Bmi1 Is Required for Hepatic Progenitor Cell Expansion and Liver Tumor Development

Lingling Fan1,3, Chuanrui Xu4, Chunmei Wang1, Junyan Tao1, Coral Ho1, Lijie Jiang1, Bing Gui1, Shiang Huang3, Matthias Evert5, Diego F. Calvisi5, Xin Chen1,2*

1 Department of Bioengineering and Therapeutic Sciences, University of California San Francisco, San Francisco, California, United States of America, 2 Liver Center, University of California San Francisco, San Francisco, California, United States of America, 3 Center for Stem Cell Research and Application, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, 4 School of Pharmacy, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, 5 Institute of Pathology, University of Greifswald, Greifswald, Germany

Abstract

Bmi1 is a polycomb group transcriptional repressor and it has been implicated in regulating self-renewal and proliferation of many types of stem or progenitor cells. In addition, Bmi1 has been shown to function as an oncogene in multiple tumor types. In this study, we investigated the functional significance of Bmi1 in regulating hepatic oval cells, the major type of bipotential progenitor cells in adult liver, as well as the role of Bmi1 during hepatocarcinogenesis using Bmi1 knockout mice. We found that loss of Bmi1 significantly restricted chemically induced oval cell expansion in the mouse liver. Concomitant deletion of Ink4a/Arf in Bmi1 deficient mice completely rescued the oval cell expansion phenotype. Furthermore, ablation of Bmi1 delayed hepatocarcinogenesis induced by AKT and Ras co-expression. This antineoplastic effect was accompanied by the loss of hepatic oval cell marker expression in the liver tumor samples. In summary, our data demonstrated that Bmi1 is required for hepatic oval cell expansion via deregulating the Ink4a/Arf locus in mice. Our study also provides the evidence, for the first time, that Bmi1 expression is required for liver cancer development in vivo, thus representing a promising target for innovative treatments against human liver cancer.

Introduction

Liver is a unique organ, being silent in normal circumstances but displaying regenerative properties following damage and/or parenchymal loss. Liver regeneration involves two different cellular compartments, depending on the nature of the injury. In response to the acute mass loss injury, such as partial hepatectomy, liver regeneration is due to the proliferation of hepatocytes from the remaining lobes [1]. Under chronic injury conditions that impair hepatocyte proliferation, a subpopulation of unique cells, which has been termed as “oval cells”, emerges and expands. Oval cells are considered to be hepatic stem or progenitor cells because of their bi-potential capability of differentiating into both hepatocytes and cholangiocytes [2,3]. Several models of oval cell reaction in rodents have been developed by exposing the animals to certain carcinogens, such as 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) [4], carbon tetrachloride (CCl4) [5], and 2-acetylaminoﬂuorene (2-AAF) [6,7], among others. In these models, oval cells arise from the portal area of the lobule and infiltrate the surrounding parenchyma. The molecular features of oval cells are still controversial, mostly due to the lack of deﬁnitive markers to identify these cells. For instance, A6, a monoclonal antibody previously described by Factor VM [8], and Cytokeratins (CKs), such as CK7 and CK19, are widely used as oval cell markers in rodents [9]. Recently, the epithelial cell adhesion molecule (EpCAM) was also found in the oval cell niche [10]. Unfortunately, all of these biomarkers can also stain the bile duct epithelium, thus limiting their usefulness as primary antibodies for oval cell isolation. Recently, a new panel of monoclonal antibodies directed against OC2s by immunization of rats with enzymatically dispersed non-parenchymal cells from the DDC-treated mouse livers in searching for oval cell specific antigens have been developed. However, whether these antibodies truly recognize oval cells or whether they recognize different oval cell sub-populations remains to be fully explored [11].

Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) are the two most common types of primary liver tumors, which originate from hepatocytes and cholangiocytes, respectively. However, some primary liver cancer showed intermediate or combined (HCC/CC) phenotypes. Those tumors were thought to be originated from transformed progenitor (oval) cells [12,13,14]. It has been hypothesized that maturation arrest might occur when a bi-potential progenitor cell is on its way to differentiation, which can give rise to tumors with a range of phenotypes with heterogeneous HCC and CC features [13]. In a previous study, we developed a mouse model in which co-activation of AKT and N-Ras oncogenes results in rapid development of HCC and CC in the mouse liver [15].
Bmi1 was initially identified as a c-Myc cooperating oncogene in murine B-cell lymphomas [16], and subsequently determined to be a member of the Polycomb group of transcriptional repressors [17,18], which participate in regulating cell cycle and senescence. Using knockout mice, it was found that Bmi1 deletion results in neurological abnormalities and severe hematopoietic defects in mice [19]. Subsequent studies revealed that Bmi1 is essential for self-renewal of both normal and leukemic hematopoietic stem cells [20,21], as well as neural stem cells [22]. Recently, the essential role of Bmi1 as an oncogene has been revealed in multiple tumor types, including breast cancer [23], melanoma [24], prostate cancer [25], non-small cell lung carcinomas [26,27], and HCC [28,29]. The Ink4a/Arf locus was identified as a critical downstream target of Bmi1. In mice, Ink4a/Arf encodes p16ink4a and p19arf genes, and both are important tumor suppressors. Of note, p16ink4a regulates cell cycle progression via modulating Cdk4/cyclin D complexes, whereas p19arf regulates cell apoptosis via the MDM2/p53 pathway. Recent studies have demonstrated that Bmi1, together with other polycomb proteins, binds throughout the Ink4a/Arf locus, and represses p16ink4a and p19arf expression [30]. Furthermore, it has been shown that ablation of Ink4a/Arf dramatically reduced the lymphoid and neurological defects in Bmi1 deficient mice [31]. However, Bmi1 and Ink4a/Arf double knockout mice remain small and unfertile, similar to that observed in Bmi1 knockout mice [32], indicating the existence of additional Ink4a/Arf independent regulatory pathways. Consistent with this hypothesis, a recent study suggested that Bmi1 also plays a role in the regulation of mitochondrial function and the DNA damage response pathway [33]. In particular, it has been shown that treatment with the antioxidant N-acetylcysteine (NAC) reduced the elevated reactive oxygen species (ROS) characteristic of Bmi1 deficient mice. Consistently, NAC rescued the defects in thymocyte maturation in Bmi1 null mice.

