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BMI1 As a Novel Target for Drug Discovery in Cancer

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ABSTRACT

Growing evidence has demonstrated that clonogenic cancer stem (initiating) cells are responsible for tumor regrowth and disease relapse. Bmi-1 plays a critical role in the self-renewal of adult stem cells. The Bmi-1 protein is elevated in many types of cancers, and experimental reduction of Bmi-1 protein levels by small interfering RNA (siRNA) causes apoptosis and/or senescence in tumor cells in vitro and increases susceptibility to cytotoxic agents. The Bmi-1 protein has no known enzymatic activity, but serves as the key regulatory component of the PRC1 complex (polycomb repressive complex-1). This complex influences chromatin structure and regulates transcriptional activity of a number of important loci including the Ink4a locus which encodes the tumor suppressor proteins $p16^{Ink4a}$ and $p14^{Arf}$. In this prospective study, we will discuss the implication of BMI1 in cancers, the biology of BMI1, and the regulatory control of BMI1 expression. The target validation and the future prospects of targeting BMI1 in cancer therapy are also discussed. J. Cell. Biochem. 112: 2729–2741, 2011.

KEY WORDS: BMI1; PLOLYCOMB REPRESSIVE PROTEIN; CANCER STEM CELLS; POST-TRANSCRIPTIONAL CONTROL; CANCER THERAPEUTIC TARGET

Current cancer therapies designed to kill proliferating cells can effectively reduce tumor mass, but the disease often relapses [Dean, 2009]. Growing evidence supports the existence of cancer stem cells (also known as tumor initiating cells) within many tumor types [Glinsky, 2007; Hubbard et al., 2009; Li et al., 2009; Gao et al., 2010; Tabor et al., 2011]. While debate continues as to the precise identity and function of cancer stem cells, there is general agreement that there is a subpopulation of cells within many tumors that are more resistant to conventional chemo- and radiation therapies [Dean, 2009]. Such cells are believed to persist in tumors as a distinct population and cause relapse and metastasis by giving rise to new tumors. Currently, there is no drug on the market that specifically targets this cell fraction. Therefore, discovery and development of such drugs holds great promise for cancer treatment since complete remission, or perhaps cure, will require targeting the cancer stem cell fraction.

BMI1 is a stem cell gene as defined by the fact that its deficiency leads to compromised adult stem cell function [Park et al., 2003]. It was first identified in the early 1990s as a component of a key insertion/activation region of the Moloney murine leukemia virus [Alkema et al., 1993]. BMI1 encodes a 37 kDa polycomb group protein (PcG) from chromosome 10p11.23 in humans and 2 A3 in mice. The BMI1 protein contains a conserved RING finger domain in its N-terminal end and a central helix-turn-helix motif (H-T-H) [Li et al., 2006]. BMI1 itself has no known enzymatic activity, but serves as the key regulatory component of the PRC1 complex (polycomb regulatory complex-1) [Cao et al., 2005]. This protein complex modulates chromatin structure and thereby regulates the transcription of a number of important genes, including the Ink4a locus which encodes two important tumor suppressor proteins $p16^{Ink4a}$ and $p14^{Arf}$ [Guney et al., 2006; Silva et al., 2006]. There is increasing evidence that deregulated expression of PcG proteins contribute to

Abbreviations used: BMI1, B cell Moloney murine leukemia virus insertion region; PRC, polycomb repressive complex: PcG, polycomb group proteins; HSC, hematopoietic stem cells; EMT, epithelial–mesenchymal transition; PTC, post-transcriptional control; UTR, untranslated region; AUE, adenine and uridine rich element in mRNA; GEMS, gene expression modulation by small molecules; H2A, histone 2A; H-RAS: Harvey rat sarcoma virus oncogene; BCR-ABL, breaking point cluster and c-abl oncogene fusion protein; PH1/2, polyhomeotic protein 1/2; CBX, chromobox homologs; HOX, homeobox genes; $P16^{Ink4a}$, Ink4a locus encoded tumor suppressor p16; $P14^{Arf}$, Ink4a encoded alternative reading frame; VEGFR, endothelial cell growth factor receptor; PTEN, phosphatase and tensin homolog deleted on chromsome 10.

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cancer development. Aberrant overexpression of PcG proteins, in particular BMI1, is associated with a number of human malignancies [Jiang et al., 2009]. Importantly, BMI1 expression is thought to promote the stem state in tumor cells and overexpression of BMI1 correlates with therapy failure in many tumor types including those in breast, prostate, lung, and ovarian cancer patients [Glinsky, 2007; Vrzalikova et al., 2008; Wang et al., 2008; Li et al., 2010]. Additionally, experimental reduction of BMI1 protein levels results in apoptosis and/or senescence in tumor cells and increases susceptibility to cytotoxic agents and radiation therapy [Wu et al., 2011b]. In this review, we will discuss the evidence of BMI1 in cancer development, the mechanisms underlying BMI1 functions, the regulation of BMI1 expression and potential approaches that could be used to target BMI1 for cancer therapy.

EXPERIMENTAL AND CLINICAL EVIDENCE FOR A BMI1 ROLE IN CANCER DEVELOPMENT

It is well known that BMI1 is an oncogene able to induce cell transformation and promote tumor growth in vitro and in animal model systems [Jiang et al., 2009]. For example, it has been shown that, in conjunction with c-myc, BMI1 can act as an oncogene that is particularly potent for the initiation of lymphoma in murine models [Haupt et al., 1993]. This role in lymphomagenesis has been attributed, in part, to transcriptional repression of the INK4A locus (containing both the $p16^{INK4A}$ and $p14^{ARF}$ genes), leading to maintenance of proliferation and prevention of differentiation [Jacobs et al., 1999b; Itahana et al., 2003]. Loss of expression of the INK4A locus due to promoter silencing has been heavily studied and is important for both the progression and prognosis of many hematologic cancers [Lessard and Sauvageau, 2003; Hosen et al., 2007]. Although the INK4A locus is occasionally lost by deletion, BMI1 has nevertheless been shown to play a role in tumorigenesis in INK4A-deficient models [Bruggeman et al., 2007], indicating that other loci important in cancer may be regulated by this protein.

Interestingly, some evidence shows that overexpression of BMI1 alone may not be sufficient to cause oncogenic transformation. Hoenerhoff et al. [2009] demonstrated that increased expression of BMI1 in the immortalized breast MCF10A cell line did not result in oncogenic transformation. However, BMI1 co-overexpression with activated H-Ras (RasG12V) resulted in efficient transformation of MCF10A cells in vitro. BMI1 collaborates with H-RAS to promote increased proliferation, invasion, and resistance to apoptosis in vitro, and an increased rate of spontaneous metastases from mammary fat pad xenografts including novel metastases to the brain [Hoenerhoff et al., 2009]. The induced transformation of MCF10A cells by BMI1 and H-Ras is through dysregulation of multiple growth pathways independent of the INK4A/ARF locus. In contrast to overexpression of BMI1, reduction of BMI1 levels in several established breast cancer cell lines leads to decreased oncogenic behavior in vitro and in vivo [Guo et al., 2011]. This observation suggests that, although BMI1 alone is not sufficient for tumor transformation, BMI1 is essential for tumor progression and cell survival and that targeting BMI1 expression could be useful for treatment of breast cancer. More recently, Rizo et al. [2010] found that BMI1 collaborates with BCR-ABL in inducing a fatal leukemia in non-obese diabetic/severe combined immunodeficiency mice transplanted with transduced human CD34+ cells. The leukemias were transplantable into secondary recipients with a shortened latency. Clonal analysis revealed that similar clones initiated leukemia in primary and secondary mice. In vivo, transformation was biased toward a lymphoid blast crisis, and in vitro, myeloid as well as lymphoid long-term, self-renewing cultures could be established. Retroviral introduction of BMI1 in primary chronic-phase CD34+ cells from CML patients elevated their proliferative capacity and self-renewal properties. Furthermore, BMI1 has also been demonstrated to cooperate with human virus, such as papillomavirus type 16 E6, to immortalize normal human keratinocytes [Kim et al., 2007]. In normal human oral keratinocytes (NHOK), exogenous BMI1 expression alone significantly extended the replicative life span without causing cellular immortalization. Immortalization of NHOK occurs only in combination with human papillomavirus type 16 E6 (HPV-16 E6). During immortalization of NHOK by sequential expression of exogenous BMI1 and E6, telomerase activation was observed after the cells had overcome crisis.

