**The polycomb group gene product Ezh2 regulates proliferation
and differentiation of murine hepatic stem/progenitor cells**

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**Number of figures and tables: 6 figures, 2 supplementary tables and 3
supplementary figures**

Abbreviations: PcG, polycomb group; PRC, polycomb repressive complex; H3K27, histone H3 at lysine 27; HCC, hepatocellular carcinoma; ED, embryonic day; EHS, Engelbreth-Holm-Swarm; OSM, oncostatin M; TNF, tumour necrosis factor; shRNA, short-hairpin RNA; ERP, enhanced red fluorescence protein; ChIP, chromatin immunoprecipitation; EGFP, enhanced green fluorescence protein; Alb, albumin; CK, cytokeratin; Epcam, epithelial cell adhesion molecule; ELISA, enzyme-linked immunosorbent assay; Gata1, GATA binding protein 1; TAT, tyrosine aminotransferase; G6P, glucose-6-phosphatase; PAS, periodic acid-Schiff; Itgb, integrin β ; ES, embryonic stem; Hnf, hepatocyte nuclear factor; Cebp, CCAAT/enhancer binding protein.

Financial support: This work was supported in part by grants for the Global COE program (Global Center for Education and Research in Immune System Regulation and Treatment) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and grants from the Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Corporation (JST), the Takeda Science Foundation, and the Uehara Memorial Foundation.

Conflict of interest: There is no conflict of interest to disclose.

Abstract

Background & Aims: Polycomb group proteins initiate and maintain gene silencing through chromatin modifications and contribute to the maintenance of self-renewal in a variety of stem cells. Among polycomb repressive complexes (PRCs), PRC2 initiates gene silencing by methylating histone H3 lysine 27, and PRC1 maintains gene silencing through monoubiquitination of histone H2A lysine 119. We have previously shown that Bmi1, a core component of PRC1, tightly regulates the self-renewal of hepatic stem/progenitor cells.

Methods: In this study, we conducted lentivirus-mediated knockdown of *Ezh2* to characterise the function of Ezh2, a major component of PRC2, in hepatic stem/progenitor cells.

Results: Loss of *Ezh2* function in embryonic murine hepatic stem/progenitor cells severely impaired proliferation and self-renewal capability. This effect was more prominent than that of *Bmi1* knockdown and was partially abrogated by the deletion of both *Ink4a* and *Arf*, major targets of PRC1 and PRC2. Importantly, *Ezh2* knockdown but not *Bmi1* knockdown promoted the differentiation and terminal maturation of hepatocytes, followed by the up-regulation of several transcriptional regulators of hepatocyte differentiation.

Conclusions: Our findings indicate that Ezh2 plays an essential role in the maintenance of both the proliferative and self-renewal capacity of hepatic stem/progenitor cells and the full execution of their differentiation.

Key words: hepatic stem/progenitor cells, Ezh2, Bmi1, Ink4a/Arf, self-renewal, differentiation

Introduction

Stem cells are generally defined as self-renewing cell populations that can differentiate into multiple distinct cell types. Liver has an enormous capacity to regenerate after injury, although the mechanism of hepatic regeneration differs depending on the proliferation of pre-existing hepatocytes, homing of bone marrow cells, and proliferation and differentiation of hepatic stem cells [1]. In the developing murine liver, endodermal-derived hepatoblasts or hepatic stem/progenitor cells differentiate into hepatocytes and cholangiocytes [1]. Although hepatic stem/progenitor cells have been successfully identified in murine foetal liver [2, 3], the molecular pathways regulating the self-renewal and differentiation of these cells are poorly understood.

Polycomb group (PcG) proteins form multiprotein complexes that play important roles in maintaining the transcriptional repression of target genes. Although PcG genes are best known for their role in maintaining the repression of *Hox* genes during development, they have been implicated in stem cell self-renewal and differentiation [4]. The PcG gene family members form two major distinct PcG complexes: one complex, known as polycomb repressive complex (PRC) 1, is composed of Ring1a/1b, Mph1, and Bmi1 or Mel18, and the other complex, PRC2, is composed of Eed, Suz12, and Ezh2. Ezh2 is a PcG protein homologous to *Drosophila* enhancer of zeste, a histone methyltransferase associated with transcriptional repression. Ezh2 has a SET domain that is typical of histone methyltransferases, and it catalyses the addition of methyl groups to histone H3 at lysine 27 (H3K27). In many cases, the methylation of H3K27 by Ezh2 results in the recruitment of PRC1, and the two PRCs cooperate in gene silencing [4]. Notably, expression of EZH2 together with BMI1 is reportedly associated with the progression and aggressiveness of hepatocellular carcinoma (HCC), and *EZH2*

knockdown inhibits the growth of cultured human HCC cell lines [5]. We previously reported that Bmi1 enhances the self-renewal capacity of hepatic stem/progenitor cells and drives cancer initiation [6]. However, the role of Ezh2 in the hepatic stem cell system remains to be clarified.

