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Leukemia and ABC Transporters

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Abstract

Acute myeloid leukemia (AML) is a heterogeneous disease caused by aberrant proliferation and/or differentiation of myeloid progenitors. However, only ~65% of AML patients respond to induction chemotherapy and the overall survival rate for AML remains low (~24% for 5-year survival). The conventional view suggests that ATP-binding cassette (ABC) transporters contribute to treatment failure due to their drug-effluxing capabilities. This might be overly simplistic. Some ABC transporters export endogenous substrates that have defined roles in normal hematopoietic progenitors. It is conceivable that these substances also provide an advantage to leukemic progenitors. This review will highlight how certain endogenous substrates impact normal hematopoietic cells and suggest that ABC transporters facilitate export of these substances to affect both normal hematopoietic and leukemic progenitors. For example, the ability to export certain endogenous ligands may facilitate leukemogenesis by modifying leukemic progenitor cell proliferation or survival. If so, the addition of ABC transporter inhibitors to traditional chemotherapy might improve therapeutic efficacy by not just increasing intracellular drug accumulation but also blocking the beneficial effects ABC transporter ligands have on cell survival.

1. HEMATOPOIESIS AND LEUKEMIA

1.1. Hematopoietic stem cells and ABC transporters

Hematopoietic stem cells (HSCs) have the potential to undergo self-renewal and differentiation into multiple lineage committed blood cells. HSCs are characterized by their ability to repopulate the bone marrow, and single HSC gives rise to all blood cell types in circulation (Osawa, Hanada, Hamada, & Nakauchi, 1996; Smith, Weissman, & Heimfeld, 1991). By transplanting genetically marked mouse bone marrow cells into lethally irradiated recipient mice, Till and McCulloch showed that each spleen colony-forming unit (CFU) originated from a single clonogenic cell (Becker, McCulloch, & Till, 1963). Subsequent studies have shown that HSCs and their progenitors can be identified and isolated using cell surface markers (Spangrude, Heimfeld, & Weissman, 1988). Long-term HSCs have an unlimited self-renewal capacity, whereas short-term HSCs have limited self-

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renewal ability. The HSC progeny gives rise to multipotent progenitors (MPPs), which then differentiate to lineage-restricted progenitors including the myeloid progenitors (Fig. 1). It is generally acknowledged that HSCs show properties such as quiescence, drug resistance (through the expression of several ATP-binding cassette (ABC) transporters), an active DNA-repair capacity, and resistance to apoptosis, which enable long life span. Murine HSCs are characterized by the absence of markers for committed blood lineage (Lin^-) and expression of c-Kit and Sca-1 ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$, LSK). Additional cell surface markers can discriminate long- and short-term HSCs as well as MPPs. Common lymphoid progenitors (CLP) express IL-7R, whereas other committed progenitors do not. Oligopotent myeloid progenitors lose Sca-1 expression, and a combination of FcγR and CD34 expression identifies common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs), and megakaryocyte/erythroid progenitors (MEPs).The hierarchy of myeloid progenitors was determined by a combination of morphology and surface markers that was identified in each progenitor population (Akashi, Traver, Miyamoto, & Weissman, 2000). For example, CMPs were capable of generating all myeloerythroid cells, whereas GMP generated only granulocyte/ macrophage (GM) cells and MEP formed only megakaryocyte/erythroid cells in their colonies.

In humans, HSCs are identified by the cell surface markers, CD34 and CD38. Lineage-negative $CD34^+CD38^-$ cells are enriched for progenitors capable of repopulating bone marrow of NOD–SCID mice with all lineages. Committed progenitors acquire the expression of CD38, and the human counterparts of CMP, GMP, and MEP were identified in the $Lin^-CD34^+CD38^+$ population. Differentiation of progenitors into the myeloid lineage requires growth factors such as interleukin-3, GM-colony-stimulating factor, steel factor (c-Kit ligand), and erythropoietin as well as various transcription factors including runt-related transcription factor-1.

HSCs capacity to extrude toxins is likely related to high levels of ABC transporters that can protect cells from accumulation of endogenous and exogenous cytotoxic compounds (Table 1). In addition, the export of small molecules might serve a signaling function (e.g., leukotriene C_4, LTC_4). Side population (SP) cells are enriched for stem cells and are characterized by their ability to efflux the fluorescent dye, Hoechst 33342 (Goodell, Brose, Paradis, Conner, & Mulligan, 1996). ABCG2 was identified as the transporter responsible for Hoechst 33342 efflux which conferred the SP phenotype (Zhou et al., 2002). Absence of Abcg2 in hematopoietic cells did not affect normal unperturbed hematopoiesis. However, absence of Abcg2 rendered HSC markedly sensitive to chemotherapeutic agents that were Abcg2 substrates (e.g., mitoxantrone). Abcg2 may not protect all stem cells as embryonic stem cells or neural stem cells lack high Abcg2 expression (Ramalho-Santos, Yoon, Matsuzaki, Mulligan, & Melton, 2002).

1.2. Leukemic stem cells

Cancer stem cells are thought of as a rare group of cells that have self-renewal capacity coupled with the ability to produce tumors from a small number of initiating cells. The frequency of these cancer stem cells reportedly ranges from 0.0001% to 0.1% of the total cells; however, they may not be that rare (in some cases, e.g., myeloma; Huff & Matsui,

2008). Cancer stem cells were first identified in leukemia, but have also been identified in solid tumors such as brain, breast, cervix, and prostate (Dean, Fojo, & Bates, 2005). The leukemia-initiating cell population was identified and characterized 20 years ago (Bonnet & Dick, 1997; Lapidot et al., 1994). In acute myeloid leukemia (AML), the cell surface of leukemic stem cells (LSCs) resembles normal HSCs by both displaying the $CD34^+CD38^-$ surface marker and possessing the ability to self-renew, proliferate, and differentiate. LSCs occur at an estimated frequency of 1 per 2×10^5 cells and produce AML when transplanted into NOD–SCID mice. In comparison, AML cells with the surface marker $CD34^+CD38^+$ have properties in common with more committed cells, i.e., limited self-renewal, and are nontumorigenic. These initial studies reported that regardless of the AML subtype (see Fig. 1), only $CD34^+CD38^-$ AML cells produced leukemia in NOD–SCID recipient mice.

However, there are exceptions to the idea that only $CD34^+CD38^-$ AML cells initiate leukemia. For instance, acute promyelocytic leukemia (French-American-British (FAB) class M3) is caused by an oncogenic PML–RARA fusion gene that is only detected in the $CD34^+CD38^+$ population. In this case, the $CD34^+CD38^-$ cells were not tumorigenic. Moreover, recent studies using mouse leukemia models have suggested that the target cells for some oncogenes, such as MLL-AF9 and MOZ-TIF2, initiate AML in more committed progenitors such as the GMP (Huntly et al., 2004; Somervaille & Cleary, 2006). These LSCs resemble committed myeloid progenitors, but appear to have acquired the capacity for self-renewal, a property absent in their normal counterparts. Regardless of the origin, LSCs are self-renewing clonogenic cells that produce AML.

