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Dysregulated haematopoietic stem cell behaviour in myeloid leukaemogenesis

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Abstract

Haematopoiesis is governed by haematopoietic stem cells (HSCs) that produce all lineages of blood and immune cells. The maintenance of blood homeostasis requires a dynamic response of HSCs to stress, and dysregulation of these adaptive-response mechanisms underlies the development of myeloid leukaemia. Leukaemogenesis often occurs in a stepwise manner, with genetic and epigenetic changes accumulating in pre-leukaemic HSCs prior to the emergence of leukaemic stem cells (LSCs) and the development of acute myeloid leukaemia. Clinical data have revealed the existence of age-related clonal haematopoiesis, or the asymptomatic clonal expansion of mutated blood cells in the elderly, and this phenomenon is connected to susceptibility to leukaemic transformation. Here we describe how selection for specific mutations that increase HSC competitive fitness, in conjunction with additional endogenous and environmental changes, drives leukaemic transformation. We review the ways in which LSCs take advantage of normal HSC properties to promote survival and expansion, thus underlying disease recurrence and resistance to conventional therapies, and we detail our current understanding of leukaemic ‘stemness’ regulation. Overall, we link the cellular and molecular mechanisms regulating HSC behaviour with the functional dysregulation of these mechanisms in myeloid leukaemia and discuss opportunities for targeting LSC-specific mechanisms for the prevention or cure of malignant diseases.

Leukaemogenesis is a lengthy, multistage process resulting in the abnormal clonal proliferation of blood cells derived from transformed primitive haematopoietic stem cells (HSCs) or from downstream progenitor cells[1,2]. Acute myeloid leukaemia (AML) is a common form of this malignant process, characterized by the accumulation of immature myeloblasts in the bone marrow and peripheral blood at the expense of the normal production of terminally differentiated blood cells. AML occurs at all ages, but incidence rates accelerate in the elderly, with a median age of diagnosis of ~70 years[3]. AML, unlike

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many other cancers, develops with rather low numbers of accumulated mutations[4]. Despite this relatively simple mutational landscape and the well-understood function of AML mutations in activating oncogenic signalling[5], the general treatment paradigm has not changed substantially in the past 30 years[6], and conventional therapies have done little to meaningfully improve overall survival, especially in older patients (>60 years of age)[3,6,7]. Although the optimization of chemotherapy regimens and allogeneic HSC transplantation (HSCT) aimed at eliminating measurable residual disease[8] have improved the 5-year survival rates in paediatric and young adult patients (<40 years of age) to 40–70%, survival has remained in the 5–15% range for the past several decades in the patient population >60 years of age[6,9,10]. Older patients with AML, who are frequently unable to tolerate intensive chemotherapeutic regimens, are often ineligible for allogeneic HSCT and thus would benefit most from effective and tolerable molecular targeted therapy[3,7].

Historically, poor outcomes in AML have been considered attributable to the post-treatment persistence of leukaemic stem cells (LSCs). LSCs are functionally defined as cells capable of regenerating and propagating disease upon transplantation in immunodeficient mice[11]. They are transformed cells that differ from leukaemic blasts in terms of their long-term maintenance and therapeutic resistance and are usually a rare leukaemic subpopulation. Analogous to how normal HSCs generate progenitors and mature blood cells, LSCs give rise to AML blasts while also retaining some biological properties of stem cells, especially their self-renewal capacity[3]. In addition, LSCs co-opt many HSC survival mechanisms in order to persist in the face of intensive cytoablative and immunologic challenges[12] and are believed to be the target cell population largely responsible for post-treatment disease relapse that must be eliminated in curative therapy. However, deep-sequencing studies have unravelled complex patterns of clonal evolution during leukaemogenesis and have highlighted the possibility that post-treatment relapse occurs as a result of existing pre-leukaemic mutated HSC clones rather than from LSCs that survive treatment[13–15], which confers a new paradigm for the management of AML relapse.

Here we examine how environmental influences, specific mutations and the distinct features of HSC biology converge to promote LSC emergence and leukaemogenesis. Moreover, we review how co-option of HSC properties contributes to the activity and resistance of LSCs to current therapies and highlight how the emergence of a more nuanced understanding of leukaemic ‘stemness’ regulation is showing promising results for the development of novel therapies and for improving patient outcomes.

Dynamic regulation of HSC activity

HSCs are a rare population of self-renewing, blood-forming stem cells that, in adults, reside in the bone marrow niche microenvironment[16]. HSCs sustain and regenerate the entire haematopoietic system through regulated fate decisions either to self-renew and maintain themselves or to differentiate into downstream progenitors, first generating multipotent progenitors (MPPs) and then more lineage-committed progenitors that gradually become more and more restricted to one of the mature blood cell types[16]. At steady state, the HSC pool is predominantly kept in a quiescent state, or the G_0 phase of the cell cycle, hence limiting HSC exposure to replication-coupled and metabolism-coupled organelle injury and

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programmed cell death that occurs in response to replication errors[17], genotoxic insults from reactive oxygen species (ROS)[18] and DNA damage checkpoint-dependent apoptosis[19,20]. However, quiescent HSCs remain highly responsive to a wide range of cell-intrinsic and cell-extrinsic stimuli and rapidly activate to produce the needed blood cells in response to regenerative stimuli[20,21]. To do so, quiescent HSCs enter the cell cycle while concomitantly switching metabolic modes from glycolysis to a mitochondrial oxidative phosphorylation (OXPHOS)-driven metabolism, to fulfil the increased demand for energy and produce the metabolic substrates required for proliferation[22,23]. The changes in metabolic cofactor bioavailability associated with this metabolic switch also control a series of DNA-modifying and histone-modifying enzymes, which, together with coordinated transcriptional changes, regulate the decision between self-renewal and differentiation[24–27] (Box 1). In addition, pro-survival signals strongly inhibit programmed cell death pathways so that activated HSCs are not eliminated through apoptosis or necroptosis during cell division[19]. Finally, the suppression of OXPHOS, in part through autophagy-dependent mitochondrial clearance[28], allows HSCs to re-enter quiescence and reacquire their resting G_0 glycolytic metabolism (FiG. 1).

The physiological requirements of haematopoiesis are altered in response to stress, where infection or injury can lead to the acute loss of mature cells and place enormous demands on the blood system to adapt its output to restore homeostasis[29]. In this process, the pool of early haematopoietic stem and multipotent progenitor cells (HSPCs) has an essential role in integrating inflammatory signals[30]. Mouse studies have indicated that blood homeostasis at steady state reflects the differential production by mostly quiescent HSCs of a small number of myeloid-biased MPP2 and MPP3 cells, as well as a large number of lymphoid-biased MPP4 cells, which contribute to both lymphoid and myeloid output[31,32]. During regeneration, mouse HSCs are induced by the inflamed bone marrow microenvironment to overproduce MPP2 and MPP3 cells through upregulation of the myeloid-priming transcription factor PU.1; in addition, MPP4 cells are reprogrammed away from lymphopoiesis and towards almost exclusive myeloid output[21,32]. A consequence of this activation is also the formation of granulocyte-macrophage progenitor (GMP) clusters in the bone marrow cavity, which drive a local burst of myeloid cell production[33]. Altogether, the remodelling of the MPP compartment and the induction of GMP clusters represent emergency myelopoiesis pathways that are transiently activated during regeneration but are continuously triggered in myeloid leukaemia[33]. Owing to this dynamic regulation between quiescent and activated states, HSCs can regulate blood cell production at steady state and in regenerative contexts, which is key to the functional maintenance of the haematopoietic system. However, persistent activation or dysregulation in this dynamic control of HSC activity can set the stage for malignant transformation.

HSC dysregulation during leukaemogenesis

Accumulating evidence from deep-sequencing studies has provided insight into the origin of LSCs in patients with AML, showing that they often emerge from an already mutated subset of pre-leukaemic HSCs upon acquisition of additional driver mutations[13–15] (FiG. 2; TABLE 1). Pre-leukaemic HSCs are present in a large proportion of patients with AML and are functionally defined by their ability to re-create a normal haematopoietic hierarchy upon

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transplantation while possessing a competitive repopulation advantage over normal HSCs[14]. On the other hand, studies of individuals without leukaemia have revealed that multilineage clones carrying somatic mutations are often present in the blood of elderly individuals (70 years of age) without obvious clinical consequence[34,35]. This finding suggests that immature haematopoietic cells, especially HSCs, gain mutations in the course of human ageing. Termed age-related clonal haematopoiesis (ARCH), this process is associated with an increased incidence of leukaemic transformation as well as with increased risk of cardiovascular disease and other forms of non-haematological morbidity and mortality[34,35] (Box 2).

Changes in the bone marrow microenvironment that accompany both leukaemia development and the ageing process (notably, an increase in inflammatory signalling from mature haematopoietic and niche cells) alter the competition between normal and mutated HSC clones, which respond differently to selective environmental pressures[36–39]. During leukaemia development, the pool of mutated HSCs becomes genetically heterogeneous, which in combination with selective environmental pressures drives the clonal evolution of pre-leukaemic HSCs[39,40] (FiG. 2). This clonal selection can lead either to expansion of the HSPC pool and overproduction of certain lineages of blood cells, in myeloproliferative neoplasms (MPNs), or to a blunting of HSC differentiation capacity and impairment of blood production, in myelodysplastic syndromes (MDSs) and bone marrow failure syndromes. Both of these pre-leukaemic states further increase the selective pressure and facilitate the acquisition of driver mutations, eventually leading to LSC emergence and full transformation to AML[41–43] (FiG. 2). Interestingly, a longitudinal study of patients with MDS and secondary AML has demonstrated that the dominant mutated HSC clone that produces the bulk of MDS cells is not necessarily the one that evolves to LSCs in secondary AML, suggesting parallel clonal evolution at the HSC level during the development of MDS and secondary AML[44]. Notably, following further transformation, LSCs can emerge from either pre-leukaemic HSCs or pre-leukaemic progenitors, such as MPPs or GMPs that acquire self-renewal properties[1,2]. In this context, a mouse study showed that targeting the self-renewal network that is aberrantly activated in pre-leukaemic *NPM1*-mutant GMPs can prevent AML development[2], highlighting the acquisition of stem cell properties in downstream progenitor cells as a mechanism of leukaemogenesis. Below we review the current knowledge on how leukaemia arises from normal haematopoiesis and discuss how dysregulation of stemness contributes to leukaemogenesis.

