from 8-week-old female BALB/c mice and were purified and cultured as described previously.^{24,25} The maintenance and passage of cultured BECs were made as described previously.^{24,25} The BECs were used during passage numbers 10 to 15 and cultured up to 8 days, as the longer culture time accelerated senescence in preliminary study. The confluency of the cells was kept less than 80%

Figure 1. Decreased expression of bmi1 and its relation with p16^{INK4a} expression in BECs at the site of chronic nonsuppurative destructive cholangitis in PBC. **A** and **B**: bmi1 was expressed in the nuclei of BECs in normal livers (**A**) and CVH livers (**B**). **C**: The expression of bmi1 was decreased (arrows) in BECs at the site of chronic nonsuppurative destructive cholangitis. PBC, stage 2. **D** and **E**: Correlated expression of bmi1 (**D**) and p16^{INK4a} (**E**) as a senescence-associated marker. The expression of bmi1 was decreased in BECs in the small bile ducts involved in chronic nonsuppurative destructive cholangitis (**D**, arrow), and the expression of p16^{INK4a} was increased in BECs in the same damaged small bile duct (**E**, arrow). PBC, stage 2. Immunostaining for bmi1 (**A–D**) and p16^{INK4a} (**E**). **F**: Dual immunostaining of bmi1 (green fluorescence) and p16^{INK4a} (red fluorescence) demonstrated the absence of bmi1 expression in nuclei of BECs expressing p16^{INK4a} (arrows). Original magnifications, ×400.

during experiments. In several experiments, mBECs were treated with H₂O₂, 112.5 μmol/L for 2 hours, washed throughout to remove H₂O₂, and cultured in fresh medium. UV irradiation (2 or 5 J/m²) was performed by a UV spectrolinker XL-1000 (Spectronics, Westbury, NY).

BECs were incubated in phosphate-buffered saline during UV irradiation, then washed and cultured in fresh media. Serum-free media was applied for BECs to make the condition of cytokine/growth factor deprivation in some experiments.

Table 1. Frequency of the Decreased Expression of *bmi1* in Small Bile Ducts in Primary Biliary Cirrhosis and Other Hepatobiliary Diseases

Diseases	No. of patients	Type of BD	No. of BD	No. of BD showing decreased expression of <i>bmi1</i> (%)
PBC, stage 1/2	12	Large, noninflamed	3	0
		Small, inflamed	51	40 (78.4%)*
		Small, noninflamed	74	0
PBC, stage 3/4	6	Large, noninflamed	3	0
		Small, inflamed	1	1 (100%)
		Small, noninflamed	22	0
CVH	14	Large, noninflamed	9	0
		Small, inflamed	50	0
		Small, noninflamed	170	0
EBO	5	Large, noninflamed	2	0
		Small, inflamed	3	0
		Small, noninflamed	60	0
Normal liver	16	Large, noninflamed	12	0
		Small, inflamed	3	0
		Small, noninflamed	146	0

PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; CVH, chronic viral hepatitis; EBO, extrahepatic biliary obstruction; BD, bile ducts.
* $P < 0.01$ versus PBC, stage 1/2, small, noninflamed and other groups.

Gene Silencing of *bmi1* and *EZH2* by Short Interfering RNA (siRNA)

The expression of *bmi1* and *EZH2* was silenced using an siRNA directed against the coding region of mouse *bmi1* and *EZH2* designed and synthesized by Qiagen (Hilden, Germany). In addition to nonlabeled siRNA, Alexa-488-labeled siRNA (Qiagen) was synthesized. A negative control siRNA labeled by Alexa-488 (Qiagen) was used as a control. The targeted sequence of mouse *bmi1* was 5'-AAGCGGGTACTACCGTTTATT-3'. The targeted sequence of the negative control, which has no homology with mammalian genes, was 5'-AATTCTCGAACGTGTCACGT-3'. One day before transfection, mBECs were plated in a type I collagen-coated 35-mm dishes (5×10^5 cells) or Lab-Tek chambers (5×10^4 cells/well), and then the cells were transiently transfected with 100 nmol/L *bmi1* siRNA by using Dreamfect transfection reagents (OZ Biosciences, Marseilles, France) according to the manufacturer's protocol.

RNA Extraction and Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the cells with the Qiagen RNeasy mini kit (Qiagen) according to the manufacturer's protocol. After cDNA was synthesized, real-time quantitative RT-PCR analysis for *bmi1*, *EZH2*, *p16^{INK4a}*, and *p21^{WAF1/Cip}* mRNAs were performed in duplicate for each sample using the ABI Prism 7700 sequence detection system instrument and software (PE Applied Biosystems, Inc., Foster City, CA). Sequence-specific primers and probes for mouse *bmi1*, *EZH2*, *p16^{INK4a}*, *p21^{WAF1/Cip}*, and *GAPDH* as the internal control were purchased from Applied Biosystems. Amplifications were performed under standard conditions.

