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p15INK4b, a Tumor Suppressor in Acute Myeloid Leukemia

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1. Introduction

p15INK4b expression is lost in a striking 80% of all patients suffering from acute myeloid leukemia (AML). Specific inactivation of the gene by aberrant promoter hypermethylation is also detected in about 50% of patients diagnosed with myelodysplastic syndromes (MDS) and almost 60% of patients with myeloproliferative disorders (MPD). More importantly, a strong correlation between the methylation levels of p15INK4b and poor prognosis is now well established in these patients. Hypermethylation levels also provide a marker for subsequent transformation and progression of the disease to a more aggressive phenotype. These clinical observations establish the repression of p15INK4b expression by promoter hypermethylation as the most prevalent genetic abnormality in myeloid leukemia. The p15INK4b gene (also referred to as CDKN2B and MTS2) encodes a 15kDa cyclin dependent kinase inhibitor (CDKI). Specific and preferential epigenetic targeting of p15INK4b for silencing over other CDKIs such as p16INK4a and p21WAF/CIP in AML, MDS and MPD patients strongly supports a role for this protein as a tumor suppressor in hematological malignancies of the myeloid lineage.

This chapter provides a review of the literature outlining the high prevalence of p15INK4b loss of expression in human myeloid malignancies, as well as the latest research carried out in mice which supports a role for p15INK4b as a tumor suppressor. It also focuses on the well established function of p15INK4b in the control of the cell cycle, as well as its role during early and late myeloid cells development. Finally, this chapter discusses the multiple mechanisms by which p15INK4b is silenced and presents a few examples of clinical studies of drugs that target p15INK4b for re-expression. These include treatments for reversing aberrant DNA methylation, and are currently being tested and used for the therapy of MDS and AML.

2. Role of p15INK4b in myeloid malignancies

2.1 Inactivation in human AML and MDS

p15INK4b silencing by promoter hypermethylation occurs almost exclusively in cancers of the hematopoietic system, and is observed in acute leukemias of myeloid (AML) and lymphoid (ALL) origins (Drexler, 1998). Aberrant hypermethylation occurs at the gene’s
CpG islands which extend throughout the promoter region, exon 1 and part of intron 1 (Herman et al., 1996). DNA methylation is the addition of methyl groups on cytosine bases on the DNA molecules of mammalian cells which affects gene expression (Deaton et al., 2011). Methylation is carried out by the enzymes DNA methyltransferases (DNMT) which catalyze the reaction converting cytosine to 5-methylcytosine (Bird et al., 2002). In the earlier studies DNA methylation was assessed by southern blotting and methylation-specific PCR technique and more recently by more sensitive assays including bisulfate pyrosequencing and genome-wide sequencing methods (Deaton et al., 2011).

Despite the broad clinical diversity of AML, with more than a hundred cytogenetic alterations described (Vardiman et al., 2002; Trost et al., 2006), aberrant methylation of \( p15^{INK4b} \) has been reported in up to 80% of patients with primary and secondary AML. Hypermethylation levels have been shown to correlate with a reduction in the mRNA and the protein expression levels of \( p15^{INK4b} \) (Cameron et al., 1999; Matsuno et al., 2005). Furthermore, density of the methylation has been shown to vary greatly between and within AML patients and its levels closely correlate with the degree of transcriptional repression (Aggerholm et al., 1999, Cameron et al., 1999). AML classification into ten different subtypes was originally defined by the French-American-British (FAB) cooperative group (Bennett et al., 1976, 1985). Numerous studies have been conducted to assess the methylation levels of \( p15^{INK4b} \) on samples of patients with AML across the different FAB subtypes (Herman et al., 1996, 1997; Aggerholm et al., 1999; Guo et al., 2000; Chim et al., 2001a, 2001b; Christiansen et al., 2003; Garcia-Manero et al., 2003; Teofili et al., 2003; Shimamoto et al., 2005). In patients with adult and childhood AML, hypermethylation of \( p15^{INK4b} \) in cells isolated from bone marrow and peripheral blood is observed in nearly all morphological FAB subtypes. Higher frequencies are generally observed in the M1, M2, M3 and M4 subtypes than in the M5, M6 or M7 subtypes and are found to occur in the vast majority of the patients’ leukemic cells (Aggerholm et al., 1999; Wong et al., 2000; Shimamoto et al., 2005; Tsellou et al., 2005). In patients with therapy-induced AML (t-AML), aberrant methylation of \( p15^{INK4b} \) (in over 90% of patients) is found to be independent from the patient’s type of previous therapy which ranges from alkylating agents, topoisomerase II inhibitors to radiotherapy (Christiansen et al., 2003).