Genomic instability and HSC mutagenesis

The earliest mutational selection events conferring competitive fitness in both ARCH and pre-leukaemic HSCs occur predominantly in enzymes that regulate DNA methylation[34,35], one of the primary epigenetic mechanisms controlling haematopoietic differentiation[45]. Even though mutations in genes such as *TET2* or *DNMT3A* are shared between ARCH and pre-leukaemic HSCs, the rate of transformation from ARCH to leukaemia remains surprisingly low relative to the transition from pre-leukaemic HSCs to fully transformed AML[34,35]. Notably, *DNMT3A*, *TET2* or *ASXL1* mutations are often found in individuals with ARCH who have low likelihood of progressing to AML[42,43]. Furthermore, the persistence of these mutations during complete remission after induction therapy does not

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have prognostic value for relapse in patients with AML[46]. These interesting observations suggest that the mutated HSCs causing ARCH are not necessarily the same as the pre-leukaemic HSCs driving leukaemia development or relapse[41,42], emphasizing the importance of risk-based stratification of individuals with ARCH. Deep-sequencing analyses of human peripheral blood samples have markedly improved the prediction of ARCH progression to AML by identifying high-risk mutational signatures that can predict with high confidence progression to AML with a median of 6 to 10 years prior to disease onset[42,43]. An increase in red blood cell distribution width (a parameter routinely measured in blood tests) occurs before the onset of AML and might also be predictive of ARCH progression to AML several years in advance[42]. These results establish a genetically and cytologically defined pre-malignant state of AML detectable in peripheral blood that probably tracks the clonal expansion of bona fide pre-leukaemic HSCs and could be used as a biomarker of AML development.

Sequencing studies have now convincingly demonstrated that human adult HSCs accumulate mutations by cell-cycle-independent mechanisms[13–15,47], indicating that founder mutations might occur directly in long-lived quiescent HSCs. Although dormancy is essential for the maintenance and reconstitution potential of HSCs, it entails vulnerability to DNA double-strand breaks (DSBs) and the accrual of chromosomal aberrations. Both mouse and human studies have shown that, owing to the inaccessibility of cell-cycle-dependent mechanisms of DNA repair, quiescent HSCs utilize error-prone non-homologous end joining (NHEJ) for repairing DSBs[19,48], and quiescent HSCs exposed to low-dose irradiation tend to acquire mutations and chromosomal abnormalities[19]. Further studies in mice suggest that a reduction in thrombopoietin-mediated signalling can similarly unleash the error-prone alternative NHEJ pathway and mutagenic retro-element insertion, which might underlie the acquisition of insertions or deletions in irradiated HSCs[48,49]. Another study has indicated that the Fanconi anaemia pathway-mediated repair process is particularly important for maintaining mouse HSC resilience to DSBs generated through DNA crosslinking induced by endogenous or alcohol-derived aldehydes[50].

Aside from genomic rearrangements derived from misrepaired DSBs, HSCs are also subject to age-related spontaneous mutation acquisition. One study has estimated that a human HSC accumulates 14 somatic mutations by single-base substitutions per year, which contributes to the acquisition of driver mutations[47]. Indeed, the mutations frequently observed in de novo and secondary AML are often missense mutations from single-base substitutions[51]. For instance, R882H and R882C, the two most frequent mutations of *DNMT3A*, are caused by a cytosine to thymidine (C→T) transition at a CpG dinucleotide, a hallmark of age-associated mutagenesis typically resulting from replication-coupled deamination of 5-methylcytosine[52]. Of note, these *DNMT3A* R882 mutations confer anthracycline resistance to HSPCs as well as to AML cells, perhaps promoting leukaemogenesis through chemotherapy-induced mutagenesis[53]. Furthermore, the majority of *TET2* mutations are the consequences of single-base substitutions resulting in loss of function[54]. Interestingly, a mouse study suggests that the loss of *TET2* increases the rate of guanine to thymidine (G→T) transversions in addition to C→T transition in HSPCs preferentially at loci where TET2 normally binds[55]. Vitamin C treatment resto res TET2 function and normal haematopoiesis from *TET2*-deficient mouse HSCs[56,57], suggesting a potential approach to suppress

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leukaemogenesis. Altogether, these findings establish that HSCs with genetic abnormalities remain or are preferentially selected for in the HSC pool, and that such vulnerability to mutation accrual is the likely basis for the emergence over time of ARCH and pre-leukaemic HSCs (FiG. 2).

Microenvironmental changes

The bone marrow niche microenvironment in which HSCs reside is complex, both in terms of its cellular composition at the endosteum and central marrow, and the regulatory factors that control HSC biology in normal, regenerative, ageing and disease conditions[39,58]. As demonstrated in mouse models, changes in the structure of the bone marrow niche contribute to clonal expansion in leukaemia, with mutant HSCs and their progeny contributing in a feedforward manner to the establishment of a microenvironment that favours LSC over HSC maintenance in both MPNs and AML[59]. In a mouse model of chronic myeloid leukaemia (CML), a progressive remodelling of the endosteal stroma is observed, leading to changes in osteoprogenitor function and fibrosis development[58]. Other mouse models of MPNs and AML exhibit instead central-marrow remodelling, resulting in vascular dysregulations and progressive neuropathy of the HSC niche, associated with the loss of specific mesenchymal stromal cell populations[60,61]. Furthermore, age-associated alterations in β-adrenergic signals promote lymphoid or myeloid skewing in a niche-dependent manner and have been linked to age-related increases in megakaryopoiesis and myelopoiesis[62]. Such changes in the functionality of bone marrow niche cells reduce the capacity of the microenvironment to support normal haematopoiesis, while allowing clonal expansion of pre-leukaemic HSCs and LSCs[59]. Other microenvironmental alterations, notably an increase in the bioavailability of growth ligands and inflammatory cytokines, also contribute to the overt transformation of normal and pre-leukaemic HSCs[39,59]. For example, an activating mutation in β-catenin in mouse osteoblasts is sufficient to promote leukaemogenesis through hyper-stimulation of Notch ligand expression, and increased β-catenin signalling in osteoblasts has been observed in ~40% of human patients with MDS or AML[38].

Dysregulated inflammatory signalling from other niche cells has similarly been shown to promote leukaemogenesis in MPNs and AML[36,37]. Experiments in haematopoietic-specific *TET2*-knockout mice have made a particularly convincing case for the role of the inflamed environment in leukaemic transformation from a pre-leukaemic state, as the MPN that develops in this model is dependent on systemic IL-6 signalling[63,64]. Intriguingly, haematopoietic-specific *TET2* loss results in defective intestinal barrier maintenance in mice, which is sufficient to promote systemic IL-6 secretion secondary to increased bacterial permeability[64]. This observation suggests that perturbed blood and immune function secondary to clonal selection events might have non-cell-autonomous roles in enabling transformation. It also implies that direct targeting of inflammatory signalling could have important preventive or therapeutic roles by limiting the oncogenic activity of pre-existing mutations. In summary, the permissiveness of the niche microenvironment to transformation, along with increased chronic inflammatory signalling, probably has an important role in dictating the risk of leukaemic progression from ARCH or pre-leukaemic HSCs, whether by disfavouring normal HSCs, directly promoting mutagenesis or increasing the oncogenic potential of mutant clones.

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Aberrant stemness properties of LSCs

Although LSCs can emerge from different stem and progenitor populations in AML, they possess common properties that reflect dysregulated aspects of normal HSCs. Indeed, the stemness transcriptional programmes in LSCs and HSCs, which share much overlap, predict the clinical outcome in AML[65]. Enrichment of LSC signature genes is highly predictive of therapy failure in AML across mutational subtypes, supporting the existence of a conserved stemness resistance programme[65,66]. AML relapse can also originate from LSCs with pre-leukaemic HSC features or blasts with a progenitor immunophenotype, both of which maintain strong stemness transcriptional signatures and functional LSC properties upon relapse[41]. These observations have been elucidated at a single-cell level[67], paralleling early observations from bulk gene signature data and reinforcing convergence on stemness as the principal mechanism endowing AML therapy resistance. Below we describe how LSCs co-opt essential stem cell mechanisms.

Cell cycle entry.—Mathematical models supported by experimental labelling strategies have estimated that ~1% or up to 3–8% of mouse HSCs enter the cell cycle on any given day[68,69]. However, HSC cycling rates are heterogeneous, with more active HSCs cycling roughly every 28–36 days[70] and highly dormant HSCs dividing quite infrequently (estimated at every 145 days in mice and 280 days in humans)[71,72]. By contrast, the cell cycle state of LSCs varies considerably between AML subtypes[73–75]. Although several experimental observations have suggested that LSCs from patients with AML are as quiescent as normal HSCs[74,75], the redefinition of LSCs by strict transplantation assays rather than phenotypic markers (that is, $CD34^{+}CD38^{-}$ in humans) indicates that actively cycling leukaemic cells with a progenitor immunophenotype can also be LSCs[11,41]. Therefore, it is conceivable that some LSCs are normally quiescent and are transiently activated in response to environmental stress, such as in M0, M1 and M2 AML subtypes with rare quiescent LSCs and a relatively deep leukaemic hierarchy. By contrast, other LSCs can be actively cycling for example in response to cell-cycle-promoting mutations such as *MLL* rearrangement or an inflamed environment and are typically found in M4 and M5 AML subtypes that are dominated by LSCs with a progenitor immunophenotype and shallow hierarchical depth[41]. In quiescent human AML LSCs and HSCs, miR-126 dampens the Pi3K-AKT-mToRC1 pathway to attenuate cell cycle entry[76]; interestingly, knockdown of miR-126 abolishes stemness in LSCs but not in HSCs[76]. This discrepancy indicates that different mechanisms regulate self-renewal in HSCs and LSCs, such as CDK3 and its inhibitor CDKN1B/p27, which have a unique role in the cell cycle entry and differentiation of LSCs but not HSCs[76]. Another example is CD93 — a functional marker for cycling LSCs in *MLL*-rearranged human AML — which suppresses the expression of CDKN2B/p15 to promote cell cycle entry in a CDK6-dependent manner[73]. Taken together, whereas HSCs are mostly quiescent, the cell cycle status of LSCs varies, in large part depending on their cell of origin and immunophenotype. Quiescent LSCs also seem to utilize mechanisms different from those in HSCs to maintain their quiescence, and cycling LSCs rely on distinct molecular pathways to promote their self-renewing cell division.

Metabolic activation.—The tight controls on HSC metabolism that regulate the switch from glycolysis-driven quiescence to OXPHOS-mediated regeneration are often deranged in

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AML LSCs. LSCs display a unique capacity to activate mitochondrial metabolism to coordinate the regenerative response, while simultaneously perturbing differentiation to drive self-renewal and the expansion of immature blasts. Indeed, quiescent ROS-low LSCs in patients with AML exist in a metabolically activated state and depend on OXPHOS[77]. These LSCs, unlike HSCs, are unable to effectively utilize glycolysis to maintain cellular energetics and redox state when mitochondrial function is inhibited with venetoclax, a selective BCL-2 inhibitor[77]. Similarly, targeting haem biosynthesis in human AML impairs mitochondrial membrane potential and sensitizes leukaemic cells to BCL-2 inhibition[78]. Additionally, LSCs are specifically dependent on amino acid catabolism for OXPHOS, and the venetoclax-5-azacitidine therapeutic regimen, which simultaneously targets OXPHOS and amino acid transporter expression, has demonstrated marked efficacy, with extensive relapse-free survival in human AML clinical trials[79,80]. Collectively, these studies show that mitochondrial apoptotic pathways and metabolism are inextricably linked in haematopoietic malignancy. Intriguingly, in human AML that has relapsed after venetoclax-5-azacitidine treatment, LSCs are no longer reliant on amino acid metabolism, as metabolic adaptation allows fatty acid oxidation (FAO) to compensate[79,81]. Adipose tissue is a reservoir of normal HSCs, and in mouse models and human myeloid malignancy this niche is exploited to increase adipose lipolysis and support a subpopulation of FAO-metabolizing CD36^{+} LSCs, which in turn drive disease recurrence[81]. Both mouse and human HSCs critically depend on both cysteine and the branched-chain amino acid (BCAA) valine for self-renewal and survival, and a diet deficient in valine reduces the cellularity of the mouse bone marrow sufficiently to allow HSCT and engraftment without irradiation[82]. In human MPNs, this dependency on valine is bypassed by increasing BCAA production through MSI2-dependent upregulation of the cytosolic aminotransferase BCAT1, the activity of which is normally suppressed by EZH2 (ReFS[83,84]).

