Exploitable metabolic dependencies in MLL-ENL–induced leukemia

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Key Points

- MLL-ENL affects glycolysis, preparing cells for high anabolic needs.
- Restricting the endogenous or exogenous Ser/Gly supply retards leukemic cell proliferation in vitro and in vivo.

Mixed-lineage leukemia (MLL) fusions are transcriptional activators that induce leukemia, with a dismal prognosis that mandates further elucidation of their transformation mechanism. In this study, knockdown of the direct MLL-ENL target gene polypyrimidine tract binding protein-1 (PTBP1) was rate limiting for cell proliferation and caused a metabolic phenotype associated with reduced glucose consumption and lactate production. This effect was accompanied by a reduction of splice isoform-2 of pyruvate kinase M (PKM2). Because PKM2 restricts glycolytic outflow to provide anabolic intermediates, we tested the consequences of glucose, energy, and Ser/Gly starvation for cell physiology. Administration of deoxyglucose, energetic decoupling with rotenone, and inhibition of Ser biosynthesis by CBR5884 had a significantly stronger influence on self-renewal and survival of transformed cells than on normal controls. In particular, inhibition of Ser synthesis, which branches off glycolysis caused accumulation of reactive oxygen species, DNA damage, and apoptosis, predominantly in leukemic cells. Depletion of exogenous Ser/Gly affected proliferation and self-renewal of murine and human leukemia samples, even though they are classified as nonessential amino acids. Response to Ser/Gly starvation correlated with glucose transport, but did not involve activation of the AMPK energy homeostasis system. Finally, survival times in transplantation experiments were significantly extended by feeding recipients a Ser/Gly-free diet. These results suggest selective starvation as an option for supportive leukemia treatment.

Introduction

Translocation-derived mixed-lineage leukemia (MLL) fusion proteins occur in up to 10% of all acute leukemias. The presence of rearranged MLL mostly confers a dismal prognosis. Functionally, chimeric MLL proteins subvert universal processes of transcriptional control by altering transcription-associated epigenetic modifications and/or the elongation activity of RNA polymerase itself.1-3 Several potential treatments have been proposed, based either on perturbation of chromatin writers and readers (eg, DOT1L,4 ENL,5 and BRD4b) or on modulating elongation (eg, by inhibiting CDK9).7 Blocking the interaction of MLL with menin, a protein that aids in genomic localization of wild-type and fused MLL, was tested as an alternative approach.8 Instead of targeting MLL fusion–associated functions directly, an alternate strategy is to probe the known transcriptional targets of MLL fusions for essential activities that may be more amenable for therapeutic intervention. This is particularly worthwhile in the case of MLL rearranged (MLL-r) leukemia, because it has been shown that the transforming potential of MLL chimeras is sufficiently strong to induce leukemic transformation, either by itself or in concert with an unusually small number of cooperating mutations.9,10 Thus, unlike disease induced by other hematopoietic oncogenes, MLL-r disease is largely the consequence of a deregulation of the MLL fusion target genes that are essential for cell proliferation and self-renewal.
fusion targets themselves, and it is not confounded by the unknown cooperative effects of several transforming inputs. Genes deregulated by MLL fusions have been identified in several studies.11–13 One likely hallmark of all cancer cells is a shift toward anabolic metabolism to provide building blocks for sustained cell proliferation. Many oncogenes rewire cells to favor glycolysis over oxidative phosphorylation, to channel carbon into biosynthesis rather than oxidizing it into carbon dioxide during oxidative phosphorylation, a phenomenon known as the Warburg effect. Thus, MLL fusions should also control genes with regulatory potential for metabolism, and if these genes are critical for transformation, it may hint of an alternative intervention strategy that targets the metabolic needs of these cells. We found that MLL-ENL activates the splicing factor polypyrimidine tract binding protein-1 (PTBP1) to induce the anabolic state characteristic of transformed cells, and we showed that MLL-ENL–induced leukemia depends on an external supply of Ser/Gly, resulting in a potentially exploitable metabolic vulnerability.