A similar pattern of aberrant methylation is also well documented in patients with MDS (Uchida et al., 1997; Quesnel et al., 1998; Aoki et al., 2000; Tien et al., 2001; Christiansen et al., 2003; Teofili et al., 2003). The FAB classification system for MDS is mainly based on the percentage of blast cells in the bone marrow and the peripheral blood and the degree of cytopenia (Bennet et al., 1982). Methylation levels have also been shown to increase during follow-up and in conversion to overt AML (Tien et al., 2001, Christiansen et al., 2003). Importantly, aberrant DNA methylation of \( p15^{INK4b} \) was found to be one of the most dominant molecular events in MDS progression to AML (Jiang et al., 2009). Similar to AML, cytogenetics of MDS is also a crucial factor in the prognosis and development of the disease (Haase et al., 2007). The World Health Organization (WHO) classification system for myeloid neoplasms was developed and takes into consideration both morphology and cytogenetic abnormalities (Harris et al., 2000). In MDS patients, methylation of \( p15^{INK4b} \) is associated with an increased percentage of immature myeloblasts in the bone marrow (Christiansen et al., 2003). The presence of DNA hypermethylation at the \( p15^{INK4b} \) promoter is found predominantly in high risk MDS patients with increased levels being reported in the subtypes characterized by advanced stages of the disease such as refractory anemia with excess blasts (RAEB). Reduced levels are reported in patients with the early
stages of MDS such as refractory anemia (RA) and refractory anemia with ringed sideroblasts (RARS) (Uchida et al., 1997; Christiansen et al., 2003). However, more recently, it was found that even in patients with the RARS subtype, which falls within the lower risk of MDS, p15INK4b was found to be the most frequently methylated gene (>20% of cases) of 25 known tumor suppressors that were evaluated in the study (Valencia et al., 2011). Although most studies have been conducted in MDS in adults, comparable levels of aberrant methylation patterns have been observed in pediatric MDS patients as well (Hasegawa et al., 2005), and a similar correlation with the disease subtypes has been established (Rodrigues et al., 2010).

In chronic myelomonocytic leukemia (CMML), aberrant methylation of p15INK4b is found in about 60% of cases and is associated with a high proportion of blastic transformation (Tessema et al., 2003). CMML is a disease that was originally categorized under myelodysplastic disorders, but is now classified by the WHO as a disorder that bridges MDS with myeloproliferative features (Harris et al., 1999). In these patients, genomic sequencing techniques have revealed that hypermethylation spans a wide area in the 5' region of the gene and is correlated with reduced expression of the mRNA levels. High variability between and within individual patients, consistent with observations in AML patients, were also reported (Tessema et al., 2003, Aggerholm et al., 1999, Cameron et al., 1999). In the pediatric form of the disease, juvenile myelomonocytic leukemia (JMML), p15INK4b hypermethylation is found to be a less frequent, however, still significant event (17% of cases) (Hasegawa et al., 2005).

With regards to cytogenetic abnormalities, p15INK4b methylation levels have been found to occur at higher frequencies in AML/MDS patients with an unfavorable karyotype (Wong et al., 2000; Galm et al., 2005; Shimamoto et al., 2005; Markus et al., 2007). Cases with unmethylated or low levels of hypermethylated p15INK4b were associated with normal karyotype or with those karyotypic abnormalities that are associated with a favorable prognosis (Wong et al., 2000; Markus et al., 2007). Studies have consistently reported an increased tendency for p15INK4b hypermethylation in unfavorable cytogenetics (Shimamoto et al., 2005). These results suggest interplay between p15INK4b loss of expression and the frequent chromosomal translocations, inversions and deletions observed in AML and MDS. The mechanisms underlying p15INK4b hypermethylation are not completely understood, but a few theories involving maintaining and de-novo DNA methylation through action of DNA methyltransferases (DNMT), as well as histone modification pathways have been suggested to play a role (Paul et al., 2010). Specifically in t-AML and therapy-induced MDS (t-MDS), deletion or loss of chromosome arm 7q, which is the most common cytogenetic abnormality in those categories, has been found to be closely associated with hypermethylation of p15INK4b (Christiansen et al., 2003).

p15INK4b is now used as an independent prognosticator in AML and MDS (Chim et al., 2001b; Teofili et al., 2003; Christiansen et al., 2003; Shimamoto et al., 2005; Chim et al., 2006). In the many categories of the diseases, aberrant p15INK4b methylation levels have been associated with a generally poor prognosis. In studies that monitor patients across all AML FAB subtypes, patients without p15INK4b hypermethylation at diagnosis had increased complete remission rates which also correlated with increased survival times (Shimamoto et al., 2005; Deneberg et al., 2010). Consistent with these observations, in APL patients, abnormal p15INK4b methylation was associated with a shorter disease-free survival (DFS) period and a higher incidence of relapse during the 5-year follow up period (Teofili et al., 2003).
In the relapsed patients, the \( p15INK4b \) hypermethylation levels remained persistent following treatment. In contrast, the patients without detectable hypermethylation displayed prolonged survival (Chim et al., 2001b). Additionally, as previously stated, patients with MDS with high methylation levels at diagnosis had a significantly higher chance of the disease progressing to AML (Tien et al., 2001; Jiang et al., 2009). It was also reported that in early stage of MDS, the \( p15INK4b \) hypermethylation is a negative risk factor for patients, closely correlating with leukemic transformation (Aggerholm et al., 2006). The same correlation has been shown in patients with t-MDS, in which methylation resulted in significantly shorter survival (Christiansen et al., 2003). A recent study showed that the high levels of methylation in lower risk MDS categories suggest a poor prognosis in those patients as well (Valencia et al, 2011). In JMML \( p15INK4b \) hypermethylation was associated with reduced overall survival rates and higher relapse of the disease following hematopoietic stem cell transplantation (Olk-Batz et al., 2011). All these results suggest that lack of \( p15INK4b \) expression, mediated by promoter hypermethylation, not only affects the prognosis in patients with AML and MDS, but can be used to predict the outcome of the diseases.

