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Bmi1 directly represses p21$^{Waf1/Cip1}$ in Shh-induced proliferation of cerebellar granule cell progenitors

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ABSTRACT

Bmi1, the main component of the Polycomb repressive complex 1, plays a key role in self-renewal of stem cells as well as in proliferation of progenitor cells and senescence, at least in part through inhibition of the Cdkn2a locus.

Bmi1 is highly expressed in the developing cerebellum, where it contributes to Shh-mediated expansion of granule cell precursors. Overexpression of Bmi1 has been described in medulloblastoma, highly aggressive brain neoplasms of childhood, which are thought to originate from deregulated proliferation of granule cell precursors.

Here, we dissected the molecular mechanisms mediating the role of Bmi1 in granule cell development by means of transcriptome analysis in loss of function mouse models in vitro and in vivo. We demonstrate that lack of Bmi1 causes significant shift in gene expression levels in Shh stimulated cerebellar granule progenitors. Our results revealed differences in the expression of a number of genes involved in TGF-β signal transduction pathway, ECM remodeling and cell adhesion, and particularly, in cell cycle control, not only the well known cell cycle inhibitors p16^{Ink4a}, p19^{Arf} but also Cdkn1a (p21$^{Waf1/Cip1}$). Finally, we demonstrate that Bmi1 directly regulates p21$^{Waf1/Cip1}$ expression through direct binding to its promoter and may therefore represent a key mechanism mediating the role of Shh in postnatal cerebellar neurogenesis.

Introduction

Bmi1 (B-cell-specific Moloney murine leukaemia virus integration site 1) is the core component of the Polycomb repressive complex 1 (PRC1). Polycomb group (PcG) proteins are epigenetic chromatin modifiers involved in heritable gene repression and maintenance of stem cell self-renewal and progenitor proliferation. They were first identified in Drosophila as maintenance factors for long-term transcriptional repression of homeotic genes. Polycomb repressive complex 2 (PRC2) is thought to be involved in initiation of gene silencing, whereas PRC1 is implicated in the stable maintenance of gene repression (Hernandez-Munoz et al., 2005). PcG genes are regarded as part of a preprogrammed memory system established during embryogenesis which marks certain key genes for repressive signals during subsequent developmental and differentiation processes (Bracken et al., 2006). PRC1 complex includes a number of proteins (Bmi1, RING1, HPH1, HPC1 and HPC2); and Bmi1 is thought to be a critical component of the complex. Although histone H2A ubiquitination by the PRC1 complex strongly depends on the Ring1b protein, the E3-ligase activity of Ring1b on histone H2A is enhanced by Bmi1 (Buchwald et al., 2006).

A well known mechanism of Bmi-mediated activation of proliferation of progenitors and self-renewal of stem cells is through repression of the Cdkn2a locus. The locus encodes for two proteins, p16^{Ink4a} and p19^{Arf}, by use of alternative reading frames (Bruggeman et al., 2005; Quelle et al., 1995; Serrano et al., 1993; Zencak et al., 2005). These proteins are important players in the retinoblastoma (RB) and p53 pathways respectively, and their activation results in growth arrest, senescence, or apoptosis (Bracken et al., 2007; Lowe and Sherr, 2003; Park et al., 2004; Sharpless and DePinho, 1999).

Cerebellar granule cells are the most numerous neurons of the adult brain, which originate from progenitors located in the rostral region of the rhombic lip, a structure found at the interface between the roof plate of the fourth ventricle and the dorsal neuroepithelium. Newly generated granule cell precursors (GCPs) migrate tangentially from the rhombic lip over the dorsal surface of the neural tube and along its anterior–posterior axis, before undergoing a centripetal radial migration from the surface of the neural tube inwards (Ono et al., 2004; Sillitoe and Joyner, 2007; Yuasa, 1995). Massive expansion of these precursors takes place in the external granule layer (EGL) of the cerebellum between postnatal days 5 and 8 of mouse development (Hatten et al., 1997). Sonic hedgehog (Shh), secreted by Purkinje neurons, plays a crucial role in regulating this

