A rare subgroup of leukemia stem cells harbours relapse-inducing potential in acute lymphoblastic leukemia

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Abstract:

After initially successful chemotherapy, relapse frequently jeopardizes the outcome of patients with acute leukemia. Leukemia stem cells, owing to their adverse characteristics of self-renewal and dormancy have been hypothesized to play a critical role in resistance to antiproliferative chemotherapy and the development of relapse. The high abundance of stem-like cells in acute lymphoblastic leukemia (ALL) however suggests that not all leukemia-initiating cells carry these adverse characteristics, complicating the biological characterization of relapse-inducing cells in this malignancy. Here, we review sources of therapy resistance and relapse in acute leukemias, which include tumor cell plasticity and reversible characteristics. We discuss the development of patient-derived (PDX) mouse models that are genetically engineered to mimic long-term dormancy and minimal residual disease in patients. These models allow the tracking and functional characterization of patient-derived ALL blasts that combine the properties of long-term dormancy, treatment resistance, and stemness. Finally, we discuss possible therapeutic avenues to target the functional plasticity of leukemia-initiating cells in ALL.

Highlights:

- Stemness and clonal architecture are insufficient criteria to define relapse-inducing cells in ALL
- Genetically engineered PDX mouse models are now available, which enable detailed studies on long-term dormant, treatment resistant leukemia-initiating cells
- Only a rare subset of leukemia-initiating cells in ALL is dormant and treatment resistant
- Dormancy and drug resistance are reversible characteristics of ALL leukemia-initiating cells

Cancer stem cells as a source for therapy resistance and relapse

Acute leukemia of lymphoid (acute lymphoblastic leukemia; ALL) or myeloid (acute myeloid leukemia; AML) lineage arise from hematopoietic stem or progenitor cells in the bone marrow. Patients with acute leukemia often achieve complete remission with induction chemotherapy, yet many will experience relapse, which poses a major obstacle to cure [1,2]. Leukemia relapse has been suggested to arise from a small subpopulation of cells that survive chemotherapy, persist as minimal residual disease (MRD) in complete remission, and ultimately re-initiate malignant growth. Although MRD detection in acute leukemia has a strong prognostic value [1,2], MRD is not an absolute marker of relapse, as not all residual cells may have the functional capability to proliferate into relapse [3]. Accordingly, relapse-inducing cells are a "subpopulation" of MRD cells with important functional properties, including the ability to repopulate the tumor. One possible explanation for this clinical behaviour can be provided by the cancer stem cell model, which is based on the idea that cancers, like normal tissues, are maintained by a rare, biologically distinct subpopulation of cells that have the capacity for long-term tumor propagation and self-renewal, and give rise to progeny that lack these characteristics. Such cancer stem cells are thought to be highly resistant to therapeutic regimens, survive chemotherapy and ultimately, lead to relapse [4,5].

The conceptual framework of a leukemia stem cell model is based on a putative analogy between normal and malignant hematopoiesis. Normal hematopoiesis is maintained by hematopoietic stem cells (HSC), a rare subpopulations of bone marrow cells with multi-lineage differentiation and self-renewal capacity. HSC give rise to highly proliferating hematopoietic progenitor cells, which generate all mature blood cells [6]. Different cell types within the bone marrow, including mesenchymal stem cells (MSCs; cells that form adipocytes, osteoblasts and chondrocytes), endothelial cells as well as nerve fibers form the stem cell niche, a specialized microenvironment that allows HSC maintenance [7,8]. At the top of the hematopoietic hierarchy are extremely slow cycling, long-term dormant HSC which carry the highest reconstitution potential [9-14]. Their contribution to steady state blood formation may be limited as it is mediated by more actively cycling HSC with restricted long-term propagation potential. The dormant HSC subsets can readily be activated upon stress, for example by interferons, lipopolysaccharides, or chemotherapy among others, and significantly contribute to repair processes [8,15]. Importantly, the transition from dormant

to active HSC is a reversible process and HSC can return into a deep quiescent state following stress [9,14], at least for a limited number of cell divisions [12]. Dormancy of adult stem cells has been suggested as a means to survive chemotherapeutic and other DNA-damaging challenges [9,16], limit the accumulation of mutations, and thus, serve as a reserve stem cell pool to maintain normal hematopoiesis following stress [8].

