



# The Roles of Cholesterol and Its Metabolites in Normal and Malignant Hematopoiesis

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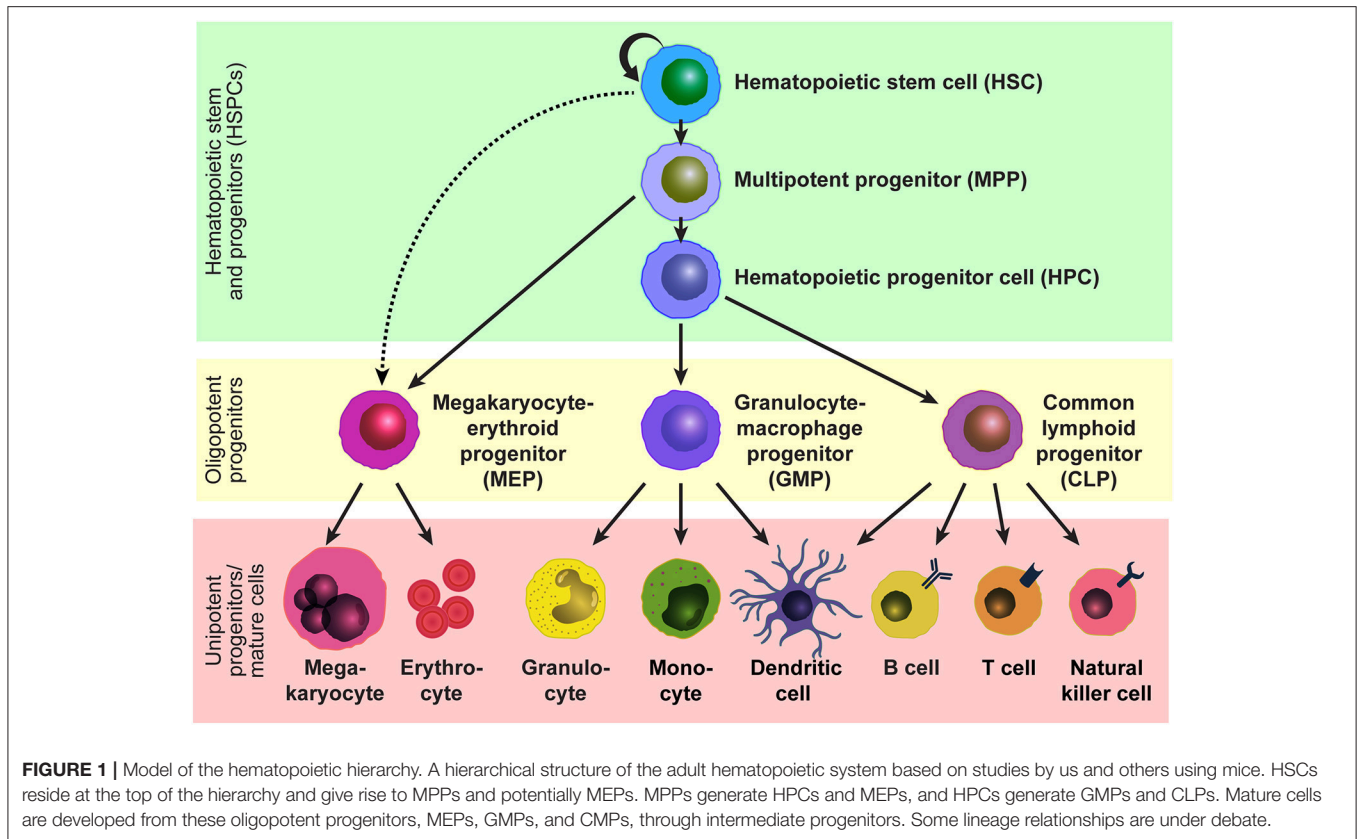
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Hematopoiesis is sustained throughout life by hematopoietic stem cells (HSCs) that are capable of self-renewal and differentiation into hematopoietic progenitor cells (HPCs). There is accumulating evidence that cholesterol homeostasis is an important factor in the regulation of hematopoiesis. Increased cholesterol levels are known to promote proliferation and mobilization of HSCs, while hypercholesterolemia is associated with expansion of myeloid cells in the peripheral blood and links hematopoiesis with cardiovascular disease. Cholesterol is a precursor to steroid hormones, oxysterols, and bile acids. Among steroid hormones,  $17\beta$ -estradiol (E2) induces HSC division and E2-estrogen receptor  $\alpha$  (ER $\alpha$ ) signaling causes sexual dimorphism of HSC division rate. Oxysterols are oxygenated derivatives of cholesterol and key substrates for bile acid synthesis and are considered to be bioactive lipids, and recent studies have begun to reveal their important roles in the hematopoietic and immune systems. 27-Hydroxycholesterol (27HC) acts as an endogenous selective estrogen receptor modulator and induces ER $\alpha$ -dependent HSC mobilization and extramedullary hematopoiesis.  $7\alpha,25$ -dihydroxycholesterol ( $7\alpha,25$ HC) acts as a ligand for Epstein-Barr virus-induced gene 2 (EBI2) and directs migration of B cells in the spleen during the adaptive immune response. Bile acids serve as chemical chaperones and alleviate endoplasmic reticulum stress in HSCs. Cholesterol metabolism is dysregulated in hematologic malignancies, and statins, which inhibit *de novo* cholesterol synthesis, have cytotoxic effects in malignant hematopoietic cells. In this review, recent advances in our understanding of the roles of cholesterol and its metabolites as signaling molecules in the regulation of hematopoiesis and hematologic malignancies are summarized.

**Keywords:** cholesterol, oxysterols, steroids, hematopoietic stem cells, hematopoiesis, hematologic malignancies

## INTRODUCTION

Hematopoietic stem cells (HSCs) sustain blood production throughout life and are the functional units of bone marrow transplantation. HSCs are capable of self-renewal to maintain their pool while producing all mature blood cells through differentiation into multipotent progenitors (MPPs) and subsequent hematopoietic progenitor cells (HPCs) with limited differentiation potentials (**Figure 1**). In adult mice, all HSCs and MPPs fall within the Lineage marker<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> (LSK) fraction (1–3), which is a heterogeneous population that contains a mixture of hematopoietic stem and progenitor cells (HSPCs), including HSCs, MPPs, and HPCs. HSCs can be further purified by selecting the CD150<sup>+</sup>CD48<sup>-/low</sup> subset (4, 5) or CD34<sup>-/low</sup>Flt3<sup>-</sup> subset (6, 7) of



