The leukaemia stem cell: similarities, differences and clinical prospects in CML and AML.

David Vetrie[1,‡],G. Vignir Helgason[1], Mhairi Copland[2,‡]

[1]Wolfson Wohl Cancer Research Centre, Institute of Cancer Sciences, University of Glasgow, Glasgow, United Kingdom, [2]Paul O'Gorman Leukaemia Research Centre, Institute of Cancer Sciences, University of Glasgow, Glasgow, United Kingdom.

‡ Corresponding authors.

Email: David.Vetrie@glasgow.ac.uk

Email: Mhairi.Copland@glasgow.ac.uk

Abstract

For two decades, leukemia stem cells (LSCs) in chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) have been advanced paradigms for the cancer stem cell field. In CML, the acquisition of the fusion tyrosine kinase BCR-ABL1 in a hematopoietic stem cell (HSC) drives its transformation to become a LSC. In AML, LSCs can arise from multiple cell types through the activity of a number of oncogenic drivers and pre-leukemic events - adding further layers of context and genetic and cellular heterogeneity to AML LSCs that is not observed in most cases of CML. Furthermore, LSCs from both AML and CML can be refractory to standard-of-care therapies and persist in patients, diversify clonally, and serve as reservoirs to drive relapse, recurrence or progression to more aggressive forms. Despite these complexities, LSCs in both diseases share biological features, making them distinct from other CML or AML progenitor cells and from normal HSCs. These features may represent Achilles' heels against which novel therapies can be developed. Here, we

review many of the similarities and differences that exist between LSCs in CML and AML and examine therapeutic strategies that could be used to eradicate them.

The authors dedicate this review to our absent colleague Tessa Laurie Holyoake, whose untimely passing in 2017 cut short the life of a brilliant clinician and scientist. Her discovery of the quiescent CML LSC in 1999, her dedication to the pre-clinical study of LSCs in the years that followed and her commitment to cure CML, paved the way for many of the scientific discoveries discussed here.

[H1] Introduction

CML and AML account for approximately half of new cases of leukemia worldwide (Surveillance, Epidemiology, and End Results Program: https://seer.cancer.gov/ and Cancer Research UK: https://www.cancerresearchuk.org/). CML, the rarer of the two by incidence, is a clonal myeloproliferative disease characterised by leukocytosis and an accumulation of granulocytes and their precursors. The Philadelphia chromosome **[G]** (Ph^+)[1-3] and the constitutive expression of the fusion protein BCR-ABL1 are the unique hallmarks of CML cells. Most CML patients present in relatively benign chronic phase (CP), but if left untreated, the disease will progress to myeloid or lymphoid blast phase (BP) where it mirrors an acute leukemia[4]. Development of tyrosine kinase inhibitors (TKIs) targeting BCR-ABL1 (hereafter referred to simply as TKIs) two decades ago has revolutionised the management of CML (Figure 1). Approximately 80% of CP patients achieve excellent disease control at five years[5, 6] on TKI therapy, and this figure rises to ~90% at 10 years[7]. Of these patients, ~10% remain in therapy-free remission (TFR) upon TKI discontinuation[8, 9]. However, TKI therapy is less successful in approximately 25% of CP patient**s** due to resistance or intolerance, and this is more common in patients with BP-CML and in those CP patients who progress to BP whilst on a TKI (close to 100%)[4]. Overall, the five-year relative survival rate **[G]** now exceeds 90% and, for this reason, CML is predicted to become the most prevalent form of leukemia within 30 years[10].

At the other extreme of the clinical and molecular spectrums is AML – one of the commonest acute leukemias. It shares similarities with BP-CML including an increased frequency of blast cells, aggressiveness and poor prognosis. However, the molecular defects underlying AML are considerably more heterogeneous than in

CML, with at least 24 different genetically-defined sub-types[11, 12]. The standard treatment for AML is cytotoxic chemotherapy that impairs DNA and RNA synthesis, and consists of a backbone of 7 days of cytarabine followed by 3 days of anthracycline. This therapy dates back to the 1970s, and novel targeted therapies for AML are limited and most have only been approved very recently[13-15] (Figure 1). For those AML patients who achieve remission, the relapse rate is ~50% and the five-year overall survival rate is a dismal ~ 30%[16].

Despite these differences, both CML and AML arise from, and are maintained by, LSCs. The earliest observational evidence of LSCs in myeloid leukemia dates back to the 1960-1980s[17-20], and seminal discoveries in the 1990s provided the first empirical evidence of LSCs in patients[21, 22] (Figure 1). Subsequent work supported the long-held view that LSCs in both CML and AML are refractory to standard-of-care therapies[23-27] – a view that has been challenged more recently[28, 29]. Disappointingly, novel therapies that eradicate AML or CML LSCs have still not reached clinical practice. This begs the question – what must we still learn in order to eradicate LSCs? In this Review we examine the experimental and clinical evidence for CML and AML LSCs and highlight many of the features they share. However, there are clear differences between LSCs in both diseases, some of which challenge consensus views in the field and raise new questions.

[H1] Defining therapy-naïve LSCs

[H2] Cell surface marker definitions. The expression of CD34 and lack of CD38 ($CD34^+CD38^-$) on the cell surface is a pattern commonly associated with hematopoietic stem cells (HSCs) residing at the apex of the hematopoietic hierarchy, and was the first immunophenotype **[G]** associated with LSCs in both diseases[21, 22,

[30]. Apart from one report to the contrary[31], $CD34^+CD38^-$ cells are generally accepted as the principal immunophenotype in which CP-CML LSCs reside. However, LSCs in BP-CML reside in multiple immunophenotypes normally associated with less primitive hematopoietic cells[32, 33], as well as within the $CD34^-$ sub-fraction[34]. In this respect, BP-CML mirrors AML, in which LSCs are also found in a variety of $CD34^+$ and $CD34^-$ immunophenotypes[23, 35-37]. Analysis of transcriptional profiles and multi-lineage differentiation capacity of AML LSCs bearing immunophenotypes other than $CD34^+CD38^-$, have demonstrated that these LSCs arise from more mature cells that have acquired self-renewal capacity and are not derived from more primitive "HSC-like" cells that have gained expression of markers of more mature myeloid cells[36, 38-40]. However, it is dangerous to infer that LSC cell surface marker expression accurately reflects the expression patterns found in normal hematopoietic cells and the identification of a growing list of LSC-selective cell surface markers (Box 1) further blurs such comparisons.

[H2] Functional definitions. LSCs are defined functionally as leukemic cells that are capable of self-renewal and are thus able to exhibit sustained survival in optimised *ex vivo* co-culture systems[41, 42], and engraftment into immunocompromised mice[21, 30, 35]. Engraftment is considered the gold-standard assay defining the leukemia initiating cell (LIC) – a term used interchangeably with LSC in the AML literature[43]. Across several studies, ~50-90% of AML patient samples have been shown to engraft in various immunocompromised mice[35, 36, 44] and engraftment levels directly correlate with LSC frequency in samples and in *ex vivo* co-cultures[45], and with poor clinical outlook[46, 47]. Patient samples are also capable of serial engraftment[21, 30, 48] thus demonstrating that AML LSCs are highly competent at self-renewal and this may exceed the competency exhibited by

HSCs[48]. Engraftment in limiting dilution experiments has also provided quantitative estimates of the frequencies of AML LSCs in samples at ~0.2 to several thousand per million mononuclear cells[21, 30, 35, 49]. The caveat here is that improvements over the last decade to immunocompromised strains that express human cytokines[50] or remove the requirement for irradiation of mice[51-53] have meant that increases in the proportion of AML samples that engraft[54] and the level to which they engraft[50, 54-56] have been observed in the newer strains. Thus, how we functionally define LSCs in AML and estimate the number of LSCs found in patient samples may be subject to change as new strains continue to be developed.

Whilst we know that all CML patients have LSCs at diagnosis[22], the rigorous functional definitions used in AML have not been possible, for the most part, in CML. This is because the vast majority of CML patient samples do not engraft well in immunocompromised mice. The exception to this is in BP-CML, where the proportion of patient samples that engraft is higher than in CP-CML and often at moderate to high levels of chimerism **[G]** (although cohort sizes in these studies were small)[33, 34, 57]. For these reasons, it remains challenging to establish correlations between engraftment potential and prognosis in CP-CML or BP-CML. Interestingly, of those CP-CML samples that do engraft, chimerism rarely exceeds a few percent[57] and the mice don't develop leukemia, confirming earlier work that CP-CML LSCs have compromised self-renewal potential[42]. This challenges the view that LSCs and LICs are functional equivalents in CML and is further supported by elegant experiments using a BCR-ABL1 transgenic mouse model[58] which revealed that some LSCs are capable of engraftment only, whilst others are capable of both engraftment and leukemogenesis[59].

[H2] Cycling versus quiescence. Slow cycling or quiescence, along with self-renewal, have long been considered requirements of both CML and AML LSCs[22, 60, 61] to prevent their exhaustion and counter the effects of leukemic proliferation. There is ample evidence which demonstrates how LSC quiescence is controlled at the molecular level, for example, by growth factors[60], miRNAs[62], or pathways involving the regulators CBP/p300-interacting transactivator 2 (CITED2) and peroxisome proliferator-activated receptor γ (PPARγ)[63]. Again there are exceptions to the assumed requirement for quiescence, as some LSCs have characteristics of actively cycling cells[35, 40, 64]. To try and reconcile these differences, studies in both diseases have shown that LSCs can be reversibly quiescent[22, 48, 59, 60] and this feature can be exploited experimentally to drive quiescent LSCs into cycle [63, 65, 66]. However, based on the limited engraftment and leukemogenic capacities of CP-CML LSCs, these may have somewhat reduced cycling potential or some may even be irreversibly quiescent.

[H2] Biochemical definitions. High reactive oxygen species (ROS) levels in bulk CML and AML cells, compared to normal haematopoietic cells, are often explained through the actions of oncogenic kinases like BCR-ABL1, FLT3 or KRAS, which increase the activities of RAC GTPases,[67, 68] or the actions of of membrane-bound NADPH oxidases,[69, 70] which correlate with increased dependence of leukemic cells on mitochondrial respiration[71, 72]. However, extremes in ROS levels (too high or too low) present problems to LSCs, as these extremes can promote differentiation and impair stem cell function[69, 73]. Indeed, LSCs in CML and AML appear to be very different in how they deal with ROS to enable their survival. CP-CML LSCs, including quiescent sub-populations, have high ROS relative to either HSC or bulk CML cells[67, 74], demonstrating they can tolerate high levels of ROS and may lack efficient

strategies to reduce them. Inhibition of BCR-ABL1 with TKIs does not significantly reduce ROS[74] – suggesting that BCR-ABL1 kinase activity alone does not determine steady-state ROS levels. Furthermore, high ROS might explain why CP-CML LSCs have impaired self-renewal potential[42]. Quiescent AML LSCs, however, generally have low ROS levels compared to cycling LSCs and bulk AML cells[75, 76] and unlike CP-CML LSCs, they appear highly proficient at reducing ROS. In some situations, they may achieve this by either reverting to glycolysis[77], thus reducing dependence on mitochondrial respiration which generates ROS, or by using mitophagy to recycle damaged mitochondria[76] that are prone to premature electron leakage to oxygen, generating ROS. Neither of these features has been explored in CML, and although CML LSCs have high levels of autophagy in murine models[66], this is clearly not sufficient to maintain a low ROS environment.

Regardless of how CML and AML LSCs regulate ROS levels, both invariably fail to effectively repair the consequences of ROS – oxidative DNA damage. Such damage drives genetic evolution of LSCs, typified by the high mutation burden and genomic instability observed in BP-CML and AML[78, 79]. Even in CP-CML, evidence points to LSCs having increased error prone repair[80] that suppresses apoptosis[81], or utilization of alternative homologous recombination repair pathways[82] (all reviewed further elsewhere[83, 84]). Importantly, impaired DNA repair also provides a mechanism to explain how quiescent AML LSCs[75] acquire mutations (albeit at lower levels) even in the absence of high ROS levels. Alternatively, the high mutation burden in AML may be driven by cycling LSCs [35, 40, 64, 85] which have higher ROS as noted above and whose clonal expansion is likely to give them competitive advantages. It's also worth noting that mutations can accrue in LSCs even in the presence of TKIs[74] since neither high ROS or aberrant DNA damage repair in CP-CML are driven by BCR-

ABL1 kinase activity[67, 74, 82]. This is at odds with one study that suggested TKI-resistant LSCs have a low mutator phenotype[86], and whether LSCs in CP-CML patients on TKIs are prone to acquiring new mutations is still an unresolved issue.

[H2] Molecular definitions. Arguably the most unequivocal definitions of LSCs lie within their global molecular profiles. Studies in AML have led the way in demonstrating that, irrespective of the oncogenic driver, or the immunophenotype, LSCs display mRNA or epigenetic signatures based on small subsets of genes that are similar to, but distinguishable from, signatures found in HSCs and normal multipotent hematopoietic progenitors[35, 87-89]. The exception to this appears to be *MLL* (also known as *KMT2A*)-rearranged AML, where LSCs possess similarities to an mRNA signature from embryonic stem cells[40]. Such signatures have excellent prognostic value[35, 87-89] and have been used as drug discovery tools to identify compounds, such as antihistamines, that target AML LSCs *in vitro*[90]. These seminal studies[35, 87-89] suggest that simple 'stemness' metrics can unify diverse AML LSC sub-classes provided they are studied in combination with engraftment readouts and undertaken in reasonably large sample cohorts. Global mRNA and epigenetic profiling has also been performed on LSCs from CP-CML and BP-CML patients, but only in small sample cohorts (n=3-6)[82, 91-93]. The limited scope of these studies, plus the issues associated with engrafting CML samples in mice, has precluded using the same approaches pioneered in AML to identify predictors of clinical outcome in CML.

