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PU.1, a Versatile Transcription Factor and a Suppressor of Myeloid Leukemia

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1. Introduction

PU.1 is a member of the Ezb transformation-specific sequence (Ets) family of transcription factors, and is expressed in granulocytic, monocytic and B-lymphoid cells (Chen et al., 1995b; Klemsz et al., 1990). PU.1 expression levels increase during the differentiation of granulocytes (Chen et al., 1995b). PU.1-deficient mice exhibit defects in the development of neutrophils, macrophages and B cells (McKercher et al., 1996; Scott et al., 1994). Therefore, PU.1 is indispensable for myeloid differentiation during normal hematopoiesis. Moreover, mice carrying hypomorphic PU.1 alleles that reduce PU.1 expression to 20% of its normal levels were reported to develop acute myeloid leukemia (AML) (Rosenbauer et al., 2004), suggesting that disruption of the function of this transcription factor plays a pivotal role in myeloid leukemia biology through the control of numerous target genes. Binding sites for PU.1 are found on almost all myeloid-specific promoters. Notable target genes are receptors for the cytokines, *macrophage colony-stimulating factor* (*M-CSF*), *granulocyte colony-stimulating factor* (*G-CSF*) and *granulocyte macrophage colony-stimulating factor* (*GM-CSF*) (Hohaus et al., 1995; Smith et al., 1996; Zhang et al., 1994), the characteristic antigens *CD11b/CD18* (Rosmarin et al., 1995), primary granule enzymes (Iwama et al., 1998) and the transcription factors c-Jun and JunB (Steidl et al., 2006). In addition, a transcriptional repressor function of PU.1 toward a certain set of genes has been demonstrated. Although the importance of the PU.1 function as a positive regulator of these myeloid genes has been widely examined and reviewed, studies on the role of PU.1 as a transcriptional repressor are relatively scarce. The author's group and others demonstrated that loss of PU.1 function resulted in upregulation of several drug-resistance or growth-regulating genes, including *metallothionein* (*MT*), *vimentin* (*VIM*), *c-myc* and *Flt3,* through disruption of the transcriptional repressor function (Imoto et al., 2010; Inomata et al., 2006; Iseki et al., 2009). This review summarizes the current understanding of the molecular functions of PU.1. Moreover, this review has a particular focus on the transcriptional function of PU.1 as an activator as well as a repressor. Furthermore, the role of PU.1 in the biology of myeloid leukemia is discussed.

2. PU.1, a member of the ETS transcription factor family

The Ets family of transcription factors is so named because the first gene was identified in the E26 avian retrovirus. Ets factors constitute a relatively large gene family, with 27

members encoded by the human genome (Sharrocks, 2001). As precisely reviewed by Oikawa and Yamada (Oikawa & Yamada, 2003), Ets family proteins can be divided into several subfamilies on the basis of their structural compositions and the similarities in their DNA-binding Ets domains. Most of them have the Ets domains in their C-terminal regions. However, several Ets family proteins, such as the ternary complex factor (TCF) subfamily members, have the Ets domains in their N-terminal regions. In addition, besides the conserved Ets domain, a subset of Ets family proteins (e.g. Ets-1, Ets-2, ERG, TEL) have another evolutionarily conserved domain called the pointed domain at their N-terminal regions, which forms a helix-loop-helix (HLH) structure for protein-protein interactions (Oikawa & Yamada, 2003). PU.1 is the most distantly related member of the Ets family proteins having only about 40% identity with Ets-1 in its DNA-binding domain (Oikawa & Yamada, 2003).

3. Structure of PU.1

Spi-1, the gene name for PU.1, was first identified by Moreau-Gachelin and colleagues as the product of a gene targeted by recurrent insertions of the Spleen Focus Forming Virus (SFFV) in Friend's erythroleukemia. An important and unusual feature of the genomic locus *Spi-1* for SFFV proviral integration is that rearrangements caused by SFFV integration were found in 95% of the erythroid tumors examined (Moreau-Gachelin et al., 1988). They subsequently isolated PU.1 (for purine-rich box 1) as a factor that binds to a purine-rich motif in the MHC class II gene promoter. Basically, monomeric PU.1 binds to the consensus DNA site 5'-AAAG(A/C/G)GGAAG-3' via its C-terminal Ets domain and activates transcription via its N-terminal glutamine-rich and acidic domains (Klemsz et al., 1990) (Figure 1).

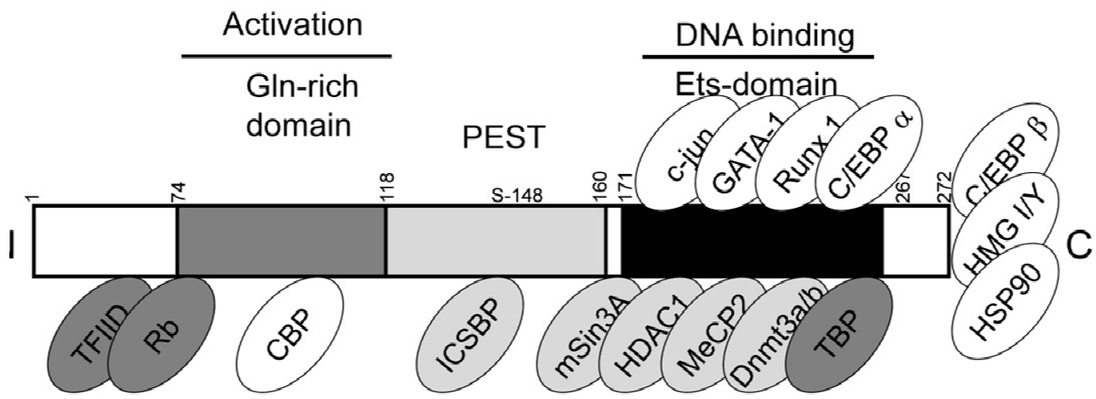

Fig. 1. Schematic presentation of PU.1 and its interacting partners. Proteins involved in transcription are shown in ovals, with transcriptional repressors shown in light gray and basic transcription factors shown in dark gray.

PU.1 is encoded by the *Spi-1 (sfpi1)* gene located on chromosome 11 in humans (chromosome 2 in mice). PU.1 transcripts are 1333 nucleotides long and are produced from the splicing of five exons (Kastner & Chan, 2008). The PU.1 protein is 272 amino acids long (predicted MW: 31 kDa). The structure of PU.1 is shown in Figure 1. The DNA-binding Ets domain shows sequence similarity with other members of the Ets family and is contained within amino acids 171–267 of the C-terminus (Klemsz et al., 1990). The Ets domain

corresponds to the DNA-binding domain and recognizes sequences harboring the core GGAA motif. This domain is also involved in the protein-protein interactions between PU.1 and other factors such as GATA-1 (Nerlov et al., 2000), c-Jun (Behre et al., 1999), Runx 1 or C/EBPα (Petrovick et al., 1998).