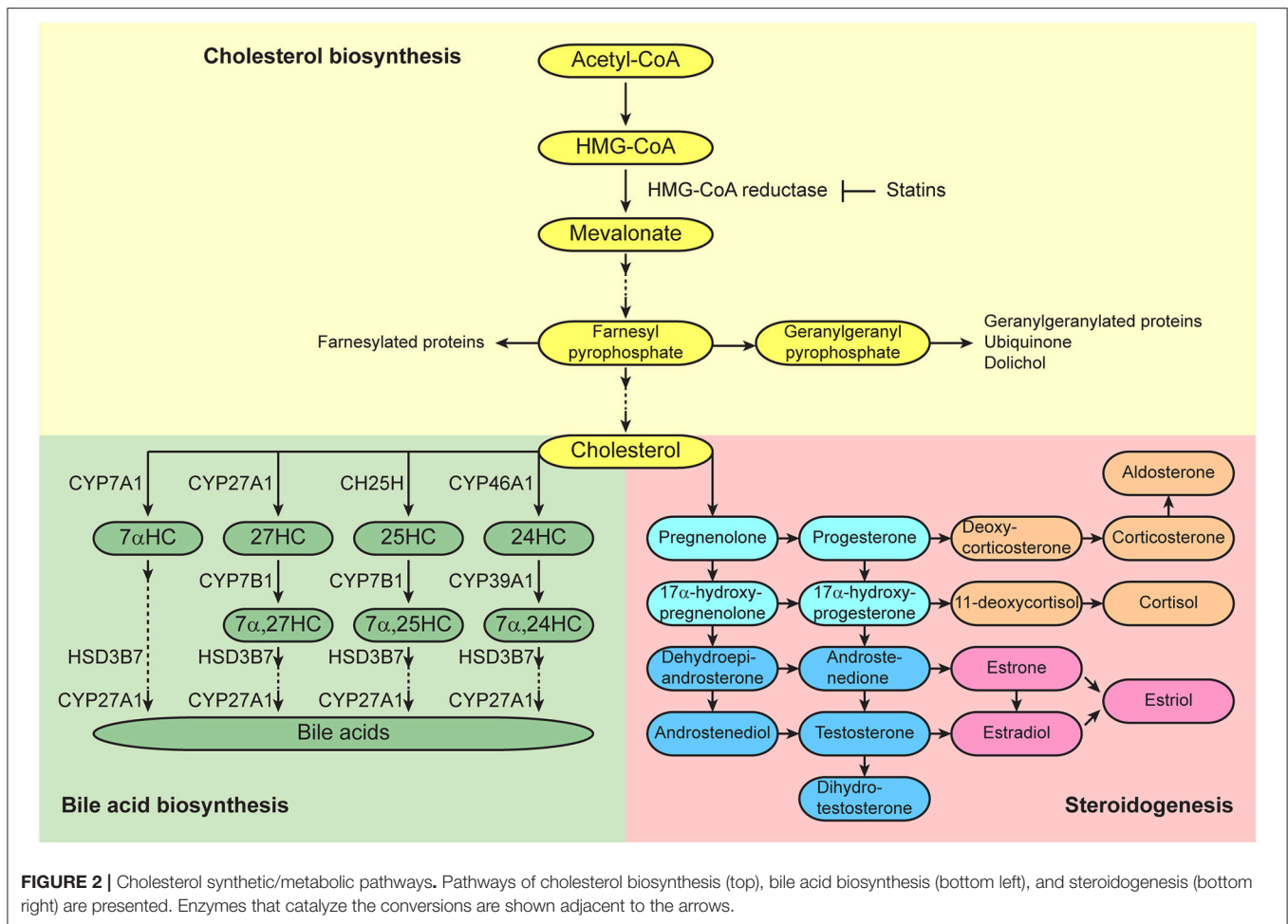
LSK cells. The HSC population is functionally heterogeneous in terms of cell-cycle kinetics, self-renewal capacity, and differentiation potential, and the heterogeneity can be distinguished by additional markers such as CD229 (5) and von Willebrand factor (8). Human HSPCs can be marked by Lineage marker<sup>-</sup>CD38<sup>-</sup>CD34<sup>+</sup> and the HSC population can be further refined by marking the CD45RA<sup>-</sup>CD49f<sup>+</sup> subset of Lineage marker<sup>-</sup>CD38<sup>-</sup>CD34<sup>+</sup> cells (9). HSCs reside in specialized niches which are local tissue microenvironments that support HSC behavior and regulate their function, such as self-renewal, differentiation, and localization, by producing factors that act directly on HSCs (10). In adults, HSCs are quiescent and localize primarily in the bone marrow, and their number is tightly regulated under steady-state conditions, comprising <0.01% of bone marrow cells in mice. In response to acute hematopoietic demands such as blood loss, myeloablation, infection, or pregnancy, HSCs change two aspects of their steady-state behaviors in order to increase production of necessary hematopoietic cells (11–14). First, quiescent HSCs re-enter the cell cycle to proliferate or differentiate through symmetric or asymmetric cell divisions, and second, they mobilize from the bone marrow to extramedullary tissues, such as the spleen, to expand the physical space for hematopoiesis.

In addition to the regulation of HSC behaviors by short-range factors, such as cytokines, cell-surface proteins, extracellular matrix components, oxygen tension, and ion levels, that are generated in their niches, HSCs are also regulated by long-range

systemic signals, such as circulating cytokines, hormones, lipids, and vitamins. Cholesterol is found in the bloodstream and within cells, and is an essential structural component of mammalian plasma membranes and is essential to maintain both membrane structural integrity and to modulate membrane fluidity (15). Cholesterol also serves as a precursor for the biosynthesis of steroid hormones, oxysterols, and bile acids (Figure 2) (16). These cholesterol metabolites have important biological roles as signal transducers and chemical chaperones, and there is accumulating evidence that these metabolites act as systemic signals that regulate normal and malignant hematopoiesis. This review discusses recent advances in understanding the roles of cholesterol and its metabolites in the regulation of hematopoiesis and hematologic malignancies.

## CHOLESTEROL LEVELS AND HEMATOPOIESIS

In the bloodstream, cholesterol is transported within lipoprotein particles, which are organized by apolipoproteins that can be recognized and bound by specific receptors on cell membranes. There are several types of lipoproteins, such as high-density lipoprotein (HDL), low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL), very-low-density lipoprotein (VLDL), and chylomicrons, in order of higher density to lower density. LDL is the major cholesterol carrier in the blood and is



**FIGURE 2 |** Cholesterol synthetic/metabolic pathways. Pathways of cholesterol biosynthesis (top), bile acid biosynthesis (bottom left), and steroidogenesis (bottom right) are presented. Enzymes that catalyze the conversions are shown adjacent to the arrows.

recognized by the LDL receptor (LDLR) in peripheral tissues. LDL is the atherogenic lipoprotein, and increased LDL levels promote cholesterol accumulation. Macrophages accumulate oxidized LDL and give rise to foam cells, and contribute to atherosclerotic plaque formation. HDL opposes this process and reduces inflammation. HDL is involved in reverse cholesterol transport, in which HDL serves to shuttle cholesterol from peripheral tissues to the liver where cholesterol is eventually converted into bile acids.

