Bmi1 – A Path to Targeting Cancer Stem Cells

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The Polycomb group (PcG) genes encode for proteins comprising two multiprotein complexes, Polycomb repressive complex 1 (PRC1) and Polycomb repressive complex 2 (PRC2). Although the initial discovery of PcG genes was made in *Drosophila*, as transcriptional repressors of homeotic (*HOX*) genes. Polycomb repressive complexes have been since implicated in regulating a wide range of cellular processes, including differentiation and self-renewal in normal and cancer stem cells. Bmi1, a subunit of PRC1, has been long implicated in driving self-renewal, the key property of stem cells. Subsequent studies showing upregulation of Bmi1 in several cancers correlated with increased aggressiveness, radioresistance and metastatic potential, provided rationale for development of targeted therapies against Bmi1. Although Bmi1 activity can be reduced through transcriptional, post-transcriptional and post-translational regulation, to date, the most promising approach has been through small molecule inhibitors targeting Bmi1 activity. The post-translational targeting of Bmi1 in colorectal carcinoma, lung adenocarcinoma, multiple myeloma and medulloblastoma have led to significant reduction of self-renewal capacity of cancer stem cells, leading to slower tumour progression and reduced extent of metastatic spread. Further value of Bmi1 targeting in cancer can be established through trials evaluating the combinatorial effect of Bmi1 inhibition with current 'gold standard' therapies.

Keywords

Polycomb group (PcG) genes, Bmi1, Mel-18, cancer stem cells (CSCs), self-renewal

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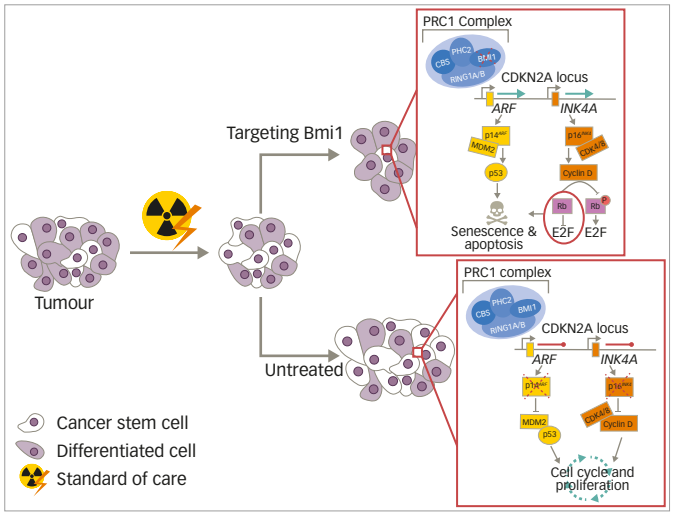
Since the discovery of the Polycomb group (PcG) gene family in *Drosophila* as repressors of homeotic (*HOX*) genes, PcG proteins have been implicated in a range of processes from chromosome X inactivation to stem cell plasticity and differentiation. PcG proteins execute their function through transcriptional repression of the promoter region of a target gene. Although PcG proteins function as two major multiprotein complexes – Polycomb repressive complex 1 (PRC1) and Polycomb repressive complex 2 (PRC2) – the existence of several context specific subcomplexes has been postulated.[1] PRC2 initiates gene silencing through the activity of histone deacetylase (HDAC), and histone methyltransferases that can methylate lysine 9 and 27 residues on histone H3 and lysine 26 on histone H1.[2–6] The stable gene repression is then maintained by PRC1 through recognition of tri-methylated H3K27 (H3K27me3).[3] The canonical repression pathway is initiated through trimethylation of H3K27 on the promoter of target gene by PRC2 subunit EZH1 or its paralog EZH2.[2–4] The H3K27me3 mark is readily recognised by PRC1 through chromatin binding ability of its subunit CBX. The repression is further maintained through ubiquitination of lysine 119 residue on histone H2A (H2AK119ub) by RING1, a subunit of PRC1, or chromatin condensation.[7–9] Promoters of the polycomb target genes have been generally characterised as CpG-rich DNA sequences that are lacking other epigenetic markers.[10–14] However, several other models of context dependent PRC2 recruitment mechanisms, including Polycomb response elements (PREs)[15–18] and ncRNAs[19–22] have been investigated.