Methods

Cells, media, nucleic acids, and reagents

Primary murine hematopoietic progenitor cells (HSPCs) transformed by MLL-ENL were generated from BALB/c mice by retroviral transduction, as described before.14 The human MLL-AF9 cell line MOLM13 was obtained from DSMZ (ACC554; Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany). Murine cells were cultivated in RPMI supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin, and 1% essential amino acids (all Thermo Fischer Scientific), and recombinant murine cytokines (5 ng/mL IL3, IL6, and GM-CSF and 50 ng/mL SCF; Milenyi, Berg-Gladbach, Germany). MOLM13 was cultivated in cytokine-free medium. In vitro starvation experiments were performed in minimum essential medium supplemented with 10% dialyzed fetal calf serum, penicillin/streptomycin, and 1× essential amino acids (all Thermo Fisher Scientific), yielding a Ser/Gly-free medium. Where appropriate Ser and or Gly (Sigma-Aldrich, Taufkirchen, Germany) were added to a final concentration of 400 μM.

The lentiviral all-in-one vector pLEPIR was modified from the miRNA30 design developed by Fellmann et al15 by standard molecular biology procedures. Constructs were verified by sequencing. Short hairpin target sequences can be cloned into the molecular biology procedures. Constructs were verified by sequencing. Short hairpin target sequences can be cloned into the molecular biology procedures.

CFC assays

For colony-forming cell (CFC) experiments, we incubated cells for 48 hours under test and control conditions. An equal number of cells was subsequently plated in triplicate into methylcellulose (Methocult M3231; Stemcell Technologies, Köln, Germany), supplemented with recombinant cytokines at concentrations identical with those in liquid growth medium. Medium for cells from patients contained recombinant hIL7, hFlt3 ligand, hIL3 (all 10 ng/mL), and murine SCF (100 ng/mL). Colonies were grown in Methocult H4035Optimum without EPO (Stemcell Technologies).

The cells were counted after staining with iodonitrotetrazolium chloride (Sigma-Aldrich) at a final concentration of 0.1 mg/mL by photographic evaluation with the software OpenCFU 3.9.0 (http://www.openfuc.sourceforge.net/). For the use of human samples, informed consent was obtained after institutional review board approval according to local laws and regulations and in accordance with the Declaration of Helsinki.

Cell cycle, apoptosis, and ROS determination

Cell cycle distribution was determined by propidium iodide staining followed by FACS analysis. Apoptosis was measured by annexin V-propidium iodide staining with an apoptosis-detection kit from eBioscience, according to the instructions of the manufacturer (Thermo Fisher Scientific). Cellular reactive oxygen species (ROS) were detected by FACS after staining with CellROX deep red reagent, as recommended by the supplier (Thermo Fisher Scientific).

Glucose and lactate determination: seahorse experiments

The glucose and lactate content of the culture supernatant was determined with a Super GL compact analyzer (Hitado, Dreiahausen, Germany), according to the manufacturer’s guidelines. Glucose transport was measured by starving cells for 2 hours in glucose-free medium followed by addition of 30 μM 2NBDG [2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose; Thermo Fisher Scientific] for 2 minutes. The cells were subsequently washed and analyzed by FACS. Bioenergetics of murine MLL-r cells were assessed with an XF96 Extracellular Flux Analyzer (Agilent Technologies, Waldbronn, Germany), as previously described.16 In brief, cells were harvested after culture and immobilized in a XF96 cell culture plate (Agilent Technologies) using CellTAK (BD, Heidelberg, Germany) at a density of 105 cells per well with 5 to 7 technical replicates. Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were recorded upon sequential addition of glucose, oligomycin, and 2DG for the glycolysis stress test, as well as oligomycin, FCCP, and antimycin A/rotenone for the mitochondrial stress test.

Nascent RNA sequencing

Nascent-RNA sequencing was performed exactly as published.17 NGS library construction was performed using a polyA priming strategy, with the NEBNext Single Cell/Well Input RNA Library Prep Kit, and libraries were sequenced commercially on an Illumina HiSeq, exactly as recommended by the manufacturers. Sequences were mapped with STAR2.5.3a,18 and expression levels were evaluated with Cufflinks2.2.1.19

Transplantation assays

For transplantation experiments, sublethally irradiated (BALB/c; 7 Gy), syngenic recipients were injected with 1 × 10E6 MLL-ENL–transduced cells and 1 × 10E6 total bone marrow cells for radioprotection. Recipients were fed ad libitum with defined chow from TestDiet (Richmond, IN). Animals were supplied with either a control diet (5WA1-BakerAA: 15% sucrose, 8% fat, 15% protein equivalent, fully supplemented with vitamins, minerals, and proteins...
Results

