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Novel Targets in Myelogenous Leukemia: The Id Family of Proteins

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1. Introduction

1.1 Myelogenous leukemia and the need for novel drug targets

The successful treatment of myelogenous leukemia depends upon the continuing coordinate efforts of the medical and research communities and patients to understand the complex nature of leukemia development and progression, and to battle drug resistant cells. Novel drug therapies, like Imatinib, have been developed which target the mutant BCR-ABL (Breakpoint Cluster Region-Abelson Kinase) protein, and have significantly changed the treatment regimens for chronic myelogenous leukemia (CML). However, while Imatinib is frequently effective against the majority of the leukemic cells, and patients often undergo remission after treatment, these patients also relapse due to a leukemic drug-resistant population (Schemionek et al., 2010). In comparison to CML, the treatment for acute myelogenous leukemia (AML) is predominantly induction therapy using strong anti-mitotic agents, followed by consolidation therapy to kill residual diseased cells (Venditti et al., 2000). These therapies are not specifically targeted to mutated proteins in the cells, and therefore can be more toxic to the patient's normal cells. Researchers have attempted to target mutated proteins in AML. However, unlike BCR-ABL, targeting FLT3-ITD (FMS-like tyrosine kinase 3-Internal Tandem Duplication) and FLT3-TKD (FMS-like tyrosine kinase 3-Tyrosine Kinase Domain) mutations have not been as successful as CML-Imatinib therapy (Kindler et al., 2010). Therefore, challenges facing patients with AML remain significant, and drug-resistant relapses remain a threat.

1.2 Transcription factor deregulation in myelogenous leukemia

To identify novel drug targets, researchers will have to gain a better understanding of the molecular mechanisms that regulate normal hematopoietic development. Hematopoiesis depends upon the activity of transcription factors and their regulators to function at the correct time and place during differentiation. Failure to control these processes can lead to deregulated proliferation and impaired normal differentiation. Ultimately, these errors can

lead to myeloproliferative diseases (MPD), myelodysplastic syndromes (MDS), and chronic or acute myelogenous leukemia (Rosenbauer et al., 2005). Current research efforts are focused on the molecular mechanism(s) that contribute to transcription factor deregulation with a goal of designing better, safer and less toxic therapies, to restore normal differentiation or initiate apoptosis of leukemic cell populations. Deregulation can result from many different types of mutations including translocations, deletions, and point mutations, which can result in loss of function, gain of function, overexpression or underexpression. The Id (Inhibitor of DNA Binding) proteins are key downstream transcriptional regulators of multiple neoplastic mutations and are deregulated (have increased expression) in many types of cancer, and therefore may be good therapeutic targets particularly for drug-resistant forms of leukemia.

2. Id proteins inhibit transcription factors that regulate cell cycle and differentiation

2.1 Tissue specific expression of Id proteins

The Id proteins belong to a subclass of the helix-loop-helix (HLH) family of proteins. There are four family members (Id1, Id2, Id3, Id4), which range in size from 13 to 20 kDa (**Figure 1**). In normal tissues, Id1 and Id3 are ubiquitously expressed; they are detectable throughout embryonic development and are present in the bone marrow, testis, kidney, brain, liver and spleen. The expression of Id2 and Id4 are more restricted (Riechmann et al., 1994). Id2, is not detectable until day 13.5 of fetal liver development. Id2 is most highly expressed in the bone

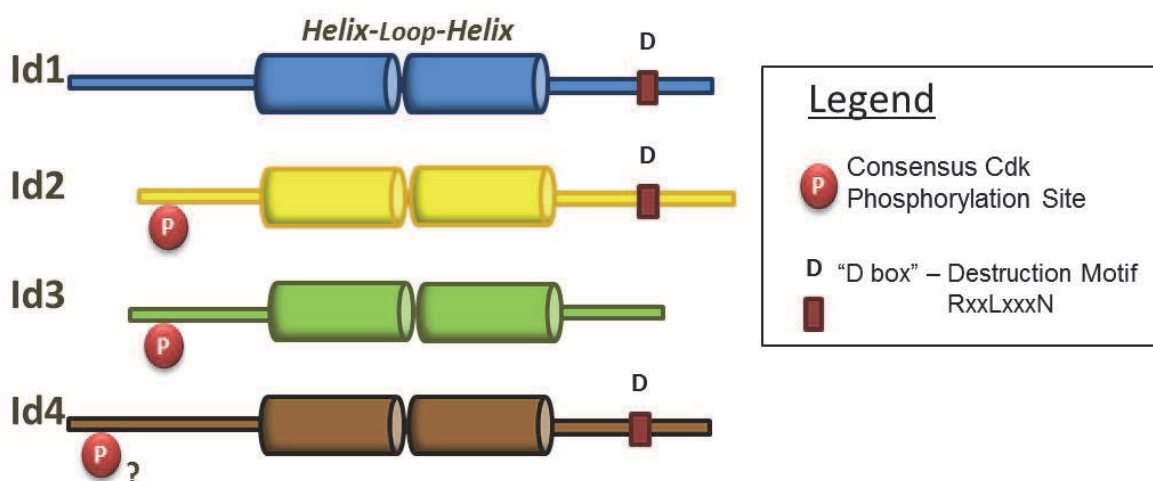


Fig. 1. Schematic representation of the Id family

Id2, Id3, and Id4 have Cdk2 phosphorylation sites, although only Id2 and Id3 are known to be phosphorylated by cyclinA/E-Cdk2. Cdk2-dependent phosphorylation of Id2 (Hara et al., 1997) and Id3 (Deed et al., 1997) inhibits binding to E proteins and Ets transcription factors. Id proteins are ubiquitinated and undergo rapid turnover during the cell cycle mediated by ubiquitination and proteosomal degradation, and two different targeting mechanisms have been reported. Id1, Id2, and Id4 have "D box" destruction motifs (RxxLxxxN) which is recognized by the anaphase promoting complex (APC) which targets the proteins for ubiquitination (Harper et al., 2002; Lasorella et al., 2006). Degradation of Id1 and Id3, is mediated by the COP9 signalosome (Berse et al., 2004).

marrow, testis and brain. Id4 is expressed during embryogenesis, but is not detectable in the fetal liver. Id4 expression is present in the adult bone marrow, testis, kidney, brain, and spleen. These data suggest that the Id proteins may be critical during development, as well as having important functions in the adult. In this regard, simultaneous deletion of any two Id gene family members results in early embryonic lethality, demonstrating that these genes are required for development (Fraidenraich et al., 2004). Therefore, in summary, as a family the Id proteins are present in many different tissues, and are expressed during early development as well as in adult tissues.