Metabolic adaptations in leukaemia can extend beyond LSCs and blasts and can even include alterations to the systemic physiology of the host. Indeed, increased insulin insensitivity and decreased insulin secretion in response to leukaemia-secreted factors increases systemic glucose levels and fuels malignant growth in mouse models and human myeloid leukaemia[85]. Collectively, these studies show that LSCs adapt their metabolism in order to allow for metabolic plasticity while uncoupling the role of activated metabolism in impairing self-renewal and driving the differentiation of normal HSCs.

Epigenetic and post-transcriptional modifications.—Mutations in the DNA methylation-regulating enzymes TET2 and DNMT3A and the disruption of HSC self-renewal programmes are common early events in leukaemogenesis. In mice, loss of either *TET2* or *DNMT3A* leads to increased HSC self-renewal and expansion of the HSPC pool[25–27], which greatly increases the risk of secondary oncogenic mutations. Pre-leukaemic HSCs with only *DNMT3A* founder mutations have been found in some patients with AML[14]. Tightly inter-regulated with DNA methylation, histone methylation and acetylation are also dysregulated in LSCs. Mouse studies have shown that the histone methyltransferase MLL (known also as KMT2A) protects *HOXA9* from methylation and silencing in LSCs, thereby promoting self-renewal and leukaemic growth[86]. *MLL* translocations are often observed in patients with AML, where they recruit the H3K79 methyltransferase DOT1L

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(which protects *MLL*-fusion target genes from SIRT1-mediated epigenetic silencing) and further promote a leukaemic state[86,87]. The polycomb repressive complex 2 (PRC2), a promoter of H3K27 methylation and gene silencing, is a negative regulator of *HOXA* gene cluster expression and is often functionally impaired in malignant haematopoiesis[88]. Mouse studies have demonstrated that ARCH-associated and pre-leukaemia-associated loss-of-function mutations in the PRC2 members ASXL1 and EZH2 enable the development of MDSs, MPNs and AML[88,89]. In contrast to the mainly tumour-suppressive role of PRC2 in leukaemia, the lysine-specific histone demethylase LSD1 (also known as KDM1A) is highly expressed in certain patients with AML, where it promotes histone demethylation and is a key effector of differentiation blockade in *MLL*-rearranged LSCs[90]. Although epigenetic dysregulation provides a selective advantage to LSCs, each epigenetic enzyme has context-dependent functions with both tumour-suppressive and oncogenic properties[89]. Thus, leukaemic mutations in epigenetic regulators will have specific synthetic lethalities, and efforts to identify and target these vulnerabilities for anti-LSC therapies are currently ongoing[91].

Post-transcriptional gene regulation, including RNA methylation and splicing, is also dysregulated in leukaemia. N^6-methyladenosine mRNA methylation, catalysed by METTL3, regulates transcriptional stability and translational efficiency, and METTL3 overexpression inhibits cell differentiation from LSCs in AML mouse models[92,93]. Mutations in the RNA-splicing machinery are common in ARCH HSCs, pre-leukaemic HSCs and AML, alongside mutations in DNA methylation enzymes[42,43]. Concurrent mutations of IDH2 and the splicing factor SRFS2 cooperatively drive the development of lethal MDS in mice[94]. The pro-tumorigenic effects of mutant SF3B1 in human MDS and in other tumours converge on aberrant splicing and mRNA degradation of the non-canonical BAF chromatin-remodelling complex member BRD9 (ReF.[95]). The emerging model suggests that dysregulation of the HSC post-transcriptional machinery contributes to leukaemogenesis, and that mutations in epigenetic regulators and spliceosome machinery components cooperate in the progression from ARCH to a pre-leukaemic state.

Pro-survival signals.—The intrinsic capacity for survival in the face of extreme insults is a hallmark of stem cells[96], and both HSCs and LSCs employ strategies to manage stress and suppress cell death. The Foxo transcription factors protect mouse HSCs from oxidative stress and maintain metabolic quiescence[97]. FOXO3A, in particular, is critical to mouse HSC survival in the context of cytokine deprivation, by directing a protective autophagy programme[98]. Both mouse and human HSCs are hardwired to resist apoptosis through high expression of MCL-1 and other pro-survival BCL-2 family members[19,99,100]. Furthermore, inflammatory cytokines that instruct the death and clearance of many progenitors and mature blood cells often promote HSC survival. For example, in mice, type I interferon depletes bone marrow cells while transiently driving HSC proliferation, and it also allows HSCs to re-enter quiescence for protection against p53-mediated apoptosis[20]. TNF, which drives the clearance of mature cells and their lineage-committed progenitors in a dose-dependent manner, promotes mouse HSC survival through upregulation of the NF-κB-p65-ciAP2 axis that primarily prevents necroptosis[101]. Thus, the privileged survival of HSCs is the default

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setting of normal haematopoiesis and is regulated through redundant mechanisms that limit stress and suppress programmed cell death mechanisms.

LSCs often highjack these HSC-specific survival mechanisms. For instance, LSCs in human AML have been shown to co-opt an AMP-dependent kinase (AMPK)-mitochondrial fission 1 (FIS1)-mediated mitophagy pathway as a means to maintain stem cell properties that might otherwise be compromised by the stress of oncogenic transformation[102]. Moreover, most human AML LSCs rely more on the overexpression of BCL-2 for their survival than do normal HSCs[77]; thus, BCL-2 family inhibitors have become an important chemotherapeutic agent in the treatment of patients with AML[103]. MCL-1 is also upregulated by oncogenic FLT3 internal tandem duplications (FLT3-iTD), inhibiting apoptosis and promoting the survival of LSCs in human FLT3-ITD AML[104]. In addition, the inhibition of RIPK3, a necroptosis mediator and inflammasome activator, is observed in human FLT3-ITD AML, likewise contributing to LSC survival[105]. Appropriation of these enhanced survival characteristics by LSCs presents a considerable clinical challenge, given their importance for HSC maintenance. Therefore, further elucidation of how LSC survival is aberrantly promoted, and especially of the types of programmed cell death mechanisms that can be activated in LSCs while sparing normal HSCs, will be important.

Immune evasion.—HSCs are protected from recognition and elimination by the immune system[106,107]. The presence of regulatory T cells (T_{regs}) in the bone marrow creates an immune-privileged microenvironment and directly regulates HSC quiescence in mice[108,109]. Indeed, the persistence of transplanted allogenic HSPCs in non-irradiated mice occurs in a T_{reg}-dependent manner, demonstrating the role of T_{regs} in suppressing immune-mediated elimination of HSCs[108]. In response to inflammatory signals, such as TNF exposure, mouse HSCs upregulate the inhibitory immune checkpoint molecules PDL1 and PDL2, allowing for direct suppression of T cell responses by HSCs[101]. When mouse or human HSCs leave the immune-privileged bone marrow niche environment, they upregulate CD47, which is a 'don't eat me' signal that inhibits phagocytosis by cells of the innate immune system and protects HSCs as they travel through the body[110]. In human AML, the constitutive upregulation of CD47 allows malignant cells to evade phagocytosis and correlates with a worse prognosis[111]. Downregulation of the human leukocyte antigen (HLA) and upregulation of inhibitory immune checkpoint molecules in AML blasts are also implicated in tumour evasion of anti-leukaemic T cells and in relapse after allogenic HSCT[112]. Additionally, human AML LSCs escape natural killer cell-mediated immunosurveillance by downregulating the expression of the NKG2D ligand via the upregulation of poly-ADP-ribose polymerase 1 (PARP1)[113]. Thus, by residing in the immune-privileged bone marrow niche and adopting the immune-evasive mechanisms that HSCs utilize under stress conditions, LSCs are able to avoid detection and elimination by the immune system.

Emerging clinical approaches

AML is increasingly recognized as not only a genetic, but also an epigenetic, metabolic and niche-dependent disease, and focusing on these multiple vulnerabilities might lead to approaches that effectively target LSCs and delay relapse12 (FiG. 3). Although selection of the appropriate combination of therapeutic agents is critical for LSC targeting, equally

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important is the time at which LSC-directed therapy is incorporated into treatment regimens for patients with AML. The frequency of LSCs has been estimated to increase 10-fold to 100-fold in patients whose disease relapses after conventional chemotherapy114. This expansion is accompanied by an increase in adaptation and genetic heterogeneity114, which both emphasize the need to target LSCs early in therapy in order to pre-empt such evolution. AML relapse can also originate from pre-leukaemic HSC clones, and effective LSC-directed therapeutic strategies must also inhibit LSC re-emergence[53,115,116]. However, defining the activity of LSCs and their response to therapeutic treatment in humans remains challenging, and although many treatments that effectively eliminate LSCs in preclinical mouse models are showing therapeutic benefits in clinical trials, it is often difficult to correlate their efficacy with direct LSC elimination. In addition, the inherent limitations of xenotransplantation and its low threshold (like any other functional assay with human cells) for detecting LSCs are increasingly being appreciated, along with the recognition that a lack of leukaemia-initiating cells might not necessarily equate with a lack of LSC activity[117]. Here we emphasize the importance of developing therapeutic approaches that target common stemness mechanisms as a method to prevent relapse. In this section, we highlight novel agents and combination regimens that are showing promise for extending the progression-free and overall survival of patients with AML by driving deep remission that effectively targets the LSC fraction (TABLE 2). We selectively highlight agents that might derive their efficacy in part from targeting the mechanisms of LSC persistence described above, related to cell cycle control, metabolic and epigenetic remodelling, cell survival and immune evasion. We also caution that the clinical literature primarily establishes correlations rather than mechanistic insight into the underlying nature of the therapeutic benefit (or lack thereof) for the highlighted agents. In reviewing these new therapeutic options for the treatment of AML, we echo prior suggestions that effective targeting of the LSC population and the elimination of measurable residual disease will be key to imparting durable remission and changing present clinical realities[12].