Xenotransplantation of AML cells in immunodeficient mice have significantly contributed to the formal verification of a hierarchical organization of cancer, as these studies demonstrated that rare stem-like cells with self-renewal capacity sustain AML. Such stem-like cells are termed leukemia-initiating cells (LIC) and are enriched within the $CD34^+CD38^-$ immunophenotype [17-19]. Although more sophisticated mouse models demonstrated that other fractions ($CD34^+CD38^+$ and also $CD34^-$) can also contain LIC in AML patient samples, it is now widely accepted that AML and numerous other malignancies are hierarchically organized [4]. In analogy to HSC, AML-LIC are functionally heterogeneous in terms of self-renewal potential [20], and heterogeneous in terms of growth kinetics, wherein at least a subset of LIC are maintained in a quiescent state [21]. Of therapeutic relevance is the fact that AML-LIC display a similar transcriptional profile as HSC, which is a powerful indicator of resistance to standard therapy [22]. Furthermore, AML-LIC frequency at diagnosis is indicative of a poor outcome [23], and frequency of LIC also increases following therapy, providing an explanation for the notorious drug resistance of relapsed AML [24]. In agreement, experiments in pre-clinical models strongly support the notion that AML-LIC are the source of relapse in AML [25,26]. Especially the quiescence of AML-LIC has been associated with resistance to anti-proliferative chemotherapy [27-29]. Consequently, efforts to break the dormancy of these cells in order to sensitize them to anti-proliferative chemotherapy were suggested as a valuable means to eradicate LIC and achieve long-term remissions [28,29].

Although various similarities in the functional behaviour of AML-LIC and HSC have been established, these results are not readily transferable to all hematological malignancies. In ALL, no markers could be identified to prospectively isolate LIC, and a high proportion of cells among various immunophenotypes display leukemia-initiating potential in xenotransplantation experiments [30-33]. Therefore, the hierarchical stem cell model of AML does not apply to ALL, where tumor progression/relapse may be a stochastic process. Although highly similar regarding the clinical course of treatment response, MRD and

relapse, the two sister malignancies ALL and AML show major differences concerning LIC biology.

Sources for therapy resistance and relapse in ALL

As stemness is an insufficient criterion to define the subpopulation of relapse-inducing cells in ALL, the basic biological conditions that determine relapse remain to be defined.

Genomic and functional analysis of ALL patient samples revealed a branching evolutionary pattern, and thus, multiple subclones co-exist at the time of diagnosis, which show functional diversity in terms of leukemia-initiating potential [34-36]. Such genetic diversity can provide a basis for the clonal selection of chemo-resistant clones by therapeutic regimens. In agreement, genomic studies of paired diagnosis and relapse samples demonstrated that at relapse, ALL blasts are derived from minor subclones present at diagnosis, or evolves from ancestral diagnostic clones, but rarely from the main diagnostic clone [37-40]. Multiple genetic variants that confer resistance to standard therapy are enriched in relapsed ALL samples. These include *NT5C2* [41,42] and *PRPS1* [43] that mediate resistance to genotoxic agents; Alterations in *CREBBP* [44] contribute to glucocorticoid resistance and CDKN2A/B [1,36,39] or RAS pathway [40,45] alterations that are associated with resistance to multiple drugs. However, sequence analysis alone may not reveal all features of relapse-inducing cells in ALL. For example, although RAS pathway mutations are associated with poor prognosis [40,45], their presence at diagnosis does not strictly predict relapse: some mutations in this pathway can be lost at relapse, or are present in diagnostic samples that never relapse [37,45,46]. This, together with the facts that i) pediatric and haematological cancers have the lowest somatic mutation rate and most cases of ALL are genetically stable [47,48], and ii) not all relapses are derived from minority subclones, but can sometimes derive from the major diagnostic clone [37-40,49,50], strongly suggest that non-genetic factors significantly contribute to relapse. While hardwired genetic mechanisms may account for refractory disease and early relapse, different mechanisms may account for late relapses that can occur even decades after the initial diagnosis [49,51]. Therefore, both clonal architecture as well as stemness remain insufficient criteria to define relapse-inducing cells in ALL.