The activation domain of PU.1 is located within the N-terminus and consists of several regions rich in either acidic amino acids or glutamines (designated the Gln-rich domain) (Klemsz & Maki, 1996). The activation domain at the N-terminal 75 amino acids of PU.1 has been shown to interact *in vitro* with the basal transcription factor IID (TFIID) and the tumor suppressor retinoblastoma (Rb) protein (Hagemeier et al., 1993). Yamamoto *et al.* (Yamamoto et al., 1999) previously found that this transcriptional activation domain of amino acids 74–122 binds to a transcriptional coactivator, cAMP response element-binding protein (CREB)-binding protein (CBP). CBP potentiated PU.1-mediated transcription of a reporter gene driven by multimerized PU.1-binding sites, suggesting that CBP functions as a coactivator for PU.1 (Yamamoto et al., 1999). A region from amino acids 118–160, which has large numbers of prolines, glutamic acids, serines and threonines, is called the PEST domain (Klemsz & Maki, 1996). This domain plays important roles in the protein-protein interactions, particularly those with interferon (IFN) consensus sequence-binding protein (ICSBP, also called IFN regulatory factor-8) (Nakano et al., 2005).

The C-terminal region of PU.1 has been reported to interact with the leucine zipper transcription factor CCAAT/enhancer-binding protein (C/EBP) β, DNA-binding protein HMG I/Y, multifunctional phosphatase MKP-1 and chaperone protein HSP90 (Nagulapalli et al., 1995) as well as the basic transcription factor TATA box-binding protein (TBP) (Kihara-Negishi et al., 2001).

The PU.1 protein is also the target of phosphorylation events, notably on serine 148, which are regulated by various extracellular signals and play critical roles in modifying the activity of this factor (Joo et al., 2004). These aspects will be discussed later in section 7.

4. Role of PU.1 in normal hematopoiesis, especially in the myeloid lineage, through the regulation of its target genes

As described in the introduction, PU.1 is expressed in B-lymphoid, granulocytic and monocytic cells (Chen et al., 1995b; Klemsz et al., 1990). PU.1 expression levels increase during the differentiation of granulocytes (Chen et al., 1995b). PU.1-deficient mice exhibit defects in the development of neutrophils, macrophages and B cells, although the generation of erythroid and megakaryocytic cells is intact (McKercher et al., 1996; Scott et al., 1994).

In the erythroid lineage, the expression of PU.1 is downregulated during terminal differentiation of murine erythroleukemia (MEL) cells by treatment with dimethylsulfoxide (Hensold et al., 1996). Enforced expression of PU.1 in MEL cells inhibits erythroid differentiation (Oikawa et al., 1999; Yamada et al., 2001). In contrast to the lack of PU.1 expression in the myelomonocytic lineage, which results in impairment of the differentiation, overexpression of PU.1 results in erythroid differentiation blockade.

Expression of PU.1 is observed in $CD34^+$ hematopoietic progenitors and differentiation commitment toward the myeloid and lymphoid lineages appears to be determined by the expression levels of PU.1, since high PU.1 levels promote macrophage differentiation and relatively low PU.1 levels induce B-cell differentiation (DeKoter & Singh, 2000).

Collectively, PU.1 functions in multiple hematopoietic lineages including myeloid, lymphoid and erythroid cells. Many reviews describing the roles of PU.1 in hematopoiesis

have been published (Gallant & Gilkeson, 2006; Kastner & Chan, 2008; Oikawa et al., 1999; Oikawa & Yamada, 2003). Therefore, this section of the present review particularly focuses on the roles of PU.1 in myeloid lineage development through the regulation of its target genes.

Early myeloid differentiation is controlled by PU.1 and other families of transcription factors, such as C/EBPs, AML1/Runx1, retinoic acid receptor α (RARα), c-Myb and others (Friedman, 2002). C/EBPα and C/EBPε knockout mice also have defects in the development of mature macrophages and neutrophils (Yamanaka et al., 1997; Zhang et al., 1999). Hence, PU.1, as well as the C/EBP family members, is a critical master regulator for the determination of the developmental pathway toward the myeloid lineage in hematopoietic cells.

Gene	Level of regulation	References
M-CSFR	Promoter	(Zhang et al., 1994) (Li et al., 2005)
GM-CSFRα	Promoter	(Hohaus et al., 1995)
G-CSFR	Promoter	(Smith et al., 1996)
CD11b	Promoter	(Chen et al., 1993)
CD18	Promoter	(Rosmarin et al., 1995) (Bottinger et al., 1994)
MPO	Enhancer	(Ford et al., 1996)
NE	Enhancer	(Nuchprayoon et al., 1999)
NE	Promoter	(Oelgeschlager et al., 1996)
PR3	Promoter	(Sturrock et al., 1996)
Lysozyme	Enhancer	(Ahne & Stratling, 1994)
$gp91^{phox}$	Promoter	(Suzuki et al., 1998)
$p47^{phox}$	Promoter	(Li et al., 1997)
c-Jun	Unknown	(Steidl et al., 2006)
JunB	Promoter	(Steidl et al., 2006)
PU.1	Promoter	(Chen et al., 1995a)

Table 1. PU.1 target genes play crucial roles in myeloid lineage development

Binding sites for PU.1 are found on almost all myeloid-specific promoters. A large collection of PU.1-dependent promoters have been identified, which control an array of genes encoding predominantly growth factor receptors, adhesion molecules and characteristic enzymes. Notable target genes are receptors for cytokines, adhesion molecules, primary granule enzymes and transcription factors.

The M-CSF receptor (M-CSFR) (Zhang et al., 1994), GM-CSF receptor α (GM-CSFRα) (Hohaus et al., 1995) and G-CSF receptor (G-CSFR) (Smith et al., 1996) gene promoters were reported to be directly regulated by PU.1. These promoters are regulated by PU.1 in combination with C/EBPα (Hohaus et al., 1995; Li et al., 2005; Smith et al., 1996). By employing sense or antisense PU.1 expression, Celada *et al.* (Celada et al., 1996) showed that PU.1 is necessary for M-CSF-dependent proliferation of macrophages, through the control of M-CSFR expression. Another group demonstrated that PU.1-deficient myeloid progenitors can proliferate *in vitro* in response to the multilineage cytokines interleukin (IL)-3, IL-6 and stem cell factor, but are unresponsive to the myeloid-specific cytokines GM-CSF, G-CSF and M-CSF (DeKoter et al., 1998). Expression of M-CSFR or G-CSFR in PU.1-deficient marrow cells did not rescue myeloid development, indicating that PU.1 is required beyond the induction of these cytokine receptors (DeKoter et al., 1998; Henkel et al., 1999).