[H1] Origins and evolution of LSCs

[H2] Pre-leukemia. Strong evidence in AML points to the origins of LSCs in pre-leukemic cells that arise through the sequential accumulation of somatic DNA mutations in HSCs. The consequences of these 'early' mutations are enhancement

or acquisition of self-renewal potential and often impairment of differentiation[94, 95], both of which can lead to variably expanded clonal populations of pre-leukemic HSCs in patients[96, 97]. 'Late' mutations in molecules within signalling pathways, for example FLT3, promote proliferation, impose a full differentiation block and drive the development of AML[98, 99]. Mutations in genes encoding epigenetic regulators (eg., DNA methyltransferase 3A (DNMT3A), the methylcytosine dioxygenases TET1, and TET2, isocitrate dehydrogenase 1 (IDH1), IDH2, the Polycomb group protein ASXL1, and cohesin complex components)[96, 97] and *TP53* (which encodes p53)[100] are some of the commonest 'early' mutational events in AML and one or several pre-leukemic mutations are found in a majority of AML patients[101]. These mutations can, in part, be attributed to the effects of compromised DNA repair leading to clonal hematopoeisis of indeterminant potential **[G]** (CHIP). CHIP is a known risk factor for hematological malignancy and has a peak incidence in the general healthy population of ~15-20% after age 70[102, 103].

A minority (~15-20%) of CP-CML patients carry putative pre-leukemic mutations[104, 105], a frequency one could attribute to CHIP and not causal or pre-disposing to CML. Two studies have shown that approximately a third of these mutations (in ~5-7% of CP-CML cases) are antecedent to the acquisition of *BCR-ABL1* in the same clone[104, 105] and these patients may have a worse clinical outcome[105]. In a separate study, the incidence of mutations often seen as pre-leukemic events in AML was shown to increase to ~50-60% in the small proportion of CP-CML patients who progressed to BP[106]. Thus, while pre-leukemic mutations may only play a relatively minor causal or predisposing role in developing CP-CML, they are likely to be a significant risk factor in developing aggressive BP disease and AML. This is consistent with the hypothesis that CP-CML primarily arises from a

single mutation “hit” (i.e, BCR-ABL1), while BP-CML and AML require two or more “hits” – one of which may be highly dependent on mutations arising from CHIP.

[H2] Emergence of LSCs and hierarchies. The wealth of immunophenotypic, genetic and functional evidence indicates that LSCs in BP-CML and AML can originate from HSCs or from cells in later stages of the hematopoietic hierarchy[48, 89, 107, 108] (Figure 2). The situation in CP-CML, however, is very different. Since the acquisition of BCR-ABL1 may impair self-renewal, this necessitates that LSCs arise only in cells with high inherent self-renewal - such as $CD34^{+}CD38^{-}$ HSCs[107], or perhaps even more primitive hemangioblasts **[G]** [109]. However, acquisition of BCR-ABL1 alone, in one mouse model, was not sufficient to induce leukemia[110], suggesting that additional genetic variants - possibly pre-leukemic ones - may also be required. Furthermore, the identification of multiple sub-types of HSCs[111], LSCs that are non-leukemogenic[59], and individuals who never develop CML but have Ph^{+} blood cells[112], suggests that the formation of a CP-CML LSC from an HSC, and the ability of this LSC to drive leukemogenesis, is highly context-dependent. Overall, the evidence points to CP-CML LSCs emerging from one or more specific sub-types of HSC, whose unique features are not yet fully understood.

The various routes by which LSCs can arise also raise the question whether there are functional hierarchies between the different LSC populations that reside in individual CML and AML patients. At one extreme are the flat LSC hierarchies; in CP-CML, these manifest as LSCs residing within the $CD34^{+}CD38^{-}$ cell population[22] and these LSCs are most similar to HSCs immunophenotypically. However, flat hierarchies also exist in both BP-CML[33] and $CD34^{-}$ AML[37], but in these situations, there are multiple seemingly independent immunophenotypes of LSCs (and in this respect these LSCs are most divergent from HSCs) that show little or no semblance

of hierarchical organization. Between these two extremes lie the majority of AML cases, which display deeper, somewhat variable, hierarchies of LSCs. In support of this, AML LSCs originating from $CD34^{+}CD38^{-}$ HSCs often show superior engraftment and self-renewal capacity compared to those found in other immunophenotypes[30, 89, 108]. Furthermore, some AML LSC immunophenotypes within a sample can give rise to other engraftable immunophenotypes, but the converse is not always true[36]. The situations described here are undoubtedly over-simplified and do not take into account LSC-selective cell surface markers (Box 1) or the stochastic effects of epigenetic plasticity[113, 114] on a continually evolving genetic background, both of which likely result in further intra- or inter- clonal hierarchies. With this in mind, it is unlikely that any AML or CML LSC hierarchy is ever truly flat.

[H2] LSCs during therapy. Studies of the effects of standard-of-care therapies on LSCs have yielded further insights into their biology and evolution. Recent evidence from AML has shown that both quiescent and cycling AML LSCs are highly sensitive to chemotherapy, refuting that AML LSCs are refractory to treatment[28, 29]. By contrast, pre-leukemic AML cells can survive chemotherapy[97, 115, 116]. In CML, TKI treatment is ineffective at targeting LSCs in all phases of disease and a population of quiescent LSCs persists in the majority of CP patients - even in those patients in very deep remission[24-26]. A plausible explanation for this, supported by the anti-proliferative effects of TKIs on LSCs in *ex vivo* studies[117, 118], points to TKIs driving an adaptive ‘quiescence’ response in LSCs that alters their gene expression profile, enabling them to survive TKI therapy. In support of this, single cell analysis has clearly shown that CP-CML patients being treated with TKIs have persisting LSC populations enriched for quiescent transcriptional signatures[119, 120] and the proportion of these cells increases in patients on more prolonged treatment[120]. Such

adaptive effects are also observed in AML - where transcriptional signatures and metabolic properties observed in chemotherapy-resistant cells [28, 29] are distinct from 'stemness' signatures found in therapy-naïve LSCs[35, 87]. Another possibility is that CML LSCs are inherently TKI-resistant because they do not require BCR-ABL1 expression for survival[121, 122]. This may also help explain why patients undergoing TKI therapy have persisting LSCs with low BCR-ABL1 expression[86]; in other words, TKIs may select for BCR-ABL1 independent LSCs.

***[H2] LSCs and disease relapse or recurrence*.** AML clonal representation at disease relapse reflects continued LSC evolution in many patients during remission. At relapse, minor clones present from diagnosis may emerge as dominant ones, or founder clones re-emerge with new sub-clonal structures[123, 124]. Even in the rare instances of very late relapse, residual LSCs from founder clones are the usual cause[125]. In many reported cases, new mutations, particularly transversions, are present in the relapse clones[123, 124], suggesting chemotherapy itself induces DNA damage that accelerates LSC clonal diversification[106, 107]. Leukemic cells that persist after chemotherapy also have increased ROS levels[28] (Figure 2), providing another mechanism to increase mutational burden and drive further clonal diversification. Although pre-leukemic HSCs have not been shown to give rise to leukemic clones driving relapse, AML patients with a high burden of pre-leukemic HSC clones show a poor clinical outcome[95, 116]. Overall, clonal evolution preceding the onset of AML and that occurring during chemotherapy are both important contributors to driving relapse and determining prognosis.

Unlike the situation in AML, a high overall burden of pre-leukemic HSCs in CML do not worsen response to TKIs or drive disease progression, but a pre-leukemic lesion in a Ph^+ clone can[105]. Moreover, whilst chemotherapy exacerbates

DNA damage that could drive further LSC clonal evolution, TKIs do not. Yet, although most CP-CML patients on TKI therapy remain in stable remission, some patients relapse or progress to BP-CML due to the emergence of Ph^+ clones carrying BCR-ABL1 kinase domain mutations (such as T315I) or mutations in other genes linked to TKI resistance[126], [105, 106]. Emergence of new mutations during therapy most likely occur from TKI-induced selective pressure that enriches for TKI-resistant LSC clones harboring mutations that arose early in tumorigenesis (as pre-leukemic lesions, in some instances)[104, 105], a view supported by clinical observations of the mutational burden present at diagnosis[106, 127, 128]. This would suggest that the clinical outlook of many CML patients may already be pre-determined even before frontline TKI therapy begins.

One poorly understood phenomenon is why half of the CML patients who qualify for TKI discontinuation have molecular disease recurrence within 12 months, whilst the rest remain in deep remission[8, 9]. LSC clones that lack leukemia-initiating potential has been discussed as one possible explanation[59] for those patients that remain in TFR. Alternatively, the immune system or the Ph^- HSCs that reside alongside LSCs may play a role. Given the recent evidence that the presence of Ph^- HSCs can discriminate TKI responders from non-responders[120], it is plausible that these HSCs may have fitness advantages allowing them to out-compete residual LSC clones in those patients who remain in remission. Reports that TKIs may facilitate Ph^- clonal hematopoiesis in some patients[129, 130], adds further support for this view.

[H1] LSC epigenetic and metabolic axes

[H2] Epigenetic re-patterning. Numerous studies point to LSCs having unique patterns of DNA methylation[88, 131], histone modifications such as methylation of histone H3 lysine 4 (H3K4)[132], H3K27[93], and H3K79[133], and chromatin accessibility[95, 108], when compared with HSCs or leukemic blasts. Importantly, there are quantifiable levels of intercellular heterogeneity associated with these changes[95, 134]. Pre-leukemic mutations in epigenetic regulators (DNMT3A, TET1, TET2, IDH1 and IDH2)[96, 97] provide the most obvious underlying basis for epigenetic re-patterning in AML and BP-CML. However, whilst DNA methylation patterns observed in patients can be specific to pre-leukemic lesions[135, 136], variable patterns within similar genetic backgrounds can also occur[137], whilst other patterns can be primarily independent of the genetic background[88, 134]. Given these different scenarios, the re-patterning that occurs in LSCs is likely to be complex. It can be driven by co-operativity or antagonism between two or more genetic mutations[131, 136], or through changes to regulatory programmes as a result of dysregulated expression of transcription factors[88, 138, 139] and epigenetic regulators such as lysine demethylase 1A (KDM1A)[140], the histone-lysine N-methyltransferases EZH2[93, 141] and DOT1L[133], the deacetylase sirtuin 1 (SIRT1)[142], and the bromodomain and extra-terminal (BET) family protein BRD4[143] amongst others. Indeed, there is ample evidence to support dysregulated expression of epigenetic regulators as a mechanism underlying epigenetic re-patterning in CP-CML (reviewed elsewhere[144]), given that most patients lack mutations other than BCR-ABL1. Predictably, there are functional consequences arising from these epigenetic changes: for example, up-regulation of self-renewal by the HOXA (AML)[88, 145] or WNT–β-catenin (CP-CML)[146, 147] pathways, blocking differentiation by GATA2-driven transcriptional programmes (AML)[131], or up-regulation of mitochondrial metabolism (CP-CML)[148]. However, epigenetic changes

also have other functional consequences in LSCs, including repression of apoptosis[93, 144].

Like clonal genetic evolution, epigenetic re-patterning has important implications in the clinic. DNA methylation signatures can predict survival in AML[88, 108] or are biomarkers of progression to BP-CML (reviewed elsewhere[144]). Furthermore, AML patients with high levels of intercellular heterogeneity in global DNA methylation have worse clinical outcome, and further increases of this heterogeneity are evident at relapse[134]. Strikingly, epigenetic signatures in AML are more predictive of outcome than genetic background[134], suggesting that underlying epigenetic mechanisms may represent a unifying feature and a priority for drug discovery. However, this enthusiasm needs to be tempered with evidence that AML LSCs are hypo-methylated compared to AML blast cells[88], suggesting that hypo-methylating agents (eg., azacytidine) may be ineffective at eradicating them. Furthermore, whilst BET inhibitors (BETi) have shown pre-clinical evidence at targeting CML LSCs[92], evidence from AML suggests that drug resistance to BETi is driven by LSC clones with altered epigenetic landscapes,[149] which are likely to arise through selection from a background of high epigenetic intercellular heterogeneity.

[H2] Metabolic alterations. The relationships between cellular metabolism and epigenetics in normal and cancer biology are now well recognized[150]. Therefore it should come as no surprise that mutations in metabolic enzymes such as IDH1 and IDH2 impact on both epigenetic re-patterning and metabolic states in LSCs. Indeed, many pathways involved in the catabolic processes regulating glucose, amino acid and free fatty acid levels are amongst those altered in LSCs[71, 75, 151, 152]. It is likely that the interplay between these metabolic processes and epigenetic readouts is

extensive in AML and CML LSCs (Figure 3), although their precise relationships and how they differ from other AML and CML cells have not yet been fully explored.

Non-mutated IDH1 and IDH2 catalyse the conversion of isocitrate to the tricarboxylic acid (TCA) cycle intermediate alpha-ketoglutarate (α-KG) and generate NADPH. Mutant IDH catalyses NADPH-dependent reduction of α-KG to the oncometabolite 2-hydroxyglutarate (2-HG), which acts as a competitive inhibitor of α-KG-dependent dioxygenases[153, 154]. This gain-of-function activity dramatically increases 2-HG levels in leukemic patients, disrupts TET2 function and leads to DNA hyper-methylation[135] and its associated down-stream effects on gene expression, cell proliferation and differentiation[155, 156]. Indeed, small molecule inhibitors targeting mutated IDH1 and IDH2 have been developed to reverse these effects (discussed in the next section).