Self-renewal, increased proliferation, and altered differentiation are key features of leukemia cells. Notably, constitutive activation of pathways that drive proliferation is often observed in LSCs. For example, Wnt/β-catenin signaling is important in normal HSCs but is highly active in chronic myelogenous leukemia (CML) cells that are rapidly proliferating during blast crisis. PI3K activates signaling pathways such as Wnt and mTOR and is activated in AML. In contrast, PTEN, a PI3K antagonist that regulates self-renewal of normal HSC, is often downregulated in AML.

1.3. AML chemotherapy and ABC transporters

Chemotherapy is the mainstream treatment for AML; however, the development of drug resistance is a major obstacle in successfully achieving remission. As exporters of a variety of classes of chemotherapeutics, ABC transporters are considered to play a role in multidrug resistance in AML. For example, ABCB1/P-glycoprotein (P-gp) exports structurally diverse cancer chemotherapeutics including anthracyclines, one of the main chemotherapeutics used in the treatment of AML, and a marker for poor prognosis in AML (reviewed extensively in Shaffer et al., 2012). Because of its clinical relevance, P-gp inhibitors have been implemented in clinical trials to investigate if their addition to standard chemotherapeutic regimens improves clinical outcome. The results have been disappointing thus far and appear due to the nonspecificity of the inhibitors as well as the pharmacokinetic effects due to altered metabolism (Libby & Hromas, 2010). A widely accepted role for ABC transporters in AML chemotherapy is associated with their capacity to export drugs, thereby

limiting leukemic cell drug exposure (Shaffer et al., 2012); however, other modes of resistance may involve intracellular drug sequestration.

Indeed, overexpression of ABCA3 increased lysosomal mass and lysosomal drug retention. This drug sequestration correlated with reduced cytotoxicity (Chapuy et al., 2009; Steinbach et al., 2006; Wulf et al., 2004). In this review, we focus on the potential role of some ABC transporters (in particular ABCC1, ABCC4, and ABCG2) in leukemia biology through their ability to modulate endogenous ligands.

2. ABC TRANSPORTERS THAT EXPORT REGULATORY MOLECULES

2.1. Cyclic nucleotides—cAMP

The cyclic nucleotide, cyclic adenosine monophosphate (cAMP), is generated by adenylate cyclase in response to extracellular ligands and mediates a broad range of cellular responses through activation of protein kinase A. Intracellular levels of cAMP are modulated by factors such as phosphodiesterases and export (Cheepala et al., 2013). However, cyclic nucleotides are also compartmentalized into domains in the plasma membrane (Li et al., 2007; Sinha et al., 2013). Cyclic AMP is produced from ATP when adenylate cyclase is activated by G protein-coupled receptors (GPCRs). In the bone marrow niche, several GPCRs including β-adrenergic receptors, prostaglandin E_2 (PGE_2) receptors, and the chemokine MIP-1α receptors play an important role in affecting hematopoietic cell cAMP levels. Cyclic nucleotide-regulated pathways are involved in hematopoietic progenitor cell (HPC) proliferation and differentiation. The bone marrow is densely innervated by β-adrenergic fibers, and PGE_2 and MIP-1α are secreted by monocytes and macrophages. Therefore, it is not surprising that HPCs contain high concentrations of cAMP-dependent PKA (Kobsar et al., 2008). Activation of PKA mediates a cascade of signaling events including PI3K/Akt and mTOR pathways.

Interestingly, cAMP inhibits proliferation of the myeloid leukemia cell lines, human Mo7e, and the murine myeloid progenitor cell line, 32D (Hendrie & Broxmeyer, 1994; Lee, 1999). Furthermore, the stable cAMP analog, 8-chloroadenosine 3′,5′-cyclic monophosphate (8-CL-cAMP), inhibited the self-renewal capacity of blast progenitors from acute myeloblastic leukemia patients (Pinto et al., 1992). Stimulation of PKA inhibits HPC proliferation in HPCs from tumor patients (Kobsar et al., 2008). Thus, it appears, in myeloid leukemia cells, that elevation of cAMP and PKA activation inhibits myeloid leukemia proliferation, unless factors counterbalance this.

2.2. MRP4 and cAMP

Within the C subfamily of ABC transporters, 13 full-length multidrug-related proteins (MRPs; i.e., encode two membrane-spanning domains and two nucleotide-binding domains in one transcript) have been identified, together with the cystic fibrosis transmembrane conductance regulator (ABCC7) and the sulfonylurea receptors (ABCC8 and ABCC9). MRP4/ ABCC4 was the first ABC transporter shown to export nucleotide monophosphates (Schuetz et al., 1999), of which one of them (PMEA aka adefovir) resembled cAMP. Thus, it was not surprising that cAMP and cGMP were identified as endogenous substrates (Chen,

Lee, & Kruh, 2001). Subsequent studies have identified other endogenous molecules such as ADP, eicosanoids, urate, bile acids, and conjugated steroid hormones as potential *in vivo* substrates (Ritter et al., 2005). Other ABCC family members MRP5 and 8 have also been shown to transport cAMP and cyclic guanosine monophosphate (cGMP), but these will not be discussed here as ABCC8 is discussed in the review by Nies et al. (Chapter 8).

MRP4 modulates the cAMP concentrations in cells in two ways: by affecting internal concentration by export and creating domains or gradients of cAMP concentration within the plasma membrane. In this context, MRP4 can reduce the local concentration of membrane by "flipping" cAMP from the membrane, to locally reduce its concentration (Li et al., 2007). MRP4 can also directly export cAMP as was originally shown by Kruh and colleagues (Chen et al., 2001). Distinguishing between MRP4 flippase and transport activity is not easily done.

Absence of MRP4 profoundly affects how cells migrate. For example, fibroblasts lacking MRP4 have both elevated membrane and intracellular cAMP and exhibit an enhanced rate of migration (Sinha et al., 2013). It is unknown if the migration of leukemic or hemapoietic cells is impacted in a similar way. Furthermore, how cAMP in the membrane versus intracellular cAMP affects proliferation and differentiation of normal hematopoietic and leukemia cells is unknown (Copsel et al., 2011).

2.3. Prostaglandins

Prostaglandins are potent lipids derived from phospholipase-released arachidonic acid (AA) that are involved in numerous homeostatic biological functions and inflammation. Prostaglandins are synthesized *de novo* by prostaglandin H synthase (PGHS; referred to as COX for cyclooxygenase) when cells are activated by various external stimuli. PGHS exists as two isoforms referred to as PGHS-1 (COX-1) and PGHS-2 (COX-2; Fig. 2). In general, COX-1 is the enzyme responsible for basal, constitutive prostaglandins synthesis, whereas COX-2 is important in various inflammatory and "induced" settings. Prostaglandins are formed and "released" by most cells to act as autocrine- or paracrine-signaling molecules. In many cases, they initiate their effects via interaction with extracellular GPCRs to affect downstream signaling pathways (Breyer & Breyer, 2000).