Immunofluorescence Staining for Cultured Cells

The mBECs growing in a Lab-Tek chamber were fixed with 5% buffered formalin and ice-cold methanol for 20 minutes each. After blocking with 3% bovine serum albumin, the cells were incubated with either of the primary antibodies for *bmi1* (described above) overnight. The cells were then incubated with Alexa-546-labeled goat anti-mouse IgG (Molecular Probes, Eugene, OR) for 30 minutes, counterstained with 10 μ g/ml 4' and 6-diamidino-2-phenylindole (DAPI), and evaluated under a conventional fluorescence microscope (Olympus, Tokyo, Japan).

Cell Growth and BrdU Incorporation

Cell proliferation activity was assessed on day 4 after treatment by using a 5-bromo-2'-deoxyuridine (BrdU) labeling and detection kit (Roche, Nonenwald, Germany), according to the manufacturer's protocol. The nuclei were simultaneously stained with DAPI. At least 1×10^3 total cells were checked and counted to assess the BrdU-labeling index with a conventional fluorescence microscope (Olympus).

Assay for Cellular Senescence

SA- β -gal activity was detected by using the senescence detection kit (BioVision, Mountain View, CA) according to the manufacturer's protocol.¹¹ The proportion of senescent cells in each condition was assessed at day 6 by counting the percentage of SA- β -gal-positive cells in at least 1×10^3 total cells using light microscopy. In addition, we performed the colony-forming assay to confirm the cellular senescence. One day after H₂O₂ treatment or siRNA transfection, the cells were harvested and counted, and 200 viable cells were seeded in type I

collagen-coated 60-mm dishes. The number of colonies (>1 mm in diameter) was counted at day 5 after the fixation with methanol and Giemsa staining.

Immunoblotting

The cell lysate samples (15 μ l) were solubilized, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane as described previously.²⁶ After transfer, the membranes were processed for immunoblotting as described previously.²⁶ The primary antibodies for bmi1 and p21^{WAF1/Cip1} are described above. P16^{INK4a} and α -tubulin were detected by using rabbit polyclonal antibody for p16^{INK4a} (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal anti- α -tubulin (clone TU-01; Zymed, South San Francisco, CA). The protein expression of bmi1, p16^{INK4a}, and p21^{WAF1/Cip1} were quantified relative to α -tubulin by using NIH image.

Statistical Analysis

Statistical analysis for the difference in human study used the Wilcoxon rank sum test. Statistical analysis of the difference in the *in vitro* assays was performed with the Student's *t*-test. A *P* value less than 0.01 was considered significant.

Results

Decreased Expression of bmi1 in Small Bile Duct Lesions in PBC

Bmi1 was expressed in the nuclei of most BECs lining small bile ducts in normal, CVH, and EBO livers (Figure 1, A and B). The expression of bmi1 was decreased in BECs in the small bile ducts involved in CNSDC in PBC stage 1/2 (Figure 1C). The decreased expression of bmi1 was observed in 10 of 12 PBC of stage 1/2 (83.3%) and 1 of 6 PBC stages 3/4 (16.7%), whereas bmi1 expression was not decreased in any of the other diseased (14 CVH and 5 EBO livers) and 16 normal livers. When small bile ducts were subdivided into inflamed and noninflamed, the decreased expression of bmi1 was detected in 40 of 51 (78.4%) and one of one (100%) inflamed small bile ducts in PBC stage 1/2 and PBC stage 3/4, respectively, whereas it was not seen in any of noninflamed small bile ducts in PBC and other livers (Table 1). Inflamed large bile ducts were not present in all liver tissue sections. The decreased expression of bmi1 was not detected in non-inflamed large bile ducts at all even in PBC stage 1/2 in which bmi1 expression was decreased in the damaged small bile ducts (Table 1). The incidence of the decreased expression of bmi1 in BECs was significantly higher in PBC stage 1/2, when compared with those in other groups (*P* < 0.01).

Bmi1 was expressed in the nuclei of bile ductules as well as in bile ducts in the control livers. The decreased expression of bmi1 was detected in bile ductules, espe-