Whilst glycolysis in the presence of oxygen is a well-known feature of most cancer cells (the Warburg effect[157]), HSCs in the oxygen-poor bone marrow microenvironment also rely on glycolysis[158], rather than oxidative phosphorylation **[G]** (OXPHOS) to fuel energy requirements. This suggests that inhibiting glycolysis will have detrimental effects on both normal hematopoiesis and leukemia cells. Indeed, depletion of lactate dehydrogenase A (LDHA), a glycolytic enzyme that catalyses the production of lactate, compromises both HSC function and leukemogenesis in mice[159]. However, recent studies have shown that primitive AML and CML cells including LSCs have higher mitochondrial mass and an increased oxygen consumption rate compared with normal cells[71, 72], making them more reliant on mitochondrial function and OXPHOS (although exceptions to this exist in AML[77]). Indeed, inhibiting mitochondrial translation of proteins required for OXPHOS, using tigecycline, has anti-LSC effects in pre-clinical AML and CML models[71, 72] More

recently, the novel electron transport chain complex I inhibitor, IACS-010759, inhibited OXPHOS and nucleotide biosynthesis by decreasing aspartate levels in AML cells[160]. Quiescent, low-ROS AML LSCs[75, 76] also overexpress BCL-2 and are dependent on amino acid uptake and OXPHOS[75, 161, 162]. These features can be targeted using a combination of a BCL-2 inhibitor (BH3-mimetic **[G]**; venetoclax) and the DNA hypo-methylating agent azacitidine to synergistically inhibit the TCA cycle, suppress OXPHOS, and disrupt energy production[163]. Primitive AML cells have low spare mitochondrial respiration capacity that renders them sensitive to increased mitochondrial oxidative stress and cell death in situations when higher levels of OXPHOS are required[164].

Fatty acid metabolism is also emerging as a critical process in LSC survival. Expression of the lipoxygenases ALOX5 and ALOX15, enzymes involved in metabolism of polyunsaturated fatty acids, are upregulated in CML and loss of either of these causes a defect in LSC function[165, 166]. Whilst the mechanism(s) of how lipid metabolites, produced by ALOX5 and ALOX15, affects LSCs is largely unknown, it has been shown that fatty acid oxidation can be utilised by a sub-population of LSCs to enable drug-resistance in atypical niches rich in adipose tissue[151]. In these situations, fatty acid oxidation is most evident in LSCs expressing the fatty acid transporter CD36 – a feature preserved in a sub-set of LSCs in both BP-CML and AML[151]. Intracellular branched-chain amino acid (BCAA) metabolism has also been shown to be altered in CML, as elevated expression of the cytosolic form of BCAA aminotransferase 1 (BCAT1) is required for propagation of BP-CML[152]. BCAT1 inhibition promotes differentiation, and impaired leukemogenesis and self-renewal of LSCs in CML mouse models, possibly through inhibition of BCAA-mediated mTOR complex 1 (mTORC1) activation[152]. BCAT1 inhibition also impairs AML cell survival

and increased mRNA levels of *BCAT1* are also found in samples from AML patients with poor prognosis[152] - suggesting that BCAT1 also plays a role in AML.

[H1] The promise of novel therapies

***[H2] Novel CML therapies*.** Although LSCs persist in most patients on TKI therapy[24-26] and a minority of these patients will achieve TFR[167], it may be that we do not need to eliminate every LSC in patients who have minimal residual disease in order to achieve TFR - providing LSC levels can be maintained below a certain threshold in patients. This could be achieved through restoration of a healthy immune system, competition with normal HSCs, or because of functional limitations of persisting LSCs discussed above. In fact, recent evidence from a TKI discontinuation trial (DESTINY) would suggest that TKI dose de-escalation prior to discontinuing treatment may be a better option than sudden TKI discontinuation for maintaining TFR, likely via effects on residual LSCs or immunological control[168].

However, the significant clinical need to develop therapies for patients with resistance to TKIs in all phases of CML has focussed research on the development of novel kinase inhibitors that are effective in the presence of BCR-ABL1 kinase domain mutations. Ponatinib was the first TKI developed that demonstrated efficacy against the T315I mutant[169, 170]. Further novel kinase inhibitors, including PF-114[171] and the allosteric BCR-ABL1 inhibitor asciminib (ABL-001)[172] have followed and are now in clinical trials (Table 1). Data is extremely limited regarding effects on LSCs for these novel agents and whether targeting BCR-ABL1 kinase activity alone will be sufficient to eliminate them is an open question.

Numerous novel compounds that target survival factors or other molecular targets in LSCs have been identified in pre-clinical studies, a proportion of which

exhibit synthetic lethality in combination with a TKI (reviewed elsewhere[83, 144]). Some of these compounds have been, or are currently being, investigated in the clinic (Table 1) to either overcome therapy resistance or to obtain even deeper molecular responses in patients who have responded optimally to TKIs with a view to achieving TFR for more patients[8, 167]. However, not all of these compounds have been tested pre-clinically for activity against LSCs – but this warrants further investigation. Whilst pre-clinical evidence from AML suggests that a window of opportunity may exist during remission where LSCs may be vulnerable to novel agents such as dopamine receptor antagonists[29], at this time, there is no evidence to suggest that CML patients in TFR would benefit from treatment with novel agents only. The alternative is safely combining these agents with TKIs used in the CML clinic, which is currently very challenging, given the high benchmark established with TKIs for patient care. There are numerous examples of CML trials investigating novel agents in combination with TKIs that closed early; reasons for this include lack of evidence for superior efficacy, increased or unacceptable toxicity or poor recruitment. For example, in preclinical studies, the Hedgehog pathway was a promising target in CML LSCs[173, 174]. However, when Smoothened (SMO) inhibitors that block the Hedgehog pathway were combined with TKIs (BMS-833923 with dasatinib, and LDE225 with nilotinib) in CML patients, no efficacy was seen and the toxicity profile was unacceptable in preliminary data from clinical trials[175, 176]. SMO antagonists, in particular glasdegib, have shown modest success for treatment of AML in the elderly, in combination with low-dose cytarabine[177, 178]. However, no clinical studies have specifically evaluated the effect of glasdegib against AML LSCs.

Despite these caveats, some clinical agents have shown promise at reducing *BCR-ABL1* mRNA levels when combined with TKIs in CML trials (Table 1). Imatinib,

the first TKI developed to target BCR-ABL1[179], combined with pulsed granulocyte colony-stimulating factor (G-CSF) reduced the number of quiescent CML LSCs in pre-clinical studies[180]. The combination of intermittent imatinib (3 weeks out of 4), with G-CSF (3 times in the week off imatinib) or intermittent imatinib alone showed modest improvements in reducing *BCR-ABL1* levels when compared to continuous imatinib therapy[181]. Interestingly, G-CSF has been combined with conventional chemotherapy in high-risk and relapsed/refractory AML cases, but results have been conflicting[182, 183]. Interferon-α was the mainstay of CML therapy prior to the introduction of TKIs (Figure 1) and it is thought to have effects on CML survival pathways, the immune system, and the bone marrow microenvironment[184]. Initial studies hinted that pre-treatment with interferon-α may result in improved TFR rates[8]; this may be by reducing LSCs levels, inhibiting their proliferation, or an effect on the immune system or bone marrow niche, and more recent clinical trials have safely combined TKI with interferon-α[185] with further studies ongoing. In pre-clinical studies, the PPARγ agonist pioglitazone in combination with one of two different TKIs demonstrated synthetic lethality by decreasing the STAT5–HIF2α–CITED2 pathway, and reducing the quiescent LSC population[63]. In a cohort study, the combination of pioglitazone with imatinib resulted in substantially deeper remissions in patients compared to those on imatinib alone[186] and this combination is now being assessed prospectively in several clinical trials (EudraCT2009-011675-79[187], NCT02889003[188], NCT02852486[189], NCT02767063[190], NCT02730195[191]). There has been limited exploration of PPARγ as a therapeutic target in AML[192].

[H2] Novel AML therapies. Arguably the greatest differences between CML and AML LSCs are evidenced by considering how treatment pathways have evolved for the two leukemias in recent years (Figure 1). Given the mutational heterogeneity in

AML, the cellular diversity within its LSC populations and their propensity to clonally evolve, identifying effective LSC therapies, particularly for relapsed/refractory AML, is hugely challenging. Thus, whilst TKIs have been standard-of-care in CML for two decades, it is only in the last two years, that we have seen successful clinical trials and regulatory approval of targeted therapies for AML[13-15] – some of which are discussed below. However, these novel agents are unlikely to be the "magic bullet" that TKIs are for CML.

[H2] Inhibitors of pre-leukemic lesions. Given the prevalence of pre-leukemic LSC clones bearing mutations in epigenetic regulators, developing approaches which target these mutant proteins is particularly attractive. Enasidenib, the clinically approved allosteric inhibitor of mutant IDH2 (present in 15-25% AML patients), or ivosidenib, an inhibitor of mutant IDH1 (present in 5-10% AML patients), reduce the levels of the onco-metabolite 2-HG, reduce the DNA hypermethylation caused by 2-HG and promote differentiation of leukaemic blasts[193-195]. However, whether either drug affects the metabolic state or DNA methylation in LSCs is not clear. Treatment with enasidenib or ivosidenib led to remission in ~40%[196, 197] or ~30%[195], respectively, of patients with relapsed/refractory AML. However, in most enasidenib or ivosidenib responsive patients, the IDH1 and IDH2 mutant clones were not eliminated[195-198], eventually resulting in relapse. A second IDH1 mutation in *cis* to the first one, or a second IDH2 lesion in *trans* to the first one, can result in patients relapsing due to acquired resistance to ivosidenib or enasidenib respectively[199]. Other mechanisms of resistance to enasidenib include mutations in IDH1, or clonal evolution of IDH2 mutant clones that have acquired other mechanisms of resistance despite continued inhibition of mutant IDH2 in patients at relapse[198]. Given the incidence of IDH1 and IDH2 mutations in BP-CML[106], lessons learned from these

inhibitors in AML, may inform therapeutic options for a proportion of BP-CML patients with these mutations.

[H2] FLT3 inhibitors. In FMS-like tyrosine kinase-3 (FLT3)-mutated AML the picture is a little different. FLT3 is a receptor tyrosine kinase expressed on primitive hematopoietic cells that activates the MAPK and PI3K–AKT pathways[200]. The FLT3-internal tandem duplication (ITD) is present in ~30% of AML cases, leads to constitutive activation of STAT5, and correlates with poor prognosis. ATP-competitive inhibitors of FLT3, e.g. midostaurin, sorafenib, quizartinib, and gilteritinib, have shown clinical efficacy in FLT3-ITD AML as single agents, but almost all patients relapse within months (reviewed elsewhere[13, 15]) – limiting their value in the clinic as monotherapies. This may be due to the fact that FLT3 mutations occur late during AML LSC evolution, and relapse could be driven by LSC clones with wild-type FLT3 antecedent to those with FLT3-ITD mutations as some researchers have suggested[15]. Combinatorial therapeutic approaches of FLT3 inhibitors with conventional chemotherapy or hypomethylating agents are also being extensively explored in AML clinical trials[13, 15, 201, 202]. As with BCR-ABL1 in CML, point mutations in the tyrosine kinase domain of FLT3 that drive resistance have been reported[203], as well as FLT3-independent resistance mechanisms, including suppression of apoptosis[204], activation of AXL, JAK and PIM kinases by cytokines produced within the bone marrow niche[205], and autocrine production of high levels of the FLT3 ligand[206]. Whether such kinase-independent resistance mechanisms share similarities in AML and CML is not known, but studies examining such cross-over warrant consideration.

[H2] Venetoclax. Although the anti-apoptotic BCL-2 family of proteins are not mutated in AML, inhibitors of these proteins, such as BCL-2 specific venetoclax, are

promising therapies[161, 207, 208]. High levels of BCL-2 are expressed in the therapy-naïve LSC population in AML[75], and there is increasing evidence that BCL-2 inhibitors can also target LSCs in CML[209-211] with clinical trials ongoing (Table 1). Furthermore, the incorporation of recently approved venetoclax[14] into therapeutic strategies in AML is expected to improve therapeutic outcomes[207, 208]. Of particular note, venetoclax has shown synergism with hypomethylating agents and low-dose cytarabine in older patients where these combinations extend median patient survival to more than 16 months[208]. As discussed above, these combinations inhibit the metabolic dependency on OXPHOS in LSCs[163] – thus providing a strategy to target this metabolic dependency in both AML and CML.

[H1] Concluding remarks

Here we have outlined the biological features of CML and AML LSCs and novel therapeutic approaches that have the potential to target these cells in the clinic. However, to fully unlock the potential of novel LSC-directed therapies, we need a greater understanding of the targetable features of individual LSC clones, their evolution and resistance mechanisms. Recent advances in single cell analysis[120, 212], cellular bar-coding[111, 213] and further application of computational models of LSC evolution[214] will become increasingly instrumental to this end. Our knowledge of the leukemic bone marrow niche must greatly improve as many LSC vulnerabilities are, in part, niche-directed (reviewed elsewhere[215, 216]). Measuring metabolic alterations in the leukemic niche[77], and using intravital microscopy[217] to visualise its three-dimensional cellular and histological sub-structures will be critical. Immune checkpoint inhibitors[218] and monoclonal antibodies against CD33[219] or against LSC-selective cell surface markers (Box 1), may hold the key to allow more patients to achieve TFR in both AML and CML. CAR-T cell-based therapies[220, 221]

may also prove critical for improving AML and BP-CML patient survival, although their efficacy at eradicating LSC is currently not known. Given all these exciting areas to explore, we look forward to a time when eradicating LSCs is no longer a current challenge, but a mere footnote in history.

Acknowledgements

D.V. and M.C. are jointly funded by Bloodwise (Ref. 14033) and the Stand Up To Cancer campaign for Cancer Research UK (Ref. C55731/A24896). G.V.H. is funded by the Kay Kendall Research Fund (Ref. KKL1069, the Howat Foundation, and Bloodwise (Ref. 18006).