Polycomb group genes and self-renewal

Over the years, in addition to *HOX* genes, numerous targets of PcG repression have been identified, with one of the examples being *CDKN2A* locus (*Figure 1*). Encoding for $p16^{INK4A}$ and $p14^{ARF}$ tumour-suppressor proteins, gene products of *CDKN2A* locus act as important mediators of the cell cycle.[23] The $p16^{INK4A}$ prevents phosphorylation of retinoblastoma protein (RB) through inhibition of cyclin D-dependent CDK4 and CDK6, which allows hypophosphorylated RB to sequester E2F transcription factor and prevent activation of genes required for DNA replication.[24] On the other hand, $p14^{ARF}$ interacts with MDM2 E3 ubiquitin ligase, preventing p53 polyubiquitination and subsequent p53 activation.[25–28] The repression of *CDKN2A* by PcG proteins in stem cells,[29,30] or its frequent deletion in cancer cells,[31,32] facilitates increased self-renewal and proliferation, and has been implicated in neoplastic transformations. A meta-analysis of 11 studies quantifying *CDKN2A* methylation in 3,440 colorectal cancer patients, has identified hypermethylation in 23% of tumours. Furthermore, *CDKN2A* promoter hypermethylation was shown to correlate with poor patient overall survival.[33] In juvenile myelomonocytic leukaemia (JMML), hypermethylation of *CDKN2A* was observed in 35% of the patients and correlated with poor outcome,[34] similarly to the patients diagnosed with non-small cell lung cancer

Figure 1: Role of Bmi1 in driving self-renewal of cancer stem cells



Cancer stem cells (CSCs) are endowed with the intrinsic ability to escape the best chemoradiotherapy regimens. Without targeted therapies against CSCs, slowly dividing cells left behind post-therapy will proliferate and drive tumour recurrence and metastatic spread. Within CSCs, Bmi1 directed self-renewal and proliferation is accomplished through transcriptional repression of the CDKN2A locus encoding for $p16^{INK4A}$ and $p14^{ARF}$. However, in the absence of Bmi1, p16-mediated inhibition of RB phosphorylation and p14-mediated prevention of p53 polyubiquitination, contribute to reduced activation of genes required for DNA replication and cell cycle progression. Through targeted therapies against Bmi1, it is possible to limit proliferation and self-renewal of the rare population of cells responsible for treatment failure and tumour recurrence. PRC1 = Polycomb repressive complex 1.

(NSCLC), hypermethylation of p16 promoter was also associated with worse outcome.[35] The notion of slowing down the replication of cancer cells, a major hallmark of tumour biology, has been of great interest, and the efforts have escalated tremendously with the conceptualisation and discovery of cancer stem cells (CSCs). The first compelling evidence of cancer cells possessing stem-like properties came from the work of Bonnet and Dick in mouse models of acute myeloid leukaemia (AML).[36] Since then, CSCs have been identified and characterised in breast,[37] brain,[38] colon[39] and lung[40,41] cancers, among others. Due to their ability to evade chemoradiotherapy along with tumour-initiating and metastatic properties, CSCs have been implicated in driving treatment failure, tumour recurrence and poor clinical outcome.[42] Although CSC populations identified in various cancer types have intrinsically different gene expression patterns, they all share the unique ability to self-renew, unlike the more differentiated cancer cells. This difference confers one of the major limitations of current therapy modalities, as they are effective against bulk tumour, consisting of highly proliferative cells, while sparing the intrinsically resistant, slow-dividing CSCs. The increasing evidence connecting self-renewal and therapeutic resistance of CSCs presents a strong rationale for developing novel therapeutic modalities as a treatment option for many aggressive malignancies. In this review, we discuss the involvement of PRC1 subunits, Bmi1 and Mel-18, in regulation of self-renewal in cancer and their potential for therapeutic targeting.