A

B

C

Figure 2. The effect of oxidative stress on bmi1, p16^{INK4a}, and p21^{WAF1/Cip1} expression in BECs. **A:** bmi1 mRNA expression was significantly lower in BECs on days 2, 3, and 4 after H₂O₂ (112.5 μ mol/L, 2 hours) treatment. P16^{INK4a} mRNA expression was increased significantly on days 3, 4, and 5 after H₂O₂ treatment. P21^{WAF1/Cip1} mRNA expression was high on day 1, gradually decreased on days 2, 3, and 4 after H₂O₂ treatment. Expression of mRNA was quantified with real-time PCR. The expression was normalized as a ratio using GAPDH as a housekeeping gene. Data are expressed as the mean $\pm$ SD. **P* < 0.01, ***P* < 0.05 compared to the control, *n* = 3 for each group. **B:** Immunoblot analysis for bmi1, p16^{INK4a}, and p21^{WAF1/Cip1} protein expression in BECs treated with H₂O₂ (112.5 μ mol/L, 2 hours) and control BECs. A pool of protein samples prepared in three independent experiments was used and analysis was performed twice. **C:** Intensity of each band in immunoblot analysis (**B**) was quantified by densitometry, normalized as a ratio using α -tubulin as an internal control, and statistically analyzed. Bmi1 protein expression was significantly lower in BECs on days 3, 4, and 5 after H₂O₂ treatment. P16^{INK4a} protein expression was significantly high after H₂O₂ treatment. P21^{WAF1/Cip1} protein expression was high on day 2 after H₂O₂ treatment and normalized thereafter. **P* < 0.01, ***P* < 0.05 compared to control.

cially in those involved in chronic portal inflammation in 4 of 12 PBC stages 1/2 and one of six PBC stages 3/4. The decreased expression of bmi1 was not seen in the other

Figure 3. Oxidative stress inhibits cell proliferation and induces cellular senescence. **A:** Cell proliferation was assessed by 5-bromo-2'-deoxyuridine (BrdU) assay on day 4 after H₂O₂ treatment. The labeling index of BrdU was counted for at least 1000 cells in each group. The labeling index of BrdU was significantly low in BECs treated with H₂O₂ (112.5 μmol/L, 2 hours), when compared with control BECs. Data are expressed as the mean ± SD. **P* < 0.01 compared to control. **B:** Cellular senescence was assessed by senescence-associated β-galactosidase activity (SA-β-gal) on day 6 after H₂O₂ treatment. Percentage of cells positive for SA-β-gal activity was significantly higher in BECs treated with H₂O₂ (112.5 μmol/L, 2 hours), when compared with the control BECs. Data are expressed as the mean ± SD. **P* < 0.01 compared to the control. **C:** Cellular senescence was assessed by colony-forming assay on day 6 after H₂O₂ treatment. Number of colonies was significantly lower in BECs treated with H₂O₂ (112.5 μmol/L, 2 hours), when compared with the control BECs. Data are expressed as the mean ± SD. **P* < 0.01 compared to the control.

diseased and normal livers. The expression of bmi1 was rather faint in hepatocytes, when compared with that in small bile ducts and bile ductules and decreased expression of bmi1 was not detectable in hepatocytes in PBC and other livers. The expression of bmi1 was detected in the nuclei of lymphocytes, but it was faint in endothelial and stromal cells in portal tracts.

When the co-related expression of bmi1 and p16^{INK4a} as a senescence-related marker was evaluated using serial sections and dual immunostaining, the expression of bmi1 was decreased in the BECs in damaged small bile ducts involved in CNSDC (Figure 1D), and the expression of p16^{INK4a} was increased in BECs at the same lesion (Figure 1E). The expression of p16^{INK4a} was observed in the cytoplasm and the nuclei of BECs (Figure 1, E and F). Dual immunostaining demonstrated the absence of bmi1 expression in nuclei of BECs expressing p16^{INK4a} (Figure 1F). In contrast, p16^{INK4a} was not expressed in small bile ducts where the expression of bmi1 was not decreased.

Oxidative Stress Decreased the Expression of bmi1 and Increased p16^{INK4a}, and p21^{WAF1/Cip} Expression in Cultured mBECs

When examined by real-time PCR, bmi1 mRNA expression was significantly lower in BECs on days 2, 3, and day 4 after H₂O₂ (112.5 μmol/L, 2 hours) treatment (Figure 2A). p16^{INK4a} mRNA expression was increased and significantly higher on days 2, 3, 4, and day 5 after H₂O₂ treatment. p21^{WAF1/Cip} mRNA expression was significantly high at day 1, and then gradually decreased. According to immunoblot analysis (Figure 2, B and C), bmi1 protein expression was significantly decreased on days 3, 4, and 5 after H₂O₂ treatment. P16^{INK4a} protein expression was significantly higher after H₂O₂ treatment. P21^{WAF1/Cip} protein expression was transiently high on day 2 after H₂O₂ treatment and normalized thereafter.