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phase of active proliferation of GCPs (Dahmane and Ruiz-i-Altaba, 1999; Wechsler-Reya and Scott, 1999). Shh binds to the transmembrane receptor Patched (Ptc) and in turn relieves Ptc-mediated inhibition of Smoothened (Smo) activity (Ingham and McMahon, 2001). Smo, a G-protein-coupled receptor, activates an inhibitory G-protein (DeCamp et al., 2000) that leads to activation of Gli transcription factors and the initiation of gene expression required for cell cycle progression. Moreover, Shh up-regulates the expression of D cyclins (Kenney and Rowitch, 2000; Pogoriler et al., 2006) via N-myc (Hatton et al., 2006), which is a direct target of the Shh pathway in cerebellar GCPs (Kenney et al., 2003). This mechanism involves other members of the Myc/Max/Mad family of basic helix–loop–helix leucine zipper (bHLHZ) DNA binding proteins, such as Mad3 protein which has been shown to play a crucial role in GCP proliferation (Yun et al., 2007).

It has been shown that N-Myc may act as a direct target of Gli1 (Browd et al., 2006; Feng et al., 2007). Binding sites for Gli1 (GBSs) have been demonstrated in a minority of Shh-dependent genes, even though a recent ChIP-on chip screen has expanded the number and location of GBSs (Vokes et al., 2007). The low number of GBSs in Shh-regulated genes raises the possibility that only a limited number of genes require direct Gli1 input. It is therefore likely that other regulatory inputs are needed to orchestrate the response of a given cell to Shh signaling.

Bmi1 is strongly expressed in proliferating cerebellar GCP in mice and humans and Bmi1-null mice displayed impaired proliferative response to Shh both in vivo and in vitro, therefore highlighting a role for Bmi1-containing polycomb complexes in proliferation control of these precursor cells (Leung et al., 2004). Notably, this defect in proliferation can be rescued by both loss of $p19^{Arf}$ and by complete deletion of the *Ink4a/Arf* locus, implicating $p19^{Arf}$ as an important downstream effector of Bmi1-mediated proliferation control of cerebellar GCPs (Bruggeman et al., 2005). Histopathological analysis of $Bmi1^{-/-}$;Ink4a/$Arf^{-/-}$ mice showed a significant rescue of the cerebellar defects observed in Bmi1 null mice but only a partial rescue of the neurological defects, resulting in a delayed onset and slower progression of the pathological process (Jacobs et al., 1999).

Genetically engineered mouse models, where genes involved in controlling proliferation of GCPs were inactivated or overexpressed, have demonstrated that abnormally proliferating GCPs can give rise to tumours closely resembling human medulloblastoma, highly aggressive embryonic tumours of childhood (reviewed in (Behesti and Marino, 2009)). Constitutive activation of Shh signaling is known to cause medulloblastoma and Bmi1 has been shown not only to be up-regulated in a majority of human medulloblastoma (Leung et al., 2004) but also to prevent Shh-induced medulloblastoma formation in the mouse (Michael et al., 2008).

Although the importance of Bmi1 in the development of the cerebellum has been demonstrated, it is currently unclear which aspects of the cellular response to Shh are regulated by Bmi1 and how. Indeed, according to recent investigations, the number of genes which can be directly controlled by PRC1 complex seems to be underestimated (Bracken et al., 2006; Bracken et al., 2007). Bmi1 may therefore be involved in the regulation of other genes then the products of Cdkn2a locus, which may account for the only partial rescue of the phenotype upon deletion of Ink4a/Arf (Bruggeman et al., 2005; Molofsky et al., 2005). Elucidating the contribution of Bmi1 to Shh-induced proliferation of GCPs might therefore be important not only to gain further knowledge on the development of the cerebellum but also to deepen our understanding of medulloblastoma pathogenesis.