Cellular plasticity in epigenetic programs (which is influenced by the genetic background as well as the tumor microenvironment) is considered the underlying cause of functional

heterogeneity in cancer [5,52,53]. In response to appropriate environmental stimuli, tumor cells may be temporarily endowed with certain traits, including tumor- / leukemia-initiating capacity and therapy resistance, as has been suggested in diverse cancers [54-59]. The reversibility of drug resistance for example has been demonstrated in various cancer cell lines in response to targeted EGFR or BRAF inhibitors or cisplatin [58], as well as in T-ALL xenografts following γ-secretase inhibition [54]. Interestingly, the number of these drug-tolerant persisting cells can be decreased and establishment of resistance prevented *in vitro* by simultaneous treatment with histone deacetylase (HDAC) inhibitors [58]. Similarly, in patient-derived xenografts of pediatric T-ALL, combined inhibition of NOTCH and BRD4, a chromatin regulator upregulated in persistent T-ALL cells, significantly prolonged survival as compared to single agent treatment [54]. The interaction of tumor cells with the microenvironment has been suggested as a major regulator of such reversible traits [5,52,53]. Evidence that environment-mediated drug resistance may protect ALL blasts from therapy comes from *in vitro* experiments, wherein the interaction of ALL blasts with stromal cells is essential for their survival *in vitro* and can protect blasts against asparaginase and prednisolone [60,61]. Accordingly, safe-haven niches that protect leukemic blasts from chemotherapy have been identified in the bone marrow *in vivo*, wherein mesenchymal stem cells [62], osteoblasts [63] or other stromal cell types in the bone marrow microenvironment [64] protect leukemic blasts from therapy. In response to chemotherapy, ALL blasts can also actively remodel their environment via the recruitment of mesenchymal cells, and the establishment of such a protective niche can be found in patient samples of partial- or non-responders [62].

Interestingly, regardless of the model tested, quiescence of cancer cells seems to be a common characteristic of therapy-resistant subpopulations: Quiescent glioblastoma stem cells survive chemotherapy and re-initiate malignant growth following remission [65], and in diverse cancer cell lines, quiescent, drug-tolerant cells persist during the course of therapy [58]. Additionally, quiescent cells survive oncogene ablation in a mouse model of pancreatic cancer [59], and in colorectal cancer xenografts, a previously dormant cell population becomes dominant following chemotherapy [55]. Similarly, slow-cycling melanoma cells display a general drug resistant phenotype [56,57] and a subpopulation of slow-cycling TGFβ-responsive squamous cell carcinoma stem cells survive chemotherapy and induce relapse in xenotransplantation experiments [66]. These studies suggest that regardless of

the presence or absence of a strict hierarchical organization, cancer cells can temporarily adapt a quiescent and drug-resistant state, which similar to adult stem cells, may be regulated by microenvironmental cues, including therapeutic regimens [5,29]. Evidence that ALL blasts survive chemotherapy by adapting a quiescent state in response to microenvironmental cues has been provided in pre-clinical models [63]. The hypothesis that quiescent therapy-persisting ALL cells may be the source for relapse is further supported by the fact that therapy resistant cells isolated from patients at MRD are predominantly quiescent [67]. Given the accumulating evidence that quiescence may be an attribute of relapse-inducing cells, we set out to develop a model that allows us to functionally characterize quiescent cells in primary ALL patient samples [68]. We hypothesized that such a model would allow us to study biological characteristics of relapse-inducing cells in ALL and help to resolve questions of translational importance: Do these traits already exist at diagnosis or are they acquired during therapy? Are these traits reversible or permanent?