Clinical implications of Bmi1 in cancer

Bmi1, a 37kDa subunit of PRC1, was first identified as a key component in the activation region of Moloney murine leukaemia virus.[43] The target gene suppression by PRC1 is primarily achieved through H2A119ub by RING1B E3 ligase, the catalytic activity of which is dramatically reduced in the absence of Bmi1. However, to date no specific enzymatic activity of Bmi1 has been reported,[44] making it a challenge to target for therapeutic intervention. In early 2000s, Bmi1 was implicated in self-renewal of haematopoietic and neural stem cell populations, and driving the proliferation of early cerebellar progenitors.[45–47] Further experimental evidence of importance of PcG gene in stem cells came from the study of *Bmi1*-deficient mice that suffered from continuous loss of haematopoietic cells and cerebellar neurons.[48] PRC1 complex was further implicated in maintaining proper function of haematopoietic stem cells (HSCs) through interaction between Mph1/Rae28 with Bmi1.[49] The oncogenic potential of Bmi1 became evident through investigation of lymphomagenesis in mouse models. Haupt et al. have demonstrated that in collaboration with c-Myc, Bmi1 contributed to lymphomagenesis in T and B cell lineages.[50] The PRC1-mediated transcriptional repression of *CDKN2A* locus during lymphomagenesis leads to maintenance of proliferative capacity and undifferentiated state,[51,52] and has since been implicated in progression and poor prognosis in a number of haematologic malignancies.[45,53] Similarly to normal stem cells, Bmi1 was implicated in maintenance of self-renewal in leukaemic stem and progenitor cells.[54] Intriguingly, there is some evidence that Bmi1 can exert its oncogenic properties even in *CDKN2A*-deficient models,[55] suggesting the existence of other gene targets repressed during tumourigenesis. In addition to haematologic malignancies,[53,56] elevated levels of Bmi1 have been shown in colorectal carcinoma, NSCLC,[57] breast carcinoma,[58,59] glioblastoma (GBM),[60] medulloblastoma[61] and prostate cancer.[62] Clinically, increased Bmi1 expression levels have been shown to correlate with poor patient prognosis in several aggressive cancer types, including colorectal carcinoma,[63] GBM,[64] and medulloblastoma.[65] The maintenance of self-renewal potential by Bmi1 contributes to the aggressive cancer phenotype by allowing CSCs to evade chemoradiotherapy regimens and drive tumour recurrence. Further contributions of Bmi1 to cancer cell survival through therapy has come from its role in promoting DNA damage repair. Both Bmi1 and Ring1B are recruited to the DNA double-strand breaks (DSB) to facilitate ubiquitination of γH2AX.[66] The continuous localisation of Bmi1 to DSB is highly dependent on functionally intact ataxia-telangiectasia mutated (ATM), in addition to ATM- and Rad-3-related (ATR) kinases.[67,68] Moreover, Bmi1-driven enhancement of DNA DSB repair allows CSCs to negate the detrimental effects of radiation and persist post therapy. For example, in GBM, a highly malignant adult brain tumour, CD133 expressing brain tumour initiating cells (BTICs)[38] were shown to have the capacity of escaping radiation through activation of DNA DSB repair mechanisms.[69] However, the loss of Bmi1-assisted DNA DSB repair increases the sensitivity of CSCs to ionizing radiation, and promotes accumulation of cells in G_2/M phase of the cell cycle. Aside from driving self-renewal and resistance to radiotherapy, Bmi1 has been implicated in promoting epithelial-mesenchymal transition (EMT), a signalling programme frequently associated with cancer invasion and metastatic potential,[70] through cooperation with Twist 1 in head and neck carcinomas.[71] By modulating SNAIL activity, Bmi1 is able to promote EMT through repression of *PTEN* and subsequent activation of AKT pathway.[72] The apparent role of Bmi1 in maintaining a stem cell-like state and an invasive phenotype, along with its clinical significance in multiple tumour types, has prompted investigation into avenues of reducing Bmi1 levels as a potential therapeutic modality.