Targeting regulators of LSC quiescence

As with most cancers, direct targeting of cell cycle and self-renewal activity has received much attention in therapeutic development for AML, although substantial survival benefits have yet to emerge (TABLE 2). For example, the FLT3 receptor tyrosine kinase stimulates cell cycle entry, and its inhibitor gilteritinib delivers a median overall survival benefit of 3.7 months in relapsed or refractory *FLT3*-mutant AML, as both primary and secondary acquired resistance remain challenges[118]. However, given that innate immune stress responses are activated via the IL-1 receptor-associated kinase 1 (IRAK1) and IRAK4 complex pathways following treatment of *FLT3*-mutant AML with FLT3 inhibitors, treatment with a dual FLT3-IRAK1-and-IRAK4 inhibitor might eliminate this adaptive resistance mechanism and promote a durable response, as has been shown in mouse xenograft models[119]. Other new targets on the horizon include inhibiting CDK6, which is critical in regulating HSC and LSC entry into the cell cycle and has had clinical success in other cancers[120,121]. Targeting AML cell proliferation using the CDK4 and CDK6 inhibitor palbociclib has shown efficacy in preclinical models[122]. In human AML with RUNX1-ETO translocations, leukaemic cells upregulate cyclin D2, making them highly sensitive to CDK4

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and CDK6 inhibition[123]. CDK6 also controls the expression of FLT3, and CDK6 inhibition suppresses oncogenic signalling in human *FLT3*-ITD AML[124].

Although inhibiting the LSC cell cycle has substantial potential to manage disease and improve outcomes, ultimately these approaches are not curative, as they enforce LSC quiescence and thereby maintain a latent pool of LSCs to drive recurrence. Conversely, approaches that can drive LSCs out of quiescence have a greater potential to eradicate measurable residual disease. Targeting promyelocytic leukaemia protein (PML) with arsenic trioxide abrogates quiescence and increases the sensitivity of human acute promyelocytic leukaemia (APL or M3 AML subset) LSCs to cytotoxic agents[125]. Mouse and human AML LSCs are dependent on EZH1 and EZH2 to maintain quiescence; thus, dual inhibition of these epigenetic modifiers forces LSCs into the cell cycle, sensitizes them to chemotherapy and depletes their numbers[126]. Similarly, the potential of autophagy inhibitors to target LSC quiescence has been demonstrated in a CML mouse model[127].

Metabolic targeting of LSC survival

Although LSCs share many metabolic parallels with HSCs, distinct aspects of metabolism — including an increased reliance on mitochondrial metabolism and non-glucose energy sources — are being exploited for therapeutic benefit in AML. The oncometabolite 2HG, which is produced by mutant IDH1 and IDH2 (Box 1), suppresses TET2 function and maintains LSC identity[24]. Ivosidenib and enasidenib, two inhibitors of mutant IDH1 and IDH2 that are approved for AML treatment, are potentially effective in relapsed or refractory AML by restoring TET2 function and promoting LSC differentiation[128,129]. Moreover, BCAT1 maintains LSC identity in human AML, at least partly by depleting αKG, thereby phenocopying loss of TET2 or IDH mutant suppression of TET activity[130]. The convergence on αKG levels and TET2 activity is instructive with respect to the need to personalize therapeutic strategies that target essential leukaemic pathways.

Early evidence using the antimicrobial tigecycline suggests that the increased reliance of AML LSCs on oxidative metabolism can be exploited therapeutically[131]. Treatment with venetoclax-5-azacitidine has demonstrated durable remission in de novo human AML, highlighting the success of selectively targeting OXPHOS fuelled by amino acid metabolism in LSCs[79,80]. However, resistance to this combination therapy occurs, owing to the upregulation of FAO metabolism in order to rescue OXPHOS in LSCs[79]. These findings emphasize the metabolic plasticity of LSCs with regard to the substrates that can fuel mitochondrial metabolism, and they support the investigation of dual metabolic-pathway-targeting strategies. Potential candidates include carnitine palmitoyl transferase 1a (CPT1a; the rate-limiting catalytic enzyme in the FAO pathway[132]), the fatty acid transporter CD36 or the fatty acid binding protein 4 (FABP4), the inhibition of which is cytotoxic in primary human AML cells as well as in preclinical xenotransplantation models[133]. These different metabolic therapeutic strategies, especially with the appropriate timing or combinations, might target the metabolic reliance of LSCs on enhanced oxidative metabolism while preventing metabolic adaptation.

The virtually ubiquitous cures following the use of all-*trans*-retinoic acid (ATRA) with arsenic trioxide observed in APL[125] also give hope that other diff eren tia tionpromoting

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therapies with broader utility might be discovered. Notably, the inhibition of dihydroorotate dehydrogenase (DHODH), an enzyme of the de novo pyrimidine biosynthesis pathway, induces differentiation of LSCs in mouse *MLL*-rearranged AML, thus sensitizing leukaemic cells to chemotherapy[134]. These agents are currently in phase I–II clinical trials for AML (TABLE 2). This differentiation-promoting effect seems to be related to the inhibition of uridine and uridine-derivative bioavailability, as well as to a global decrease in protein-N-acetylglycosylation and its implications for post-translational modifications of AKT, the TET family of proteins and c-MYC in LSCs[134].

Epigenetic targets

The fact that most mutations in epigenetic modification enzymes are loss-of-function mutations has limited the development of therapeutic options. However, broad epigenetic modulation approaches using metabolism-targeting agents that affect the activity of epigenetic enzyme cofactors are starting to show efficacy. The DNA hypomethylation agents 5-azacitidine and decitabine achieve clinical responses in a subset of patients with AML, although the basis for their ability to treat AML or MDS is still largely unknown, and they are not associated with a clear survival advantage as monotherapies[116,135]. However, the remarkable efficacy of hypomethylating agents in *TP53*-mutant AML[116] should be noted, especially as these compounds have become the backbone of AML combination therapy trials with agents such as venetoclax and immune checkpoint blockade (TABLE 2). Histone-modifying enzymes have also become targets of active drug development (TABLE 2). Preclinical studies with human AML cells demonstrate that LSD1 inhibitors efficiently induce LSC differentiation but spare normal myelopoiesis, both by re-commissioning the PU.1 and C/EBPα enhancers[136] and by disrupting negative regulatory interactions with the transcription factor GFI1B[137]. Additional evidence suggests that the use of LSD1 inhibitors in combination with ATRA might unlock ATRA efficacy in non-APL AML, by providing access to retinoic acid receptor α (RARα)-driven transcriptional programmes[138]. Inhibition of the H3K79 methyltransferase DOT1L (another purported enzymatic regulator of the LSC-transformed state, particularly in *MLL*-rearranged and *DNMT3A*-mutant AML) is another approach that has shown some promise[139,140]. With several additional epigenetic targets being tested in clinical trials (TABLE 2), the emerging focus of these approaches is on restoring LSC differentiation and targeting the downstream survival, metabolic and immunogenic dependencies that result from a perturbed epigenetic leukaemic state.

Immunotherapeutic approaches

Allogeneic HSCT, the current therapeutic option with the most potent anti-leukaemic effect, is predominantly an immunotherapeutic approach, as its curative potential is predicated on the graft-versus-leukaemia effect of allogeneic T cells on measurable residual disease. However, the toxicity of the current conditioning regimens often precludes their utilization in the elderly or in patients with comorbidities. Emerging non-myeloablative conditioning regimens, such as those using antibody-drug conjugates that target CD117 (c-KIT)[141], are effective in preclinical models and might help redefine the eligibility calculus for transplantation. Whether or not alternative immunotherapeutic strategies can similarly harness anti-leukaemic immune responses remains to be determined, but represents an active area of drug development. To this end, antibody-drug conjugates, bispecific T cell engagers

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(BITEs) and chimeric antigen receptor (CAR)-T cells targeting leukaemia antigens are in development and might become clinically relevant in the near future (TABLE 2).

Cell surface antigens with differential abundances in leukaemic and normal cells offer latch points for immunotherapy. CD33 is a transmembrane receptor normally expressed by cells of myeloid origin, but not in HSCs, and with widespread expression in AML blasts[142]. Gemtuzumab ozogamicin, an antibody-drug conjugate that recognizes CD33, was the first CD33-targeted therapy approved for AML; however, despite evidence supporting improved event-free survival, on-target and off-target toxicities (including myelosuppression and liver toxicity) have limited its therapeutic index[143]. Another approach with an unconjugated anti-CD33 antibody, lintuzumab, was less toxic to the liver but did not improve survival in patients with relapsed or refractory AML[144]. New approaches combining CD33-directed CAR-T cell therapy and allogeneic transplantation of gene-edited CD33-null bone marrow HSCs, which might selectively eliminate leukaemic cells while preserving the host immune system, are now emerging[145]. However, it should be noted that liver toxicity might result at least in part from an on-target effect on CD33-expressing hepatocytes, and it remains to be evaluated whether CD33 CAR-T cell therapy will be similarly problematic. Additional immunophenotypic targets overexpressed in LSCs with active clinical development include CD123 (ReF.[146]), CD47 (ReF.[147]) and IL-1 receptor accessory protein (IL1RAP)[148] (TABLE 2).

In contrast to the success of checkpoint blockade in solid tumours, AML has not proven to be sufficiently immunogenic to benefit from this therapy[149]. However, certain combination therapies might elicit a strong anti-AML T cell response and eventually unlock the long-lasting remissions associated with immune checkpoint inhibitor treatment (TABLE 2). In particular, 5-azacitidine activates broad anti-tumour immune signalling pathways, increases the expression of HLA class-1 antigens and leads to PD1 upregulation on T cells, which might underlie early signals of overall survival benefit in responders to 5-azacitidine and anti-PD1 combination therapy[150]. Early clinical evidence also suggests that CTLA4 therapy might prove clinically useful in the post-allogeneic HSCT setting, in which the anti-tumour immune response threshold is lowered[151]. In fact, results from a trial of personalized dendritic cell-based vaccines have provided additional evidence that antigen-specific T cell responses can be successfully generated in order to impart long-term disease control[152]. Moreover, a phase I–II clinical trial with adoptive transfer of T cell receptor (TCR)-transgenic T cells targeting WT-1 achieved 100% relapse-free survival at a median of 44 months post-therapy when combined with allogenic HSCT[153], compared with 54% relapse-free survival in similar-risk post-transplant AML (without CAR-T cell treatment) over the same time period, suggesting that antigen-specific adoptive T cell transfer might further promote the elimination of measurable residual disease. This proof of principle offers hope that other shared high-affinity antigens can be identified for TCR targeting. Additional preclinical data for allogeneic natural killer cell therapy is similarly encouraging in this respect and reinforces the potential for immunotherapy in AML (TABLE 2).