The studies described above confirm that aberrant hypermethylation levels of \( p15INK4b \) have important prognostic implications for clinical monitoring in MDS and assessment of risk of progression into AML. However, its potential use as a biomarker in leukemia excluded estimation of minimal residual disease in patients who have achieved clinical remission, and its implications in terms of subsequent relapse. A study aiming at addressing this issue, evaluated \( p15INK4b \) methylation levels in AML patients in complete clinical remission (Agrawal et al., 2007). The study reported that even in remission, leukemia patients that harbored a significant amount of methylation in the bone marrow cells had a higher risk for leukemia relapse. Moreover, the time of disease-free survival was found to be significantly reduced in correlation with the amount of residual hypermethylation of the \( p15INK4b \) gene. Concurrently, low levels of \( p15INK4b \) methylation during complete remission were associated with reduced relapse rates during the 12 month follow-up. It was suggested that analysis of \( p15INK4b \) methylation levels during clinical remission can be potentially used as a prognosticator for the occurrence of relapse (Agrawal et al., 2007).

In recent years, it has been suggested in a number of studies that DNA methylation of \( p15INK4b \) could also help predict response to therapy (Grovdal et al., 2007; Shen et al., 2009). Grövdal et al. (2007) studied DNA methylation patterns in older patients with high risk MDS and AML following MDS. Patients were treated with conventional induction therapy. Methylation levels of \( p15INK4b \), \( E\)-cadherin, and \( HIC1 \) (hypermethylated in cancer 1), were assessed prior to initiation of treatment. Abnormal levels of methylation of \( p15INK4b \) alone did not correlate with decreased complete remission (CR), but all patients with all three genes methylated did not achieve CR. Another study, in which patients with MDS and AML were treated with the DNA methyltransferase inhibitor 5-azacytidine (5-aza-C), reported consistent results with these observations (Raj et al., 2007; Tran et al., 2011). Patients with levels exceeding 24% methylation in the \( p15INK4b \) promoter region did not respond to treatment (Raj et al., 2007). The possibility of using methylation of \( p15INK4b \) as an indicator for treatment outcome is still under investigation. However, results suggest that studying \( p15INK4b \) methylation density in conjunction with other altered genes at diagnosis and monitoring its levels following treatment might have predictive information with respect to the patient’s response to treatment.
Although hypermethylation is the most common mode of inactivation of p15INK4b in myeloid neoplasms, other silencing mechanisms have also been described. In AML with chromosome 16 inversion (inv16), the overall p15INK4b methylation levels are found to be very low and almost comparable to levels in normal patients. However, expression of the gene is severely suppressed. In this type of AML, the inversion (16) results in a fusion protein between the core binding factor (CBFβ) and the smooth myosin heavy chain gene (SMMHC). This chimeric transcription factor CBFβ-SMMHC binds directly to the promoter of p15INK4b and represses its expression (Markus et al., 2007). These results further emphasize an important role of p15INK4b silencing in leukemogenesis of the myeloid lineage, and suggest, that in the absence of a repressive epigenetic event, other mechanisms may result in inhibition of p15INK4b expression (Markus et al., 2007).

2.2 Inactivation in other types of human leukemias
In B and T cell acute lymphoblastic leukemias (B-ALL, T-ALL), p15INK4b hypermethylation as well as deletion of the entire 9p21 locus which includes p15INK4b, p16INK4a and ARF genes has been reported (Roussel, 1999; Ruas et al., 1998). Homozygous deletions of p16INK4a and p15INK4b are found in approximately 30% of childhood acute lymphoblastic leukemia at first presentation, with striking rates in T-ALL (60 to 80%), and lower rates in B-cell precursor ALL (5 to 20%) (Drexler HG, 1998; Chim et al., 2001a). Further studies have analyzed methylation levels of the two genes in these disease categories specifically in terms of overall survival and absence of relapse at 6 years of follow-up. p15INK4b and p16INK4a methylation levels were found to occur at similar rates (35%) in adults and children with mature B-ALL (Graf-Einsiedel et al., 2002). Deletion of the entire locus was observed in 12% and 30% of children and adults, respectively. Interestingly, results show that deletion is associated with poor overall survival (OS) in adults only, but not in children (Van Zutven et al., 2005; Mirebeau et al., 2006; Kim et al., 2009). Furthermore, it did not affect the type of relapse or DFS time in children. In untreated adult patients with precursor B-ALL, high methylation levels of p15INK4b (found in 43% of patients) were significantly associated with decreased DFS at 4 years (Hoshino et al., 2002). Recent methylation profiles in 95 children with ALL supported older studies showing that methylation of p15INK4b occurred predominantly in T-ALL as opposed to B-ALL, and p15INK4b is one of the most commonly methylated genes among the 14 genes analyzed (Takeuchi et al., 2011). A clear correlation between increased methylation and prognoses in T-ALL has not been established.

2.3 p15Ink4b as a tumor suppressor in mice
To define the role of p15INK4b as a tumor suppressor in AML, mouse models have been developed and characterized (Lатres et al., 2000; Wolff et al., 2003a, 2004; Bies et al., 2010). These have provided strong experimental evidence to support the hypothesis that loss of p15INK4b function plays an important role in the development of myeloid leukemia.