2.4. Prostaglandin and HSCs

PGE_2 is the most abundant eicosanoid and has a variety of salutary effects in hematopoietic cells. In 1974, PGE_2 was shown to regulate myeloid progenitor differentiation (Feher & Gidali, 1974). In the bone marrow, myeloid cells, as well as osteoclasts in particular, contain a high PGE_2 synthetic capacity. Osteoclasts are an important component of the HSC niche and PGE_2 released from these cells may regulate HSC and HPC function. PGE_2 specifically binds to EP1–4 receptors, which are coupled to their respective G proteins to mediate distinct downstream pathways (Sugimoto & Narumiya, 2007). While EP3 receptor activation inhibits adenylate cyclase, EP2 and EP4 receptors activate it, which results in cAMP elevation. PGE_2 dose-dependently inhibits growth of human and colony-forming units granulocyte/macrophage (CFU-GM) in vitro (Pelus, Broxmeyer, Kurland, & Moore, 1979; Pelus, Broxmeyer, & Moore, 1981) and myelopoiesis *in vivo* (Gentile, Byer, & Pelus,

1983) but stimulates erythroid and multilineage progenitor cells (Lu, Pelus, & Broxmeyer, 1984; Lu et al., 1987). Short-term *ex vivo* treatment of bone marrow cells with PGE_2 increases the proportion of mouse colony-forming units spleen (CFU-S) (Feher & Gidali, 1974). In humans, PGE_2 treatment increases the proportion of cycling CFU-GM from quiescent cells that appear to be stem cells. This effect is critically dependent on timing, duration of exposure, and concentration (Pelus, 1982).

Signal transduction after PGE_2 engages in EP receptor can be either positive or negative, depending upon cell type. Positive signal transduction receptors include EP1, which triggers Ca^{2+} mobilization, and EP2 and EP4, which stimulate cAMP production. EP3 reduces cAMP concentration. PGE_2 also enhances survival, proliferation, and homing of HSCs. Brief exposures to $dmPGE_2$, a long-acting derivative of PGE_2, *ex vivo* increase HSC frequency in murine bone marrow cells and enhance recovery of hematopoiesis in zebrafish following sublethal irradiation (Hoggatt, Singh, Sampath, & Pelus, 2009; Northet al., 2007). Based on these promising preclinical studies, $dmPGE_2$ is being investigated in phase II clinical trials for patients with leukemia receiving umbilical cord blood transplantation to expand and improve engraftment of HSCs (Cutler et al., 2013; Goessling et al., 2011).

Hematopoietic progenitors show a competitive advantage in repopulation assays after a brief $dmPGE_2$ treatment, due to an increase in the homing, survival, and proliferation of HSCs (Hoggatt et al., 2009). The increased homing capacity of HSCs was due to enhanced expression of the chemokine receptor, CXCR4. CXCR4 is a receptor for the chemoattractant stromal cell-derived factor-1α (SDF-1α)/CXCL12, which has a key role in the trafficking and homing of HSCs and HPCs to the bone marrow niche. The CXCL12/ CXCR4 axis plays an important role in progression of various tumors as CXCR4 increases metastasis of tumor cells into various organs, and CXCL12 can support the survival and proliferation of tumor cells. Notably, expression of CXCR4 is a poor prognostic marker in AML.

In addition, PGE_2 signaling through the EP4 receptor increases β-catenin signaling, suggesting synergistic cross talk between prostaglandin and Wnt pathways (Wang, Mann, & DuBois, 2004). This is consistent with the finding showing PGE_2-mediated increases in cAMP associated with β-catenin stabilization in HSC promoting survival. The *in vivo* significance of the PGE_2/Wnt interaction is that HSC survival is promoted (Goessling et al., 2009). cAMP elevation by forskolin phenocopied the increase in HSC proliferation as well as demonstrated the requirement for Wnt/ β-catenin. This PGE_2 pathway might also be relevant in AML as downregulation of β-catenin by short hairpin RNA in both AML cell lines and AML blasts reduced proliferation of AML (Siapati et al., 2011).

2.5. MRP4 and prostaglandins

The understanding of how prostaglandins are extruded and taken up into cells is only a recent development because the conventional view was that these molecules entered and left cells by diffusion. Organic anion transporters mediate the uptake of prostaglandins (Schuster, 2002). The Borst lab was the first to demonstrate an ABC transporter (MRP4) actively export PGE_1 and PGE_2 (in the absence of additional cofactors, e.g., glutathione; Reid et al., 2003; Fig. 2). It is interesting that nonsteroidal anti-inflammatory drugs potently inhibit MRP4 and therefore the export of these proinflammatory prostaglandins. Cellular

retention of PGE_2 is related to MRP4 level with greater expression producing greater reductions in intracellular PGE_2 concentrations (Reid et al., 2003). PGE_2 export is blocked by inhibition or knockdown of MRP4 (Reid et al., 2003). Furthermore, MRP4 deficiency and RNA interference-mediated MRP4 knockdown significantly reduced extracellular PGE_2. cAMP-dependent protein kinase activity and COX-2 expression are reduced in MRP4-deficient cells, suggesting that prostaglandin synthesis might be restrained along with a lack of prostaglandin transport (Lin et al., 2008). It is likely that MRP4-mediated export of PGE_2 engages the EP receptors to modulate cAMP signaling and restrain prostaglandin synthesis. One could speculate that in the absence of MRP4, PGE_2 levels in the bone marrow niche might be lower, producing reduced HSC function and/or homing. Furthermore, in HSC, MRP4 absence might also reduce PGE_2 activation of EP2 and EP4, thereby reducing intracellular cAMP and producing a net reduction in HSC numbers.

2.6. Leukotrienes in hematopoietic cells

The leukotrienes are biosynthesized by oxygenation of AA by 5-lipoxygenase (5-LOX) and converted into the unstable intermediate LTA_4 (Fig. 2). LTA_4 is either enzymatically hydrolyzed to LTB_4 or conjugated to glutathione forming the cysteinyl leukotriene LTC_4. LTC_4 is then converted to LTD_4 and LTE_4. Leukotrienes, like other eicosanoids, are mostly produced in myeloid cells (Lindgren & Edenius, 1993). 5-LOX is found in HSC (Bautz, Denzlinger, Kanz, & Mohle, 2001) and myeloid cells; however, loss of the *Alox5* gene does not affect normal HSC function (Chen, Hu, Zhang, Peng, & Li, 2009). Notably, the leukotrienes LTB_4, LTC_4, andLTD_4 increase the number of HSC both in mouse and in human (Braccioni et al., 2002; Elsas et al., 2008; Vore, Eling, Danilowicz, Tucker, & Luster, 1989). For LTB_4, the increase in HSC is likely due to an increase in proliferation as well as a reduction in apoptosis (Chung et al., 2005).