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Conclusions

LSCs have long been thought to be the ultimate target for eradicating leukaemia and have been studied intensively. Even though many functional properties seem to overlap between LSCs and HSCs, the misappropriation of a large array of regulatory mechanisms by LSCs represents a clear vulnerability to target these transformed cells for elimination while sparing normal HSCs. Clinical studies have revealed the existence of ARCH in the elderly and the presence of competing pre-leukaemic HSC clones that carry epigenetic driver mutations with an increased propensity for leukaemic transformation. These observations have raised the important question of whether eradicating LSCs will be sufficient to cure patients with leukaemia and whether patients with ARCH or pre-leukaemic HSCs would benefit from earlier therapeutic interventions. Thus, individuals prospectively identified as being at high risk of developing AML might benefit from preventive targeted epigenetic therapy in the deterministic but still pre-symptomatic phase of the disease. Ultimately, the risk:benefit ratio of any preventive therapy will have to be evaluated in clinical trials that stratify individuals on the basis of mutational and cytogenetic risk signatures. However, the identification of a deterministic molecular signature of ARCH progression to AML with a long latency period offers paradigm-shifting opportunities for understanding leukaemogenesis and identifying vulnerabilities to target. Exploiting this critical opportunity to change the present realities of AML treatment will require an in-depth understanding of HSC biology and its perturbation in pre-leukaemic HSCs and LSCs. As multiple layers of HSC dysregulation contribute to leukaemogenesis, studies of how these aberrations crosstalk and synergize with each other are warranted in order to better strategize treatment options. Many specific vulnerabilities of LSCs have now been identified and can be targeted, whether by focusing on their metabolic dependencies, epigenetic state and differentiation potential, immunophenotype, or immunogenicity. Predicting and pre-empting resistance mechanisms with rational therapeutic sequencing and combinations should therefore promote durable remission in AML, with the potential to open the door to additional curative treatment options for more patients.

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Glossary

Haematopoietic stem cells (HSCs)

Blood-forming stem cells that possess self-renewal and multilineage differentiation capacity, giving rise to all types of blood cells, including granulocytes, monocytes, dendritic cells, erythrocytes, platelets and lymphocytes

Acute myeloid leukaemia (AML)

Malignant disease with rapid onset in which haematopoietic progenitors are arrested in an early stage of myeloid differentiation

Bone marrow

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The soft, spongy tissue that fills the inner cavities of most bones; in adult mammals, it is the place where haematopoiesis occurs and where most of the blood cells are produced

Peripheral blood

Fluid (plasma) and blood cells circulating throughout the body; it contains white blood cells (leukocytes), red blood cells (erythrocytes) and platelets

HSC transplantation (HSCT)

Transplantation of HSCs from donors matched in human leukocyte antigen to the recipient; HSCs are usually derived from bone marrow, peripheral blood or umbilical cord blood. The therapeutic effect derives from myeloablative conditioning and the graft-versus-tumour effect of the donor immune system

Measurable residual disease

A small number of leukaemic cells that remain in the body during or after treatment; this is a major cause of leukaemia relapse and is typically detected by PCR or flow cytometry

Leukaemic stem cells (LSCs)

Subset of leukaemic cells that share many features with HSCs, including self-renewal capacity and resistance to anti-cancer drugs, thereby considered as a major contributor to leukaemia development and relapse

Multipotent progenitors (MPPs)

early haematopoietic progenitors generated by HSCs that can differentiate into all types of blood cells, but that do not have self-renewal capacity

G_0 phase

An inactive, non-proliferative state of the cell cycle from which HSCs can reversibly exit to enter the cell cycle in response to physiological stimuli (also referred to as quiescence)

Reactive oxygen species (ROS)

oxygen-containing compounds that easily react with other molecules. They are generated by many biological processes, including mitochondrial oxidative phosphorylation, and can damage cellular components such as DNA, RNA, proteins and lipids. RoS can also act as signalling molecules

Oxidative phosphorylation (OXPHOS)

Process by which ATP, a usable form of energy in a cell, is produced through the phosphorylation of ADP as a result of the oxygen-driven electron transfer chain in mitochondria

Granulocyte-macrophage progenitor (GMP)

A committed myeloid progenitor, generated by HSCs, that can only differentiate to the granulocytic and monocytic lineages

Pre-leukaemic HSCs

Subset of HSCs that are mutated but still capable of differentiating to all lineages of blood cells. They can be found in patients with leukaemia at diagnosis and in clinical remission

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Age-related clonal haematopoiesis (ARCH)

Asymptomatic, age-associated clonal expansion of blood cells derived from mutated HSCs, associated with increases in the risk of haematological malignancy, cardiovascular disease and all-cause mortality

Myeloproliferative neoplasms (MPNs)

Clonal haematopoietic disorders characterized by the overproduction of mature cells. They include chronic myeloid leukaemia, polycythemia vera, essential thrombocythemia and primary myelofibrosis. Sometimes these disorders transform to AML

Myelodysplastic syndromes (MDSs)

Haematological disorders characterized by cytopenia in one or more blood lineages, production of abnormal blood cells and destruction of the bone marrow niche microenvironment. Sometimes these also transform to AML

TET2

Gene encoding the enzyme that converts 5-methylcytosine (5-mC) into 5-hydroxymethylcytosine in an α-ketoglutarate (αKG) and Fe^{2+}-dependent manner. essential for DNA demethylation

DNMT3A

Gene encoding the enzyme that promotes DNA methylation by catalysing the transfer of methyl groups from *S*-adenosylmethionine to specific CpG sites of DNA, resulting in the conversion of cytosine into 5-mC

DNA double-strand breaks (DSBs)

Severe form of DNA damage in which both strands of DNA are broken, typically caused by ionizing radiation and replication fork collapse. its misrepair results in chromosomal aberration

Non-homologous end joining (NHEJ)

Mutation-prone repair process in which the two DSB ends are directly ligated. Does not require sister-chromatid-l ike homology repair and can occur throughout the cell cycle, including in G_0 phase

PI3K-AKT-mTORC1 pathway

intracellular signalling pathway that is critical for metabolic activation and cell cycle entry. Regulates various cellular functions, including nutrient uptake, anabolic reactions, autophagy inhibition, cell growth and survival

BCL-2

Protein that controls cell survival by blocking the mitochondrial apoptosis pathway. Also involved in non-apoptotic processes such as improving oxPHOS and inhibiting autophagy

Venetoclax-5-azacitidine

Treatment approach combining a BCL-2 inhibitor and a DNA demethylating agent that effectively targets LSCs in AML. Such treatment might have profound implications for changing the ‘standard of care’ currently used in the clinic to treat patients with AML

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Fatty acid oxidation (FAO)

Process by which fatty acids are broken down in mitochondria by sequential oxidation in order to produce acetyl-CoA and generate ATP via electron transport chain

Branched-chain amino acid (BCAA)

one of three essential amino acids with branched-chain structures (e.g., valine, leucine and isoleucine) that cannot be synthesized de novo in the body and therefore must be obtained from food

FOXO transcription factors

Transcription factors involved in cellular metabolism and resistance to oxidative stress. Their activation can promote quiescence, autophagy induction and RoS detoxification. They are directly phosphorylated and inhibited by the Pi3K–AKT pathway

TNF

inflammatory cytokine that can induce various intracellular signalling pathways upon recognition by its receptors. TNF is also known as a death ligand that causes apoptosis and necroptosis

NF-κB-p65-cIAP2 axis

intracellular signalling pathway that promotes cell survival by ubiquitylating RiPK1 and stabilizing pro-survival TNF receptor complex i upon TNF ligation, activation of an NF-κB subunit p65 and transactivation of ciAP2

FLT3-ITD

A poor prognostic mutation common in AML that leads to constitutive activation of the FLT3 receptor tyrosine kinase

Regulatory T cells (T_{regs})

Subpopulation of T cells that act by suppressing the proliferation and cytokine production of other immune cells; they are essential for preventing autoimmunity

Human leukocyte antigen (HLA)

A major histocompatibility complex in humans, expressed on the cell surface to present antigenic peptides to the T cell receptor on T cells. it is involved in acquired immunity activation and self-versus-nonself discrimination

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Box 1 |

Metabolism and epigenetic enzymes in HSC fate decisions

Haematopoietic metabolism fuels the expansion of progenitors and mature cells and regulates the metabolically intensive epigenetic remodelling that drives haematopoietic differentiation. In this manner, the TeT2 and DNmT3A enzymes integrate metabolic activation and haematopoietic differentiation. Inactivation of these enzymes allows leukaemic stem cells (lSCs) to utilize oxidative phosphorylation (oXPHoS) without impairing self-renewal[190]. DNA demethylation by TeT2 depends on the tricarboxylic acid (TCA) cycle substrate α-ketoglutarate (αKG), which is generated by the TCA enzyme isocitrate dehydrogenase (IDH) under conditions of active oXPHoS[24]. *S*-adenosylmethionine (SAm), the cofactor necessary for the methylation by DNmT3A, is synthesized from the amino acid methionine and ATP and is similarly dependent on a metabolically active cell state[190]. IDH1 and IDH2 are often mutated in Aml, creating neomorphic enzymatic activity that generates 2-hydroxyglutarate (2HG) rather than αKG[24]. The oncometabolite 2HG inhibits TeT2 and phenocopies TeT2 mutation[24]. Additionally, the BCAT1-dependent production of branched-chain amino acids in lSCs also uses αKG as a substrate, thereby depleting the metabolite and impairing TeT2 function[130]. Thus, under normal conditions, haematopoietic stem cell (HSC) differentiation depends on the activation of oXPHoS, generation of αKG and alterations to DNA methylation by TeT2 and DmNT3A; in malignancy, this metabolic to epigenetic reprogramming is disrupted at many different levels to drive transformation and aberrant lSC function[191]. Histone methylation and acetylation are also tightly regulated in HSCs and similarly depend on metabolic cofactors, although much remains to be learned about the mechanisms by which these histone modifications instruct normal and malignant haematopoiesis. Histone acetyltransferases (HAT), including CReB-binding protein (CBP), the monocytic leukaemia zinc finger (moZ) and the HAT cofactor TRRAP, are essential for HSC self-renewal and haematopoietic maintenance and are dependent on the TCA cycle metabolite acetyl-CoA[45,190]. Histone deacetylases (HDACs) have important functions in regulating haematopoietic maintenance: the loss of class I HDACs is associated with decreased bone marrow and progenitor cellularity, and the loss of several sirtuin class III HDACs is associated with HSC ageing phenotypes[39,45]. Metabolism and epigenetic regulation are increasingly being appreciated as tightly intertwined in development and disease, representing an active area of ongoing research in the field.

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Box 2 |

Ageing HSCs and age-related clonal haematopoiesis

Approximately 10% of individuals   70 years of age experience an expansion of mutated clones of blood cells that meets the diagnostic criteria for age-related clonal haematopoiesis (ARCH)[34,35]. The mutations observed in ARCH include many mutations associated with acute myeloid leukaemia (Aml)[34,35] but are found without any associated dysplasia or cytopenia in patients, which distinguishes ARCH haematopoietic stem cells (HSCs) from the pre-leukaemic HSCs found in a myeloproliferative neoplasms (mPNs) or myelodysplastic syndromes (mDSs). ARCH has been taken as direct evidence of clonal selection of mutated HSCs in humans. So far, the same findings have not been documented in mice. Importantly, individuals with ARCH are more likely to develop leukaemia[40], and mouse models have been used to demonstrate the progression of ARCH to lethal mPN and Aml by the sequential activation of *DNMT3A* and nucleophosmin (*NPM1*) mutations[192]. Deep-sequencing techniques with high resolution of variant allele fractions have identified the presence of Aml-associated mutations in ~95% of a population of healthy 50–60 year olds[193], suggesting that these mutations occur frequently but are only selected in specific conditions. Although the development of ARCH might be merely a progression of these barely detectable clones, equally plausible is that ARCH occurs as a result of bone marrow niche degradation with age, functional declines in aged HSCs, or a bottleneck selection event that favours the relative expansion of mutated HSC clones[40]. In fact, most of the features of HSC ageing seem to result from global epigenetic changes, which are indicative of HSCs' replicative history and contribute to the declining fitness of aged HSCs, rather than from the acquisition of specific mutations[194]. In this context, ARCH might increase the competitive fitness of mutated HSCs as compared with other aged HSCs, allowing them to dominate in a remodelled and inflammatory aged bone marrow niche microenvironment. Age-associated epigenetic heterogeneity might also directly contribute to the transformation of ARCH HSCs to pre-leukaemic HSCs. evidence in support of this selection model includes studies that have demonstrated that chronic stressors (such as infection) deplete normal haematopoietic stem and multipotent progenitor cells in an interferon γ-dependent manner[195], and that elevated levels of TNF selectively favour the expansion of *TET2*-mutant HSCs[196]. Treatment with chemotherapy also provides a fitness advantage for HSC clones with mutations in DNA damage regulators such as *TP53* and *PPM1D*[197,198], and the development of clonal haematopoiesis is now well documented in patients treated for non-haematological cancers[198].

