Inhibitor of DNA binding proteins revealed as orchestrators of steady state, stress and malignant hematopoiesis

Shweta Singh[1†], Tanmoy Sarkar[1†], Brad Jakubison[1,2], Stephen Gadomski[1], Andrew Spradlin[1], Kristbjorn O. Gudmundsson[1,2] and Jonathan R. Keller[1,2*]

[1]Mouse Cancer Genetics Program, Center for Cancer Research, National Cancer Institute (NCI)-Frederick, Frederick, MD, United States, [2]Basic Science Program, Frederick National Laboratory for Cancer Research, Frederick, MD, United States

Adult mammalian hematopoiesis is a dynamic cellular process that provides a continuous supply of myeloid, lymphoid, erythroid/megakaryocyte cells for host survival. This process is sustained by regulating hematopoietic stem cells (HSCs) quiescence, proliferation and activation under homeostasis and stress, and regulating the proliferation and differentiation of downstream multipotent progenitor (MPP) and more committed progenitor cells. Inhibitor of DNA binding (ID) proteins are small helix-loop-helix (HLH) proteins that lack a basic (b) DNA binding domain present in other family members, and function as dominant-negative regulators of other bHLH proteins (E proteins) by inhibiting their transcriptional activity. ID proteins are required for normal T cell, B cell, NK and innate lymphoid cells, dendritic cell, and myeloid cell differentiation and development. However, recent evidence suggests that ID proteins are important regulators of normal and leukemic hematopoietic stem and progenitor cells (HSPCs). This chapter will review our current understanding of the function of ID proteins in HSPC development and highlight future areas of scientific investigation.

KEYWORDS

ID proteins, hematopoiesis, stem cells, quiescence, stress

Introduction

Hematopoiesis is sustained by a limited number of hematopoietic stem cells (HSCs) that reside in a quiescent state protected from proliferation-induced damage and exhaustion (1, 2). HSCs proliferate and differentiate to give rise to multipotent progenitor (MPP) cells with limited self-renewal potential that give rise to more

restricted progenitor cells to maintain normal numbers of differentiated blood cells (3, 4). Recent evidence suggests that HSCs have a limited number of self-renewing divisions indicating that HSC quiescence and proliferation is a tightly regulated process that protects the host from HSC exhaustion and hematopoietic failure (5, 6). While HSCs are one of the best characterized vertebrate stem cells, a significant effort is still focused on understanding the molecular and cellular mechanisms that regulate HSC quiescence, self-renewal and differentiation to 1) improve methods to expand adult HSCs and their differentiated progeny for bone marrow transplantation (BMT) and cell therapies, 2) identify molecular pathways that direct adult HSC development and expansion from pluripotential stem cells, 3) improve gene editing technology in hematopoietic stem and progenitor cells (HSPCs), and 4) develop new methods to detect, prevent and treat hematologic malignancies.

Inhibitor of DNA binding proteins (ID1-4) are members of the helix-loop-helix (HLH) family of proteins that regulate cell proliferation and differentiation (7–10). ID proteins function as dominant negative regulators of other HLH proteins (E proteins) by inhibiting their DNA binding and transcriptional activity, which is essential for the differentiation and proliferation of normal tissues including muscle, nerve, lymphoid, and embryonic stem cells (11–14). ID proteins also inhibit the transcriptional activity of erythroblast transformation specific (ETS), retinoblastoma (RB), and paired box proteins (PAX) proteins, which affect cell growth and differentiation (15–17). ID proteins are critical transcriptional regulators of hematopoietic cell differentiation, and are required for the proper development of T cells, B cells, dendritic and NK and innate lymphoid cells (13, 14, 18–23). ID proteins have emerged as critical regulators of HSPC quiescence, proliferation and differentiation under homeostasis, inflammatory and genotoxic stress, and aging. These findings suggest that ID proteins could have therapeutic potential to treat myeloid proliferative neoplasia's (MPN), myelodysplastic syndromes (MDS), and clonal hematopoiesis. Therefore, this prospective will review data related to the function of *Id* genes in regulating normal HSPC quiescence and differentiation, and their roles in hematopoietic stress and hematologic malignancies.

Id gene expression in hematopoietic cells

Id gene expression in hematopoietic cells was first reported in the murine erythroleukemia cell line, MEL cells, and its expression was shown to decrease during erythroid differentiation (11, 24–26). Subsequently, *Id1* was detected in
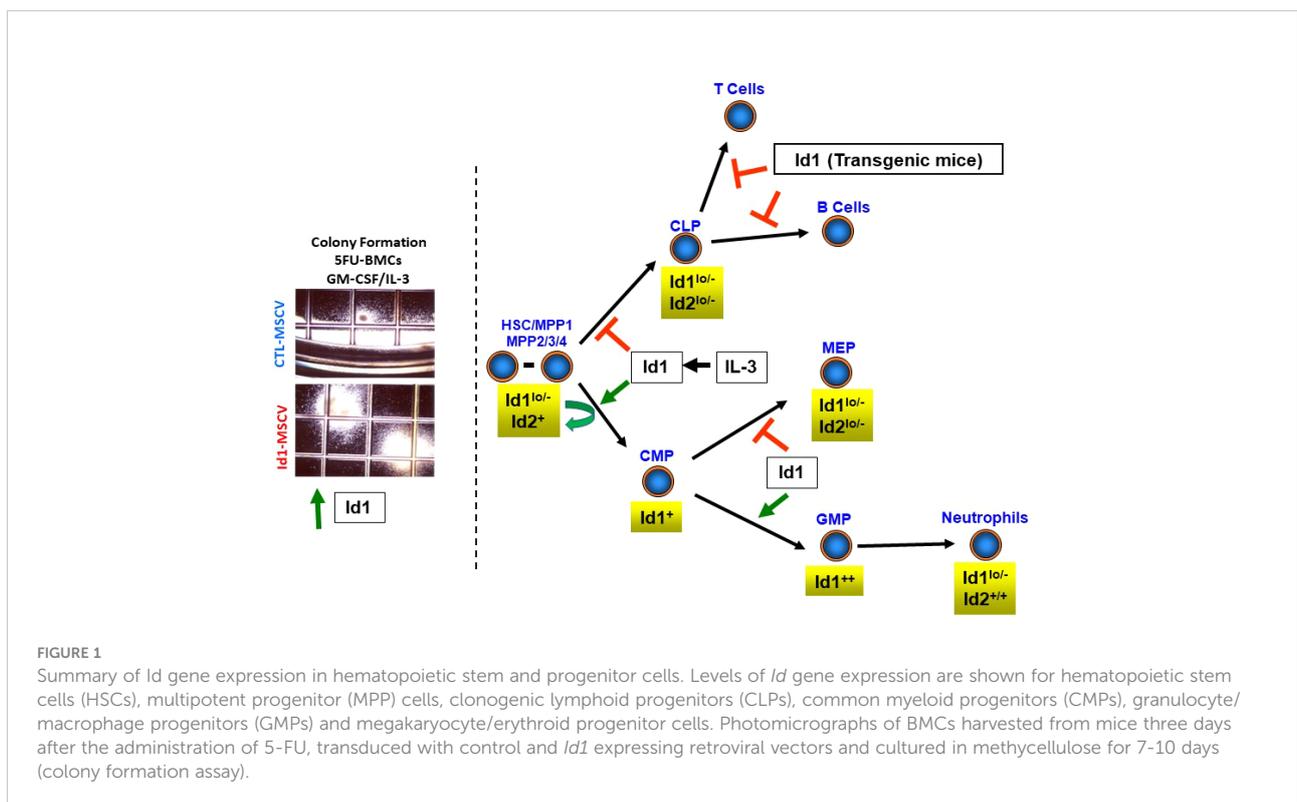
an interleukin-3 (IL-3) dependent myeloid progenitor cell line, 32Dcl3 cells, which can be induced to differentiate into neutrophils when cultured in media containing granulocyte-colony stimulating factor (G-CSF) (27). The expression of *Id1* decreased during G-CSF-induced differentiation of 32Dcl3 cells, while the binding of bHLH proteins to a canonical E-box motif increased. Furthermore, enforced expression of *Id1* blocked G-CSF-induced differentiation of 32Dcl3 cells. Taken together, these results suggested that ID1 may function during myeloid cell differentiation by disrupting bHLH protein function, the canonical target of ID proteins. Subsequent studies demonstrated that IL-3 and other myeloid growth factors (G-CSF and granulocyte/macrophage CSF, GM-CSF) induce *Id1* expression and cell proliferation in other IL-3-dependent progenitor cell lines including NFS-60 and FDC-P1 cells, while Id2 expression increases upon withdrawal of IL-3 and cell cycle arrest or differentiation (28–30). The HSC/MPP-like stem cell factor (SCF) -dependent progenitor cell line, EML cells, express ID2 but not ID1, while more committed IL-3-dependent progenitors cell lines derived from EML cells express ID1 but not ID2, suggesting ID2 may function in more primitive hematopoietic progenitor cells. Taken together, these studies demonstrated that ID1 is correlated with increased prolifersin/growth and decreased differentiation of hematopoietic progenitor cell (HPC) lines, which is consistent with results from previous studies in other tissues suggesting that ID1 promotes cell proliferation and inhibits cellular differentiation (7, 9, 10, 31, 32). In addition, these studies suggest that ID2 may have functions distinct from ID1 in HSPCs.