Although Bmi1 is known to play critical roles in regulating multiple types of stem or progenitor cells, its functional significance in regulating hepatic oval cells and hepatocarcinogenesis remains poorly understood. In the present study, using Bmi1 null mice, we demonstrated that Bmi1 is required for DDC-induced oval cell expansion in vivo. To investigate the molecular mechanism underlying this phenotype, we evaluated the oval cell expansion in Bmi1 and Ink4a/Arf double knockout mice as well as Bmi1 null mice treated with NAC. Our study clearly demonstrates that loss of Ink4a/Arf rescues the oval cell expansion defects in Bmi1 null mice, supporting the hypothesis that Bmi1 regulates hepatic oval cells via modulation of the Ink4a/Arf locus. Furthermore, we co-expressed activated forms of AKT and Ras in Bmi1 null mice to evaluate the role of Bmi1 in hepatocarcinogenesis. The results indicate that ablation of Bmi1 dramatically delays liver tumor development driven by AKT and Ras co-expression. Delayed hepatocarcinogenesis was accompanied by the loss of hepatic oval cell marker expression in the AKT/Ras liver tumor samples. Altogether, our study provides novel insights into the role of Bmi1 in regulating hepatic progenitor cell proliferation and hepatocarcinogenesis.

Results

Bmi1 is expressed in hepatic oval cells and is required from oval cell expansion

Despite the fact that Bmi1 is considered to be an important stem cell marker, it remains unknown whether Bmi1 is expressed in hepatic oval cells. We therefore investigated the expression of Bmi1 in hepatic oval cells. To establish a stable oval cell expansion model for this study, adult wild-type mice were randomized to normal diet or DDC diet for 3 weeks. Consistent with the previous reports, typical histological changes were detected in all DDC treated mouse livers. H&E staining revealed a population of small cells with a large nucleus to cytoplasm ratio in the periporal area of the liver lobule, in the DDC treated mouse livers. Many of these small cells had an atypical duct-like morphology, which is a well-known oval cell phenotype [4,37] (Figure 1). Immunohistochemical staining showed the nuclear Bmi1 staining in these oval cells (Figure 1 and Figure S1). In contrast, Bmi1 expression was undetectable in normal liver tissues (Figure 1). Consistent with these data, Bmi1 mRNA level was higher in DDC treated liver tissues compared with that in untreated liver tissues (Figure S2).

Next, we subjected Bmi1−/− mice (n = 5) and their littersmates with Bmi1+/+ or Bmi1+/− genotypes (n = 9) to the DDC treatment. Oval cell expansion could be clearly visualized in DDC treated Bmi1+/+ or Bmi1−/− mice (Figure 2A and data not shown). By immunofluorescence staining, we detected positive staining for the ductal oval cell markers A6, CK19 and EpCAM in both untreated and DDC treated mouse livers (Figure 2B). However, in the untreated liver sections, cells positive for A6, CK19 and EpCAM markers were limited to bile duct cells in the periportal region. In DDC treated livers, A6-, CK19-, and EpCAM-stained cells were characterized by atypical ductal proliferation, and stretched from the periportal area to the central area (Figure 2B). In addition, the extensive staining of periductal marker OC2-2A6 without overlapping with ductal marker A6 (Figure 2D) further confirmed the oval cell expansive pattern. In striking contrast, we found that the oval cell expansion was significantly reduced in DDC treated Bmi1−/− mouse livers (Figure 2A). Few atypical duct-like cells were detected in Bmi1−/− mice. Using A6 and CK19 staining, we found that areas positive for of A6 and CK19 staining were significantly decreased in the DDC treated Bmi1−/− livers, when compared with Bmi1+/+ controls (Figure 2B and 2C). Similar results were obtained with EpCAM (Figure 2B). Interestingly, we observed the loss of the periductal cell marker OC2-2A6 in the DDC treated Bmi1−/− livers (Figure 2B), suggesting the defective expansion of these periductal cells in Bmi1 deleted mice.

Altogether, our results provide solid evidence that Bmi1 is expressed in hepatic oval cell population, and ablation of Bmi1 significantly inhibits the oval cell expansion induced by DDC treatment. The data indicate that Bmi1 is essential for hepatic oval cell expansion in the DDC mouse model.

Antioxidant treatment does not rescue the oval cell expansion in Bmi1−/− Mice

Next, we investigated the molecular mechanisms that underlie the Bmi1 mediated hepatic oval cell expansion. It has been reported that increased ROS levels contribute to the multiple in vivo defects observed in Bmi1−/− mice [33]. To examine whether Bmi1 regulates oval cell expansion via the ROS pathway, we performed pharmacological treatment with the antioxidant NAC as previously described [33]. We found that there was an increased fluorescent signal of A6 and CK19 in the Bmi1−/− DDC livers supplemented with NAC when compared with untreated Bmi1−/− DDC livers (Figure 3C). However, the typical oval cell expansion phenotypic appearance that could be readily observed in Bmi1+/+ DDC livers (n = 8) was not apparent in the NAC treated Bmi1−/− DDC livers (n = 5) (Figure 3A and 4B). Intriguingly, morphological evaluation of the livers revealed that an enlargement of bile ducts occurred in response to NAC treatment (Figure 3A), which was presumably the cause of the increased overall fluorescent signal for A6 and CK19 antibodies in these mice. Consistent with this result, lipid peroxidation which is commonly used as the indicator of oxidative stress in tissues, assessed by MDA content, was not significantly inhibited in the NAC treated DDC livers compared with that in untreated liver tissues (Figure S2).
Bmi1 in Hepatic Oval Cell and Hepatocarcinogenesis

Figure 1. Hepatic oval cell expresses Bmi1. H&E staining (upper row) and immunostaining of Bmi1 (lower row) in the wildtype (left) and DDC treated (right) mouse liver tissues. Arrows indicate expanding oval cells. At least 3 animals in each group were assayed.
doi:10.1371/journal.pone.0046472.g001