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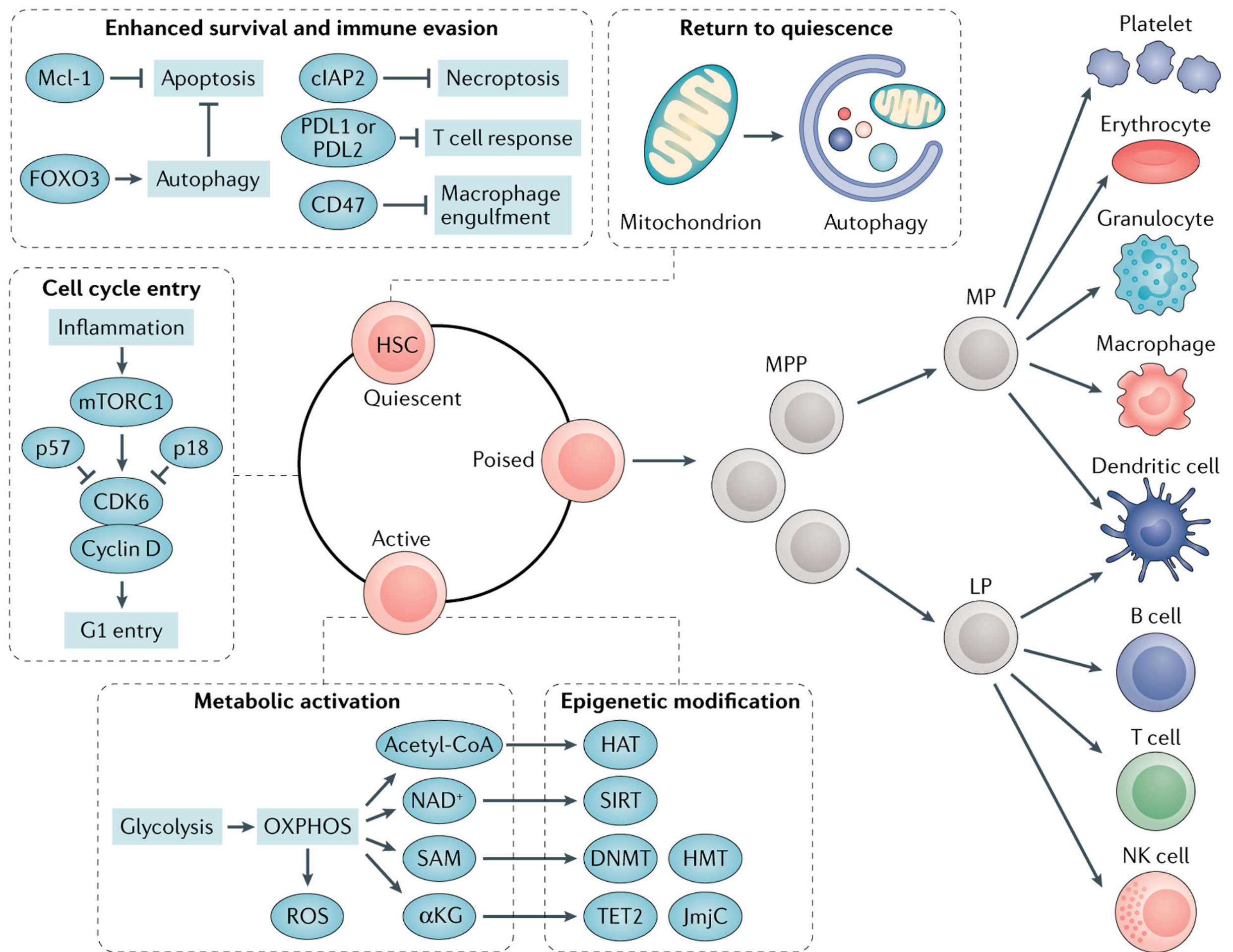

Fig. 1 |. Dynamic and coordinated regulation of HSC activity.

In the steady state, the majority of haematopoietic stem cells (HSCs) are maintained in a quiescent, G_0 state. The cell cycle of quiescent HSCs is reversibly arrested owing to limited activity of the cyclin D-CDK6 complex, which is mediated by low *CDK6* expression or high levels of endogenous CDK6 inhibitors such as p57 (encoded by *CDKN1C*) and p18 (encoded by *CDKN2C*). Once stimulated by regenerative signals, CDK6 activity is upregulated at least in part through mTORC1 activity, and HSCs enter the cell cycle. Quiescent HSCs rely on glycolysis for their metabolic needs, whereas cycling HSCs activate mitochondrial oxidative phosphorylation (OXPHOS). This metabolic switch results in increasing the amounts of tricarboxylic acid (TCA) cycle products, including acetyl-CoA, nicotinamide adenine dinucleotide (NAD^+), *S*-adenosylmethionine (SAM) and α-ketoglutarate (αKG), which are essential to key epigenetic modifiers, such as histone acetyl transferase (HAT), sirtuins (SIRT), DNA methyltransferases (DNMT), histone methyltransferases (HMT), ten–eleven translocation 2 (TET2) and jumonji C domain-containing histone lysine demethylases (JmjC). Epigenetic alteration controls HSC fate decisions, such as self-renewal versus differentiation, through modulation of key transcription factor activity. HSCs are robustly protected from programmed cell death mechanisms, such as apoptosis and necroptosis, via the upregulation of pro-survival BCL-2 genes and the TNF-NF-κB-p65-cIAP2 axis. HSCs return to the quiescent state by

deactivating their cell cycle machinery and switching their metabolism back to glycolysis, in part through autophagy-dependent mitochondrial clearance. LP, lymphoid progenitor; MP, myeloid progenitor; MPP, multipotent progenitor; NK cell, natural killer cell; ROS, reactive oxygen species.

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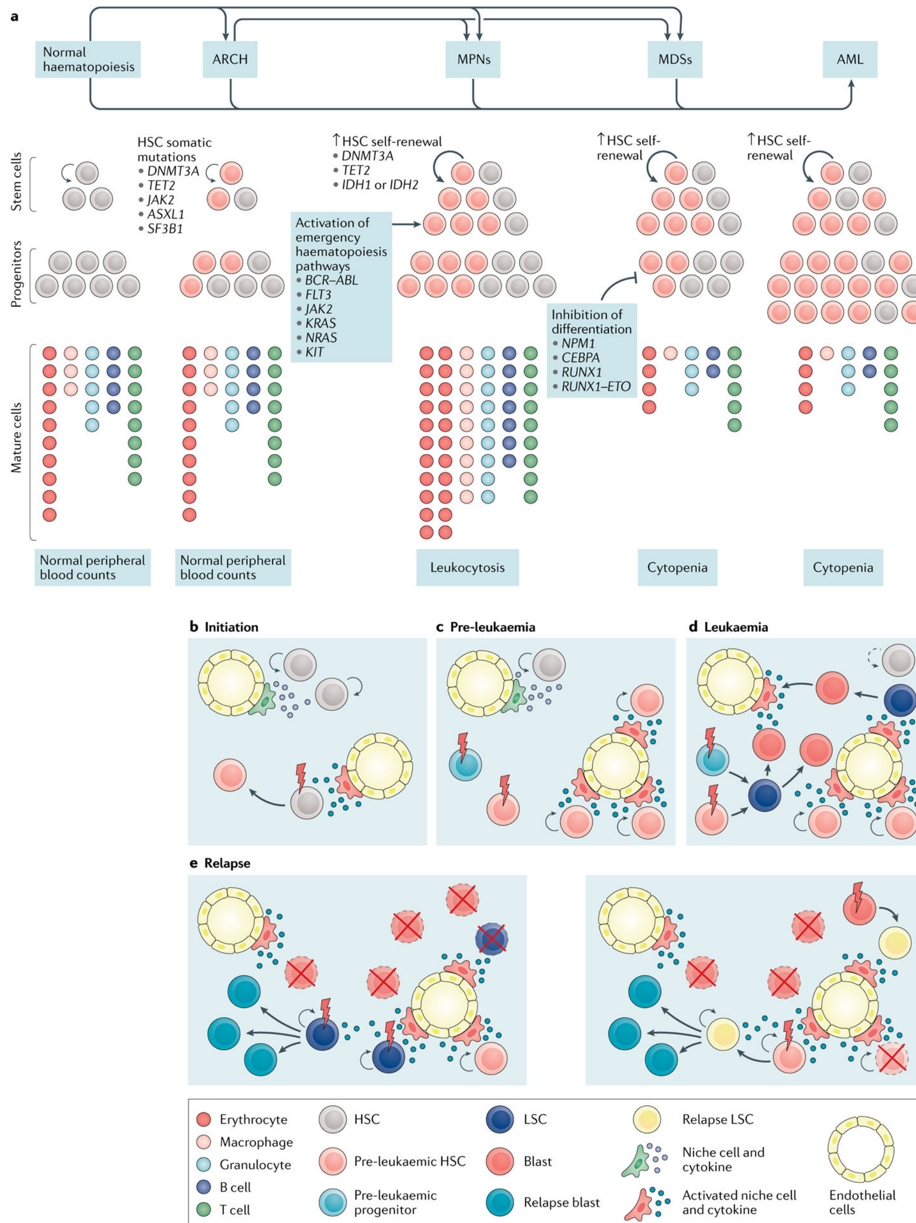

Fig. 2 |. Dysregulation of HSC properties during LSC emergence and leukaemia development.

a | Leukaemia develops through the accumulation of mutations that dysregulate haematopoietic stem cell (HSC) self-renewal, activate HSC proliferation and inhibit differentiation into progenitor cells. In age-related clonal haematopoiesis (ARCH), somatic mutations in HSCs lead to competitive fitness and cause the relative expansion of single clones, without obvious changes to the lineage output of the haematopoietic system. In myeloproliferative neoplasms (MPNs), HSCs accrue mutations that enhance self-renewal and activate emergency haematopoiesis pathways, leading to excessive production of mature cells. Separately, myelodysplastic syndromes (MDSs) arise when increased HSC self-renewal and the inhibition of progenitor differentiation cause dysplasia of the haematopoietic stem and multipotent progenitor cell (HSPC) compartment and mature cell