Id1 gene expression was increased in normal murine progenitor cells during the early proliferative phase of colony formation in soft agar assays stimulated by myeloid growth factors (IL-3/SCF/IL-1/erythropoietin, EPO), while *Id2* was induced to a lesser extent and no effect on *Id3 or Id4* expression was observed in these assays (29). In comparison, *Id2* levels were increased and *Id1* levels decreased in cells from colonies that contained differentiated progeny after 7-10 days. Thus, *Id1* is expressed in normal proliferating HPCs, while *Id2* expression increases and *Id1* levels decrease as cells exit the cell cycle and differentiate into myeloid cells. Subsequent studies confirmed that myeloid hematopoietic growth factors (HGFs) (SCF, IL-3 and GM-CSF) but not lymphoid HGFs (IL-7) and erythroid/megakaryocyte HGFs (EPO/thrombopoietin, TPO) induce *Id1* expression in purified lineage-negative, Lin-/Sca-1 +/c-Kit+ (LSK) BMCs that are enriched for HSCs/MPPs, suggesting that *Id1* may be required for myeloid development, but not lymphoid or erythroid development (33, 34). Analysis of *Id* gene expression in purified HSPC populations showed low levels of *Id1* expression in LSK cells and clonogenic lymphoid progenitors (CLPs), while *Id1* expression was increased in

common myeloid progenitors (CMP), and further increased in more differentiated granulocyte/macrophage progenitors (GMPs), but not in megakaryocyte/erythroid progenitors (MEP), indicating a potential role for *Id1* in myeloid cell development (Figure 1) (33–35). Subsequent analysis of purified HSCs demonstrated that roughly 5-10% of normal HSCs defined as LSK/Flk2-/CD150+/CD48- express *Id1* during steady state hematopoiesis, and that myeloid HGFs induce *Id1* in HSCs (36, 37). While the expression of *Id1* increases in committed myeloid progenitor cells (CMP and GMP), the expression of *Id1* decreases during the final stages of neutrophil maturation. Specifically, FACS purified normal neutrophils express low levels of ID1 protein compared to Lin-cells that are comprised of LSK cells and more committed Lin-/cKit+/Sca-1- (LK) progenitor cells that include CMPs/GMPs (33). ID1 expression decreases during G-CSF- and GM-CSF-induced neutrophil differentiation of myeloid progenitor cell lines, 32Dcl3 and MPRO, respectively (27, 33). In contrast, mature neutrophils in *Id1^{EGFP}* reporter mice show high levels of ID1/EGFP expression compared to CMP's and GMP's, suggesting that neutrophils express high levels of ID1 (34, 36). However, EGFP might not accurately reflect ID1 protein levels in neutrophils since ID proteins are rapidly degraded and the stability of EGFP and Id1 may differ significantly in these terminally differentiated cells. Future studies are needed to

define the precise expression and function of ID1 and other ID genes in maturing neutrophils and macrophages.

ID2 expression is 3-fold higher than *ID1* and 6.5-fold higher than *ID3* in purified human cord blood (CB) HSCs suggesting that human HSCs express high levels of ID2 (38). Little or no expression of ID1 was detected in purified CD34+38- human bone marrow HSPCs, but was induced by myeloid HGFs including GM-CSF/IL-3, but not SCF/EPO (erythroid) and SCF/TPO (megakaryocytes) HGFs (39). Thus, ID1 is expressed at low levels in human HSPCs and is induced by myeloid HGFs, and is consistent with results obtained with murine bone marrow cells (BMCs). Similarly, ID1 protein expression is induced during the early proliferative phase of normal human neutrophil/eosinophil differentiation in culture; after which, ID1 expression rapidly declines, while ID2 steadily increases (40). Furthermore, gain and loss of function studies showed that ID2 expression promotes eosinophil/neutrophil differentiation, and ID1 expression promotes neutrophil differentiation of human CD34+ progenitor cells *in vitro* and *in vivo* (40). Collectively, these results demonstrate that ID1 functions during the early proliferative stages of myeloid development, while ID2 may be required for the final stages of myeloid cell development. Further studies are needed to more precisely define, 1) the cell-specific and temporal expression of *Id1* and *Id2*, 2) how *Id1* and *Id2* gene expression is regulated, and 3) their molecular mechanism(s) of

FIGURE 1
Summary of Id gene expression in hematopoietic stem and progenitor cells. Levels of *Id* gene expression are shown for hematopoietic stem cells (HSCs), multipotent progenitor (MPP) cells, clonogenic lymphoid progenitors (CLPs), common myeloid progenitors (CMPs), granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythroid progenitor cells. Photomicrographs of BMCs harvested from mice three days after the administration of 5-FU, transduced with control and *Id1* expressing retroviral vectors and cultured in methylcellulose for 7-10 days (colony formation assay).

action during myeloid cell differentiation in normal human and murine cells.

Insights into *Id* gene function in hematopoietic stem and progenitor cells through the lens of enforced expression

Initial studies to define the physiological function of *Id* genes in hematopoietic cells *in vivo* demonstrated that transgenic mice overexpressing *Id1* or *Id2* during T cell differentiation manifest a severe block in T cell development (Figure 1) (41, 42). Similarly, transgenic mice that overexpress *Id1* during B cell differentiation show impaired B cell development (43). These studies are consistent with the strict requirement for E2A homodimers in B cell development and E2A homo and heterodimers in T cell development, and the dominant negative regulation of E protein function by ID proteins (20, 44).

To study *Id* gene function in HSPCs, *Id* genes were overexpressed in normal murine HSPCs using retroviral vectors and analyzed for growth and differentiation *in vitro* (33). Enforced expression of *Id1* in HSPCs resulted in a significant increase in the size and number of colonies in soft agar in the presence of SCF, IL-3 and GM-CSF (Figure 1) (39). Furthermore, these colonies contained increased numbers of primitive and differentiating myeloid cells, suggesting that *Id1* promotes myeloid cell hyperplasia without blocking differentiation *in vitro*. However, immortalized hematopoietic progenitor cell (HPC) lines that escape normal senescence and differentiation could be readily derived from BMCs overexpressing *Id1* in liquid culture containing SCF (39). The HPC lines resembled CMP-like progenitors by immunophenotype and lineage specific gene expression, and continued to divide in SCF; however, these cells were not arrested in differentiation and retained the ability to differentiate in response to G-CSF and GM-CSF *in vitro*. In other studies, overexpression of *Id1* in purified LSK cells inhibited B cell development and promoted myeloid/dendritic cell development in cultures supplemented with SCF/FLT3/IL-7 (34). Furthermore, LSK/Flk2- and lymphoid-primed multipotent progenitor (LMPP) cells isolated from mice that overexpress a dominant form of E protein (ET2) that prevents ID protein binding, showed increased B cell, and reduced myeloid cell development when cultured *in vitro*. Collectively these studies demonstrate that *Id1* promotes the proliferation of murine HSPCs and myeloid development, while inhibiting lymphoid development *in vitro*.