### Hypercholesterolemia Induces Proliferation and Mobilization of Mouse Hematopoietic Stem and Progenitor Cells

Several studies have shown a strong correlation between plasma cholesterol levels and the mobilized HSC number in the peripheral blood using mouse models. Gomes et al. showed that mice fed a high-fat/high-cholesterol (HFHC) diet for 30 days displayed thrombocytosis, lymphocytosis, and an increase in the number of HSPCs mobilized into the peripheral blood, while the HSPC number in the bone marrow decreased (17). They also found that the HFHC diet induced increased plasma levels of C-X-C motif chemokine ligand 12/ stromal cell-derived

factor 1 (CXCL12/SDF1), a chemokine which is chemotactic for HSCs that express its receptor C-X-C motif chemokine receptor 4 (CXCR4) (18). Apolipoprotein E (APOE) is a key component in cholesterol metabolism and *ApoE*-deficient mice cause hypercholesterolemia (19, 20). *ApoE*-deficient mice fed an HFHC diet developed monocytosis (21–23), and Murphy et al. also reported neutrophilia associated with the proliferation and expansion of HSPCs in the bone marrow (23). Interestingly, APOE was expressed on the surface of HSPCs and acted cell autonomously to control HSPC proliferation, monocytosis, neutrophilia, and monocyte accumulation in atherosclerotic lesions, as revealed by transplantation of *ApoE*-deficient bone marrow cells. LDLR deficiency causes impaired LDL clearance, resulting in high plasma LDL-cholesterol levels and causing familial hypercholesterolemia. LDL receptor-deficient (*Ldlr*<sup>-/-</sup>) mice fed an HFHC diet displayed hypercholesterolemia associated with increased HSPCs in both bone marrow and peripheral blood, and increased monocytes and granulocytes in the peripheral blood (24, 25). In addition to their mobilization, more HSPCs in the bone marrow incorporated the DNA synthesis marker 5-bromo-2'-deoxyuridine (BrdU) in *Ldlr*<sup>-/-</sup> mice on an HFHC diet as compared to in *Ldlr*<sup>-/-</sup> mice on a normal diet, indicating that hypercholesterolemia promoted

HSPC proliferation. In contrast, infusion with reconstituted HDL reduced the frequency and proliferation rate of HSPCs in the bone marrow, highlighting the opposing effects of LDL and HDL on HSPC proliferation. Scavenger receptor type BI (SR-BI, encoded by *Scarb1* gene) is a HDL receptor, and *Scarb1*<sup>-/-</sup> mice showed increased plasma total cholesterol levels with unchanged plasma concentration of apoA-I, the major protein in HDL (26). Gao et al. reported that *Scarb1*<sup>-/-</sup> mice fed an HFHC diet showed significantly increased the number of HSPCs in the bone marrow, spleen, and peripheral blood, as well as the proliferation of HSPCs as compared to wild-type mice fed an HFHC diet (27). Interestingly, HSPCs in *Scarb1*<sup>-/-</sup> mice fed an HFHC diet displayed increased levels of reactive oxygen species (ROS). Elevation of ROS levels hinders HSC quiescence and self-renewal, and accelerates HSC exhaustion (28). Injection of ROS inhibitor N-acetylcysteine attenuated HSPC expansion and leukocytosis in *Scarb1*<sup>-/-</sup> mice fed an HFHC diet, suggesting a correlation between ROS levels and HSPC proliferation. Tie et al. also reported that *ApoE*-deficiency increased the number and ROS levels of HSPCs, and these were further increased by the HFHC diet (29). They also observed shorter telomere length in HSPCs in *ApoE*<sup>-/-</sup> mice as compared to wild-type mice, suggesting accelerated aging of HSPCs, and this phenotype was reversed by treating *ApoE*<sup>-/-</sup> mice with N-acetylcysteine. These studies clearly indicate increased systemic cholesterol levels promote proliferation and mobilization of HSPCs.

## Cholesterol Efflux Pathways Regulate Proliferation and Mobilization of Mouse Hematopoietic Stem and Progenitor Cells

ABCA1 and ABCG1, adenosine triphosphate-binding cassette transporters, play a key role in promoting active cellular cholesterol efflux (30). Yvan-Charvet et al. reported that *Ldlr*<sup>-/-</sup> mice on an HFHC diet that were transplanted with *Abca1*<sup>-/-</sup>*Abcg1*<sup>-/-</sup> bone marrow cells showed accelerated atherosclerosis and extensive infiltration of myocardium and spleen with macrophage foam cells as compared to transplantation with wild-type bone marrow cells (31). The same group subsequently reported that *Abca1*<sup>-/-</sup>*Abcg1*<sup>-/-</sup> mice on a normal diet displayed five-fold increase of HSPCs (including the CD34<sup>+</sup>CD150<sup>+</sup>Flt3<sup>-</sup> highly-pure HSC population) in the bone marrow, as well as the increase in the S/G2/M fraction in HSPCs (32). The overall BrdU incorporation of *Abca1*<sup>-/-</sup>*Abcg1*<sup>-/-</sup> bone marrow cells was increased *in vitro*, whereas when wild-type bone marrow cells were mixed with *Abca1*<sup>-/-</sup>*Abcg1*<sup>-/-</sup> bone marrow cells, the overall BrdU incorporation of wild-type bone marrow cells was not increased, suggesting that HSPC proliferation in *Abca1*<sup>-/-</sup>*Abcg1*<sup>-/-</sup> mice was caused by cell autonomous effects. Interestingly, in their next report, they showed that *Abca1*<sup>-/-</sup>*Abcg1*<sup>-/-</sup> mice also displayed an increase in HSPCs in the peripheral blood, spleen, and liver, indicating HSPC mobilization and extramedullary hematopoiesis (33). In this study, they performed a competitive bone marrow transplantation experiment by transplanting a mixture of equal numbers of bone marrow cells from wild-type and *Abca1*<sup>-/-</sup>