A PDX model to track quiescent ALL cells over the course of therapy

In order to be able to track and isolate small numbers of viable PDX ALL cells from the bone marrow of mice, we utilized our previously established lentiviral transduction protocols and ectopically expressed luciferase [69], a truncated nerve growth factor receptor (NGFR) and a red fluorophore [70] in patient-derived xenograft (PDX) ALL blasts. This triple labelling of ALL PDX cells allows us to track their *in vivo* growth in mice and enables the isolation of minute numbers of viable human ALL cells from the murine bone marrow [68]. Following one passage in mice for enrichment of transgene expressing cells, lentivirally transduced PDX ALL cells were stained with carboxyfluorescein succinimidyl ester (CFSE). This fluorescent dye has been useful to track cell divisions of HSC in mice over several month [14]. CSFE labels cytoplasmic proteins and is thus independent of the cell cycle status, is equally distributed to daughter cells with each cell division, and importantly, does in contrast to other proliferation dyes (e.g. BrdU) not require permeabilization of cells for staining [14]. Therefore, the combined use of lentiviral transduction and CSFE labelling allows for tracking and isolation of non-dividing viable cells from the *in vivo* environment.

Upon transplantation into immunodeficient recipient mice, we identified ALL cells that retained the CSFE dye up to three weeks, shortly before mice had to be sacrificed due to high leukemia burden. We could identify a rare subpopulation of these label-retaining cells

(LRC), which had undergone no more than three cell divisions, from all nine transgenic ALL-PDX samples analysed [68]. Immunohistochemical analysis revealed that LRC, as compared to non-LRC, are preferentially found close to the endosteum, a niche that has previously been suggested to regulate quiescence and resistance to therapy of LIC *in vivo* [27,28,63,71].

Functional characterization of LRC in ALL-PDX

Given the putative link between quiescence, stemness and chemoresistance, we first characterized leukemia-initiating potential of LRC and evaluated how LRC respond to chemotherapy [68]. LIC frequency was very similar between the LRC and non-LRC compartments, which is in agreement with the stochastic stem cell model proposed for ALL, wherein most cells are capable to propagate the tumor [33]. Yet, these results demonstrate phenotypic heterogeneity within the LIC compartment, wherein a rare subpopulation of LIC is quiescent. To test whether treatment resistance and dormancy are linked, we treated leukemia-bearing mice with chemotherapy, which strongly selected for LRC, while most non-LRC were eradicated [68]. Importantly, surviving LRC retained the ability to propagate the leukemia upon re-transplantation into secondary mice, and formed tumors with similar growth kinetics as untreated LRC. Thus, LRC share the combined features of dormancy, drug resistance and stemness, indicating that these cells may serve as preclinical surrogates for relapse-inducing cells in ALL [68] (**Figure 1**).

To further characterize molecular features of LRC and strengthen the link to MRD, LRC and MRD cells established from our PDX model (PDX-MRD) were subjected to single cell RNA sequencing. Gene expression profiles of single or bulk leukemia cells of untreated LRC and PDX-MRD demonstrated that overall RNA content was decreased in LRC, which is indicative of a low metabolic activity, a defining characteristic of dormant cells. Furthermore, in both LRC and PDX-MRD, cell cycle and DNA replication were the most downregulated pathways. Thus, transcriptomic analysis reinforced the presence of a dormancy phenotype in LRC and PDX-MRD (**Figure 1**). Interestingly, the most upregulated gene network in LRC and PDX-MRD was cell adhesion, indicating that the interaction with the microenvironment might mediate the observed phenotypes [68] (**Figure 1**). The established LRC signature was also identified in primary MRD cells isolated from adult or pediatric patients, and differentially regulated genes of published transcriptomes of CD34-positive chronic myeloid leukemia cells, of acute leukemia stem cells, of hematopoietic stem cells, as well as of pediatric ALL cells with high

risk of relapse were significantly enriched in LRC [68]. Importantly, the phenotypes of both dormancy and therapy resistance of LRC were reversible: when LRC and non-LRC were transplanted into secondary mice, LRC gave rise to tumors with similar growth kinetics as non-LRC and vice versa, non-LRC gave rise to tumors that contained a quiescent subpopulation at a similar frequency as observed following transplantation of unsorted leukemic cells (**Figure 1**). When LRC or non-LRC were treated with chemotherapeutic drugs *in vitro*, they demonstrated similar cell death rates, indicating that the release from the bone marrow microenvironment sensitizes LRC to therapy. Accordingly, co-culture of LRC, non-LRC or unsorted bulk cells with feeder cells reduced drug sensitivity, strongly suggesting a role for the bone marrow microenvironment in determining drug resistance.