In this study, we investigated the function of Ezh2 in foetal liver Dlk⁺ hepatic stem/progenitor cells by knocking down *Ezh2* using lentivirus-mediated stable shRNA expression. *Ezh2* knockdown profoundly inhibited the proliferation of Dlk⁺ hepatic stem/progenitor cells and promoted differentiation into hepatocytes. These findings provide the first evidence of an essential role for Ezh2 in the homeostasis of the hepatic stem cell system.

Materials and Methods

Mice

Pregnant C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). *Ink4a-Arf*^{+/-} mice (Strain code 01XB1) obtained from Mouse Models of Human Cancers Consortium in NCI-Frederick (Frederick, MD, USA) were bred and maintained in accordance with our institutional guidelines for the use of laboratory animals.

Purification and culture of Dlk⁺ cells

Dlk⁺ cells were prepared from liver cell suspensions of embryonic day (ED) 14.5 foetal livers as described previously [2, 3]. Briefly, cells were stained with rat anti-mouse Dlk monoclonal antibody (MBL, Nagoya, Japan) followed by anti-rat IgG-conjugated magnetic beads. Dlk⁺ cells were purified by passage through cell separation columns in a magnetic field (Miltenyi Biotec, Bergisch Gladbach, Germany). They were plated at 1×10^3 cells/well on collagen type IV-coated 6-well plates (Becton Dickinson, Franklin lakes, NJ, USA) and cultured as described elsewhere [2, 3]. Colony assays were performed in at least three independent triplicate experiments. To evaluate the potential to differentiate into hepatocytes, Dlk⁺ cells were placed on an Engelbreth-Holm-Swarm (EHS) gel (Becton Dickinson) in the presence of oncostatin M (OSM, R&D Systems, Minneapolis, MN, USA) [7]. Collagen type I gel culture (Nitta Gelatin, Osaka, Japan) in the presence of tumour necrosis factor (TNF)- α (Peprotech, Rocky Hill, NJ, USA) was also conducted to examine the ability to differentiate into cholangiocytes [8].

Viral production and transduction

Lentiviral vectors (CS-H1-shRNA-EF-1 α -EGFP) expressing short-hairpin RNAs (shRNAs) against murine *Ezh2* (target sequence: sh-*Ezh2*-1, 5'-GGAAAGAACTGAAACCTTA -3'; sh-*Ezh2*-2, 5'-GGTAAATGCTCTTGGTCAA -3') were constructed. Lentiviral vectors (CS-H1-shRNA-EF-1 α -EGFP) expressing shRNAs against *Bmi1* and *luciferase* were also used [6]. A lentiviral vector carrying enhanced red fluorescent protein (ERP) (CS-H1-shRNA-RfA-ERP) expressing shRNA against *Bmi1* was also constructed for the double knockdown of *Ezh2* and *Bmi1*. Recombinant lentiviruses were produced as described previously [6]. Purified cells were transduced with indicated viruses 12–18 hours after pre-incubation.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as reported previously [9]. Briefly, cross-linked chromatin was sonicated into 200- to 500-bp fragments. The chromatin was immunoprecipitated using anti-*Ezh2* (clone AC22, a gift from Dr. Kristian Helin) and anti-H3K27me3 (Millipore, Bedford, MA, USA) antibodies. Normal mouse IgG was used as a negative control. Quantitative PCR was conducted using SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan). Primer sequences are listed in Supplementary Table 2 [10].

Statistics

Data are presented as the mean $\pm$ SEM. Statistical differences were analysed using the Mann-Whitney U test. *P* values less than 0.05 were considered significant.