In the BCR–ABL-mouse model of CML, 5-Lox/*Alox5* is upregulated in LSCs. Interestingly, the increased 5-Lox activity is accompanied by an elevated plasma level of LTB_4 (Chen et al., 2009). In a mouse model of CML, BCR–ABL failed to develop CML when bone marrow cells lacking *Alox5* were used. The LSCs exhibited impaired differentiation, division, and displayed increased apoptosis (Chen et al., 2009). Accordingly, 5-LOX inhibitor, Zileuton, was more effective than the conventional therapeutic in prolonging the survival of CML mice. LTB_4 has been implicated in reducing HSC self-renewal capacity and favoring differentiation (Chung et al., 2005); however, its precise role is not yet defined. Although it is not clear how *Alox5* specifically regulates LSC, loss of *Alox5* correlated with reduction of β-catenin expression in BCR–ABL-expressing hematopoietic cells. Furthermore, *Alox5* function has been implicated in many signaling pathways including PI3K, which has an important role in AML.

2.7. MRP1 and leukotrienes

MRP1 was the second major drug transporter to be identified by the laboratories of Cole et al. (1992). MRP1 overexpression confers resistance to many drugs including anthracyclines and vinca alkaloids. MRP1 can act as a cotransporter with glutathione (GSH) of amphipathic organic anions, as well as an exporter of glutathione-, glucuronate-, or sulfate-conjugated drugs. Therefore, it is not surprising that the high-affinity endogenous substrate for MRP1

includes the GSH-conjugated leukotriene, LTC_4. Using membrane vesicles prepared from MRP1 overexpressing HeLa cells, it was shown that MRP1 mediates ATP-dependent transport of LTC_4, LTD_4, and LTE_4, with the highest affinity being for LTC_4 (Leier et al., 1994).

A biological role for MRP1 as a LTC_4 transporter *in vivo* was shown in the *Mrp1*-null mouse model (Wijnholds et al., 1997). Higher LTC_4 concentrations were discovered in mast cells from *Mrp1*-null mice, but there was no obvious hematopoietic defect. Importantly, a dramatically attenuated LTC_4-mediated inflammatory response was observed in mast cells from the *Mrp1*-null mice. These results showed that LTC_4 export required MRP1 to mediate a normal mast cell inflammatory response. Accumulation of intracellular LTC_4 resulted in increased LTB_4 due to product inhibition of LTC_4 synthase (Schultz, 2001). It is interesting to note that MRP4 has been shown to transport LTB_4 in the presence of GSH (Rius, Hummel-Eisenbeiss, & Keppler, 2008). It is unknown if cross talk between MRP1 and MRP4 plays a role in inflammatory responses. However, because leukotriene biosynthesis is critical for LSC survival and self-renewal, LTC_4 export by MRP1 might play a role in mediating signaling in leukemia cells (Fig. 2).

2.8. Porphyrin and ABCG2

One of the physiologically relevant substrates for ABCG2, pheophorbide a, a plant-derived chlorophyll metabolite, was identified using an *Abcg2*-null mouse model (Jonker et al., 2002). Although pheophorbide a is a chlorophyll metabolic breakdown product, it is a tetrapyrrole and structurally resembles endogenous porphyrins such as heme and protoporphyrin IX (PPIX). Heme is an essential cofactor to many proteins that regulate cell proliferation, death, and differentiation. Heme synthesis begins in the mitochondrial matrix with glycine and succinyl-CoA forming δ-aminolevulinic acid (ALA). Four enzymatic steps convert ALA to coproporphyrinogen III in the cytoplasm. The mitochondrial ABC transporter ABCB6 mediates the ATP-dependent import of coproporphyrinogen III back into the mitochondria where it is converted further to PPIX (Krishnamurthy et al., 2006; Lynch, Fukuda, Krishnamurthy, Du, & Schuetz, 2009). Iron is then enzymatically inserted into this tetrapyrrole to produce heme. Although heme is critical for multiple cell functions, its precursors, porphyrins, can be photoactivated to generate reactive oxygen species from molecular oxygen (see review by Ishikawa and colleagues, Chapter 7, Critical role of ABCG2 in ALA-photodynamic diagnosis and therapy of human brain tumor). In addition, PPIX can also induce cell death independent of photoactivation (Bednarz, ZawackaPankau, & Kowalska, 2007). The mechanism accounting for this nonphotoactivatable cell death is unknown but might be due to alterations in mitochondrial function. It is plausible that ABCG2 plays a role in modulating the excess intracellular PPIX to protect leukemic cells.

HSCs reside in a region of the bone marrow that has reduced oxygen level. The hypoxia-inducible factors (HIFs) are transcription factors, activated during hypoxia, that alter the metabolic pathways required for adaption to low oxygen environments (e.g., glycolytic enzymes). Hypoxia induces *Abcg2* via HIFs (Krishnamurthy et al., 2004; Martin et al., 2008). The hematopoietic progenitors from Abcg2 KO mice exhibited reduced self-renewal of myeloid progenitors under hypoxia and chemical inhibition of ABCG2 in wild-type (WT)

progenitors produced the same impairment in self-renewal. Conversely, overexpression of ABCG2 in a myeloid cell line (OCI-AML) resulted in increased hypoxic survival. The survival advantage conferred by ABCG2 was associated with its ability to export the heme precursor, PPIX (Krishnamurthy et al., 2004). During hypoxia, heme production is upregulated via increased ALAS and CPOX (Hofer, Wenger, Kramer, Ferreira, & Gassmann, 2003; Klinkenberg, Mennella, Luetkenhaus, & Zitomer, 2005; Vasconcelles et al., 2001). Therefore, ABCG2 may protect the hematopoietic and LSCs from cytotoxic PPIX over accumulation during hypoxia.

2.9. ABC transporters and AML

Much like normal hematopoiesis, AML blasts fall into a hierarchy, and depending on the progenitors stage, differentiation is arrested. AML can be divided into eight subtypes (M0–M7) according to the FAB classification based on the histopathology of the blasts (see Fig. 1). *ABCG2* expression has been reported to correlate with an immature immunophenotype in normal hematopoietic cells, and its expression is downregulated upon differentiation of hematopoietic progenitors. One exception appears to be the erythroid-lineage which constitutively expresses ABCG2 (Raaijmakers, Van Den Bosch, Boezeman, De Witte, & Raymakers, 2002; Raaijmakers, van Emst, de Witte, Mensink, & Raymakers, 2002; Scharenberg, Harkey, & Torok-Storb, 2002; Zhouet al., 2002, 2001). We interrogated the Oncomine database (www.oncomine.org) to determine *ABCG2* expression in various AML subtypes (Fig. 3). No significant expression was detected in myelocytic or monoblastic subtypes of AML (M3–M5). In contrast, *ABCG2* was highly expressed in the myeloblastic leukemia subtypes (M0, without maturation) as well as in acute erythroleukemia (M6), a leukemia of red cell lineage. In pediatric AML M7, *ABCG2* is highly expressed compared to other AML subtypes and a promoter unique to this subtype has been identified (Campbell et al., 2011).