Id1-overexpressing HSPCs showed increased myeloid and decreased B cell repopulation potential when transplanted into γ-irradiated (IR) recipients *in vivo* (Figure 1) (33, 39). In addition, thymocyte repopulation was inhibited during the early stages of T cell development after transplantation; however, no effect on T cell repopulation was observed in PBCs 4-6 months after BMT. A comparison of Id1/EGFP expression in repopulating T cells and myeloid cells in the same mouse by flow cytometry showed a significant reduction in Id1/EGFP expression in T cells compared to myeloid cells. Thus, it was reasoned that T cells expressing high levels of *Id1* do not repopulate, while low levels of *Id1* expression are permissive for T cell development *in vivo*, and account for the recovered T cell development over time. Historically, murine BMCs cultured in HGFs cocktails that included IL-3 to promote HSC cycling and increase retroviral vector transduction, show significantly reduced lymphoid repopulation when transplanted *in vivo*. It is tempting to speculate that inhibition of lymphoid cell repopulation in these BMT experiments was due to the induction of *Id1* by IL-3. In this regard, HGFs that promote *Id1* expression and inhibit lymphoid development of HSCs should be avoided in HSC expansion media. Finally, mice transplanted with *Id1*-overexpressing HSPCs become moribund within a year after transplantation and develop a myeloid proliferative neoplasia (MPN) including 1) increased myeloid blasts in BM, 2) splenomegaly and extramedullary hematopoiesis (myeloid and erythroid), and 3) myeloid cell infiltration in liver, which does not progress to acute myeloid leukemia (AML) (39). Collectively, enforced expression of *Id1* regulates HSPC fate by promoting myeloid and inhibiting lymphoid development *in vitro* and *in vivo*, and *Id1* can promote HSPC proliferation and immortalize HSPCs *in vitro*, and promote a myeloproliferative disease *in vivo*.

HPC lines were also derived from murine BMCs transduced with lentiviral vectors expressing inducible *Id2* (Id2-HPCs), which were established in conditions that support primitive B cell growth including SCF, FLT-3 and IL-7 on S17 stromal cells (45). The Id2-HPC lines express c-Kit, CD43 and low levels of B220, but lack the expression of other cell surface markers expressed on more mature myeloid and lymphoid lineage cells. Id2-HPC lines are multipotential and differentiate into myeloid cells in the presence of IL-3, GM-CSF, and M-CSF *in vitro* and *in vivo*, under conditions that maintain *Id2* expression. Id2-HPCs differentiate into lymphoid cells (B cell and T cell) upon withdrawal of ID2 *in vitro* and *in vivo* consistent with the ability of ID2 to inhibit E protein function. The lymphoid potential of the Id2-HPC lines was lost with cell passaging suggesting that the Id2-HPC lines may undergo further myeloid differentiation and commitment *in vitro*. It is possible that the Id2-HPC lines that have lost lymphoid potential may resemble the SCF-dependent CMP-like HPC lines that overexpress *Id1* (39). The Id2-HPC line transcriptome resembles the transcriptome of the previously described *E2a*$^{-/-}$ multipotent cell lines (pre-pro-B cells) that were established in SCF, FLT-3, IL-7 and stromal cells (45), which is consistent with the ability of ID2 to inhibit the function of E2A and prevent lymphoid development. In comparison, over expression of *Id2* in

human cord blood CD34+ HSPCs resulted in expansion of CD34+/CD38-/CD90+/CD45RA- HSCs, and a skewing toward myeloid (CMP/MEP) versus lymphoid (B/NK/GMP) by immunophenotype analysis *in vitro*, and reduced lymphoid development when transplanted into NSG mice (38). HSPCs isolated from NSG mice transplanted with ID2-overexpressing CB-HSPCs show increased expression of stem cell genes and genes expressed in primitive myeloid differentiation programs, and decreased expression of B cell factors, *EBF1* and *FOXO1*, which are E2A target genes (46). ID2-mediated expansion of HSCs was rescued by overexpressing E2A *in vitro*, and overexpression of E2A promoted lymphoid development *in vivo*. Knock down of the *E2A* target gene, *EBF1*, which is required for lymphoid development, resulted in an increase in HSC numbers. These results suggested that ID2 expands or maintains HSC numbers by inhibiting lymphoid lineage priming, which leads to a reduction in HSC numbers (38). Taken together, overexpression of *ID2* expands HSCs and restrains B cell development in human HSPCs. These studies suggest that ID2 may function to regulate human and mouse HSCs function *in vivo*.

Hematopoietic progenitor cell lines were also established by overexpressing *Id3* in fetal liver cells and BMC cultures containing SCF, FLT-3, IL-7, and stromal cells, and resemble pre-pro B cells (Id3-HPC lines) (47). Id3-HPC lines are multipotential and retain myeloid, B cell and T cell potential *in vitro* and *in vivo*. The lymphoid potential of Id3-HPC lines was induced by down regulation of *Id3* expression in developing B cells *in vivo*, supporting previous studies that high levels of *Id3* impair B and T cell development (48–51). Secondary transplantation of BMCs from primary BMT recipients that received Id3-HPCs showed residual myeloid reconstitution, but no lymphoid reconstitution suggesting that Id3-HPCs are not HSC/MPP-like cells (47). Id3-HPC lines could not be established from human CB HSPCs; however, *Id3* overexpressing CB HSPCs cells showed enhanced proliferation *in vitro* and limited repopulation potential in NSG mice. Additional experiments are required to further understand the mechanism(s) of *Id3*-mediated immortalization in murine and human HSPCs compared to *Id1* and *Id2* immortalization.

Hematopoietic phenotypes in mouse models of *Id* gene loss of function

Conventional *Id1$^{-/-}$* mice

The first *Id1*- deficient (*Id1$^{-/-}$*) mouse model was generated by gene targeting, which replaced the first exon and part of the promoter of *Id1* with a neomycin resistance gene cassette. Conventional *Id1$^{-/-}$* mice were born at normal mendelian

frequencies, were fertile and showed normal lifespan with no overt abnormalities (52). No significant difference in the number of mature hematopoietic cells was reported in PBCs, BMCs and spleen cells of *Id1$^{-/-}$* mice, suggesting that *Id1* is not required for normal hematopoietic development. However, a more detailed analysis of these mice revealed that *Id1$^{-/-}$* mice have impaired hematopoietic development including 1) increased myeloid cells and decreased lymphoid cells in PBCs, 2) decreased BM cellularity, 3) decreased numbers of LSK cells, 4) decreased numbers of LSK/CD34-/Flk2- cells enriched for HSCs, 5) increased cycling of LSK cells, and 6) increased proliferation and differentiation of LSK cells *in vitro* (35). No difference in the repopulation potential of *Id1$^{-/-}$* and *Id1$^{+/+}$* BMCs was observed in primary BMT recipients; however, *Id1$^{-/-}$* BMCs showed impaired secondary repopulation ability albeit with low statistical significance, suggesting that *Id1$^{-/-}$* HSCs have a defect in self-renewal. Collectively, these investigators concluded that *Id1$^{-/-}$* HSCs show increased cycling and increased myeloid commitment, which resulted in decreased HSC self-renewal in secondary BMT recipients, suggesting that *Id1* is required to maintain HSCs.