In summary, our studies demonstrated that there is significant functional heterogeneity within the abundant LIC population in ALL, wherein only a rare subpopulation of ALL-LIC displays the adverse phenotypes of quiescence and drug resistance. These LRC exist before the onset of therapy, and their adverse phenotypes are reversible. Additionally, the high similarity of LRC and MRD cells isolated from paediatric and adult patients strongly suggests that LRC can be used as surrogates for relapse-inducing cells in patients [68,71]. The presence of a quiescent and therapy-resistant subpopulation of LIC in ALL has important implications for therapy. Especially the fact the adverse phenotypes of LIC are reversible, once released from the protective bone marrow environment, offers therapeutic opportunities.

Therapeutic implications:

Environment-mediated drug resistance provides an explanation for the clinical observation that maintenance therapy with low dose oral chemotherapy for about two years is required, even in low-risk patient groups, to prevent relapse in ALL [72]. However, maintenance therapy is not always sufficient and relapse might occur under treatment. As even patients that maintained complete remission over several years can relapse, targeting long-term dormant residual cells represents an important unmet clinical need in order to increase the chance for long-term remission and cure (**Figure 2**).

Multiple routes can be envisioned to target LRC (**Figure 2**): It has long been known that leukemic cells depend on stromal support for growth and survival, which is mediated by

direct adhesion and soluble factors, that can affect treatment response [60-64,73-77] and our data also strongly suggests that the release of ALL cells from the bone marrow environment sensitizes leukemic blasts to conventional therapies [68]. Inspired by the discovery of agents that can activate dormant HSC [29], blocking the CXCL12/CXCR4 axis, inhibition of osteopontin, cell adhesion molecules, and treatment with G-CSF have been demonstrated to affect the homing of leukemic cells to the bone marrow and influence therapeutic response in experimental models [28,63,76,78-80] (**Figure 2**). Plerixafor and G-CSF, which are approved for HSC mobilization, or Natalizumab, a monoclonal antibody to block integrin α_4, are promising candidates to evaluate in LRC-specific combination therapy [81-85]. However, as leukemic growth and chemotherapy induces massive remodelling of the stromal bone marrow environment [62,86-89], signals that regulate LIC homing likely differ from those regulating HSC. The remodelled niches can furthermore be specific for distinct leukemia subtypes [89,90], and may change over the course of disease progression or following therapy. Accordingly, leukemia-specific signals will need to be identified in order to efficiently induce cell cycle entry of all leukemic blasts. In this context, CRISPR-Cas9 edited NGS mice may serve useful in elucidating the role of individual niche-derived factors [91]. Another point to consider is that real-time *in vivo* monitoring of T-ALL cells within the bone marrow demonstrated a highly motile phenotype of ALL blasts, suggesting that leukemic cells may not permanently engraft in a specific niche [90]. Therefore, interaction between leukemic blasts and their environment are versatile and multiple leukemia-specific factors may need to be targeted therapeutically to achieve an efficient release of all blasts from the bone marrow. Alternatively, one may envision targeting leukemia cell-intrinsic adhesion pathways, with one putative target being focal adhesion kinase (FAK) (**Figure 2**). Small molecule FAK inhibitors such as Dafactinib or GSK2256098 are currently under clinical investigation in solid tumors [92,93] and can be tested for their use to sensitize LRC towards chemotherapy. Inhibition of FAK signalling has recently been demonstrated to disrupt cell adhesion in highly aggressive Philadelphia-Chromosome positive, IKZF1-mutated ALL mouse models and FAK inhibition combined with a BCR-ABL1 inhibitor effectively abrogated leukemic cell growth *in vivo* [94].