Knockdown of PTBP1 causes a metabolic phenotype

To individually assess the role of direct MLL-ENL targets, as identified in our previous work, we modified an available lentiviral all-in-one tetracycline-inducible short hairpin (shRNA) expression system (Figure 1A). A puromycin cassette was coupled via an IRES sequence to a reverse tetracycline-inducible transactivator to allow for antibiotic selection of the integrated provirus. In addition, the shRNA processing cassette was placed into the 3’ untranslated region of cDNA coding for the extracellular part of the low-affinity nerve growth factor receptor (LNGFR), enabling specific magnetic enrichment and surface staining for quantification of shRNA-expressing cells. This strategy became necessary because we noted that integrated lentiviruses showed strongly variegated expression with respect to the tet-inducible gene. Despite the 100% puromycin-resistant population, not all cells expressed the respective shRNA/LNGFR, which would preclude accurate quantification of knockdown efficiency by quantitative PCR (qPCR), which was therefore performed on LNGFR-selected cell populations. Cell sorting confirmed that the expression state (shRNA on/off) within a cell remained stable over time (supplemental Figure 1A).

After knockdown of the confirmed MLL-ENL target gene and splicing factor Ptbp1/PTBP1 (henceforth PTBP1, if of human and murine origin) in murine MLL-ENL–transformed cells and in the human cell line MOLM13 (derived from an MLL-AF9 leukemia), we saw an unusual phenotype. Reduction of PTBP1 (Figure 1B) not only led to a gradual loss of shRNA-expressing (LNGFR+) cells from mixed cultures (Figure 1C), but shRNA+ cells also displayed a clear metabolic phenotype (Figure 1D). Despite identical density, PTBP1 knockdown cells showed delayed medium acidification compared with the shRNA/LNGFR+ controls. Because lactate is a major acidic metabolite, we examined this phenomenon further by quantifying glucose and lactate levels in supernatants of equally dense cultures of knockdown and control cells (Figure 1E). These experiments corroborated the previous visual observations, indicating that a reduction in PTBP1 reduces glucose consumption and lactate production. Knockdown was accompanied by a weak-to-moderate arrest in G1, but no increase in apoptosis, when compared to the controls (supplemental Figure 1B).

PTBP1 controls splicing of PKM

PTBP1 is a known splicing factor that binds to HNRNPA1 (heterogeneous nuclear RNA binding protein 1). The resulting dimer regulates alternative splicing of many transcripts, including the mRNA for pyruvate kinase M (PKM; M is the muscle type, named according to the tissue of initial characterization). PKM is present in 2 isoforms that differ in alternative inclusion of either exon 9 or exon 10 (Figure 2A). High levels of PTBP1/HNRNPA1 cause skipping of exon 9, leading to predominant translation of isoform 2 (PKM2), whereas absence of these proteins favors production of PKM1, which skips exon 10. To see whether the presence of MLL fusion forces a shift toward PKM2 through upregulation of PTBP1, we determined splicing patterns in knockdown and control cells. The identification of these patterns was possible because the coding sequence of exon 10 contains a restriction enzyme site not present in exon 9. Digestion with PstI selectively cleaves the cDNA for PKM2. Splicing patterns were determined in murine and in MOLM13 cells by using 3 different shRNAs in total (Figure 2B), demonstrating a reduction of the PKM2/PKM1 ratio upon loss of PTBP1. This was confirmed at the protein level with isofrom-specific antibodies detecting a shift from PKM2 to PKM1 after knockdown (Figure 2C). In human cells, total PKM protein was upregulated as a response. A close correlation of MLL-ENL activity, Ptbp1 transcription, and Pkm2/Pkm1 ratios was also detectable in primary murine cells transformed by a tamoxifen-inducible derivative of MLL-ENL integrated into the genome under control of the endogenous Mll control elements, derived from MLL-ENL-ER mice, as described previously (Figure 2D).