A p15Ink4b−/− mouse model was first described by Latres et al. (2000). Mice were generated by genetic targeting with elimination of the second coding exon of the p15Ink4b gene. Knockout mice were viable, fertile, and did not exhibit any behavioral abnormalities. Mouse embryonic fibroblasts were found to have a higher proliferation rate and plating efficiency when compared to their wild type counterparts. More importantly, they were more susceptible to transformation with c-myc and ras oncogenes, confirming the reported results that p15Ink4b participates in the tumor suppressor activity triggered after inappropriate
oncogenic ras activation of the Raf-Mek-Erk pathway (Malumbres et al., 2000). However, with respect to AML, deleting p15Ink4b did not result in leukemogenesis in these mice. Of note, extramedullary hematopoiesis and lymphoid hyperplasia in the spleen were observed in mice aged less than 9 months and resulted in death of over 75% of the mice at an older age. Taken together, these experimental results were the first to suggest that p15Ink4b might be playing a tumor suppressor role in AML (Latres et al., 2000).

A role for p15Ink4b in myeloid neoplasia in mice was first supported by the finding that retrovirus-induced AML had hypermethylation of the CpG promoter region of the p15Ink4b gene. Based upon this, further mouse models were developed to determine if loss of p15Ink4b increases susceptibility to myeloid leukemia when additional oncogenic events were provided by retroviral insertional mutagenesis. For these studies, a specific retrovirus with a broad tropism and the capability of inducing a high incidence of myeloid leukemia was constructed (Wolff et al., 2003b and 2004). It consists of a recombinant virus incorporating Moloney murine leukemia virus (Mo-MuLV) sequences, and regulatory LTR sequences of retrovirus 4070A. The recombinant virus, named MOL4070LTR, combines the capacity of 4070A to accelerate myeloid disease with the wide tropism of Mo-MuLV, and successfully produced myeloid disease when inoculated intraperitoneally into wild-type FVB and BALB/c mice as neonates (Wolff et al., 2003b). The p15Ink4b knockout mice were developed using the same targeting vector as described by Latres et al. (2000), and MOL4070LTR was inoculated into neonates. While there was no incidence of disease in control wild-type mice (p15Ink4b+/+), a significant percentage of heterozygous mice (p15Ink4b+/-) developed myeloid leukemia within a year. Surprisingly, a smaller percentage of homozygous knockout mice (p15Ink4b-/-) developed myeloid tumors (Wolff et al., 2003a).

Further experiments demonstrated that in heterozygous p15Ink4b+/- mice, the second remaining p15Ink4b allele was actually hypermethylated, with a reduction of its mRNA expression. This data supported the fact that p15Ink4b functions as a tumor suppressor for myeloid leukemia, however, it was difficult to explain why mice heterozygous for the null allele were more susceptible than homozygous null mice. One explanation might be that in the homozygous null mice, p15Ink4b is lost in all the tissues and loss of expression in one tissue may have compensating effects on loss in another tissue.

A new mouse system in which deletion of the gene is restricted to the myeloid lineage was developed to mimic more closely myeloid lineage disease in man (Bies et al., 2010). The mouse strain utilizes a Cre-loxP system for conditional deletion of the p15Ink4b gene through action of Cre recombinase exclusively expressed in blood cells of the myeloid lineage (Clausen et al., 1999). In this model, Cre recombinase specifically recognizes loxP sites to mediate efficient excision of exon 2 of the p15Ink4b gene in myeloid cells. In order to monitor disease development in mice with targeted p15Ink4b deletion (p15Ink4bfl/flLysMcre), white cell counts were performed in circulating blood from targeted and wild-type animals from different age groups. Interestingly, p15Ink4bfl/flLysMcre mice showed a significant increase in the number of circulating monocytes compared to control mice (p15Ink4bw/wLysMcre), whereas neutrophils, lymphocytes, platelets and red blood cell counts were not affected. Monocytosis remained in targeted mice beyond 8 months of age, while wild-type mice showed a marked decrease in monocytes resulting in an even greater significance in the statistical comparisons (Figure 3A). Expansion of myelomonocytic cells in the bone marrow (BM) of p15Ink4bw/wLysMcre mice was also observed. Analysis of BM cells for the cell surface markers Gr-1, Mac-1 and c-Kit revealed that BM cells from
p15lnk4b\(^{fl/fl}\)/LysMcre mice had a significant increase in both mature myeloid (Gr-1\(^+\)/Mac-1\(^+\)) and monocytic (Gr-1\(^-\)/Mac-1\(^+\)) cells. This increase correlated with a significantly higher proportion of immature myeloid (Mac-1\(^+/c\)-Kit\(^+\)) cells in the BM. Inactivation of \textit{p15lnk4b} in myeloid cells promoted a mild preleukemic myeloproliferative-like disease (Bies et al., 2010). A small percentage of the targeted mice spontaneously progressed to a form of leukemia featuring an increased number of mature circulating myeloid cells in the peripheral blood, as well as an increase in the number of progenitors in the BM. The disease observed in mice most closely resembled an advanced form of CMML (Bies et al., 2010). However, the disease did not progress to an acute form of leukemia over the period of 15 months in any of the mice. These results were in agreement with studies carried on in the embryonal p15lnk4b\(^{-/-}\) mice, and suggested that inactivation of the \textit{p15lnk4b} gene without an additional genetic/epigenetic hit is not sufficient to cause acute leukemia. Retrovirus-induced mutagenesis in p15lnk4b\(^{fl/fl}\)/LysMcre mice was used to identify genetic changes that