Clinically, a growing list of human malignancies have been shown to express high levels of BMI1 including lymphoma, acute myeloid leukemia, colorectal carcinoma, liver carcinoma, non-small cell lung cancer, breast carcinoma, prostate cancer, head and neck cancer, medulloblastoma, and glioblastoma [Hosen et al., 2007; Merkerova et al., 2007; Vrzalikova et al., 2008; Li et al., 2009; Honig et al., 2010; Guo et al., 2011; Tabor et al., 2011]. Importantly, the elevated of levels of BMI1 have been shown to have prognostic relevance in a number of tumor types. For example, Chowdhury et al. [2007] assessed the prognostic value of high BMI1 protein expression in 64 acute myeloid leukemia patients. They divided the patients into two groups on the basis of median BMI1 protein expression in circulating tumor cells and analyzed survival. Patients with lower BMI1 ($<55\%$, $n=33$) had significantly longer overall survival ($P=0.0001$), relapse-free survival ($P=0.0072$), and remission duration ($P=0.0065$) when compared to the patients with higher BMI1 ($>55\%$, $n=31$), regardless of age group. Similarly, van Galen et al. [2007] showed that BMI1 expression is highly prognostic in diffuse large B cell lymphomas (DLBCL), a disease in which neoplastic cells originate from germinal center B (GCB) cells or their descendents. Other groups have linked elevated BMI1 expression with poor prognosis in other leukemias, as well as neuroblastomas, glioblastomas, hepatocellular carcinomas, and breast, colorectal, prostate, lung, gastric, and salivary gland cancers [Glinsky, 2007; Vrzalikova et al., 2008; Wang et al., 2008; Li et al., 2010; Song et al., 2010]. Collectively, these data implicate BMI1 in cancer and suggest that reducing BMI1 protein levels may have a beneficial effect in multiple types of cancers.

BMI1 AND CANCER STEM CELLS

Ample evidence exists linking BMI1 expression levels to self-renewal and maintenance of both normal and cancer stem cells [Bruggeman et al., 2007a; Chiba et al., 2010; Yu et al., 2011]. The

study of BMI1 knockout mice revealed that BMI1 is indispensable for the self-renewal of normal hematopoietic stem cells (HSC) [Park et al., 2003]. The number of HSCs in the fetal liver of $BMI1^{-/-}$ mice was normal. In postnatal $Bmi1^{-/-}$ mice, the number of HSCs was markedly reduced. Transplanted fetal liver and bone marrow cells obtained from $Bmi1^{-/-}$ mice were able to contribute only transiently to hematopoiesis. There was no detectable self-renewal of adult HSCs, indicating a cell autonomous defect in $Bmi1^{-/-}$ mice. More recently, the Schuringa laboratory demonstrated that BMI1 expression is required for maintenance and self-renewal of not only normal but also leukemic stem and progenitor cells [Schuringa and Vellenga, 2010]. In an assessment of acute myeloid leukemia stem cell (LSC) populations, van Gosilga et al. [2007] showed that $CD34^{+}/CD38^{-}$ cells capable of forming leukemic-cobblestone colonies on a bone marrow substrate through at least two rounds of expansions. This cell population expressed high levels of BMI1 mRNA and was capable of establishing an aggressive leukemia in mice, while those cells that had lower levels of BMI1 could not. Further studies have demonstrated that BMI1 also regulates self-renewal, proliferation, and senescence of cancer stem cells from other type of tumors including breast cancer, glioblastoma, and hepatocellular carcinoma [Chiba et al., 2010; Yu et al., 2011]. For instance, Chiba et al. [2010] demonstrated the polycomb gene product BMI1 contributes to the maintenance of tumor-initiating side population cells in hepatocellular carcinoma, while Abdouh et al. [2009] showed BMI1 protein is involved in GBM tumor growth and is required to sustain cancer initiating stem cell renewal. More interestingly, Yang et al. [2010] recently demonstrated that Bmi1 is essential in Twist1-induced epithelial–mesenchymal transition (EMT), a key developmental program often activated during cancer invasion and metastasis [Singh and Settleman, 2010]. Induction of EMT results in the acquisition of mesenchymal traits and in the expression of stem cell markers in epithelial cells in vitro. In patients with head and neck cancers, increased levels of both Twist1 and BMI1 correlated with downregulation of E-cadherin and $p16^{Ink4a}$, and this expression pattern was associated with the poorest prognosis. Twist1-induced EMT and tumor-initiating capability in cancer cells occurs through BMI1 mediated-chromatin remodeling, which leads to unfavorable clinical outcomes. Collectively, these studies implicate BMI1 in tumor growth and cell survival and suggest a critical function in tumor initiation or maintenance of tumor stem cells.

THE FUNCTION OF BMI1 AND THE UNDERLYING MECHANISMS

The BMI1 protein has no known enzymatic activity, but serves as the key regulatory component of the PRC1 complex, which is required to maintain the transcriptionally repressive state of many genes throughout development via chromatin remodeling and modification of histones [Simon and Kingston, 2009]. In addition to BMI1, PRC1 consists of several other subunits, including RING1A/B, PH1, PH2, and CBX2, 4, 6, 7, 8 [Kerppola, 2009]. The composition of PRC1 complexes can differ among specific cell types. Little is known of how this is regulated and how the composition of PRC1 complexes impact silencing of specific subsets of genes within a specific cell type. In HSCs, BMI1 expression decreases upon cell differentiation and maturation [Schuringa and Vellenga, 2010]. In contrast, other PRC1 components, including MEL18, CBX, and PH2 are expressed at rather low levels in HSCs, but increase upon differentiation [Park et al., 2003]. This suggested that not all PRC1 subunits are required for appropriate chromatin remodeling by PRC1 or that the components of the complex may impart differential activity/selectivity. Recently, it has been suggested that the presence of BMI1 and MEL18, as well as CBX7 and CBX8, in the PRC1 complex is mutually exclusive [Kajiume et al., 2009; Maertens et al., 2009]. To date, no information has been published on the functional consequences of these differences in PRC1 composition.