A second *Id1$^{-/-}$* mouse model was generated by inserting the EGFP coding sequence downstream of the Id1 transcriptional start site, which results in an *Id1* null allele (*Id1$^{EGFP/EGFP}$*) (37). In comparison to the Jankovic et al. *Id1$^{-/-}$* mouse model described above, the hematopoietic phenotypes observed in *Id1$^{EGFP/EGFP}$* mice included decreased numbers of HSCs (LSK/CD150+/CD48-); however, this report showed no effect on the cycling of HSC enriched populations, no difference in the development of mature lymphoid and myeloid cell populations in BMCs or PBCs. *Id1$^{EGFP/EGFP}$* mice showed no difference in repopulation in primary BMT recipients, but reduced secondary repopulation potential suggesting that *Id1* was required for HSC development and maintenance. However, it should be noted that the secondary BMT was performed 16 days after primary BMT, which is a significant deviation from the typical secondary BMT protocol, which is usually performed 10-16 weeks after primary BMT, when hematopoiesis resembles more steady-state conditions (37). Therefore, it is difficult to conclude from these studies if *Id1* is required for HSC self-renewal.

A third study also analyzed hematopoietic development in the *Id1$^{-/-}$* mouse model used in the Jankovic et al. study discussed above (35) and confirmed that 1) myeloid cells were increased and lymphoid cells decreased in BMCs and PBCs, 2) BM cellularity was decreased, and 3) LSK cycling was increased (53). This study showed no difference in the number of HSC-enriched cells (LSK/CD34- cells) and HSC function *in vivo*; however, the two studies used different cell surface antigens to immunophenotype the HSCs, which could explain the differences in HSC numbers. Finally, the two studies agreed and showed no difference in HSC repopulation potential in primary BMT recipient mice and differed in serial repopulation

potential leaving open the question whether *Id1* functions in HSC self-renewal.

Based on the known function of *Id1* in other cellular contexts, it might be predicted that loss of *Id1* would lead to reduced cell cycling and increased B cell development in the hematopoietic compartment of *Id1*$^{-/-}$ mice. However, *Id1*$^{-/-}$ mice show the opposite hematopoietic phenotypes including increased LSK cycling and proliferation, and decreased B cell development (35, 53). Since *Id1* expression is ablated in all cells in conventional *Id1*$^{-/-}$ mice, and *Id* genes are widely expressed in other tissues including endothelial cells (ECs) and skeletal stem cells (SSCs) and their progeny, which are cellular constituents of the hematopoietic microenvironment (HME) (8, 54–57), it is possible that loss of *Id1* function in the HME could contribute to the hematopoietic phenotypes observed in conventional *Id1*$^{-/-}$. Therefore, in the third study, *Id1*$^{+/+}$ and *Id1*$^{-/-}$ BMCs were transplanted into γ-IR *Id1*$^{-/-}$ or *Id1*$^{+/+}$ recipient mice and monitored for hematopoietic development (53). *Id1*$^{+/+}$ BMCs transplanted into *Id1*$^{-/-}$ recipients showed impaired hematopoietic development similar to the hematopoietic phenotypes observed in the conventional *Id1*$^{-/-}$ mice including decreased BM cellularity, increased myeloid and decreased erythroid development. Importantly, *Id1*$^{-/-}$ BMCs showed normal hematopoietic development when transplanted into *Id1*$^{+/+}$ recipient mice. These results were confirmed in a recent study using a mouse model that lacked *Id1* and *Id3* expression in the HME, since HME phenotype was less severe in mice on a pure C57BL/6 background and *Id3* can compensate for loss of *Id1* in some models (58). Transplantation of normal BMCs into γ-IR *Id1*$^{-/-}$;*Id3*$^{-/-}$ recipient mice showed a significant decrease in BM cellularity, decreased B cell development, increased HSC cycling and decreased HSC numbers (56). Finally, *Id1*$^{-/-}$ stromal cells show altered cytokine production *in vitro*, and cytokine levels were deregulated in conventional *Id1*$^{-/-}$ mice *in vivo*. Collectively, these results demonstrated that *Id1* is required for the proper function of the HME, and that the hematopoietic phenotypes observed in conventional *Id1*$^{-/-}$ mice could, in part, be explained by the loss of *Id1* function in the HME.

Conditional loss of *Id1* in endothelial cells

Endothelial cells are critical cellular components of the HME and are required to maintain steady state hematopoiesis (59–63). SECs are critical cellular components of the HME and are required to maintain steady state hematopoiesis, in part, through angiocrine signaling (64, 65). Lineage tracing studies using vascular endothelial cadherin (VE-Cad) transgenic mice *Cdh5(PAC)-CreERT2* bred to *Rosa26-mT/mG* mice showed that transition endothelial vessels (H vessels), proliferate to regenerate diaphyseal sinusoidal ECs (SECs) within forty days under homeostasis (66). Recent evidence suggest that *Id* genes

promote EC proliferation and vessel regeneration under stress (67–69); thus, it was hypothesized that *Id* genes may be required for proper HME function by maintaining ECs under steady state conditions and stress (56). Therefore, *Cdh5(PAC)-CreERT2* mice were bred to *Id1*$^{F/F}$;*Id3*$^{-/-}$ mice to specifically ablate *Id1* and *Id3* expression in ECs, since *Id1* and *Id3* are required for proper HME in mice on a pure C57/BL6 background. Loss of sinusoidal integrity was observed in *Id1*$^{-/-}$; *Id3*$^{-/-}$ mice characterized by dilated, leaky, and apoptotic BM SECs that increased in severity over time (56). The proliferation of *Id1*$^{-/-}$; *Id3*$^{-/-}$ SECs, and transition endothelial vessels was significantly reduced *in vitro* and *in vivo*, leading to impaired vascular integrity under steady state conditions, which was more severe following acute stress. The disruption in sinusoidal integrity and neovascularization in *Id1*$^{-/-}$;*Id3*$^{-/-}$ mice led to a progressive decline in hematopoiesis, marked by increased HSC activation, proliferation, differentiation, migration, and exhaustion. Thus, *Id1* and *Id3* are required for the survival and steady state regeneration of BM SECs, which provide a supportive niche for HSC quiescence and survival. Future studies are needed to examine if *Id* genes regulate other cells in the HME including subtypes of SSCs (Leptin-cre+, Nestin-ER-cre+, and NG2-cre+), which functionally support hematopoiesis, and their downstream progeny including osteoblasts and chondrocytes (60, 63, 70). Future studies are needed to examine Id gene function in the neural niche, since nerve fibers such as adrenergic and cholinergic nerves are instructive for hematopoietic mobilization and quiescence respectively.

Conventional *Id3*$^{-/-}$ mice

Id3$^{-/-}$ mice are born at normal mendelian frequencies, are fertile, and young mice show no overt phenotypes. *Id3*$^{-/-}$ mice have normal numbers of developing B cells, but show impaired humoral immunity, B-cell proliferation, and develop a unique autoimmune disease, Sjogren's syndrome, and γδ-T cell hyperplasia with age (49, 50, 71, 72). In addition, *Id3*$^{-/-}$ mice show severely impaired positive and negative thymocyte selection (50). Transplantation of *Id3*$^{-/-}$ BMCs into γ-IR mice show normal myeloid and B cell repopulation, but impaired T cell repopulation in secondary recipient mice, suggesting that *Id3*$^{-/-}$ is not required for HSC maintenance (37). However, further studies are needed to examine if *Id3* is required for HSC self-renewal in serial BMT assays separated by 10-12 weeks (37). In other studies, no difference in donor B cells, T cells, neutrophils, HSCs and MPP repopulation were observed in mice 12 weeks after competitive BMT of *Id1*$^{-/-}$*Id3*$^{-/-}$ BMCs compared to controls, confirming that *Id3* is not required for HSC repopulation of primary BMT recipient mice; however, HSC self-renewal was not evaluated in these assays (56). Therefore, additional studies are needed to examine the requirement of *Id3* in HSC development.

