The hallmarks of hematopoietic stem cells (HSC) are their ability to self-renew and to develop into multiple lineages through differentiation (Fig. 1A; ref. 1). HSCs can be functionally defined as either long-term (LT-HSC) or short-term (ST-HSC) repopulating stem cells by their capacity to provide life-long or transient hematopoiesis. Furthermore, LT-HSCs primarily reside in bone marrow niches, whereas ST-HSC may be mobilized (Fig. 1A; ref. 2). Strict regulation of HSCs is crucial to ensure maintenance of regenerating cells without abnormal outgrowth of immature cells. Dysregulated expansion and growth of HSCs is likely to play a critical role in leukemogenesis (Fig. 1A and B; refs. 3, 4).

The existence of leukemic stem cells (LSC) was proposed more than three decades ago following the development of clonogenic growth assays with the capacity for clonal growth of leukemia in vitro. There was no definitive proof of LSCs, however, until Dick and colleagues showed that the engraftment of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice with primary acute myeloid leukemia (AML) samples could only be accomplished using cells that were phenotypically similar to normal HSCs by expressing CD34 and lacking markers of lineage commitment such as CD38 (5). Furthermore, these primitive cells produced leukemic blasts within engrafting animals that phenotypically matched each patient’s original AML. These results suggested that the cellular organization of AML is similar to normal hematopoiesis with immature stem cells that have clonogenic growth potential giving rise to differentiated cells with little long-term growth potential. Importantly, LSCs could be isolated and transplanted into secondary recipients, showing that they had the capacity to self-renew (5). Due to a high degree of phenotypic and functional similarity, it has been hypothesized that most human leukemias arise from transformation of HSCs (Fig. 1A; refs. 5, 6). Conversely, recent studies have shown that transduction of the MLL-ENL or MOZ-TIF2 fusion genes into HSCs, common myeloid progenitors, and granulocyte-macrophage progenitors resulted in the identical leukemia (4, 7). These results indicate that committed progenitors with little long-term replicative potential may acquire self-renewal capability and transform into LSCs (Fig. 1A and B). It is unclear, however, what proportion of human myeloid leukemias arise from committed progenitors or by a single oncogenic event such as MLL-ENL and MOZ-TIF2, and whether these models can be useful in identifying key regulatory pathways that represent therapeutic targets.

In the following sections of this review, we will therefore highlight regulatory pathways of LSCs with validated and treatable LSC targets (Table 1).

Clinical Translational Advances

Regulatory pathways in LSCs

HSCs and LSCs share common features: self-renewal, the capacity to differentiate, resistance to apoptosis, and limitless proliferative potential. The pathways regulating these functional properties can be categorized into self-renewal, developmental, and miscellaneous pathways, each of which is governed by a distinct set of critical genes that have emerged from molecular profiling and can be associated with “stemness” (Fig. 1).

Self-renewal pathways

**BMI.** BMI-1 is a polycomb group (PcG) RING-finger protein that has an essential function in the maintenance of HSCs and LSCs. The BMI-1 gene was originally identified as an oncogene inducing B-cell or T-cell leukemia. Recent
experiments with Bmi-1−/− mice showed that leukemic (AML) stem/progenitor cells lacking Bmi-1 were unable to engraft and proliferate and displayed signs of differentiation and apoptosis. Conversely, the reconstitution of the Bmi-1 gene was found to completely abrogate these proliferative defects (8). Functional-ly, BMI-1 forms a heterodimeric complex with another PcG protein, Ring1b. PcG complexes bind to chromatin and initiate and maintain gene repression, which is thought to be mediated by methylation, deacetylation, and ubiquitination of core histones. BMI-1 and Ring1b reconstitute an ubiquitin E3 ligase activity with histone H2A as its ubiquitination substrate (9). Thus, inhibitors of methylation, histone deacetylase inhibitors, or inhibitors of the ubiquitin-proteasome system could be exploited as anti–Bmi-1 strategies in LSCs. Interestingly, a recent study showed that BMI-1 localization to PcG bodies can be interdicted by the DNA methylation inhibitor 5-azacytidine (Table 1; ref. 10). Further studies are required to assess the capacity of 5-azacytidine to modulate BMI-1 as well as the role of BMI-1 (and epigenetics) in LSCs.