PRC1, via the chromodomain of the CBX proteins, recognizes the H3K27 trimethylation mark (resulting from PRC2 initiation) and leads to chromatin association. In genome-wide screens in human embryonic fibroblasts and murine embryonic stem cells, PRC1 co-occupied more than 1,000 genes with a strong bias for embryonic development and cell fate decisions [Bracken et al., 2006]. Target genes included many homeotic genes found in the HOX (homeobox) clusters and most members of the distal-less homebox (DLX), iroquois homeobox (IRX), and paired box (PAX) gene families, which regulate early developmental steps in neurogenesis, hematopoiesis, tissue patterning, and cell fate specification. Less is known about PcG targets in human adult cells, but one of the key targets regulated is the Ink4a locus that encodes the tumor suppressor proteins $p16^{Ink4a}$ and $p14^{Arf}$ [Jacobs et al., 1999a]. The repression of $p16^{Ink4a}$ and $p14^{Arf}$ is required to bypass senescence of embryonic fibroblasts. It was believed that PRC1 suppresses the target gene expression through mono-ubiquitination of histone 2A via RING1B E3 ligase activity [Cao et al., 2005]. RING1B alone has very little E3 liagase activity, but when BMI1 is associated within the PRC1 complex the RING1B ligase activity is dramatically enhanced. It was also shown that PRC1 suppresses gene expression through methylation of CpG islands via association with methyltransferase 1 (DNMT1) at promoter regions [Hernandez-Munoz et al., 2005]. Agherbi et al. [2009b] demonstrated that BMI1 in young proliferating embryonic fibroblasts was recruited to the regulatory domain (RD) region of Ink4a/Arf through CDC6 to regulate this locus. More recently, Meng et al. [2010] demonstrated that BMI1 regulates the expression of p16 in HeLa cells by binding directly to the BMI1-responding element (BRE) within the p16 promoter. The BRE resided at bp −821 to −732 upstream of the p16 initiator ATG codon in the human genome, while the RD region resides in a ~35 kb upstream translation start site of p16. This difference may reflect different cell systems were used by these two groups. It is also possible that BMI1 may regulate Ink4a/Arf via binding to both the BRE and RD regions, a possibility that needs to be further investigated.

BMI1 has also been shown to play a role in tumorigenesis in Ink4a-deficient models [Bruggeman et al., 2007], indicating that other loci or pathways (Fig. 1) important in cancer are regulated by this protein. Our own data (see below and Fig. 3) confirm that loss of the BMI1 protein induces growth arrest and senescence in HT1080 fibrosarcoma cells that are lacking Ink4a [Kuerbitz et al., 1999]. It has been reported that BMI1 regulates expression of other genes, such as that of the Hox gene and tumor suppressors PTEN and

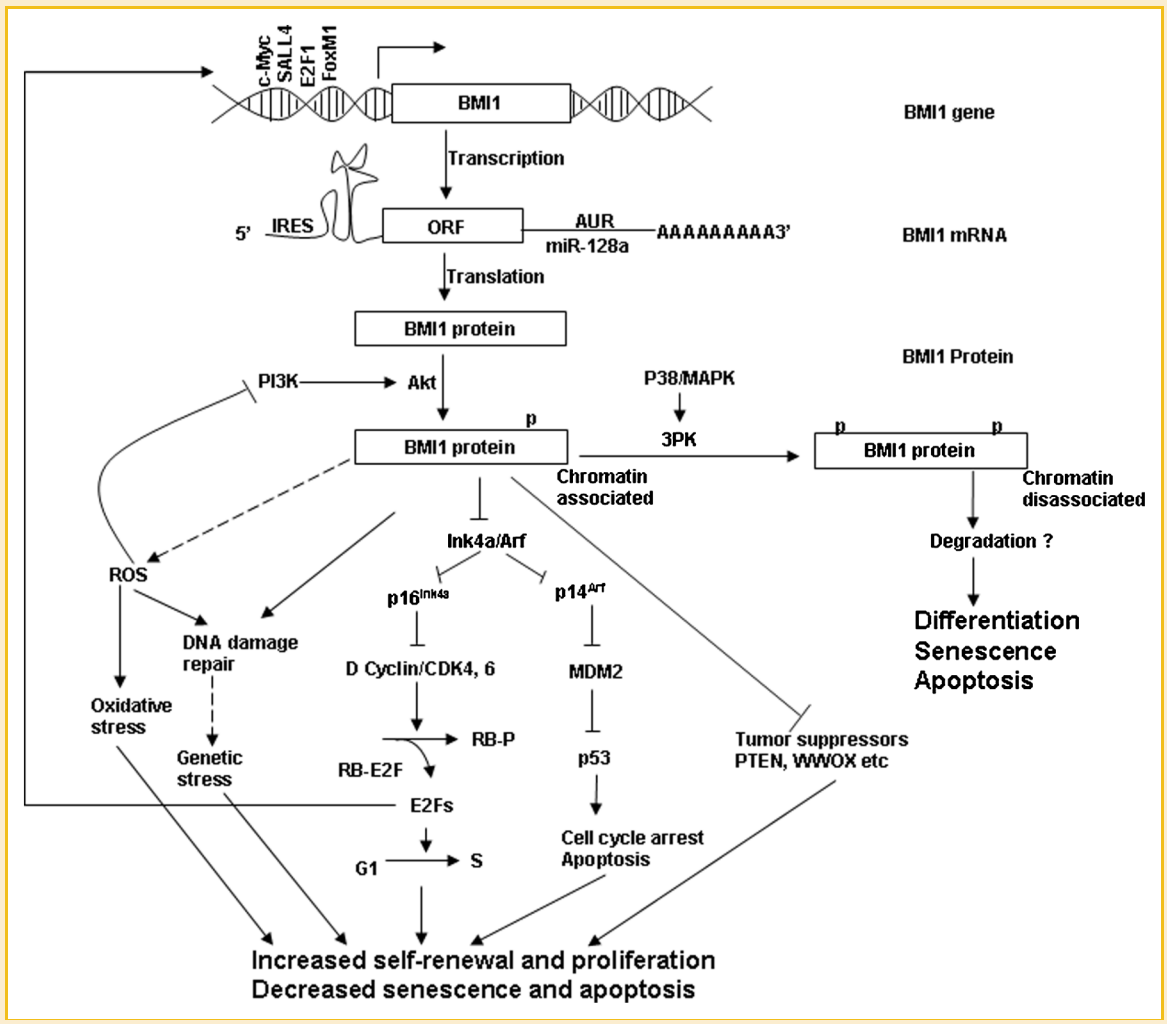

Fig. 1. Regulation of BMI1 expression and downstream signaling pathways. Details see the discussion in the text.

WWOX [Bracken et al., 2006; Fan et al., 2009; Kimura et al., 2011]. Knockdown of BMI1 caused a global and loci-specific loss of H2A ubiquitination, upregulation of the HoxC5 gene, and slowed cell growth [van der Lugt et al., 1996]. Another study demonstrated that E2F6 and BMI1 cooperate in the regulation of Hox gene expression (particularly Hox C10 and B9), and consequently affect axial skeleton development, but not repression of the Ink4a-Arf locus [Courel et al., 2008]. Trimarchi et al. [2001] reported that the transcription factor E2F6 is actually a component of the mammalian BMI1-containing polycomb complex. Their results suggested that the biological properties of E2F6 are mediated through its ability to recruit the polycomb transcriptional repressor complex. These findings underscore the significance of the E2F6–BMI1 interaction and suggest that the Hox and Ink4a-Arf loci are regulated by somewhat different, though BMI1-dependent mechanisms.