In a recent study, we developed a novel liver tumor model by co-expressing myr-AKT (with C-terminal HA tag) and N-RasV12 genes via hydrodynamic injection [38], which will be referred to as AKT/Ras mice in this paper. In this mouse model, co-activation of AKT and Ras rapidly induced liver tumors in five to six weeks after injection (Figure 5A). Histological evaluation of the liver showed that both HCC, representing ~70% of the liver lesions and CC, ~30% of the liver lesions, were present in AKT/Ras mice (Figure 5B), in accordance with our previous report [15]. Because AKT/Ras induced both HCC and CC, we investigated whether the oval cell marker A6 was expressed in these tumor cells. We found that double staining of HA-tag, which labeled ectopically injected AKT, and A6 consistently co-localize in HCC cells. We found that double staining of HA-tag, which labeled ectopically injected AKT, and A6 consistently co-localize in HCC cells. We found that double staining of HA-tag, which labeled ectopically injected AKT, and A6 consistently co-localize in HCC cells.

Loss of Bmi1 has been previously shown to inhibit the tumor development in multiple mouse models, such as K-Ras induced lung cancer [30] or hedgehog pathway-driven medulloblastoma [39]. In addition, the present study supports the critical role of Bmi1 in regulating A6 positive liver progenitor cell expansion. Thus, we sought to determine whether Bmi1 expression is elevated in liver tissues from DDC treated Bmi1−/− mice when comparing with liver tissues from DDC treated wildtype mice (Figure S3). Altogether, our data suggest that the role of Bmi1 in oval cell expansion is most likely unrelated to the role of Bmi1 in regulating mitochondrial function and ROS levels.

Ink4a/Arf deletion completely rescues the oval cell expansion in Bmi1−/− mice

The Ink4a/Arf locus is a well-characterized downstream target of Bmi1. Indeed, we found that expression levels of p16Ink4A and p19Arf were higher in liver tissues from DDC treated Bmi1−/− mice compared with liver tissues from DDC treated wildtype mice (Figure S4). We therefore hypothesized that Bmi1 regulates hepatic oval cell expansion in an Ink4a/Arf-dependent manner. To test this hypothesis generated Bmi1 and Ink4a/Arf double knockout mice (Bmi1−/−; Ink4a/Arf−/−) to explore whether the deletion of Ink4a/Arf locus could rescue the defective oval cell expansion phenotype observed in Bmi1−/− mice.

As a first step, we determined whether Ink4a/Arf locus affects oval cell expansion. For this purpose, we compared the oval cell expansion in Ink4a/Arf−/− DDC livers and Ink4a/Arf−/− DDC livers. Using H&E morphological analysis as well as immunofluorescence staining for A6 and CK19, we found similar oval cell expansion phenotypes in Ink4a/Arf−/− and Ink4a/Arf−/− mice (data not shown), indicating that loss of Ink4a/Arf locus has no effect per se on oval cell proliferation. Subsequently, we generated Bmi1−/−;Ink4a/Arf−/− mice and treated the mice with DDC diet (n = 4). Histological analysis and A6 and CK19 staining showed that, when compared to the Bmi1−/−; Ink4a/Arf−/− DDC livers (n = 4), the typical oval cell expansion pattern was restored in Bmi1−/−; Ink4a/Arf−/− DDC livers, with a similar labeling pattern observed in Bmi1−/−; Ink4a/Arf−/− (n = 4) DDC livers (Figure 4A and 4B). Quantification analysis demonstrated that the percentage of A6 and CK19 positive areas on Bmi1−/−; Ink4a/Arf−/− DDC livers was restored to the same level of Bmi1−/−; Ink4a/Arf−/− DDC liver sections (Figure 4C).

In summary, the present results indicate that the ablation of Ink4a/Arf locus in Bmi1 deficient mice can completely restore the oval cell expansion. Therefore, our in vivo study demonstrates that Bmi1 plays its essential role in hepatic oval cell proliferation in an Ink4a/Arf-dependent manner.

Ablation of Bmi1 inhibited AKT/Ras induced hepatocarcinogenesis

In a recent study, we developed a novel liver tumor model by co-expressing myr-AKT (with C-terminal HA tag) and N-RasV12 genes via hydrodynamic injection [38], which will be referred to as AKT/Ras mice in this paper. In this mice model, co-activation of AKT and Ras rapidly induced liver tumors in five to six weeks after injection (Figure 3A). Histological evaluation of the liver showed that both HCC, representing ~70% of the liver lesions and CC, ~30% of the liver lesions, were present in AKT/Ras mice (Figure 5B), in accordance with our previous report [15]. Because AKT/Ras induced both HCC and CC, we investigated whether the oval cell marker A6 was expressed in these tumor cells. We found that double staining of HA-tag, which labeled ectopically injected AKT, and A6 consistently co-localize in HCC and CC lesions (Figure 5D). These data, therefore, suggest that AKT/Ras hepatocarcinogenesis might depend on hepatic progenitor cells.

Thus, we sought to determine whether Bmi1 expression is
required for AKT/Ras induced liver tumor development. As the first step, we evaluated Bmi1 expression in AKT/Ras tumor cells, and we found that expression of Bmi1 in both HCC and CC lesions (Figure 5C). Hydrodynamic injection of AKT/Ras into Bmi1−/− mice (n = 6) as well as Bmi1+/+ control littermates (n = 5) was performed (Table 1). In all the control mice, massive abdomen enlargement was evident within 4 weeks after injection and mice became moribund, requiring to be euthanized by 6 to 7 weeks.