*Abcg1*<sup>-/-</sup> mice into wild-type recipient mice, and found that HSPC mobilization of both the *Abca1*<sup>-/-</sup>*Abcg1*<sup>-/-</sup> and wild-type donor cells was induced, suggesting that there is a cell-extrinsic factor that induces HSPC mobilization of wild-type donor cells from *Abca1*<sup>-/-</sup>*Abcg1*<sup>-/-</sup> donor cells. Plasma levels of granulocyte colony-stimulating factor (G-CSF) were significantly increased in recipients of *Abca1*<sup>-/-</sup>*Abcg1*<sup>-/-</sup> bone marrow cells and the mobilization of *Abca1*<sup>-/-</sup>*Abcg1*<sup>-/-</sup> HSPCs was reduced by injection of G-CSF-neutralizing antibody. Interleukin-17 (IL-17) is a potent inducer of G-CSF (34), and the production of IL-17 can be mediated by the secretion of interleukin-23 (IL-23) from splenic phagocytic macrophages and dendritic cells (35). In the recipients of *Abca1*<sup>-/-</sup>*Abcg1*<sup>-/-</sup> bone marrow cells, plasma G-CSF levels and colony-forming HSPC numbers in the blood were normalized by an IL-17-blocking antibody, and plasma levels of IL-17 and G-CSF, as well as colony-forming HSPCs in the blood, were reduced by administration of IL-23 receptor-neutralizing antibody. Both myeloid cell (including macrophages) -specific and dendritic-cell specific deletion of *Abca1* and *Abcg1* using *lysM-cre; Abca1*<sup>fl/fl</sup>; *Abcg1*<sup>fl/fl</sup> mice, and *CD11c-cre; Abca1*<sup>fl/fl</sup>; *Abcg1*<sup>fl/fl</sup> mice, respectively, exhibited increased levels of splenic IL-23, plasma IL-17 and G-CSF, and colony-forming HSPCs in the blood, suggesting that IL-23/IL-17/G-CSF signaling is associated with enhanced HSPC mobilization in *Abca1*<sup>-/-</sup>*Abcg1*<sup>-/-</sup> mice. They further reported that CXCL12 levels and the number of N-Cadherin<sup>+</sup> osteoblasts, one of the CXCL12-expressing cell populations in the bone marrow (36), were decreased in the bone marrow of *Abca1*<sup>-/-</sup>*Abcg1*<sup>-/-</sup> mice, an effect that might be caused by depletion of bone marrow macrophage populations due to an enhanced IL-23/IL-17/G-CSF signaling axis. Thus, this study supports a step-wise mechanism by which increased intracellular cholesterol levels lead to mobilization of HSCs: (1) increased cholesterol initially promotes secretion of pro-inflammatory cytokines from immune cells, (2) this increases production of G-CSF by bone marrow stromal cells, (3) reducing osteoblast number and osteoblast production of CXCL12, a chemokine which attracts HSCs, and (4) leads to HSC mobilization into the bloodstream. They subsequently reported that *Ldlr*<sup>-/-</sup> recipient mice that received *lysM-cre; Abca1*<sup>fl/fl</sup>; *Abcg1*<sup>fl/fl</sup> bone marrow cells and were fed an HFHC diet developed atherosclerosis associated with monocytosis and neutrophilia (37). The authors demonstrated a cell-extrinsic mechanism in which the expression of macrophage colony-stimulating factor (M-CSF) and G-CSF were increased in the spleen, and this might cause monocyte and neutrophil production in the bone marrow.

## Cholesterol Levels and Human Hematopoiesis

Cholesterol homeostasis also affects human hematopoiesis. Crysandt et al. performed a retrospective analysis of a variety of clinical parameters in 83 patients following high-dose cyclophosphamide and G-CSF treatment and found that patients with hypercholesterolemia showed a substantially higher number of harvested CD34<sup>+</sup> HSPCs in the peripheral blood

as compared to normocholesterolemic patients (38). 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is a rate-limiting enzyme of *de novo* cholesterol synthesis, and statins, as inhibitors of HMG-CoA reductase, prevent the conversion of HMG-CoA to L-mevalonate and inhibit downstream cholesterol biosynthesis (Figure 2). Cimato et al. treated human subjects with different statins, atorvastatin, pravastatin, and rosuvastatin, to vary cholesterol levels and analyzed the number of mobilized CD34<sup>+</sup> HSPCs in the peripheral blood (39). They found a positive correlation between CD34<sup>+</sup> HSPC number and both total and LDL-cholesterol levels. In addition, G-CSF and its upstream regulator IL-17 both correlated positively with LDL-cholesterol levels. Gao et al. studied the correlation between HDL and white blood cell levels in patients with coronary heart disease (27). They found negative correlations between HDL levels and both total white blood cell and neutrophil counts in the peripheral blood, and patients with low HDL-cholesterol had more mobilized Lineage<sup>-</sup>CD34<sup>+</sup>CD38<sup>-</sup>CD45RA<sup>-/low</sup> HSCs in the peripheral blood as compared to the patients with normal HDL-cholesterol. Tolani et al. analyzed data from a clinical trial of rosuvastatin in children with heterozygous familial hypercholesterolemia and found that the children with the lowest HDL-cholesterol levels had higher monocyte counts in the peripheral blood, and there was an inverse correlation between HDL levels and monocyte percentage (40). Thus, increased cholesterol levels induce mobilization of not only mouse HSCs but human HSCs, which suggests that cholesterol level is a factor that should be considered when mobilizing HSCs for clinical transplantation.

## ROLES OF CHOLESTEROL METABOLITES IN HEMATOPOIESIS

### Sex Steroid Hormones

Estrogens and androgens are classically recognized as sex steroid hormones, and progesterone are recognized as a third class of sex steroid hormones. Each of these sex steroid hormones is synthesized from cholesterol, and the first and rate-limiting step of the steroidogenic pathway is the cleavage of the cholesterol side chain by P450<sub>scc</sub> (CYP11A1) to convert into pregnenolone (Figure 2) (41). Estrogens are produced in gonadal and extra-gonadal tissues. In females, 17 $\beta$ -estradiol (E2), a most potent estrogen, is produced primarily by theca and granulosa cells in the ovaries. Androstenedione is generated from cholesterol and is converted into testosterone by aromatase in theca cells, and they are further converted into E2 by aromatase in granulosa cells. Testosterone is the primary androgen secreted from Leydig cells in the testes, and small amounts are also secreted from theca cells in the ovaries. Progesterone is a critical progesterone to establish and maintain pregnancy. Progesterone is produced from cholesterol in the corpus luteum of the ovary during early pregnancy and the production is sustained by the placenta in humans and rodents. In addition to their well-recognized effects on reproductive tissues, the sex steroid hormones are also being recognized as having broad physiological effects on non-reproductive tissues, such as nervous, cardiovascular, skeletal,

immune, and hematopoietic systems. It is known that females and males differ in innate and adaptive immune responses, and these sex-biased differences in the immune system contribute to variations in the prevalence of autoimmune diseases and malignancies, susceptibility to infectious diseases, and responses to vaccines (42).

### Androgens and Lympho-Hematopoiesis

Several studies have shown that androgens negatively regulate B lymphopoiesis. Castration of male mice leads to spleen enlargement and expansion of the B-cell population in the bone marrow and spleen (43–45). This effect is reversed by androgen replacement with either testosterone or dihydrotestosterone (DHT) (46). Androgen-resistant “testicular feminization” mutant male mice also show expansion of B-cell populations in the bone marrow and spleen (45, 47). In addition to the regulation of B lymphopoiesis, castrated mice and testicular feminization male mice also show thymic hypertrophy, which can be rescued by DHT administration (43, 45, 48–50). Experiments transplanting wild-type bone marrow cells into testicular feminization male mice suggest that androgen receptors expressed by bone marrow stromal cells or thymic epithelium modulate B-cell development or thymus size, respectively (50, 51). Velardi et al. showed that one mechanism by which androgens influence thymopoiesis is through direct inhibition of the Notch ligand *Dll4* in cortical thymic epithelial cells (52). Immune function progressively declines with age in mice and humans (53). In male mice, castration rejuvenates aged bone marrow and thymus, enhances peripheral T- and B-cell functions, and promotes immune recovery following chemotherapy-induced immunodepletion and HSC transplantation (54–62). These studies indicate that androgens are critical mediators of age-related lymphoid decline. Castration also enhances the recovery of bone marrow-resident HSCs after chemotherapy-induced immunodepletion (62). Khong et al. demonstrated that the number of HSCs marked by CD34<sup>-</sup>Flt3<sup>-</sup>LSK was significantly increased at 7 days after castration of 9-month-old mice as compared to sham-treated mice, and the repopulation potential during serial bone marrow transplantations was enhanced when using these mice as donors (63). Their gene expression analyses suggest that castration induces qualitative changes in both HSCs and their bone marrow environment.