The CD11b (or macrophage-1 antigen; MAC-1) leukocyte integrin subunit exists on the surface of human granulocytes and monocytes/macrophages coupled with the CD18 (β) subunit in a heterodimer. This heterodimer mediates multifaceted adherence reactivity of these myeloid cells, including the ability to adhere to endothelial cells (Arnaout, 1990). The CD11b promoter was reported to be positively regulated by both Sp1 and PU.1 (Chen et al., 1993). CD18 promoter activity is also regulated by PU.1 (Bottinger et al., 1994; Rosmarin et al., 1995). Consistently, CD11b/CD18 are undetectable on the surfaces of cells lacking PU.1 (Henkel et al., 1996; Olson et al., 1995). These molecules are from the β2 integrin chain, otherwise known as complement receptor 3 (CR3) (Ehlers, 2000). The CD11b subunit of CR3 promotes phagocytosis and macrophage migration by binding to extracellular matrix proteins, coagulation proteins and a variety of microbial antigens (Ehlers, 2000).

Primary granule enzymes are a group of serine proteases or closely related molecules found in cells of the granulocyte series. PU.1-deficient myeloid promoters lack or exhibit low expression of primary granule enzymes, including myeloperoxidase (MPO), neutrophil elastase (NE), proteinase 3 (PR3) and lysozyme (Iwama et al., 1998). Ford *et al.* (Ford et al., 1996) demonstrated that the MPO upstream enhancer is accessible in multipotent cell chromatin, but functionally incompetent before granulocyte commitment. Multipotent cells contain both PU.1 and C/EBPα as enhancer-binding activities. It was reported that a 220-bp portion of the second intron of the *PR3* gene, which contains an Sp1 consensus site flanked by two Ets family consensus sequences that bind PU.1, functions as an enhancer of NE expression (Nuchprayoon et al., 1999). In addition, PU.1 cooperates with C/EBP α and c-Myb to regulate the NE promoter (Oelgeschlager et al., 1996). PR3 expression has also been shown to be regulated by PU.1 (Sturrock et al., 1996). PU.1-deficient neutrophils do not express secondary granule components, including collagenase, lysozyme and lactoferrin, and have limited ability to ingest and kill bacteria (Anderson et al., 1998; Iwama et al., 1998). The activity of the lysozyme gene enhancer was demonstrated to be controlled by PU.1 in avian macrophages (Ahne & Stratling, 1994). In addition, the expression of gp91^{phox} and p47^{phox}, which are both components of nicotinamide adenine dinucleotide phosphate (NADPH) phagocyte oxidase, is positively regulated by PU.1 (Li et al., 1997; Suzuki et al., 1998).

In addition to these myeloid-specific genes, several transcription factors play crucial roles in the phenotype of PU.1-deficient mice. Steidl *et al.* (Steidl et al., 2006) examined the

transcriptome of preleukemic hematopoietic stem cells (HSCs) from mice in which PU.1 was knocked down to identify transcriptional changes. They demonstrated that the transcription factors c-Jun and JunB were among the top downregulated targets. They concluded that the decreased levels of c-Jun and especially JunB contribute to the development of PU.1 knockdown-induced AML by blocking differentiation and increasing self-renewal. It was previously reported that PU.1 autoregulates its expression in myeloid cells (Chen et al., 1995a). However, autoregulation by PU.1 remains uncertain, since the expression of GFP reporters driven by the endogenous PU.1 promoter remains high in PU.1-null hematopoietic cells (Back et al., 2005; Dakic et al., 2005).

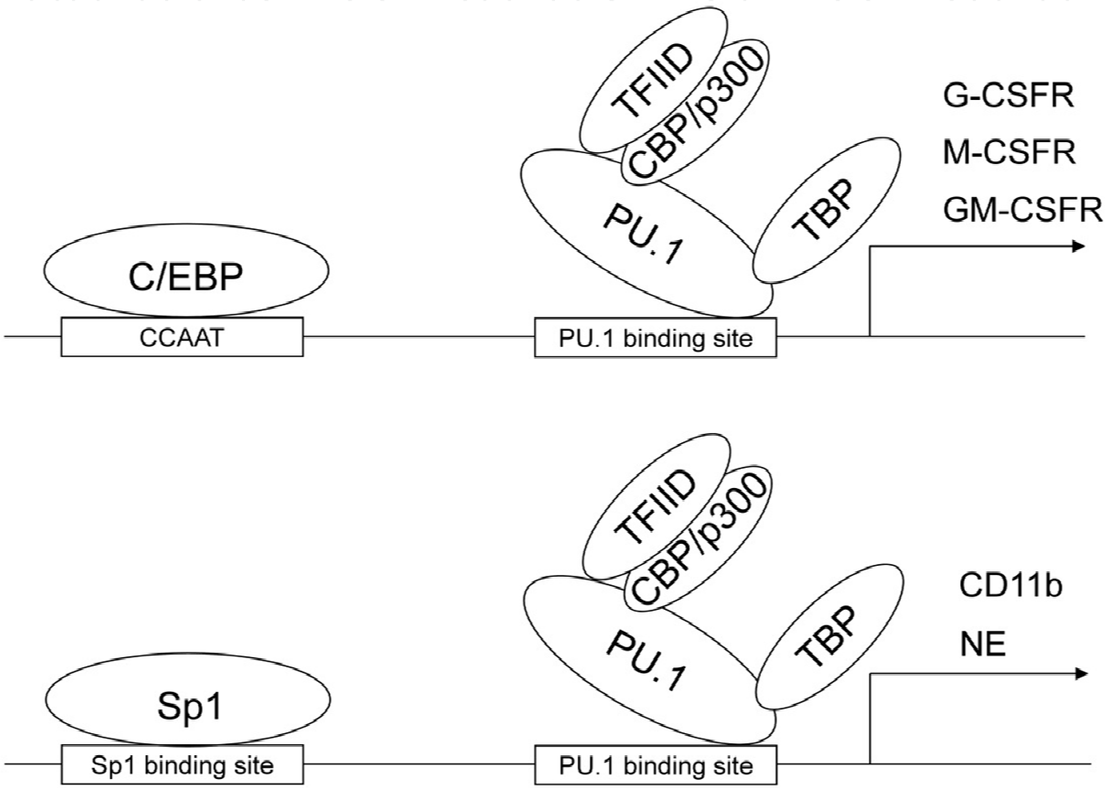

Fig. 2. PU.1 as a transcriptional activator. The known myeloid promoters regulated by a set of transcription factors in combination with PU.1 are depicted.