Results

Basal expression of Ezh2 and stable knockdown of Ezh2 in hepatic stem/progenitor cells

We first analysed the mRNA expression of *Ezh2* in hepatic stem/progenitor cells, which are enriched in the Dlk⁺ cell fraction [2,3] in ED14.5 foetal liver. Haematopoietic cells were excluded by gating the CD45⁺Ter119⁺ cell fraction, and liver cells were divided into the Dlk⁺ hepatic stem/progenitor fraction and the Dlk⁻ non-stem/progenitor fraction. *Ezh2* expression was readily detected in both fractions, but quantitative RT-PCR and western blot analyses revealed a higher level of *Ezh2* expression in the Dlk⁺ than the Dlk⁻ fraction (Fig. 1A and B).

To investigate the function of *Ezh2* in Dlk⁺ hepatic stem/progenitor cells, we used lentivirus-mediated *Ezh2* knockdown. Dlk⁺ cells prepared from ED14.5 wild-type foetal livers were infected with sh-*Ezh2* viruses and allowed to propagate for 5 days. Flow-cytometric analyses revealed that the majority (more than 90%) of cells were positive for enhanced green fluorescent protein (EGFP), a marker of lentiviral integration (Fig. 1C). We compared the effect of the two anti-*Ezh2* shRNAs (sh-*Ezh2*-1 and sh-*Ezh2*-2) by real-time RT-PCR and western blot analyses. Real-time RT-PCR showed that the level of endogenous *Ezh2* was markedly reduced in cells infected with lentivirus expressing shRNA against *Ezh2* compared with the control cells expressing shRNA against *luciferase* (sh-*Luc*) at multiple time points (Fig. 1D). Both shRNA severely reduced *Ezh2* expression, although sh-*Ezh2*-2 was less effective than sh-*Ezh2*-1 (Fig. 1D). Concordant with this, the western blot analysis of cells at day 5 of culture showed that sh-*Ezh2*-1 was more effective in knocking down *Ezh2* than sh-*Ezh2*-2 (Fig. 1E). Therefore, we mainly used sh-*Ezh2*-1 in the following

experiments, but we also obtained very similar results with sh-*Ezh2*-2 (Fig. 2C, D and data not shown).

Impaired proliferation and self-renewal of Dlk⁺ cells following Ezh2 knockdown

It has been reported that approximately 15% of purified Dlk⁺ cells gave rise to colonies at day 5 of culture. Among of them, Dlk⁺ cells with the ability to form large colonies consisting of more than 100 cells at day 5 of culture possess the properties of hepatic stem/progenitor cells. Because Dlk⁺ cells produce a large number of Dlk⁻ progeny, the proportion of Dlk⁺ cells declines to less than 1% at day 5 of culture. Nonetheless, Dlk⁺ cells in culture retain clonogenic activity [2, 3]. Corresponding to these reports, almost 15% of Dlk⁺ cells gave rise to colonies, which included a significant number of large colonies, while Dlk⁻ cells scarcely gave rise to colonies, and no Dlk⁻ cells generated large colonies at day 5 of culture (Fig. 2A). Next, we performed loss-of-function assays of *Ezh2* and/or *Bmi1* in Dlk⁺ cells. Knockdown efficiencies were confirmed by western blot (Fig. 1E and 2B). *Ezh2* knockdown modestly decreased the total number of colonies formed at day 5 of culture (Fig. 2C). By contrast, the number of large colonies derived from *Ezh2*-knockdown Dlk⁺ cells was significantly decreased compared with the control, and most of the *Ezh2*-knockdown cells did not proliferate beyond 14 days (Fig. 2D). The effect of *Bmi1* knockdown was milder than that of *Ezh2* knockdown, and double knockdown of *Ezh2* and *Bmi1* had a limited advantage over single knockdown of *Ezh2* in inhibiting the proliferation of Dlk⁺ cells (Fig. 2C and D). Although *Ezh2* knockdown enhanced apoptotic cell death in cultures of Dlk⁺ cells, levels of apoptosis were not prominent in control or *Ezh2*-knockdown cultures (1.1 ± 0.2 and $3.6 \pm 0.5\%$, respectively, Fig. 2E).