Figure 2. Loss of Bmi1 inhibits DDC induced hepatic oval cell expansion in mice. H&E staining (A) and immunostaining of A6, CK19, EpCAM and OC2-2A6 (B) on sections of Bmi1 wild-type (WT) liver without DDC treatment; Bmi1 WT liver with 3wk-DDC treatment; and Bmi1 knockout (KO) liver with 3wk-DDC treatment. Representative images of three independent experiments are shown. Expanded views of A6 and CK19 staining are shown on the lower right corner; (C) Quantification of A6 and CK19 immunostaining using ImageJ software. ***P<0.001 by Student’s t test. (D) Dual staining of OC2-2A6 (periductal) and A6 (ductal) showing different oval cell populations in DDC treated mouse liver. Yellow arrows in (A) represents expanded oval cells. At least 3 animals in each group were assayed.

doi:10.1371/journal.pone.0046472.g002
In contrast, all Bmi1−/− mice appeared to be normal with no palpable abdominal mass 6 to 8 weeks post-injection (Table 1). Unfortunately, we were unable to maintain the AKT/Ras injected Bmi1−/− mice beyond 8 weeks post injection since these mice started to die due to infections consequent to their severe immunodeficiency.

Upon dissection, tumor nodules were present throughout the liver of AKT/Ras injected wild-type mice (Figure 6A). The average liver weight was 11.6 g, and the liver to body ratio of approximately 0.34 in AKT/Ras wild-type mice (Table 1 and Figure 6B). In contrast, no visible nodular lesions could be identified in the livers from AKT/Ras injected Bmi1−/− mice, although the liver appeared to be paler and spottier than normal liver (Figure 6A). Liver weight average was approximately 1 g, and liver to body ratio 0.06 in AKT/Ras injected Bmi1−/− mice (Table 1 and Figure 6B).

Histological analysis revealed that neoplastic lesions occupied the majority of the liver parenchyma in AKT/Ras injected wild-type mice (Figure 6A). The average liver weight was ~11.6 g, and the liver to body ratio of approximately 0.34 in AKT/Ras wild-type mice (Table 1 and Figure 6B). In contrast, no visible nodular lesions could be identified in the livers from AKT/Ras injected Bmi1−/− mice, although the liver appeared to be paler and spottier than normal liver (Figure 6A). Liver weight average was approximately 1 g, and liver to body ratio ~0.06 in AKT/Ras injected Bmi1−/− mice (Table 1 and Figure 6B).

Discussion

Bmi1, a member of the Polycomb group protein family, is required for maintaining self-renewal of stem or progenitor cell in multiple organs, particularly in the neural and hematopoietic system. In the current study, we showed Bmi1 is expressed in hepatic oval cells and the oval cell expansion is significantly inhibited when Bmi1 is deleted, supporting the critical role of Bmi1 in regulating this type of liver progenitor cells. In order to
investigate the mechanisms whereby Bmi1 influences oval cell expansion and proliferation, we designed two rescue experiments based on the functional roles that have been ascribed to Bmi1. The first mechanism is the regulation of mitochondrial function played by Bmi1 via modulation of ROS levels [33,40]. To investigate this issue, we treated the mice with the NAC antioxidant, which has been demonstrated to rescue the Bmi1\(^{-/}\) thymocyte development and other defects. The results showed that the typical hepatic oval cell expansion phenotype was absent in Bmi1\(^{-/}\) mice treated with DCC and NAC. However, a marked bile duct enlargement phenotype occurred in these mice. This observation indicates that ROS level does not affect hepatic oval cell expansion, but may influence the biliary tract development. The mechanism leading to the bile duct enlargement in response to NAC remains unclear.

![Image of experimental results](image)

**Figure 4. Deletion of Ink4a/Arf rescues oval cell expansion defects in Bmi1\(^{-/}\) mice.** H&E staining (A) and A6 and CK19 immunostaining (B) on Bmi1\(^{+/+}\); Ink4a/Arf\(^{+/+}\), Bmi1\(^{-/-}\); Ink4a/Arf\(^{+/+}\) and Bmi1\(^{-/-}\); Ink4a/Arf\(^{-/-}\) DDC treated mouse livers. Representative images of three independent experiments are shown. (C) Quantification of A6 and CK19 staining using ImageJ software. At least 3 animals in each group were assayed. ***P<0.001 by Student’s t test.

doi:10.1371/journal.pone.0046472.g004
and requires further investigation. As concerns the second mechanism, Bmi1 has been previously shown to modulate stem cell proliferation via repressing the Ink4a/Arf locus. For instance, multiple studies have demonstrated that loss of Ink4a/Arf locus rescued the hematopoietic stem cell defects [31,41,42] and neurological stem cell abnormalities [32,43,44] that were observed in Bmi1 mutant mice. Pietersen AM et al. reported that co-deletion of the Ink4a/Arf locus can rescue severe mammary-epithelium growth defects observed in Bmi1 deficient mice [45]. In the present study, we found that double deletion of Bmi1 and Ink4a/Arf in mice rescued the oval cell expansion defects induced by the loss of Bmi1. Therefore, our data imply that Bmi1, similar to that described in other tissue types, regulates hepatic oval cell expansion in an Ink4a/Arf-dependent manner.

While our studies showed that Bmi1 is required for hepatic progenitor cell expansion, it remains unknown whether Bmi1 is required for adult hepatocyte proliferation. To investigate this question, we performed 2/3 partial hepatectomy on Bmi1−/− mice. However, none of the Bmi1−/− mice survived the surgery and all animals died within 24 hours. In contrast, all control Bmi1+/+ and Bmi1+/− mice were still alive 48 to 72 hours post surgery (Fan L, unpublished observation). This is likely due to the severe hematopoietic defects characteristic of Bmi1−/− mice. Thus, we used an alternative approach by comparing the proliferative rates of hepatocytes from 2-week-old young mice, when hepatocytes are actively proliferating, in Bmi1−/− and Bmi1+/− mice. BrdU incorporation assay and Ki67 staining were used to assess hepatocyte proliferation. We found that there was no significant difference between Bmi1−/− and their littermate control mice (Figure S5). The data suggest that it is likely Bmi1 is not required for hepatocyte proliferation. However, more studies are required before the definitive conclusion can be reached on this issue. The most appropriate approach is to generate liver specific Bmi1 knockout mice by crossing AlbCre mice [46] with
Bmi1fl/+ mice [47] Partial hepatectomy should be performed on these conditional knockout mice, and whether Bmi1 is required for hepatocyte proliferation can be definitively determined.