Table 1 shows a list of myeloid genes that are known to be positively regulated by PU.1. Collectively, PU.1 plays a pivotal role in myeloid development through the regulation of cytokines, receptors, transcription factors and various myeloid specific genes.

The majority of the PU.1-dependent promoters in the myeloid lineage share several structural features, including a PU.1-binding site close to the site(s) of transcriptional initiation, no TATA box and a cluster of binding sites for Sp1 and members of the C/EBP or core-binding factor (CBF) families (Fisher & Scott, 1998; Tenen et al., 1997). A schematic layout of the transcription factors binding to the proximal promoter regions is shown in Figure 2. The G-CSFR, M-CSFR and GM-CSFR promoters are regulated by PU.1 and C/EBPs, whereas the CD11b and NE promoters are regulated by PU.1 in combination with Sp1.

In contrast to PU.1 or C/EBPs, the other transcription factors are widely expressed outside of the hematopoietic system, indicating that myeloid-specific expression may be principally caused by PU.1 or unique combinations of adjoining transcription factors working in concert with PU.1 (Fisher & Scott., 1998). In this group of myeloid promoters, as depicted in Figure 2, binding of PU.1 close to the start point of transcription may facilitate recruitment to the TFIID complex and promote the binding of adjoining transcription factors. TFIID is a protein complex consisting of TBP and a set of TBP-associated factors (TAFs) (Pugh, 1996). Binding of TFIID to the promoter region is the first step in the assembly process that forms the transcription initiation complex. Not only does the N-terminal of PU.1 interact with TFIID, but its C-terminal was reported to interact with TBP (Kihara-Negishi et al., 2001) (Figures 1 and 2). Even though PU.1 may make the initial contact with TFIID, other transcriptional factors (e.g. C/EBPs, Sp1) are likely to help stabilize the TFIID complex through TAF interactions. The PU.1-initiated cooperative stabilization of TFIID may be sufficient to evoke myeloid-specific expression for this group of genes.

5. Regulation of PU.1

As described in the previous section, expression of PU.1 is critical for hematopoiesis. Therefore, the regulatory mechanisms of PU.1 expression have been extensively studied. To date, most aspects of PU.1 regulation have been explained by invoking two regulatory elements, the promoter and an upstream regulatory element (URE) at –14 kb upstream of the transcription start site of the *Spi-1* gene encoding PU.1.

The *Spi-1* promoter contains octamer-binding sites that affect B cell expression (Chen et al., 1996). As described in the previous section, PU.1 was demonstrated to bind to its own promoter to regulate itself in myeloid cells (Chen et al., 1995a). *Spi-1* promoter activity can also be directed in myeloid cells by C/EBPα and AP-1 (Cai et al., 2008) (Figure 3). Although the promoter alone cannot drive reporter expression in a chromatin context, a search for added regulatory function yielded the conserved URE at around –14 kb, reported to be a myeloid-specific enhancer, that enhanced the promoter activity in a myeloid cell line (Li et al., 2001). Huang *et al.* (Huang et al., 2008) demonstrated that AML1/Runx1 binds to functionally important sites within the URE and regulates PU.1 expression at both embryonic and adult stages of development. As described later in section 8, NF-κB was reported to regulate PU.1 through a novel site within the upstream URE (Bonadies et al., 2010). Zarnegar *et al.* (Zarnegar et al., 2010) recently uncovered a set of conserved *cis*-regulatory regions for *Spi-1*. One is a novel enhancer (–10 kb) that can amplify myeloid cell-specific expression of PU.1. The other is a separate pro-T-cell-specific silencer element (–9 kb). The enhancer element at –9 kb functions as a weak silencer in myeloid cells. The schematic layout is presented in Figure 3. Using a combination of transgenic studies, global chromatin assays and detailed molecular analyses, Leddin *et al.* (Leddin et al., 2011) presented evidence that PU.1 is regulated by a novel mechanism involving crosstalk between different *cis*-elements together with lineage-restricted autoregulation. They revealed that myeloid progenitors express C/EBPα, which binds to the URE and induces activation of an enhancer at –12 kb to allow the formation of a second PU.1 autoregulatory loop and binding of additional PU.1 driven-transcription factors, such as early growth response 2 (EGR2). In this model, PU.1 regulates its expression in B cells and macrophages by differently associating with cell type-specific transcription factors at one of its *cis*-regulatory elements to establish differential activity patterns at other elements.

Other mechanisms underlying the regulation of PU.1 expression have been demonstrated. Ebralidze *et al.* (Ebralidze et al., 2008) identified naturally occurring antisense transcripts overlapping the PU.1 coding region. They demonstrated that noncoding antisense RNAs are regulated by shared evolutionarily conserved *cis*-regulatory elements, and further showed that antisense RNAs inhibit PU.1 expression by modulating RNA translation. Vigorito *et al.* (Vigorito et al., 2007) carried out gene expression profiling of activated B cells and validated PU.1 as a direct target of microRNA-155 (miR-155)-mediated inhibition. Using mouse bone marrow (BM) cells, Hu *et al.* (Hu et al., 2010) revealed HOXA9-mediated upregulation, which is frequently observed in AML, of miR-155 in fractionated bone marrow progenitors. Furthermore, they revealed that ectopic expression of miR-155 resulted in a decrease in PU.1 protein (Hu et al., 2010). These findings suggest that the expression of PU.1 is controlled both transcriptionally and post-transcriptionally.