Myelodysplastic syndrome (MDS) is a heterogeneous disorder with abnormal proliferation, morphology, and differentiation of myeloid progenitors and carries a high risk of transforming to AML. ABCC4 and ABCG2 were both highly expressed in this population considered to be “leukemic precursors” in two studies where one study included patients with MDS. Whether ABCC4 and ABCG2 play a role in a malignant transformation remains to be elucidated. Interestingly, there have been some rare, but well-documented, cases of MDS and AML that arose from therapy with the thiopurine, azathiopurine (Arnold, Ranson, & Abdalla, 1999). ABCC4 has been shown to transport thiopurine-derived nucleotides, and mice lacking *Abcc4/Mrp4* displayed greater hematopoietic toxicity after thiopurine administration compared to the WT mice (Krishnamurthy et al., 2008). One could speculate that ABCC4 deficiency primes hematopoietic progenitors to DNA damage by thiopurines. This damage might be followed by an oncogenic second hit producing MDS or AML. Therefore, it is conceivable that patients with a single nucleotide polymorphism (SNP) in *ABCC4* that alters its function might be more vulnerable to thiopurine-associated AML. Among several nonsynonymous SNPs found in *ABCC4*, 2269G>A (E757K) has been shown to result in a lower surface expression and results in a greater cellular accumulation of thiopurines (Krishnamurthy et al., 2008). This SNP is found at a high frequency in the Japanese population (>18%) as well as in Asians (~4%). Indeed, this SNP was associated

with greater hematopoietic toxicity in the Japanese IBD patients receiving thiopurine treatment (Ban et al., 2010). In addition, other ABCC4 non-synonymous SNPs (i.e., G187W and G487E) were shown to have reduced MRP4 function (Abla et al., 2008), although it is unknown if these are risk alleles for increased thiopurine toxicity.

Incomplete eradication of leukemic stems cells is considered to be one source of relapse in AML. Correlation between high *ABCG2* expression and relapse has been reported in several studies. For example, a higher relapse rate was observed among one-third of the patients with high ABCG2 (Damiani et al., 2006). Higher *ABCG2* expression was also associated with secondary AML and lower complete remission (van den Heuvel-Eibrink et al., 2007). In some AML patient samples, ABCG2 expression and function were higher at relapse compared to diagnosis (van der Kolk et al., 2002). A similar observation was made in 11 AML patient samples where five patients expressed higher ABCG2 at the time of relapse compared to diagnosis (Patel et al., 2013). In a childhood AML cohort, *ABCG2* expression was higher at the time of relapse and the high *ABCG2* expression correlated with worse overall survival. In addition, *ABCG2* was ~10-fold higher in patients who were refractory to treatment (Steinbach et al., 2002), and its expression was higher especially in $CD34^{+}CD38^{-}$ leukemic progenitors (Ho, Hogge, & Ling, 2008). Unsupervised clustering based on gene expression profiles of samples from 170 AML patients resulted in six clusters with high *ABCG2* expression (ranked 18th in the upregulated genes) found in a group with the poorest outcome (Wilson et al., 2006). However, *ABCG2* expression did not predict complete remission (Uggla et al., 2005). These results imply that ABCG2 expression is probably higher in a rare LSC population that hastens relapse. Consistent with this is the finding that SP cells (high ABCG2-expressing cells) were more abundant in the bone marrow cells from *de novo* and refractory/relapsed adult AML patients than those from the normal or AML patients in remission (Huang et al., 2013).

3. KINASES IMPACT TRANSPORTER LOCATION AND FUNCTION

3.1. Kinases and ABC transporters

Phosphorylation regulates cellular processes by modulating protein function, localization, half-life, and protein–protein interaction. Protein kinases mediate the addition of phosphate groups onto their target proteins at tyrosine, threonine, or serine residues. Because of the wide range of target proteins, kinases are involved in the modulation of many cellular functions. ABC transporters are no exception where LC/MS analyses have identified *bona fide* phosphorylation of many of the ABC transporter family members (Stolarczyk, Reiling, & Paumi, 2011). In cancer cells including AML, kinases are often constitutively active and confer survival advantage. Therefore, specific kinase inhibitors targeted against these survival pathways, especially tyrosine kinase inhibitors (TKIs), have been widely employed in AML therapy.

Many TKIs, such as imatinib and nilotinib, have been shown to be substrates and/or inhibitors for ABC transporters (reviewed in Shukla, Chen, & Ambudkar, 2012). The mode of interaction of these was not known until recently. By using structure–activity relationships combined with mutagenesis, Shukla and colleagues showed that key residues of P-gp/ABCB1 bound nilotinib (a BCR–ABL kinase inhibitor), in the substrate-binding

pocket rather than at the nucleotide-binding domain (Shukla et al., 2014). In addition, several serine/threonine kinases have been shown to modulate ABC transporter cellular localization and/or function. Because the relationship between ABC transporters and TKIs has been discussed in details elsewhere (Brozik et al., 2011; Shukla et al., 2012), we will focus on serine/threonine kinases as potential modulators of ABC transporter function.

3.2. Serine/threonine kinases Pim-1 and Akt affect ABCG2 location

The proto-oncogene *pim-1* encodes a serine/threonine protein kinase with a wide array of substrates and regulates such processes as cell cycle and apoptosis. Recent studies suggest that it might regulate ABCG2, which is relevant because Pim-1 is highly expressed in AML (Chen, Redkar, Taverna, Cortes, & Gandhi, 2011). Pim-1L interacts directly with and phosphorylates ABCG2 at T362, which resides in the linker region between the nucleotide-binding domain and the membrane-spanning domain (Xie et al., 2008). Phosphorylation of ABCG2 at T362 increases the transport capability, and the coexpression of ABCG2 and Pim-1L, but not phosphorylation-incompetent ABCG2 (T362A), increased drug resistance. The importance of T362 phosphorylation was confirmed by the ABCG2 mutant harboring a T362D substitution to mimic the phosphorylation. This mutant ABCG2 showed increased drug resistance independent of Pim-1L activity. The phosphorylation of T362 regulates membrane localization as well as a homodimer formation, resulting in an increase in ABCG2 transport function. The Pim-1 kinase inhibitor SGI-1776 reduced ABCG2 activity through reduction in its surface expression. Thus, one therapeutic advantage of the Pim-1 kinase inhibitor is that it modulates ABCG2 function by affecting its cellular location.