Liver cancer has two major types, namely HCC and CC, which are believed to be derived from hepatocytes and cholangiocytes, respectively. However, the concept of hepatic progenitor cells as targets of hepatocarcinogenesis has recently been elicited [48,49,50], even though it is still quite controversial. Increasing evidence indicated the link between hepatic oval cells and HCC/CC mixed liver tumors. For example, in a recent study by Samira et al., a progressive expansion of oval cells was induced by liver specific deletion of Nf2 (neurofibromatosis type 2) tumor suppressor gene. Importantly, all those mice eventually developed both HCCs and CCs, suggesting that Nf2−/− progenitors can be a cell of origin for these tumors [51]. In another study by Lee et al., a similar HCC/CC mixed phenotype was developed in mice heterozygous for the tumor suppressor WW45 or in mice with liver-specific WW45 ablation. All the tumors were positive for the oval cell marker A6, supporting the progenitor cell origin of the mixed HCC/CC tumor cells [46].

In a recent study, we developed a novel mouse model in which HCC/CC combined liver tumors were developed by co-activating AKT and N-Ras oncogenes via hydrodynamic injection [15]. We now show that the AKT/Ras tumor cells overexpressed the oval cell marker A6. The expansion of cells positive for the A6 oval cell marker dramatically decreases the liver tumor burden, while the Bmi1 null mice overexpressing AKT/Ras developed only lipid-rich preneoplastic lesions, with no visible signs of malignancy. Of note, the absence of frankly malignant tumors was paralleled by the reduced expression of the A6 oval cell marker in Bmi1 null mice. Thus, these data suggest that Bmi1 play an important role in AKT/Ras hepatocarcinogenesis, most likely via regulating hepatic progenitor cell proliferation. Furthermore, as we have shown that Bmi1 regulates hepatic oval cell expansion via regulating the Ink4A/Arf locus, it would be important to determine whether loss of Ink4A/Arf locus can rescue the tumor phenotype in Bmi1 null mice. This experiment is currently in process and will be reported separately.

Our previous studies showed Bmi1 cooperates with activated Ras pathways to promote hepatic carcinogenesis in vivo [28]. In addition, a recent study by Chiba T et al demonstrated that overexpression of Bmi1 in Ink4a/Arf−/−;Dlk(+) liver progenitor cells led to tumor formation in the Xenograft model [52]. Our current study adds to all these previous studies, providing further evidence that Bmi1 functions as an oncogene and is required for liver cancer development. Altogether, these studies suggest that targeting Bmi1 may be novel therapeutic strategy for the treatment of liver cancer.

### Table 1. Ablation of Bmi1 inhibits AKT/Ras induced hepatocarcinogenesis.

| Code                  | Genotype | Gender | W.P.I. (*) | liver weight | body weight | Ratio (##) |
|-----------------------|----------|--------|------------|--------------|-------------|------------|
| WT AKT Ras 1          | Bmi1+/+  | Male   | 5          | 13.3         | 38.9        | 0.34       |
| WT AKT Ras 2          | Bmi1+/+  | Male   | 5          | 13.8         | 37.2        | 0.37       |
| WT AKT Ras 3          | Bmi1+/+  | Female | 6          | 8.8          | 31.3        | 0.28       |
| WT AKT Ras 4          | Bmi1+/+  | Female | 6          | 8.6          | 28.3        | 0.30       |
| WT AKT Ras 5          | Bmi1+/+  | Female | 7.5        | 13.3         | 35.1        | 0.38       |
| KO AKT Ras 1          | Bmi1−/−  | Male   | 8          | 1.2          | 19.3        | 0.06       |
| KO AKT Ras 2          | Bmi1−/−  | Male   | 8          | 1.1          | 15.4        | 0.07       |
| KO AKT Ras 3          | Bmi1−/−  | Male   | 8          | 1.1          | 16.2        | 0.07       |
| KO AKT Ras 4          | Bmi1−/−  | Female | 6          | 0.5          | 8.7         | 0.06       |
| KO AKT Ras 5          | Bmi1−/−  | Female | 8          | 1           | 17.7        | 0.06       |
| KO AKT Ras 6          | Bmi1−/−  | Female | 8          | 0.9         | 11.5        | 0.08       |

*refers to weeks post injection; # refers to the ratio of liver weight to body weight.

doi:10.1371/journal.pone.0046472.t001

### Materials and Methods

#### Constructs and reagents

All the constructs, including pT3-EF1α-RasV12, pT3-EF1α-myr-AKT and pCMV-SB used for mouse injection were previously described [34,35,36]. DDC was purchased from Deans Animal Feed Inc. (Redwood City, CA) and NAC from Sigma-Aldrich (St. Louis, MO).

#### Mice

Ink4a/Arf−/− mice were purchased from the NCI Mouse Models of Human Cancers Consortium (MMHCC). Bmi1+/− mice were kindly provided to us by Dr. Carla Kim of Harvard University. Bmi1+/− mice were intercrossed to generate Bmi1 null mice. Ink4a/Arf−/− mice and Bmi1+/− mice were mated and the offspring were backcrossed to generate double mutant mice (Bmi1−/−; Ink4a/Arf−/−). Briefly, Bmi1+/−; Ink4a/Arf−/− mice and Bmi1+/−; Ink4a/Arf−/− mice were initially mated to generate Bmi1−/−; Ink4a/Arf−/− mice. These mice were further mated with Bmi1−/−; Ink4a/Arf−/− mice to generate Bmi1−/−; Ink4a/Arf−/− mice. Bmi1−/−; Ink4a/Arf−/− mice were intercrossed to generate Bmi1−/−; Ink4a/Arf−/− and Bmi1−/−; Ink4a/Arf−/− littermates. Genotyping was performed by polymerase chain reaction (PCR) on genomic DNA from tail clips.

---

PLOS ONE | www.plosone.org 8 September 2012 | Volume 7 | Issue 9 | e46472

Bmi1 in Hepatic Oval Cell and Hepatocarcinogenesis
Sulfamethoxazole and trimethoprim combination (TMS) was added to drinking water at 1:40 ratio to prevent potential infection in Bmi1 mutant mice. For hepatic oval cell expansion, 6-week-old mice were supplied, continuously for 3 weeks, with a diet containing 0.1% DDC. For the in vivo administration of NAC, water containing NAC at 1 mg ml⁻¹ (0.1%) was supplied to animals starting from at 5 weeks of age (one week before DDC treatment) and administration of NAC continued throughout the DDC treatment course. To examine the hepatocyte proliferation, BrdU (100 mg/kg body weight) was injected i.p. 2 hours before the mice were euthanized.