Here, we investigated the effect of lack of Bmi1 on Shh-mediated shift in gene expression levels in GCPs using transcriptome analysis. Our results revealed significant difference in the expression of a number of genes involved in TGF-β signal transduction pathway as well as in ECM remodeling and cell adhesion, which are known to play important roles in cerebellar development. Particularly striking was the difference in expression levels of genes involved in cell cycle control, such as inhibitors of cell cycle, not only $p16^{Ink4a}$ and $p19^{Arf}$ but also Cdkn1a ($p21^{Waf1/Cip1}$) which were up-regulated in $Bmi1^{-/-}$ GCPs. While it has been previously shown that inhibition of $p21^{Waf1/Cip1}$ expression through Bmi1 is important during the embryonic development of the neocortex (Fasano et al., 2007), we show here for the first time that regulation of $p21^{Waf1/Cip1}$ expression through Bmi1 plays an important role also in postnatal granule cell development. Moreover we show that transcriptional regulation of $p21^{Waf1/Cip1}$ through Bmi1 occurs through direct interaction and independently on Cdkn2b/p53 pathway.

Results and discussion

Gene expression analysis of the response to Shh of GCPs lacking Bmi1

To investigate the molecular mechanisms regulated by Bmi1 in the context of the response to Shh, we examined gene expression levels in cerebellar GCPs isolated from Bmi1-deficient cerebella and from control littermates at postnatal day 7 (P7) in the presence or absence of Shh (3 μg/ml) for 24 h (Fig. 1A). This time point was chosen as it represents a good level of induction of proliferation obtained upon Shh stimulation of these cultures ((Zhao et al., 2002) and our own data) and also because it coincides with the maximal up-regulation of Bmi1 expression under the same conditions (Leung et al., 2004). To account for inter-Chip variability and biological sample variability all experiments were performed in triplicates on three independently derived cultures for each genotype and in triplicates on two independently derived cultures for each genotype treated with Shh. Differentially regulated genes were identified as described in Experimental methods section and the analysis – comparison between $Bmi1^{-/-}$ GCPs and control GCPs as well as between $Bmi1^{-/-}$ GCPs treated with Shh and control GCPs treated with Shh – revealed significant changes in the expression levels of more than 3000 genes.

Validation of the data obtained with the array analysis was carried out by real-time PCR using RT² Profiler™ PCR Array System (SABioscience) and custom designed primers for genes which were up-regulated in $Bmi1^{-/-}$ GPCs and selected on the basis of their biological relevance, as explained later in this manuscript. 10 genes were confirmed by custom qPCR analysis and other 25 genes were confirmed using PCR Arrays for Cell Cycle, Wnt-, Tgf-beta pathways with small number of genes demonstrated contradictive results; some typical examples are shown in Table 1.

Genes differentially expressed between the various conditions and their overlaps are shown in a Venn diagram (Fig. 1B) and hierarchical clustering of differentially expressed genes is visualized by means of a heat map (Fig. 1C). The comparison between $Bmi1^{-/-}$ GCPs and control GCPs revealed 197 genes, the expression of which was altered by lack of Bmi1 and which were therefore regulated by basal level of Bmi1. Shh treatment dramatically shifted the gene expression profiles of both control GCPs (2882 genes) and $Bmi1^{-/-}$ GCPs (2397 genes); though comparison between the latter conditions allowed us to identify subgroups of genes, the expression of which was either regulated by Shh independently of Bmi1 (1699 genes) or by Shh through Bmi1 (1183 genes). Additionally we identified a forth subgroup of genes, the expression of which was regulated by Shh solely in GCPs lacking Bmi1 (698 genes).

Next we interrogated the data set acquired through this analysis to gain insights into the role of Bmi1 as a downstream mediator of Shh signaling in GCPs. To this end we have taken two complementary approaches. Firstly, we concentrated our attention on genes, the expression levels of which were most prominently different between the subgroups. Secondly, we have used the Metacore software to try and identify pathways and networks affected in $Bmi1^{-/-}$ GCPs and in $Bmi1^{-/-}$ GCPs upon Shh stimulation.

A list of genes differentially expressed in $Bmi1^{-/-}$ GCPs is shown in Tables 1 and 2. As expected, taking into account the role of Bmi1 as