2.2 Id protein function: Regulation of transcription factors and cell cycle regulators

The Id proteins function by blocking the DNA binding activity of three types of essential transcription factors: (1) The basic Helix-Loop-Helix (bHLH) proteins (E proteins), (2) Helix-Turn-Helix ETS transcription factors, and (3) Pax transcription factors (Pax-5 in B cells) (Figure 2). In addition, specific members of the Id family decrease the activity of Rb tumor suppressors (Iavarone et al., 1994; Lasorella et al., 1996).

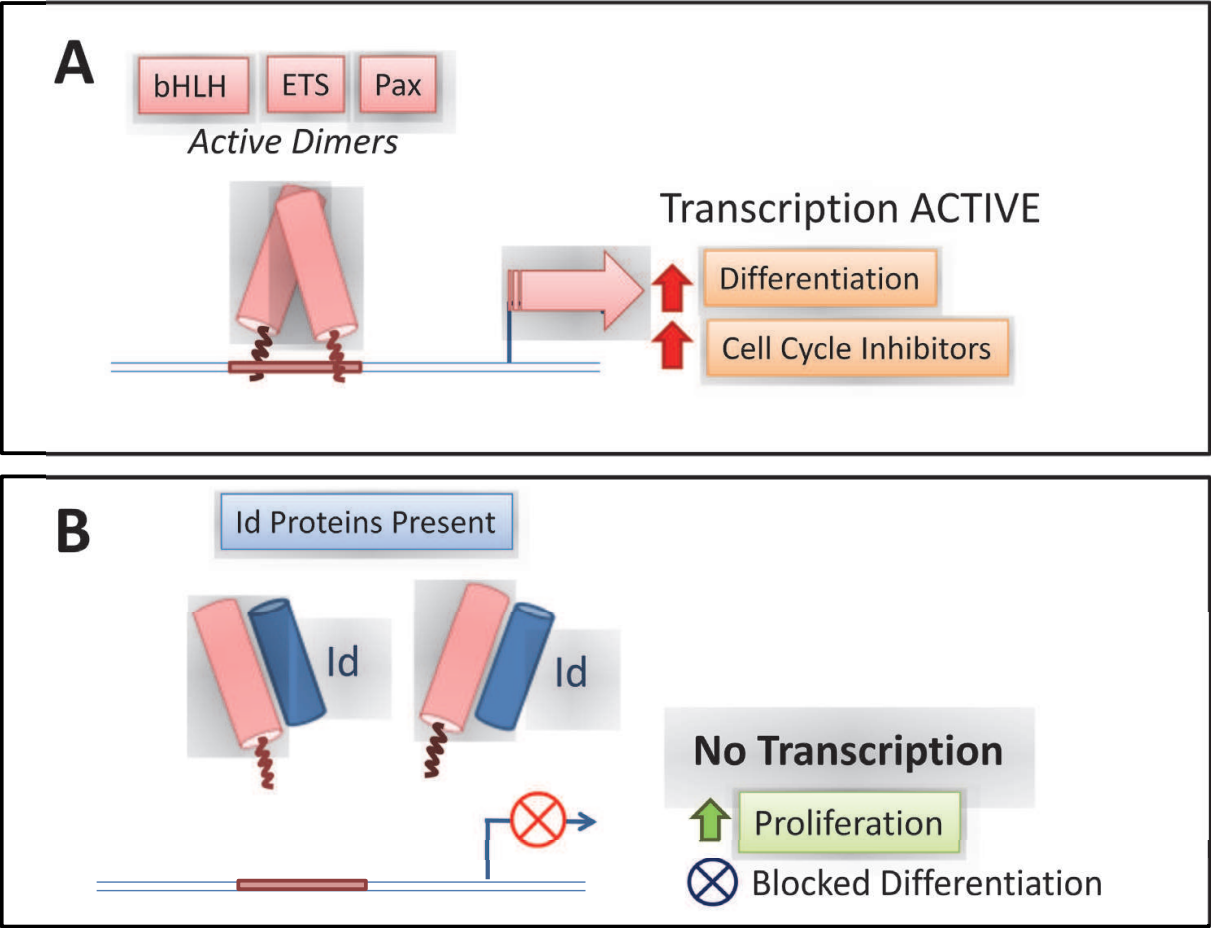


Fig. 2. Id proteins bind bHLH, ETS, and Pax transcription factors and block their function. (A) bHLH, ETS, and Pax factors dimerize and bind to and activate the promoter regions of genes essential for differentiation and cell cycle arrest. (B) When Id proteins are present, they bind to bHLH, ETS and Pax transcription factors, blocking their dimerization and inhibiting their ability to activate transcription.

E proteins and regulation of differentiation. The HLH transcription factors are essential for regulating the differentiation programs of multiple tissues types. The E proteins, also known as "Class A" HLH family members, include *E2A* (encoding E12 and E47 proteins), *HEB*, and *E2-2*. E proteins homodimerize or heterodimerize with "Class B" bHLH family members, tissue specific proteins such as *SCL/tal1* (hematopoiesis), *HAND*, *MyoD*, myogenin (myogenesis), *NeuroD/BETA2* (pancreatic development and neurogenesis), *HES-1*, and *MASH-1* (neurogenesis). When dimerized, these bHLH proteins bind to E box consensus sequence (CANNTG, where "N" is any nucleotide) through their basic N-terminal regions. When Id proteins are present, they bind to the E proteins and disrupt their dimerization with the Class B binding partners (Langlands et al., 1997), and block the differentiation of muscle, neuronal, pancreatic and hematopoietic cells. The Id proteins homodimerize poorly with each other and preferentially bind to the Class A HLH factors (Sun et al., 1991). In summary, the effect of Id proteins, prevents or arrests differentiation by blocking the E protein transcription activating function in many different cell types.