Fig. 1. Cell cycle regulation by the CDKI families. CDKs are switched on and off at different times during the cell cycle; the cyclins Ds-dependent kinases 4 and 6, and CDK2/cyclin E, CDK2/cyclin A regulate G1 progression and entry into the S phase. CDK1/cyclin B regulates entry and exit from mitosis. The CDK/cyclin complexes phosphorylate pRb to allow for the transcription of genes under the control of E2Fs which include factors necessary for cell cycle progression. CIP/KIP family members of CDKI can be either activators or inhibitors of cyclin/CDK assembly (Sherr & Roberts, 1999).
could cooperate with the loss of p15Ink4b in leukemia development. Mice inoculated with the MOL4070LTR retrovirus were monitored for 15 months for signs of disease. Control mice developed leukemia with low penetrance, whereas the incidence of retrovirus-induced leukemia was statistically highly significant for the p15Ink4b<sup>fl/fl</sup>LysMcre animals. Additionally, phenotypic analyses of tumor cells demonstrated a strong bias towards the development of AML in the knockout animals. Myeloid-specific inactivation of p15Ink4b results in retrovirus-induced development of tumors mostly monocytic (F4/80<sup>+</sup>, F4/80<sup>+</sup>/Mac1<sup>+</sup>) and myelomonocytic (F4/80<sup>+</sup>/Mac1<sup>+</sup>/Gr-1<sup>+</sup>), whereas there was an equal distribution of lymphoid and monocytic tumors in the control mice (Figure 3B). Results generated using this model complement the embryonal p15nk4b<sup>−/−</sup> studies and demonstrate an active role for p15Ink4b silencing in promoting the establishment of preleukemic conditions. These results also provided strong experimental evidence that p15Ink4b functions as a tumor suppressor for myeloid leukemia development (Bies et al., 2010).

3. Conventional and novel functions of p15INK4b

3.1 p15INK4B as a regulator of the cell cycle

p15INK4b belongs to the INK4 family of cyclin-dependent kinases inhibitors (CDKIs). The family comprises three other members – p16INK4a, p18INK4c and p19INK4d and is one of two families of CDKIs that have been identified and defined based on their structure and CDK specificities. INK4 proteins all show a high and exclusive specificity towards the activity of cyclin-dependent kinase 4 (CDK 4) and cyclin-dependent kinase 6 (CDK6) during the early and mid-G1 phase of the cell cycle (Sherr & Roberts, 1999). The cell cycle is comprised of 4 phases, G1, S, G2 (also referred to as interphase), and M phase (mitosis). Entrance of cells from the quiescent G0/G1 phase into cycle is governed by the actions of kinases CDK4/CDK6 during the transition to S phase, CDKs hyperphosphorylate pRb causing its dissociation from the nuclear transcription factors E2Fs. E2Fs regulate the transcription of genes which are required for the completion of the cell cycle and include cyclins A and E, thymidine synthetase and PCNA (Korenjak & Brehm, 2005). Throughout the S, G2 and M phases, pRb is kept in a hyperphosphorylated state by an orchestrated mechanism that involves sequential activities of multiple cyclins/CDKs (Sherr & Roberts, 1999). INK4 proteins inhibit CDKs/cyclin Ds complexes and, therefore, function in G1-S checkpoint control. When INK4 proteins block formation of these complexes, the pRb is in a hypophosphorylated, active state and interacts with E2F to inhibit its function (Korenjak & Brehm, 2005). Structural studies have demonstrated that INK4 proteins perform their inhibitory activity by allosteric competition with cyclins Ds to bind CDK4 and CDK6. CDK4/6-INK4s protein complexes have reduced affinity toward the D-type cyclins (Jeffrey et al., 2000; Yuan et al 2000).

p15INK4b and p16INK4a are tandemly linked on human chromosome 9p21 within a 40kb DNA region, whereas p18INK4c and p19INK4d are located in the chromosomal regions 1p32.3 and 19p13.2, respectively. The 9p21 chromosomal locus is referred to as the INK4/ARF locus (Baron et al., 1994). In addition to p15INK4b and p16INK4a, it also encodes a third gene called p14ARF, originally identified as an alternative transcript of p16INK4a (Figure 2). p14ARF is transcribed from exon 1β and exons 2 and 3 of p16INK4a, but using a different reading frame (Figure 2). The p14ARF protein (p19Arf in mouse) is immunologically and functionally unrelated to the p16INK4a protein; they are not considered to be isoforms and do not share
sequence homology or overlapping roles in the cell (Ozenne et al., 2010; Sherr CJ, 2006). Furthermore, p14ARF bears little or no structural similarities with the INK4 family members, and is unable to bind or inhibit CDKs. It is not considered to be part of the INK4 family of inhibitors, but it still participates in the negative regulation of the cell cycle by antagonizing the effects of MDM2, a ubiquitin ligase that targets the tumor suppressor protein p53 for degradation by the 26S proteasome (Ruas et al., 1998).

Fig. 2. 9p21 chromosomal locus showing the promoters and exons that are involved in the transcription of p15INK4b, p14ARF and p16INK4a genes. The CpG island is depicted for the p15INK4b gene only and extends throughout the promoter region, exon 1 and part of intron 1 (Herman et al., 1996).