Instead of addressing the interaction between leukemic blasts and the environment, direct targeting of quiescent cells is another possibility to eradicate long-term persisting LIC (**Figure 2**). Metabolic vulnerabilities of quiescent drug-tolerant persisting cells have been identified,

and targeting for example their reliance on either oxidative phosphorylation (OXPHOS) [56,57,59] or an increased antioxidant capacity [66] may eradicate these cells or render them sensitive to conventional therapy. The bone marrow microenvironment also mediates metabolic adaptions of ALL blasts that support their survival [74], and microenvironment-mediated redox adaptions in ALL blasts reportedly mediate therapy resistance in cell culture models [95]. A multitude of drugs that target either OXPHOS or inhibit antioxidant production are available and have been approved for cancer treatment or non-oncological indications [96]. Possible drugs to test for their capacity to deplete LRCs can include the OXPHOS inhibitors metformin [97,98], or arsenic trioxide [96]. Furthermore, the anti-rheumatic drugs sulfasalazine and auranofin together with the GSH-depleting drug buthionine sulfoximine may serve to sensitize therapy resistant cells to genotoxic agents [99]. The antibiotic tigecycline as well as inhibition of Bcl-2 antiapoptotic proteins have also been shown to excerpt their antitumor-activity by impairing mitochondrial function and increasing oxidative stress [100-102]. These findings provide a rationale for testing tigecycline or the BH3-mimetics navitoclax, venetoclax or S63845 [103-105] for their use as LRC-specific therapies.

Finally, interfering with the epigenetic programs that allow persisting cells to reversibly enter/escape a quiescent and therapy resistant state may be of therapeutic merit (**Figure 2**), as demonstrated in diverse cancer cell lines and a T-ALL xenograft model [54,58]. Several epigenetic therapeutics are either approved or under clinical evaluation, including BET bromodomain inhibitors [106-108].

One important consideration regarding the plasticity of the therapy resistant state is the onset and timing of therapeutic approaches. Although our study has clearly demonstrated that a quiescent and therapy resistant subpopulation of ALL cells exist before the onset of therapy, our experimental setup did not allow to monitor *de novo* emergence of quiescent cells upon treatment initiation [68]. However, as LRC can be derived from non-LRC upon re-transplantation into secondary recipients, it is likely that the quiescent and therapy resistant population can be continuously replenished. If innovative therapeutic strategies succeed in retrieving resistant leukaemia cells from protective niches, sensitive leukaemia cells might most probably take over empty slots and acquire a resistant phenotype. Thus, therapeutic regimens will likely be most effective when administered throughout the course of therapy,

as also suggested by *in vitro* experiments in non-small cell lung cancer cell lines, where only continuous administration of HDAC inhibitors prevented the onset of resistance to EGFR inhibitors [58].

Concluding Remarks:

After initially successful chemotherapy, relapse frequently jeopardizes the outcome of cancer patients. To improve the prognosis of ALL patients, treatment strategies that eliminate tumor cells at MRD and prevent relapse are urgently required. Although ALL lack a clear hierarchical organisation, several adverse phenotypes, including quiescence and chemoresistance, are maintained in a rare subset of ALL-LIC, which are otherwise generally attributed to stem cells. The fact that dormancy and resistance are reversible upon release from the microenvironment indicates that transcriptional reprogramming of ALL-LIC is an early event in the generation of therapy resistance. This process may precede the onset of hardwired irreversible resistance and relapse, and a similar multistep-mechanism of the emergence of resistant disease has recently been suggested under treatment with the immunotoxin Moxetumomab pseudotox [109]. The *in vivo* model described in our recent study [68], in combination with CRISPR-Cas9 edited NGS mice [91] can facilitate dissecting regulatory factors within the bone marrow microenvironment that endow LIC with adverse characteristics. Our models can serve as preclinical tools to test therapeutic strategies that aim to interfere with the interaction between leukemic blasts and their niche in order to prevent relapse and ultimately increase prognosis and cure rate of patients with ALL.

Conflict of interest disclosure

The authors declare no competing financial interest.