**Telomerase.** Telomerase is a ribonucleoprotein enzyme composed of the human telomerase reverse transcriptase (hTERT) and the human telomerase RNA component (hTERC), which synthesizes telomeric repeats onto chromosomal ends and thereby prevents replicative senescence (11). Although stem cells in general possess limitless proliferative capacity and long telomeres, LT-HSCs are unable to prevent telomere shortening on serial transplantation because of low levels of telomerase expression. Conventional cytotoxic chemotherapy can only achieve tumor debulking by killing mature leukemia cells. LSCs are resistant to conventional treatment strategies and will often repopulate, resulting in recurrence of the disease. LSC-directed therapies (see Table 1), when given together with debulking agents, are hoped to yield durable remissions and ultimately cures. *, LSC targets that can be treated with drugs.
 niches, leading to stem cell proliferation in vitro and excessive hair growth in vivo (14, 15), whereas short telomeres cause stem cell failure and limit tissue renewal capacity (16). In human chronic myeloid leukemia, LSCs and HSCs were comparatively analyzed for telomere length, and it was found that telomeres in LSCs were significantly shorter than in HSCs and that telomerase activity in LSCs was high (Fig. 2; ref. 12, 13). This differential in telomere length and telomerase activity between HSCs and LSCs (Fig. 2) opens avenues for exploiting telomerase-directed treatments as stem cell therapeutics. Drugs with telomerase-modulating properties that are already in clinical trials in leukemia include arsenic trioxide, the hTERC antisense oligonucleotide GRN163L, and hTERT vaccines (Table 1.; refs. 17–21).

**Developmental pathways**

**Notch signaling pathway.** The Notch signaling pathway regulates the specification of cell fate and differentiation (22). Four Notch transmembrane heterodimeric receptors (Notch 1-Notch 4) and five ligands are known. The ligands Jagged 1, Jagged 2, and Adulting 1 to Adulting 3 can initiate Notch signaling by releasing the intracellular domain of the receptor (Notch-IC) through proteolytic cleavage involving α-secretase and γ-secretase (Fig. 3). Notch-IC then enters the nucleus and induces the transcription of Notch-responsive genes (22, 23). Overexpression of constitutively active Notch 1 in HSCs results in a complete inhibition of B-cell development. In T-cell acute lymphoblastic leukemia, Notch 1 is found to be constitutively activated in LSCs (Fig. 1A; ref. 34). This differential in telomere length and telomerase activity between HSCs and LSCs (Fig. 2) opens avenues for exploiting telomerase-directed treatments as stem cell therapeutics. Drugs with telomerase-modulating properties that are already in clinical trials in leukemia include arsenic trioxide, the hTERC antisense oligonucleotide GRN163L, and hTERT vaccines (Table 1.; refs. 17–21).

**Table 1.** Experimental and available LSC-directed therapies

<table>
<thead>
<tr>
<th>Target</th>
<th>Agents</th>
<th>Leukemic type</th>
<th>Phase of clinical development</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD33</td>
<td>Gemtuzumab ozogamicin (Mylotarg)*</td>
<td>AML</td>
<td>In clinical use</td>
<td>(55, 54)</td>
</tr>
<tr>
<td>Telomeres and telomerase</td>
<td>Arsenic trioxide (Trisenox)*</td>
<td>APL</td>
<td>In clinical use</td>
<td>(17, 18)</td>
</tr>
<tr>
<td></td>
<td>GRN163L (hTERC inhibitor)</td>
<td>CLL</td>
<td>I/II</td>
<td>Recruiting: NCT00124189</td>
</tr>
<tr>
<td></td>
<td>hTERT vaccine (TVAX)</td>
<td>Hematologic malignancies</td>
<td>I</td>
<td>Recruiting: NCT00305773</td>
</tr>
<tr>
<td></td>
<td>Vorinostat (Zolinza)</td>
<td>AML</td>
<td>II</td>
<td>Recruiting: NCT0005737</td>
</tr>
<tr>
<td></td>
<td>Azacitidine (Vidaza)</td>
<td>CML</td>
<td>II</td>
<td>(56)</td>
</tr>
<tr>
<td>Epigenetics (BMI-1)</td>
<td>Decitabine (Dacogen)</td>
<td>AML, ALL, CML</td>
<td>I</td>
<td>(57)</td>
</tr>
<tr>
<td>FLT3</td>
<td>CEP701 (Lestauring)</td>
<td>AML</td>
<td>II</td>
<td>(45)</td>
</tr>
<tr>
<td></td>
<td>PKC412 everolimus</td>
<td>AML hematologic malignancies</td>
<td>II</td>
<td>(58)</td>
</tr>
<tr>
<td></td>
<td>Temsirolimus</td>
<td>CML and CLL</td>
<td>I/II</td>
<td>(49)</td>
</tr>
<tr>
<td></td>
<td>Rapamycin</td>
<td>AML</td>
<td>I and II</td>
<td>Recruiting: NCT00101088, NCT00290472</td>
</tr>
<tr>
<td></td>
<td>P-Glycoprotein</td>
<td>Zosuquidar</td>
<td>Advance malignancies</td>
<td>I/II</td>
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<tr>
<td></td>
<td>γ-Secretase (Notch)</td>
<td>MK-0752</td>
<td>T-cell ALL and other leukemia</td>
<td>I</td>
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<tr>
<td></td>
<td>NF-κB</td>
<td>NPI-0052 (salinosporamides A)</td>
<td>Hematologic malignancies: NHL, CLL, and Ph+ ALL</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>TDZD-8 (parthenolide)</td>
<td>Leukemia</td>
<td>Recruiting: NCT0035773</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(36)</td>
</tr>
</tbody>
</table>

Abbreviations: APL, acute promyelocytic leukemia; CLL; chronic lymphocytic leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; NHL, non-Hodgkin’s lymphoma.