Author contributions

D.V., G.V.H. and M.C. researched the data for the article, provided substantial contributions to discussions of its content, wrote the article and reviewed and/or edited the manuscript before submission.

Competing interests

The authors declare no competing interests.

Table of contents summary

This Review discusses many of the similarities and differences between leukemia stem cells (LSCs) in chronic myeloid leukemia and acute myeloid leukemia and examines therapeutic strategies that could be used to eradicate these LSCs.

References

1. Nowell, P.C. & Hungerford, D.A. Chromosome studies in human leukemia. II. Chronic granulocytic leukemia. *J Natl Cancer Inst* **27**, 1013-1035 (1961).
2. Rowley, J.D. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature* **243**, 290-293 (1973).
3. Heisterkamp, N. et al. Localization of the c-ab1 oncogene adjacent to a translocation break point in chronic myelocytic leukaemia. *Nature* **306**, 239-242 (1983).
4. Hehlmann, R. How I treat CML blast crisis. *Blood* **120**, 737-747 (2012).
5. Cortes, J.E. et al. Final 5-Year Study Results of DASISION: The Dasatinib Versus Imatinib Study in Treatment-Naive Chronic Myeloid Leukemia Patients Trial. *Journal of Clinical Oncology* **34**, 2333-2340 (2016).
6. Hochhaus, A. et al. Long-term benefits and risks of frontline nilotinib vs imatinib for chronic myeloid leukemia in chronic phase: 5-year update of the randomized ENESTnd trial. *Leukemia* **30**, 1044-1054 (2016).
7. Hochhaus, A. et al. Long-Term Outcomes of Imatinib Treatment for Chronic Myeloid Leukemia. *New England Journal of Medicine* **376**, 917-927 (2017).
8. Mahon, F.X. et al. Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective, multicentre Stop Imatinib (STIM) trial. *Lancet Oncol* **11**, 1029-1035 (2010).
9. Ross, D.M. et al. Safety and efficacy of imatinib cessation for CML patients with stable undetectable minimal residual disease: results from the TWISTER study. *Blood* **122**, 515-522 (2013).
10. Huang, X., Cortes, J. & Kantarjian, H. Estimations of the increasing prevalence and plateau prevalence of chronic myeloid leukemia in the era of tyrosine kinase inhibitor therapy. *Cancer* **118**, 3123-3127 (2012).
11. Papaemmanuil, E. et al. Genomic Classification and Prognosis in Acute Myeloid Leukemia. *N Engl J Med* **374**, 2209-2221 (2016).
12. Arber, D.A. et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood* **127**, 2391-2405 (2016).
13. Short, N.J., Kantarjian, H., Ravandi, F. & Daver, N. Emerging treatment paradigms with FLT3 inhibitors in acute myeloid leukemia. *Therapeutic Advances in Hematology* **10**, 1-18 (2019).
14. Wei, A.H. & Tiong, I.S. Midostaurin, enasidenib, CPX-351, gemtuzumab ozogamicin, and venetoclax bring new hope to AML. *Blood* **130**, 2469-2474 (2017).
15. Daver, N., Schlenk, R.F., Russell, N.H. & Levis, M.J. Targeting FLT3 mutations in AML: review of current knowledge and evidence. *Leukemia* **33**, 299-312 (2019).
16. Medeiros, B.C., Chan, S.M., Daver, N.G., Jonas, B.A. & Pollyea, D.A. Optimizing survival outcomes with post-remission therapy in acute myeloid leukemia. *American Journal of Hematology* **94**, 803-811 (2019).
17. Levin, R.H. et al. Persistent Mitosis of Transfused Homologous Leukocytes in Children Receiving Antileukemic Therapy. *Science* **142**, 1305-1311 (1963).
18. Clarkson, B., Ohkita, T., Ota, K. & Fried, J. Studies of Cellular Proliferation in Human Leukemia .I. Estimation of Growth Rates of Leukemic and Normal Hematopoietic Cells in 2 Adults with Acute Leukemia Given Single Injections of Tritiated Thymidine. *Journal of Clinical Investigation* **46**, 506-529 (1967).
19. Fialkow, P.J. Origin and Development of Human Tumors Studied with Cell Markers. *New England Journal of Medicine* **291**, 26-34 (1974).

20. Griffin, J.D. & Lowenberg, B. Clonogenic Cells in Acute Myeloblastic-Leukemia. *Blood* **68**, 1185-1195 (1986).
21. Lapidot, T. et al. A Cell Initiating Human Acute Myeloid-Leukemia after Transplantation into Scid Mice. *Nature* **367**, 645-648 (1994).
22. Holyoake, T., Jiang, X., Eaves, C. & Eaves, A. Isolation of a highly quiescent subpopulation of primitive leukemic cells in chronic myeloid leukemia. *Blood* **94**, 2056-2064 (1999).
23. Ishikawa, F. et al. Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nature Biotechnology* **25**, 1315-1321 (2007).
24. Chu, S. et al. Persistence of leukemia stem cells in chronic myelogenous leukemia patients in prolonged remission with imatinib treatment. *Blood* **118**, 5565-5572 (2011).
25. Chomel, J.C. et al. Leukemic stem cell persistence in chronic myeloid leukemia patients with sustained undetectable molecular residual disease. *Blood* **118**, 3657-3660 (2011).
26. Bhatia, R. et al. Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment. *Blood* **101**, 4701-4707 (2003).
27. Jordan, C.T., Guzman, M.L. & Noble, M. Mechanisms of disease - Cancer stem cells. *New England Journal of Medicine* **355**, 1253-1261 (2006).
28. Farge, T. et al. Chemotherapy-Resistant Human Acute Myeloid Leukemia Cells Are Not Enriched for Leukemic Stem Cells but Require Oxidative Metabolism. *Cancer Discovery* **7**, 716-735 (2017).
29. Boyd, A.L. et al. Identification of Chemotherapy-Induced Leukemic-Regenerating Cells Reveals a Transient Vulnerability of Human AML Recurrence. *Cancer Cell* **34**, 483-498 (2018).
30. Bonnet, D. & Dick, J.E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Medicine* **3**, 730-737 (1997).
31. Lemoli, R.M. et al. Molecular and functional analysis of the stem cell compartment of chronic myelogenous leukemia reveals the presence of a CD34(-) cell population with intrinsic resistance to imatinib. *Blood* **114**, 5191-5200 (2009).
32. Jamieson, C.H.M. et al. Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *New England Journal of Medicine* **351**, 657-667 (2004).
33. Kinstrie, R. et al. Heterogeneous leukemia stem cells in myeloid blast phase chronic myeloid leukemia. *Blood Advances* **1**, 160-169 (2016).
34. Tanizaki, R. et al. Irrespective of CD34 expression, lineage-committed cell fraction reconstitutes and re-establishes transformed Philadelphia chromosome-positive leukemia in NOD/SCID/IL-2R gamma c(-/-) mice. *Cancer Science* **101**, 631-638 (2010).
35. Eppert, K. et al. Stem cell gene expression programs influence clinical outcome in human leukemia. *Nature Medicine* **17**, 1086-1093 (2011).
36. Goardon, N. et al. Coexistence of LMPP-like and GMP-like Leukemia Stem Cells in Acute Myeloid Leukemia. *Cancer Cell* **19**, 138-152 (2011).
37. Quek, L. et al. Genetically distinct leukemic stem cells in human CD34(-) acute myeloid leukemia are arrested at a hemopoietic precursor-like stage. *Journal of Experimental Medicine* **213**, 1513-1535 (2016).
38. Somervaille, T.C.P. & Cleary, M.L. Identification and characterization of leukemia stem cells in murine MLL-AF9 acute myeloid leukemia. *Cancer Cell* **10**, 257-268 (2006).
39. Kirstetter, P. et al. Modeling of C/EBP alpha mutant acute myeloid leukemia reveals a common expression signature of committed myeloid leukemia-initiating cells. *Cancer Cell* **13**, 299-310 (2008).
40. Somervaille, T.C.P. et al. Hierarchical Maintenance of MLL Myeloid Leukemia Stem Cells Employs a Transcriptional Program Shared with Embryonic Rather Than Adult Stem Cells. *Cell Stem Cell* **4**, 129-140 (2009).

41. Ailles, L.E., Gerhard, B. & Hogge, D.E. Detection and characterization of primitive malignant and normal progenitors in patients with acute myelogenous leukemia using long-term coculture with supportive feeder layers and cytokines. *Blood* **90**, 2555-2564 (1997).
42. Udomsakdi, C. et al. Rapid decline of chronic myeloid leukemic cells in long-term culture due to a defect at the leukemic stem cell level. *Proc Natl Acad Sci U S A* **89**, 6192-6196 (1992).
43. Dick, J.E. Stem cell concepts renew cancer research. *Blood* **112**, 4793-4807 (2008).
44. Reinisch, A. et al. A humanized bone marrow ossicle xenotransplantation model enables improved engraftment of healthy and leukemic human hematopoietic cells. *Nature Medicine* **22**, 812-821 (2016).
45. Griessinger, E. et al. Acute myeloid leukemia xenograft success prediction: Saving time. *Experimental Hematology* **59**, 66-71 (2018).
46. Pearce, D.J. et al. AML engraftment in the NOD/SCID assay reflects the outcome of AML: implications for our understanding of the heterogeneity of AML. *Blood* **107**, 1166-1173 (2006).
47. Ran, D. et al. Aldehyde dehydrogenase activity among primary leukemia cells is associated with stem cell features and correlates with adverse clinical outcomes. *Exp Hematol* **37**, 1423-1434 (2009).
48. Hope, K.J., Jin, L.Q. & Dick, J.E. Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. *Nature Immunology* **5**, 738-743 (2004).
49. Vargaftig, J. et al. Frequency of leukemic initiating cells does not depend on the xenotransplantation model used. *Leukemia* **26**, 858-860 (2012).
50. Wunderlich, M. et al. AML xenograft efficiency is significantly improved in NOD/SCID-IL2RG mice constitutively expressing human SCF, GM-CSF and IL-3. *Leukemia* **24**, 1785-1788 (2010).
51. Cosgun, K.N. et al. Kit Regulates HSC Engraftment across the Human-Mouse Species Barrier. *Cell Stem Cell* **15**, 227-238 (2014).
52. McIntosh, B.E. et al. Nonirradiated NOD,B6.SCID Il2r gamma(-/-) Kit(W41/W41) (NBSGW) Mice Support Multilineage Engraftment of Human Hematopoietic Cells. *Stem Cell Reports* **4**, 171-180 (2015).
53. Miller, P.H. et al. Analysis of parameters that affect human hematopoietic cell outputs in mutant c-kit-immunodeficient mice. *Experimental Hematology* **48**, 41-49 (2017).
54. Krevvata, M. et al. Cytokines increase engraftment of human acute myeloid leukemia cells in immunocompromised mice but not engraftment of human myelodysplastic syndrome cells. *Haematologica* **103**, 959-971 (2018).
55. Klco, J.M. et al. Functional Heterogeneity of Genetically Defined Subclones in Acute Myeloid Leukemia. *Cancer Cell* **25**, 379-392 (2014).
56. Nakamichi, N. et al. Enhanced and Accelerated Repopulation of Mutant C-Kit Immunodeficient Mice By Transplants of Primary Chronic and Acute Myeloid Leukemic Human Cells. *Blood* **128**, 4537-4537 (2016).
57. Gerber, J.M. et al. Characterization of chronic myeloid leukemia stem cells. *Am J Hematol* **86**, 31-37 (2011).
58. Koschmieder, S. et al. Inducible chronic phase of myeloid leukemia with expansion of hematopoietic stem cells in a transgenic model of BCR-ABL leukemogenesis. *Blood* **105**, 324-334 (2005).
59. Zhang, B. et al. Heterogeneity of leukemia-initiating capacity of chronic myelogenous leukemia stem cells. *J Clin Invest* **126**, 975-991 (2016).
60. Guan, Y., Gerhard, B. & Hogge, D.E. Detection, isolation, and stimulation of quiescent primitive leukemic progenitor cells from patients with acute myeloid leukemia (AML). *Blood* **101**, 3142-3149 (2003).
61. Terpstra, W. et al. Fluorouracil selectively spares acute myeloid leukemia cells with long-term growth abilities in immunodeficient mice and in culture. *Blood* **88**, 1944-1950 (1996).