It was recently demonstrated that Twist1 and BMI1 were mutually essential to promote EMT and tumor initiating capability [Yang et al., 2010]. PTEN may play a critical role in BMI1-mediated EMT. Song et al. [2009] found that upregulation of BMI1-induced EMT and enhanced the motility and invasiveness of human nasopharyngeal epithelial cells, whereas silencing endogenous BMI1 expression reversed EMT and reduced motility. They demonstrated that BMI1 transcriptionally downregulated expression of the tumor suppressor PTEN in tumor cells through direct association with the PTEN locus. This in vitro analysis was consistent with the statistical inverse correlation detected between BMI1 and PTEN expression in a cohort of human nasopharyngeal carcinoma biopsies. Moreover, ablation of PTEN expression partially rescued the migratory/invasive phenotype of BMI silenced cells, indicating that PTEN might be a major mediator of BMI1-induced EMT. More recently, another tumor suppressor, WWOX (WW domain-containing oxidoreductase), was identified as a BMI1 target in lung cancer cells by chromatin immunoprecipitation and quantitative real-time PCR [Kimura et al., 2011]. The underlying mechanism for suppressing WWOX by BMI1 has yet to be elucidated.

There is also evidence that BMI1 is important for the hedgehog (Hh) pathway in breast cancer [Liu et al., 2006b]. Additional genes

regulated by BMI1 remain to be identified, but the available data suggest that BMI1 plays key roles in various cell types and developmental stages.

BMI1 function is not limited to the silencing of tumor suppressor genes. BMI1 also functions in protection against oxidative and DNA damage stresses. Downregulation of BMI1 with BMI1 RNAi vector transduction resulted in an accumulation of ROS levels both in knockout mouse models and in human cells, while the enhanced expression of BMI1 also protect LSC from oxidative stress [Dong et al., 2011]. Schuringa's group demonstrated that down-modulation of BMI1 in cord blood CD34+ cells impaired long-term expansion and progenitor-forming capacity, both in cytokine-driven liquid cultures as well as in bone marrow stromal cocultures [Rizo et al., 2009]. The long-term culture-initiating cell frequencies were dramatically decreased upon knockdown of BMI1, indicating an impaired maintenance of stem and progenitor cells. The reduced progenitor and stem cell frequencies were associated with increased expression of $p14^{Arf}$ and $p16^{Ink4a}$ and increased levels of intracellular reactive oxygen species (ROS). In AML CD34+ cells, down-modulation of BMI1 also impaired long-term expansion and self-renewal capacity, as determined by the loss of replating capacity of the cultures. These phenotypes were also associated with the increased intracellular ROS.

In non-hematopoietic model systems, it was also shown that downregulation of BMI1 results in p53-mediated apoptosis and increased ROS levels in cells [Chatoo et al., 2009]. The induction of ROS via the reduction of BMI1 could be counteracted by treatment with antioxidants such as *N*-acetylcysteine (NAC). Venkataraman et al. [2010] demonstrated that reducing BMI1 expression by microRNA (miR) 128a increases intracellular ROS levels in medulloblastoma cells. They examined the expression of miR in medulloblastoma and then investigated the functional role of miR-128a in regulating medulloblastoma cell growth. miR-128a inhibits the growth of medulloblastoma cells by reducing the BMI1 expression, which resulted in changes of the intracellular redox state of the tumor cells, thus promoting cellular senescence. The underlying molecular mechanism of increased ROS is thought to be through reducing p53 levels via BMI1-mediated repression of the Ink4a/Arf locus and/or via modulation of the oxidative stress response by impairing mitochondrial functions. The understanding of this function of BMI1 is still evolving.

In ATM-deficient astrocytes, oxidative stress resulted in an increase in ROS levels, which inhibited cell growth via a MEK–ERK1–BMI1–p16-dependent pathway [Kim and Wong, 2009b]. In the absence of BMI1, ROS accumulate and cause activation of DNA damage response pathways. In $Bmi1^{-/-}$ mice, the increase in ROS coincided with an increase in DNA damage and an activation of the DNA damage repair pathways [Liu et al., 2009]. A number of genes that regulate intracellular redox homeostasis were found to be de-repressed in $Bmi1^{-/-}$ mice. Thus, increased BMI1 might protect cancer cells from apoptosis induced by oxidative stress conditions.

The mechanism underlying BMI1 promotion of DNA damage repair was investigated by Ismail et al. [2010], who found that BMI1 and RING1B are recruited to the sites of DNA double-strand breaks (DSBs) where they contribute to the ubiquitination of gamma-H2AX. In the absence of BMI1, the recruitment of several proteins, such as 53 BP1 and BRCA1, to DSBs is impaired. Loss of BMI1 protein sensitizes cells to ionizing radiation to the same extent as loss of ring finger protein 8 (RNF8). The simultaneous depletion of both proteins revealed an additive increase in radiation sensitivity. Facchino et al. [2010] demonstrated that the CD133+ cancer initiating neural stem cell (NSC) population from glioblastoma multiforme (GBM) was resistant to gamma radiation through preferential activation of the DSB response machinery, including the ataxia-telangiectasia-mutated (ATM) kinase. BMI1 co-purifies with DNA DSB response and non-homologous end joining (NHEJ) repair proteins in GBM cells. BMI1 is enriched at chromatin after irradiation and co-localized and co-purified with ATM and the histone gamma H2AX. BMI1 deficiency in GBM cells severely impaired DNA DSB response, resulting in increased sensitivity to radiation. In turn, BMI1 overexpression in normal NSCs enhanced ATM recruitment to the chromatin, the rate of gamma H2AX foci resolution, and resistance to radiation. More recently, Ginjala et al. [2011] reported that BMI1 is rapidly recruited to sites of DNA damage and the sustained localization of BMI1 to damage sites is dependent on intact ATM and ATR and requires H2AX phosphorylation and recruitment of RNF8. Loss of BMI1 leads to impaired repair of DNA DSBs by homologous recombination and accumulation of cells in G_2/M. Moreover, BMI1 may regulate DNA replication timing at the Ink4a/Arf locus. Agherbi et al. [2009] identified the replication licensing factor CDC6 as a new partner of the polycomb group member BMI1. Their results suggest that, in young cells, polycomb proteins are recruited to the Ink4a/Arf locus through CDC6 and the resulting silent locus is replicated during late S-phase. Together, these results suggest a unified model that integrates replication, transcription, and epigenetic regulation at the Ink4a/Arf locus.

REGULATION OF BMI1 EXPRESSION

Since BMI1 plays key roles in the development of cancers, modulation of BMI1 expression has attracted attention recently as a potential approach to cancer therapy. As outlined in Figure 1, regulation of BMI1 expression and function is controlled at multiple levels, including gene amplification, transcriptional, post-transcriptional, and post-translational levels.

GENE AMPLIFICATION

Gene amplification is one of the mechanisms for oncogene activation in cancer, and amplification and/or overexpression of oncogenes are commonly linked to tumor progression and poor clinical prognosis. BMI1 gene amplification was originally identified in mantle cell lymphomas (MCL) [Bea et al., 2001]. The authors in this study examined 160 lymphoproliferative disorders, 13 myeloid leukemias, and 89 carcinomas by Southern blot analysis and detected BMI1 gene amplification (3- to 7-fold) in 4 of 36 (11%) MCLs with no alterations in the Ink4a/Arf locus. The four tumors with gene amplification showed significantly higher mRNA levels than other MCLs and NHLs with the BMI1 gene in germline configuration. A good correlation between BMI1 mRNA and protein

levels was observed in all types of lymphomas. Increased gene copy number of BMI1 has also been found in primary and recurrent human glioma. Hayry et al. [2008] investigated the genetic status and the corresponding expression patterns of BMI1 in a series of 100 low- and high-grade primary and recurrent gliomas. Chromogenic in situ hybridization (CISH) with probes directed against BMI1 at 10p13 and the centromere of chromosome 10 was used in the analyses. Increased copy numbers of the BMI1 locus (3–5 copies) were found in all histological types, especially in high-grade astrocytomas. However, no difference in prognosis between cases with normal copy numbers and cases with increased copy numbers could be observed. It is worth noting that the authors in this study did not determine the overall expression levels of BMI1 protein in those patient samples. Nevertheless, these findings suggest that BMI1 gene amplification in human neoplasm is uncommon, but they may contribute to the pathogenesis in a subset of malignant cancer, particularly of mantle cell type.