MLL-ENL–transformed cells are sensitive to nutrient restriction

PKM1/2 are major regulators of the cellular metabolic state. PKM2 has reduced catalytic activity compared with PKM1, and therefore the ratio of PKM2/PKM1 controls the outflow of phosphoenolpyruvate from glycolysis. Slowing down phosphoenolpyruvate conversion causes accumulation of glycolytic intermediates as the anabolic precursors necessary for proliferation (Figure 3A). This shifts metabolism away from the normally predominant oxidative phosphorylation, and it is part of the Warburg effect. A complete genomic deletion of PKM2 prevents leukemogenesis in general. In addition, PKM2 causes cells to adapt to increased Ser demand, because in contrast to PKM1, PKM2 is an allosteric enzyme that needs to be bound to Ser for full activity. Thus Ser deprivation reduces PKM2 activity and blocks glycolytic outflow further and thus increases the concentration of 3-phosphoglycerate as the starting point for de novo Ser biosynthesis. To ascertain the involvement of glycolytic control in the observed phenotype, we performed glycolytic and mitochondrial stress tests (Figure 3B). Because the consequences of PTBP1 knockdown were very similar in murine and human cells, these and all further experiments were performed with murine cells only. PTBP1-knockdown cells displayed a larger OCR, congruent with a higher mitochondrial influx and increased oxidative phosphorylation, as expected of cells with higher PKM1 levels. Interestingly, the ATP synthesis rate was not correspondingly increased. Rather, energy seemed to be dissipated by mitochondrial uncoupling. This finding goes along with the previous observation that PTBP1 knockdown slowed proliferation and hence concomitantly also slowed energy consumption. ECARs, mainly caused by lactate production, were also diminished after knockdown, corroborating the previous lactate measurements.

That even a moderate perturbation of the anabolic efficiency of glycolysis correlated with a significant reduction in cell proliferation indicated that transformed cells were particularly dependent on an...
Figure 1. Knockdown of Ptbp1/PTBP1 causes a metabolic phenotype. (A) Overview of the pLEPIR lentiviral all-in-one vector for tetracycline-inducible expression of shRNAs. (B) Knockdown efficiency after shRNA treatment in murine MLL-ENL–transformed primary HSPCs and human MOLM13 cells. Shown are qPCR results obtained with RNA isolated from cells selected for the presence of LNGFR from shRNA-expressing and vector-only (nontargeting shRNA) populations. (C) Ptbp1/PTBP1 knockdown affected long-term fitness of the cells. The graph depicts the percentage of shRNA+ (knockdown) cells, as detected by coupled expression of a truncated LNGFR in FACS analysis. Cultures were induced at day 0. Further explanations are given in the text. A representative of 2 experiments is depicted. (D) Visual aspect of Ptbp1 knockdown. An equal number of selected knockdown (shRNA) and control (vec) cells was cultivated overnight demonstrating reduced medium acidification as a consequence of shRNA activity. This phenotype was observed in murine and human cells; only the murine sample is shown. (E) Glucose and lactate metabolism changed after knockdown of Ptbp1/PTBP1. An equal number of enriched LNGFR+ cells from cultures expressing shRNA or vector as controls were seeded, and glucose and lactate concentrations were determined in supernatant medium sampled at the indicated time points.
Therefore, we investigated whether they would be particularly sensitive to starvation. For this purpose, we determined sensitivity to 2-deoxyglucose (DG), CBR5884, and rotenone. DG competes for glucose entry into glycolysis but cannot be metabolized, thus restricting total glycolysis rates. CBR5884 inhibits phosphoglycerate dehydrogenase (PHGDH) and therefore

![Diagram](image1)

Figure 2. Knockdown of Ptbp1/PTBP1 affects splicing of PKM in MLL-transformed cells. (A) Schematic depiction of alternative splicing options for PKM RNA. Concentrations of available Ptbp1/Hnrnpa1 dimers altered exon retention in PKM RNA. High levels of Ptbp1/Hnrnpa1 favored alternative inclusion of exon 10, thus preferentially producing isoform PKM2. (B) Analysis of PKM splice isoforms. RNA was isolated from selected shRNA (LNGFR)-positive and -negative murine and human cells. After reverse transcription, the relevant PKM region was amplified by PCR and digested with the PstI enzyme, which exclusively cuts the cDNA of the PKM2 isoform. Digested and undigested PCR products are shown. Please note that an additional murine shRNA (shRNA2275) was included in this experiment. (C) PKM protein isoform analysis. Proteins were extracted from cells as in panel B and subjected to western blot analysis with antibodies specific for PKM1, PKM2, or total PKM, as indicated. (D) MLL-ENL activity correlated with Ptbp1 expression and Pkm2/Pkm1 ratio. Left: RNA was isolated from primary cells transformed by a tamoxifen-inducible version of MLL-ENL at the indicated time points (0 days [0d], TAM present and MLL-ENL active). Pkm1/2 splice analysis was performed by detection of the Pkm2-specific RFLP through digestion with PstI. Ptbp1 transcript levels in the same RNA samples were determined by quantitative reverse transcription-PCR.
Figure 3.