Conventional *Id2*⁻/⁻ mice

The majority of conventional *Id2*⁻/⁻ mice show perinatal lethality and are born at less than ten percent of the normal mendelian frequencies. The surviving mice lack Langerhans cells, splenic dendritic cells, and NK cells, and show absence of lymph nodes and Peyer's patches, which demonstrates the requirement for ID2 in the development of these cells (18, 22). Surviving *Id2*⁻/⁻ mice also show increased B cell development, decreased erythroid cells and no effect on myeloid cell development (73). Gain and loss of *Id2* function studies in normal HSPCs confirmed that *Id2* intrinsically inhibits B cell development *in vivo* by negatively regulating E2A. In addition, these studies showed that ID2 binds to PU.1 and interferes with PU.1's ability to inhibit GATA-1 transcriptional activity, suggesting a potential mechanism of action for how ID2 promotes erythroid development (73). Additional experiments are needed to further explore this mechanism of action, and more precisely define the progenitors that express *Id2*, and where cell fate is determined and how the expression of *Id2* is regulated. In this regard, *Gfi-1* has been identified as a direct transcriptional repressor of *Id2*, and high levels of *Id2* are expressed in *Gfi-1*⁻/⁻ BMCs, and these mice show defects in B cell, T cell, and neutrophil development (74, 75). In addition, these mice have impaired short term reconstituting cell (STRC) activity or ability to radio-protect lethally irradiated recipient mice, and have significantly reduced numbers of HSCs (74–76). Reducing *Id2* levels in *Gfi-1*⁻/⁻ mice (*Gfi-1*⁻/⁻;*Id2*⁺/⁻ mice) partially restores B cell development by overcoming the block in B cell development at the pro-B cell stage, and reduces myeloid hyperplasia, but does not rescue T cell development. These results provide evidence for a direct link between *Gfi-1* and the B cell transcriptional network *via Id2*, which inhibits E2A function required for B cell development (76). While reducing the levels of *Id2* in *Gfi-1*⁻/⁻ mice rescued the myeloid hyperplasia in the spleen, it did not rescue neutrophil differentiation. Additional studies are needed to uncover how *Id2* promotes myeloid expansion in *Gfi-1*⁻/⁻ mice and why normal myeloid development is not restored. Finally, reducing *Id2* levels in *Gfi-1*⁻/⁻ mice partially restores the number of STRCs, CMP and MEP progenitors and differentiating erythroid cells in the BM bone marrow, which is sufficient to radio-protect lethally irradiated BMT recipient mice (77). Increased red cell production in *Gfi-1*⁻/⁻;*Id2*⁺/⁻ mice was correlated with increased expression of *Gata1*, *Eklf* and *EpoR*, which are required for erythroid development. It was proposed that ID2 inhibits E2A/Scl complexes that regulate erythroid gene expression *via* a multiprotein transcription factor complex that binds paired E-box/GATA sites in the promoters of *Gata1, Eklf* and *EpoR*. However; the precise molecular mechanism(s) of *Id2* action that rescue the erythroid lineage in *Gfi-1*⁻/⁻ mice remain to be defined.

Finally, reducing *Id2* levels in *Gfi-1*⁻/⁻ mice does not increase HSC numbers and rescue the defect in HSC function in competitive repopulation assays indicating that other genes mediate HSC loss in *Gfi-1*⁻/⁻ mice. Collectively, these observations suggest that *Id2* regulates HSPC fate at multiple cellular levels, but leaves open the question whether *Id2* regulates HSC development.

Conditional *Id2*⁻/⁻ mice

Few conventional *Id2*⁻/⁻ mice survive beyond birth suggesting that the surviving *Id2*⁻/⁻ mice may have compensated in some way to promote the survival of *Id2*⁻/⁻ mice. Therefore, the intrinsic requirement of *Id2* in HSCs was evaluated in conditional *Id2*^F/F mice. Specifically, BMCs from *Mx1-cre;Id2*^F/F mice (78) were transplanted in γ-IR recipient mice (chimeric mice) to reconstitute the host hematopoietic system and eliminate any contribution that loss of *Id2* function might have in the HME. Chimeric mice were treated with *pIpC* to induce interferon production and ablate *Id2* expression in hematopoietic cells six weeks after BMT, and then examined for hematopoietic development after ten weeks. Chimeric mice showed a significant reduction in the total number immunophenotypic HSCs, and decreased donor reconstitution of competitively transplanted primary recipient mice confirming that *Id2* is required to maintain HSCs in chimeric mice (Figure 2). Furthermore, *Id2*⁻/⁻ chimeric mice showed reduced overall survival due to anemia and BM failure compared to *Id2*⁺/⁺ chimeric mice indicating that ID2 is intrinsically required for HSC maintenance. Mechanistically, *Id2*⁻/⁻ HSCs showed increased proliferation and cycling, mitochondrial activation, reactive oxygen species (ROS) production and differentiation *in vitro* and *in vivo*. Pathway analysis of differentially expressed genes in *Id2*⁺/⁺ and *Id2*⁻/⁻ HSCs revealed increased expression of genes that regulate cellular proliferation and genes that regulate oxidative phosphorylation in *Id2*⁻/⁻ HSCs compared to *Id2*⁺/⁺ HSCs. In addition, gene set expression analysis (GSEA) and pathway analysis revealed that HIF-1α target genes were decreased *Id2*⁻/⁻ HSCs suggesting that ID2 might affect the levels or function of HIF-1α. In this regard, *HIF-1α*⁻/⁻ mice show similar hematopoietic phenotypes with *Id2*⁻/⁻ mice including increased cycling, decreased quiescence, and increased susceptibility to 5-FU treatment (79). HIF-1α protein levels were reduced in purified *Id2*⁻/⁻ HSCs, and loss of HSC function in *Id2*⁻/⁻ mice could be restored by chemically stabilizing HIF-1α and overexpression of stabilized HIF-1α *in vitro* and *in vivo*. Mechanistically, ID2 stabilizes HIF-1α by binding to VHL and interfering with HIF-1α ubiquitination and proteasomal degradation (Figure 2). Collectively, ID2 is required to maintain HSC quiescence, a function that is distinct from ID1,
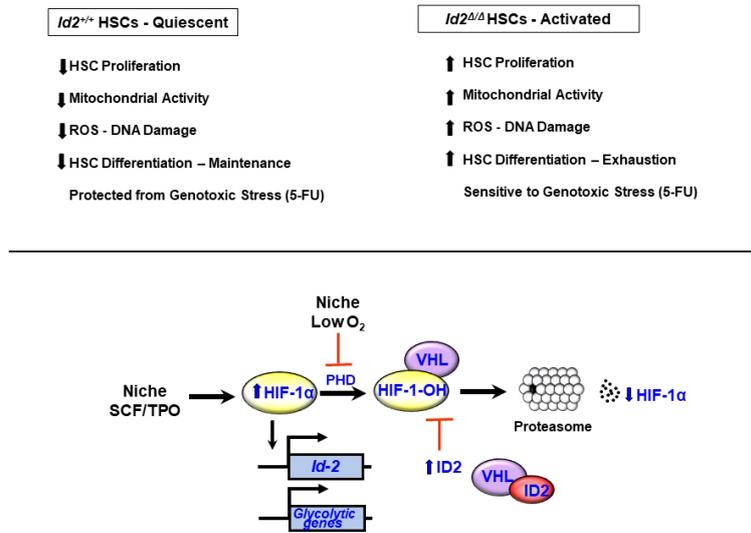
FIGURE 2

Id2 is required for HSC quiescence and maintenance. Summary of hematopoietic phenotypes in *Id2* ablated mice. *Hif-1α* expression is induced in HSCs by stem cell factor (SCF) and thombopoietin (TPO). HIF-1α protein levels are maintained at low levels under normoxic conditions *via* the action of proline hydroxylases (PHDs), which hydroxylate HIF-1α and promotes its association with the VHL complex, ubiquitination and proteasomal degradation. Under low O_2 conditions, PHD is inhibited resulting in reduced levels of hydroxylated HIF-1α, and reduced ubiquitination and stabilization of HIF-1α. ID2 also acts to stabilize HIF-1α by binding to VHL, which prevents ubiquination and proteosomal degradation of HIF-1α and promotes HSC quiescence.