62. Lechman, E.R. et al. miR-126 Regulates Distinct Self-Renewal Outcomes in Normal and Malignant Hematopoietic Stem Cells. *Cancer Cell* **29**, 214-228 (2016).
63. Prost, S. et al. Erosion of the chronic myeloid leukaemia stem cell pool by PPARgamma agonists. *Nature* **525**, 380-383 (2015).
64. Iwasaki, M., Liedtke, M., Gentles, A.J. & Cleary, M.L. CD93 Marks a Non-Quiescent Human Leukemia Stem Cell Population and Is Required for Development of MLL-Rearranged Acute Myeloid Leukemia. *Cell Stem Cell* **17**, 412-421 (2015).
65. Saito, Y. et al. Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML. *Nature Biotechnology* **28**, 275-280 (2010).
66. Baquero, P. et al. Targeting quiescent leukemic stem cells using second generation autophagy inhibitors. *Leukemia* **33**, 981-994 (2019).
67. Nieborowska-Skorska, M. et al. Rac2-MRC-cIII-generated ROS cause genomic instability in chronic myeloid leukemia stem cells and primitive progenitors. *Blood* **119**, 4253-4263 (2012).
68. Sallmyr, A. et al. Internal tandem duplication of FLT3 (FLT3/ITD) induces increased ROS production, DNA damage, and misrepair: implications for poor prognosis in AML. *Blood* **111**, 3173-3182 (2008).
69. Adane, B. et al. The Hematopoietic Oxidase NOX2 Regulates Self-Renewal of Leukemic Stem Cells. *Cell Reports* **27**, 238-254 (2019).
70. Moloney, J.N., Stanicka, J. & Cotter, T.G. Subcellular localization of the FLT3-ITD oncogene plays a significant role in the production of NOX- and p22(phox)-derived reactive oxygen species in acute myeloid leukemia. *Leukemia Research* **52**, 34-42 (2017).
71. Kuntz, E.M. et al. Targeting mitochondrial oxidative phosphorylation eradicates therapy-resistant chronic myeloid leukemia stem cells. *Nature Medicine* **23**, 1234-1240 (2017).
72. Skrtic, M. et al. Inhibition of Mitochondrial Translation as a Therapeutic Strategy for Human Acute Myeloid Leukemia. *Cancer Cell* **20**, 674-688 (2011).
73. Ito, K. et al. Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. *Nature Medicine* **12**, 446-451 (2006).
74. Bolton-Gillespie, E. et al. Genomic instability may originate from imatinib-refractory chronic myeloid leukemia stem cells. *Blood* **121**, 4175-4183 (2013).
75. Lagadinou, E.D. et al. BCL-2 Inhibition Targets Oxidative Phosphorylation and Selectively Eradicates Quiescent Human Leukemia Stem Cells. *Cell Stem Cell* **12**, 329-341 (2013).
76. Pei, S. et al. AMPK/FIS1-Mediated Mitophagy Is Required for Self-Renewal of Human AML Stem Cells. *Cell Stem Cell* **23**, 86-100 (2018).
77. Hao, X.X. et al. Metabolic Imaging Reveals a Unique Preference of Symmetric Cell Division and Homing of Leukemia-Initiating Cells in an Endosteal Niche. *Cell Metabolism* **29**, 950-965 (2019).
78. Grossmann, V. et al. A deep-sequencing study of chronic myeloid leukemia patients in blast crisis (BC-CML) detects mutations in 76.9% of cases. *Leukemia* **25**, 557-560 (2011).
79. Rebechi, M.T. & Pratz, K.W. Genomic instability is a principle pathologic feature of FLT3 ITD kinase activity in acute myeloid leukemia leading to clonal evolution and disease progression. *Leukemia & Lymphoma* **58**, 2040-2050 (2017).
80. Cramer, K. et al. BCR/ABL and other kinases from chronic myeloproliferative disorders stimulate single-strand annealing, an unfaithful DNA double-strand break repair. *Cancer Res* **68**, 6884-6888 (2008).
81. Stoklosa, T. et al. BCR/ABL inhibits mismatch repair to protect from apoptosis and induce point mutations. *Cancer Res* **68**, 2576-2580 (2008).
82. Cramer-Morales, K. et al. Personalized synthetic lethality induced by targeting RAD52 in leukemias identified by gene mutation and expression profile. *Blood* **122**, 1293-1304 (2013).
83. Holyoake, T.L. & Vetrie, D. The chronic myeloid leukemia stem cell: stemming the tide of persistence. *Blood* **129**, 1595-1606 (2017).

84. Pearsall, E.A., Lincz, L.F. & Skelding, K.A. The Role of DNA Repair Pathways in AML Chemosensitivity. *Current Drug Targets* **19**, 1205-1219 (2018).
85. Ho, T.C. et al. Evolution of acute myelogenous leukemia stem cell properties after treatment and progression. *Blood* **128**, 1671-1678 (2016).
86. Kumari, A., Brendel, C., Hochhaus, A., Neubauer, A. & Burchert, A. Low BCR-ABL expression levels in hematopoietic precursor cells enable persistence of chronic myeloid leukemia under imatinib. *Blood* **119**, 530-539 (2012).
87. Ng, S.W.K. et al. A 17-gene stemness score for rapid determination of risk in acute leukaemia. *Nature* **540**, 433-437 (2016).
88. Jung, N., Dai, B., Gentles, A.J., Majeti, R. & Feinberg, A.P. An LSC epigenetic signature is largely mutation independent and implicates the HOXA cluster in AML pathogenesis. *Nature Communications* **6**, 8489 (2015).
89. Krivtsov, A.V. et al. Cell of origin determines clinically relevant subtypes of MLL-rearranged AML. *Leukemia* **27**, 852-860 (2013).
90. Laverdiere, I. et al. Leukemic stem cell signatures identify novel therapeutics targeting acute myeloid leukemia. *Blood Cancer Journal* **8**, 52 (2018).
91. Graham, S.M., Vass, J.K., Holyoake, T.L. & Graham, G.J. Transcriptional analysis of quiescent and proliferating CD34+ human hemopoietic cells from normal and chronic myeloid leukemia sources. *Stem Cells* **25**, 3111-3120 (2007).
92. Abraham, S.A. et al. Dual targeting of p53 and c-MYC selectively eliminates leukaemic stem cells. *Nature* **534**, 341-346 (2016).
93. Scott, M.T. et al. Epigenetic Reprogramming Sensitizes CML Stem Cells to Combined EZH2 and Tyrosine Kinase Inhibition. *Cancer Discov* **6**, 1248-1257 (2016).
94. Lu, C. et al. IDH mutation impairs histone demethylation and results in a block to cell differentiation. *Nature* **483**, 474-478 (2012).
95. Corces, M.R. et al. Lineage-specific and single-cell chromatin accessibility charts human hematopoiesis and leukemia evolution. *Nature Genetics* **48**, 1193-1203 (2016).
96. Jan, M. et al. Clonal evolution of preleukemic hematopoietic stem cells precedes human acute myeloid leukemia. *Sci Transl Med* **4**, 149ra118 (2012).
97. Shlush, L.I. et al. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature* **506**, 328-333 (2014).
98. Gilliland, D.G. & Griffin, J.D. The roles of FLT3 in hematopoiesis and leukemia. *Blood* **100**, 1532-1542 (2002).
99. Zheng, R. & Small, D. Mutant FLT3 signaling contributes to a block in myeloid differentiation. *Leukemia & Lymphoma* **46**, 1679-1687 (2005).
100. Lal, R. et al. Somatic TP53 mutations characterize preleukemic stem cells in acute myeloid leukemia. *Blood* **129**, 2587-2591 (2017).
101. Ley, T.J. et al. Genomic and Epigenomic Landscapes of Adult De Novo Acute Myeloid Leukemia. *New England Journal of Medicine* **368**, 2059-2074 (2013).
102. Genovese, G. et al. Clonal Hematopoiesis and Blood-Cancer Risk Inferred from Blood DNA Sequence. *New England Journal of Medicine* **371**, 2477-2487 (2014).
103. Jaiswal, S. et al. Age-Related Clonal Hematopoiesis Associated with Adverse Outcomes. *New England Journal of Medicine* **371**, 2488-2498 (2014).
104. Schmidt, M. et al. Molecular-defined clonal evolution in patients with chronic myeloid leukemia independent of the BCR-ABL status. *Leukemia* **28**, 2292-2299 (2014).
105. Kim, T. et al. Spectrum of somatic mutation dynamics in chronic myeloid leukemia following tyrosine kinase inhibitor therapy. *Blood* **129**, 38-47 (2017).
106. Branford, S. et al. Integrative genomic analysis reveals cancer-associated mutations at diagnosis of CML in patients with high-risk disease. *Blood* **132**, 948-961 (2018).
107. Huntly, B.J. et al. MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. *Cancer Cell* **6**, 587-596 (2004).

108. George, J. et al. Leukaemia cell of origin identified by chromatin landscape of bulk tumour cells. *Nature Communications* **7**, 12166 (2016).
109. Gunsilius, E. et al. Evidence from a leukaemia model for maintenance of vascular endothelium by bone-marrow-derived endothelial cells. *Lancet* **355**, 1688-1691 (2000).
110. Foley, S.B. et al. Expression of BCR/ABL p210 from a knockin allele enhances bone marrow engraftment without inducing neoplasia. *Cell Rep* **5**, 51-60 (2013).
111. Cheung, A.M. et al. Analysis of the clonal growth and differentiation dynamics of primitive barcoded human cord blood cells in NSG mice. *Blood* **122**, 3129-3137 (2013).
112. Biernaux, C., Loos, M., Sels, A., Huez, G. & Stryckmans, P. Detection of major bcr-abl gene expression at a very low level in blood cells of some healthy individuals. *Blood* **86**, 3118-3122 (1995).
113. Easwaran, H., Tsai, H.C. & Baylin, S.B. Cancer Epigenetics: Tumor Heterogeneity, Plasticity of Stem-like States, and Drug Resistance. *Molecular Cell* **54**, 716-727 (2014).
114. Flavahan, W.A., Gaskell, E. & Bernstein, B.E. Epigenetic plasticity and the hallmarks of cancer. *Science* **357**, pii: eaal2380 (2017).
115. Corces-Zimmerman, M.R., Hong, W.J., Weissman, I.L., Medeiros, B.C. & Majeti, R. Preleukemic mutations in human acute myeloid leukemia affect epigenetic regulators and persist in remission. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 2548-2553 (2014).
116. Rothenberg-Thurley, M. et al. Persistence of pre-leukemic clones during first remission and risk of relapse in acute myeloid leukemia. *Leukemia* **32**, 1598-1608 (2018).
117. Graham, S.M. et al. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. *Blood* **99**, 319-325 (2002).
118. Jorgensen, H.G., Allan, E.K., Jordanides, N.E., Mountford, J.C. & Holyoake, T.L. Nilotinib exerts equipotent antiproliferative effects to imatinib and does not induce apoptosis in CD34+ CML cells. *Blood* **109**, 4016-4019 (2007).
119. Warfvinge, R. et al. Single-cell molecular analysis defines therapy response and immunophenotype of stem cell subpopulations in CML. *Blood* **129**, 2384-2394 (2017).
120. Giustacchini, A. et al. Single-cell transcriptomics uncovers distinct molecular signatures of stem cells in chronic myeloid leukemia. *Nature Medicine* **23**, 692-702 (2017).
121. Corbin, A.S. et al. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *J Clin Invest* **121**, 396-409 (2011).
122. Hamilton, A. et al. Chronic myeloid leukemia stem cells are not dependent on Bcr-Abl kinase activity for their survival. *Blood* **119**, 1501-1510 (2012).
123. Shlush, L.I. et al. Tracing the origins of relapse in acute myeloid leukaemia to stem cells. *Nature* **547**, 104-108 (2017).
124. Ding, L. et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature* **481**, 506-510 (2012).
125. Yilmaz, M. et al. Late relapse in acute myeloid leukemia (AML): clonal evolution or therapy-related leukemia? *Blood Cancer Journal* **9**, 7 (2019).
126. Soverini, S., de Benedittis, C., Mancini, M. & Martinelli, G. Mutations in the BCR-ABL1 Kinase Domain and Elsewhere in Chronic Myeloid Leukemia. *Clin Lymphoma Myeloma Leuk* **15 Suppl**, S120-S128 (2015).
127. Shah, N.P. et al. Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. *Cancer Cell* **2**, 117-125 (2002).
128. Franke, G.N. et al. Incidence of Low Level Mutations in Newly Diagnosed CML Patients: A Substudy of the German Tiger Trial. *Blood* **130** (2017).
129. Bumm, T. et al. Emergence of clonal cytogenetic abnormalities in Ph- cells in some CML patients in cytogenetic remission to imatinib but restoration of polyclonal hematopoiesis in the majority. *Blood* **101**, 1941-1949 (2003).

130. De Melo, V.A.S. et al. Philadelphia-negative clonal hematopoiesis is a significant feature of dasatinib therapy for chronic myeloid leukemia. *Blood* **110**, 3086-3087 (2007).
131. Shih, A.H. et al. Mutational Cooperativity Linked to Combinatorial Epigenetic Gain of Function in Acute Myeloid Leukemia. *Cancer Cell* **27**, 502-515 (2015).
132. Wong, S.H.K. et al. The H3K4-Methyl Epigenome Regulates Leukemia Stem Cell Oncogenic Potential. *Cancer Cell* **28**, 198-209 (2015).
133. Bernt, K.M. et al. MLL-Rearranged Leukemia Is Dependent on Aberrant H3K79 Methylation by DOT1L. *Cancer Cell* **20**, 66-78 (2011).
134. Li, S. et al. Distinct evolution and dynamics of epigenetic and genetic heterogeneity in acute myeloid leukemia. *Nature Medicine* **22**, 792-799 (2016).
135. Figueroa, M.E. et al. Leukemic IDH1 and IDH2 Mutations Result in a Hypermethylation Phenotype, Disrupt TET2 Function, and Impair Hematopoietic Differentiation. *Cancer Cell* **18**, 553-567 (2010).
136. Glass, J.L. et al. Epigenetic Identity in AML Depends on Disruption of Nonpromoter Regulatory Elements and Is Affected by Antagonistic Effects of Mutations in Epigenetic Modifiers. *Cancer Discovery* **7**, 868-883 (2017).
137. Figueroa, M.E. et al. DNA Methylation Signatures Identify Biologically Distinct Subtypes in Acute Myeloid Leukemia. *Cancer Cell* **17**, 13-27 (2010).
138. Will, B. et al. Minimal PU.1 reduction induces a preleukemic state and promotes development of acute myeloid leukemia. *Nature Medicine* **21**, 1172-1181 (2015).
139. Assi, S.A. et al. Subtype-specific regulatory network rewiring in acute myeloid leukemia. *Nature Genetics* **51**, 151-162 (2019).
140. Harris, W.J. et al. The Histone Demethylase KDM1A Sustains the Oncogenic Potential of MLL-AF9 Leukemia Stem Cells. *Cancer Cell* **21**, 473-487 (2012).
141. Basheer, F. et al. Contrasting requirements during disease evolution identify EZH2 as a therapeutic target in AML. *Journal of Experimental Medicine* **216**, 966-981 (2019).
142. Li, L. et al. Activation of p53 by SIRT1 inhibition enhances elimination of CML leukemia stem cells in combination with imatinib. *Cancer Cell* **21**, 266-281 (2012).
143. Zuber, J. et al. RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. *Nature* **478**, 524-528 (2011).
144. Koschmieder, S. & Vetrie, D. Epigenetic dysregulation in chronic myeloid leukaemia: A myriad of mechanisms and therapeutic options. *Seminars in Cancer Biology* **51**, 180-197 (2018).
145. Deshpande, A.J. et al. AF10 Regulates Progressive H3K79 Methylation and HOX Gene Expression in Diverse AML Subtypes. *Cancer Cell* **26**, 896-908 (2014).
146. Riether, C. et al. Tyrosine kinase inhibitor-induced CD70 expression mediates drug resistance in leukemia stem cells by activating Wnt signaling. *Sci Transl Med* **7**, 298ra119 (2015).
147. Jin, Y. et al. Targeting methyltransferase PRMT5 eliminates leukemia stem cells in chronic myelogenous leukemia. *J Clin Invest* **126**, 3961-3980 (2016).
148. Abraham, A. et al. SIRT1 regulates metabolism and leukemogenic potential in CML stem cells. *Journal of Clinical Investigation* **129**, 2685-2701 (2019).
149. Fong, C.Y. et al. BET inhibitor resistance emerges from leukaemia stem cells. *Nature* **525**, 538-542 (2015).
150. Reid, M.A., Dai, Z.W. & Locasale, J.W. The impact of cellular metabolism on chromatin dynamics and epigenetics. *Nature Cell Biology* **19**, 1298-1306 (2017).
151. Ye, H. et al. Leukemic Stem Cells Evade Chemotherapy by Metabolic Adaptation to an Adipose Tissue Niche. *Cell Stem Cell* **19**, 23-37 (2016).
152. Hattori, A. et al. Cancer progression by reprogrammed BCAA metabolism in myeloid leukaemia. *Nature* **545**, 500-504 (2017).