Among the colonies derived from hepatic stem/progenitor cells, those that keep

growing beyond 28 days retain a significant number of hepatic stem/progenitor cells and efficiently generate secondary colonies in replating assays [6]. Because it is difficult to culture *Ezh2*-knockdown Dlk^+ cells beyond 14 days, we recovered cells from uninfected colonies that kept growing beyond 28 days and infected them with sh-*Ezh2* viruses. At day 7 of culture, $\text{Dlk}^+\text{EGFP}^+$ cells expressing sh-*Ezh2*-1 were collected by cell sorting and replated to permit the formation of colonies. The frequencies of total secondary colonies and large secondary colonies were markedly reduced by *Ezh2* knockdown compared to the control (Fig. 2F). Again, the inhibitory effect of *Ezh2* knockdown was greater than that of *Bmi1* knockdown, and double knockdown minimally enhanced the inhibitory effect of *Ezh2* knockdown. These results suggest that *Ezh2* plays an essential role in the maintenance of both the proliferative and self-renewal capacity in hepatic stem/progenitor cells. Colony PCR demonstrated that secondary large colonies expressed both hepatocyte markers and cholangiocyte markers (Supplementary Fig. 1), indicating that the ability to differentiate into either hepatocytes or cholangiocytes was maintained in replated Dlk^+ cells. Of interest, the *Ezh2*-knockdown colonies showed a higher expression level of *albumin* (*Alb*) than the control colonies, whereas *Bmi1* knockdown caused a mild increase in the expression of *Sall4*, which is a regulator of cholangiocyte differentiation [11].

Role of Ezh2 in differentiation of hepatic stem/progenitor cells

Ezh2 has recently been implicated in the differentiation of neural stem cells and epidermal stem cells [12,13]. However, its role in hepatic stem/progenitor cells remains to be addressed. In the control colonies, cytokeratin (CK) 7⁺ cholangiocytes predominantly differentiated compared to Alb^+ hepatocytes. By contrast, comparable numbers of Alb^+ hepatocytes were detected in the colonies generated from

Ezh2-knockdown Dlk⁺ cells (Fig. 3A and B). Although *Bmi1* knockdown did not significantly compromise the differentiation of Dlk⁺ cells, double-knockdown colonies contained a greater number of Alb⁺ hepatocytes than in *Ezh2* knockdown (Fig. 3A and B). These results suggest that *Ezh2* knockdown promotes the differentiation of hepatic stem/progenitor cells towards the hepatocyte lineage. Consistent with this finding, an enzyme-linked immunosorbent assay (ELISA) assay detected a significant increase in Alb secretion from culture of *Ezh2*-knockdown Dlk⁺ cells compared to that of control Dlk⁺ cells (Supplementary Fig. 2A). The expression of liver-enriched transcription factors was up-regulated in both *Ezh2*- and *Bmi1*-knockdown Dlk⁺ cells at day 5 of culture (Fig. 3C). However, the increase was mild in the *Bmi1*-knockdown Dlk⁺ cells compared to the *Ezh2*-knockdown or double-knockdown cells. A slight increase in the expression of *Sall4* was also observed in *Ezh2*-knockdown and double-knockdown cells. In clear contrast, neither *Ezh2*- nor *Bmi1*-knockdown Dlk⁺ cells showed remarkable changes in the expression of *Gata1* (*GATA binding protein 1*), a haematopoietic transcription factor gene (Fig. 3C).

Regulation of the Ink4a/Arf gene by Ezh2

Bmi1, a core component of PRC1, regulates the self-renewal of various somatic stem cells, targeting *p16^{Ink4a}* and *p19^{Arf}* in particular [14,15]. *Ezh2* likewise contributes to the repression of the *Ink4a/Arf* locus in mouse embryonic fibroblasts [16].

To examine whether *Ezh2* knockdown results in derepression of the *Ink4a* and *Arf* genes, we performed real-time RT-PCR analyses. Expression of both *p16^{Ink4a}* and *p19^{Arf}* was up-regulated in *Ezh2*-knockdown Dlk⁺ cells at day 5 of culture, although their derepression levels were moderate compared with those in *Bmi1*-knockdown cells (Fig. 4A). To address whether *Ezh2* is involved in transcriptional repression through

histone modifications at the *Ink4a/Arf* locus, we conducted ChIP analyses in wild-type Dlk⁺ cells. ChIP assays demonstrated the binding of Ezh2 across the *Ink4a/Arf* locus and an increase in H3K27me3 levels (Fig. 4B).