TRANSCRIPTIONAL REGULATION

BMI1 is transcriptionally regulated by a number of factors, including SALL4, FoxM1, c-Myc, E2F-1, Mel18, and HDAC [Nowak et al., 2006; Guo et al., 2007; Yang et al., 2007; Li et al., 2008; Jung et al., 2010]. Like BMI1, SALL4 is a putative oncogene that appears to modulate stem cell pluripotency and play a role in leukemogenesis. Murine Sall4 plays an essential role in maintaining the properties of ES cells and in governing the fate of the primitive inner cell mass. Transcription from the BMI1 promoter is markedly activated by SALL4 in a dose-dependent manner [Yang et al., 2007]. The Forkhead box transcription factor FoxM1 is expressed in proliferating cells and has been shown to upregulate BMI1 expression in transformed NIH 3T3 cells in response to oxidative stress through c-myc activation [Li et al., 2008]. The BMI1 homolog, Mel18, is a potent repressor of BMI1 expression. The BMI1 promoter region contains a functional E-box through which c-Myc and Mel-18 can regulate BMI1 expression. Because Mel-18 down-regulates c-Myc expression and BMI1 is a c-Myc target, these data suggest that Mel-18 regulates expression of BMI1 via repression of c-Myc during cellular senescence and thus links c-Myc and polycomb function [Guo et al., 2007]. Similarly, a recent report suggests that E2F-1 may also regulate BMI1 expression in neuroblastoma [Nowak et al., 2006]. The BMI1 promoter contains a putative E2F-binding site required for the activation of a BMI1 promoter-dependent reporter construct by E2F-1. HDACs are also reported to regulate BMI1 expression [Jung et al., 2010]. It is believed that HDACs modulate RB phosphorylation status and hence regulate binding to the E2F-binding site on the c-MYC P2 promoter. In turn, c-MYC is thought to bind to the BMI1 promoter and regulate expression of BMI1.

POST-TRANSCRIPTIONAL REGULATION OF BMI1 EXPRESSION

It has become increasingly understood over the last decade that appropriate expression of many genes requires extensive post-transcriptional control [Peltz et al., 2009]. The main steps in post-transcriptional regulation are: RNA processing and maturation, RNA transport/subcellular localization; mRNA translation; and mRNA degradation. The largest number of known post-transcriptional control determinants map to the mRNA 5′- and 3′-untranslated regions (UTRs). Post-transcriptional regulation occurs through interaction of cellular factors with sequence elements including secondary structures, protein-binding sites, upstream open reading frames (uORFs), internal ribosome entry sites (IRESs), and the poly(A) tail, which are all located within the 5′- and 3′-UTRs of an mRNA. mRNA UTRs and UTR-dependent processes comprise very attractive and novel targets for the therapeutic modulation of target gene expression across disease areas.

A previous study suggested that BMI1 expression is enhanced through post-transcriptional regulation during the progression of chronic myeloid leukemia [Bhattacharyya et al., 2009]. For these experiments, the expression of BMI1 in CD34+ cells at each of the chronic phase (CP), the accelerated phase (AP), and blastic crisis (BC) was assessed by flow cytometry. Interestingly, the level of BMI1 expression was significantly higher in CP than in controls and was further increased during the course of the disease progression. However, mRNA levels for BMI1 were almost constant during the disease progression from CP to BC. Further, overexpression of BCR-ABL in human embryonic kidney, 293 cells enhanced BMI1 expression, and BMI1 expression was increased in K562 cells derived from patients with BC in the presence of proteasomal inhibitors. BMI1 was presumed to be positively regulated by BCR-ABL and further by post-transcriptional modification in the course of disease progression.

The 3′-UTR of the BMI1 mRNA is long (1,766 nucleotides) and contains two polyadenylation (poly A) signals and multiple adenosine and uridine rich (AUR) elements. These elements have been demonstrated to regulate the stability of the mRNA due to regulatory factor binding. The BMI1 5′-UTR is considerably longer (506 nucleotides) than those of most eukaryotic mRNAs and contains several unique features, including: (1) 67% GC content, indicative of the possible formation of stable secondary structures, (2) several putative alternative translation initiation codons (CUG) in frame with the authentic initiation codon, and (3) several short uORFs in front of the full-length BMI1 protein coding region. Additionally, the unique 5′-UTR structure suggests that cap-dependent translation could be repressed, and an IRES may allow alternative translation initiation within the BMI1 5′-UTR. To test the potential regulatory role of the BMI1 UTRs, a series of constructs were synthesized with a reporter gene (firefly luciferase) flanked by the BMI1 or control UTRs (Fig. 2A). HT1080 fibrosarcoma cells were then transiently transfected with these constructs to determine the relative expression of the reporter genes under the control of the various UTRs. A GFP expression vector was co-transfected to normalize for transfection efficiency. As shown in Figure 2B, the construct containing the BMI1 3́-UTR increased normalized luciferase expression by more than twofold in comparison to the vector control. However, in the presence of both the 5′-UTR and 3′-UTR, luciferase expression was only modestly increased over the control. These data suggest that the BMI1 3′-UTR enhances gene expression while the 5′-UTR controls