153. Ward, P.S. et al. The Common Feature of Leukemia-Associated IDH1 and IDH2 Mutations Is a Neomorphic Enzyme Activity Converting alpha-Ketoglutarate to 2-Hydroxyglutarate. *Cancer Cell* **17**, 225-234 (2010).
154. Xu, W. et al. Oncometabolite 2-Hydroxyglutarate Is a Competitive Inhibitor of alpha-Ketoglutarate-Dependent Dioxygenases. *Cancer Cell* **19**, 17-30 (2011).
155. Losman, J.A. et al. (R)-2-Hydroxyglutarate Is Sufficient to Promote Leukemogenesis and Its Effects Are Reversible. *Science* **339**, 1621-1625 (2013).
156. Wang, F. et al. Targeted Inhibition of Mutant IDH2 in Leukemia Cells Induces Cellular Differentiation. *Science* **340**, 622-626 (2013).
157. Warburg, O., Wind, F. & Negelein, E. The metabolism of tumors in the body. *Journal of General Physiology* **8**, 519-530 (1927).
158. Simsek, T. et al. The Distinct Metabolic Profile of Hematopoietic Stem Cells Reflects Their Location in a Hypoxic Niche. *Cell Stem Cell* **7**, 380-390 (2010).
159. Wang, Y.H. et al. Cell-State-Specific Metabolic Dependency in Hematopoiesis and Leukemogenesis. *Cell* **158**, 1309-1323 (2014).
160. Molina, J.R. et al. An inhibitor of oxidative phosphorylation exploits cancer vulnerability. *Nat Med* **24**, 1036-1046 (2018).
161. Konopleva, M. et al. Efficacy and Biological Correlates of Response in a Phase II Study of Venetoclax Monotherapy in Patients with Acute Myelogenous Leukemia. *Cancer Discovery* **6**, 1106-1117 (2016).
162. Jones, C.L. et al. Inhibition of Amino Acid Metabolism Selectively Targets Human Leukemia Stem Cells. *Cancer Cell* **34**, 724-740.e4 (2018).
163. Pollyea, D.A. et al. Venetoclax with azacitidine disrupts energy metabolism and targets leukemia stem cells in patients with acute myeloid leukemia. *Nat Med* **24**, 1859-1866 (2018).
164. Sriskanthadevan, S. et al. AML cells have low spare reserve capacity in their respiratory chain that renders them susceptible to oxidative metabolic stress. *Blood* **125**, 2120-2130 (2015).
165. Chen, Y., Hu, Y., Zhang, H., Peng, C. & Li, S. Loss of the Alox5 gene impairs leukemia stem cells and prevents chronic myeloid leukemia. *Nat Genet* **41**, 783-792 (2009).
166. Chen, Y. et al. Arachidonate 15-lipoxygenase is required for chronic myeloid leukemia stem cell survival. *J Clin Invest* **124**, 3847-3862 (2014).
167. Saussele, S. et al. Discontinuation of tyrosine kinase inhibitor therapy in chronic myeloid leukaemia (EURO-SKI): a prespecified interim analysis of a prospective, multicentre, non-randomised, trial. *Lancet Oncology* **19**, 747-757 (2018).
168. Clark, R.E. et al. De-escalation of tyrosine kinase inhibitor therapy before complete treatment discontinuation in patients with chronic myeloid leukaemia (DESTINY): a non-randomised, phase 2 trial. *Lancet Haematology* **6**, E375-E383 (2019).
169. O'Hare, T. et al. AP24534, a Pan-BCR-ABL Inhibitor for Chronic Myeloid Leukemia, Potently Inhibits the T315I Mutant and Overcomes Mutation-Based Resistance. *Cancer Cell* **16**, 401-412 (2009).
170. Cortes, J.E. et al. Ponatinib in Refractory Philadelphia Chromosome-Positive Leukemias. *New England Journal of Medicine* **367**, 2075-2088 (2012).
171. Mian, A.A. et al. PF-114, a potent and selective inhibitor of native and mutated BCR/ABL is active against Philadelphia chromosome-positive (Ph+) leukemias harboring the T315I mutation. *Leukemia* **29**, 1104-1114 (2015).
172. Wylie, A.A. et al. The allosteric inhibitor ABL001 enables dual targeting of BCR-ABL1. *Nature* **543**, 733-737 (2017).
173. Dierks, C. et al. Expansion of Bcr-Abl-positive leukemic stem cells is dependent on Hedgehog pathway activation. *Cancer Cell* **14**, 238-249 (2008).
174. Irvine, D.A. et al. Deregulated hedgehog pathway signaling is inhibited by the smoothened antagonist LDE225 (Sonidegib) in chronic phase chronic myeloid leukaemia. *Sci Rep* **6**, 25476 (2016).

175. Shah, N.P. et al. Dasatinib Plus Smoothened (SMO) Inhibitor BMS-833923 in Chronic Myeloid Leukemia (CML) with Resistance or Suboptimal Response to a Prior Tyrosine Kinase Inhibitor (TKI): Phase I Study CA180323. *Blood* **124**, 4539-4539 (2014).
176. Ottmann, O. et al. Smoothened (Smo) Inhibitor Lde225 Combined with Nilotinib in Patients with Chronic Myeloid Leukemia (Cml) Resistant/Intolerant (R/I) to at Least 1 Prior Tyrosine Kinase Inhibitor: A Phase 1b Study. *Haematologica* **100**, 62-63 (2015).
177. Cortes, J.E. et al. Randomized comparison of low dose cytarabine with or without glasdegib in patients with newly diagnosed acute myeloid leukemia or high-risk myelodysplastic syndrome. *Leukemia* **33**, 379-389 (2019).
178. Savona, M.R. et al. Phase Ib Study of Glasdegib, a Hedgehog Pathway Inhibitor, in Combination with Standard Chemotherapy in Patients with AML or High-Risk MDS. *Clinical Cancer Research* **24**, 2294-2303 (2018).
179. Druker, B.J. et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat Med* **2**, 561-566 (1996).
180. Jorgensen, H.G. et al. Intermittent exposure of primitive quiescent chronic myeloid leukemia cells to granulocyte-colony stimulating factor in vitro promotes their elimination by imatinib mesylate. *Clinical Cancer Research* **12**, 626-633 (2006).
181. Gallipoli, P. et al. Safety and efficacy of pulsed imatinib with or without G-CSF versus continuous imatinib in chronic phase chronic myeloid leukaemia patients at 5 years follow-up. *British Journal of Haematology* **163**, 674-676 (2013).
182. Milligan, D.W. et al. Fludarabine and cytosine are less effective than standard ADE chemotherapy in high-risk acute myeloid leukemia, and addition of G-CSF and ATRA are not beneficial: results of the MRC AML-HR randomized trial. *Blood* **107**, 4614-4622 (2006).
183. Ossenkoppele, G.J. et al. The value of fludarabine in addition to ARA-C and G-CSF in the treatment of patients with high-risk myelodysplastic syndromes and AML in elderly patients. *Blood* **103**, 2908-2913 (2004).
184. Bhatia, R., Wayner, E.A., McGlave, P.B. & Verfaillie, C.M. Interferon-alpha restores normal adhesion of chronic myelogenous leukemia hematopoietic progenitors to bone marrow stroma by correcting impaired beta 1 integrin receptor function. *J Clin Invest* **94**, 384-391 (1994).
185. Hjorth-Hansen, H. et al. Safety and efficacy of the combination of pegylated interferon-alpha2b and dasatinib in newly diagnosed chronic-phase chronic myeloid leukemia patients. *Leukemia* **30**, 1853-1860 (2016).
186. Rousselot, P. et al. Pioglitazone together with imatinib in chronic myeloid leukemia: A proof of concept study. *Cancer* **123**, 1791-1799 (2017).
187. EU Clinical Trials Register. clinicaltrialsregister.eu, https://www.clinicaltrialsregister.eu/ctr-search/search?query=eudract_number:2009-011675-79. (2009).
188. US National Library of Medicine. ClinicalTrials.gov, https://www.clinicaltrials.gov/ct2/show/NCT02889003?term=NCT02889003&rank=1. (2016).
189. US National Library of Medicine. ClinicalTrials.gov, https://www.clinicaltrials.gov/ct2/show/NCT02852486?term=NCT02852486&rank=1. (2016).
190. US National Library of Medicine. ClinicalTrials.gov, https://www.clinicaltrials.gov/ct2/show/NCT02767063?term=NCT02767063&rank=1. (2016).
191. US National Library of Medicine. ClinicalTrials.gov, https://www.clinicaltrials.gov/ct2/show/study/NCT02730195?term=NCT02730195&rank=1. (2016).
192. Faber, K. et al. CDX2-driven leukemogenesis involves KLF4 repression and deregulated PPAR gamma signaling. *Journal of Clinical Investigation* **123**, 299-314 (2013).

193. Yen, K. et al. AG-221, a First-in-Class Therapy Targeting Acute Myeloid Leukemia Harboring Oncogenic IDH2 Mutations. *Cancer Discovery* **7**, 478-493 (2017).
194. Shih, A.H. et al. Combination Targeted Therapy to Disrupt Aberrant Oncogenic Signaling and Reverse Epigenetic Dysfunction in IDH2- and TET2-Mutant Acute Myeloid Leukemia. *Cancer Discovery* **7**, 494-505 (2017).
195. DiNardo, C.D. et al. Durable Remissions with Ivosidenib in IDH1-Mutated Relapsed or Refractory AML. *New England Journal of Medicine* **378**, 2386-2398 (2018).
196. Stein, E.M. et al. Enasidenib in mutant IDH2 relapsed or refractory acute myeloid leukemia. *Blood* **130**, 722-731 (2017).
197. Amatangelo, M.D. et al. Enasidenib induces acute myeloid leukemia cell differentiation to promote clinical response. *Blood* **130**, 732-741 (2017).
198. Quek, L. et al. Clonal heterogeneity of acute myeloid leukemia treated with the IDH2 inhibitor enasidenib. *Nature Medicine* **24**, 1167-1177 (2018).
199. Intlekofer, A.M. et al. Acquired resistance to IDH inhibition through trans or cis dimer-interface mutations. *Nature* **559**, 125-129 (2018).
200. Tsapogas, P., Mooney, C.J., Brown, G. & Rolink, A. The Cytokine Flt3-Ligand in Normal and Malignant Hematopoiesis. *International Journal of Molecular Sciences* **18**, pii: E1115 (2017).
201. Stone, R.M. et al. Midostaurin plus Chemotherapy for Acute Myeloid Leukemia with a FLT3 Mutation. *New England Journal of Medicine* **377**, 454-464 (2017).
202. Swaminathan, M. et al. The Combination of Quizartinib with Azacitidine or Low Dose Cytarabine Is Highly Active in Patients (Pts) with FLT3-ITD Mutated Myeloid Leukemias: Interim Report of a Phase I/II Trial. *Blood* **130**, 723-723 (2017).
203. Zhang, W.G. et al. Reversal of Acquired Drug Resistance in FLT3-Mutated Acute Myeloid Leukemia Cells via Distinct Drug Combination Strategies. *Clinical Cancer Research* **20**, 2363-2374 (2014).
204. Stolzel, F. et al. Mechanisms of resistance against PKC412 in resistant FLT3-ITD positive human acute myeloid leukemia cells. *Annals of Hematology* **89**, 653-662 (2010).
205. Sung, P.J., Sugita, M., Koblish, H., Perl, A.E. & Carroll, M. Hematopoietic cytokines mediate resistance to targeted therapy in FLT3-ITD acute myeloid leukemia. *Blood Advances* **3**, 1061-1072 (2019).
206. Sato, T. et al. FLT3 ligand impedes the efficacy of FLT3 inhibitors in vitro and in vivo. *Blood* **117**, 3286-3293 (2011).
207. Wei, A.H. et al. Venetoclax Combined With Low-Dose Cytarabine for Previously Untreated Patients With Acute Myeloid Leukemia: Results From a Phase Ib/II Study. *Journal of Clinical Oncology* **37**, 1277-1284 (2019).
208. DiNardo, C.D. et al. Venetoclax combined with decitabine or azacitidine in treatment-naive, elderly patients with acute myeloid leukemia. *Blood* **133**, 7-17 (2019).
209. Goff, D.J. et al. A Pan-BCL2 Inhibitor Renders Bone-Marrow-Resident Human Leukemia Stem Cells Sensitive to Tyrosine Kinase Inhibition. *Cell Stem Cell* **12**, 316-328 (2013).
210. Ko, T.K., Chuah, C.T.H., Huang, J.W.J., Ng, K.P. & Ong, S.T. The BCL2 inhibitor ABT-199 significantly enhances imatinib-induced cell death in chronic myeloid leukemia progenitors. *Oncotarget* **5**, 9033-9038 (2014).
211. Carter, B.Z. et al. Combined targeting of BCL-2 and BCR-ABL tyrosine kinase eradicates chronic myeloid leukemia stem cells. *Science Translational Medicine* **8**, 355ra117 (2016).
212. Baryawno, N. et al. A Cellular Taxonomy of the Bone Marrow Stroma in Homeostasis and Leukemia. *Cell* **177**, 1915-1932.e16 (2019).
213. Nguyen, L.V. et al. Barcoding reveals complex clonal dynamics of de novo transformed human mammary cells. *Nature* **528**, 267-271 (2015).
214. Roeder, I. et al. Dynamic modeling of imatinib-treated chronic myeloid leukemia: functional insights and clinical implications. *Nat Med* **12**, 1181-1184 (2006).