E proteins and cell cycle control. The E proteins also induce the expression of cell cycle inhibitors, such as *p16^{INK4a}* and *p21^{Cip1}*. The cell cycle inhibitors cyclin dependent kinases 4 and 6 (*Cdk4*, *Cdk6*), are necessary for the cell to progress to S phase. Therefore, Id proteins inhibit the transcriptional activity of the E proteins, which decrease the expression of cell cycle inhibitors, and ultimately results in increased cellular proliferation (Alani et al., 2001; Zheng et al., 2004; Prabhu et al., 1997). In support of this, when Id1 is knocked-out in mice, mouse embryo fibroblasts that lack Id1 (*Id1*^{-/-}) undergo premature senescence, due in part to increased expression of *p16^{INK4a}* (Alani et al., 2001). Thus, increased expression of Id1 proteins promotes cell proliferation by inhibiting the function of E protein transcriptional activity, by blocking their ability to induce the expression of key cell cycle inhibitors.

Regulation of ETS proteins by Ids. The Id proteins also regulate the activity of the ETS (E twenty-six) helix-turn-helix family of transcription factors. The ETS proteins comprise one of the largest families of transcription factors, and they bind the canonical sequence GGAA/T. The ETS proteins are characterized by the presence of the ETS DNA binding domain, and the family includes *PU.1* (*SPI1*), *Ets1*, *Ets2*, *TEL*, and *TEL2* as well as the ternary complex factors (TCF): *Elk1*, *Elk3* (*Net/SAP2*), and *Elk4* (*SAP1*). The Id family members interact with the ETS protein DNA binding domain, blocking the ability of the ETS proteins to bind DNA, and repressing their ability to activate transcription. Id1, Id2 and Id3 have all been shown to bind to the TCF proteins *Elk-1* and *Elk-4*, and Id1 and Id3 both suppress the expression of the cell cycle inhibitor *p27^{Kip1}* by blocking *Elk-1*'s ability to activate transcription (Yates et al., 1999; Chassot et al., 2007). As previously described, Ids can decrease *p16^{INK4a}* protein expression by repressing E protein activity, in addition, Id1 has also been shown to reduce *p16^{INK4a}* expression by the inhibiting the activation of its expression by *Ets1* and *Ets2* (Ohtani et al., 2001). Interestingly, the ability of the Id proteins to bind to the ETS transcription factors can be inhibited by phosphorylation of the Ids. Within the Id family, only Id2 and Id3 undergo phosphorylation, and phosphorylation of these Ids by cyclin A/E-*Cdk2* decreases their ability to bind to ETS proteins (specifically, *Elk-1*), resulting in cell cycle arrest (Stinson et al., 2003). This mechanism may compose part of a normal cell cycle regulatory feedback mechanism in cells that permits cell populations to expand and then return to a growth arrested state. In summary, Id proteins inhibit the function of the ETS proteins, blocking their ability to activate transcription of cell cycle inhibitors, which results in increased proliferation.

Retinoblastoma (Rb) and p53 tumor suppressors. The retinoblastoma protein (Rb), is an important part of normal cell cycle control. When Rb is hypo-phosphorylated, it is active and bound to E2F, causing cell cycle arrest by preventing E2F from inducing the expression of genes necessary for cell proliferation (Sherr and McCormick, 2002). When Rb is phosphorylated by the cyclin dependent kinases (Cdks), it dissociates from E2F, and this allows E2F to activate the expression of genes necessary for DNA replication. Rb is frequently mutated in many different malignancies, and finding ways to restore Rb function remains a goal in cancer research. Interestingly, Id2 alone of the Id family binds to the hypo-phosphorylated Rb, as well as related proteins p107 and p130 and inhibits their growth suppressive activities. The Id proteins do not affect the expression levels or phosphorylation state of Rb (Lasorella et al., 1996). However, when Id2 binds in the pocket-binding of Rb site it prevents it from arresting the cell cycle (Iavarone et al., 1994; Lasorella et al., 1996). There are also reports that Id1 can decrease p53 activity, although it is not known whether this is a direct or an indirect effect. The reduction of p53 activity results in decreased PTEN tumor suppressor expression, and ultimately results in increased cellular proliferation (Lee et al., 2009).

In summary, the Id family of proteins affects both differentiation and cell cycle progression by inhibiting the DNA binding activity of the E and ETS protein transcription factors, by binding to and disrupting the activity of Rb and related proteins, and by decreasing p53 activity.

3. The role of Id proteins in normal myelopoiesis

3.1 Id protein levels during myelopoiesis

Id proteins were first identified in a mouse leukemic cell line in the search for novel factors containing HLH domains. Benezra et al. identified the first Id protein in murine erythroleukemia (MEL) cells in 1990 (Benezra et al., 1990). They observed that Id1 was present in less-differentiated cell types, and found that Id expression decreased after differentiation was induced in erythroid, muscle, and endothelial cell lines. Subsequently, using a myeloid progenitor cell line, 32DCI3, it was found that endogenous Id1 expression rapidly decreased in cells induced to differentiate to neutrophilic granulocytes by granulocyte colony stimulating factor (G-CSF), then rose again during the later stages of differentiation. Id2 expression also decreased following G-CSF treatment of 32DCI3 cells (Leeanansaksiri et al., 2005), as well as in multiple other myeloid cell lines (Ishiguro et al., 1996). When Id1 concentration was artificially elevated in the 32DCI3 cells, the cells failed to differentiate in response to G-CSF (Kreider et al., 1992). Similarly, overexpression of Id1 in MEL cells also resulted in a block in differentiation (Shoji et al., 1994). Therefore, in normal cells, Id1 expression needs to decrease for a defined period after the induction of differentiation. If Id proteins are overexpressed during this phase, the cells do not differentiate in spite of the extracellular signals instructing them to mature.