Another kinase appears to also regulate ABCG2 intracellular location. The serine/threonine kinase, Akt, is activated in AML. Loss of Akt in mice produced a phenotype in the bone marrow compartment that suggested ABCG2 function was lost. In contrast, exogenous expression of Akt increased ABCG2 transport phenotype (Mogi et al., 2003). Furthermore, chemical inhibition of PI3K by LY294002 reduced ABCG2 transport activity. It is unknown how PI3K/Akt phosphorylation affects translocation or retention of ABCG2 at the plasma membrane (Mogi et al., 2003; Takada, Suzuki, Gotoh, & Sugiyama, 2005). In the BCR–ABL-expressing leukemia cell line, K562, inhibition of the TKI, imatinib, reduced ABCG2 protein levels (Nakanishi, Shiozawa, Hassel, & Ross, 2006). Although it is still unclear how Akt alters ABCG2 localization, it is likely that it or another kinase mediates phosphorylation of ABCG2.

3.3. Casein kinase 2 modulates MRP1 function

Casein kinase 2 (CK2) is a ubiquitously expressed and constitutively active serine/threonine protein kinase, which regulates numerous cellular processes such as cell growth, proliferation, and survival owing to its large number of substrates (Meggio & Pinna, 2003). CK2 is comprised of two catalytic subunits (α/α or α/α′) and two regulatory β subunits. CK2 expression is upregulated in many cancers, including AML. One of the substrates for CK2α is MRP1 and an increase in its transport function upon phosphorylation has been reported (Stolarczyk et al., 2012). The direct phosphorylation at Thr249 by CK2α increases doxorubicin efflux by ABCC1/MRP1. The Thr249 is located in the cytoplasmic loop between N-terminal extension (MSD1), which is unique to MRPs and the ABC core

domains. In adult AML patients, high expression CK2α correlated with lower disease and overall survival compared to patients with low CK2α expression (Kim et al., 2007). CK2 selective inhibitors, tetrabromobenzotriazole or apigenin, induced cell death in primary AML blasts with a preference for cells with high CK2α expression. Thus, CK2α is a viable therapeutic target for AML and its inhibition will have an added benefit of inactivating MRP1.

4. FUTURE PERSPECTIVE

Efficient treatment of AML depends on elucidating the signaling pathways that provide LSCs with a survival advantage and unlimited self-renewal capacity. The conventional view functionally circumscribes ABC transporters as proteins that simply reduce treatment efficacy by either extruding chemotherapeutic drugs or sequestrating drugs within organelles such as lysosomes. However, recent discoveries of roles for ABC transporter ligands in HSCs suggest that export of these molecules, by ABC transporters, might have undefined salutary roles in leukemia (Fig. 4). The ligands for ABC transporters discussed here such as cAMP, PGE_2, LTC_4, and PPIX affect normal hematopoietic cell survival, differentiation, and/or self-renewal. It is unknown if these ligands have an analogous in LSCs. The future challenge is to discover these roles. For example, because exogenous leukotrienes maintain the LSC pool, it is conceivable that MRP1 might contribute to LSC function by supplying LTC_4 to its receptor. Moreover, MRP4 might contribute to increased proliferation and metastatic capacity of leukemic cells, by extruding PGE_2 enabling, it to affect (via its receptors) tumorigenicity in an autocrine or paracrine manner. ABCG2 may have a protective role for LSC by modulating the levels of cytotoxic porphyrins in the hypoxic niche or under increased demand for heme production. Some of these ABC transporters are regulated in part by serine/threonine protein kinases that are also upregulated in AML. Specific inhibitors for these kinases might be a viable addition to AML therapy because they might not only interfere with these serine/threonine kinases but also render ABC transporters nonfunctional, thus altering pathways important for LSC proliferation and survival.

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ABBREVIATIONS

ABC	ATP-binding cassette
ALA	δ-aminolevulinic acid
AML	acute myeloid leukemia
CK	casein kinase
CML	chronic myelogenous leukemia
CMP	common myeloid progenitor

COX	cyclooxygenase
GMP	granulocyte-macrophage progenitor
GPCR	G protein-coupled receptor
HIF	hypoxia-inducible factor
HPC	hematopoietic progenitor cell
HSC	hematopoietic stem cell
IBD	inflammatory bowel disease
LSC	leukemic stem cell
LSK	Lin $^{-}$Sca-1^{+}c-Kit^{+}
LTC_4	leukotriene C_4
MDS	myelodysplastic syndrome
MEP	megakaryocyte/erythroid progenitor
MPP	multipotent progenitors
MRP	multidrug-related protein
PGE_2	prostaglandin E_2
PPIX	protoporphyrin IX
SNP	single nucleotide polymorphism

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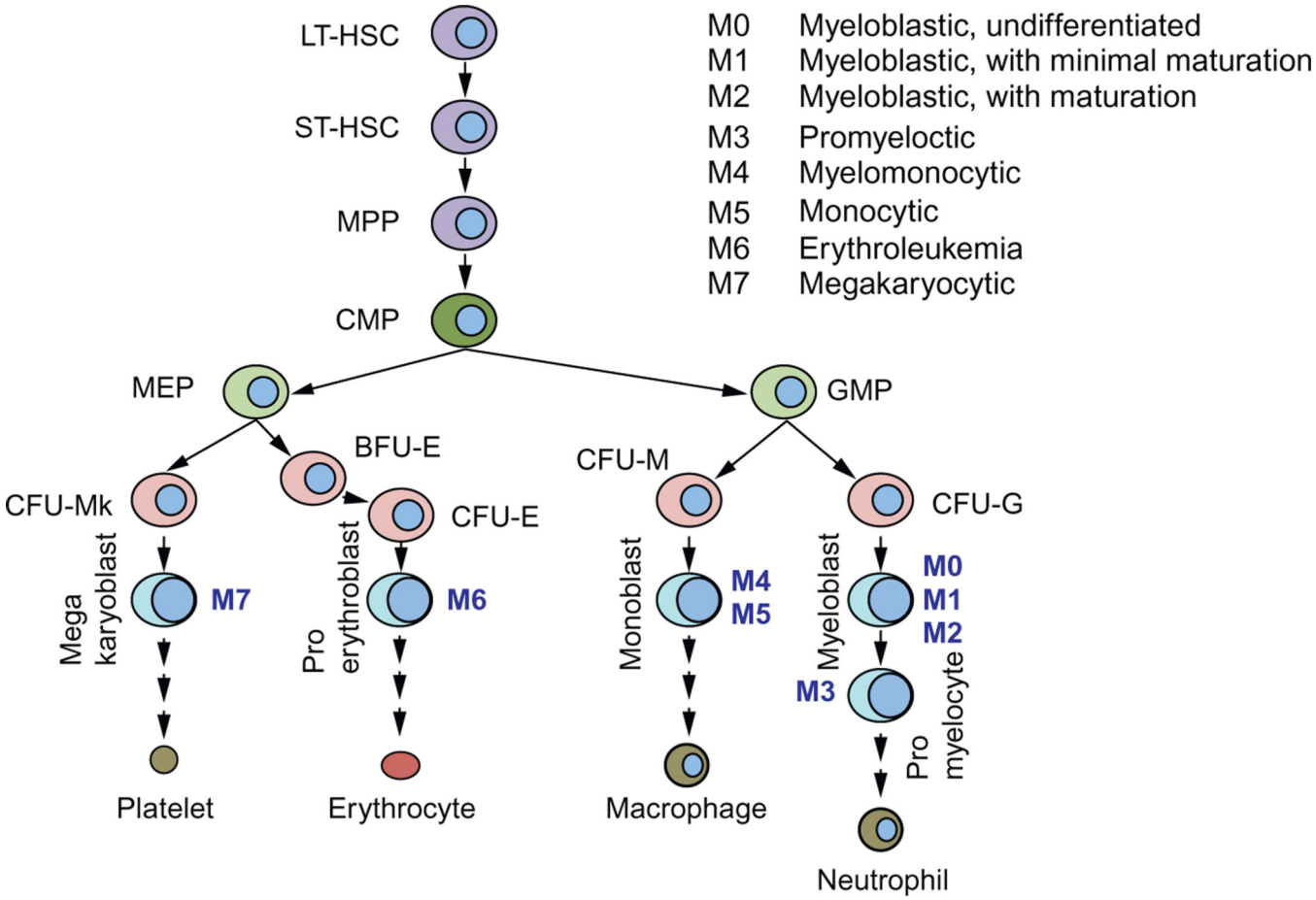