Figure 6. Absence of Bmi1 decelerates AKT/Ras induced liver tumor development. Gross images (A), Ratio of liver to body weight (B), H&E staining (C) and Ki67 staining (D) of Bmi1 WT AKT/Ras liver and Bmi1 KO AKT/Ras liver (6 weeks post injection). At least 3 animals in each group were assayed. Representative images of three independent experiments are shown.

doi:10.1371/journal.pone.0046472.g006
For the mouse liver tumor model, Bmi1<sup>+/−</sup> mice were backcrossed with wild-type FVB/N mice obtained from Charles River (Wilmington, MA) for five passages. The Bmi1<sup>+/−</sup> mice were inter-crossed to obtain Bmi1<sup>−/−</sup> mice as well as control littermates. The hydrodynamic injection procedures were performed as previously described [36]. In brief, pT3-EF1α-RasV12, pT3-EF1α-myr-AKT, and pCMV-SB were mixed at the ratio of 25:25:2 in saline and injected at 1/10 volume of mouse weight in 5 to 7 seconds.

All mice were housed, fed and treated in accordance with protocols approved by the committee for animal research at the University of California, San Francisco.

**Immunohistochemistry and Immunofluorescence**

For immunohistochemistry staining, liver tissue was divided and fixed in 4% paraformaldehyde overnight at 4°C, then in 75% ethanol overnight at 4°C and processed to be embedded in paraffin blocks. Paraffin slides were dewaxed by xylene, followed by rehydrating through a series of washes with incrementally decreasing percentages of ethanol. Antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0) by placement in a microwave on high for 10 min, followed by a 20-min cool down at room temperature. After a blocking step with the 5% goat-serum and Avidin-Biotin blocking kit (Vector Laboratories, Burlingame, CA). The slides were incubated with a primary antibody: anti-Ki67 (Labvision, Fremont, CA) at a 1:150 dilution overnight at 4°C; anti-Bmi1 (Bethyl) at a 1:100 dilution overnight at room temperature. Slides were then subjected to 3% hydrogen peroxide for 10 min to quench endogenous peroxidase activity and subsequently the biotin conjugated secondary antibody at a 1:400 dilution for 30 min at room temperature. Detection was performed with the ABC-Elite peroxidase kit (Vector Laboratories) by using the DAB substrate kit (DakoCytomation).

For immunofluorescence staining, liver tissue was freshly isolated from euthanized animals and directly embedded in O.C.T. compound and frozen in cold 2-Methylbutane. Frozen sections were cut at 5 μm, blocked with 5% goat serum, labeled with primary antibodies (A6, CK19, OC2-2A6) at 1:200 dilution overnight at 4°C and secondary antibody Alexa Fluor®594 or Alexa Fluor®488 goat anti-rat IgG (Invitrogen, Carlsbad, CA) at 1:500 dilution for 30 min at room temperature. A6 antibody was kindly supplied by Dr. V. Factor (Laboratory of Experimental Carcinogenesis, NCI, NIH). OC2-A6 antibody was a generous gift from Dr. Markus Grompe (Oregon Health and Science University). CK19 antibody was purchased from Developmental Studies Hybridoma Bank (Iowa City, IA). For Brdu staining, two additional steps of 30-min incubation with 2N HCl at 37°C followed by a 10-min rinsing in 0.1 M borate acid buffer at room temperature were performed before incubation with primary antibody (anti-Brdu, 1:100, Labvision). The immunofluorescence signal was visualized by a immunofluorescence microscope after the sections were mounted with VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories, Inc). Antibody negative controls were performed by omitting the primary antibody from the protocol.

**Quantitative Real-time RT–PCR**

Sybergreen based quantitative real-time RT-PCR was performed using SYBR Green master mix (Life Technologies, Grand Island, NY) according to the manufacturer’s protocol. Primer sequences are the same as previous published [28].

**Malondialdehyde (MDA) Assay**

Formation of MDA in the mouse liver tissues were analyzed using the Thiobarbituric Acid Reactive Substances (TBARS) kit (Cayman Chemical Company, Ann Arbor, MI).

**Data Analysis**

The threshold of positive staining area related to positive staining nuclei by DAPI was measured by ImageJ software (http://rsb.info.nih.gov/ij/). Three randomly selected areas of each slide were analyzed at 40× magnification. Each experiment was repeated at least three times and data were expressed as means ± standard deviation (SD). Student’s t test was used to evaluate statistical significance. Values of P<0.05 were considered to be statistically significant.
Bmi1 in Hepatic Oval Cell and Hepatocarcinogenesis

Supporting Information

Figure S1 Representative immunohistochemistry of Bmi1 (A, C, E) and CK19 (B, D) in wild-type mouse livers treated with DDC. Note that both antibodies stain cells located in the liver periportal regions. As shown in (E), these cells tend to form pseudo-ductular structures (thin arrows) and are morphologically distinct from surrounding hepatocytes (thick arrows), exhibiting the prototypical features of oval cells (small and oval nuclei). (TIF)

Figure S2 Bmi1 is overexpressed in oval cells expanded liver. Relative expression of mBmi1 in Bmi1 WT livers without DDC treatment (n = 3) and with 3 weeks DDC treatment (n = 3). (TIF)

Figure S3 ROS activation assessed by MDA content was not elevated in Bmi1 KO mice (n = 3) livers compared with Bmi1 WT mice (n = 3) livers after 3 weeks DDC treatment. (TIF)

Figure S4 Ink4a/Arf encoded P16 and P19 are increased in oval cells expanded liver. Relative expression (Log2) of mP16 (A) and mP19 (B) in Bmi1 WT livers (n = 3) and Bmi1 KO livers (n = 3) after 3 weeks DDC treatment. (TIF)

Figure S5 Loss of Bmi1 does not affect hepatocytes proliferation. (A) Ki67 (upper) and Brdu (lower) staining of hepatocytes of 2-weeks-old Bmi1 WT and KO mouse liver. Representative images of three independent experiments are shown. (B) Quantification of Ki67 and Brdu staining. At least three random fields of each section were counted for quantification and a total of three batches of mice were analyzed. (TIF)

Acknowledgments

We would like to thank Sandra Huling of the UCSF Liver Center Morphology Core for histology support; Dr. Carla Kim for Bmi1+/− mice; Dr. Y. Factor and Dr. Markus Grome for antibodies used in the study.