### Estrogens and Lympho-Hematopoiesis

B lymphopoiesis in the bone marrow and T lymphopoiesis in thymus are drastically reduced during pregnancy (64, 65). Ovariectomy stimulates B lymphopoiesis and results in increased numbers of B cells in the bone marrow (66, 67), and it is reversed by administration of E2 (68). Genetically hypogonadal female mice which have a partial deletion of the hypothalamic gonadotropin-releasing hormone gene have a secondary deficiency in gonadal steroidogenesis and show expansion of B cell progenitors. Estrogen replacement with E2 reversed the increased numbers of B cell progenitors in these hypogonadal mice (69). Exogenous E2 treatment suppresses B cell development in both male and female mice (68, 70, 71).

Very early lymphoid precursors marked by Lineage marker<sup>-</sup>IL-7R $\alpha^+$ c-Kit<sup>lo</sup>Terminal deoxynucleotidyl transferase<sup>+</sup> in the bone marrow are selectively depleted by exogenous E2 treatment (72). Both stromal-dependent and independent pathways of estrogen action on developing B cells have been postulated. It is proposed that bone marrow stromal cells expressing estrogen receptors mediate negative regulatory effects of E2 on early pre-B development (47, 73). A study of estrogen receptor  $\alpha$  (ER $\alpha$ ) male knockout mice by Thurmond et al. indicated that ER $\alpha$  is necessary for development of normal numbers of B cells in the bone marrow (74). They investigated the effect of E2 on lymphopoiesis by performing bone marrow transplantation using ER $\alpha$ -deficient mice as donors, recipients, or both, and treated with E2. They demonstrated that exogenous E2-induced alteration of B cell populations was primarily caused by a hematopoietic cell-intrinsic mechanism rather than by their environment.

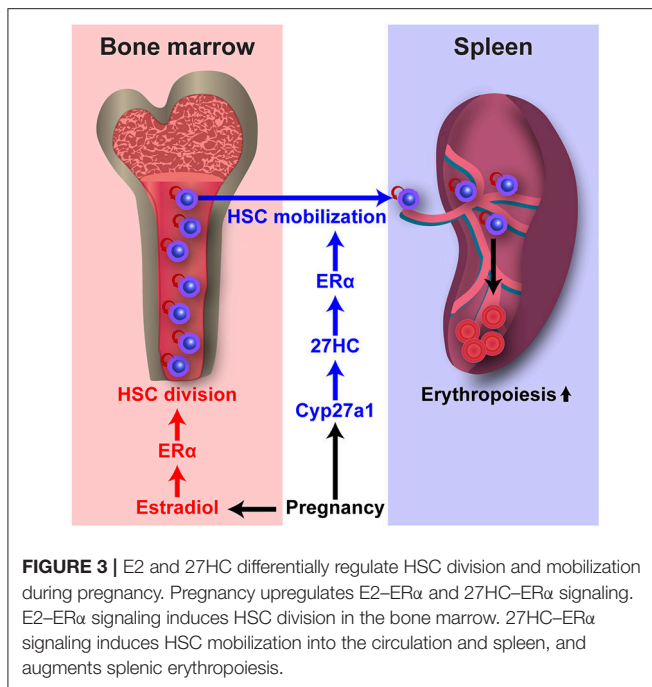
Estrogens also regulate other hematopoietic lineages. Administration of a high-dose of estrogen induces anemia in rodents and dogs, regardless of their sex (75–77). Schroeder et al. reported that E2 sustained proliferation of erythroid progenitors from chick bone marrow, but E2 also caused erythroid differentiation arrest and blocked erythrocyte gene expression (78). Blobel et al. also showed that E2 added to the culture also reduced the number of erythroid progenitors from human bone marrow (79). They demonstrated that the transcriptional activity of GATA1, an erythroid master transcription factor that is necessary for full maturation of erythrocytes, was strongly repressed by direct binding of ER in an E2-dependent manner in NIH 3T3 and COS cell lines. The same group subsequently reported that inhibition of GATA1 activity by ER induced apoptosis in a murine Friend virus-induced erythroleukemia cell line (80). E2 treatment also stimulates the megakaryocyte colony formation potential of CD34<sup>+</sup> human cord blood cells *in vitro* (81). E2 promotes megakaryocyte polyploidization and maturation via activation of ER $\beta$  accompanied by a significant upregulation of the expression of GATA1, which is also a key regulator of megakaryocyte differentiation (82). Differentiation of dendritic cells with characteristics of Langerhans cells from myeloid progenitors in culture, as induced by granulocyte-macrophage colony-stimulating factor (GM-CSF), is promoted by addition of E2 and inhibited by ER antagonists and ER $\alpha$ -deficiency (83). Interferon regulatory factor 4 (IRF4), a transcription factor induced by GM-CSF and critical for Langerhans cell development, is a target of ER $\alpha$  signaling during this process (84). In contrast, E2 significantly inhibits Flt3-ligand-induced plasmacytoid and conventional dendritic-cell differentiation in culture by decreasing numbers of viable differentiated cells (85). Thus, the effects of E2 are likely to be dependent on the cytokine pathways that might be operative in the steady state or during inflammation and disease.