which promotes HSC proliferation. *Id2* and *Id1* expression are inversely correlated during hematopoietic stress, where *Id2* expression decreases in HSCs as they exit quiescence and *Id1* expression increases; after which, *Id1* decreases and *Id2* increases when HSCs resume quiescence (Figure 3). Future experiments are needed to determine if *Id2* regulates quiescence of leukemic stem cells (LSCs), and if there are additional mechanism(s) of *Id2* action in normal and LSCs.
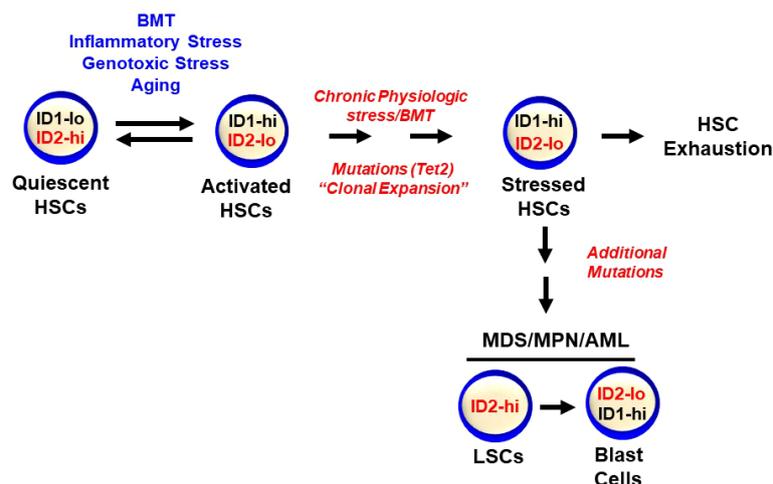

FIGURE 3

Id1 and *Id2* are differentially expressed during HSC activation and quiescence. HSCs express low levels of *Id1* that are induced during stress including BMT, genotoxic and inflammatory stress, and aging. *Id2* is required to maintain HSC quiescence and *Id2* decreases during HSC proliferation and activation, after which, *Id1* levels decrease and *Id2* levels increase as HSCs return to quiescence. Chronic stress can lead to HSC exhaustion and clonal hematopoiesis and hematopoietic malignancies.
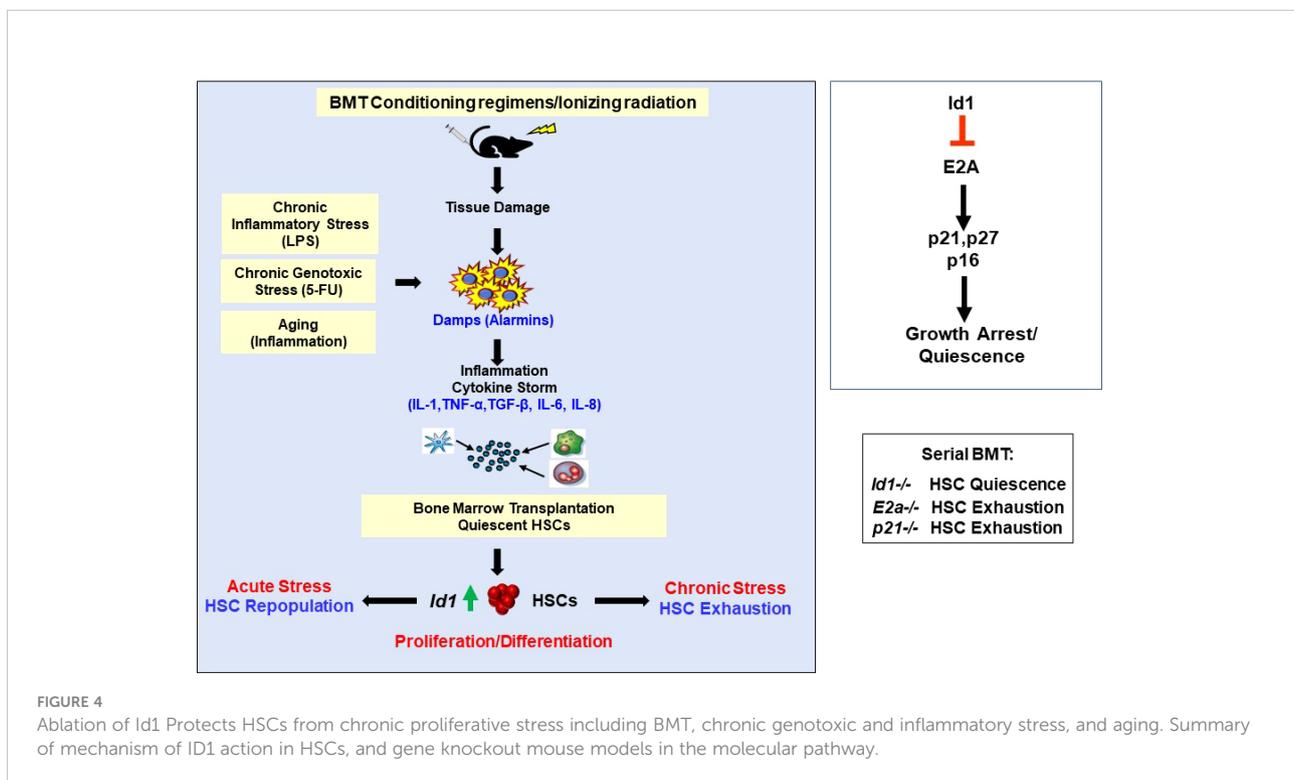
ID1 and stress hematopoiesis

Id genes are early response genes that are induced in NIH-3T3 fibroblasts by serum and growth factors, and are required for re-entry of serum-starved cells into cell cycle, demonstrating the requirement for *Id* genes in HGF-induced proliferation (stress) (80). *Id* genes are expressed at low levels in most adult tissues, and are induced by a wide array of extracellular signals in response to stress or injury to promote tissue repair and regeneration (10, 67, 68). HSCs express low levels of *Id1* under steady state conditions, and *Id1* is induced by HGFs that promote myeloid proliferation and differentiation including IL-3 (33, 34). Furthermore, enforced expression of *Id1* in HSPCs promotes myeloid cell proliferation at the expense of lymphoid development, suggesting that ID1 may function during hematopoietic stress (33, 39). Therefore, to evaluate the role of *Id1* in hematopoietic stress, $Id1^{-/-}$ BMCs were serially transplanted in γ-IR recipient mice. No significant difference in the repopulation potential of $Id1^{-/-}$ BMCs compared to $Id1^{-/-}$ BMCs was observed in primary BMT recipients. However, $Id1^{-/-}$ BMCs showed enhanced self-renewal potential and promoted the survival of serially transplanted mice significantly beyond the potential of $Id1^{+/+}$ BMCs (Figure 4) (36). Increased numbers of HSCs were detected in mice serially transplanted with $Id1^{-/-}$ BMCs compared to $Id1^{+/+}$ BMCs, demonstrating that $Id1^{-/-}$ HSCs are maintained and protected from exhaustion during chronic stress. Furthermore, $Id1^{-/-}$ HSCs in serially transplanted mice showed reduced cycling, DNA damage (γ-H2AX phosphorylation), mitochondrial biogenesis and activation, and ROS levels compared to $Id1^{+/+}$ HSCs, indicating that $Id1^{-/-}$ HSCs show increased quiescence during stress (36). Comparative transcriptome analysis of purified donor $Id1^{-/-}$ and $Id1^{+/+}$ HSCs confirmed that $Id1^{-/-}$ HSCs have an increased quiescent molecular signature including reduced expression of genes involved in cell cycle, oxidative phosphorylation, ribosomal biogenesis, and protein synthesis. Taken together, $Id1^{-/-}$ HSCs show increased quiescence and reduced proliferation and activation during hematopoietic stress compared to $Id1^{+/+}$ HSCs.