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cytopenia. In acute myeloid leukaemia (AML), the activation of emergency haematopoiesis pathways and the inhibition of differentiation combine to drive aggressive proliferation and expansion of leukaemic blasts. Although the interrelated malignancies can progress in a stepwise manner, MPNs, MDSs and AML can also arise de novo from normal haematopoiesis, and ARCH can probably progress directly to AML. Commonly mutated genes that drive each dysregulated process in HSPCs are listed. **b–e** | Leukaemia also hijacks the bone marrow niche microenvironment to drive leukaemic stem cell (LSC) emergence and disease evolution. **b** | Initiation phase: HSCs reside in a specialized niche that provides supportive factors for maintaining quiescence and stemness. However, quiescent HSCs are intrinsically vulnerable to mutagenesis driven by erroneous DNA repair and age-associated single-base substitutions. In this context, pre-leukaemic HSCs gain founder mutations that confer competitive fitness over normal HSCs in the changing selective pressure provided by the bone marrow niche microenvironment. **c** | Pre-leukaemic phase: mutated pre-leukaemic HSCs gradually expand in an increasingly inflammatory bone marrow milieu, which also allows for the accumulation of additional mutations, leading to the eventual transformation of pre-leukaemic HSCs into LSCs. **d** | Leukaemic phase: LSCs emerge from either pre-leukaemic HSCs or their progeny, produce leukaemic blasts and further remodel the bone marrow niche, thereby suppressing normal HSC function and haematopoiesis. **e** | Relapse phase: relapse can occur through different mechanisms. Upon treatment with conventional chemotherapeutics, leukaemic blasts but not LSCs are killed, allowing pre-existing LSCs to re-establish the leukaemic hierarchy. Alternatively, after successful eradication of LSCs, the therapy-resistant pre-leukaemic HSC clones can acquire other driver mutations and transform into new LSC clones. Leukaemic blasts, if not killed by the therapy, can also become LSCs by acquiring additional mutations conferring self-renewal.

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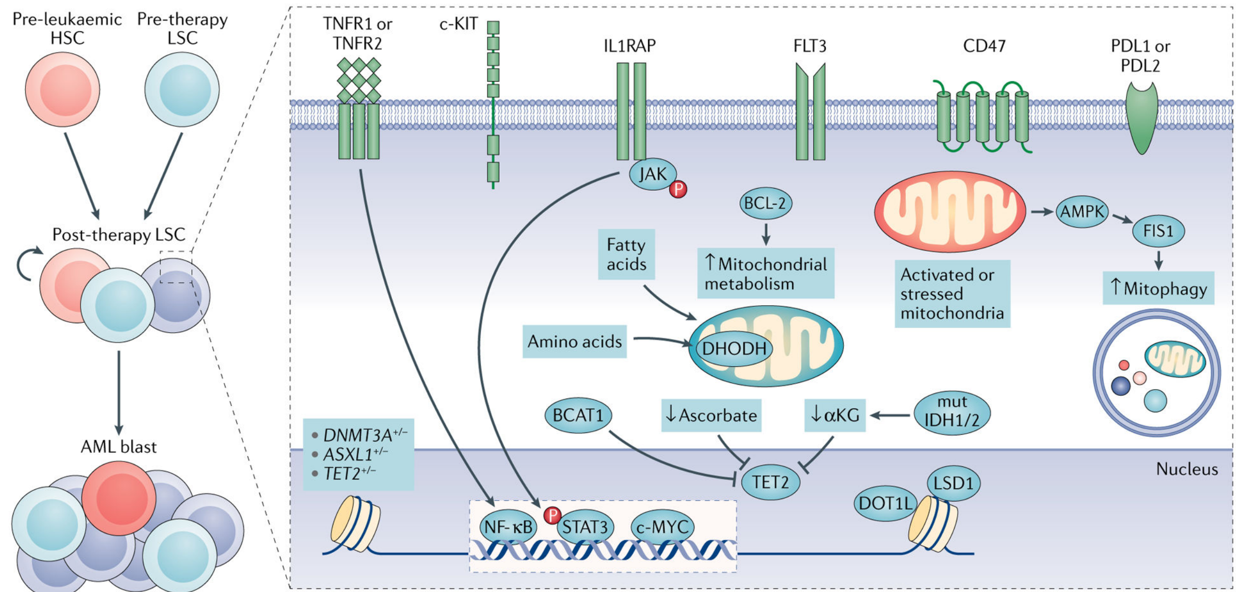

Fig. 3 |. Mechanisms of LSC resistance.

Leukaemic stem cells (LSCs) are both intrinsically genetically heterogeneous and functionally plastic, features that underlie therapeutic resistance in acute myeloid leukaemia (AML). LSCs expand 10-fold to 100-fold following initial therapy and become immunophenotypically and molecularly more heterogeneous, which makes subsequent targeting more challenging. Many of the haematopoietic stem cell (HSC)-intrinsic pro-survival and immune-evasion pathways are co-opted by LSCs. Signalling through several cell surface receptors is upregulated in LSCs compared with HSCs, including the potentiation of chronic inflammatory signalling pathways via TNF receptors (TNFRs) or IL-1 receptor accessory protein (IL1RAP), along with proliferation and survival signalling via c-KIT and FLT3. The upregulation of CD47, PDL1 and PDL2 might also have an immune-inhibitory role in LSCs. The metabolism of LSCs is also rewired, most notably by increased utilization of mitochondrial metabolism and non-glucose energy substrates such as amino acids and fatty acids. BCL-2 is upregulated in LSCs and is involved in maintaining mitochondrial oxidative phosphorylation (OXPHOS)[77]. The mitochondrial enzyme dihydroorotate dehydrogenase (DHODH), which catalyses pyrimidine synthesis and downstream anabolic synthesis, is dependent on amino acid utilization by the mitochondria and has a key role in blocking the differentiation of LSCs[134,189]. Metabolic rewiring and specific mutations in proteins such as IDH1 and IDH2 can lead to the depletion of α-ketoglutarate (αKG), restricting TET2 activity. BCAT1 upregulation and ascorbate depletion also restrict TET2 function[130]. Direct mutations in *TET2*, *DNMT3A* and *ASXL1*, alongside upregulation of LSD1 and DOT1L activity, profoundly alter the epigenetic landscape of AML stem cells and promote LSC survival and differentiation blockade. Enhanced mitophagy also has an important role in maintaining AML stem cell self-renewal[102].

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Table 1 |

Genes frequently mutated in AML and their normal function in HSCs

Gene	Frequency (%)[5]	Protein function in HSCs	Refs
NPM1	27–35	NPM1 is important for maintaining HSC quiescence and self-renewal by regulating ribosomal RNA 2′-O-methylation-dependent translation	154
DNMT3A	26	DNMT3A is critical for HSC differentiation by silencing genes involved in HSC self-renewal	25
FLT3	19–28	Signalling through FLT3 receptor activates HSCs in response to the FLT3 ligand while promoting proliferation, differentiation and progenitor expansion, particularly towards lymphoid lineages	155
ASXL1	17–19	ASXL1 is a member of the PRC2 complex that promotes HSC differentiation by repressing *HOXA* gene expression	156
IDH1/2	17–18	IDH1 and IDH2 are TCA-cycle enzymes that generate αKG and in this manner support TET2 function, DNA methylation and HSC differentiation	24
TET2	8–27	TET2 is an epigenetic-modifying enzyme that promotes HSC differentiation through its DNA demethylating activity	26
NRAS	8–9	NRAS encodes a GTPase, the signalling of which activates HSCs and promotes either self-renewal or differentiation	157
WT-1	6–7	WT-1 is an intracellular protein that normally is embryogenically restricted but is overexpressed 10–1,000 times in AML, including in LSCs; WT-1 promotes HSC quiescence and increases competitiveness in transplantation; WT-1-mutant AML has reduced levels of 5-hmC and is associated with PRC2 dysregulation	158,159
RUNX1	5–10	RUNX1 is a transcription factor involved in developmental generation of HSCs in the embryo; in adults, RUNX1 drives the differentiation of HSCs to MPPs	160
MLL	5	MLL is a histone methyltransferase that promotes HSC quiescence and competitive fitness in transplantation	161
CEBPA	4–6	CEBPA is a transcription factor critical to instructing myeloid lineage differentiation and for maintaining HSC quiescence	162
PHF6	3	PHF6 promotes HSC activation and differentiation	163
TP53	2–8	TP53 is an essential tumour suppressor that coordinates the response to DNA damage and cellular stress; TP53 has a role in promoting HSC quiescence; in AML, TP53 signalling is often repressed via overexpression of its endogenous inhibitors MDM2 and MDMX	164,165
KIT	2–4	Signalling through the tyrosine-protein kinase KIT receptor promotes HSC quiescence, survival and self-renewal in response to the SCF ligand while promoting progenitor expansion	166
KRAS	2–4	KRAS signalling drives the proliferation and expansion of HSCs	167
EZH2	2	EZH2 is part of the PRC2 complex and prevents HSC exhaustion after replicative stress	168
JAK2	1–3	JAK2 is a non-receptor tyrosine kinase that promotes HSC activation in response to inflammatory cytokines and instructs differentiation towards erythroid and myeloid lineages	169

AML, acute myeloid leukaemia; HSC, haematopoietic stem cell; MPP, multipotent progenitor; PRC2, polycomb repressor complex 2; TCA, tricarboxylic acid.