Oxidative Stress by H₂O₂ Treatment Decreased Cell Proliferation and Increased Cellular Senescence in Cultured mBECs

The BrdU labeling index on day 4 after H₂O₂ treatment was significantly lower in BECs with H₂O₂ treatment (6.9 ± 3.0%), when compared with control mBECs (15.3 ± 8.1%; *P* < 0.01) (Figure 3A). These findings suggest that oxidative stress decreased cell proliferation of mBECs. Cellular senescence of mBECs with and without H₂O₂ treatment was assessed by the detection of SA-β-gal. As shown in Figure 3B, the SA-β-gal-labeling index on day 6 after H₂O₂ treatment was significantly higher in BECs with H₂O₂ treatment (31.6 ± 6.38%), when compared with control BECs (9.9 ± 4.0%) (*P* < 0.01) (Figure 3B). The BECs with H₂O₂ treatment showed lower colony-forming activity (21.3 ± 2.4% control) after passaging (*P* < 0.01) (Figure 3C). These findings confirmed our previous report⁸ showing the increased cellular senescence in mBECs by oxidative stress with H₂O₂ treatment.

The Effect of UV Irradiation and Serum Deprivation on *bmi1* Expression

Apoptotic cells recognized as cells with fragmented and/or condensed nuclei by nuclear staining with DAPI were significantly increased on day 1 after UV irradiation (5 J/m^2) ($4.48 \pm 1.38\%$), when compared with control ($2.29 \pm 1.56\%$) ($P < 0.05$). Apoptotic cells were not significantly increased in UV irradiation (2 J/m^2) ($2.61 \pm 0.63\%$) and serum deprivation ($3.96 \pm 1.75\%$). When examined by real-time PCR, *bmi1* mRNA expression was significantly lower in BECs on days 1 and 2 after serum deprivation ($P < 0.01$) (Figure 4). *bmi1* mRNA expression was slightly lower in BECs on day 2 after UV irradiation (2 J/m^2) ($P < 0.05$) (Figure 4).

Knockdown of *bmi1* Induced the Up-Regulation of $p16^{\text{INK4a}}$, Decreased Cell Proliferation, and Increased Cellular Senescence in Cultured mBECs

Validation of siRNA Targeting *bmi1*

To verify the significance of decreased *bmi1* in BECs for the induction of cellular senescence, we took advantage of siRNA targeting *bmi1* to inhibit *bmi1* expression in mBECs. *bmi1* mRNA expression was significantly lower in BECs transfected with siRNA targeting *bmi1* on days 2, 3, and 4 (Figure 5A). Immunoblot analysis for *bmi1* confirmed the decreased expression of *bmi1* in BECs transfected with siRNA targeting *bmi1* at the protein level (Figure 5B). *Bmi1* protein expression was suppressed in the cells transfected with *bmi1* siRNA, but not those with control siRNA (Figure 5C). These findings indicate that the siRNA targeting *bmi1* designed in this study successfully knocked down *bmi1* expression in mBECs.

The Effect of *bmi1* Knockdown on $p16^{\text{INK4a}}$ and $p21^{\text{WAF1/Cip}}$ Expression

It is conceivable that knockdown of *bmi1* increases $p16^{\text{INK4a}}$ expression, which is repressed by *bmi1* in the basal state. As expected, $p16^{\text{INK4a}}$ mRNA expression was significantly higher in BECs transfected with siRNA targeting *bmi1* on days 3, 4, and 5 (Figure 6A). $p16^{\text{INK4a}}$ protein expression was significantly higher in mBECs on days 3, 4, and 5 after *bmi1* siRNA transfection (Figure 6B). $p21^{\text{WAF1/Cip}}$ mRNA was significantly higher on days 3 and 5 (Figure 5A). $p21^{\text{WAF1/Cip}}$ protein expression was rather low on day 3 after siRNA transfection.

Bmi1 Knockdown Inhibits Cell Proliferation

Knockdown of *bmi1* should decrease cell proliferation by inducing $p16^{\text{INK4a}}$ expression as a CDK inhibitor. As expected, the labeling index of BrdU was significantly low in BECs transfected with *bmi1* siRNA ($20.3 \pm 7.8\%$), when compared with BECs transfected with the control siRNA ($31.4 \pm 5.6\%$; $P < 0.01$) (Figure 7A). Most BECs

Figure 4. The effect of UV irradiation and serum deprivation on *bmi1* expression in BECs. *bmi1* mRNA expression was significantly lower in BECs on days 1 and 2 after serum deprivation and on day 1 after UV irradiation (2 J/m^2). *bmi1* mRNA was quantified with real-time PCR and the expression was normalized as a ratio using *GAPDH* as a housekeeping gene. Data are expressed as the mean $\pm$ SD. * $P < 0.01$, ** $P < 0.05$ compared to the control, $n = 3$ for each group. Serum dep., serum deprivation.

transfected with *bmi1* siRNA were negative for BrdU labeling, as shown in Figure 7B. These findings clearly suggest that the knockdown of *bmi1* inhibits cell proliferation.