Therapeutic targeting of Bmi1

The extensive research on Bmi1 function in numerous malignancies has created a large amount of empirical data, correlating reduced Bmi1 expression levels with less proliferative, more therapy-sensitive and less tumourigenic phenotype of cancer cells. The development of small molecule inhibitors against Bmi1 might create an opportunity for designing therapies that not only target the highly proliferative cancer cells, but also the slowly dividing, therapy-evading CSCs. Additionally, Bmi1 targeting within CSCs can allow for modulation of two key tumour suppressor pathways driven by Rb and p53. In its turn, the combinatorial

approach might allow for de-escalation of existing chemoradiotherapy protocols and minimise the associated toxicity and side effects. Decreased Bmi1 activity can be achieved through transcriptional, post-transcriptional or post-translational regulation.[73] In breast cancer cells, broad spectrum HDAC inhibitors have been shown to inhibit expression of Bmi1 and the activity of PRC1 complex as measured through a decrease of H1AK119ub.[74,75] However, HDAC inhibitor-mediated reduction of Bmi1 expression is unlikely to be useful as a targeted therapy against cancer cells, and thus is likely to be associated with toxicity and side effects. In a recent publication by Kaneta et al., researchers have isolated and identified a series of naturally occurring compounds targeting Bmi1 promoter activity. The most active compound, wallichoside, was shown to decrease Bmi1 protein levels in colon carcinoma cells and reduced self-renewing capacity of human hepatocellular carcinoma cells.[76] However, further *in vivo* studies in human-mouse xenograft models are warranted to generate better understanding of the compound's therapeutic value. Additionally, molecules targeting Bmi1 transcript have been shown to hold a promising therapeutic potential in reducing oncogenic potential of prostate CSCs[77] and hepatocellular carcinoma.[78] Another potential way to modulate protein function, localisation and half-life is through post-translational modifications.[73] Targeting Bmi1 post-translationally, using small molecule inhibitors can present a more clinically relevant therapy modality with its potential to be selective for CSCs. The first experimental evidence demonstrating the imminent value of targeting Bmi1 through post-translational modification, came from the study conducted by Voncken et al.[79] in 1999, where researchers observed fluctuating phosphorylation levels of Bmi1 through its progression in the cell cycle. In G_1/S phase, hypophosphorylated Bmi1 is present in the chromatin-bound state, whereas the phosphorylation of Bmi1 in the G_2/M phase reduces its chromatin association. Mechanistic insight into kinases involved in post-translational modification of Bmi1 came from the yeast two-hybrid interaction assay, which identified MAPKAP kinase 3pK as a regulator of Bmi1 chromatin association, among other PcG proteins.[80] In recent years, small molecule inhibitors developed by PTC Therapeutics (South Plainfield, NJ, US), were designed to promote phosphorylation of Bmi1, and have been tested in colorectal carcinomas,[81] lung adenocarcinomas,[82] multiple myeloma (MM),[83] prostate cancer[77] and medulloblastoma (unpublished data). In all cases, small molecule inhibitor resulted in decreased Bmi1 protein levels and reduced activity of the PRC1 complex. More importantly, diminished Bmi1 levels reduced the self-renewal capacity of CSCs, which in turn correlated with lowered tumourigenic potential *in vitro* and *in vivo*. In keeping with the original observations, cells treated with a Bmi1 inhibitor became more apoptotic[77,81–83] and underwent cell cycle arrest at G_0 phase.[81,82] Collectively, these studies have provided a strong rationale for including Bmi1-targeted therapy in the treatment strategies for patients presenting with malignancies displaying elevated Bmi1 expression. Currently, the lead compound developed by PTC Therapeutics, PTC-596, is being examined in a phase I clinical trial (ClinicalTrials.gov identifier NCT02404480) for recurrent solid malignancies in adults. Another example of post-translational regulation of Bmi1 is through beta-transducin repeat containing protein (βTrCP) mediated ubiquitination and subsequent degradation of Bmi1. In their work, Sahasrabuddhe et al. were able to demonstrate that wild-type Bmi1 is readily recognised and bound to by βTrCP, a subunit of SCF (SKP1-cullin F-box) E3-ubiquitin ligase, and is destined for ubiquitin-proteasome mediated degradation.[84] Development of therapies increasing the extent of Bmi1 ubiquitination might present an avenue to continuously reduce Bmi1 protein levels within the cell, and thus ensure un-inhibited transcription of *CDKN2A* locus.