## Estrogens Regulate HSC Division Rate

Estrogens also regulate HSC behavior. In our previous study, we observed that HSCs divide more often in female mice as compared to male mice (14). Ovariectomy in females (which

depletes both estrogens and progesterone) significantly reduced HSC division to male levels, while castration of males has no effect on HSC division. Conversely, administration of exogenous E2, but not progesterone or dihydrotestosterone, significantly increased HSC division in both female and male mice. Because E2 treatment also increased HSC division in ovariectomized female mice and castrated male mice, its action is independent of the gonads. Although HSC division rate was increased in untreated female mice as compared to male mice and in E2-treated mice of either sex as compared to vehicle-treated control mice, we did not observe an increase in absolute HSC numbers in those untreated female mice or E2-treated mice. Instead, we observed an increased generation of megakaryocyte-erythroid progenitors (MEPs) in female mice as compared to male mice, and increased splenic erythropoiesis in E2-treated mice of both sexes as compared to vehicle-treated control mice. Given that increased myeloid progenitors including MEPs may arise directly from the asymmetric division of HSCs (Figure 1) (86), our observations raise the possibility that the increased MEPs in female mice reflects increased asymmetric division of an HSC to produce one HSC and one MEP in response to E2. ER $\alpha$ , but not ER $\beta$ , is highly expressed by HSCs. Conditional deletion of *Esr1*, which encodes ER $\alpha$ , from hematopoietic cells significantly reduced the HSC division rate in female mice, but not in male mice. *Esr1*-deficient HSCs of both sexes were insensitive to exogenous E2 treatment. Moreover, E2 treatment of chimeric recipient mice transplanted with equal numbers of wild-type and *Esr1*-deficient hematopoietic cells revealed that E2 significantly induced division of wild-type HSCs but not *Esr1*-deficient HSCs in the same recipient mice, indicating that E2 acts directly on HSCs, rather than acting indirectly by stimulating secondary signals from other cells. E2 levels increase in females during pregnancy (87), when extramedullary hematopoiesis is induced to increase the production of red blood cells. Notably, pregnant mice exhibit significantly increased HSC division rate relative to non-pregnant female mice, and the deletion of *Esr1* in hematopoietic cells significantly reduced the normal increase in HSC division during pregnancy (Figure 3). Increased spleen size is observed during pregnancy in mice and humans (88, 89). In addition to the increased HSC division rate, we found that pregnant mice exhibited significantly increased cellularity, erythropoiesis, myelopoiesis, and HSC number in the spleen, indicating extramedullary hematopoiesis and HSC mobilization, and these processes also depend upon ER $\alpha$  function in HSCs. Pregnant mice also had increased absolute HSC numbers in the bone marrow, but deletion of *Esr1* in hematopoietic cells did not reverse this phenomenon, suggesting the existence of ER $\alpha$ -independent factor(s) that increase(s) HSC numbers in pregnant mice. Nevertheless, ER $\alpha$  signaling is critical for the induction of HSC division and mobilization to the spleen for the expansion of splenic erythropoiesis.

Illing et al. also reported the effects of E2 on HSC function (90). In this study, they treated mice with E2 at a dose of 0.24 mg/kg/day for 30 days, which is a higher dose and longer treatment than our study at a dose of 0.1 mg/kg/day for 7 days. In this condition, they observed a profound reduction



in bone marrow cellularity by E2 treatment. E2 treatment caused more HSPCs to enter the S phase. They also observed an increased frequency of long-term reconstituting HSCs in E2-treated mice by performing bone marrow transplantation with a limiting dilution assay. However, donor-derived HSPCs of the bone marrow of the recipient mice after tertiary transplantation was decreased in the recipients that received bone marrow cells from E2-treated mice, suggesting exhaustion of reconstituting cells during serial transplantation. Deletion of *Esr1* reversed the reduction of bone marrow cellularity by E2 treatment, however it did not reverse the increased frequency of long-term reconstituting HSCs by E2 treatment. Sanchez-Aguilera et al. reported the effect of tamoxifen, a selective estrogen receptor modulator (SERM), on HSC function (91). Tamoxifen induced HSC division as well as apoptosis in MPPs, and these effects are mediated by ER $\alpha$  but not by ER $\beta$ . Tamoxifen treatment significantly reduced MPP number, but not HSC number, and compromised activation of hematopoiesis after chemotherapy. They observed increased expression of *Myc* in HSCs after tamoxifen treatment, and *Myc*-deficient MPPs did not undergo apoptosis upon tamoxifen treatment. Interestingly, female immunodeficient recipient mice support reconstitution of the blood system by transplanted human HSCs more efficiently than male immunodeficient recipient mice (92, 93). Since female mice have higher E2 levels than male mice and E2 induces HSC division, it is anticipated that higher E2 levels in female recipient mice will promote proliferation and differentiation of transplanted HSCs as compared to male recipient mice with very low E2 levels. The molecular mechanisms by which E2 regulates HSCs are not fully understood. Chapple et al. proposed that HSCs from mice treated with E2 had increased regenerative capacity after

transplantation or irradiation (94). They demonstrated that E2-ER $\alpha$  signaling induced expression of *Ern1*, which encodes Ire1 $\alpha$ , to activate the Ire1 $\alpha$ -Xbp1 pathway of the unfolded protein response, and promoted resistance of HSCs against proteotoxic stress.

## 27-Hydroxycholesterol Regulates HSC Mobilization

Oxysterols are oxygenated derivatives of cholesterol and key substrates for bile acid synthesis (Figure 2) (16). In the classic pathway of bile acid synthesis, cholesterol is converted into 7 $\alpha$ -hydroxycholesterol (7 $\alpha$ HC) by cholesterol 7 $\alpha$ -hydroxylase CYP7A1, a rate limiting step that occurs in the liver. In alternative pathways that occur primarily in extrahepatic tissues, cholesterol is converted into 24-hydroxycholesterol (24HC), 25-hydroxycholesterol (25HC), and 27-hydroxycholesterol (27HC) by cholesterol 24-hydroxylase CYP46A1, cholesterol 25-hydroxylase CH25H, and sterol 27-hydroxylase CYP27A1, respectively. Oxysterols are considered to be bioactive lipids and recent studies have started to reveal their important roles in both the hematopoietic and immune systems. 27HC is the most abundant circulating oxysterol and acts as an endogenous SERM which can bind to ERs and regulate their function (95). Plasma 27HC levels strongly correlate with total cholesterol levels (96), as 27HC is generated directly from cholesterol by the sterol 27-hydroxylase CYP27A1. Plasma 27HC levels are greatly reduced in *Cyp27a1*-deficient mice (97). CYP27A1 is abundant in the liver, but it is also expressed in extrahepatic tissues (16). Dietary or genetic changes that elevate 27HC levels modulate ER activity, thereby inhibiting vascular repair in cardiovascular disease (95), promoting ER-positive breast cancer growth (98, 99), and increasing the severity of atherosclerosis (100).