A. Diagram showing metabolic pathways with annotations.

B. Graphs showing OCR and ECAR measurements with various treatments.

C. Graphs showing proliferation and ECAR changes with different treatments.

D. Bar graph showing relative colony number with different treatments.

- **ECAR** and **OCR** measurements are used to test mitochondrial stress.
- **Mito stress test** involves using **oligomycin**, **FCCP**, and **antimycin A**.
- **Glycolysis stress test** uses 2-deoxy-glucose (**2DG**) to assess glycolytic stress.

- **PKM1** and **PKM2** enzymes are involved in glycolysis.
- **PKM1** is active in normal cells, while **PKM2** is active in cancer cells.

- **MLLENL** and **C72h treatment** are related to proliferation and colony formation.
- **DMSO**, **2d-gluc**, **CBR5884**, and **rotenone** are used as treatments.

- **p-values** are indicated for treatment comparisons.
blocks Ser biosynthesis. In addition, Ser is the precursor of glycine and cysteine synthesis, important components of the ROS neutralizer glutathione. The mitochondrial decoupling agent rotenone was included because energy production in mitochondria is limited in PKM2-high cells, and further reduction of ATP production may therefore be particularly deleterious. The 50% inhibitory values for these substances were determined by MTT test to be 0.3 mM (DG), 2 μM (CBR5884), and 15 nM (rotenone) (Figure 3C). For effective treatment, it is more important to eradicate the leukemia repopulating unit, rather than the bulk cell population. As repopulating cells are often more resistant and because they can hide within the major population, we determined the effect of starvation specifically on the repopulating units. For this purpose, the clonogenic activity of transformed cells in comparison with normal bone marrow–derived HSPCs (kit/CD117+ subfraction including GMPs) was recorded after a transient 48-hour treatment with 2DG, CBR5884, and rotenone (Figure 3D). The evaluation of CFC assays revealed that transformed cells were significantly more sensitive to the inhibitors than were their normal counterparts.

**Inhibition of Ser synthesis causes increased cellular stress and apoptosis in transformed cells**

With respect to practical in vivo applications, glucose and energy starvation are difficult to achieve because neuronal cells are especially sensitive to reduced glucose and energy levels. Long-lasting energy deprivation is neurotoxic, and application of rotenone to animals causes Parkinsoni
tic disease.27 In addition, low energy levels trigger AMP-activated protein kinase (AMPK), effectively protecting cells from further stress.28 Therefore, we decided to investigate Ser metabolism as a potentially exploitable vulnerability of leukemic cells. To further examine the preferential sensitivity of leukemic CFCs for Ser deprivation, we determined the cell cycle in leukemic/normal cultures after a 48-hour exposure to CBR5884 (Figure 4A). Whereas controls did not show significant changes in cycle distribution, sub-G1 cells, confirmed to be apoptotic by annexin/propidium iodide staining (supplemental Figure 2), increased to more than 30% in the leukemic population. In comparison with normal HSPCs, this effect was accompanied by an increase in ROS and DNA damage, as detected by γ-H2AX (Figure 4B-C). To test whether ROS overproduction is the main reason for the loss of self-replicating capacity after CBR5884 treatment, CFC assays were repeated, quenching ROS with N-acetyl-cysteine (NAC; Figure 4D). Addition of 0.4 mM NAC was sufficient to reverse the effects of CBR5884, indicating that ROS was the major stressor that affected CFC viability.