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Figures and legends

Figure 1. Plasticity of ALL-LIC:

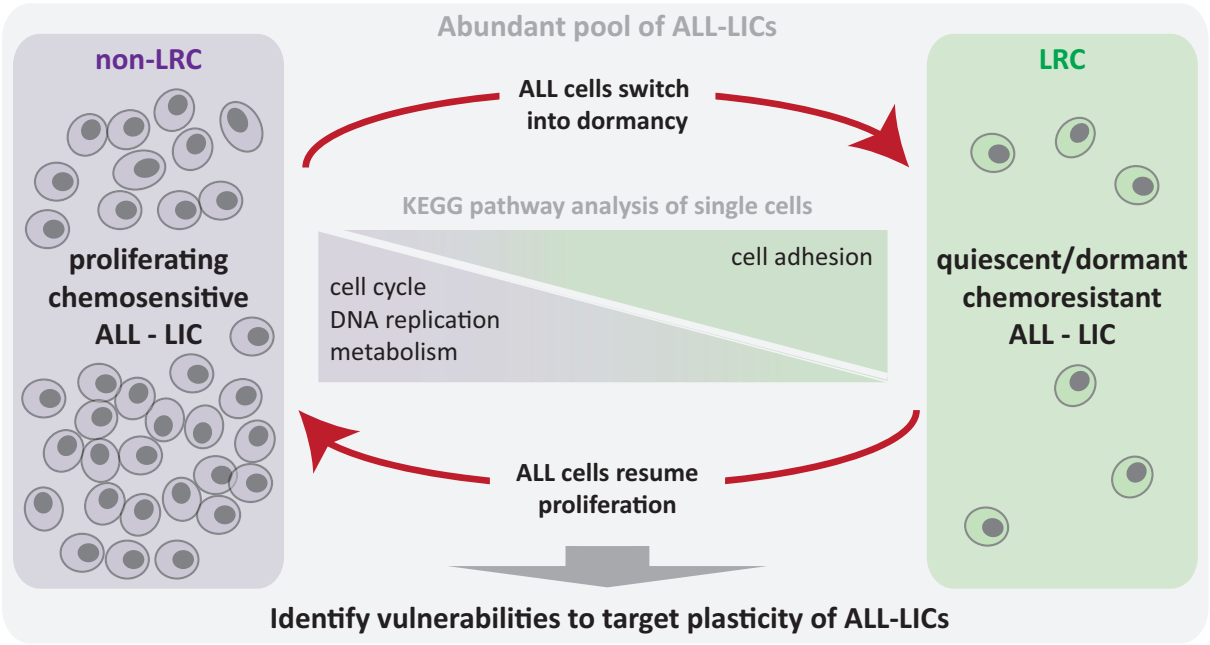

Figure 1. Plasticity of ALL-LIC: Characterization of label-retaining cells (LRC) in acute lymphoblastic leukemia (ALL) patient-derived xenografts revealed that leukemia-initiating cells (LIC) harbour functional plasticity and exist in at least two states within the bone marrow microenvironment; (i) a proliferative, chemosensitive state and (ii) a dormant, chemoresistant state. The adverse phenotypes of dormancy and therapy resistance are reversible upon release from the bone marrow microenvironment, while stemness remains a constant feature. Single cell RNA sequencing analysis demonstrated that the rare subpopulation of LRC is quiescent and the most upregulated pathway is cell adhesion, indicating that the interaction with the microenvironment might influence the dormant phenotype.

Figure 2. Rationale and possible approaches for targeting dormant, therapy resistant ALL-LIC.