BMT conditioning regimens including γ-IR damages the HME resulting in acute and chronic inflammation due to the production of alarmins, recruitment of inflammatory cells and the production of pro-inflammatory cytokines (cytokine storm) (81–85). Initial reports demonstrated that myeloid HGFs induce *Id1* expression and proliferation of HSPCs *in vitro* (33, 34). Subsequent studies showed that *Id1* can be induced in HSCs by a variety of proinflammatory cytokines *in vitro*, and HSCs that express *Id1* are actively proliferating (36). In addition, these studies found that *Id1* is induced in HSCs by proinflammatory cytokines *in vivo*, and in HSCs after 6 Gy γ-IR. Thus, during chronic stress $Id1^{-/-}$ HSCs fail to properly respond to cytokine-induced proliferation and differentiation and are protected from exhaustion *in vitro* and *vivo* during serial BMT.

It is difficult to predict which cytokines in the cytokine storm induce *Id1* in HSCs after BMT *in vivo*; however, many cytokines

FIGURE 4

Ablation of Id1 Protects HSCs from chronic proliferative stress including BMT, chronic genotoxic and inflammatory stress, and aging. Summary of mechanism of ID1 action in HSCs, and gene knockout mouse models in the molecular pathway.

signal *via* the Jak/Stat pathway suggesting that JAK inhibitors could protect HSCs from chronic proliferative stress. In this regard, JAK1 is an intracellular tyrosine kinase signaling molecule required for HSCs to respond to stress cytokines including IFN-α/β/γ and IL-3 (86). *Jak1* deficient HSCs exhibited increased quiescence, inability to enter cell cycle, reduced response to type I interferons and IL-3, and impaired ability to reconstitute hematopoiesis during BMT and stress (86). Since ID1 proteins are induced by IL-3 in normal HSPCs and cell lines (33), the increased quiescence of *Jak1*$^{-/-}$ HSCs during stress may be mediated, in part, by reduced expression of *Id1*. Therefore, mice were treated with small molecule inhibitors of the JAK/STAT pathway after BMT to inhibit *Id1* induction in HSCs. JAK/STAT inhibitors partially inhibited the induction of *Id1* in HSCs after BMT in γ-IR recipient mice demonstrating that pro-inflammatory cytokines produced after γ-IR induce *Id1* in HSCs *in vivo* (36). Future studies could target other relevant ligands (cytokines), receptors, and downstream signaling pathways involved in proinflammatory cytokine signaling during BMT including IL-6, IL-1, tumor necrosis factor (TNF), IL-8 and others to prevent HSC exhaustion and improve BMT.

It was hypothesized that since *Id1*$^{-/-}$ HSCs are protected from chronic proliferative stress and exhaustion during serial BMT, ablation of *Id1* expression in HSPCs during other conditions of chronic stress including inflammatory and genotoxic stress, and aging could prevent HSC exhaustion (Figure 4). Indeed, studies found that *Id1*$^{-/-}$ HSCs were also preserved in models of chronic proliferative stress including chronic inflammatory stress (LPS) and genotoxic stress (5-FU) (36, 87). In addition, HSCs from aged (2 years old) *Id1*$^{-/-}$ mice resemble more youthful HSCs including, 1) an immunophenotype of young HSCs, 2) increased HSC function in serial BMT assays, 3) increased HSCs in G0, 4) decreased HSC DNA damage, 4) reduced HSC ROS levels, and 5) reduced HSC mitochondrial stress compared to *Id1*$^{+/+}$ HSCs. Future experiments are needed to further define the molecular mechanism(s) that protect *Id1*$^{-/-}$ HSCs from aging. Collectively, these results demonstrate that *Id1*$^{-/-}$ HSCs are more quiescent and resistant to chronic proliferative stress including inflammatory and genotoxic stress and aging. Clonal hematopoiesis, MPN and MDS are associated with increased inflammation (84, 88–90), which could induce *Id1*, promote HSPC proliferation during clonal hematopoiesis, and increase mutational load and genomic instability over time.

Mechanism(s) of Id1 action in hematopoietic stem and progenitor cell growth and quiescence

Id1$^{-/-}$ HSCs fail to properly respond to cytokine-induced proliferation and differentiation and show decreased BrdU incorporation and cell cycling after stress *in vitro* and *in vivo* (36). Since E proteins inhibit cell proliferation, in part, by promoting the expression of cyclin dependent kinase inhibitors (Cdki's) (91–94), and ID proteins inhibit E-proteins resulting in increased proliferation, it is reasonable to hypothesize that the reduced cycling of *Id1*$^{-/-}$ HSCs is due to unrestrained E protein transcriptional activity and increased Cdki expression (Id1-E2a-Cdki pathway) (Figure 4). Increased *p21* expression was observed in *Id1*$^{-/-}$ LSK cells compared to *Id1*$^{+/+}$ HSCs (35). Subsequent reports demonstrated that *Id1*$^{-/-}$ HSCs show increased *p27* and *p16* expression and reduced proliferation in expansion cultures compared to *Id1*$^{+/+}$ HSCs, and *Id1*$^{-/-}$ HSCs show increased expression of *p21* and *p27* after 14 weeks in competitive BMT assays compared to *Id1*$^{+/+}$ HSCs (36). Furthermore, knock down of *E2a* and *p16* expression in *Id1*$^{-/-}$ HSPC expansion cultures increased HSC proliferation suggesting that *Id1* increases HSC proliferation, in part, by restraining E protein function and reducing *p16* expression (36). Importantly, *E2a*$^{-/-}$ HSCs show increased cycling, decreased serial repopulation potential, and decreased expression of *p21* and *p27* expression (95–97), and *p21*$^{-/-}$ HSCs have decreased serial repopulation populations potential (98). Thus, *Id1*$^{-/-}$ HSCs demonstrate opposite hematopoietic phenotypes to *E2a*$^{-/-}$ and *p21*$^{-/-}$ HSCs, whereby *Id1*$^{-/-}$ HSCs show increased quiescence and are protected from hematopoietic stress and exhaustion, and *E2a*$^{-/-}$ and *p21*$^{-/-}$ HSCs show increased proliferation and activation and are sensitive to hematopoietic stress and premature exhaustion. Thus, the Id-E-Cdki pathway critically regulates HSC cycling during stress. In other studies, the E protein(E47) – Cdki (p21) pathway was shown to be important in preventing HSC exhaustion during BMT and 5-FU mediated stress (99). Specifically, genetic experiments examining the requirement for E47- p21 pathway in maintaining HSCs under stress showed that *E47*^{het}*p21*^{het} HSCs exhibit decreased serial BMT repopulation when compared to *E47*^{het}*p21*^{WT}, which have increased p21 levels. The E-Cdki pathway is conserved in endothelial cells (ECs), where *p21* and *p27* RNA expression is increased in ECs that lack *Id* gene expression, and growth inhibition of *Id1*$^{-/-}$*Id3*$^{-/-}$ ECs *in vitro* was rescued by knocking down *E2-2* expression (56). In comparison, *E2a* and *p16* shRNAs partially restore the proliferation of *Id1*$^{-/-}$ HSCs *in vitro*, suggesting that other E and Cdki proteins may regulate quiescence in *Id1* ablated HSCs, or *Id1* regulates HSC functions through other target genes and pathways. Therefore, it will be important to evaluate if knock down of other E proteins, *E2-2* and *Heb*, and other Cdki's rescue *Id1*$^{-/-}$ HSC proliferation *in vitro*. In this regard, the role of *E2-2* and *Heb* in HSC development in conditional mouse models has not been evaluated. Furthermore, it would be important to know if reducing the expression of *p21* and other Cdki's *via* genetic experiments can rescue the hematopoietic phenotypes in *Id1*$^{-/-}$ mice *in vivo*. Finally, additional transcriptomic, proteomic, and

single cell analysis of normal, stressed, or aged $Id1^{-/-}$ HSCs might uncover additional molecular pathways that regulate HSC quiescence.