Bmi1 Knockdown Induces Cellular Senescence

Bmi1 knockdown should increase cellular senescence via the increased expression of $p16^{\text{INK4a}}$ in BECs. As shown in Figure 7C, the SA- β -gal-labeling index was significantly higher in BECs transfected with *bmi1* siRNA ($13.7 \pm 3.4\%$), when compared with BECs transfected with control siRNA ($6.1 \pm 1.7\%$) ($P < 0.01$). The BECs transfected with *bmi1* siRNA showed lower colony-forming activity ($53.4 \pm 3.3\%$ control) after passaging ($P < 0.01$) (Figure 7D). These findings indicate that the knockdown of *bmi1* increases cellular senescence in mBECs.

The Knockdown of *EZH2* Decreases the Nuclear Expression of *bmi1* but Did Not Affect Cell Proliferation and Cellular Senescence

Since recent studies show that the other polycomb protein, *EZH2*, is essential for *bmi1* recruitment to the polycomb body,¹⁷ we examined the association of *EZH2* with *bmi1* expression and the involvement in the induction of cellular senescence.

The Effect of Oxidative Stress, UV Irradiation, and Serum Deprivation on *EZH2* Expression in BECs

When examined by real-time PCR, *EZH2* mRNA expression was significantly lower ($P < 0.01$) in BECs on day 4 after H_2O_2 ($112.5 \mu\text{mol/L}$, 2 hours) treatment (Figure 8A). *EZH2* mRNA expression was significantly lower ($P < 0.01$) in BECs on days 1 and 2 after serum deprivation and on day 2 after UV irradiation (5 J/m^2) (Figure 8B).

Figure 6. The effect of bmi1 knockdown on p16^{INK4a} and p21^{WAF1/Cip} expression. **A:** P16^{INK4a} mRNA expression was significantly higher in BECs transfected with siRNA targeting bmi1 on days 3, 4, and 5 (left). P21^{WAF1/Cip} mRNA was significantly higher on days 3 and 5 after siRNA transfection. P16^{INK4a} and p21^{WAF1/Cip} mRNA was quantified with real-time PCR. The expression was normalized as a ratio using GAPDH as a housekeeping gene and was shown as percentage of control. Data are expressed as the mean $\pm$ SD. * $P < 0.01$, ** $P < 0.05$ compared to the control, $n = 3$ for each group. **B:** Immunoblot for p16^{INK4a} and p21^{WAF1/Cip} protein expression in BECs transfected with siRNA targeting bmi1 and a control siRNA. The intensity of each band in immunoblot analysis was quantified by densitometry, normalized as a ratio using α -tubulin as an internal control and statistically analyzed. P16^{INK4a} protein expression was significantly higher in BECs on days 3, 4, and 5 after bmi1 siRNA transfection (left). P21^{WAF1/Cip} protein expression was low on day 3 after siRNA transfection. * $P < 0.01$, ** $P < 0.05$ compared to the control. Three independent experiments showed similar results.

The Knockdown of EZH2 Slightly Decreases the Nuclear Expression of bmi1

EZH2 mRNA expression was significantly lower in BECs transfected with siRNA targeting EZH2 on day 2 (Figure 9A). bmi1 mRNA expression was slightly lower in BECs transfected with siRNA targeting EZH2 on days 2, 3, and 4, but there was no statistical difference (Figure 9B). When the expression of bmi1 was detected by immunocytochemistry, the nuclear expression of bmi1 was slightly decreased in the cells transfected with EZH2

siRNA, when compared with the cells transfected with control siRNA on day 4 after transfection (Figure 9C).

The Knockdown of EZH2 Does Not Affect Cell Proliferation and Cellular Senescence

The labeling index of BrdU on day 4 was not significantly different in BECs transfected with EZH2 siRNA ($16.3 \pm 8.8\%$), when compared with BECs transfected with control siRNA ($12.9 \pm 6.0\%$; $P > 0.01$) (Figure 9D).

B

Bmi1 siRNA (green)/ BrdU (red)

DAPI

Figure 7. The knockdown of *bmi1* inhibits cell proliferation and induces cellular senescence. **A:** Cell proliferation was assessed by 5-bromo-2'-deoxyuridine (BrdU) assay. The labeling index of BrdU was counted for at least 1000 cells in each group. The labeling index of BrdU was significantly low in BECs transfected with *bmi1* siRNA, when compared with BECs transfected with control siRNA. Data are expressed as the mean $\pm$ SD. * $P < 0.01$. Day 4 after transfection. **B:** Most BECs transfected with *bmi1* siRNA showing green fluorescence in cytoplasm were negative for BrdU-labeling (red fluorescence). **C:** Cellular senescence was assessed by the senescence-associated β -galactosidase activity (SA- β -gal). Percentage of cells for positive for SA- β -Gal activity was significantly higher in BECs transfected with *bmi1* siRNA, when compared with BECs transfected with control siRNA. Data are expressed as the mean $\pm$ SD. * $P < 0.01$. Day 6 after transfection. **D:** Number of colonies was significantly lower in BECs transfected with *bmi1* siRNA, when compared with BECs transfected with control siRNA. Data are expressed as the mean $\pm$ SD. * $P < 0.01$. Day 6 after transfection.