INK4 family members are highly conserved among species, with over 90% identity between the human and the corresponding mouse proteins. In human, the four proteins share roughly 40% sequence homology with each other and have a very closely related structure, characterized by the presence of ankyrin motif tandem repeats. Four ankyrin repeats are found in p15INK4b and p16INK4a, and five repeats in p18INK4c and p19INK4d (Yuan et al., 2000). Ankyrin motif repeats consist of a helix-loop-helix structure that promotes protein-protein interaction (Li et al., 1999). Structural studies involving programmed mutations and generation of chimeric proteins have revealed that the third ankyrin repeat is necessary for the interaction with CDK4 and CDK6, and is responsible for the inhibitory activity of these CDKIs. Crystallography work on the CDK6 bound p16INK4a, p18INK4c and p19INK4d suggested that the INK4 proteins bind to one side of the catalytic cleft, opposite to the cyclin binding site, and that binding and recognition are mediated mostly via hydrogen bonds (Brotherton et al., 1998; Noh et al., 1999; Russo et al., 1998). INK4 proteins were found not to interfere with the cyclin binding site which is consistent with the presence of INK4s/cyclin Ds/CDK4 or CDK6 ternary complexes. When bound in the absence of cyclin D, they cause a conformation switch in CDK4 and CDK6 which distorts the cyclin and the ATP-binding sites leading to rapid recycling of unbound D-type cyclins by the ubiquitin-dependent 26S proteasome. The specificity towards CDK 4 and CDK6 was found to be due primarily to the critical residues involved in the hydrogen bonds with INK4 proteins which are conserved exclusively in CDK4 and CDK6, but not in the other CDKs (Russo et al., 1998). Interestingly, several of the residues necessary for recognition and binding have been reported to be mutated in cancer (Li et al., 1999).

The structural similarities observed between the INK4 proteins are consistent with the shared biological and biochemical properties of these molecules. However, the expression
pattern of each of the human INK4 proteins appears to be highly specific to the cell type and tissue localization, as well as the differentiation stage of the cells (Shwaller et al., 1997; Thullberg et al., 2000). For instance, in normal hematopoietic cells, the expression of \( p15\text{INK4b} \) is shown to be lineage restricted, and is mainly detected in monocytes and lymphocytes, but not in any of the erythroid precursors (Teofili et al., 1998). Myeloid-restricted expression of \( p15\text{INK4b} \) is observed in peripheral blood and bone marrow and its levels are reported to be increased during megakaryocyte and monocyte/macrophage differentiation (Furukawa et al., 2000; Teofili et al., 2001; Haviernik et al., 2003).

\( p15\text{INK4b} \), in particular, is an important downstream effector of anti-proliferative signaling by the transforming growth factor-\( \beta \)1 (TGF-\( \beta \)) (Hannon & Beach, 1994). In different human and mouse cell lines, treatment with this negative growth factor, as well as interleukin 6 (IL-6) or Interferon \( \beta \) (IFN-\( \beta \)) leads to G0/G1 cell cycle arrest (Schmidt et al., 2004; Haviernik et al., 2003). Treatment with TGF-\( \beta \)1 induces a significant increase in the transcription levels of \( p15\text{INK4b} \), but also induces a major increase in \( p15\text{INK4b} \) protein stability (Sandhu et al., 1997). In contrast to \( p15\text{INK4b} \), whose expression is absent in hematopoietic stem cells, but increases as the cells mature along the myeloid lineage, \( p16\text{INK4a} \) is highly expressed in hematopoietic stem cells, and down-regulated with differentiation of all lineages (Furukawa et al., 2000).

\( p18\text{INK4c} \) is found to be the most homogeneously and abundantly expressed member of the family, whereas \( p19\text{INK4d} \) is the most restricted, and its expression is limited to lymphoid cell, epithelial cells, seminiferous tubes and adrenal gland cells (Thullberg et al., 2000). The differential expression patterns of the INK4 proteins, suggest non-overlapping physiological functions.

Despite their common function in regulation of the cell cycle, the four members are found to be differentially involved in tumorigenesis. Mouse knock-out models along with genetic screenings of human tumors and gene expression profiling of cell lines have been used to help elucidate the role of these cell cycle inhibitors in the establishment and progression of cancer (Cánepa et al., 2007). In concordance with the molecular analysis of human tumor tissues, mice deficient in different Ink4 proteins display an increased susceptibility to the development of various types of tumors with variable penetrance. \( p16\text{INK4a} \) is a family member that has a prominent role in carcinomas of the pancreas and the bladder, glioblastomas, leukemias and melanomas among others. Its expression is lost by several mechanisms including point mutations, small deletion and epigenetic modifications which have been reported in thousands of human cancers (Serrano et al., 1996; Krimpenfort et al., 2001). On the other hand, as previously described, \( p15\text{INK4b} \) is noted to be silenced primarily by an epigenetic mechanism in human cancers, and loss of its expression through hypermethylation of its promoter region is well documented in hematologic neoplasms in particular (Drexler HG, 1998; Roussel, 1999). In these types of cancers, inactivation of \( p15\text{INK4b} \) has been reported in the absence of aberrant modification or deletion of \( p16\text{INK4a} \). In contrast with \( p15\text{INK4b} \) and \( p16\text{INK4a} \), \( p18\text{INK4c} \) was originally found to play a more limited role as a human tumor suppressor, and \( p19\text{INK4d} \) is not thought to be involved in the pathogenesis of cancer (Thullberg et al., 2000). \( p18\text{INK4c} \)-null mice are viable but display an unusual phenotype with pronounced gigantism, lymphomas and more importantly pituitary hyperplasia (Franklin et al., 1998). Later examination of these mice revealed that \( p18\text{INK4c} \) is a haploinsufficient tumor suppressor for spontaneous and carcinogen-induced
pituitary tumors and lymphomas (Bai et al., 2003). Loss of p16Ink4a expression was shown to be a necessary event in conjunction with loss of p18Ink4c for the mice to develop aggressive advanced stages of pituitary carcinoma (Morishita et al., 2004). Furthermore, it is now clear that p18Ink4c is a tumor suppressor in human glioblastoma multiform and hepatocellular carcinoma (Solomon, 2008a and 2008b). Interestingly, studies in mice deficient for p19Ink4d have not revealed increased susceptibility to any cancer or other proliferative diseases, suggesting a limited or nonexistent role in carcinogenesis (Buchof et al. 2007, Zindy et al, 2000).