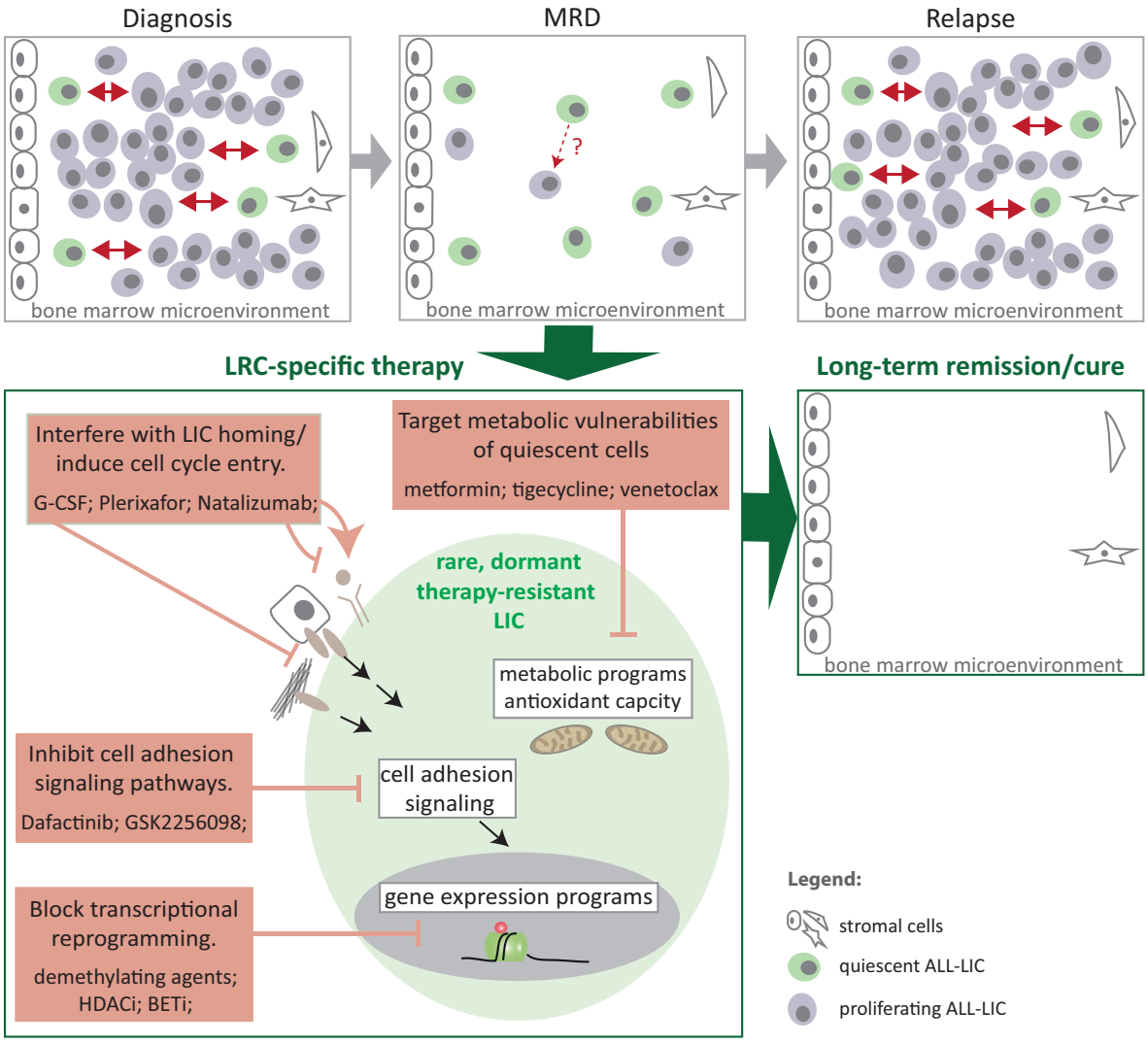

Figure 2. Rationale and possible approaches for targeting dormant, therapy resistant ALL-LIC. The characterization of ALL-PDX models during the course of therapy indicated that quiescent, label-retaining cells (LRC) preferably survive chemotherapy and persist at minimal residual disease (MRD). Dormant cells are already present at the time of diagnosis. Occasional transitions from the quiescent into a chemosensitive, proliferative state (dashed arrow) may explain that long-term maintenance therapy is required to prevent relapse in ALL patients. However, reversibility of the quiescent phenotype (red arrows) allows these LIC to resume proliferation at later time points and proliferate into relapse. Accordingly, therapeutic targeting of this dormant subpopulation of LIC may be necessary and sufficient to prevent the development of relapse in ALL. Possible routes to target therapy-persisting LIC are depicted in the lower left box. Inhibition of LIC-stroma or LIC-ECM interaction, or downstream adhesion signalling, or interfering with soluble factors that home LIC into the bone marrow microenvironment may release cells from the protective niche and render them sensitive to standard chemotherapy. Metabolic vulnerabilities of quiescent LIC provide an alternative target, which may allow eradication of LIC without the need to induce their proliferation. To prevent the *de novo* generation of quiescent, drug-resistant LIC during the

course of therapy, epigenetic programs could be targeted. Some putative drugs to achieve each goal are depicted.

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