When the cellular senescence was assessed by the SA- β -Gal labeling index (Figure 9E) and colony-forming assays (Figure 9F), there was no significant differ-

ence between BECs transfected with *EZH2* siRNA and BECs transfected with control siRNA in both indexes of cellular senescence.

Figure 8. The effect of oxidative stress, UV irradiation, and serum deprivation on EZH2 expression in BECs. **A:** EZH2 mRNA expression was significantly lower in BECs on day 4 after H_2O_2 ($112.5 \mu\text{mol/L}$, 2 hours) treatment. **B:** EZH2 mRNA expression was significantly lower in BECs on days 1 and 2 after serum deprivation and on day 2 after UV irradiation (5 J/m^2). **A** and **B:** EZH2 mRNA was quantified with real-time PCR and the expression was normalized as a ratio using *GAPDH* as a housekeeping gene. Data are expressed as the mean $\pm$ SD. * $P < 0.01$ compared to the control, $n = 3$ for each group. Serum dep, serum deprivation.

Discussion

Cellular senescence is a state in which a cell no longer has the ability to proliferate. We have reported previously that the BECs involved in bile duct damage in PBC frequently show markers of cellular senescence and that oxidative stress may be a candidate cause of cellular senescence in BECs in PBC.⁸ Cellular senescence of BECs may be critical for progressive bile duct loss in PBC, because cellular senescence can compromise the regenerative capacity of tissues.¹¹ Recently, accumulating data suggest that *bmi1*, a polycomb group repressor, is critical for the inhibition of $p16^{\text{INK4a}}$ expression and the inhibition of cellular senescence. We hypothesized that the expression of *bmi1* may be decreased at the site of bile duct damage by oxidative stress, resulting in cellular senescence with $p16^{\text{INK4a}}$ up-regulation. Therefore, we investigated the involvement of *bmi1* and its relation to

oxidative stress in the pathogenesis of cellular senescence in PBC.

A major conclusion of this study is that the expression of *bmi1* is decreased in BECs involved in bile duct damage in PBC, whereas BECs in normal small bile ducts express *bmi1* in other diseased liver and normal livers. In the late stage of PBC, the number of the remaining small bile ducts was limited and the decreased expression of *bmi1* was detected in only one small bile duct involved in CNSDC. Therefore, it may be one possibility that the *bmi1*-expressing bile ducts were spared and survive in the late stage of PBC. It is of interest that the decreased expression of *bmi1* co-localized the increased expression of $p16^{\text{INK4a}}$ in damaged bile ducts in PBC. Taken together, it is conceivable that the decreased expression of *bmi1* may be responsible for the cellular senescence of BECs with the increased expression of $p16^{\text{INK4a}}$ in damaged bile ducts in PBC. Hepatic abnormality has not been reported so far in *bmi1* knockout mice in which hematological impairment and neurological abnormality are major features. However, one possibility is that additional environmental factors might be required to trigger the development of PBC in *bmi1* knockout mice, because both genetic and environmental factors are thought to be involved in the pathogenesis of PBC. Another possibility is that other compensatory mechanism(s) may relieve the influence of *bmi1* defect in several organs in *bmi1* knockout mice because the impact of *bmi1* defect is various in each organ. When *bmi1* is disrupted by siRNA, such compensatory mechanism(s) may not be induced promptly.

There have so far been few reports regarding factors that down-regulate *bmi1* expression.²⁷ A proliferation associated SNF-2-like gene (PASG) in mutant mice demonstrates a markedly increased expression of $p16^{\text{INK4a}}$ that is associated with the down-regulation of *bmi1*,²⁷ although the mechanism remains to be determined.²⁷ Because the involvement of oxidative stress has been suggested in the pathogenesis of cellular senescence in PBC,^{8,28,29} we examined the influence of oxidative stress in the expression of *bmi1* and cellular senescence using cultured mBECs. We took advantage of mBECs, because nonneoplastic human BECs are not always conveniently available and the features of senescent mBECs are reportedly similar to those of senescent human BECs.³⁰ Immortalized human BECs³¹ and cholangiocarcinoma cell lines³² appear to be inappropriate for the present study, because genetic and/or epigenetic alterations of $p16^{\text{INK4a}}$ gene and SV40-T-antigen-transformation affects directly the pathway of cellular senescence.