In addition to development of small molecules directly affecting Bmi1 transcription or protein levels, novel compounds modulating Bmi1 through inhibition of proteins that contribute to normal regulation of Bmi1, have been tested. Polo-like kinase 1 (PLK1) is overexpressed in several cancer subtypes, correlates with poor patient outcomes and has been shown to play an important role in driving tumour cell growth.[85,86] In breast cancer, small molecule inhibition of PLK1 resulted in marked induction of cellular senescence. Further experimentation revealed that downregulation of PLK1 activity caused upregulation of miR-200c and miR-141, which in turn, post-transcriptionally inhibited expression of Bmi1.[87]

Negating Bmi1 effects by upregulation of Mel-18

Despite the highly similar amino acid sequences and functional redundancy between Bmi1 and its paralog Mel-18,[88] the two proteins differ in their regulation of PRC1 complex. Both Bmi1-PRC1 complex and Mel-18-PRC1 target genes with high levels H3K27me3 and contribute to the canonical PcG-mediated gene repression.[12] However, *in vitro* studies in MCF7 cells indicated that Mel-18-PRC1 complexes have reduced ubiquitination activity compared to Bmi1-PRC1 complexes.[44,89] It has also been postulated that increased levels of Mel-18 allows it to outcompete Bmi1 for integration into PRC1 and thus reduce the extent of PRC1 mediated gene repression.[90] Interestingly, the expression pattern of *Bmi1* and *Mel-18* varies in adult tissues suggesting regulation of distinct cellular programmes.[12,91] In contrast to *Bmi1*, expression levels of *Mel-18* escalate during differentiation of HSCs,[46,54,91] suggesting that while Bmi1 is essential in preserving HSCs, Mel-18 ensures proper differentiation.[92,93] Further functional differences between Bmi1 and Mel-18 extend to their role in cancer cells. Unlike Bmi1, that is often upregulated in aggressive cancers, Mel-18 is often downregulated,[94–100] suggesting a tumour suppressive role. Studies in human fibroblasts identified Mel-18 contribution to transcriptional regulation of *Bmi1* through modulation of c-Myc levels. The Mel-18-driven downregulation of c-Myc during cellular senescence reduces its binding to the Bmi1 promoter, and thus stalls *Bmi1* gene expression.[96,101–103] Several other pathways modulated by Mel-18 include Wnt signalling, E-cadherin and angiogenesis.[104–106] From a therapeutic perspective, Mel-18 presents an intriguing possibility to negate Bmi1 activity through Mel-18 agonists. The increased levels of Mel-18 will allow for formation of more Mel-18-PRC1 complexes that have opposing effects to Bmi1-PRC1 complexes and thus will reduce the extent to which Bmi1 is able to contribute to therapy evasion and self-renewal ability of CSCs.

Concluding remarks

A combinatorial approach of targeting the bulk tumour population, along with the CSC fraction can potentially not only lead to de-escalation of current treatment protocols, but also address the root cause of tumour recurrence. However, since Bmi1 is ubiquitously expressed throughout the human body, the effects of Bmi1 targeting must be carefully and methodically evaluated. In the pre-clinical models investigating the efficacy of Bmi1 targeting with small molecule inhibitors for treatment of childhood medulloblastoma, our lab has shown a higher sensitivity of tumour cells to the Bmi1 inhibitor, relative to neural stem cells (unpublished data). These results highlight the importance of further elucidating different Bmi1 roles and its regulation in cancer cells, when compared to normal cells. The increasing evidence demonstrating Bmi1-driven therapy evasion and tumour recurrence warrants routine profiling of Bmi1 levels in oncologic patients, in order to identify patients who have the potential to benefit from combining Bmi1 targeted therapies with today's gold standard chemoradiotherapies. Moreover, the relationship between Bmi1 and Mel-18 requires further investigation as indirect modulation of Bmi1 levels by Mel-18 can create new therapeutic avenues for minimising oncogenic effects of Bmi1. ❐

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