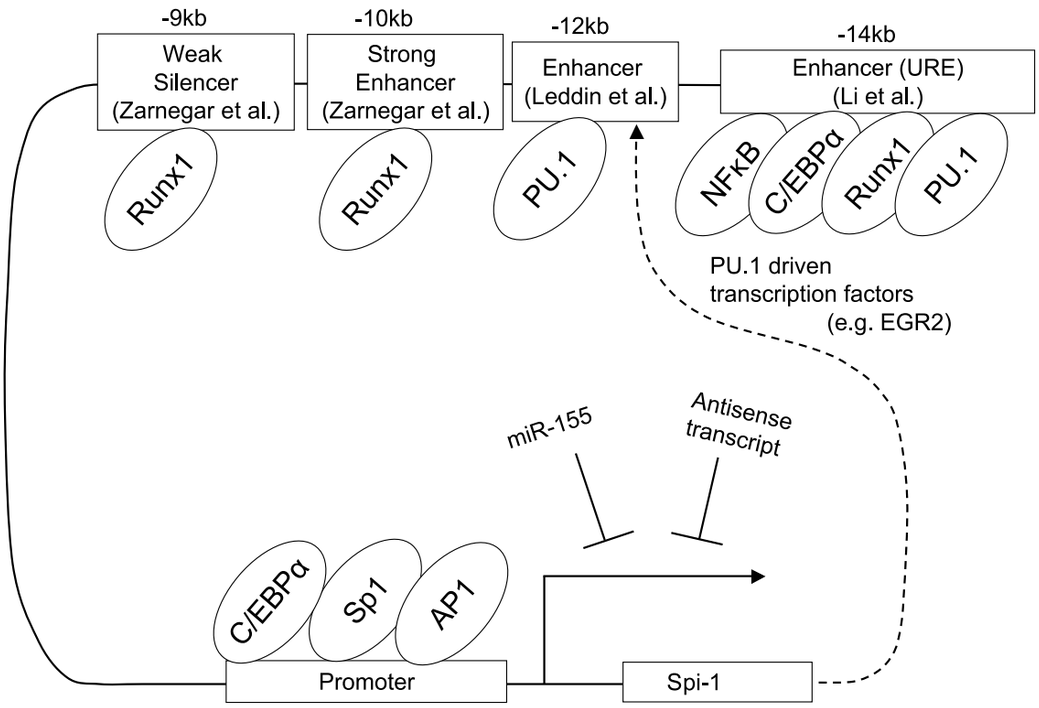

Fig. 3. Regulation of PU.1 expression.

6. PU.1 suppressive targets

Stiedl *et al.* (Steidl et al., 2006) revealed that ~21,500 of 45,000 transcripts were expressed in PU.1-knockdown HSCs, among which 225 transcripts were downregulated and 97 were upregulated in PU.1-knockdown HSCs. These findings indicate that PU.1 not only activates but also represses a substantial number of genes in HSCs. Consistent with this notion, several PU.1 suppressive targets have been identified and reported to date. Table 2 shows a list of the known PU.1 suppressive targets.

Borras *et al.* (Borras et al., 1995) first identified PU.1 as a transcriptional repressor. They examined the ability of PU.1 to regulate the expression of the *major histocompatibility complex I-Ab* gene. Their results indicated that PU.1 represses *I-Ab* expression, possibly by binding to a PU.1 box that is located between the Y-box and the transcription start site (Borras et al., 1995). The *c-myc* promoter (–138 to +517) was found to be a target for downregulation by PU.1 in a dose-dependent manner by luciferase assays (Kihara-Negishi et al., 2001). These authors also found that the *c-fos*, *SV-40*, *β-actin* and *PCNA* promoters are suppressed by expression of PU.1, without binding to a PU.1-binding site. They further showed that the *c-myc* minimal promoter including a TATA box is sufficient to exert PU.1 repression. Using luciferase assays, they demonstrated that a restricted region of PU.1 including the PEST domain is responsible for PU.1-mediated transcriptional repression. They clearly demonstrated that C-terminal amino acids 101–272 of PU.1 formed a complex with mSin3A and HDAC1 *in vivo* (Kihara-Negishi et al., 2001) (Figure 1). By affinity purification assays, Suzuki *et al.* (Suzuki et al., 2003) found that PU.1 bound directly to methyl-CpG-binding protein (MeCP) 2 via the C-terminal Ets domain. They revealed that MeCP2 was integrated into the PU.1–mSin3A–HDAC complex. The same group clearly revealed an interaction between PU.1 and DNA methyltransferase (Dnmt) 3a and Dnmt3b via the Ets domain (Suzuki et al., 2006). They further showed that the CpG sites in the *p16(INK4A)* promoter were methylated by PU.1 overexpression in NIH3T3 cells, accompanied by downregulation of *p16* (*INK4A*) gene expression (Suzuki et al., 2006). Moreover, hematopoietic precursor cells expressing PU.1 showed downregulation of the c-Myb protein levels. Transient expression of PU.1 in cotransfection assays in different cell lines resulted in inhibition of *c-myb* promoter activity through the PU.1-binding site (Bellon et al., 1997).

Gene	Level of regulation	References
Major histocompatibility complex I-Ab	Promoter	(Borras et al., 1995)
c-myc	Promoter	(Kihara-Negishi et al., 2001) (Kihara-Negishi et al., 2005)
c-fos, SV-40, β-actin, PCNA	Promoter	(Kihara-Negishi et al., 2001)
p16 (INK4A)	Promoter	(Suzuki et al., 2006)
c-Myb	Promoter	(Bellon et al., 1997)
CD11c	Promoter	(Lopez-Rodriguez & Corbi, 1997)
MT-1s, VIM	Promoter	(Imoto et al., 2010)
Annexin 1	Unknown	(Iseki et al., 2009)
Flt3	Promoter	(Inomata et al., 2006)

Table 2. Suppressive gene targets regulated by PU.1

It was reported that PU.1 negatively regulates the *CD11c integrin* gene promoter through recognition of the PU.1-binding site at the major transcriptional start site (Lopez-Rodriguez

& Corbi, 1997). The inhibitory action of PU.1 on CD11c is in contrast to its positive regulatory effect on the other integrin-related *CD11b*/*CD18* gene promoters (Rosmarin et al., 1995), as described in section 4. The opposite changes in CD11b and CD11c expression that take place in extravasating and maturing blood monocytes (Prieto et al., 1994) might be explained by the differential actions of PU.1 on the regulatory regions of the *CD11b* and *CD11c* genes.

The author's group recently revealed that *MT-1* and *VIM* gene expression is directly regulated by PU.1 (Imoto et al., 2010). The MT proteins comprise a group of low molecular weight cysteine-rich intracellular proteins that can be activated by a variety of stimuli, including metal ions, oxidative stress, cytokines, glucocorticoids and growth factors (Cherian et al., 2003). MT was reported to be a potential negative regulator of apoptosis (Shimoda et al., 2003). VIM is a cytoskeletal protein that belongs to the intermediate filament family (Lazarides, 1982). VIM suppresses the apoptotic effect of chemotherapeutic treatment (Belichenko et al., 2001). These findings imply that induced expression of MT, as well as VIM, may function in anti-apoptosis mechanisms in leukemia cells. We demonstrated that MeCP2 and PU.1 bind to the CpG-rich regions in the *MT-1* and *VIM* promoters. Bisulfite sequencing analyses of the PU.1-bound regions of these promoters revealed that the proportions of methylated CpG sites are tightly correlated with the PU.1 expression levels (Imoto et al., 2010). Importantly, the mRNA expression levels of the *MT-1* and *VIM* genes were inversely correlated with PU.1 mRNA expression in 43 primary AML specimens (MT-1G: $R=-0.50$, $p<0.001$; MT-1A: $R=-0.58$, $p<0.0005$; VIM: $R=-0.39$, $p<0.01$) (Imoto et al., 2010).