In the present study, we have reported firstly that oxidative stress decreased *bmi1* expression. There have been no reports describing the down-regulation of *bmi1* by oxidative stress to our knowledge. After H_2O_2 treatment, *bmi1* mRNA expression was decreased, being followed by the decreased *bmi1* protein expression in mBECs. $p16^{\text{INK4a}}$ expression was gradually increased in mBECs after H_2O_2 treatment, corresponding to the decreased *bmi1* expression. The early increase of $p16^{\text{INK4a}}$ expression 1 day after H_2O_2 treatment may occur in a *bmi1*-independent manner (as discussed below). The

present study also demonstrated the decreased cell proliferation activity and increased rate of cellular senescence after H_2O_2 treatment. These findings suggest that oxidative stress down-regulates *bmi1* expression and causes the following increased expression of $p16^{INK4a}$, the decreased cell proliferation activity, and increased rate of cellular senescence. In addition, we demonstrated that *bmi1* mRNA expression was decreased by serum deprivation and UV irradiation ($2 J/m^2$). This finding suggests that decreased *bmi1* expression may be involved also in senescence induced by other factors.

To clarify the role of *bmi1* in the regulation of $p16^{INK4a}$ expression and cellular senescence in BECs, we took advantage of siRNA targeting *bmi1*. The designed siRNA targeting *bmi1* successfully inhibited the expression of *bmi1* in the present study. As a result, the knockdown of *bmi1* using siRNA induced the up-regulation of $p16^{INK4a}$, decreased cell proliferation, and increased cellular senescence in BECs. The induction of cellular senescence in BECs by *bmi1* inhibition is similar to the earlier premature senescence in cells from *bmi1* knockout mice accompanied by the increased expression of $p16^{INK4a}$.¹⁴ These results also support our hypothesis that decreased *bmi1* expression is responsible for the induction of $p16^{INK4a}$ and cellular senescence in BECs.

The findings in the present study suggest that at least a part of cellular senescence by oxidative stress is induced via a *bmi1*-dependent pathway. In addition to the *bmi1*-dependent pathway, the immediate increase of $p21^{WAF1/Cip}$ and $p16^{INK4a}$ after H_2O_2 treatment observed in the present study indicated that oxidative stress influenced $p21^{WAF1/Cip}$ and $p16^{INK4a}$ expression via another pathway. Cells subjected to DNA damage induced by ultraviolet or X-radiation, to oxidative stress (induced by H_2O_2 or hyperoxia)³³ induces $p21^{WAF1/Cip}$ expression rapidly via a p53-dependent pathway^{33,34} and enter stress-induced premature senescence. Therefore, it is likely that the immediate increase of $p21^{WAF1/Cip}$ expression in mBECs after H_2O_2 treatment may occur in a p53-dependent manner. In fact, the increased expression of $p21^{WAF1/Cip}$ as well as $p16^{INK4a}$ is also seen in damaged small bile ducts in PBC.⁸ Although several studies suggest that oxidative stress acts mainly through the p53- $p21^{WAF1/Cip}$ -Rb arm to induce senescence,³⁴ other studies indicate that $p16^{INK4a}$ can be activated in response to oxidative stress, possibly through the action of the p38-MAPK protein.³⁵ In the present study, an immediate increase of $p16^{INK4a}$ protein expression was seen on day 2 after H_2O_2 treatment without preceding *bmi1* inhibition. This finding agreed with the latter studies.

A transient response before day 1 might be responsible for the increased $p16^{INK4a}$ protein expression because $p16^{INK4a}$ mRNA expression on day 1 was rather low. Reportedly, an increased expression of $p21^{WAF1/Cip}$ is involved in the induction of senescence, whereas the $p16^{INK4a}$ accumulated in senescent cells is involved in the maintenance of senescence.^{36–38} Taken together, the immediate increase of $p21^{WAF1/Cip}$ as well as $p16^{INK4a}$ by oxidative stress seems to contribute to the induction of cellular senescence of mBECs in coordination with the *bmi1*-dependent increase of $p16^{INK4a}$ expression.

There have so far been no studies showing the effect of *bmi1* inhibition on $p21^{WAF1/Cip}$ expression. However, it is suspected that $p21^{WAF1/Cip}$ is up-regulated by *bmi1*-inhibition through the pathway involving $p19^{Arf}$, mouse double minute 2 (MDM2), and p53, resulting in cell-cycle arrest.^{13,39} In the present study, $p21^{WAF1/Cip}$ mRNA expression increased on days 3 and 5 because of *bmi1* inhibition, although the up-regulation of $p21^{WAF1/Cip}$ protein is obscure. The increased $p21^{WAF1/Cip}$ mRNA expression on days 3 and 5 might be induced via a $p19^{Arf}$ -p53-dependent pathway. However, the reason why $p21^{WAF1/Cip}$ expression fluctuated due to *bmi1* inhibition remains unclear and it may suggest the presence of a complex regulatory system between *bmi1* and $p21^{WAF1/Cip}$ expression.