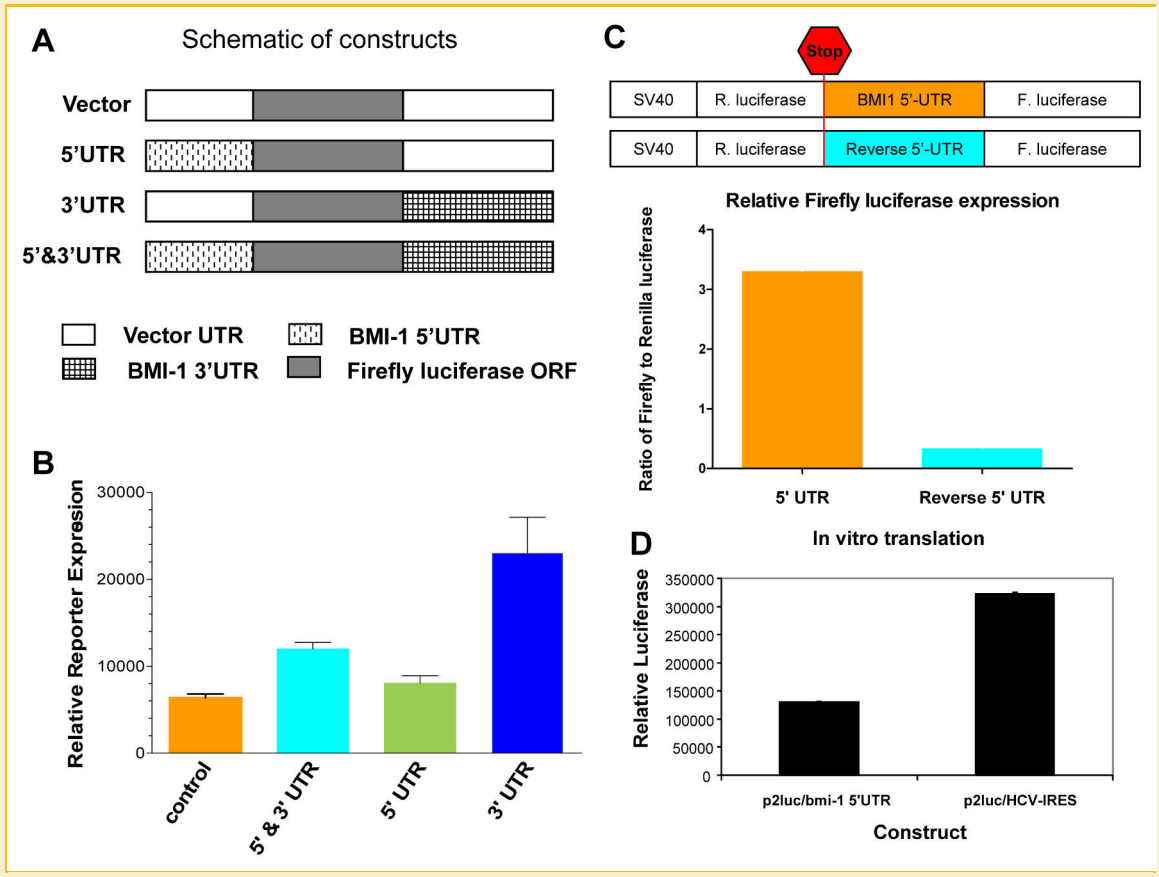

Fig. 2. Regulation of gene expression by Bmi-1 UTRs. HT1080 cells were co-transfected with the plasmids depicted in panel A with a GFP expression vector. After 24 h of transfection, cells were lysed and luciferase activity was determined with Steady-Glow substrate (Promega). The results (normalized to the co-transfected GFP) are presented in the Panel B. Alternative translation initiation by Bmi-1 5′-UTR is presented in panels C and D. 293T cells were transfected with the bicistronic vectors that contain the Bmi-1 5′-UTR or its reverse sequence between the two reporter genes. A stop code inserted immediately after the first reporter ORF (top of panel C). The firefly luciferase expressed only if the inserted UTR is in right orientation (bottom of panel C). In vitro translation of purified dicistronic mRNA that was transcribed from plasmid p2lu/Bmi-1 5′-UTR or a positive control vector p2luc/HCV-IRES is shown in panel D.

the positive effect of the 3′-UTR. Moreover, the BMI1 5́-UTR efficiently maintained the expression levels of the reporter gene even though the 5′-UTR is long, GC rich, and likely to be highly structured, suggesting alternative translation initiation may occur.

To explore the possibility that there is an IRES in the BMI1 5′-UTR, we cloned the 5′-UTR into the dicistronic luciferase vector p2luc [Bhattacharyya et al., 2009]. In this dicistronic vector, the translation of Renilla luciferase is cap-dependent. Cap-dependent read through of the firefly luciferase is blocked by a stop codon. Thus, the expression of firefly luciferase occurs only if there is IRES activity in the inserted sequence between the two reporter genes. Additionally, as shown in Figure 2C, only the vector containing the correct orientation of the BMI1 5′-UTR sequence expressed strong firefly luciferase activity, whereas transfection of either of the plasmids resulted in similar levels of Renilla luciferase expression. The orientation requirement indicates a potential IRES activity in the BMI1 5′-UTR.

Alternative explanations for the above data include either cryptic promoter activity or a cryptic splice acceptor site in the 5′-UTR. To exclude these possibilities, we first generated the promoterless vector, NP-2luc/BMI1 5UTR. Transfection with this construct resulted in nearly complete loss of expression of both Renilla luciferase and Firefly luciferase activity (our unpublished data), as compared to the wild-type vector containing the intact SV40 promoter. These results indicate that no cryptic promoter exists in the BMI1 5′-UTR.

To confirm the presence of IRES activity in the BMI1 5′-UTR, we then performed in vitro translation with purified dicistronic mRNA in a rabbit reticulocyte extract, a widely accepted standard for the presence of strong viral IRESs (though most cellular IRESs show poor activity in this assay). Dicistronic plasmids were linearized, transcribed in vitro, and the synthesized mRNA was purified. Cryptic promoter or cryptic splice acceptor activity is not possible in this assay. As shown in Figure 2D, the mRNA from the p2luc/BMI1 5′-UTR vector demonstrated significant expression levels of the second cistronic reporter gene, firefly luciferase. These in vitro translation data strongly support a robust IRES within the BMI1 5′-UTR. These observations constitute the first evidence of significant