The author's group also demonstrated an inverse correlation between the mRNA expression of Annexin 1 and PU.1 in AML specimens (Iseki et al., 2009). In addition, the author and colleagues have reported that the *Flt3* gene promoter is suppressed by overexpression of PU.1, and that there is a significant negative correlation between FLT3 and PU.1 ($r=-0.43$, $p<0.05$) (Inomata et al., 2006). Therefore, PU.1 downregulation, which is important in the pathogenesis of AML as described in section 8, may impact on the overexpression of FLT3, which is frequently observed in AML, leading to activation of the FLT3 pathway (Ozeki et al., 2004; Takahashi, 2011).

Together with these findings, PU.1 represses several genes through its epigenetic activity under certain circumstances. The repression of PU.1 is directed in at least two ways. The author's group demonstrated that PU.1 binds to the methylated CpG-rich region with MeCP2, where no consensus PU.1-binding sites were found (Imoto et al., 2010). The other way is direct binding of PU.1 to PU.1-binding sites, as demonstrated in the promoters of the *CD11c*, *p16 (INK4A)*, *major histocompatibility complex I-Ab, c-myb* and other genes (Borras et al., 1995; Lopez-Rodriguez & Corbi, 1997; Suzuki et al., 2006). In addition, some suppressive target genes of PU.1 may be indirect effects (e.g. Iseki et al., 2009), because direct binding to their promoters has not been demonstrated. A schematic layout of the complex of PU.1 and its corepressors binding to these promoters is shown in Figure 4.

7. Putative regulatory mechanisms for PU.1 as a transcriptional activator or repressor

As mentioned above, Kihara-Negishi *et al.* (Kihara-Negishi et al., 2001) showed a direct interaction of PU.1 with mSin3A. On the other hand, the same group previously demonstrated a direct interaction of PU.1 with the transcriptional coactivator CBP, which

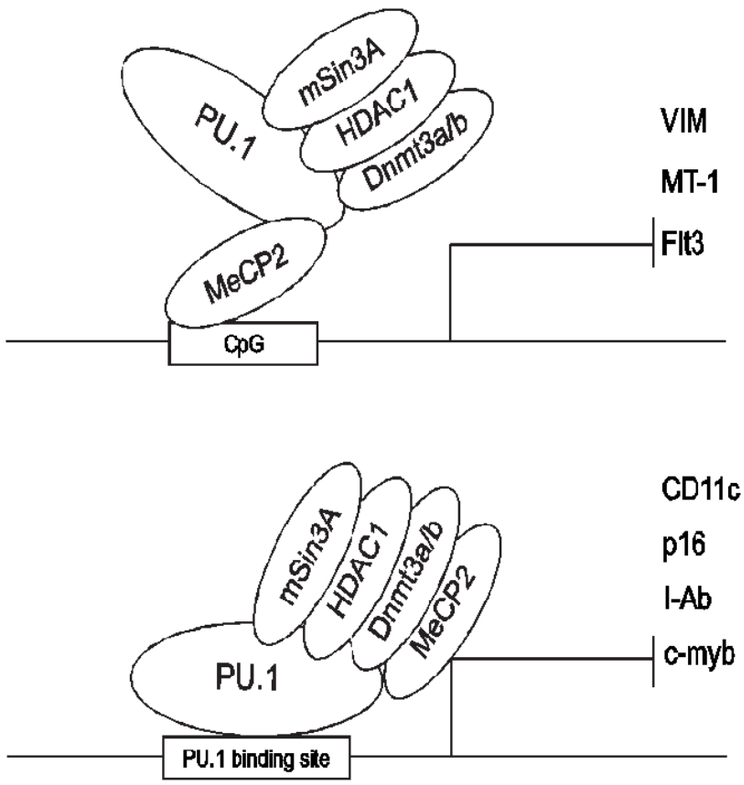

Fig. 4. PU.1 as a transcriptional repressor. This is a schematic picture about the hypothetical formation of the PU.1 repressor complexes, because some of these informations are putative. Since the *Flt3 gene* promoter has a CpG-rich region without a functional PU.1-binding site (Inomata et al., 2006), Flt3 may be classified into the upper group, although direct binding of PU.1 to the *Flt3 gene* promoter has not been examined.

enhances the transcriptional activity of PU.1 (Yamamoto et al., 1999). Of particular interest is the regulatory mechanism of the selection of the interacting partners, CBP and mSin3A-HDAC, which possess opposite functions in the regulation of gene expression. Posttranslational modification of PU.1, such as phosphorylation or acetylation, may be involved in the preferential recruitment of coactivators or corepressors.

In general, the expression of PU.1 is usually unaffected by most stimuli that regulate gene activation and its expression levels remain relatively constant (Yordy & Muise-Helmericks 2000). PU.1 is primarily regulated post-transcriptionally by phosphorylation (Lloberas et al., 1999; Yordy & Muise-Helmericks, 2000). PU.1 is potentially phosphorylated on five separate serine residues (Ser41, Ser45, Ser132, Ser133 and Ser148) and is an *in vitro* substrate for casein kinase II (CKII) (Lodie et al., 1997). Lodie *et al.* (Lodie et al., 1997) revealed that lipopolysaccharide stimulation induces phosphorylation of PU.1 at Ser148 (Figure 1), located within a CKII consensus motif, which increases the transactivation

function of PU.1. PU.1 is also phosphorylated at Ser142 by stimulation of IL-3-induced p38 MAPK-mediated pathways (Wang et al., 2003). Mutation of Ser142 to alanine attenuates the IL-3-enhanced transactivation activity of PU.1. In contrast, Breig *et al.* (Breig et al., 2010) recently described that Ser41, but not Ser148, is necessary for Spi-1/PU.1-mediated repression of hemoglobin expression in Friend erythroleukemia cells. Phosphorylation of critical residues in PU.1 may switch the function of this transcription factor from an activator to a repressor. Kihara-Negishi *et al.* (Kihara-Negishi et al., 2005) examined whether acetylation regulates the physical and functional activities of PU.1 in MEL cells. They found that PU.1 is acetylated *in vivo* and that its repressor activity is reduced when the putative acetylation motifs in the Ets domain are mutated. The mutant cooperates with the coactivator CBP, similar to the case for wild-type PU.1, but shows insufficient cooperation with the corepressor mSin3A, which binds to the Ets domain of PU.1. These observations are summarized in Table 3.

The modifications of each functional domain (as depicted in Figure 1), which are required for transcriptional activation or repression, may result in the dissociation of cofactors, leading to changes in the transcriptional ability of PU.1.

However, a question still remains. As described in the previous section, in a same-cell population like HSCs (Steidl et al., 2006), some genes are suppressed while other genes are upregulated by PU.1. How are these genes differently regulated even in the same cells? It is possible that the context of the promoter may be important, and that promoter-specific cofactors may modify PU.1 and regulate the activity of this protein. These mechanisms remain to be elucidated in future studies.