Author Contributions

Conceived and designed the experiments: XC SH LF DC. Performed the experiments: LF CX CW CH LJ BG JT ME DC. Analyzed the data: LF CX SH XC ME DC. Contributed reagents/materials/analysis tools: SH CX. Wrote the paper: LF XC DC.

References

1. Laconi S, Carrelli F, Diana S, Pasciu D, De Filippo G, et al. (1999) Liver regeneration in response to partial hepatectomy in rats treated with retinovir: a kinetic study. J Hepatol 31: 1069–1074.
2. Evarts RP, Nagy P, Marden D, Thorogirsson SS (1987) A precursor-product relationship exists between oval cells and hepatocytes in rat liver. Carcinogenesis 8: 1737–1740.
3. Sc1 (2001) Heterogeneity and plasticity of hepatocyte lineage cells. Hepatology 33: 738–750.
4. Prieiogkhe KH, Factor VM, Fuchsiicher A, Stumpner C, Denk H, et al. (1999) Atypical ductular proliferation and its inhibition by transforming growth factor beta1 in the 3,3-dioxytocarbonyl-4,4-dihydrocollidine mouse model for chronic alcoholic liver disease. Lab Invest 79: 103–109.
5. Pritchard MF, Nagy LE (2010) Hepatic fibrosis is enhanced and accompanied by robust oval cell activation after chronic carbon tetrachloride administration to Egr-1-deficient mice. Am J Pathol 176: 2743–2752.
6. Zheng D, Oh SH, Jung Y, Petersen BE (2006) Oval cell response in 2-acetylamino-6-azauracil-induced hepatic regeneration rat. Hepatology 43: 262–269.
7. Roskams T (2006) Liver stem cells and their implication in hepatocellular and cholangiocarcinoma. Oncogene 25: 3818–3822.
8. Fujita VM, Enggfrardt NV, Iazova AK, Lazareva MN, Poltoranina VS, et al. (2005) Polycomb group protein Bmi1 in Heptic Oval Cell and Hepatocarcinogenesis. Biochim Biophys Acta 1782: 642–648.
9. Fan C, He L, Kapoor A, Gillis A, Rybak AP, et al. (2009) Bmi1 promotes prostate tumorigenesis via inhibiting p16(INK4A) and p14(ARF) expression. Biochim Biophys Acta 1782: 642–648.
10. Brunk BP, Martin EC, Adler PN (1994) Drosophila genes Posterior Sex Combs and Suppressor two of rose encode proteins with homology to the murine bmi-1 oncogene. Nature 353: 351–353.
11. van Lohuizen M, Frisch M, Wientoens E, Berns A (1991) Sequence similarity between the mammalian bmi-1 proto-oncogene and the Drosophila regulatory genes Psc and Su(z)2. Nature 353: 333–335.
12. van der Lugt NM, Donen J, Linders K, van Roon M, Robanus-Maandag E, et al. (1994) Posterior transformation, neurological abnormalities, and severe hematopoietic defects in mice with a targeted deletion of the bmi-1 proto-oncogene. Genes Dev 8: 757–769.
13. Park JM, Qian D, Kiel M, Becker MW, Pihalja M, et al. (2003) Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. Nature 423: 302–305.
14. Lessard J, Sauvageau G (2003) Bmi-1 determines the proliferative capacity of normal and leukemic stem cells. Nature 423: 205–209.
15. Molotkov AV, Pardal R, Iwashita T, Park IK, Clarke MF, et al. (2003) Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. Nature 425: 962–967.
16. Silva J, Garcia JM, Pena C, Garcia V, Dominguez G, et al. (2006) Implication of polycomb members Bmi-1, Mel-18, and Hpc2 in the regulation of p16INK4a, p14ARF, h-TERT, and c-Myc expression in primary breast carcinomas. Clin Cancer Res 12: 6929–6936.
17. Brunk RP, Martin EC, Adler PN (1994) Drosophila genes Posterior Sex Combs and Suppressor two of rose encode proteins with homology to the murine bmi-1 oncogene. Nature 353: 351–353.
18. van Lohuizen M, Frisch M, Wientoens E, Berns A (1991) Sequence similarity between the mammalian bmi-1 proto-oncogene and the Drosophila regulatory genes Psc and Su(z)2. Nature 353: 333–335.
19. van der Lugt NM, Donen J, Linders K, van Roon M, Robanus-Maandag E, et al. (1994) Posterior transformation, neurological abnormalities, and severe hematopoietic defects in mice with a targeted deletion of the bmi-1 proto-oncogene. Genes Dev 8: 757–769.
20. Park JM, Qian D, Kiel M, Becker MW, Pihalja M, et al. (2003) Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. Nature 423: 302–305.
21. Lessard J, Sauvageau G (2003) Bmi-1 determines the proliferative capacity of normal and leukemic stem cells. Nature 423: 205–209.
22. Molotkov AV, Pardal R, Iwashita T, Park IK, Clarke MF, et al. (2003) Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. Nature 425: 962–967.
23. Silva J, Garcia JM, Pena C, Garcia V, Dominguez G, et al. (2006) Implication of polycomb members Bmi-1, Mel-18, and Hpc2 in the regulation of p16INK4a, p14ARF, h-TERT, and c-Myc expression in primary breast carcinomas. Clin Cancer Res 12: 6929–6936.
24. Mikie-Probst B, Kuster A, Kilgus S, Bode-Lenziewiska B, Ingold-Heggen B, et al. (2007) Consistent expression of the stem cell renewal factor BMI-1 in primary and metastatic melanoma. Int J Cancer 121: 1764–1770.
25. Fan C, He L, Kapoor A, Gillis A, Rybak AP, et al. (2009) Bmi1 promotes prostate tumorigenesis via inhibiting p16(INK4A) and p14(ARF) expression. Biochim Biophys Acta 1782: 642–648.
26. Becker M, Korn C, Sierentz AR, Voswinckel R, Luekenhaus K, et al. (2009) Polycomb group protein Bmi1 is required for growth of RAF driven non-small-cell lung cancer. PLoS One 4: e4230.
27. Rouaulten S, Haghayi J, Altermatt HJ, Guiguer M, Kappeler A, et al. (2001) The bmi-1 oncogene is differentially expressed in non-small cell lung cancer and correlates with INK4A-ARF locus expression. Br J Cancer 84: 1372–1376.
28. Xu CR, Lee S, Ho C, Bunnii P, Huang SA, et al. (2009) Bmi1 functions as an oncogene independent of Ink4a/Arf repression in hepatic carcinogenesis. Mol Cancer Res 7: 1937–1945.
29. Chibata T, Miyagi S, Saraya A, Aoki R, Seki A, et al. (2008) The polycomb gene product BMI1 contributes to the maintenance of tumor-initiating side population cells in hepatic cell carcinoma. Cancer Res 68: 7742–7749.
30. Brucken AP, Kleine-Kohlbrecher D, Dietrich N, Pasini D, Gargiulo G, et al. (2007) The Polycomb group proteins bind throughout the INK4A-ARF locus expression. Br J Cancer 84: 1372–1376.
31. Jacobs J, Kierboum K, Marino S, DePinho RA, van Lohuizen M (1999) The oncoproteins and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. Nature 397: 164–168.
32. Molotkov AV, He S, Bylon M, Morrison SJ, Pardal R (2003) Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and differentiation. Cell 115: 93–105.