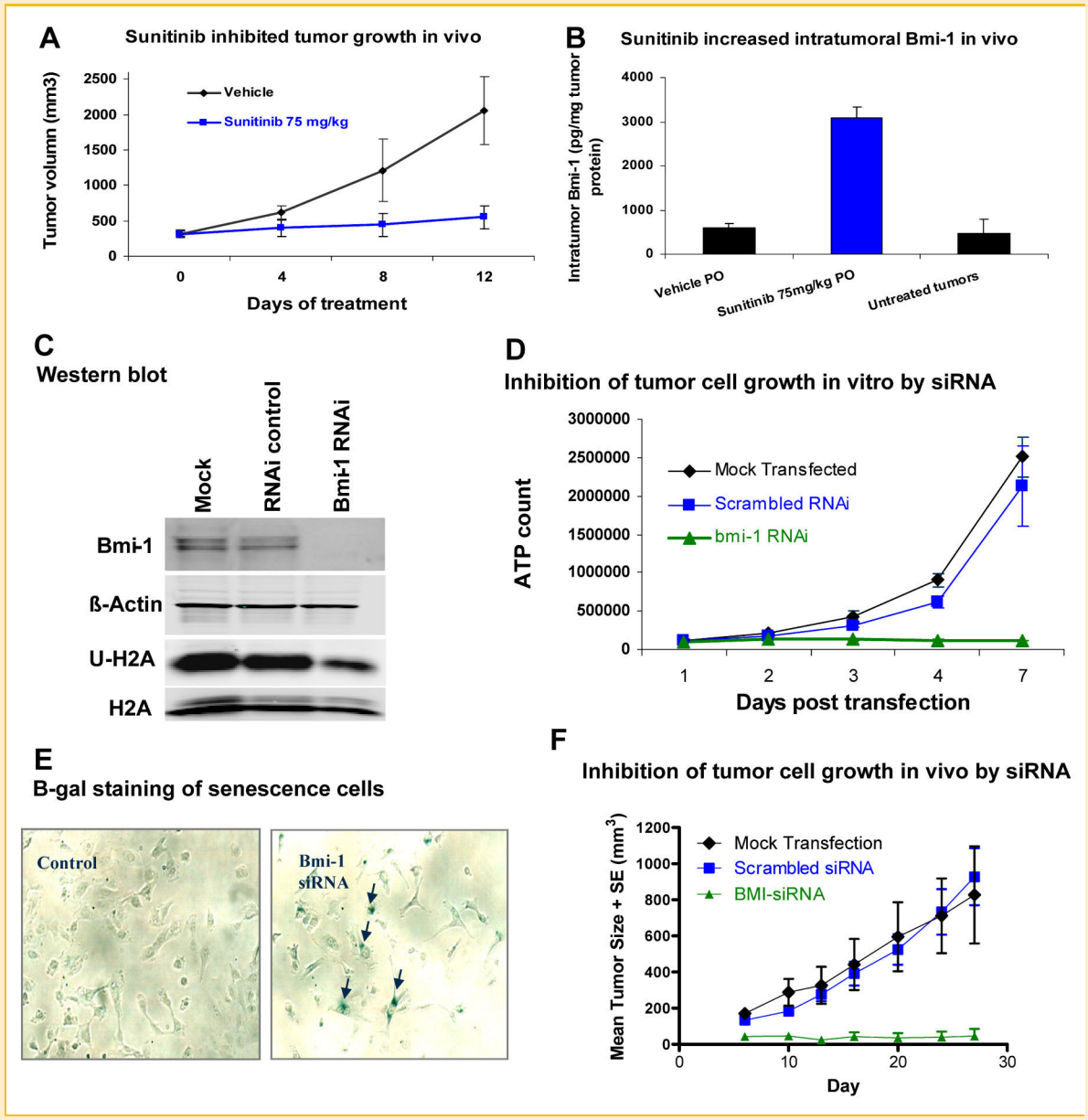

Fig. 3. Validation of BMI1 as an anti-cancer target. A,B: Effects of anti-angiogenesis drug Sunitinib on tumor growth and Bmi-1 production in K562 xenograft tumor model in nude mice. 5×10^6 cells were inoculated subcutaneously. When the tumors reached about 250 mm^3, mice were randomized and treated with vehicle or Sunitinib 75 mg/kg, twice per day, 10 mice per group (untreated group only with three mice). The tumor volume was measured every 4 days. At the end of the study, tumors were taken down and homogenized with gentle MACS. Intratumoral BMI1 levels were assessed by a BMI1 sandwich ELISA and normalized to total protein concentration. C,D: Reduction of Bmi-1 by siRNA inhibited proliferation of tumor cells in vitro and in vivo. C: Western blot demonstrated Bmi-1 siRNA effectively reduced Bmi-1 expression and ubiquitinization of H2A in HT1080 cells. D: Bmi-1 or control siRNA transfected cells were cultured in vitro, and the cell proliferation was assessed with the ATP assay (Promega) at the time points indicated. E: B-gal positive senescence cells (arrow) were found in Bmi-1 siRNA transfected culture. F: 5×10^6 of Bmi-1 or control siRNA transfected cells (cell viability >97%) were inoculated into nude mice 16 h after transfection. Tumor volume was measured at indicated time points during the experiment.

post-transcriptional regulation of the BMI1 gene and provided the rationale to identify small molecule inhibitors for cancer therapy.

POST-TRANSLATIONAL REGULATION OF BMI1

The post-translational modulation of a protein can alter its intrinsic biological activity, subcellular localization, half-life, and docking with ligands. It was first reported in 1999 that phosphorylation of BMI1 is inversely correlated with its chromatin association [Voncken et al., 1999]. In both primary and tumor cell lines, a marked cell cycle regulation of Pc-G-chromatin interaction is observed: nuclear BMI1 staining dissipates in late S-phase and is re-established early in G_1-phase. Chromatin association of BMI1 inversely correlates with its phosphorylation status in a cell cycle-dependent fashion: at G_1/S, hypophosphorylated BMI1 is

specifically retained in the chromatin-associated nuclear protein fraction, whereas during G_2/M, phosphorylated BMI1 is not chromatin-bound. Subsequently, it was reported that MAPKAP kinase 3pK phosphorylates and regulates chromatin association of the PcG Bmi1 [Voncken et al., 2005]. Using yeast two-hybrid interaction and co-immunoprecipitation, 3pK was identified as an interaction partner of PcG proteins in vitro and in vivo. Activation or overexpression of 3pK resulted in phosphorylation of Bmi1 and other PcG members and their dissociation from chromatin. Additionally, cells overexpressing 3pK showed concomitant re-expression of p14(ARF).

3pk is not the only kinase reported to phosphorylate BMI1. Kim et al. [2011] recently demonstrated that epidermal growth factor (EGF)-induced Akt activation phosphorylated BMI1 and that this modification renders BMI1 resistant to proteasomal degradation, leading to its stabilization and accumulation in the nucleus. Quantitative analysis of the human spindle phosphoproteome revealed that three serine residues (S251, S253, and S255) at the C-terminal of the BMI1 protein were phosphorylated in extracts from HeLa cells [Malik et al., 2009]. These three amino acids are in the BMI1 PS domain (proline and serine rich domain) implicated in BMI1 stability [Yadav et al., 2010]. Deletion of the C-terminal PS domain of BMI1 increases the stability of BMI1, and promoted its pro-oncogenic activities in human mammary epithelial cells (HMECs). Overexpression of a PS region deleted-mutant of BMI1 increased proliferation of HMECs and promoted an EMT phenotype in the HMECs. Furthermore, when compared to wild-type BMI1, exogenous expression of the PS domain-deleted BMI1 led to a significant downregulation of $p16^{Ink4a}$ and an efficient bypass of cellular senescence in human diploid fibroblasts. These data suggest that Akt activation induced-phosphorylation of BMI1 at its C-terminal PS domain may be essential for its stability and its oncogenic activity.

On the other hand, chronic oxidative stress results in the activation of p38 and leads to inhibition of the Akt-dependent BMI1 stabilizing process, thus causing BMI1 to degrade more rapidly [Kim and Wong, 2009a]. NSCs isolated from the subventricular zone (SVZ) of $ATM^{-/-}$ mouse brains show defective self-renewal and proliferation, which is accompanied by activation of chronic p38 mitogen-activated protein kinase (MAPK) and a lower level of the polycomb protein BMI1. Overexpression of BMI1 increases the self-renewal and proliferation of $ATM^{-/-}$ NSCs to normal levels, indicating that defective proliferation in $ATM^{-/-}$ NSCs is a consequence of downregulation of BMI1. However, treatment of the $ATM^{-/-}$ NSCs with a specific p38 MAPK inhibitor SB203580 extended BMI1 protein half-life and subsequently increased global H2A ubiquitination in $ATM^{-/-}$ NSCs, suggesting inhibition of the Akt-dependent BMI1 stabilizing process reduces the levels of BMI1 [Kim et al., 2011]. Based on the results from this $ATM^{-/-}$ model, the authors hypothesize that a function of ATM is to act as a redox modulator. As a consequence of increased ROS levels, p38 is activated, which may block the Akt-dependent BMI1 stabilizing process. This event accelerates proteasome-mediated degradation of BMI1, which in turn upregulates p21 and suppresses proliferation.

THERAPEUTIC TARGETING OF BMI1 FOR THE TREATMENT OF CANCER

Tumor stem cells that are quiescent with the expression of multifunctional efflux transporters are the root of drug resistant and treatment failure to chemotherapy [Dean, 2009]. We found that similar refractory sub-populations play a role in resistance to anti-angiogenic therapy, an approach once thought to lack classical induction of resistance. In a tumor xenograft study with leukemia K562 cells in nude mice, the VEGFR kinase inhibitor Sunitinib effectively inhibited tumor growth, but also elevated intratumoral levels of BMI1 more than fivefold compared to vehicle treated or non-treated controls (Fig. 3A,B). It is possible that Sunitinib increases BMI1 expression, or more likely, as reported with cytotoxic therapies, it preferentially targets the rapidly proliferating cancer cells and thus enriches the fraction of the cancer stem cells remaining. Nevertheless, our result is consistent with treatment failure, as the benefit of anti-angiogenic therapy is often short-lived and the majority of cancer patients eventually relapse and progress [Prince et al., 2009]. Indeed, increasing evidence indicates that better outcomes may be realized by targeting this chemo-resistant tumor cell fraction [Dean, 2009].