Because the tumor suppressor TP53 is central for cellular stress response, we checked the level of TP53 in untreated normal HSPCs and leukemic cells (Figure 4E). TP53 was higher in transformed cells, an unexpected result, because TP53 activates transcription of the cell-cycle inhibitor p21, the presence of which should be incompatible with active proliferation. TP53 was not mutated in MLL-ENL cells, as confirmed by sequencing (not shown); however, its transcriptional response was blunted. Despite the higher level of TP53 protein in transformed cells, TP53 targets p21 and Mdm2 were significantly lower or equal to the levels observed in primary bone marrow HSPCs (Figure 4F). A potential explanation for this reduced TP53 activity in leukemia was found in much higher expression of Myc, a gene that is not only known to be stimulated by MLL fusions but also to act as a repressor of TP53–induced transcription. In addition, it has been shown that MLL fusions can directly bind and inhibit p53.29

**MLL-ENL–transformed cells are dependent on exogenous Ser/Gly in vitro and in vivo**

The pharmacodynamic properties of CBR5884 are not ideal for in vivo applications. Yet, it has been reported that the increased demand for Ser and glycine of some cancers require an additional exogenous supply of these normally nonessential amino acids.30,31 Because selective starvation for these amino acids would be a very simple and enticing option to influence leukemia development, we tested MLL-ENL–transformed cells for their reliance on exogenous Ser and/or glycine (Figure 5A). Although depletion of glycine did not have effects on cells in MTT tests, proliferation was reduced in Ser-free medium, and this reduction was exacerbated by combined omission of Ser and glycine. Because Ser is the biosynthetic precursor of glycine, a reduction of exogenous glycine depletes Ser stores even further. Remarkably, a transient 48-hour period of Ser/Gly starvation was sufficient to reduce clonogenic activity of leukemic cells to ~10% of the control value. In contrast, the same treatment had no effect on CFC capability of normal HSPC (Figure 5B-C). Exogenous Ser/Gly starvation increased cellular ROS only moderately, and it did not affect cell-cycle distribution (Figure 5D-E). However, cells responded with a differentiation stimulus, as detected by an induction of the maturation marker Gr-1 (Ly6C/G; Figure 5F). By subjecting 2 human samples to the same treatment, we confirmed that sensitivity to Ser/Gly starvation is not restricted to murine cells (Figure 5G). CFC assays demonstrated that a 48-hour starvation for Ser/Gly causes a strong reduction in clonogenic activity in original human cells, as well.
Finally, we tested the effects of selective Ser/Gly starvation on development of leukemia in vivo. A transplantation experiment was performed by injecting MLL-ENL transduced syngenic HSPCs into sublethally irradiated recipients. Animals were fed either a special Ser/Gly-free chow or an identical diet including those amino acids. Health was monitored by an institutionally standardized scoring system.

![Graph showing cell cycle distribution and ROS levels](image)

**Figure 4. Inhibition of Ser synthesis causes cellular stress in leukemic cells** (A) Cell cycle distribution of normal and leukemic HSPCs after a 48-hour block of Ser biosynthesis. Shown are means and standard deviations of 3 independent experiments. Cell cycle phases are colored as indicated. (B) Cellular ROS in cells treated as in panel A. The cells were stained with CellROX deep red reagent and evaluated by FACS. A representative example of 2 experiments is depicted. (C) DNA damage after Ser synthesis blockade. Nuclear proteins were extracted from cells treated as before and immunoblotted with antibodies against the damage-specific histone derivative γ-H2AX and for histone H3 as the control. (D) ROS quenching restores CFC activity after CBR5884 treatment. MLL-ENL–transformed cells were incubated for 48 hours with 8 μM CBR5884 and either solvent or the indicated concentrations of the ROS-quencher NAC. The number of CFCs was recorded in triplicate experiments, with results depicted as mean and standard deviation. An example of the CFC assay is shown in the right panel. DMSO (left columns) difference between NAC 0 mM to 0.4 mM is not significant; difference between 0 mM and 1 mM NAC is P < .03. CBR5884 (right columns) difference between 0 mM and 0.4 mM NAC is P < .00002; difference between 0 mM and 1 mM NAC is P < .002. (E) Steady state levels of p53 in normal and leukemic HSPCs, as detected by western blot. (F) Blunted p53 response in leukemic cells. Expression levels of the major p53 target genes Cdkn1a/p21 and Mdm2, as well as those of the p53 antagonist Myc were determined by qPCR in normal and leukemic HSPCs, in steady state and after CBR5884 treatment.
procedure. Upon persistent signs of disease-like hunched posture, alterations in fur hygiene, and labored breathing, the animals were euthanized and examined for signs of leukemia, such as increased and infiltrated spleen, pale liver, enlarged lymph nodes, and bone marrow blasts. Leukemia was also confirmed if permanently proliferating myeloid blast cultures were established from spleen samples, which was the case in all animals tested (not shown). Remarkably, dietary Ser/Gly restriction was sufficient to prolong overall survival by ~30% in the Ser/Gly-starved population (Figure 5H). The only observable side effect of feeding a Ser/Gly-free diet was...
moderate alopecia, most likely caused by mutual trichophagy. Apart from that, no influence on well-being or behavior was observed. Spleen weights were not significantly different between groups indicating that experimental groups were euthanized at comparable states of leukemia development (Figure 5I), corroborating the leukemia-delaying effect of Ser/Gly-free nutrition.