Although these cell cycle regulators exhibit overlapping and surely compensatory activities mainly due to their structural similarities (Krimpenfort et al., 2007), their temporal and tissue-restricted expression patterns as well as their differential involvement in the pathogenesis of human cancers clearly suggest that they can harbor specific and distinct functions during development.

3.2 Function as a regulator of cell fate during early myelopoiesis

The strikingly high prevalence of p15INK4b loss of expression during the development of myeloid disease in human patients has triggered scientists to explore alternate functions for this CDKI. As a cell cycle regulator, p15INK4b was suggested to be one of the players determining the fine balances in differentiation and proliferation of myeloid cells. To test this hypothesis, the previously described p15Ink4b germline knockout mice as well as murine transplant models were used. Results revealed a novel role for the protein during early and late stages of myeloid cells development (Rosu-Myles et al., 2007, 2008).

Myelopoiesis is the process by which an undifferentiated progenitor cell gives rise to mature differentiated functional myeloid cells. The process takes place in the bone marrow and is driven by a pool of hematopoietic cytokines that have different binding specificities to cell surface markers in a stage and lineage dependent manner. Committed progenitors with a restricted myeloid lineage fate arise from earlier common myeloid progenitor cells (CMP), who themselves originate from hematopoietic stem cells (HSC) (Akashi et al. 2000).

In p15Ink4b knockout mice (p15Ink4b−/−), loss of p15Ink4b was found to favor the differentiation of CMPs into granulocyte macrophage progenitors (GMP) which results in an imbalance between the erythroid and myeloid compartments (Rosu-Myles et al 2007). This defect was in the bipotent differentiation capacity of the CMP, and did not affect the frequency of early long-term HSCs, or their ability to self-renew and proliferate. Therefore, this finding differs from the traditional role of p15Ink4b in regulating the cell cycle. As shown in Figure 3A, the increased number of myeloid progenitors was found to occur at the expense of differentiation of CMPs towards the erythroid progenitors. Furthermore, competitive repopulating assays have shown that the defect is intrinsic to the cells. Loss of p15Ink4b provided a competitive advantage over the wild-type cells within the myeloid compartment (Rosu-Myles et al., 2007).

Interestingly, an earlier study carried out in p18Ink4c−/− mice, had shown that this CDKI also impacts cell fate but targets a different cell type. Deletion of p18Ink4c was found to result in a long-term engraftment advantage in HSCs. The observed effects were not due to increased proliferation capacity of the cells, but rather to an enhanced potential of self-renewal of these cells as opposed to differentiation. This lead to an efficient expansion of HSCs as well as hematopoietic progenitor pools, which fully retained their multi-lineage differentiation potential (Yuan et al., 2004).
3.3 Function in cell cycle arrest in late myeloid cell development
In addition to its hypothesized role in cell fate decision of early myeloid progenitors, p15Ink4b is implicated during the late stages of myelopoiesis. In this case its role appears to be the induction of cell-cycle arrest. p15Ink4b expression has been shown to increase specifically during myeloid differentiation in vivo both in human bone marrow and peripheral blood cells (Teofili et al. 2000); and in vitro, in murine M1 myeloblastic cells which undergo monocytic differentiation following treatment with IL-6 (Schmidt et al 2004).

Fig. 3. The loss of p15Ink4b in myeloid lineage results in: A. Imbalance in the myeloid progenitor pools and nonreactive monocytosis; B. Increased incidence of the retrovirus-induced leukemia with preference toward the myeloid phenotype.
The M1 leukemia cell line, does not express p53, proliferates autonomously and is often used as a model for monocytic differentiation. When terminal differentiation is induced in these cells, upregulation of p15Ink4b expression is accompanied by inhibition of Cdk4 kinase activity as well as by a decrease in levels of phosphorylated Rb (Bergh et al., 1999). Furthermore, over-expression of p15Ink4b in M1 cells causes cell cycle arrest in the G1 phase, providing additional evidence for the implication of the protein in maturation and cell cycle inhibition of late stage progenitors (Haviernik et al., 2003). This function is further supported by studies in human CD34+ hematopoietic progenitor cells. When expression of p15INK4b is triggered, higher levels of the protein are associated with transcriptional upregulation of genes known to induce myeloid differentiation, such as the colony stimulating factor 1 receptor gene (c-fms), the myeloperoxidase gene (mpo) and lactoferrin (lf), among others (Furukawa et al 2000). Overexpression of p15INK4b is also linked with a dramatic decrease in early blast progenitor populations and an increase in the numbers of cells that adopt a myeloid morphology (Teofili et al., 2000).