Loss-of-function assays of Ezh2 in Ink4a/Arf^{-/-} Dlk⁺ cells

To evaluate directly the involvement of the *Ink4a/Arf* locus in Ezh2 function in hepatic stem/progenitor cells, we isolated Dlk⁺ cells from *Ink4a-Arf^{-/-}* embryos. Deletion of the *Ink4a/Arf* locus significantly enhanced the propagation of colonies compared with the wild-type Dlk⁺ cells, and most of the colonies at day 5 of culture kept expanding up to day 14 (Fig. 5A and B). Flow-cytometric analysis at day 14 of culture revealed that the percentage of Dlk⁺ cells in *Ink4a/Arf^{-/-}* colonies was approximately 9-fold higher than in wild-type colonies (data not shown), indicating that deletion of the *Ink4a/Arf* locus enhanced the self-renewal capability of Dlk⁺ cells. Notably, however, not only the total number of colonies but also the number of large colonies (consisting of more than 100 cells at day 5 of culture) was significantly reduced by *Ezh2* knockdown (Fig. 5A and B). Consistent with this, the diameter of large *Ezh2*-knockdown colonies at day 14 of culture was significantly smaller than in the control (Fig. 5C). Of interest, the effect of *Bmi1* knockdown was largely cancelled by the deletion of *Ink4a/Arf*, while that of *Ezh2* knockdown was only partially abrogated (Fig. 5A-C). Immunostaining revealed that large colonies derived from *Ezh2*-knockdown *Ink4a-Arf^{-/-}* Dlk⁺ cells still harboured a similar number of Alb⁺ hepatocytes as those derived from *Ezh2*-knockdown wild-type Dlk⁺ cells (Fig. 5D and E).

We next performed replating assays. At day 14 of culture, *Ink4a-Arf^{-/-}* Dlk⁺ EGFP⁺ cells transduced with knockdown vectors were collected by cell sorting and replated to allow colonies to form. The frequency of total secondary colonies and of large

secondary colonies was markedly reduced by *Ezh2* knockdown compared to the control (Supplementary Fig. 3A). Deletion of the *Ink4a/Arf* locus cancelled out the effect of *Ezh2* knockdown to a lesser extent than that of *Bmi1* knockdown. Secondary colonies were generated in a similar fashion to the original colonies and exhibited enhanced differentiation towards the hepatocyte lineage (Supplementary Fig. 3B).

Role of Ezh2 in terminal differentiation and maturation in wild-type and Ink4a/Arf^{-/-} Dlk⁺ cells

We cultured Dlk⁺ cells in EHS gel supplemented with OSM to selectively induce hepatocyte terminal maturation. By day 5 of culture, multiple cell clusters with tight cell–cell contact were formed. The *Ezh2*-knockdown clusters consisted of mature hepatocytes with a highly condensed, granulated cytosol and clear round nuclei compared with the control clusters (Fig. 6A). In addition, real-time RT-PCR revealed that tyrosine aminotransferase (*TAT*) and glucose-6-phosphatase (*G6P*), the metabolic enzyme genes highly expressed in terminally differentiated hepatocytes, were significantly up-regulated in *Ezh2*-knockdown cells (Fig. 6B). In addition, periodic acid-Schiff (PAS) staining successfully detected intracellular glycogen accumulation in *Ezh2*-knockdown cells, which indicated the functional maturation of the hepatocytes (Supplementary Fig. 2B). Although the deletion of *Ink4a/Arf* in Dlk⁺ cells slightly promoted hepatocyte maturation in EHS gel cultures compared to wild-type Dlk⁺ cells, *Ezh2* knockdown further promoted hepatocyte maturation (Fig. 6A and B). Together, these findings indicate that post-commitment hepatocyte maturation is facilitated by *Ezh2* knockdown.

Next, Dlk⁺ cells were cultured on collagen type I gel in the presence of TNF- α to selectively induce differentiation into cholangiocytes. Both the control and

Ezh2-knockdown cells similarly formed tube-like structures (Fig. 6C). Expression of *CK7*, *CK19* and integrin $\beta 4$ (*Itgb4*), useful marker genes of cholangiocyte maturation, was mildly upregulated in *Ezh2*-knockdown cells, but another marker gene, *Sall4*, did not show any changes in expression (Fig. 6D). The deletion of the *Ink4a/Arf* genes caused no remarkable changes in collagen type I gel cultures in terms of cholangiocyte maturation (Fig. 6C and D).