We have demonstrated that the increases in HSC division, HSC mobilization, and extramedullary hematopoiesis during pregnancy require ER $\alpha$  in HSCs, and E2 treatment induces HSC division as described above (14). Interestingly, our recent study revealed that treatment of mice with E2 did not increase HSC number in the spleen, indicating that E2 treatment does not induce HSC mobilization (101). In contrast, treatment with 27HC, another endogenous ER ligand, increased HSC number in the spleen but not HSC division in the bone marrow, indicating a role in inducing HSC mobilization. We demonstrated that the effect of 27HC on HSC mobilization is nullified by deletion of *Esr1* in hematopoietic cells, indicating that 27HC-induced HSC mobilization is dependent on ER $\alpha$ . Plasma cholesterol levels increase in humans during pregnancy (102). During pregnancy in mice, we observed significant increases in 27HC levels in HSPCs. *Cyp27a1*-deficient mice had significantly reduced HSC mobilization and extramedullary hematopoiesis during pregnancy, while the increased rate of HSC division in the bone marrow during pregnancy was not affected. These findings indicate that 27HC acts in concert with E2 to promote hematopoiesis during pregnancy by regulating ER $\alpha$  signaling in HSCs (Figure 3). As described above, increased cholesterol

levels promote HSC mobilization in mice and humans, and increased HSC mobilization in *Abca1<sup>-/-</sup>Abcg1<sup>-/-</sup>* mice is associated with elevated serum G-CSF levels (33). In our study, we observed that 27HC treatment significantly induced HSC mobilization in mice deficient for *Csf3*, which encodes G-CSF (101). Together, 27HC and G-CSF additively increased the numbers of colony-forming HSPCs in the peripheral blood. Therefore, 27HC and G-CSF likely act through distinct mechanisms to induce HSC mobilization. These findings suggest an alternative model that the ability of elevated cholesterol levels to promote HSC mobilization is mediated by increases in 27HC production, because treating mice with 27HC induces HSC mobilization and 27HC levels increase as cholesterol levels increase.

## E2, 27HC, and ER $\alpha$

ER $\alpha$  is a nuclear receptor transcription factor and E2 is the most potent endogenous estrogen. Different ER ligands are known to have distinct effects on ER $\alpha$ -mediated regulation of gene expression, and ER ligands differ in their structures and their effects on ER conformation (103–106). For example, Wardell et al. used breast cancer cell lines to test E2 and five different synthetic ER ligands and observed different gene expression patterns regulated by different ER–ligand complexes (106). 27HC induces a unique conformational change in the ER that is different from that mediated by E2 and other ER ligands (107). Different ER–ligand complexes also engage functionally distinct coregulators by selective recruitment of coactivators and corepressors to activate and repress expression of target genes, respectively (108). Thus, as different ER ligands can act through the ER $\alpha$  to differentially regulate gene expression, this may explain why E2 and 27HC have distinct effects on HSCs even though both act through ER $\alpha$  (Figure 3). It will be interesting to explore the nature of the ER $\alpha$  target genes as well as the mechanism by which ER $\alpha$  function is differentially regulated by E2 and 27HC, two major endogenous ER ligands.

## Bile Acids

Bile acids are synthesized from cholesterol in the liver (Figure 2), secreted into the bile, and delivered to the lumen of the small intestine where they act as emulsifiers of dietary lipids, cholesterol, and fat-soluble vitamins (16). However, bile acids have additional roles (109). Tauroursodeoxycholic acid (TUDCA) serves as a chemical chaperone and reduces endoplasmic reticulum stress (110). HSCs are predisposed to apoptosis through misfolded protein accumulation in the endoplasmic reticulum caused by cellular stress that subsequently activates the unfolded protein response (111). Addition of TUDCA to cultured mouse HSCs alleviates endoplasmic reticulum stress and increases their capacity to reconstitute the hematopoietic system in recipient mice upon transplantation (112). In contrast to the quiescent state of adult HSCs in the bone marrow, HSCs undergo a rapid expansion in the fetal liver during development (113). Adult HSCs in the bone marrow have a lower rate of protein synthesis as compared to most other hematopoietic cells (114). Sigurdsson et al.

reported that although fetal-liver HSCs had a higher rate of protein synthesis as compared to adult HSCs in the bone marrow, fetal-liver HSCs had lower expression of endoplasmic reticulum stress response genes (115). In addition to the role of CYP27A1 for side chain oxidation of cholesterol for the generation of 27HC to initiate the alternative acidic bile acid biosynthetic pathway, CYP27A1 also catalyzes another side chain oxidation after the ring modification step of both classic and alternative acidic bile acid synthesis pathways (Figure 2) (16). *Cyp27a1*-deficient mice have significantly decreased bile acids (97). Sigurdsson et al. also reported that fetuses in *Cyp27a1*-deficient mothers displayed significantly reduced levels of total bile acids as well as secondary bile acids in the fetal liver, suggesting that maternal bile acids are transferred to the fetus during pregnancy (115). Interestingly, the livers of *Cyp27a1*-deficient fetuses in *Cyp27a1*-deficient mothers contained a significantly lower number of long-term reconstituting HSCs, as assessed by limiting dilution transplantation assay, and these fetal HSCs showed significantly higher levels of protein aggregation. These findings imply that bile acids enable fetal-liver HSCs to have a higher level of protein synthesis without activating a stress response, allowing expansion of the HSC pool during fetal development.

## 25-Hydroxycholesterol

25HC is generated directly from cholesterol by CH25H, and 25HC is further converted into 7 $\alpha$ ,25-dihydroxycholesterol (7 $\alpha$ ,25HC) by the oxysterol 7 $\alpha$ -hydroxylase CYP7B1-mediated hydroxylation (Figure 2) (116). Expression of *Ch25h* is upregulated in macrophages and dendritic cells when they are exposed to various inflammatory mediators (117–120). 25HC augments the production of inflammatory cytokines in macrophages, and mediates feedback inhibition of *IL1b* expression and inflammasome activation in activated macrophages in the DNA sensor protein absent in melanoma 2 (AIM2)-dependent manner (121–124). 25HC is strongly induced following viral infection and by interferon, and it inhibits the replication of a wide range of enveloped viruses (125, 126). 25HC also promotes macrophage foam cell formation (127). Epstein-Barr virus-induced gene 2 (EBI2), a G protein-coupled receptor also known as GPR183, controls follicular B-cell migration and T-cell-dependent antibody production (128, 129). 7 $\alpha$ ,25HC acts as a ligand for EBI2 and directs migration of B cells in the spleen during the adaptive immune response (130, 131). EBI2 and 7 $\alpha$ ,25HC also regulate splenic CD4<sup>+</sup> dendritic cells for positioning in marginal zone bridging channels to maintain their homeostasis and mount a response against certain antigens, and positioning of activated CD4 T cells at the interface of the follicle and T zone to interact with activated dendritic cells (132–134). Thus, 25HC broadly regulates innate and adaptive immune cell behavior. The 7 $\alpha$ ,25HC/EBI2 axis also regulates bone mass homeostasis (135). EBI2 is expressed in monocyte/osteoclast precursors, and 7 $\alpha$ ,25HC is secreted by osteoblasts. EBI2 guides osteoclast precursors toward bone surfaces by promoting their movement and positioning, which facilitates fusion of osteoclasts and enhances the development of large osteoclasts to maintain bone mass homeostasis.