Figure 1.
Hierarchy of hematopoiesis and leukemia in myeloid lineage. AML subtypes according to FAB classification (M0–M7) are indicated.

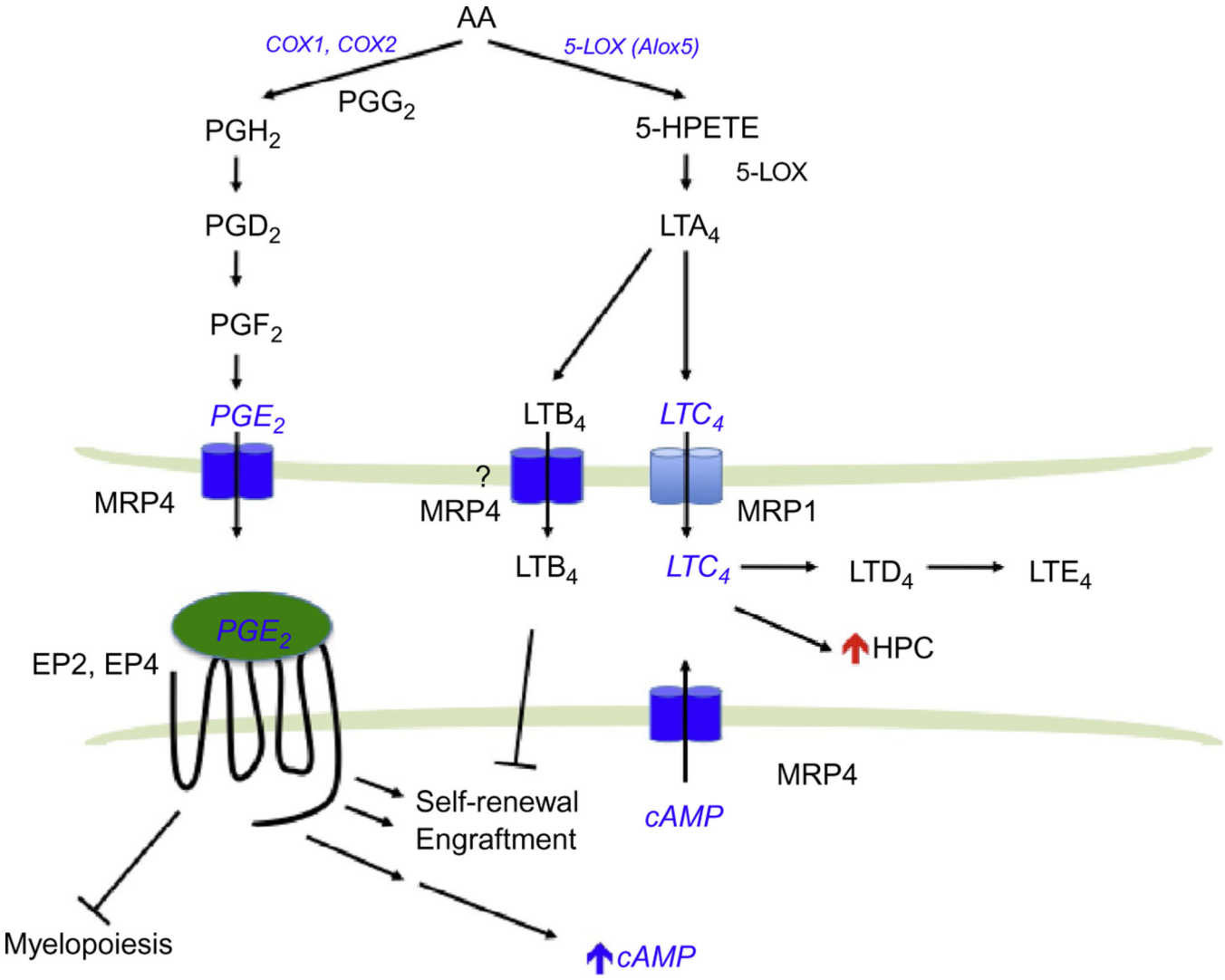

Figure 2.

Eicosanoid biosynthesis pathways. Prostaglandins and leukotrienes are synthesized from a common precursor, arachidonic acid (AA). Molecules implicated in leukemia are highlighted in blue.