215. Schepers, K., Campbell, T.B. & Passegue, E. Normal and leukemic stem cell niches: insights and therapeutic opportunities. *Cell Stem Cell* **16**, 254-267 (2015).
216. Medyouf, H. The microenvironment in human myeloid malignancies: emerging concepts and therapeutic implications. *Blood* **129**, 1617-1626 (2017).
217. Pittet, M.J. & Weissleder, R. Intravital Imaging. *Cell* **147**, 983-991 (2011).
218. Ribas, A. & Wolchok, J.D. Cancer immunotherapy using checkpoint blockade. *Science* **359**, 1350-1355 (2018).
219. Godwin, C.D., Gale, R.P. & Walter, R.B. Gemtuzumab ozogamicin in acute myeloid leukemia. *Leukemia* **31**, 1855-1868 (2017).
220. Pizzitola, I. et al. Chimeric antigen receptors against CD33/CD123 antigens efficiently target primary acute myeloid leukemia cells in vivo. *Leukemia* **28**, 1596-1605 (2014).
221. Kenderian, S.S. et al. CD33-specific chimeric antigen receptor T cells exhibit potent preclinical activity against human acute myeloid leukemia. *Leukemia* **29**, 1637-1647 (2015).
222. Eide, C.A. et al. Combining the Allosteric Inhibitor Asciminib with Ponatinib Suppresses Emergence of and Restores Efficacy against Highly Resistant BCR-ABL1 Mutants. *Cancer Cell* **36**, 431-443.e5 (2019).
223. Gallipoli, P. et al. JAK2/STAT5 inhibition by nilotinib with ruxolitinib contributes to the elimination of CML CD34+ cells in vitro and in vivo. *Blood* **124**, 1492-1501 (2014).
224. Lim, S. et al. Targeting of the MNK-eIF4E axis in blast crisis chronic myeloid leukemia inhibits leukemia stem cell function. *Proceedings of the National Academy of Sciences of the United States of America* **110**, E2298-E2307 (2013).
225. Essers, M.A.G. et al. IFN alpha activates dormant haematopoietic stem cells in vivo. *Nature* **458**, 904-908 (2009).
226. Chen, M. et al. Targeting BCR-ABL(+) stem/progenitor cells and BCR-ABL-T315I mutant cells by effective inhibition of the BCR-ABL-Tyr177-GRB2 complex. *Oncotarget* **8**, 43662-43677 (2017).
227. Pemovska, T. et al. Axitinib effectively inhibits BCR-ABL1(T315I) with a distinct binding conformation. *Nature* **519**, 102-105 (2015).
228. Riether, C., Gschwend, T., Huguenin, A.L., Schurch, C.M. & Ochsenbein, A.F. Blocking programmed cell death 1 in combination with adoptive cytotoxic T-cell transfer eradicates chronic myelogenous leukemia stem cells. *Leukemia* **29**, 1781-1785 (2015).
229. Turhan, A.G. et al. Vitamin D3 analog inecalcitol synergizes with tyrosine kinase inhibitors (TKI) and selectively inhibit the growth of chronic myeloid leukemia (CML) progenitors: Development of a clinically applicable leukemic stem cell targeting strategy. *Cancer Research* **75**, 3981-3981 (2015).
230. Galton, D.A.G. Myleran in Chronic Myeloid Leukaemia - Results of Treatment. *Lancet* **264**, 208-213 (1953).
231. Fishbein, W.N., Frei, E., Freireich, E., Carbone, P.P. & Misra, D. Clinical Trials of Hydroxyurea in Patients with Cancer + Leukemia. *Clinical Pharmacology & Therapeutics* **5**, 574-580 (1964).
232. Doney, K. et al. Treatment of Chronic Granulocytic Leukemia by Chemotherapy, Total-Body Irradiation and Allogeneic Bone-Marrow Transplantation. *Experimental Hematology* **6**, 738-747 (1978).
233. Talpaz, M., Mccredie, K.B., Mavligit, G.M. & Gutterman, J.U. Leukocyte Interferon-Induced Myeloid Cytoreduction in Chronic Myelogenous Leukemia. *Blood* **62**, 689-692 (1983).
234. Shah, N.P. et al. Overriding imatinib resistance with a novel ABL kinase inhibitor. *Science* **305**, 399-401 (2004).
235. Weisberg, E. et al. Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. *Cancer Cell* **7**, 129-141 (2005).
236. Puttini, M. et al. In vitro and in vivo activity of SKI-606, a novel Src-Abl inhibitor, against imatinib-resistant Bcr-Abl(+) neoplastic cells. *Cancer Research* **66**, 11314-11322 (2006).

237. Rodriguez, V. et al. Pomp Combination Chemotherapy of Adult Acute Leukemia. *Cancer* **32**, 69-75 (1973).
238. Lichtman, M.A. A historical perspective on the development of the cytarabine (7 days) and daunorubicin (3 days) treatment regimen for acute myelogenous leukemia: 2013 the 40th anniversary of 7+3. *Blood Cells Molecules and Diseases* **50**, 119-130 (2013).
239. Thomas, E.D. et al. 100 Patients with Acute-Leukemia Treated by Chemotherapy, Total-Body Irradiation, and Allogeneic Marrow Transplantation. *Blood* **49**, 511-533 (1977).
240. Weisberg, E. et al. Inhibition of mutant FLT3 receptors in leukemia cells by the small molecule tyrosine kinase inhibitor PKC412. *Cancer Cell* **1**, 433-443 (2002).
241. Lu, C. & Thompson, C.B. Metabolic Regulation of Epigenetics. *Cell Metabolism* **16**, 9-17 (2012).
242. Maddocks, O.D.K., Labuschagne, C.F., Adams, P.D. & Vousden, K.H. Serine Metabolism Supports the Methionine Cycle and DNA/RNA Methylation through De Novo ATP Synthesis in Cancer Cells. *Molecular Cell* **61**, 210-221 (2016).
243. Gallipoli, P., Giotopoulos, G. & Huntly, B.J.P. Epigenetic regulators as promising therapeutic targets in acute myeloid leukemia. *Therapeutic Advances in Hematology* **6**, 103-119 (2015).
244. Gross, S. et al. Cancer-associated metabolite 2-hydroxyglutarate accumulates in acute myelogenous leukemia with isocitrate dehydrogenase 1 and 2 mutations. *Journal of Experimental Medicine* **207**, 339-344 (2010).
245. Lo-Coco, F. et al. Gemtuzumab ozogamicin (Mylotarg) as a single agent for molecularly relapsed acute promyelocytic leukemia. *Blood* **104**, 1995-1999 (2004).
246. Walter, R.B., Appelbaum, F.R., Estey, E.H. & Bernstein, I.D. Acute myeloid leukemia stem cells and CD33-targeted immunotherapy. *Blood* **119**, 6198-6208 (2012).
247. Borot, F. et al. Gene-edited stem cells enable CD33-directed immune therapy for myeloid malignancies. *Proc Natl Acad Sci U S A* **116**, 11978-11987 (2019).
248. Herrmann, H. et al. CD34(+)/CD38(-) stem cells in chronic myeloid leukemia express Siglec-3 (CD33) and are responsive to the CD33-targeting drug gemtuzumab/ozogamicin. *Haematologica-the Hematology Journal* **97**, 219-226 (2012).
249. Jabbour, E. et al. Twice-Daily Fludarabine and Cytarabine Combination With or Without Gentuzumab Ozogamicin is Effective in Patients With Relapsed/Refractory Acute Myeloid Leukemia, High-Risk Myelodysplastic Syndrome, and Blast- Phase Chronic Myeloid Leukemia. *Clinical Lymphoma Myeloma & Leukemia* **12**, 244-251 (2012).
250. Sadovnik, I. et al. Expression of CD25 on leukemic stem cells in BCR-ABL1(+) CML: Potential diagnostic value and functional implications. *Experimental Hematology* **51**, 17-24 (2017).
251. Touzet, L. et al. CD9 in acute myeloid leukemia: Prognostic role and usefulness to target leukemic stem cells. *Cancer Medicine* **8**, 1279-1288 (2019).
252. Sadovnik, I. et al. Identification of CD25 as STAT5-Dependent Growth Regulator of Leukemic Stem Cells in Ph+ CML. *Clin Cancer Res* **22**, 2051-2061 (2016).
253. Saito, Y. et al. Identification of Therapeutic Targets for Quiescent, Chemotherapy-Resistant Human Leukemia Stem Cells. *Science Translational Medicine* **2**, 17ra9 (2010).
254. Valent, P. et al. DPPIV (CD26) as a novel stem cell marker in Ph+ chronic myeloid leukaemia. *Eur J Clin Invest* **44**, 1239-1245 (2014).
255. Jilani, I. et al. Differences in CD33 intensity between various myeloid neoplasms. *American Journal of Clinical Pathology* **118**, 560-566 (2002).
256. Chao, M.P., Weissman, I.L. & Majeti, R. The CD47-SIRPalpha pathway in cancer immune evasion and potential therapeutic implications. *Curr Opin Immunol* **24**, 225-232 (2012).
257. Jaiswal, S. et al. CD47 Is Upregulated on Circulating Hematopoietic Stem Cells and Leukemia Cells to Avoid Phagocytosis. *Cell* **138**, 271-285 (2009).
258. Oehler, V.G. et al. CD52 Expression In Leukemic Stem/Progenitor Cells. *Blood* **116**, 1131-1132 (2010).

259. de Boer, B. et al. Prospective Isolation and Characterization of Genetically and Functionally Distinct AML Subclones. *Cancer Cell* **34**, 674-689.e8 (2018).
260. Greenlee-Wacker, M.C., Galvan, M.D. & Bohlson, S.S. CD93: recent advances and implications in disease. *Curr Drug Targets* **13**, 411-420 (2012).
261. Kinstrie, R. et al. CD93 Is a Novel Biomarker of Leukemia Stem Cells in Chronic Myeloid Leukemia. *Blood* **126**, 49-49 (2015).
262. Hosen, N. et al. CD96 is a leukemic stem cell-specific marker in human acute myeloid leukemia. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 11008-11013 (2007).
263. Chung, S.S. et al. CD99 is a therapeutic target on disease stem cells in myeloid malignancies. *Science Translational Medicine* **9**, pii: eaaj2025 (2017).
264. Celik, H. et al. Discovery of first-in-class small molecule CD99 inhibitors for targeted therapy of Ewing sarcoma. *Cancer Research* **77**, Abstract nr 1933 (2017).
265. van Rhenen, A. et al. The novel AML stem cell-associated antigen CLL-1 aids in discrimination between normal and leukemic stem cells. *Blood* **110**, 2659-2666 (2007).
266. Jaras, M. et al. Isolation and killing of candidate chronic myeloid leukemia stem cells by antibody targeting of IL-1 receptor accessory protein. *Proc Natl Acad Sci U S A* **107**, 16280-16285 (2010).
267. Askmyr, M. et al. Selective killing of candidate AML stem cells by antibody targeting of IL1RAP. *Blood* **121**, 3709-3713 (2013).
268. Kikushige, Y. et al. TIM-3 Is a Promising Target to Selectively Kill Acute Myeloid Leukemia Stem Cells. *Cell Stem Cell* **7**, 708-717 (2010).

Table 1: Ongoing CML clinical trials evaluating novel agents.*

DRUG‡	TARGET	PATIENT POPULATION	TRIAL REFERENCE§	PRE-CLINICAL EVIDENCE†
Asciminib (ABL001)	BCR-ABL1	CML (all phases) Ph^+ ALL (relapse/refractory) CP CML (TKI failure)	NCT02081378 NCT03106779	[222]
PF-114	BCR-ABL1	CML (all phases; TKI-resistant or T315I)	NCT02885766	[171]
Ruxolitinib	JAK1 and JAK2	CP CML CML (all phases) CP CML CP CML CML	NCT01702064 NCT02253277 NCT02973711 NCT03654768 NCT01751425	[223]
ETC-1907206	MNK1 and MNK2	AP and BP CML Ph^+ and Ph^- ALL	NCT03414450	[224]
IFN-α (AOP2014 or P1101)	IFNα receptor (IFNAR)	CML (in remission) CP CML (newly diagnosed) CP CML (newly diagnosed)	NCT03117816 NCT02201459 NCT01657604	[225]

		CP CML	NCT01933906	
BP1001 (antisense oligonucleotide)	GRB2 mRNA	CML	NCT02923986	226
Pioglitazone	PPARγ	CML (relapsed) CP CML (in deep remission; TFR) CP CML CML (all phases) and Ph⁺ ALL	NCT02889003 NCT02852486 NCT02767063 NCT02730195	63
Axitinib	BCR-ABL1	CML (all phases - resistance)	NCT02782403	227
Venetoclax	BCL-2	CP CML (newly diagnosed)	NCT02689440	210
Avelumab	PD-L1	CP CML	NCT02767063	228
Nivolumab	PD-1	CP and AP CML (TKI failure)	NCT02011945	228
Inecalcitol	Vitamin D analogue	CP CML (not in deep remission)	NCT02949570	229

*A detailed description of all current/potential AML therapies in clinical trials is beyond the scope of this review.