In mouse bone marrow, moderate levels of Id1 are present in the hematopoietic stem cells (HSC) and common lymphoid progenitors (CLP). Id1 expression is increased in the more differentiated common myeloid progenitors (CMP), and further increased in the granulocyte macrophage progenitors (GMP), however Id1 levels do not increase in megakaryocyte erythroid progenitors (MEP). The dynamic levels of Id1 expression during these early stages of hematopoiesis suggest that Id1 may regulate cell fate decisions during myeloid, erythroid, and lymphoid development (Leeanansaksiri et al., 2005). In support of this

hypothesis, culture of mouse bone marrow cells in the presence of interleukin-3 (IL-3) or IL-3 with stem cell factor (SCF), or GM-CSF, which promote granulocyte and macrophage development, all increased Id1 expression. Correspondingly, culture of human CD34⁺ bone marrow cells with myeloid growth factors SCF and IL-3, SCF and macrophage colony stimulating factor (M-CSF), SCF and granulocyte macrophage colony stimulating factor (GM-CSF), or with GM-CSF alone also induced Id1 expression (Suh et al., 2008). However, SCF alone, SCF and erythropoietin (EPO), or SCF and thrombopoietin (TPO), which promote erythroid and megakaryocyte development, did not induce Id1 expression in murine or human hematopoietic progenitors. Furthermore, initial reports showed that Id2 expression increases during differentiation of myeloid cells (Ishiguro et al., 1996), although the onset of its expression in myelopoiesis is later in hematopoietic development than Id1 (Cooper et al., 1997). In summary, in normal mouse and human progenitor cells, hematopoietic growth factors that promote granulocyte and macrophage development induce the expression of Id1.

3.2 Id protein levels rise in later stages of differentiation

The study by Kreider et al., which examined Id1 expression during induction of differentiation in 32DC13 cells by G-CSF showed that Id1 levels also increased during terminal neutrophil differentiation of 32DC13 (Kreider et al., 1992). In agreement with this, Shoji et al. demonstrated that if Id1 is overexpressed in later stages of myeloid differentiation, that Id1 did not block myeloid maturation, as in the early stages of myeloid differentiation (Shoji et al., 1994). These reports suggest that Id protein expression may have an additional role in terminal myeloid differentiation. This model is supported by studies in human CD34⁺ cord blood cells that show ectopic expression of Id1 can enhance neutrophil development; and expression of Id2 accelerates final maturation of eosinophils and neutrophils (Buitenhuis et al., 2005). In addition, restoring Id1 expression (along with C/EBP α expression) in cells isolated from patients with low-risk myelodysplastic syndrome (MDS) restores neutrophil maturation *in vitro* (Geest et al., 2009). Thus, the expression of the Id proteins is biphasic during normal hematopoietic development, appearing first in the stem and progenitor stages, decreases during the intermediate developmental stages, and then rises again during terminal differentiation.

3.3 Loss of Id proteins disrupts normal hematopoietic development

The Id proteins are necessary for proper hematopoietic development, including progenitor expansion and fate determination. While Id1 knockout mice are viable and show no overt developmental defects, several groups have reported that hematopoietic stem and progenitor cells in these mice show impaired development (Yan et al., 1997; Perry et al., 2007; Suh et al., 2009). Specifically, the hematopoietic stem cells responsible for long term engraftment and repopulation (LT-HSC) fail to compete against co-transplanted bone marrow cells as well as normal control bone marrow LT-HSC (Jankovic et al., 2007; Perry et al., 2007). However, four serial transplantations of Id1^{-/-} bone marrow, without the addition of co-transplanted competitor bone marrow, supported equal engraftment and viability of the transplant recipients as wild type mouse bone marrow cells, suggesting the intrinsic loss of Id1 does not impair the fundamental long-term self-renewal ability of LT-HSC (Suh et al., 2009). In addition, these studies also showed that the loss of Id1 also significantly alters the bone marrow microenvironment, specifically the stromal cells and the hematopoietic

growth factors they produce, reducing the amount of SCF and SDF produced, and increasing the amount of GM-CSF, G-CSF, and M-CSF produced by the stromal cells (Suh et al., 2009). These changes in the stromal microenvironment may explain the myeloid-priming effect reported in the Id1-null LT-HSC (Jankovic et al., 2007). In summary, the loss of Id1 can disrupt normal hematopoiesis, causing significant changes in the bone marrow microenvironment as well as in the hematopoietic stem and progenitor populations.

While Id1 expression is important for maintenance of the hematopoietic microenvironment, and affects the competitive repopulation ability of LT-HSC, Id2 is essential for normal hematopoietic development as demonstrated by deletion of Id2 in mouse models. Id2 knockout mouse models have defects in natural killer (NK) and dendritic cell development, as well as impaired lymphopoiesis and erythroid defects (Yokota et al., 1999). Id2 regulates fate determination in myeloid development by interaction with the ETS transcription factor PU.1. Id2 relieves PU.1 repression of the GATA-1 transcription factor, which shifts progenitor cell development from a monocyte/granulocyte differentiation program to an erythroid program (Ji et al., 2008). In summary, Id2 is essential for normal hematopoietic development and is a key regulatory factor in the fate determination of B and erythroid cell development.

In contrast to its family members Id1 and Id2, the normal physiological role of Id3 appears to be regulation of lymphoid development. Specifically Id3 has a major role in supporting the proper maturation of B cells, as mice that are Id3 null have defects in B cell proliferation and maturation (Pan et al., 1999; Rivera et al., 2000). However, Id3 is not normally expressed in myeloid development, nor is Id4 (Ishiguro et al., 1995; Ishiguro et al., 1996). The levels at which the Id proteins are expressed in the cells and the stage in differentiation at which they are expressed as well as the type of cell in which they are expressed affect how Id proteins function "normally" in the cells. Altogether, the Id family of proteins has two functions in normal cells: to regulate the process of fate determination and differentiation, and to control cell proliferation.

4. Id proteins and leukemia

4.1 Ids and leukemogenesis

Id proteins have oncogenic properties in myeloid cells. Overexpression of the Id genes is associated with malignancy and poor prognosis in many different tissues (**Table 1**). Whether increased expression of Id proteins are a by-product of deregulated growth or are causative is still under investigation. Id gene overexpression in cell lines results in hyperproliferation and blocked differentiation. For example, Shoji et al., demonstrated that Id1 overexpression inhibits differentiation of erythroleukemia cell lines (Shoji et al., 1994) and Kreider et al. demonstrated Id1 blocks 32DC13 myeloid progenitor differentiation (Kreider et al., 1992).