Ser/Gly sensitivity in MLL-ENL–transformed cells correlates with glucose transport

To investigate the molecular details of Ser/Gly requirement more closely, we made use of a starvation-resistant population of MLL-ENL-transformed cells that was serendipitously identified after prolonged cultivation of the cells in Ser/Gly-depleted medium. The cells proliferated in the absence of Ser/Gly at nearly the same rates as the parental population in fully supplemented medium (Figure 6A), demonstrating that the requirement for exogenous supplementation with Ser/Gly is not simply a property of every proliferating cell. Genes that modulate the response to Ser/Gly deprivation were identified by isolating and sequencing nascent RNA from MLL-r cells in Ser/Gly-supplemented or -depleted medium and also from starvation-resistant cells. Genes were ordered according to the up- or downregulation during starvation and their further change in expression in resistant cells. The top 20 genes, with a minimum threshold of a twofold expression difference between supplemented/starved conditions and an at least twofold further increase after acquiring resistance, included the glucose transporter Slc2a3/Slc2a3/Slc2a3 (Figure 6B). Concomitantly, uptake of a fluorescently labeled glucose analog (2NBDG) was higher in the transforming oncogene. The MLL-ENL fusion protein restricts driver oncogene needs to perturb normal metabolic reactions to starvation resistance (supplemental Figure 3).

In this regard, it is important to stress that selective Ser/Gly starvation is different from simply restricting calories or glucose. A comparable transplantation model revealed that a ketogenic diet forcing energy production from fatty acids and thus depleting glycolytic intermediates exacerbated the development of MLL-induced leukemia.34 Cells have developed various mechanisms to protect them from metabolic stress during phases of “natural” nutrient paucity. The AMP-activated kinase AMPK is one of these alert systems. In previous work,28 AMPK deletion or inhibition was identified lactate dehydrogenase as an MLL-ENL entity. This adds selective nutrient deprivation to the approach (eg, by restricting glucose), may circumvent endogenous specific branches of anabolic metabolism, rather than a broader therapy breaks, for example. The ease of clinical implementation makes this selective starvation an enticing possibility for future trials.
Figure 6. The sensitivity of leukemic cells toward amino acid starvation correlates with sugar transport. (A) Spontaneous emergence of a Ser/Gly starvation-resistant population. Proliferation rates of the parental and resistant (res. clone) cells under cultivation conditions as indicated. (B) Heat map depicting the top 20 genes selected according to upregulation under starvation conditions and in starvation-resistant cells. The glucose transporter Slc2a3 (also known as Glut3) is highlighted. (C) Ser/Gly starvation resistance correlated with glucose transport capacity. Glucose uptake was measured by incubation with 2NBDG, a fluorescent glucose analogue, and measured by FACS. (D) Relative expression of Slc2a3 determined by qPCR in leukemic, resistant, and normal cells in complete (blue bars) or Ser/Gly-deficient (red bars) medium. (E) AMPK was not activated by Ser/Gly starvation. MLL-ENL transformed cells were incubated for 48 hours under the indicated conditions, and cellular extracts were analyzed for the presence of total and active/phosphorylated AMPK by western blot.
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Authorship

Contribution: M.-P.G.-C., J.L., M.B., and R.K.S. performed and analyzed experiments; D.M. advised on metabolite determination and Seahorse experiments; M.M. provided human cell samples; R.K.S. conceived and supervised experiments and wrote the manuscript; and all authors read, discussed, and modified the manuscript.

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