Taken together, these findings indicate that post-commitment hepatocyte maturation rather than cholangiocyte maturation is accelerated by *Ezh2* knockdown. Moreover, these results suggest that the *Ink4a/Arf* locus is also a major target of *Ezh2*, but there exist additional targets of *Ezh2* in the regulation of hepatic stem/progenitor cell growth and self-renewal.

Discussion

Ezh2 plays an important role in gene silencing through the trimethylation of H3K27. In embryonic stem (ES) cells, most developmental genes are reversibly silenced through the bivalent domain in their transcriptional regulatory region, which consists of large regions of trimethylated H3K27 harbouring smaller regions of trimethylated H3K4 [17, 18]. Therefore, PRC2 is critical in maintaining the pluripotency of embryonic stem (ES) cells. However, little is known about the role of PRC2 in somatic stem cells, especially in primary hepatic stem/progenitor cells. Our loss-of-function analysis of *Ezh2* clearly showed that *Ezh2* regulates the proliferation of hepatic stem/progenitor cells in *Ink4a/Arf*-dependent and -independent manners. Given that *Ezh2* knockdown profoundly affected the replating efficiency of hepatic stem/progenitor cells, *Ezh2* might also be needed to maintain the self-renewal capacity of these cells. These functional

characteristics of *Ezh2* are very similar to those of *Bmi1*, but *Ezh2* behaved quite differently from *Bmi1* in the regulation of hepatic stem/progenitor cell differentiation. *Ezh2* knockdown promoted the differentiation of hepatic stem/progenitor cells into hepatocytes and further enhanced the maturation of hepatocytes. However, *Bmi1* knockdown did not compromise their differentiation. These findings unveil distinct functions of PRC1 and PRC2 in the regulation of hepatic stem/progenitor cell differentiation. A similar finding has been reported in breast cancer, in which EZH2 and BMI1 inversely correlate with prognosis and TP53 mutation [19].

Ink4a and *Arf* are major target genes of PcG proteins such as *Bmi1* and *Ezh2* [16]. *Ezh2* contributes to the proliferation of pancreatic β -cells during regeneration by suppressing the *Ink4a/Arf* locus [20]. Overexpression of EZH2 reportedly decreases expression of $p16^{\text{INK4a}}$ through hypermethylation of the $p16^{\text{INK4a}}$ promoter in cholangiocarcinogenesis in hepatolithiasis [21]. The present ChIP analyses showed that *Ezh2* binds to the *Ink4a/Arf* locus, accompanied by increased levels of trimethylated H3K27 in hepatic stem/progenitor cells. However, deletion of the *Ink4a/Arf* locus only partially abrogated the inhibitory effect of *Ezh2* knockdown on the expansion of hepatic stem/progenitor cells. On the other hand, the inhibitory effect of *Bmi1* knockdown was largely cancelled by deletion of *Ink4a/Arf*. These results indicate that *Ezh2* regulates hepatic stem/progenitor cells in both *Ink4a/Arf*-dependent and -independent manners. Actually, double knockdown of *Ezh2* and *Bmi1* had a limited advantage over single knockdown of *Ezh2* in inhibiting hepatic stem/progenitor cell proliferation, further indicating additional functions of *Ezh2* independent of *Ink4a/Arf*.

A number of liver-enriched transcription factors, including hepatocyte nuclear factors (Hnfs) and CCAAT/enhancer binding proteins (Cebps), play a central role in normal

hepatogenesis [22]. It has been reported that Hnf3 β , a marker of definitive endoderm, is indispensable for early liver development [23]. Consistent with this, enforced expression of Hnf3 β in ES cells and mesenchymal stem cells promotes differentiation towards the hepatocyte lineage [24,25]. Recent studies have shown that Cebp α , a nuclear transcription factor of the bZIP protein family, is essential for hepatocyte differentiation [26]. In addition, suppression of Cebp α expression stimulates biliary cell differentiation, with reduced expression of Hnf6 and Hnf1 β [27].