When Id protein expression is deregulated, normal progenitor expansion and differentiation becomes unbalanced and shifts towards progenitor hyperproliferation. In the myeloid lineage, forced expression of Id1 or Id2 can impair monocyte, granulocyte, and erythroid maturation (Jen et al., 1996; Kreider et al., 1992; Leraanansaksiri et al., 2005; Lister et al., 1995). Furthermore, in mouse bone marrow cells, overexpression of either Id1 or Id3 can immortalize growth factor-dependent hematopoietic progenitors *in vitro*, resulting in cells with an AML-like morphology. Immunophenotypic and gene expression analyses of these

Id Family Member	Tumor Type	Reference
Id1, Id3	Glioblastoma, Medulloblastoma, Neuroblastoma	(Lyden et al., 1999)
Id1, Id2, Id3	Pancreatic Cancer	(Kleeff et al., 1998; Maruyama et al., 1999)
Id1, Id2, Id3, Id4	Testicular Seminoma	(Sablitzky et al., 1998)
Id1	Thyroid Cancer	(Kebebew et al., 2000, 2003)
Id1, Id2, Id3	Squamous Cell Carcinoma	(Hu et al., 2001; Langlands et al., 2000; Nishimine et al., 2003; Wang et al., 2002)
Id1	Breast Cancer	(Lin et al., 2000; Schoppmann et al., 2003)
Id1	Endometrial Cancer	(Takai et al., 2001)
Id1	Cervical Cancer	(Schindl et al., 2001)
Id1	Melanoma	(Polsky et al., 2001)
Id2	Neuroblastoma	(Lasorella et al., 2002; Lasorella et al., 2000)
Id2	Ewing Sarcoma	(Fukuma et al., 2003; Nishimori et al., 2002)
Id1, Id2, Id3	Astrocytic Tumor	(Vandeputte et al., 2002)
Id1	Basal Cell Carcinoma	(Chaturvedi et al., 2003)
Id1, Id2, Id3	Colorectal Carcinoma	(Norton, 2000; Wilson et al., 2001)
Id1	Hepatocellular Carcinoma	(Lee et al., 2003)
Id1	Kaposi's Sarcoma	(Tang et al., 2003)
Id1	Ovarian Cancer	(Schindl et al., 2003)
Id1	Prostate Cancer	(Ouyang et al., 2002)
Id1	AML subsets	(Suh et al., 2008; Tang et al., 2009)

Table 1. Id Protein Expression in Cancer

immortalized cells suggest they are developmentally arrested at the CMP/GMP phase of maturation (Suh et al., 2008). The bone marrow cells immortalized by Id1 overexpression showed decreased levels of p15^{INK}, p16^{INK4a}, p19^{ARF} and p21^{Cip1} during culture (Suh et al., 2008), indicating that Id1 expression reduced the expression of multiple cell cycle inhibitors to cause increased growth in deregulated myelopoiesis.

These results showed that overexpression of Id1 immortalized normal bone marrow cells *in vitro*, and led researchers to investigate if deregulated Id1 expression cells would cause a myeloproliferative disease *in vivo*. Transplantation of Id1 overexpressing mouse HSPC resulted in the development of a myeloproliferative disease in mice, causing the animals to become moribund within a year. Complete blood cell counts showed the mice had monocytosis, and low levels of hemoglobin and hematocrit, as well as the presence of cells with leukemic-blast morphology. Pathology revealed that the mice had myeloid and erythroid hyperplasia in their bone marrow and spleens which lead to splenomegaly (Suh et al., 2008). Therefore, overexpression of Id1 is sufficient to immortalize mouse progenitor cells *in vitro*, and leads to a lethal myeloproliferative disease *in vivo*.

Similar to Id1, overexpression of Id2 has also been shown to cause the development of leukemia in mouse models. Transgenic overexpression of Id2 in mouse models leads to a block in lymphoid differentiation, aberrant apoptosis, and development of T cell lymphoma (Morrow et al., 1999). Id2 also blocks differentiation in human cells, specifically, ectopic expression of Id2 in human CD34⁺ myeloid progenitor cells inhibits the acquisition of monocyte characteristics, suggesting that Id2 can block myeloid as well as lymphoid differentiation (Heinz et al., 2006). It is important to note, that the effects of Id1 or Id2 overexpression depend upon the developmental stage and lineage in which overexpression occurs. In summary, overexpression of both Id1 and Id2 can block differentiation, over-ride senescence, and lead to the development of myelo- and lymphoproliferative disease, and ultimately leukemogenesis.

Increased Id1 and Id2 expression is associated with human AML. The finding that Ids immortalize primary mouse bone marrow cells *in vitro*, and promote MPD *in vivo*, led investigators to ask if Id expression levels are elevated in myelogenous leukemia. A microarray performed on 285 AML patient samples demonstrated an increase in Id1 and Id2 in AML subsets (Suh et al., 2008). Specifically, 17.5% of the mRNA samples from AML patients had increased Id1 expression, and 19.2% had increased Id2 mRNA levels. Surprisingly, most of the patients with elevated Id1 and Id2 levels had a normal karyotype, and were evenly distributed across the French-American-British (FAB) subtype classifications: M0-M6. 61% and 50% of patients with 5q and/or 7q deletion and 28 and 22% of patients with t(15;17) showed increased expression of Id1 and Id2, suggesting that elevated Id expression levels contribute to a block in granulocyte development. Seventy-eight patients had high levels of FLT3-ITD mutations, and 23 of those (29%) had elevated levels of Id1 expression. Twenty-three patients had EVI1 deregulation, of which 8 patients (35%) had increased Id1 expression. Id2 expression was found in patients with deregulated NRAS (30%), KRAS (44%), C/EBP α (6%) and EVI1 (30%) expression. Altogether, the analysis suggests that Id gene expression may be induced downstream of multiple signal transduction pathways of activated oncogenes. A separate analysis of a different cohort of AML samples showed that patients with the highest levels of Id1 had a poor prognosis compared to those with lower levels of Id1 (Tang et al., 2009). The causes of Id1 and Id2 overexpression in AML are still under investigation. Thus, elevated levels of Id gene expression could be used diagnostically to elucidate the severity and prognosis of AML patients.

Expression of Id1 and Id2 are increased by known leukemic oncogenes. It is possible that in many cases Id1 and Id2 mRNA levels increase due to mutations in upstream regulatory pathways. Id1 is a downstream target of multiple leukemia-associated oncogenic tyrosine kinases, including BCR-ABL and FLT3-ITD (Tam et al., 2008). In addition, the JAK2V617F – STAT5, and AML-ETO mutations can also increase Id1 expression (Cammenga et al., 2003; Wood et al., 2009). PLZF and the PLZF translocation products, associated with APL (acute promyelocytic leukemia), activate Id1 and Id3 expression (Bernardo et al., 2007; Doulatov et al., 2009; Rice et al., 2009). In addition, overexpression of HOXA9 and HOXA10, both of which are associated with myeloid hyperplasia, directly activate expression of Id2 (Nagel et al., 2010). Furthermore, viruses also induce Id expression, for example, Id1 is upregulated by Epstein-Barr viral protein LMP1 (NH) (Li et al., 2004). Therefore, the Id proteins are likely to be part of the mechanism by which several of the known oncogenic mutations impair growth control.