Site	Cells	Responsible Signaling	Effect	Ref.
Ser41, Ser45, Ser132, Ser133 and Ser 148	Raw264.7 (macrophage) cells	Casein Kinase II	Phosphorylation of Ser 148 increases transactivation function.	(Lodie et al., 1997)
Ser 142	Ba/F3 (IL-3 dependent pro B cells)	p38MAP kinase	Phosphorylation of Ser 142 increases transactivation of PU.1, resulted in the stimulation of mcl-1 gene transcription.	(Wang et al., 2003)
Ser 41	Friend erythro-leukemia cells	PI3 kinase /Akt	Ser 41 is responsible for the PU.1 mediated repression, and phosphorylation of this site impairs its repression activity.	(Breig et al., 2010)
Lysine -rich acetylation motifs (a.a.221-223, 244-249)	Zinc inducible PU.1 transgenic MELB8/3 cells, 293T cells	Unknown	The repressor activity of PU.1 was impaired by mutations of both two acetylation motifs	(Kihara -Negishi et al., 2005)

Table 3. List of the post-translational modifications of PU.1

8. Role of PU.1 in the pathogenesis of AML

For many years, it has been known that irradiated mice frequently develop AML after a long latent period, and that nearly all of their tumors contain an interstitial deletion in one copy of chromosome 2 that includes the *PU.1* gene (Hayata et al., 1983; Silver et al., 1999; Trakhtenbrot et al., 1988). Subsequent studies revealed that mice with γ-irradiation-induced myeloid leukemia acquired both a deletion in one copy of chromosome 2 and a point mutation in the ETS domain of PU.1, which impaired DNA binding of the other PU.1 allele (Cook et al., 2004; Suraweera et al., 2005). These single amino acid substitutions were shown to alter the function of PU.1 by reducing its transactivation activity. Grisolano *et al* (Grisolano et al., 1997) previously showed that 10–20% of transgenic mice expressing PML-RARα in their early myeloid cells develop acute promyelocytic leukemia (APL) after a long latent period. The penetrance of APL development increases substantially when RARα-PML is coexpressed in early myeloid cells (Pollock et al., 1999) or when young mice are sublethally irradiated (Walter et al., 2004). In both scenarios, the vast majority of APL tumors contain a large (>20 Mb) interstitial deletion in one copy of chromosome 2 that invariably includes the *PU.1* gene (Walter et al., 2004; Zimonjic et al., 2000). These data strongly suggest that PU.1 acts in a similar manner to a tumor suppressor in myeloid progenitors, and that deletion of one allele, followed by a reduction in the function of the other allele, is relevant for AML pathogenesis. Indeed, PU.1 is expressed at low levels in most cases of human AML (Steidl et al., 2006).

PU.1 expression and/or function appear to be suppressed by several leukemogenic fusion products, such as RUNX1-ETO (Vangala et al., 2003), FLT3-ITD (Mizuki et al., 2003) and PML-RARα (Mueller et al., 2006a). Following these initial reports of suppressed PU.1 function in human AML, the leukemic potential of tumor progenitors with critically reduced PU.1 function has been illustrated by a series of mouse models. The level of PU.1 expression is critical for specifying cell fate and, if perturbed, even modest decreases in PU.1 can lead to leukemias and lymphomas (DeKoter & Singh, 2000a; Rosenbauer et al., 2004). Mice with an engineered deletion of the URE of PU.1 show decreased PU.1 expression and subsequently develop AML, through suppression of the AP-1 transcription factors JunB and c-Jun, by blocking differentiation and increasing self-renewal as described in the previous section. However, while these observations indicate that low but detectable amounts of PU.1 might favor the malignant potential of leukemic cells, this view was challenged by a report suggesting that complete loss of PU.1 can also lead to AML (Metcalf et al., 2006).

As described in section 5, the URE is important for the regulation of PU.1. Several reports have described associations between genetic abnormalities of this region and AML biology. In an analysis of 209 AML patient specimens, Steidl *et al.* (Steidl et al., 2007) revealed that a distal single nucleotide polymorphism (SNP) alters the long-range regulation of the *PU.1* gene in AML. The homozygous SNP within a highly conserved distal enhancer element in humans, which is 2.4-fold more frequent in AML with a complex karyotype (24.3%) than in AML with a normal karyotype (10.0%), leads to decreased enhancer activity and reduces PU.1 expression in myeloid progenitors in a development-dependent manner (Steidl et al., 2007). Bonadies *et al.* (Bonadies et al., 2010a) examined the URE sequence in 120 AML patients. They identified one AML patient with heterozygous loss of the entire URE sequence with markedly reduced PU.1 expression. The link between PU.1 URE mutations and AML pathogenesis might be explained by another model. The same group also identified NF-κB as an activator of PU.1 expression through a novel site within the upstream

URE (Bonadies et al., 2010b). They found sequence variations of this particular NF-κB site in four of 120 AML patients. These variant NF-κB sequences failed to mediate activation of PU.1, and AML patients with such variant sequences had suppressed PU.1 mRNA expression. These findings suggest that the change of a single base pair in a distal element critically affects the regulation of the tumor suppressor gene *PU.1*, thereby contributing to the development of AML.

Mutations of PU.1 have previously been examined by several groups. Mueller *et al.* (Mueller et al., 2002; Mueller et al., 2003) identified 10 mutant alleles of the *PU.1* gene in 9 of 126 AML patients. However, these findings could not be confirmed in two subsequent studies. Vegesna *et al.* (Vegesna et al., 2002) analyzed 381 samples of hematopoietic and solid malignancies, including 60 cases of *de novo* AML and 60 cases of myelodysplastic syndromes (MDS), and were not able to identify *PU.1* coding region mutations. Similarly, Lamandin *et al.* (Lamandin et al., 2002) failed to detect *PU.1* gene mutations in 77 primary AML samples. Collectively, direct genetic inactivation of the *PU.1* gene in myeloid leukemia has only been found in very rare cases. Although there is no doubt that a reduction in PU.1 expression plays a very important role in the pathogenesis of AML, its mutations in human myeloid leukemia biology remain unclear.

9. Perspectives and conclusions

The author has summarized the recent advances in the molecular biology of the PU.1 transcription factor, with a focus on its functions as both a transcriptional activator and repressor. In addition, the role of PU.1 in AML biology has been described.

In 2009, inactivating mutations of the *Ten-Eleven-Translocation oncogene family member 2* (*TET2*) gene were identified in about 15% of patients with various myeloid malignancies, such as MDS (19%), myeloproliferative disorders (12%), secondary AML (24%) and chronic myelomonocytic leukemia (22%) (Delhommeau et al., 2009). TET2 can convert 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) (Ko et al., 2010), which is hypothesized to be an intermediate in the demethylation of DNA. Ko *et al.* (Ko et al., 2010) demonstrated that disruption of TET2 enzymatic activity favors myeloid tumorigenesis.