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Table 2 |

Selected therapeutics targeting LSC vulnerabilities

Target	Description	Therapy	Stage of development	Active clinical trial identifier
Quiescence and cell cycle targets				
CDK inhibitors	CDK9 is dysregulated in AML, and its inhibition downregulates cell survival genes regulated by MYC and cyclin D1 (REF.[165]). CDK1-CDK2 and CDK4-CDK6 inhibitors are also being tested for synergy with chemotherapy in refractory AML	Alvocidib (CDK9)	Phase I and Phase II	NCT02520011; NCT03441555
		AZD4573 (CDK9)	Phase I	NCT03263637
		Voruciclib (CDK9)	Phase I	NCT03547115
		Prexasertib (CDK1/2)	Phase I	NCT02649764
		Palbociclib	Phase I/II	NCT03844997
Hedgehog pathway inhibitors	Evidence exists that activation of the hedgehog pathway is important in persistence of LSCs and resistance to cytoablative therapy. The first smoothened receptor inhibitor has been approved in combination with low dose cytarabine in patients with refractory AML who cannot tolerate intensive chemotherapy. Initial monotherapy trials have failed; additional combination trials are ongoing	Glasdegib	Phase III	NCT03416179
		Sonidegib	Approved	NA
		Vismodegib	Terminated; Unknown	NCT01880437; NCT02073838
Aurora kinase inhibitors	Increased expression of this cell division regulating kinase occurs in AML[170]. Encouraging data exist for use of alisertib in upfront induction in combination with chemotherapy, with rates of remission >80% in patients with high risk disease. A modest response rate as monotherapy has been observed in advanced AML[171]	Alisertib	Phase II	NCT02560025
PI3K inhibitors	Initial trials were terminated following failure to see an objective response	PKI-587	Terminated	NCT02438761
FLT3 inhibitors	FLT3 mutations are generally observed as internal tandem duplications that lead to hyperactivity. Therapies have demonstrated decreased relapse risk and improved survival[118]. Several FLT3 inhibitors (midostaurin, sorafenib, sunitinib and crenolanib) are multi-targeted kinase inhibitors that also inhibit PDGFR (which promotes AML cell proliferation[172]) and c-KIT (which supports LSC survival[173]). Next-generation FLT3 inhibitors (quizartinib) have increased specificity and potency[174]. Anti-FLT3/CD3 bispecific T cell engaging antibodies (AMG-427) are also in development	Midostaurin	Approved	NA
		Sorafenib	Phase II	NCT01253070
		Sunitinib	Phase II	NCT01620216
		Crenolanib	Phase III	NCT02298166
		Quizartinib	Phase III	NCT02668653
		Gliteritinib	Phase II	NCT03836209
		AMG-427	Phase I	NCT03541369
mTOR inhibitors	Pilot studies of mTOR inhibitors yielded no evidence of clinical benefit[175]; no active development currently noted	Sirolimus, Everolimus, Temsiorlimus	NA	NA
PIM-kinase inhibitors	PIM kinases are oncogenic FLT3-ITD targets. Dual inhibition of FLT3 and PIM has the potential to modulate resistance to FLT3 tyrosine kinase inhibitors[176]. Results of clinical activity are still pending	SEL24/MEN1703	Phase I/II	NCT03008187
Metabolic targets				
IDH1 and IDH2 inhibitors	Mutations in *IDH* genes result in overproduction of the onco-metabolite 2HG, which mediates epigenetic changes in part through depletion of αKG and suppression of TET2 activity[24]	Ivosidenib (IDH1 mutant)	Approved	NA

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Target	Description	Therapy	Stage of development	Active clinical trial identifier
		Enasidenib (IDH2 mutant)	Approved	
BCL-2 inhibitors	BCL-2 inhibition suppresses oxidative metabolism in LSCs, in part by limiting amino acid uptake and glutathione metabolism. Durable remissions have been observed when combined with the hypomethylating agent 5-azacitidine[80]	Venetoclax	Approved	NA
DHODH inhibitors	DHODH is an enzyme in the pyrimidine synthesis pathway. Its inhibition promotes differentiation of LSCs in preclinical AML models and has encouraging responses in early settings[134]	ASLAN003	Phase II	NCT03451084
		BAY2402234	Phase I	NCT03404726
		AG-636	Phase I	NCT03834584
		PTC299	Phase I	NCT03761069
Glutaminase inhibitors	Glutaminase is important in the generation of glutathione, which fuels OXPHOS in LSCs, a key metabolic dependency[177]	Telaglenstat	Phase I	NCT03047993
Epigenetic targets				
Hypomethylating agents	Hypomethylaying agents are the backbone therapy for combination regimens in AML, both approved and in clinical development. 5-azacitidine has moderate single agent activity[178], but powerfully sensitizes LSCs to pro-differentiative, anti-proliferative, metabolic perturbing, cell-death promoting and immune agonizing therapies	Azacitidine	Approved	NA
		Decitabine	Approved	NA
		SGI-110	Phase I	NCT02293993
Histone deacetylase inhibitors	Histone deacetylase inhibitors have failed to demonstrate meaningful clinical activity as a monotherapy or in combination with azacitidine[179]	Vorinostat	Suspended	NA
		Panobinostatv	Suspended	
DOT1L inhibitors	DOT1L inhibition is selective against MLL-rearranged cells by repressing abnormal methylation of H3K79 (REF.[180]). Trials are ongoing in MLL-rearranged AML, though treatment emergent resistance is a concern[181]	EPZ-5676	Phase I/II	NCT03724084; NCT03701295
LSD1 inhibitors	The lysine-specific demethylase LSD1 is a regulator of stem cell potential in AML[90,138]. Initial development was terminated owing to an unfavourable risk:benefit profile. Additional trials in combination with 5-azacitidine and ATRA are ongoing	GSK2879552	Terminated	NCT02177812
		ORY-1001	Phase I	2018–000482-36
		Tranylcypromine	Unknown	NCT02261779
		IMG-7289	Phase I	NCT02842827
Bromodomain inhibitors	BET proteins bind acetylated histones and activate super-enhancer complexes important in oncogenic processes, including c-MYC, RAS and Hedgehog. Despite not being directly dysregulated by mutation or overexpression in AML, BET-bromodomain inhibition might be important for suppressing key transcription factors that support LSC activity[182] and enhance 5-azacitidine efficacy[183]. Some evidence of activity has been noted in initial clinical trials[184]. These agents are in dose finding studies	BI 894999	Phase I	NCT02516553
		OTX015/MK-8628	Phase I	NCT02698189
PRMT5-MEP50	PRMT5-MEP50 can post-transcriptionally suppress p53 activity in AML[185]	GSK3326595	Phase I	NCT03614728
		JNJ-64619178	Phase I	NCT03573310
Immunophenotypic cell surface antigen targets				
CD33	Multiple therapeutic modalities are used to target the LSC surface antigen CD33. Targeting of CD33 is limited by associated liver toxicity and neutropenia. Therapy in conjunction with autologous CD33 CRISPR-knockout bone marrow transplantation has been proposed[145]	Gemtuzumab ozogamicin (ADC)	Approved	NA
		SGN-CD33A (ADC)	Phase I	NCT01902329

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Target	Description	Therapy	Stage of development	Active clinical trial identifier
		AMG 330 (anti-CD33 and CD3 BITE)	Phase I	NCT02520427
		CD33 CAR-T cells	Phase I/II	NCT03971799
CD123	CD123 is an IL-3R subunit and an established LSC marker[146]	XmAb1404	Phase I	NCT02730312
		Flotetuzumab	Phase I/II	NCT02152956
		SGN-CD123A	Terminated	NCT02848248
		CD123 CAR-T cells	Phase II/III; Phase I; Phase I	NCT03631576; NCT03796390; NCT04014881
CLL-1	CLL-1 has prevalent expression in AML, but not on HSCs[186]	CLL1-CD33 cCAR	Phase I	NCT03795779
		CD123/CLL1 CAR-T	Phase II/III	NCT03631576
CD47	CD47 is an anti-phagocytic surface receptor that is proposed to regulate LSC immune escape and is associated with poor prognosis[110,111]. Initial clinical experience has demonstrated high objective response rates in MDS and AML[187]	Hu5F9-G4	Phase I/II	NCT03248479
		IBI188	Phase I	NCT03717103
		TTI-621	Phase I	NCT02663518
IL1RAP	IL1RAP potentiates oncogenic activity in AML through activation of the MyD88-IRAK-TRAF innate immune signalling pathway. IL1RAP is upregulated in pre-LSC and LSC-containing immunophenotypic populations[148]	CAR-LMC	Phase I in CML (preclinical in AML)	NCT02842320
E-selectin	E-selectin binding to CD15 on LSCs activates survival signalling and promotes chemotherapy resistance in AML. E-selectin antagonism improves MRD negative remission rates in early AML clinical trials	Uproleselan	Phase II/III	NCT03701308
Immune and cell death therapies				
Anti-PD1 combination therapies	PDL1 is frequently expressed in AML and might mediate immune evasion[112]. Initial indications suggest that anti-PD1 might have utility in combination with 5-azacitidine[150]. Anti-PD1 therapy is being tested to eliminate MRD following remission with chemotherapy	Pembrolizumab	Phase II Phase I/II	NCT02845297; NCT02996474
		Nivolumab	Phase II	NCT02275533
Anti-CTLA4 combination therapies	CTLA4 is constitutively expressed in 80% of AML at diagnosis and might mediate immune evasion[188]. Studies of anti-CTLA4, or combined checkpoint inhibition following allogeneic HSCT, are ongoing[151]	Ipilimumab	Phase I	NCT02846376
WT-1 T cell receptor gene therapy	Infusion of WT-1 antigen-specific T cells has shown promise in extending post-allogeneic transplant relapse-free and overall survival in AML[153]	WT-1-sensitized allogeneic T-lymphocytes	Phase I/II	NCT01640301
Personalized dendritic cell vaccines	These vaccines have been developed via fusion of patient-derived AML cells with autologous dendritic cells and infused into patients whose AML is in remission. Evidence from an uncontrolled study shows vaccination to be correlated with long-lived antigen-specific T cell immunity and median relapse-free survival of 57 months in 12 of 17 patients[152]. A randomized trial is now enrolling	DC/AML fusion cell vaccine	Phase II; Phase I	NCT03059485; NCT03679650
NK cell therapy	NKG2D ligand expression is specifically lost in LSC populations downstream of PARP1 activity, which is important in LSC immune escape. Transfer of polyclonal alloreactive NK cells can suppress leukaemogenesis in PDXs in combination with PARP inhibition[113]. NK cells are also being used to generate CAR therapies	Alloreactive NK cell infusion	NA	NCT03955848

Target	Description	Therapy	Stage of development	Active clinical trial identifier
PARP inhibitors	PARP inhibition sensitizes tumour cells to DNA damage induced cell death and inflammation and has been suggested to increase NK cell mediated cytotoxicity against LSCs[113]	Talozaporib	Phase I/II	NCT02878785
		Olaparib	Phase I	NCT01139970
		Veliparib	Phase I	NCT01139970
MDM2/MDMX inhibitors	In addition to being mutated, p53 can be inactivated by the endogenous inhibitors MDM2 or MDMX, which are frequently overexpressed in AML Inhibition of MDM2 or MDMX can sensitize LSCs to apoptosis and disrupt their clonogenic and serial replicating capacity[165]	ALRN-6924	Phase I	NCT03654716
		AMG-232	Phase I	NCT03041688
		Idasanutlin	Phase I/II	NCT03850535
Mutant p53 reactivation	Reactivation of mutant p53 via the use of chaperone stabilizing proteins might sensitize AML and MDS to death. Trials are ongoing in combination with 5-azacitidine	APR-246	Phase III	NCT03745716

This table selectively highlights approaches that have entered clinical trials and might derive their efficacy by targeting LSC persistence mechanisms. AML, acute myeloid leukaemia; ATRA, all-*trans*-retinoic acid; BET, bromodomain (BRD) and extra terminal; CDK, cyclin-dependent kinase; CML, chronic myeloid leukaemia; FLT3-ITD, FLT3 internal tandem duplications; HSC, haematopoietic stem cell; HSCT, haematopoietic stem cell transplantation; IL1RAP, IL-1 receptor accessory protein; LSC, leukaemic stem cell; MDS, myelodysplastic syndrome; MRD, measurable residual disease; NA, not available; NK cell, natural killer cell; OXPHOS, oxidative phosphorylation; PDX, patient-derived xenotransplant models; PI3K, phosphatidylinositol 3-kinase; PRMT5-MEP50, protein arginine methyltransferase 5-methylosome protein 50 complex.

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