Id2 has both oncogenic and tumor suppressor properties. Interestingly, although Id2 has been shown to be upregulated in AML patient samples (as described above), Id2 may have both oncogenic and tumor suppressor properties. Specifically, there are data demonstrating that the loss of Id2 *in vivo* results in the development of a lethal myeloproliferative disorder (Ko et al., 2008). This effect was seen in Id2 knockout mice backcrossed onto a C57Bl/6 background, but not in 129/sv Id2 knockout, although the reasons for this difference are not yet known. Altogether, the data suggest that Id2 is important for functional hematopoiesis, but that its function as an oncogene or as a tumor suppressor depend upon the cellular environment in which it is expressed.

Id1 causes genomic instability. Of the Id family of proteins, overexpression of Id1 is most consistently reported as associated with oncogenesis. A possible explanation for this may be that Id1 has been associated with genomic instability. Interestingly, it was shown that Id1 contributes to the acquisition of secondary mutations by causing centrosome abnormalities. One mechanism by which this has been shown to occur is by Id1 binding to the S5A protein. Loss of S5A has been associated with mitotic defects resulting in abnormal chromosome segregation (Szlanika et al., 2003). When Id1 is overexpressed it interacts with S5A, and this interaction causes overduplication of the centrosomes (Hasskarl et al., 2004; Hasskarl et al., 2008). Leukemogenic translocation mutations such as BCR-ABL can induce genomic instability (Dierov et al., 2009). Based on the data showing that BCR-ABL can induce expression of Id1, it is possible that part of the mechanism by which BCR-ABL causes genomic instability is by inducing overexpression of Id1 (Dierov et al., 2009). Thus, the presence of overexpressed Id1 can not only block differentiation and override senescence; it can actively contribute to the acquisition of additional mutations.

Id family overexpression is associated with disruption of apoptosis. In addition to causing genomic instability, another mechanism by which cancer cells survive is by avoiding programmed cell death. Multiple reports have shown that Id1, Id2, and Id3 can influence apoptosis. However, whether the Id proteins behave as pro-apoptotic or anti-apoptotic factors depends upon the cell context. Transgenic expression of Id1 in developing T cells in a mouse model resulted in a large percentage of the developing thymocytes to undergo apoptosis. The authors of the study speculated that this was because the thymocytes were blocked in maturation and apoptosis was initiated during the process of V(D)J recombination (Kim et al., 1999). Interestingly, lymphomas developed from the surviving thymocytes, suggesting that overexpression of Id1 alone did not drive apoptosis. In

addition, Id1 overexpression in prostate cancer cells actually protects malignant cells from apoptosis (Ling et al., 2003; Wong et al., 2004). Furthermore, decreasing the activity of the Id proteins promotes cell cycle arrest and apoptosis in both breast and ovarian cancer cells (Mern et al., 2010a; Mern et al., 2010b). In summary, the Id proteins can have variable effects on apoptosis, depending upon the developmental stage and microenvironment of the cell.

Id4 functions as a tumor suppressor in myeloid leukemia. Id4 is interesting because of reports that suggest it may function *not* as an oncogene, but as a tumor suppressor in leukemia (in contrast to its siblings). Methylation of the Id4 promoter is associated with poor prognosis in high risk MDS patients, who have a greater probability of developing leukemia (Wang et al., 2010b). In addition, high risk myelodysplastic patients whose Id4 promoters are methylated have an increased risk of leukemic transformation, an observation which would support for the use of demethylating agents in MDS/AML treatment (Chen et al., 2011; Wang et al., 2010b). Id4 methylation also correlates with CML progression. Specifically, Id4 is un-methylated in the chronic phase of CML, but is frequently methylated in patients in the accelerated and blast crisis stages (Wang et al., 2010a).

Reports also show that Id4 promoter is methylated in AML cell lines and in AML primary patient samples (Yu et al., 2005). This study also demonstrated that Id4 promoter methylation resulted in decreased Id4 mRNA expression. At the same time, the role of Id4 as a tumor suppressor in myelodysplasia appears to be cell type specific, as Id4 overexpression increases the proliferation of other cell types (breast cancer) (Dell'Orso et al., 2010). Therefore, of all of the Id family members, Id4 is the only one that is consistently reported to be a tumor suppressor in myeloid cells, although how it functions mechanistically in that role is not yet understood.

4.2 Ids and drug resistance in leukemia

Altogether, the reports described above suggest a strong link between Id expression levels and malignancy. It is possible, therefore, that Ids may be part of a mechanism that helps the malignant cells evade standard chemotherapeutic treatments. A search of the Gene Expression Omnibus (GEO; (Barrett and Edgar, 2006)) database reveals studies, which show a correlation between increased Id expression and drug resistance in AML and CML cell lines. Specifically, these analyses show that Id1 expression is increased in cytarabine resistant AML cell lines (**Figure 3A**) (GDS 1907), and that Id1 is also increased in cyclophosphamide resistant CML (**Figure 3B**) (GDS2729) (Bao et al., 2007). In addition, Id3 mRNA levels are increased in cyclophosphamide resistant CML lines (**Figure 3C**) (GDS2729) (Bao et al., 2007). Altogether, these analyses indicate that high levels of Id1 and/or Id3 expression may correlate with a drug resistant phenotype in myelogenous leukemia.

These analyses agree with many other studies that show Id1 functions as an anti-apoptotic factor in cancerous cells from non-hematopoietic tissues. Id1 expression has been shown to prevent malignant cells from undergoing programmed cell death when treated with chemotherapeutic agents (Summarized in (Zhang et al., 2007)). Decreasing Id1 expression with Id1-targeted siRNA restored drug sensitivity in many of these malignant cell lines, however, it has not yet been tested in hematopoietic cells (Zhang et al., 2007). In summary, the data from these studies suggest that high levels of Id1 expression may be indicative of drug resistance in leukemic cells, or may possibly be part of the mechanism underlying drug resistance, which will likely be a focus of future studies with both AML and CML.