Consistent with this observation, it was reported that Dnmt3a mutations were detected in 62 of 281 (22.1%) AML patients (Ley et al., 2010). These mutations were highly enriched in a group of patients with an intermediate-risk cytogenetic profile, as well as in FLT3 receptor mutations (25 of 61 (41.0%) patients; $p<0.003$) (Ley et al., 2010). Walter *et al.* (Walter et al., 2011) also described relatively frequent mutations of Dnmt3a in MDS. They performed sequencing in patients with MDS and identified 13 heterozygous mutations with predicted translational consequences in 12 of 150 (8.0%) patients. Yan *et al.* (Yan et al., 2011) discovered mutations in DNMT3a in 23 of 112 (20.5%) cases with the M5 subtype of AML. The DNMT mutants showed reduced enzymatic activity or aberrant affinity for histone H3 *in vitro*. Notably, there were alterations in the DNA methylation patterns and/or gene expression profiles, such as *HOXB* genes, in samples with DNMT3a mutations compared with those without such mutations (Yan et al., 2011). These recent findings strongly suggest a link between recurrent genetic alterations and aberrant DNA methylation status in myeloid malignancies.

As shown in Figures 1 and 4, PU.1 interacts with DNMT3a and DNMT3b (Suzuki et al., 2006) and suppresses its target genes through Dnmt activity (Imoto et al., 2010; Suzuki et al., 2006). Since the expression of DNMT3s is constant in AML specimens (Ley et al., 2010), it is tempting to speculate that DNMT3a mutations exert their leukemogenic potential, at least in

part, by functional impairment of PU.1 epigenetic activities as a transcriptional repressor, mainly through downregulation of its expression. It is possible that this may also play an important role in AML pathogenesis (Figure 5).

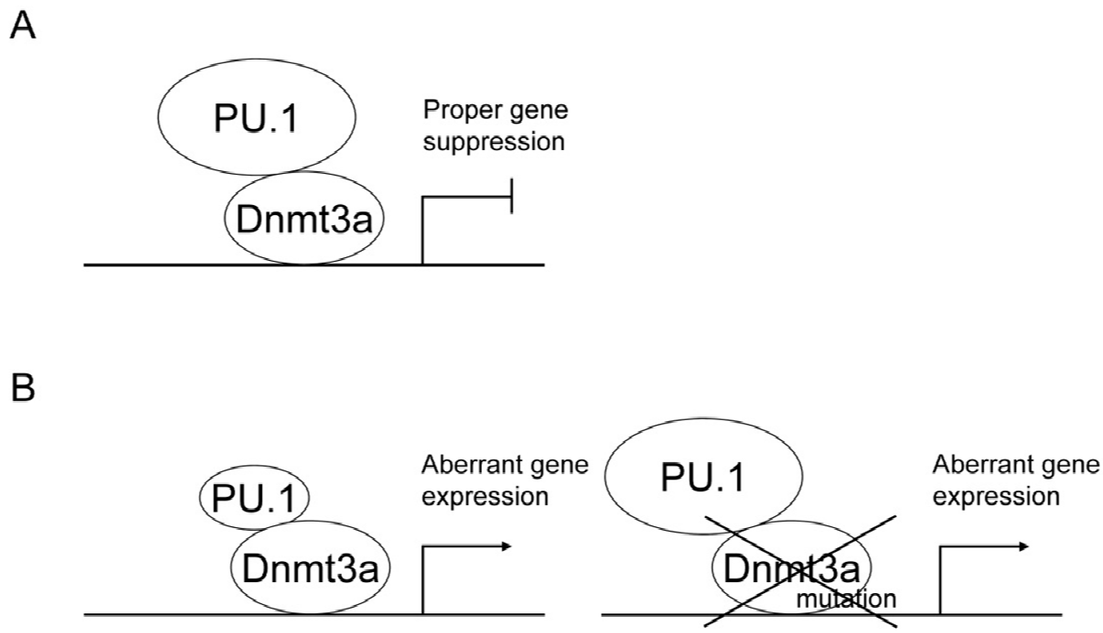

Fig. 5. Putative mechanisms of deregulated PU.1 repressor activity in AML. A, Normal cells. B, AML cells. A reduction in PU.1 expression (left panel), as well as mutations in Dnmt3a (right panel), result in aberrant target gene expression, which plays a role in the pathogenesis of AML.

Collectively, not only deregulation of proper myeloid-specific gene expression by downregulation of PU.1, but also insufficient recruitment of such epigenetic factors may play important roles in leukemia pathogenesis. Further clarification of the functions of this versatile transcription factor, with a particular focus on its function as a transcriptional repressor, may reveal the molecular biology of myeloid leukemia.

10. Acknowledgments

I thank Dr. Alison Sherwin (Edanz Editing) for helpful advice on the English language and editing of this manuscript. This work was supported in part by Grants-in-Aid for Scientific Research (No. 23590687) from the Ministry of Education, Science and Culture, Japan, Mitsubishi Pharma Research Foundation, Nakayama Foundation for Human Science, Takeda Science Foundation, and a foundation from Kitasato University School of Allied Health Sciences (Grant-in-Aid for Research Project, No. 2010-1004, No. 2011-1001).

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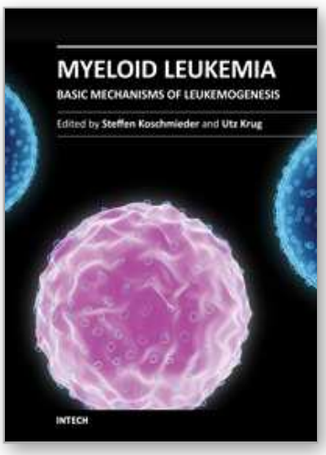

Myeloid Leukemia - Basic Mechanisms of Leukemogenesis
Edited by Dr Steffen Koschmieder

ISBN 978-953-307-789-5
Hard cover, 484 pages
Publisher InTech
Published online 14, December, 2011
Published in print edition December, 2011

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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Shinichiro Takahashi (2011). PU.1, a Versatile Transcription Factor and a Suppressor of Myeloid Leukemia, Myeloid Leukemia - Basic Mechanisms of Leukemogenesis, Dr Steffen Koschmieder (Ed.), ISBN: 978-953-307-789-5, InTech, Available from: http://www.intechopen.com/books/myeloid-leukemia-basic-mechanisms-of-leukemogenesis/pu-1-a-versatile-transcription-factor-and-a-suppressor-of-myeloid-leukemia

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