Recently, Hernandez-Munoz and colleagues¹⁷ reported that the other polycomb group protein EZH2 is required for the recruitment of *bmi1* to polycomb group bodies. Therefore, we also examined the involvement of EZH2 in the regulation of *bmi1* expression and the induction of cellular senescence. We have obtained several interesting results regarding this issue. Decreased EZH2 expression on day 4 after oxidative stress appears to have less impact on the induction of cellular senescence, when compared with the prompt decrease of *bmi1*. It is of interest that the knockdown of EZH2 by siRNA slightly decreased the nuclear expression of *bmi1*. This result agreed with the recent report by Hernandez-Munoz and colleagues.¹⁷ However, the knockdown of EZH2 does not affect cell proliferation and cellular senescence. Because the efficiency of EZH2 knockdown by siRNA was not complete in the present study, the decreased expression of EZH2 may not be enough to induce cellular senescence. Further studies are needed to clarify the significance of EZH2 and its association with *bmi1* in the stress-induced cellular senescence.

Accumulating data suggest the importance of cellular senescence in the pathogenesis of several types of

Figure 9. The knockdown of EZH2 slightly decreases the nuclear expression of *bmi1* but does not affect cell proliferation and cellular senescence. **A:** *EZH2* mRNA expression was significantly lower in BECs transfected with siRNA targeting EZH2 on day 2. *EZH2* mRNA was quantified with real-time PCR. The expression was normalized as a ratio using *GAPDH* as a housekeeping gene. Data are expressed as the mean $\pm$ SD. * $P < 0.01$ compared to the control, $n = 3$ for each group. **B:** *bmi1* mRNA expression was slightly lower in BECs transfected with siRNA targeting EZH2 on days 2, 3, and 4, but there was no statistical difference. *bmi1* mRNA was quantified with real-time PCR. The expression was normalized as a ratio using *GAPDH* as a housekeeping gene. Data are expressed as the mean $\pm$ SD. **C:** The nuclear expression of *bmi1* labeled by red fluorescence was decreased in the cells transfected with *EZH2* siRNA (left), when compared with the cells transfected with control siRNA (right). Day 4 after transfection. **D:** The labeling index of BrdU was not significantly different in BECs transfected with *EZH2* siRNA ($16.3 \pm 8.8\%$), when compared with BECs transfected with control siRNA ($12.9 \pm 6.0\%$). Data are expressed as the mean $\pm$ SD. The labeling index of BrdU was counted for at least 1000 cells in each group. Day 4 after transfection. **E:** Percentage of cells positive for SA- β -Gal activity was not significantly different in BECs transfected with *EZH2* siRNA, when compared with BECs transfected with control siRNA. Data are expressed as the mean $\pm$ SD. Day 6 after transfection. **F:** Number of colonies was not significantly different in BECs transfected with *EZH2* siRNA, when compared with BECs transfected with control siRNA. Data are expressed as the mean $\pm$ SD. Day 6 after transfection.

chronic liver diseases. We have reported cellular senescence in small bile ducts in PBC.⁸ Lunz and colleagues³⁰ have previously implicated the stress-induced expression of p21^{WAF1/Cip} in BECs as an important mediator of cellular senescence that precedes bile duct loss in chronic liver allograft rejection. Marshall and colleagues⁴⁰ have recently reported the relation between hepatocyte G₁ arrest, impaired regeneration, and fibrosis in chronic HCV infection. P21^{WAF1/Cip}-mediated cell-cycle arrest may be a direct viral effect or may be induced by interferon- γ and transforming growth factor- β under conditions of oxidative stress and DNA damage.⁴⁰ Hepatocytes showing SA- β -gal have been detected in chronic hepatitis due to HCV.⁴¹ It is conceivable that the oxidative stress-induced, bmi1-mediated up-regulation of p16^{INK4a} may also impair the hepatocellular and biliary function, limit regeneration of BECs and hepatocytes, and contribute to the progression of chronic liver diseases, as well as PBC.

In conclusion, the decreased expression of bmi1 is closely associated with the cellular senescence of damaged small bile ducts in PBC. The *in vitro* study demonstrated that oxidative stress induced the decreased expression of bmi1 and the subsequent increased p16^{INK4a} expression, decreased cell proliferation activity, and cellular senescence. Taken together, the decreased expression of bmi1 caused by oxidative stress may be critical for the induction of cellular senescence of BECs in PBC. Given that the decreased expression of bmi1 may be responsible for the increased expression of p16^{INK4a} and cellular senescence, therapeutic agents to maintain bmi1 expression may be effective for the prevention of cellular senescence of BECs and for the prevention of bile duct loss in PBC.

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