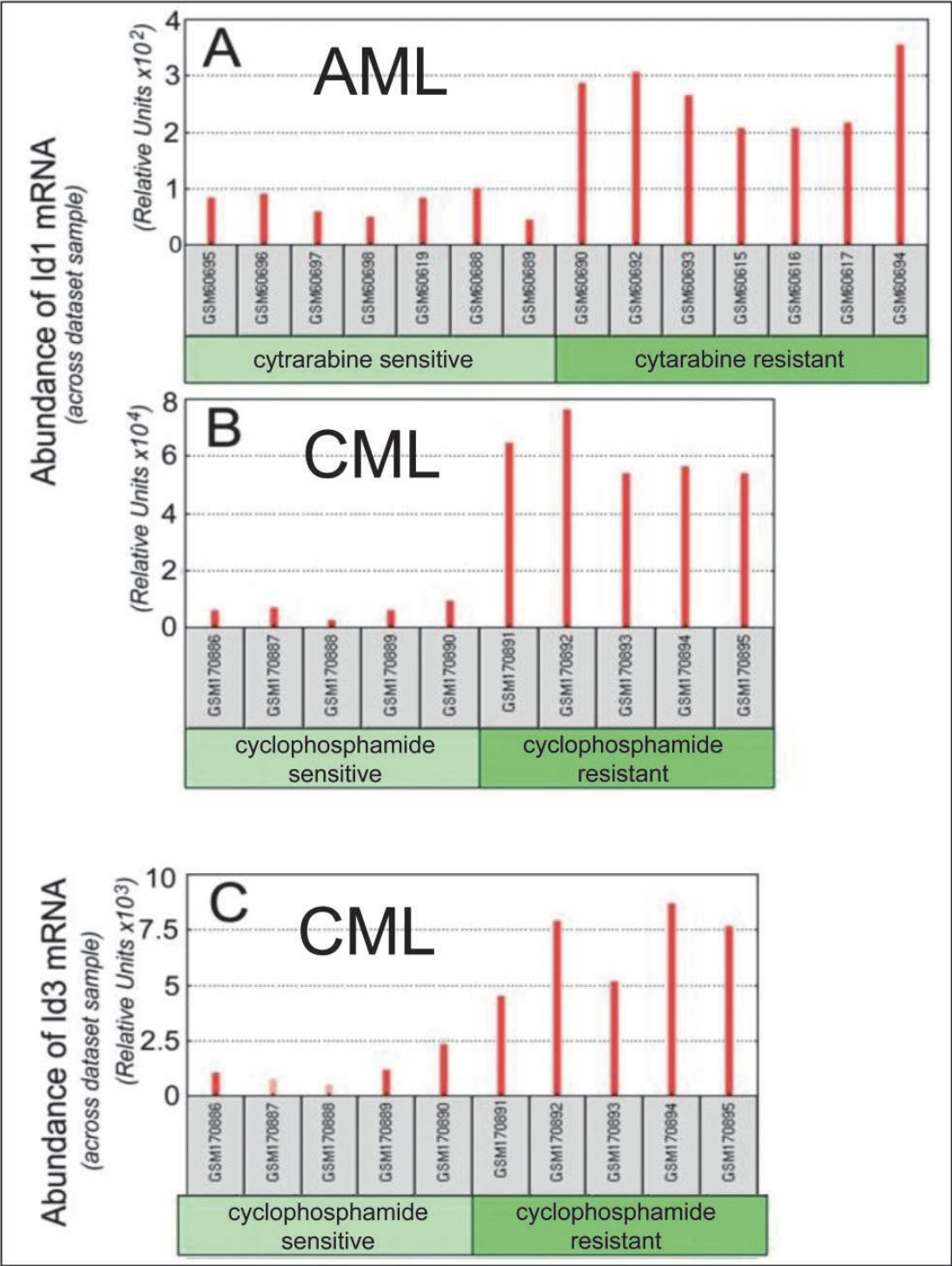


Fig. 3. Increased Id levels associated with drug resistance in leukemic cell lines (A) Id1 mRNA expression is increased in cytarabine resistant AML cell lines (GDS 1907); (B) Id1 is also increased in cyclophosphamide resistant CML (GDS2729), and (C) Id3 mRNA is increased in cyclophosphamide resistant CML lines (GDS2729)

4.3 Ids and Leukemia Initiating Cells

The results shown above lead to the question of whether or not the Ids might be expressed in the "Leukemia Initiating Cell" (LIC) population that is resistant to drug treatment. These quiescent leukemic stem cells have been termed the "Holy Grail of Leukemia Therapy" (Misaghian et al., 2009). However, identifying and understanding the regulation of these cells depends on a better understanding of normal transcription factors in stem cells; and how they may be deregulated, and become leukemia stem cells (Rosenbauer et al. 2005).

The process of normal stem-cell self-renewal must be tightly regulated to prevent the creation of leukemic stem cells. Therefore, the proteins that regulate the HSC self-renewal process are currently under intense investigation. E proteins maintain the stem cell pool and promote myeloid progenitor differentiation, and because Id proteins control E protein function, variations in Id protein levels can significantly affect HSC self-renewal (Semerad et al., 2009). In support of this concept, Id proteins were shown to have a role in maintaining the self-renewal capacity of stem cells. Specifically, BMP induction of Id proteins suppresses differentiation and sustains ES cell self-renewal in collaboration with STAT3 (Ying et al., 2003). In addition, in glioma cancer stem cells, decreasing expression of Id1 and Id3 effectively targeted and reduced the malignant stem cell population (Anido et al., 2010). There are also reports that loss of Id1 significantly decreases hematopoietic stem cell function, although reports vary on whether this is an environmental effect or a result of changes in the bone marrow microenvironment (Jankovic et al., 2007; Perry et al., 2007; Suh et al., 2009). In addition, it has been observed that loss of Id2 significantly decreases the long-term self-renewal capacity of hematopoietic stem cells (Ming Ji et al., unpublished). All of these studies suggest that Id genes contribute to the control of self-renewal of normal HSC and deregulation of the Id proteins may contribute to the generation of leukemic stem cells. Future studies will likely focus on this important aspect of Id protein function, as they may provide a novel approach to target leukemic stem cells.