‡All novel agents shown in the table, with the exception of PF-114, are being tested in combination with at least one or more standard-of-care TKIs that target BCR-ABL1 (imatinib, dasatinib, nilotinib, bosutinib or ponatinib).

† With the exception of Asciminib, PF-114 and Axitinib, pre-clinical evidence includes evidence of activity against LSCs. .

§Trial reference refers to IDs found at https://clinicaltrials.gov/.

CML milestones	Type	Date	Reference
busulfan	clinical	1953	230
Philadelphia chromosome identified	scientific	1961	1, 2
hydroxyurea	clinical	1964	231
allogeneic stem cell transplant	clinical	1978	232
evidence of LSC clonal origin	scientific	1974	19
interferon α	clinical	1983	233
BCR-ABL1 fusion identified	scientific	1983	3
evidence of LSCs in long-term co-culture	scientific	1992	42
quiescent Ph⁺ LSCs found in patients	scientific	1999	22
imatinib	clinical	2002	179
LSCs insensitive to TKIs *in vitro*	scientific	2002	117
LSCs detected in patients on TKIs	scientific	2003	26
dasatinib and nilotinib	clinical	2006-2007	234, 235
~10% CML patients safely discontinue TKI treatment	clinical	2010	8, 9
BCR-ABL1 kinase independent LSCs	scientific	2011	121, 122
bosutinib and ponatinib	clinical	2012	169, 236
altered epigenomes in LSCs	scientific	2016	93
LSCs dependent on OXPHOS	scientific	2017	71
single cell LSC transcriptomes	scientific	2017	120
CML patient 10-year survival > 90%	clinical	2017	7

AML milestones	Type	Date	Reference
prednisone, 6-mercaptopurine, methotrexate, vincristine	clinical	1965	237
slow cycling cells in patients	scientific	1963	17
7 + 3 chemotherapy	clinical	1973	238
allogeneic stem cell transplants	clinical	1977	239
engraftment of human LSCs in mice	scientific	1994	21
evidence of LSCs in long-term co-culture	scientific	1997	41
LSC clones are hierarchical	scientific	1997	30
CD33 Ab-drug conjugate gemtuzumab ozogamicin	clinical	2000	219
LSC mRNA signatures predict survival	scientific	2011	35
altered epigenomes in LSCs	scientific	2011	133
pre-leukemic HSCs identified	scientific	2012	96, 97
LSCs dependent on OXPHOS	scientific	2013	75
LSC clonal evolution at relapse	scientific	2017	123
quiescent LSCs sensitive to therapy	scientific	2017	28, 29
midostaurin (FLT3), enasidenib (IDH2), ivosidenib (IDH1), venetoclax (BCL-2)	clinical	2017	163, 195-197, 207, 208, 240
AML patient 5-year survival < 30%	clinical	2019	16

Figure 1. Scientific and clinical milestones relevant to CML and AML LSCs. The timelines highlight important milestones in CML and AML LSC research and track them chronologically alongside milestones linked to clinical care – some of which are referred to in the text. Relevant references linked to scientific and clinical milestones are noted on the figure. Clinical milestones for the various treatments shown on the timeline are dated from when they were first approved for, or became routinely used as, standard-of-care. 7 + 3 chemotherapy used in the treatment of AML refers to 7 days of cytarabine followed by 3 days of anthracycline. Currently, imatinib is front-line therapy for CML, whilst dasatinib, nilotinib, bosutinib and ponatinib are approved second-line therapies for patients with resistance or intolerance to imatinib. All five of these TKIs inhibit BCR-ABL1 kinase activity and have efficacy, to varying degrees, against BCR-ABL1 kinase domain mutations. Ponatinib is the only TKI with efficacy against the BCR-ABL1 T315I mutation.

Figure 2. The evolving LSC. Models for how CML and AML LSCs arise, and evolve during disease progression and/or as a result of therapies (see further discussion and references in the main text). CP-CML LSCs arise when BCR-ABL1 is acquired

in normal hemangioblasts and HSCs, which decreases their self-renewal potential. In BP-CML or AML, LSCs can arise from either HSCs or more mature normal progenitor cells. When arising from progenitors, evidence suggests that LSCs would need to acquire self-renewal capacity first via pre-leukemic 1st hits (for example, mutations in DNMT3A or IDH1 amongst others), followed by acquisition of oncogenic 2nd hits (for example, the BCR-ABL1 fusion protein or FLT3 mutations). Alternatively, BP-CML can arise via disease progression from a minor CP-CML clone that has acquired one or more 2nd hits (such as mutations in DNMT3A or IDH1). The possibility also exists that LSCs arising from HSCs can also acquire new mutations in their more mature progeny giving rise to additional LSC clones – but this is not depicted in this figure for simplicity. As a result of increased ROS and DNA damage, LSC clones diversify further by acquiring additional mutations – the exception being most cases of CP-CML, where mutation burden remains low. In the absence of additional mutations in CP-CML LSCs, robust responses to TKI treatment lead to residual disease being maintained by TKI-insensitive quiescent LSCs. Complete or partial resistance to TKIs can also arise from clonal selection of a minor clone carrying BCR-ABL1 kinase mutations such as T315I that expand during TKI therapy. In AML, disease relapse after 7 days of cytarabine followed by 3 days of anthracycline (7 + 3 chemotherapy) is driven by the major founder clone which has acquired additional mutations, or through expansion of a minor founder clone. In BP-CML, response to a TKI is usually transient, and relapsed BP-CML is likely to follow similar clonal evolution as relapsed AML.

Figure 3. The epigenetic and metabolic axis in LSCs. The schematic diagram depicts many of the known relationships between glycolysis, the TCA cycle, fatty acid and amino acid metabolism, and epigenetic processes[150, 241, 242] in eukaryotic

cells. Exemplar proteins that have been shown to be survival factors or mutated in CML and/or AML (reviewed elsewhere[83, 144, 243]) and with known roles in metabolism or epigenetic processes are shown (purple ovals). In CML LSCs, free fatty acid (linoleic acid, stearic acid and oleic acid) levels are reduced, while carnitine and acetylcarnitine are increased – suggestive of increased lipolysis and fatty acid oxidation[71]. This process is believed to be linked to expression of the fatty acid transporter CD36 in atypical LSC niches[151]. Enzymes involved in fatty acid metabolism (ALOX5 and ALOX15) are known survival factors in CML[165, 166] – although their roles in CML LSC metabolism is not known. Production of acetyl CoA in CML LSCs is thought to be derived from both fatty acid oxidation and shunting of pyruvate (an NAD^+-dependent process) derived from glycolysis[71] - the net effects of which are increased reliance on the TCA cycle and OXPHOS. Within the TCA cycle, the effects of wild type (WT) and mutant (MUT) IDH1 and IDH2 forms are well studied in AML[153, 154, 244]. In this and other respects, effects of altered levels of metabolites impact directly on the function of epigenetic regulators in CML and AML. These include SIRT1, which mediates NAD+-dependent histone and non-histone deacetylation; histone acetyltransferases (KAT3A and KAT3B), which utilise acetyl groups (Ac) from acetyl CoA to acetylate histone and non-histone targets; the inhibitory effects of 2-hydroxyglutarate (2-HG) on histone demethylation (KDM6A) and the hydroxylation (OH) of 5-methylcytosine (by TET1 and TET2) that leads to DNA demethylation; DNA and histone methyltransferases (DNMT3A, EZH2, DOT1L) which utilise methyl groups (Me) from S-adenosyl methionine produced via amino acid metabolism. DNA damage arising as a consequence of OXPHOS-driven ROS production by mitochondria is also shown. α-KG, alpha-ketoglutarate.

Box 1. Selective cell surface markers in CML and AML LSCs.

For many years, normal HSCs and LSCs from CML or AML patient samples have been purified by fluorescence-activated cell sorting (FACS) using the same sets of cell surface markers - making them immunophenotypically indistinguishable. In recent years, several LSC-selective cell surface markers have been identified that may serve as biomarkers for LSC enrichment or for disease progression, or as drug targets. The potential utility of these cell surface markers as drug targets is exemplified by gemtuzumab ozogamicin (GO) - the first humanised anti-CD33 monoclonal antibody conjugated to the toxic antibiotic calicheamicin (see the table). GO leads to improved outcomes in combination with conventional chemotherapy in favourable and intermediate risk AML patients[219]. Although pre-clinical evidence in acute promyelocytic leukemia would predict that GO is more effective on leukemias where the LSCs have emerged from more mature normal progenitor cells[245], the situation is likely to be different in favourable risk AML patients. In these patients, the efficacy of GO could be explained if the $CD33^-$ pre-leukemic LSCs are non-proliferative, and further mutations leading to leukemic transformation occur in a more mature immunophenotypic $CD33^+$ LSC compartment[246]. In this scenario, the mature LSCs would be eradicated by GO, but the non-proliferative pre-LSCs would remain, resulting in relapse in the absence of additional consolidation therapy. Recent preclinical studies have also explored the possibility of using gene-edited stem cells to enable CD33-directed immunotherapies in AML[247]. Interestingly, preliminary preclinical studies of GO in CML LSCs showed efficacy[248], and isolated case reports have demonstrated clinical responses[249].

MARKER	ALIAS	DESCRIPTION	INHIBITOR(S)	LEUKEMIA TYPE	REF.
CD9	TSPAN29	leukocyte antigen MIC3	ALB6 (anti-CD9 monoclonal Ab);	CML, AML	250, 251

			PAINS-13 (anti-CD9 monoclonal Ab)		
CD25	IL2RA	α-chain of the high-affinity IL-2 receptor	denileukin diftitox (IL-2 and diphtheria toxin fusion protein)	CML, AML	252, 253
CD26	DPP4	serine exopeptidase S9B family member.	gliptins (e.g. sitagliptin)	CML, AML (FLT3-ITD)	254
CD32	FCGR2A, FCGR2B, FCGR2C	Fc-γ receptor II	clone 2B6 (anti-FCGR2B monoclonal Ab); MGD010 (CD32B and CD79B DART bi-specific Ab-based molecule)	AML	253
CD33	SIGLEC3	myeloid cell surface antigen	vadastuximab talirine (Ab-drug conjugate); gemtuzumab ozogamicin (Ab-drug conjugate)	AML, CML	250, 255
CD47	MER6	receptor for SIRPα	Hu5F9-G4 (anti-CD47 monoclonal Ab); TTI-621 (SIRPα binding domain and IgG Fc fusion protein); INBRX-103 (anti-CD47 monoclonal Ab)	BP-CML, AML	256, 257
CD52	HE5	Campath-1 antigen	alemtuzumab (anti-CD52 monoclonal Ab)	BP-CML, AML	258
CD82	TSPAN27	tetraspanin-27	none	AML	259
CD93	MXRA4	complement component 1 Q subcomponent receptor 1	none	CML, AML	260, 261
CD96	TACTILE	T-cell surface protein tactile	none	AML	262
CD97	ADGRE5	adhesion G protein-coupled receptor E5	troglitazone; retinoic acid	AML	259
CD99	MIC2	T-cell surface glycoprotein E2	clofarabine; cladribine	AML	263, 264
CD103	ITGAE	integrin subunit αE	none	AML	259
CD123	IL3RA	interleukin 3 receptor subunit α	SGN-123A (Ab-drug conjugate); IMGN632 (Ab-drug conjugate)	AML, CML	250
CD371	CLEC12A	C-type lectin	MCLA-117 (bi-	AML, CML	250, 265

		domain family 12 member A	specific CLEC12A and CD3 Ab)		
IL1RAP	IL1R3	interleukin 1 receptor accessory protein	IL1RAP monoclonal Ab	CML, AML	266, 267
TIM3	HAVCR2	hepatitis A virus cellular receptor 2	TSR-022 (anti-TIM3 monoclonal Ab)	AML	268

Glossary

Philadelphia chromosome: The chromosomal abnormality characteristic of CML cells that arises from a reciprocal translocation between chromosomes 9 and 22.

relative survival rate: the percentage survival of individuals with leukemia after taking into account death rates of individuals without leukemia.

immunophenotype: a population of cells characterized by markers expressed on the cell surface as determined by flow cytometry.

chimerism: the relative proportions of human to mouse cells that result when human cells are engrafted into the bone marrow of immunocompromised mice.

clonal hematopoeisis of indeterminant potential: a process that occurs in some older individuals whereby DNA mutations in HSCs result in the accumulation of clonally expanded populations of hematopoietic cells**.**

hemangioblasts: multipotent precursor cells that can give rise to both HSCs and enthothelial cells.

oxidative phosphorylation: the mitochondrial process in which electrons are transferred from NADH or $FADH_2$ to oxygen (O_2) through a series of carriers and protein complexes to produce ATP from ADP and phosphate.

BH3-mimetic: one of a class of compounds that bind to a hydrophobic groove found in anti-apoptotic proteins and prevents them from binding to the BH3 domain of the BCL-2 family of proteins.