In the present study, *Ezh2* knockdown in hepatic stem/progenitor cells resulted in enhanced expression of Hnfs. Likewise, increased expression of *Hnf6* and *Hnf1b* was simultaneously observed. Of interest, chromatin immunoprecipitation combined with DNA microarray (ChIP-on-chip) analyses reveal that both PRC1 and PRC2 could bind to the promoter regions of *Hnf3b*, *Cebpa*, and *Hnf6* in ES cells [28]. As *Hnf3b* and *Cebpa* have bivalent domains in their promoter regions [17,18], we hypothesised that *Ezh2* mediates the silencing of these genes to maintain hepatic stem/progenitor cells in an immature state. Unexpectedly, the present ChIP analyses using purified Dlk⁺ cells failed to demonstrate the binding of Ezh2 at these loci (data not shown). It is possible that the Dlk⁺ fraction was inappropriate for detecting the recruitment of Ezh2 for repression of target genes because it contained not only stem/progenitor cells but also cells in different stages of differentiation. Intriguingly, a recent study showed that Ezh2-dependent trimethylated H3K27 prevents the recruitment of AP1 and other transcription factors required for terminal differentiation [13]. It is also possible that Ezh2-dependent H3K27me3 on hepatocyte differentiation-related genes likewise prevents the recruitment of liver-enriched transcription factors in hepatic stem/progenitor cells.

Although PcG proteins have been characterised as self-renewal factors of embryonic

as well as somatic stem cells, Ezh2 has recently been implicated in the differentiation of neural stem cells and epidermal stem cells [12,13]. Our findings further support an essential role for PcG proteins in the precise regulation of stem cell differentiation. The composition of PcG complexes is highly dynamic and differs with different cell types and even at different gene loci. Given that the complexes exhibit differences in specificity for histone substrates, the target genes regulated by PcG proteins are quite diverse among different cell types [29]. Efforts to unravel the molecular machinery of PcG proteins, including Ezh2, would facilitate our overall understanding of the hepatic stem cell system and contribute to the establishment of liver regeneration therapy.

Acknowledgements

The authors thank Dr. Kristian Helin for the anti-Ezh2 antibody, Dr. Hiroyuki Miyoshi for the CS-H1-shRNA-EF-1 α -EGFP and CS-H1-shRNA-RfA-ERP vectors, Dr. Akihide Kamiya for technical assistance and Mieko Tanemura for laboratory assistance.

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Figure Legends

Fig. 1. Basal expression of *Ezh2* in wild-type and *Ezh2*-knockdown hepatic stem/progenitor cells. (A) Real-time RT-PCR analysis of *Dlk* and *Ezh2* expression in freshly purified *Dlk*⁺ cells. *, Statistically significant ($p < 0.05$). (B) western blot analysis of *Ezh2* expression in freshly purified *Dlk*⁺ cells compared to *Dlk*⁻ cells. (C) The EGFP-positivity of cells transduced with indicated viruses. (D) Real-time RT-PCR analysis of *Ezh2* expression in *Dlk*⁺ cells transduced with sh-*Ezh2*-1 or sh-*Ezh2*-2. A lentiviral vector expressing shRNA against *luciferase* (sh-*Luc*) was used as a control. Expression relative to the control is depicted. (E) *Dlk*⁺ cells transduced with sh-*Ezh2*-1 or sh-*Ezh2*-2 were subjected to western blot analysis at day 5 of culture.

Fig. 2. Loss-of-function analyses of *Bmi1* and *Ezh2* on hepatic stem/progenitor cell proliferation. (A) The number of total colonies and large colonies consisting of more than 100 cells generated from 1,000 *Dlk*⁺ or *Dlk*⁻ cells was counted at day 5 of culture. (B) *Dlk*⁺ cells expressing anti-*Bmi1* shRNA together with *EGFP* or *RFP* as a marker gene were subjected to western blot analysis at day 5 of culture. (C) Numbers of colonies generated from 1,000 *Dlk*⁺ cells transduced with indicated viruses. Total numbers of colonies were counted at day 5 of culture. (D) The number of large colonies containing more than 100 cells at days 5 and 14. *, Statistically significant ($p < 0.05$). (E) TUNEL assays (upper panels) and fluorescence images (lower panels) of colonies at day 5 of culture. Nuclear DAPI staining (blue) is shown in the insets (upper panels). *, Statistically significant ($p < 0.05$). Scale bar = 500 μ m. (F) Effect of *Ezh2* knockdown on replating capacity. Among the colonies derived from uninfected *Dlk*⁺ cells, those which kept growing beyond 28 days were infected with

viruses expressing shRNAs. Seven days after infection, Dlk⁺EGFP⁺ cells were collected by cell sorting and replated to allow colonies to form. The total number of total colonies and large colonies consisting of more than 100 cells were counted at day 7 of subculture.