Id Genes and hematopoietic malignancies

Id gene expression has been correlated with the initiation, progression, and metastasis of cancer in many tissues (7, 10, 100). *Id* genes are frequently overexpressed in advanced stage chronic myeloid leukemia (CML), AML and MDS patient samples while low or no levels of ID1 were detected in normal CD34+ HSPCs (39, 101–110). Knock down of *ID1* expression in the AML cell line, MO-7e, resulted in decreased growth and suggested that some AML cells may require ID1 for growth. Furthermore, analysis of *ID* gene expression in an AML patient cohort showed that high levels of *ID* gene expression were correlated with *FLT3-ITD, RAS, EVI-1* and *C/EBPA* mutations, suggesting *ID* gene expression may be induced downstream of oncogene activated signal transduction pathways (111). *ID1* was identified as a common downstream target of oncogenic tyrosine kinases including FLT3-ITD and BCR-ABL (112). Stable KD of *ID1* in K562 (BCR-ABL) and Molm14 (FLT3-ITD) AML cell lines inhibited their growth *in vitro* confirming that *ID1* is required for the growth of some AML cell lines. Analysis of *Id1* expression in a 237 AML patient cohort demonstrated that AML patients with high levels of *ID1* gene expression were less likely to achieve complete remission and were correlated with shorter disease-free survival and overall survival (113). Thus, it was suggested that *ID1* expression levels may provide a molecular tool for refining risk classification of AML. In addition, these studies suggest that Id1 may represent a candidate for targeted therapy to treat AMLs (106).

To explore the intrinsic role of *Id* genes in hematopoietic malignancies, *Id1*-overexpressing HSPCs were transplanted into lethally irradiated recipient mice and monitored for survival. *Id1*-overexpressing mice died roughly one year after BMT compared to control mice, and sick mice showed 1) myeloid/erythroid cell hyperplasia and increased numbers of immature cells in the BM, 2) splenomegaly and extramedullary hematopoiesis, and 3) peripheral blood monocytosis, indicating that these mice succumbed to a MPN that did not progress to AML (39). *ID1* expression is increased in leukemic cells from AML patients with t (8, 21) translocations, and AML1-ETO regulates the *ID1* promoter, suggesting a role for *ID1* in AML1-ETO leukemia (114). Ablation of *Id1* in AML1-ETO transduced murine fetal liver cells delays the onset and development of leukemia by roughly 130 days after transplantation *in vivo* (115). Furthermore, conditional loss of *Id1* in established AML-ETO-leukemia's slows the development of leukemia and promotes animal survival, suggesting that *Id1* is also required for the maintenance of leukemia. Mechanistically,

these studies provided evidence that *Id1* promotes AML-ETO leukemic cell growth by interacting with Akt and increasing its activity. In other studies, the progression of leukemia was significantly delayed after transplantation of MLL-AF9-transduced $Id1^{-/-}$ FL cells compared to MLL-AF9-transduced $Id1^{+/+}$ fetal liver cells (116). The delay in $Id1^{-/-}$ MLL-AF9 leukemia was reversed in FL cells that lack *p21*, which is consistent with previous studies demonstrating that ID proteins promote HSPC proliferation by inhibiting the E-Cdki pathway. Interestingly, loss of *Id1* accelerated the progression of MLL-AF9-induced leukemia of BMCs, suggesting that *Id1* is not required for MLL-AF9-induced leukemia of BMCs, and that leukemogenesis may differ significantly for FL and BM HSPCs. The cellular and molecular mechanism(s) that account for the differences in the progression of leukemia in these models is not currently known. Taken together, these studies provide evidence that *ID1* is required for the initiation and progression of oncogene driven leukemias. Future studies are needed to determine if *ID1* is required for AML cell line growth in xenotransplantation and human AML PDX models, and if the recently identified inhibitors of ID1 including cannabidiol and AGX51 show any therapeutic benefit in these models (117–119). Since MPN and MDS and myeloid malignancies that develop with age are strongly correlated with inflammation and *Id1* is induced in HSPCs downstream of pro-inflammatory signals, future studies are needed to better understand the role of *ID1* in these diseases and the molecular mechanism(s) of ID1 action (84, 89, 120).

Ablation of *Id2* in HSCs results in increased proliferation and HSC activation suggesting that ID2 may function as a tumor suppressor. Interestingly, Ko et al. showed that mice transplanted with $Id2^{-/-}$ fetal liver cells develop leukocytosis after 6 months that resembles a myeloproliferative disorder, and that over expression of ID2 delays the onset BCR-ABL-induced CML *in vivo* (121). In addition, loss of *Id2* expression is associated with increased MLL-AF9-induced leukemia in mice, and over expression of *Id2* inhibits the growth of MV4-11 and MOLM-13 AML cell lines that express MLL-AF9, and Kasumi AML cells that express AML-ETO (122). Together, these results suggest that ID2 may function as a tumor suppressor in hematopoietic malignancies. In addition, mice that lack *Id2* develop intestinal adenomas, and show a hyperproliferation of colon stem cells during embryonic development due to increased Wnt/B-catenin signaling, suggesting that ID2 may function as a tumor suppressor in other cell types (123, 124). Analysis of ID2 expression in 145 AML patient BMCs showed that AML patient cells with high levels of ID2 expression were correlated with lower complete remission and shorter overall survival, and was a predictor of poor chemotherapy response (103). Analysis of *ID2* expression in a subset of MLL-rearranged AML patient cells indicated that MLL patients (35 patients) with high levels of *ID2* expression had a significantly better overall and event free survival than patients with low levels of *ID2* (122). Further

studies are needed to examine if levels of *ID2* expression are prognostic for AML patient subsets, and to determine if ID2 is expressed in LSCs and functions to regulate their quiescence and survival (Figure 3).

Conclusion and perspectives

ID proteins have emerged as important regulators of HSPC quiescence (ID2) and proliferation (ID1). Specifically, low levels of ID1 are expressed in primitive HSPCs, but are induced in HSPC after acute stress including BMT, inflammatory and genotoxic stress, and promote HSPC proliferation and myeloid development, while inhibiting lymphoid development (Figure 3). Upon resolution of an acute stress, HSCs return to quiescence with low levels of ID1 and hematopoiesis resumes under steady state conditions. However, under chronic proliferative stress ID1 levels remain high and HSCs undergo excessive proliferation, exhaustion and bone marrow failure. Reducing ID1 levels during serial BMT, chronic inflammatory and genotoxic stress and aging may be therapeutic to protect HSCs from exhaustion. In addition, since hematopoietic malignancies and bone marrow failure syndromes are often accompanied by inflammation and increased ID1 expression, reducing ID1 levels could be therapeutic by reducing preleukemic proliferation and clonal expansion, which could delay the onset and reduce the incidence of hematopoietic malignancies and bone marrow failure syndromes. ID2 has emerged as a critical regulator of normal HSC quiescence and shows opposite expression to ID1, where ID2 levels decrease as HSCs exit quiescence and ID1 levels increase during cell proliferation. Maintaining high levels of ID2 *in vitro* and *in vivo* could be exploited to expand HSCs for gene and cell therapies including BMT. Opposing expression of ID1 and ID2 is also observed during the final stages of myeloid development, where ID1 expression is increased in myeloid progenitors (CMP/GMP), and then decreases as cells exit the cell cycle and differentiate, while ID2 expression is increased in mature neutrophils. Furthermore, current evidence suggests that ID1 and ID2 may function during neutrophil and eosinophil development, however, gain and loss of function studies are needed to reveal if these genes are required for the differentiation and function of these cells. Since ID2 regulates normal HSC quiescence, the potential role of ID2 in LSC quiescence and resistance to current therapies remains to be explored.

Author contributions

SS, TS, BJ, SG, AS, KG, and JK contributed to writing and editing the review and figures. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare 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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