## CHOLESTEROL SYNTHESIS/METABOLISM AND HEMATOLOGIC MALIGNANCIES

Cholesterol metabolism is dysregulated in hematologic malignancies. The rate of cholesterol synthesis is higher in cells from acute myeloid leukemia (AML) patients as compared to healthy subjects (136). Hypocholesterolemia is frequently observed due to elevated LDL uptake by leukemia cells (137–139), but elevated cholesterol levels in leukemia cells have also been reported (140–143). Yvan-Charvet et al. reported that HFHC diet administered *Ldlr*<sup>+/-</sup> mice transplanted with cellular cholesterol efflux pathway-deficient *Abca1*<sup>-/-</sup>*Abcg1*<sup>-/-</sup> bone marrow cells displayed a myeloproliferative neoplasm (MPN)-like phenotype, and expression of an *APOA1* transgene that elevates HDL levels suppressed this phenotype (32). Thus, changes in intracellular cholesterol levels are associated with the development and maintenance of hematologic malignancies. Statins have cytotoxic effects in various types of malignant hematopoietic cells including AML (144–158), chronic myeloid leukemia (CML) (153, 159–161), MPNs (162), acute lymphocytic leukemia (ALL) (163, 164), chronic lymphocytic leukemia (CLL) (165–167), adult T-cell leukemia (ATL) (168), lymphoma (169, 170), and multiple myeloma (171–176). To identify compounds that can inhibit the stem cell activity of leukemia-initiating cells (LICs), Hartwell et al. performed a high-throughput screen in a bone marrow-mimicking culture system in which LICs expressing the *MLL-AF9* fusion oncogene were co-cultured with a bone marrow stromal cell line (177). Among the compounds that selectively inhibited LICs but not normal HSPCs, lovastatin also inhibited LIC stem cell activity in an *in vivo* bone marrow transplantation model. Although these reports demonstrate the effectiveness of statins, the mechanisms of their anticancer effects are not fully understood. Griner et al. reported that MPN-associated *JAK2*<sup>V617F</sup> localized to lipid rafts, subdomains of the plasma membrane that contain protein receptors and a high concentration of cholesterol, and simvastatin inhibited this localization and *JAK2*<sup>V617F</sup>-dependent cell growth in MPN model cell lines (162). Simvastatin also inhibited erythroid colony formation of primary cells from MPN patients, but had no effect on cells from healthy individuals. Other than the cholesterol-lowering effect, inhibition of the mevalonate pathway by statins also reduces the levels of farnesyl pyrophosphate and geranylgeranyl pyrophosphate and thereby inhibits protein farnesylation and geranylgeranylation, modifications that are important for a variety of cellular processes including cell proliferation, survival, and migration (Figure 2). Thus, the anticancer effects of statins could also be rendered through changes in these other cellular processes (157, 158, 170, 176).

Among the metabolites of cholesterol, oxysterols such as 7 $\beta$ HC, 25HC, 7 $\beta$ ,25HC, 7-ketocholestanol, and 7-ketocholestanol have cytotoxic effects on leukemia and lymphoma cells (178–183). Tsujioka et al. reported that DNA methyltransferase inhibitors induced *CH25H* expression with enhanced 25HC production and promoted apoptosis in leukemia and myelodysplastic syndrome (MDS) cell lines, while

exogenous 25HC treatment suppressed cell growth of leukemia and MDS cell lines (184). Other than oxysterol, Sanchez-Aguilera et al. reported that tamoxifen treatment blocked development of *JAK2*<sup>V617F</sup>-induced MPN in mice and induced apoptosis of human MPN cells from patients with *JAK2*<sup>V617F</sup> mutation in a xenograft model (91). They also demonstrated that tamoxifen treatment reduced leukemic burden in a mouse model of AML using mice transplanted with bone marrow cells expressing the *MLL-AF9* oncogene. Their findings have uncovered the potential role of estrogen signaling in leukemia and suggest the potential use of SERMs as a treatment for leukemia. The roles of cholesterol metabolites in hematologic malignancies are not yet fully explored, and further studies of cholesterol metabolites are expected to elucidate their roles in hematologic malignancies and their potential in preventing and treating hematologic malignancies.

## CONCLUSION AND FUTURE DIRECTIONS

Cholesterol and its metabolites are now being recognized to have important roles in broad biological processes by regulating a wide variety of molecular machinery. Advances in understanding these molecular mechanisms will benefit human health. One potential clinical application of molecules that regulate or are regulated by cholesterol metabolism is to enhance mobilization of HSCs for transplantation. To enable efficient collection of mobilized HSCs from the peripheral blood for HSC transplantation, donors are treated with HSC-mobilizing agents such as G-CSF (185). However, a significant proportion of donors fail to reach the minimum HSC collection threshold required for transplantation using traditional strategies (186). The failure of mobilization can increase patient morbidity because patients cannot proceed to transplantation. Thus, advances in mobilization strategies that could increase the success of HSC collection without introducing additional side effects are needed to improve patient outcomes. For example, administration of 27HC enhances the mobilization of HSPCs by G-CSF (101). Identification of the genes downstream of 27HC-ER $\alpha$  signaling that mediate HSC mobilization may contribute to the development of new methods that improve the yield of mobilized HSCs for transplantation, while also offering an explanation for the long-standing observation that increased cholesterol levels are associated with increased HSC mobilization in mice and humans.

High blood cholesterol levels are associated with the development of atherosclerosis. Atherosclerosis is a progressive disease in which the inside of the artery become thick and stiff due to the buildup of the atheromatous plaque which consists of cholesterol, fat and other substances, and restricts blood flow and causes complications including myocardial infarction, peripheral artery disease, and stroke. In addition to lipids, various types of leukocytes also accumulate in the atheromatous plaque. Hypercholesterolemia causes monocytosis, and these monocytes give rise to macrophages which eventually turn into foam cells by ingesting LDL in the plaque, and promotes plaque growth and inflammation. Other than monocytes, diverse immune-cell subsets, such as neutrophils, mast cells,

B and T lymphocytes, are associated with atherosclerosis [reviewed in (187, 188)]. Oxysterols are formed and accumulate in the plaque as a result of LDL oxidation due to the inflammatory response. Although atherosclerotic properties of oxysterols have been tested, it is still unclear whether oxysterols have pro- or anti-atherosclerotic properties [reviewed in (189)]. Estrogens also affect atherogenesis. Despite of reports that support the atheroprotective effects of estrogens, it is also controversial whether they have pro- or anti-atherosclerotic properties [reviewed in (190)]. After myocardial infarction, monocyte recruitment is increased, and sustained and accelerated atherosclerosis is observed in a mouse model. Interestingly, myocardial infarction causes HSPC mobilization into the spleen and sustains augmented monocyteopoiesis, providing a possibility of novel therapy to mitigate progression of atherosclerosis (191, 192). Thus, cholesterol and its metabolites link hematopoiesis with cardiovascular health, and deciphering this link is critical for developing new targeted therapies.

The molecular mechanisms underlying the regulation of normal and malignant hematopoiesis by cholesterol and its metabolites are not yet fully understood. There are many drugs that target cholesterol synthetic and metabolic

pathways, and further studies are expected to generate novel strategies for enhancing hematopoiesis, augmenting hematopoietic recovery after hematopoietic injuries, improving collection of mobilized HSCs for transplantation, and treating hematologic malignancies.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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**Conflict of Interest Statement:** The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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