Fig. 3. Effects of *Ezh2* and *Bmi1* knockdown on the differentiation of hepatic stem/progenitor cells. (A) Bright-field images and fluorescence micrographs of large colonies (containing more than 100 cells) transduced with indicated viruses at days 5 of culture. Dual immunostaining was performed to detect the expression of Alb (red) and CK7 (green) in clonal colonies. EGFP expression in single-knockdown colonies, EGFP and RFP expression in a double-knockdown colony (upper panels) and nuclear DAPI staining (middle panels) are shown in the insets. Scale bar = 200 μ m. (B) The percentages of Alb⁺CK7⁻, Alb⁻CK7⁺, Alb⁺CK7⁺, and Alb⁻CK7⁻ cells in large colonies containing more than 100 cells were calculated at day 5 of culture. The average for 10 colonies is presented as the mean $\pm$ SD. *, Statistically significant ($p < 0.05$). (C) Real-time RT-PCR analysis of the expression of liver-enriched transcription factors and *Gata1* in colonies derived from Dlk⁺ cells transduced with indicated viruses at day 5 of culture. *, Statistically significant ($p < 0.05$).

Fig. 4. Regulation of the *Ink4a/Arf* genes by *Ezh2* in hepatic stem/progenitor cells.

(A) Real-time RT-PCR analyses of *p16^{Ink4a}* and *p19^{Arf}* expression in colonies derived from Dlk⁺ cells transduced with indicated viruses at day 5 of culture. *, Statistically significant ($p < 0.05$). (B) ChIP analyses of freshly purified Dlk⁺ cells were conducted on the *Ink4a/Arf* locus (primer set 1-4) and the β -actin (*Actb*) control promoter region using indicated antibodies.

Fig. 5. Loss-of-function analyses of *Bmi1* and *Ezh2* on the proliferation of *Ink4a-Arf*^{-/-} hepatic stem/progenitor cells. (A) Numbers of colonies generated from 1,000 *Ink4a-Arf*^{-/-}Dlk⁺ cells transduced with indicated viruses. Total numbers of colonies were counted at day 5 of culture. (B) The number of large colonies containing more than 100 cells at days 5 and 14. *, Statistically significant ($p < 0.05$). (C) The diameter of large colonies at day 14 of culture. *, Statistically significant ($p < 0.05$). (D) Bright-field images and fluorescence micrographs of large colonies (containing more than 100 cells) transduced with indicated viruses at day 5 of culture. Dual immunostaining was performed to detect the expression of Alb (red) and CK7 (green) in clonal colonies. EGFP expression (upper panels) and nuclear DAPI staining (middle panels) are shown in the insets. Scale bar = 200 μ m. (E) The percentages of Alb⁺CK7⁻, Alb⁻CK7⁺, Alb⁺CK7⁺, and Alb⁻CK7⁻ cells in large colonies containing more than 100 cells were calculated at day 5 of culture. The average for 10 colonies is presented as the mean $\pm$ SD. *, Statistically significant ($p < 0.05$).

Fig. 6. Terminal differentiation of *Ezh2*-knockdown Dlk⁺ cells towards hepatocytes and cholangiocytes. (A) To evaluate the terminal differentiation of wild-type or *Ink4a-Arf*^{-/-}Dlk⁺ cells towards hepatocytes, *Ezh2*-knockdown Dlk⁺ cells were placed on EHS gel in the presence of OSM. Bright-field images and fluorescence micrographs (inset panels) of cells in EHS gel at day 5 of culture are presented. Scale bar = 100 μ m. (B) Real-time RT-PCR analyses of hepatocyte differentiation and maturation marker genes. *, Statistically significant ($p < 0.05$). (C) To evaluate the terminal differentiation of wild-type or *Ink4a-Arf*^{-/-}Dlk⁺ cells towards cholangiocytes, *Ezh2*-knockdown Dlk⁺ cells were subjected to collagen gel culture in the presence of TNF- α . Bright-field

images and fluorescence micrographs (inset panels) of cells in collagen type I gel at day 5 of culture are presented. Scale bar = 100 μm . (D) Real-time RT-PCR analyses of cholangiocyte differentiation and maturation marker genes. *, Statistically significant ($p < 0.05$).

Figure 1_ **B** **C** **D** **E**

Figure 3 **B** **C**

Figure 4 **B**

Figure 5