*Food and Drug Administration approved for treatment of leukemia.

*Food and Drug Administration approved for different indication.
NF-κB by stabilizing its suppressor IκB, and parthenolide, a natural product that can directly target NF-κB, are being investigated as LSC treatments (Table 1; refs. 35, 36).

**FLT3.** Under normal conditions, FMS-like tyrosine kinase 3 (FLT3) receptor is expressed in CD34+ short-term reconstituting hematopoietic stem and progenitor cells (37–39). FLT3 mutations are among the most common genetic abnormalities in AML and are present in ~30% of all cases. It is associated with poor prognosis and increased relapse rates (39–43). Recent data showed that the FLT3 internal tandem duplication mutation is present in CD34+/CD34- LSCs (Fig. 1A; ref. 44). Furthermore, inhibition of FLT3 signaling with CEP701 resulted in failure of FLT3 internal tandem duplication LSCs to engraft in NOD/SCID mice. Currently, several FLT3 inhibitors, including CEP701 and PKC41 2, are in phase II clinical trials in AML (Table 1; refs. 45, 46).

**Phosphatidylinositide-3-kinase/AKT/mammalian target of rapamycin.** The phosphatidylinositide-3-kinase/AKT/mammalian target of rapamycin (mTOR) pathway is constitutively activated in most AMLs and is essential for AML blast survival (47). Unlike in short-term repopulating leukemia cells, the activation of mTOR is required in LT-HSCs, suggesting an essential role of this pathway in LSCs (Fig. 1A). Interestingly, coadministration of the mTOR inhibitor rapamycin was found to potentiate the ability of etoposide to prevent the engraftment of AML cells in NOD/SCID mice (48). Three mTOR inhibitors, rapamycin, temsirolimus, and everolimus, are under clinical investigation in leukemia, albeit not as LSC targeting strategies (Table 1; refs. 48, 49).

**Cell-surface proteins.** An intriguing property of normal and LSCs is the expression of high levels of the drug efflux pumps P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2; ref. 50). Whereas the ABC transporters provide a mechanism of self-protection in HSCs, they are involved in multidrug resistance of leukemia to a wide variety of cytotoxic agents. P-glycoprotein inhibition using cyclosporine A was found to enhance clinical outcomes in combination with standard cytotoxic chemotherapy in poor-risk patients with AML (51). Subsequent clinical studies with the P-glycoprotein inhibitor valsapar (PSC-833), however, failed to show benefit (52). The P-glycoprotein inhibitor zosuquidar is currently in clinical trials in leukemias in combination with standard cytotoxic agents. Breast cancer resistance protein inhibitors will likely be more selective and effective, but are still in preclinical development (Table 1; refs. 50, 53).

Another cell-surface molecule that could be exploited as a LSC target is CD33. Bonnet et al. have shown that leukemia-initiating cells in NOD/SCID mice express CD33 and proposed that anti-CD33 antibodies might be useful to direct cytotoxic drugs to LSCs. Mylotarg is such a preparation that is Food and Drug Administration approved for clinical use in AML (Table 1; refs. 54, 55).

### Clinical Implications of LSC-Directed Therapies

Initial evidence for the existence of LSCs suggested that these highly clonogenic cells must be eradicated to achieve durable remission or cure (6). However, the molecular understanding to identify relevant pathways and produce novel targeted therapeutics was lacking. In current clinical practice, standard anticancer agents are still used with the intent to kill the bulk tumor mass. However, most of these fail to eradicate cancer stem cells, resulting in disease relapse. Because cancer stem cells are rare, it is likely that novel clinical trial designs must be used that consider LSC biology.
and the potential of delayed responses. These strategies may also require combining different classes of agents to target both mature cells and LSCs (Fig. 1B; Table 1). For example, differentiated leukemic cells that make up the bulk of the disease could be treated with conventional chemotherapy to alleviate patients’ symptoms. Subsequently, when tumor burden is low, LSC-directed treatments should be initiated (Fig. 1B). To ultimately prove the validity and efficacy of LSC-tailored chemotherapy regimens, stem cell markers must be used to investigate the effects of LSC-targeting agents on this rare cell population.

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