5. Id proteins and the bone marrow microenvironment

Chemotherapy-resistant human leukemia stem cells home to and engraft within the bone-marrow endosteal region (Ishikawa et al., 2007), and reports indicate that the Id proteins may also have an effect on this microenvironment. As previously described, the loss of Id1 in the bone marrow niche leads to increased progenitor cycling caused by a change in the cytokine milieu (Suh et al., 2009). Thus, Id genes may additionally contribute to the initiation or progression of AML by affecting the stromal cells that constitute the hematopoietic niche. Interestingly, in addition to affecting the local cytokine milieu, the Ids regulate the fundamental structure of the bones by controlling osteoblast and osteoclast activity. For example, ectopic Id4 expression promotes osteoblast differentiation and was suggested as a possible preventative treatment for senile osteoporosis (Tokuzawa et al., 2010). Id1, Id2, and Id3 are all upregulated in response to BMP9 treatment of mesenchymal progenitor cells (via Smad4), which results in increased proliferation and a block in differentiation of osteogenic progenitors (Peng et al., 2004). In addition, Id proteins have been shown to regulate osteogenic transcription factor activity (Ogata and Noda, 1991; Tamura and Noda, 1994; Zhang et al., 2008). Id2, and Id4 genes also regulate adipogenesis by regulating the proliferation pre-adipocyte progenitors and their ability to undergo differentiation (Murad et al., 2010; Park et al., 2008). Altogether, these results suggest that the Id family members

not only regulate hematopoietic growth factor expression, but the fundamental structure of the niche itself.

6. Targeting Id proteins

The identification of Id1 as a common downstream effector of oncogenic mutations such as BCR-ABL and FLT3-ITD in CML and AML, as well as their upregulation in multiple types of cancer, suggest that the Id proteins represent potential targets for therapeutic intervention (Suh et al., 2008; Tam et al., 2008). Inhibiting Id1 protein expression in human induced pluripotential stem cells using with small inhibitory RNAs (siRNA) increases differentiation into committed progenitors suggesting that decreasing Id expression can support cell maturation (Hong et al., 2011). To date, there are three novel inhibitors of Id function currently under investigation.

6.1 Peptide conjugated antisense oligonucleotides

Henke et al. generated an anti-sense oligonucleotide that is covalently coupled to an "address-peptide," Id1-PCAO (peptide-conjugated anti-sense oligonucleotide) (Henke et al., 2008). The peptide used in this study was designed specifically to target endothelial cells; however, other targeting peptides are available and could be linked to the Id1 anti-sense molecule to direct the anti-sense molecule to hematopoietic cells. While the Id1-PCAO strategy has not been tested in hematopoietic cells, it is a feasible approach for use in the hematopoietic malignancies. Targeting peptides have been identified which home to bone marrow and bind primitive hematopoietic stem cells (Nowakowski et al., 2004). The benefit of this procedure would be that it does not require the use of any viral transduction procedures, and the targeting peptide should limit potential toxicity by localizing the anti-sense molecule to the diseased area.

6.2 Peptide aptamer

Another small molecule which has recently been developed is a peptide that binds both Id1 and Id3. Mern et al. identified a peptide aptamer (a short peptide), Id1/Id3-PA7, from a randomized combinatorial expression library using yeast and mammalian two-hybrid systems. When the aptamer is fused to a "cell-penetrating protein transduction domain" or PTD (truncated VP22 ORF), and tested on ovarian cancer cell lines, it causes increased expression of p16^{INK4a}, and it induced apoptosis (as indicated by PARP cleavage) (Mern et al., 2010a). The aptamer colocalized with Id1 and Id3 staining based on immunohistochemistry, suggesting that its effects result from its direct interaction with Id1 and/or Id3. To date, the authors have tested this molecule on ovarian and breast cancer cell lines *in vitro* and demonstrated growth inhibition, but have not yet examined its effects on normal cells nor in hematopoiesis (Mern et al., 2010a; Mern et al., 2010b).

6.3 Dominant interfering molecule 13I

In an effort to better understand the rules of HLH protein interaction, Ciarapica and colleagues screened a phage display library to identify and isolate mutant domains with could interfere with HLH domain interactions. They discovered a dominant interfering HLH domain, 13I, which selectively binds to Ids (Id1, Id2, Id3) as opposed to their E protein HLH binding partners, and also impairs complex formation with Rb (Id2). Expression of 13I

in a human embryonic kidney cell line (293) and in neuroblastoma cell lines restored the ability of the E protein E47 to bind and activate the promoters of cell cycle inhibitors. In addition, 13I was also able to induce differentiation in neuroblastoma cells suggesting it can over-ride both the cell cycle promotion and differentiation blocking functions of the Id proteins (Ciarapica et al., 2009). To date, 13I has not yet been tested on normal or hematopoietic cells.

Each of these inhibitors represents a hopeful step forward to reducing aberrant Id levels and restoring proper differentiation to hyperproliferative cells. The attractive feature of the Ids as potential therapeutic targets is that their expression does not need to be completely reduced, just suppressed to low enough levels to restore differentiation, as seen in normal tissues.

7. Summary

In summary, the Id proteins are potential targets in myelogenous leukemia. Much of the work that has gone into characterizing this family of small proteins indicates that if the expression of Id1, Id2, and Id3 is maintained at high levels in progenitor cells, and if their expression is not reduced at the correct time during maturation or differentiation that this could contribute to hyperplasia or neoplasia. Currently there are three targeting molecules available that are undergoing testing at the basic research level.

The discovery of novel mediators of oncogenesis, and biomarkers for disease identification and progression, such as the Id family of proteins, will contribute to the design of better drugs and more effective therapies for myelogenous leukemia.

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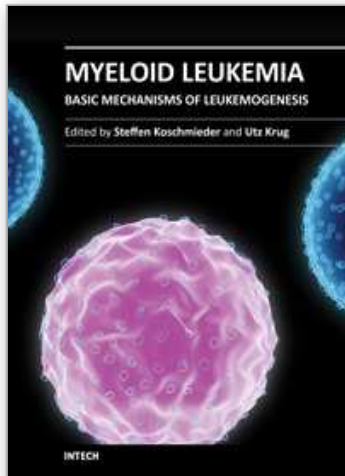
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