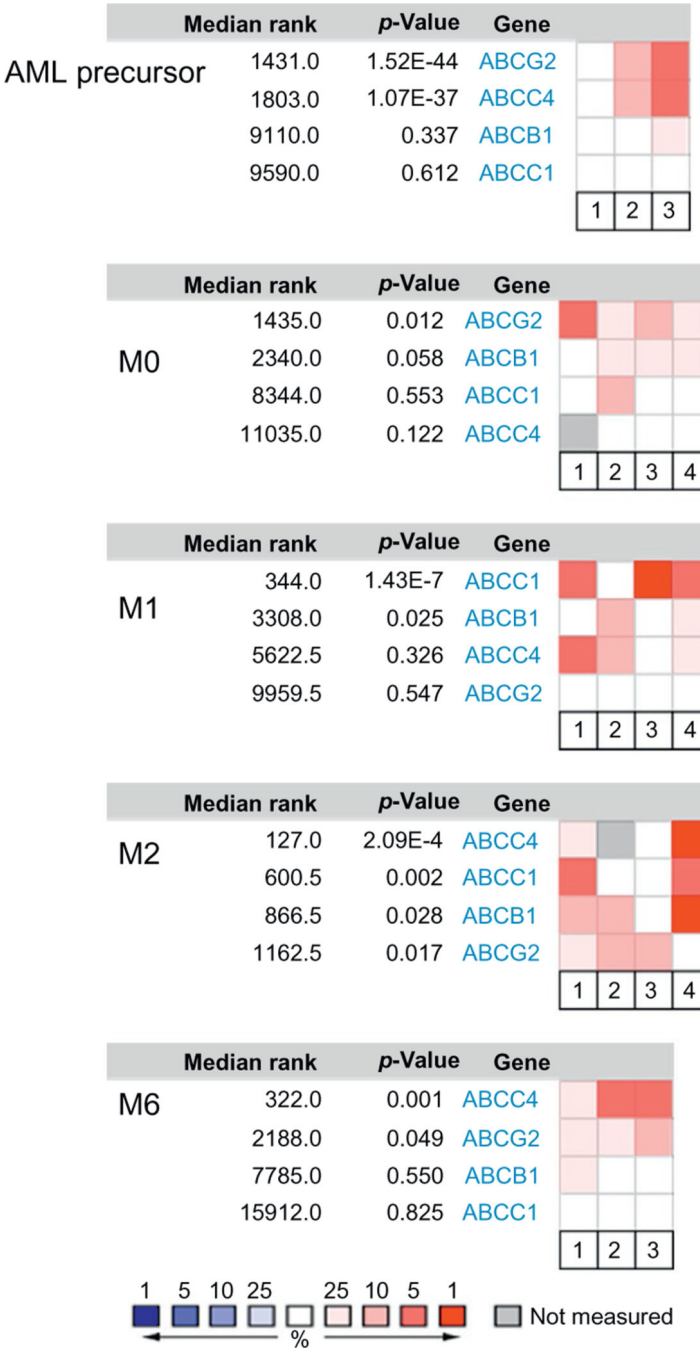

Figure 3.

Gene expression pattern of MRP1, MRP4, and ABCG2 in human AML. Expression of MRP1, MRP4, and ABCG2 in adult AML patients was queried in Oncomine database for different FAB subtypes. Only the subtypes with significant expression of at least one of these transporters are shown. Red indicates high expression, blue indicates low expression, and gray indicates not measured. *p*-Value is for the median-ranked analysis.

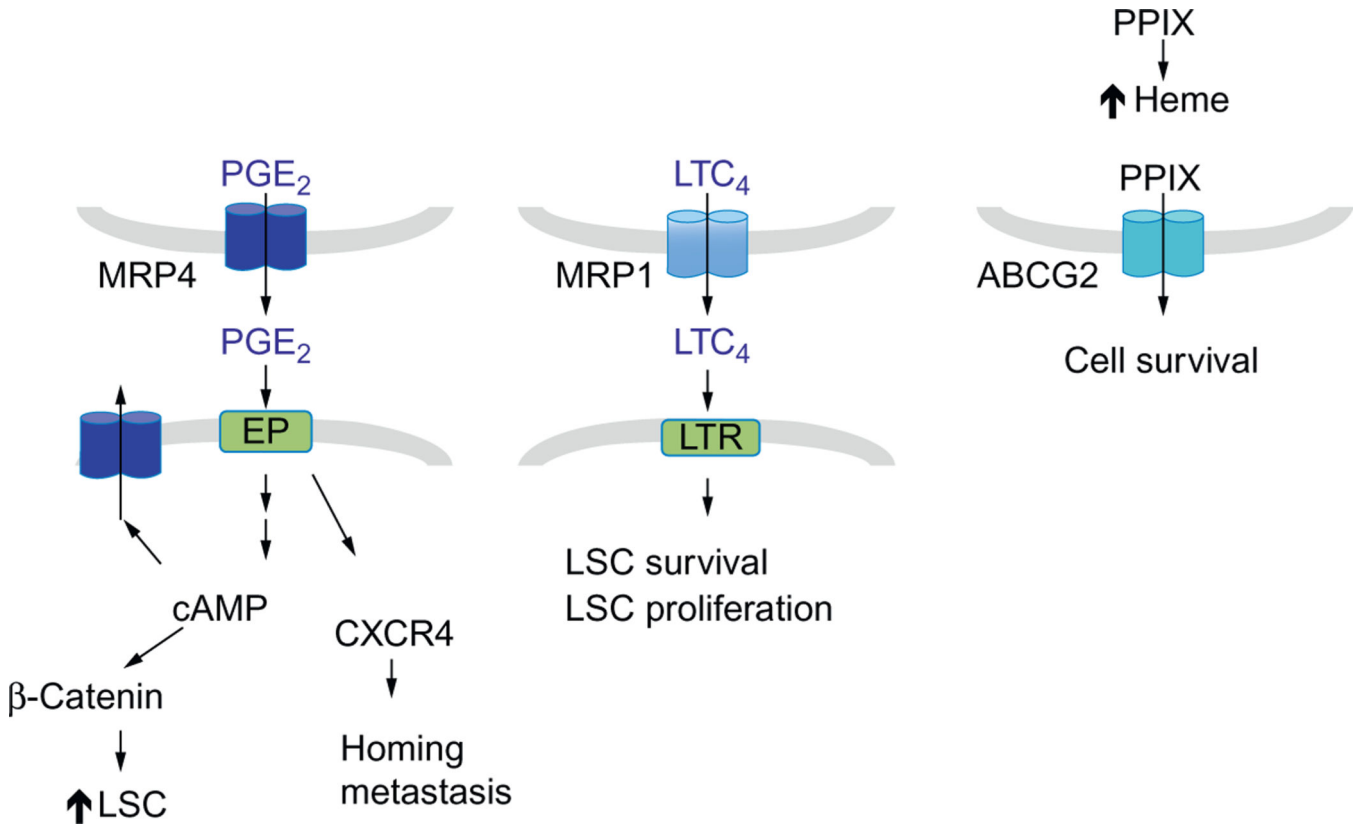

Figure 4.

Models depicting possible roles for ABC transporters in promoting AML through transport of endogenous ligands. MRP4 effluxes PGE2, which acts on EP receptors to increase LSC and metastasis. MRP4 also modulates cAMP levels. LTC_4 effluxed by MRP1 can act on CysLTR to promote LSC survival and proliferation. Heme biosynthesis is upregulated in highly proliferating cells. ABCG2 might alleviate toxic effect of excess PPIX when heme production is inadequate.

Table 1

ABC transporters and endogenous ligands

Transporter	Ligand	Function
MRP1 (ABCC1)	LTC_4, LTD_4, LTE_4	Inflammatory response
	GSH, GSSG, bilirubin, S1P	Redox
MRP4 (ABCC4)	PGE_1, PGE_2, PGF_2	Cell migration
	cAMP, cGMP, TXB_2, LTB_4, LTC_4	CFTR function modulation Inflammatory response
BCRP (ABCG2)	Flavonoids, porphyrins (PPIX) cGMP, folic acid	Survival under hypoxia