PLOS ONE | www.plosone.org 11 September 2012 | Volume 7 | Issue 9 | e46472
Bmi1 in Hepatic Oval Cell and Hepatocarcinogenesis

Liu J, Cao L, Chen J, Song S, Lee H, et al. (2009) Bmi1 regulates mitochondrial function and the DNA damage response pathway. Nature 459: 367–372.

Carlson CM, Frandsen JL, Kirchhof N, Mclvor RS, Largaespada DA (2005) Somatic integration of an oncogene-harboring Sleeping Beauty transposon models liver tumor development in the mouse. Proc Natl Acad Sci U S A 102: 17059–17064.

Calvisi DF, Wang C, Ho C, Ladu S, Lee S, et al. Increased lipogenesis, induced by AKT-mTORC1-RPS6 signaling, promotes development of human hepatocellular carcinoma. Gastroenterology 140: 1071–1083.

Lee S, Ho C, Roy R, Kosinski C, Patil MA, et al. (2008) Integration of genomic analysis and in vivo transfection to identify sprouty 2 as a candidate tumor suppressor in liver cancer. Hepatology 47: 1200–1210.

Wang X, Foster M, Al-Dhalimi M, Lagasse E, Finegold M, et al. (2003) The origin and liver repopulating capacity of murine oval cells. Proc Natl Acad Sci U S A 100 Suppl 1: 11801–11809.

Dovey JS, Zacharek SJ, Kim CF, Lees JA (2008) Bmi1 is critical for lung tumorigenesis and bronchioalveolar stem cell expansion. Proc Natl Acad Sci U S A 105: 11857–11862.

Michael LE, Westerman BA, Ermilov AN, Wang A, Ferris J, et al. (2008) Bmi1 is required for Hedgehog pathway-driven medulloblastoma expansion. Neoplasia 10: 1343–1349, 1343a following 1349.

Schuriging J, Vellenga E. (2010) Role of the polycomb group gene BMI1 in normal and leukemic hematopoietic stem and progenitor cells. Curr Opin Hematol 17: 294–299.

Smith KS, Chanda SK, Lingbeek M, Ross DT, Boitstein D, et al. (2005) Bmi-1 regulation of INK4A-ARF is a downstream requirement for transformation of hematopoietic progenitors by E2a-Pbx1. Mol Cell 12: 393–400.

Lessard J, Schumacher A, Thorsteinsson U, van Lohuizen M, Magnuson T, et al. (1999) Functional antagonism of the Polycomb-Group genes eed and Bmi1 in hematopoietic cell proliferation. Genes Dev 13: 2691–2703.

Lee KP, Lee JH, Kim TS, Kim TH, Park HJ, et al. (2010) The Hippo-Salvador pathway restrains hepatic oval cell proliferation, liver size, and liver tumorigenesis. Proc Natl Acad Sci U S A 107: 8248–8253.

Arranz L, Herrera-Merchan A, Lagos JM, de Molina A, Dominguez O, et al. (2012) Bmi1 is critical to prevent Ikaros-mediated lymphoid priming in hematopoietic stem cells. Cell Cycle 11: 65–78.

Ji J, Yamashita T, Budhu A, Forgues M, Jia HL, et al. (2009) Identification of microRNA-181b by genome-wide screening as a critical player in EpCAM-positive hepatic cancer stem cells. Hepatology 50: 472–480.

Yamashita T, Ji J, Budhu A, Forgues M, Yang W, et al. (2009) EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. Gastroenterology 136: 1012–1024.

Yang W, Yan JX, Chen L, Liu Q, He YQ, et al. (2008) Wnt/beta-catenin signaling contributes to activation of normal and tumorigenic liver progenitor cells. Cancer Res 68: 4287–4295.

Benhamouche S, Curto M, Saotome I, Glaudin AB, Liu CH, et al. (2010) NF2/Merlin controls progenitor homeostasis and tumorigenesis in the liver. Genes Dev 24: 1716–1730.

Chiba T, Seki A, Aoki R, Ichikawa H, Negishi M, et al. (2010) Bmi1 promotes hepatic stem cell expansion and tumorigenicity in both Ink4a/Arf-dependent and -independent manners in mice. Hepatology 52: 1111–1123.