Since it is overexpressed in many cancer types and plays an essential role in stem cell self-renewal and survival, BMI1 stands out as a promising target in the list of comparatively few genes shown to control cancer stem cell function.

To evaluate the possibility of BMI1 as a cancer therapy target, we have determined the effect of reducing BMI1 levels in vitro and in vivo. As shown in Figure 3C, BMI1 small interfering RNA (siRNA) selectively reduced the levels of BMI1 and one of its downstream targets, mono-ubiquitinated histone H2A, in HT1080 fibrosarcoma cells. This resulted in the inhibition of tumor cell proliferation in vitro (Fig. 3D) and elicited signs of tumor cell senescence (Fig. 3E). To evaluate further the impact of BMI1 reduction over a longer period of time and under selective pressure, we performed a xenograft study to assess tumor growth in vivo. Briefly, HT1080 cells were transfected with BMI1 or control siRNA. After 16 h, cells were collected and 5×10^6 cells (with >97% viability) were inoculated into nude mice. Tumor growth in nude mice was monitored during the 27 days experiment. As shown in Figure 3F, a single transient transfection with BMI1 siRNA completely inhibited the ability of these cells to form tumors, suggesting that transient reduction of BMI1 levels could cause tumor cells to terminally differentiate or senescence. Similar results have been demonstrated by other groups [Liu et al., 2006a]. Therefore, small molecular weight compounds that inhibit BMI1 may have clinical potential as cancer therapeutic agents.

Several known drugs have been reported to reduce BMI1 expression. For examples, Bommi et al. [2010] investigated the effects of HDACIs on BMI1 expression and downstream targets in human breast cancer cells. They found that exposure of cells to the broad spectrum histone deacetylase (HDAC) inhibitors, such as sodium butyrate and valproic acid resulted in marked down-regulation of BMI1 (and EZH2) through a transcriptional mechanism, accompanied by diminished activity of BMI1-related polycomb repressive complexes, manifested by decrease in histone

2A lysine 119 ubiquitination (H2AK119Ub). Specifically, primary transcription and promoter activity of BMI1 is suppressed upon treatment with HDAC inhibitors. These events were accompanied by re-expression of growth inhibitory proteins and putative tumor suppressor genes, resulting in cell death by apoptosis or senescence. Similar results were also obtained by another group [Jung et al., 2010].

Recently, the drug Artemisinin was also shown to inhibit the BMI1 expression [Wu et al., 2011a]. Artemisinin and its derivatives are particularly useful for the treatment of infection of resistant *Plasmodium falciparum* malaria parasites. This drug has an endoperoxide group that is activated by intraparasitic heme–iron to form free radicals, which kill malaria parasites by alkylating biomolecules [Nakase et al., 2008]. Artemisinin inhibited BMI1 at both protein and transcript levels. BMI1 knockdown made the cells more sensitive to artemisinin with an increase in accumulation at G_1-phase, but overexpression of BMI1 only partially reversed the artemisinin-induced G_1 cell cycle arrest, indicating artemsinin is not a specific BMI1 inhibitor [Wu et al., 2011a].

The identification of BMI1 as a target of HDACIs and artemisinin may provide a rationale for combination studies including these types of agents. However, it is evident that the mode of action for both HDACi and artemisinin are highly pleiotropic, and could cause many unwanted side-effects. To date, no small molecules with acceptable specificities have been reported. Thus, discovery of novel compounds that selectively inhibit BMI1 expression is warranted.

As a non-enzymatic protein, targeting of BMI1 by traditional drug discovery methods is considered to be a challenge, requiring an innovative approach. Alternative therapeutic strategies might directly or indirectly impact the expression of BMI1 protein. At PTC Therapeutics, we have demonstrated that BMI1 expression is tightly controlled by post-transcriptional processes (see above). The BMI1 3′-UTR contains multiple AREs (A–U rich elements) and can significantly upregulate reporter gene expression, while its 5′-UTR contains a strong IRES activity. Considering its role in many cancers, the post-transcriptional regulation of BMI1 presents a chemotherapeutic intervention point that could be exploited to target the expression of this important protein. Using PTC's proprietary GEMS^{TM} technology platform, we have screened our chemical library and identified molecules that can reduce the production of BMI1 protein by modulating its post-transcriptional regulation. These molecules selectively inhibit proliferation of tumor cells to a much greater extent than normal human progenitor cells or HSCs, suggesting a possible therapeutic safety window. While this program is still in early stages, we have recently, demonstrated that compounds from one of our top series inhibited tumor growth and significantly reduced intratumoral levels of BMI1 (our unpublished data). Our goal is to develop a novel low molecular weight drug that can be used alone or in combination with conventional therapy for treatment of cancer. The hope is that small molecule drugs that reduce BMI1 protein levels will target the chemoresistant cancer stem cell fraction within the tumor and that this will result in a more long-lasting therapeutic benefit than currently available therapies.

In addition to reducing BMI1 protein expression by targeting its post-transcriptional regulation as described above, other therapeutic strategies may also be applied to therapeutically target BMI1. A small molecule agonist of MAPKAP kinase 3pK could increase BMI1 phosphorylation and should cause the PRC1 complex to dissociate from chromatin resulting in re-expression of tumor suppressor genes such as p16, p14, and PTEN. Alternatively, BMI1 siRNA might prove useful. siRNAs are emerging as possible approaches for treatment of otherwise incurable diseases such as cancers. In theory, the development of siRNA to inhibit gene expression is straightforward. However, there have been delivery challenges inherent in the development of siRNAs that are yet to be resolved.

CONCLUSION AND PERSPECTIVE

It has now been clearly established that BMI1 is a bonafide oncogene and plays critical roles in promoting cancer stem cell self-renewal and tumorogenesis. Small molecule drugs that reduce BMI1 protein levels will target cancer stem cells and may provide more long-lasting therapeutic benefit when added to existing therapeutic treatments. However, the safety and tolerability of targeting BMI1 systemically over the course of treatment is a question which remains to be fully addressed. Toxicity to normal cell proliferation is a critical issue for any drug discovery effort. To date, the safety of transient reduction of BMI1 on adult patients is unknown. However, BMI1 null mice may provide some insight into potential side effects. BMI1-deficient mice are born with a relatively normal phenotype but demonstrate progressive loss of their adult hematopoietic and neuronal stem cell fractions and typically die due to lack of repopulation of these tissues by 6 months of age. Therefore, such effects on the normal adult stem cell compartment would be a primary on-target side effect to be monitored in any trial. Studies with siRNA and PTC lead molecules suggest that tumor cells are less tolerant to modest reductions of BMI1 protein expression than normal human progenitor cells [Liu et al., 2006a]. The reason for that is not clear. We observed that the normal hematopoietic stem/progenitor cells express BMI1 by Western blot, but the levels are much lower than those in many types of tumor cells. Additionally, mice dosed with our compounds for weeks demonstrated effective tumor growth control with no overt toxicity and no observable effects on blood cell counts or ratios (our unpublished data). These observations suggest a potential therapeutics window for a small molecule that reduces BMI1 protein expression post-transcriptionally. Though there is no specific BMI1 inhibitor in the clinical currently, the observation that HDAC inhibitors that target BMI1 expression are well tolerated in patients also bodes well for development of a specific BMI1 inhibitor for treatment of cancer. Future work will need to be focused on understanding the differences of BMI1 function and expression regulation between normal and cancer stem cells, which may inform further efforts to therapeutically target this important protein.

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