This data suggests that p15INK4b has functions during both the early and late stages of myelopoiesis; in early progenitors, p15Ink4b influences cell fate by altering the balance between myeloid and erythroid progenitors, whereas during late myelopoiesis, p15INK4b appears to be causing withdrawal from the cell-cycle in response to cytokines.

4. Targeting p15INK4b for re-expression in AML and MDS

The reversible nature of epigenetic alterations makes them very attractive therapeutic targets for AML and MDS. In the case of p15INK4b, these epigenetic alterations consist of DNA methylation as well as chromatin remodeling by post-transcriptional histone modifications. Studying the association of p15INK4b promoter DNA methylation with histone modifications revealed important insight into the interplay of these two types of epigenetic mechanisms (Paul et al., 2010). Histones undergo post-transcriptional modifications that target primarily the N-terminal tail regions, and involve the attachment of phospho, acetyl, methyl, ribosyl, and small ubiquitin-like modifier or ubiquitin groups on the side chains of the different amino acids residues of histone molecules (Biancotto et al., 2010). Acetylation and de-acetylation of lysine residues are catalyzed by two groups of enzymes with opposing actions: histone acetyltransferases (HAT) and histone deacetylases (HDAC) (Jenuwein & Allis, 2001), whereas histone methyltransferases (HMT), and histone demethylases (HDM) control the balance of histone methylations. Similar to DNA methylation, histone modifications are fully biochemically reversible, result in changes in the protein structure and affect the affinity of histone tails to DNA molecules (Varier & Timmers, 2011). Paul et al. (2010) found that in AML cell lines with aberrant p15INK4b DNA hypermethylation, the histone 3 trimethylated at lysine 4 (H3K4me3), which is a transcriptional activation mark, was at lower levels than in AML cell lines without hypermethylation. Interestingly, irrespective of the methylation status of p15INK4b, this study also reported the presence of the repressive mark H3K27me3 (histone 3 trimethylated at lysine 27) at the 9p21 locus. Human AML blasts with hypermethylation of p15INK4b were similarly found to have H3K27me3, but lacked H3K4me3 at the gene.

The tight collaboration of the different epigenetic alterations in silencing the p15INK4b gene makes combinatorial therapeutics a promising approach for its reexpression. Importantly, removal of methyl groups from hypermethylated CpG clusters associated with the gene promoter reverses the inhibitory effects and restores normal gene expression (Jones &
Baylin, 2002). The DNMT inhibitor 5-azacytidine (5-aza-C) and its analogue 5-aza-deoxycytidine (5-aza-dC, Decitabine) are powerful hypomethylating agents that are used in the therapy of high-risk MDS and AML. It has been demonstrated that they can lead to the reversal of hypermethylation and subsequent reexpression of the p15INK4b gene in patients with MDS (Daskalakis et al., 2002; Farinha et al., 2004; Gore et al., 2006; Santos et al., 2010). It was shown that treatment of patient blasts with hypomethylating agent Decitabine also affects histone modifications. Paul et al. (2010) provided evidence that the levels of H3K4me3 increased with retention of H3K27me3, thus inducing a state of bivalency. The use of other HMT inhibitors such as 3-Deazaneplanocin A (DZNep) have been reported to successfully decrease global DNA methylation levels, but not to induce re-expression of specific genes including p15INK4b (Flotho et al., 2009; Miranda et al., 2009).

HDAC inhibitors such as Trichostatin A (TSA) have been used for targeting of histone acetylations. In the case of AML samples with p15INK4b hypermethylation, it was shown that TSA treatment alone is insufficient to increase p15INK4b expression levels (Scott et al., 2007; Paul et al., 2010). However, studies that incorporated the use of both HDAC and DNMT inhibitors have proven successful at inducing higher levels of p15INK4b expression (Cameron et al., 1999; Scott et al., 2007; Paul et al., 2010).

5. Conclusion

p15INK4b is a CDK inhibitor whose expression is lost in a very high proportion of patients with MDS and AML. This implicates an important role for the loss of the protein expression in the development of myeloid disease in humans. Its role in myeloid leukemogenesis as a tumor suppressor is now confirmed by research carried out in several mouse models. In addition to its role in inhibiting CDKs to arrest the cell cycle during late myeloid development, it is now known to affect cell fate decisions during early myelopoiesis. In myeloid progenitors, loss of p15Ink4b results in an imbalance in the progenitor pools, and favors the expansion of GMPs at the expense of MEPs. Importantly, when deleted in the cells of the myeloid lineage only, mice develop nonreactive monocytosis and are strongly predisposed to succumb to retrovirus-induced leukemia, especially of myeloid origin. These results validate a tight link between loss of p15INK4b and human myeloid neoplasia. Clinical observations that associate p15INK4b demethylation and re-expression with improved prognosis and survival, further stress the importance of this gene in AML and MDS treatment.

6. References


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The current book comprises a series of chapters from experts in the field of myeloid cell biology and myeloid leukemia pathogenesis. It is meant to provide reviews about current knowledge in the area of basic science of acute (AML) and chronic myeloid leukemia (CML) as well as original publications covering specific aspects of these important diseases. Covering the specifics of leukemia biology and pathogenesis by authors from different parts of the World, including America, Europe, Africa, and Asia, this book provides a colorful view on research activities in this field around the globe.

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