proportion of patients develop early blast crisis even when treated first line with a more potent TKI, and for those individuals, novel therapies that might carry an increased risk of toxicity could be justified. In a machine learning logistic regression model, the authors developed a classifier for blast crisis with no false positives, and a sensitivity of 50% to 67%.[1] This cohort included only 6 patients, and the cases were diverse with respect to latency of blast crisis (76-2936 days) as well as lineage and genetics, so validation of these findings in a larger cohort is required. If patients destined for transformation or treatment failure could be identified at diagnosis, there would be an opportunity to design clinical trials of intensified first-line treatment to reduce this risk.

Recent studies have begun to dissect the relevance of genomic lesions in addition to *BCR::ABL1*, showing that some variants associated with blast crisis are already present at diagnosis.[7] Variants in *ASXL1* were found in 9% of 222 patients with chronic phase CML in a clinical trial using the more potent TKI, nilotinib, and were associated with a reduced incidence of major molecular response (*BCR::ABL1* ≤0.1%).[8] Variants in *ASXL1* were detected in 23.8% of patients by Krishnan et al (whose series was enriched for higher-risk individuals), but were identified across all response cohorts, including those with optimal response.[1] One of the limitations of bulk sequencing is that it cannot distinguish between the 3 cellular contexts in which a mutation might occur. Additional variants could represent clonal evolution (*BCR::ABL1* first), a preleukemic clone (*BCR::ABL1* second), or independent clonal hematopoiesis (*BCR::ABL1* wild type). In Ph-negative myeloproliferative neoplasms, it has been shown that the order of acquisition of mutations can influence disease biology.[9] Larger single-cell sequencing studies may help to determine whether the prognostic effect of an additional variant in CML is influenced by its clonal relationship to *BCR::ABL1*.

Broader application of single-cell sequencing for clinical purposes is currently limited by cost and analytical complexity. Recognizing the need for simpler, more widely applicable methods to validate and apply these findings, Krishnan et al showed that flow cytometry or mass cytometry could be used to detect the predictive signatures from their transcriptomic analysis. Although larger single-cell sequencing studies may be undertaken in the future, the data contained in this report present multiple hypotheses that can be tested now. Some of the biological pathways that influence optimal response and TFR overlap with those that lead to treatment failure and blast crisis. Understanding and targeting these pathways has the potential to lead to improved outcomes at both ends of the response spectrum in CML.

Conflict-of-interest disclosure: D.M.R. has received research funding from Novartis and BMS and honoraria from Novartis, BMS, Takeda, Keros, and Menarini. ■

REFERENCES

1. Krishnan V, Schmidt F, Nawaz Z, et al. A single-cell atlas identifies pretreatment features of primary imatinib resistance in chronic myeloid leukemia. *Blood*. 2023;141(22):2738-2755.
2. Giustacchini A, Thongjuea S, Barkas N, et al. Single-cell transcriptomics uncovers distinct molecular signatures of stem cells in chronic myeloid leukemia. *Nat Med*. 2017;23(6):692-702.
3. Ilander M, Olsson-Stromberg U, Schlums H, et al. Increased proportion of mature NK cells is associated with successful imatinib discontinuation in chronic myeloid leukemia. *Leukemia*. 2017;31(5):1108-1116.
4. Irani YD, Hughes A, Clarson J, et al. Successful treatment-free remission in chronic myeloid leukaemia and its association with reduced immune suppressors and increased natural killer cells. *Br J Haematol*. 2020;191(3):433-441.
5. Shanmuganathan N, Pagani IS, Ross DM, et al. Early BCR-ABL1 kinetics are predictive of subsequent achievement of treatment-free remission in chronic myeloid leukemia. *Blood*. 2021;137(9):1196-1207.
6. Yeung DT, Osborn MP, White DL, et al. TIDEL-II: first-line use of imatinib in CML with early switch to nilotinib for failure to achieve time-dependent molecular targets. *Blood*. 2015;125(6):915-923.
7. Branford S, Wang P, Yeung DT, et al. Integrative genomic analysis reveals cancer-associated mutations at diagnosis of CML in patients with high-risk disease. *Blood*. 2018;132(9):948-961.
8. Schonfeld L, Rinke J, Hinze A, et al. ASXL1 mutations predict inferior molecular response to nilotinib treatment in chronic myeloid leukemia. *Leukemia*. 2022;36(9):2242-2249.
9. Ortmann CA, Kent DG, Nangalia J, et al. Effect of mutation order on myeloproliferative neoplasms. *N Engl J Med*. 2015;372(7):601-612.

https://doi.org/10.1182/blood.2023020165

RED CELLS, IRON, AND ERYTHROPOIESIS

Comment on Qin et al, page 2756

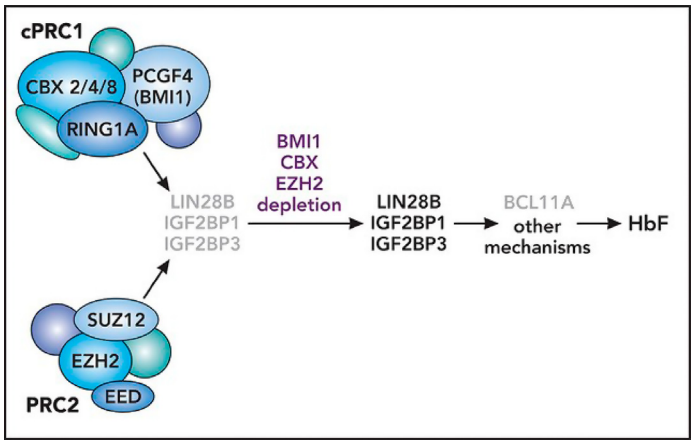
Help on the way to unsilence HbF

Ann Dean | National Institute of Diabetes and Digestive and Kidney Diseases

In this issue of *Blood*, Qin et al[1] show that BMI1 (PCGF4), a polycomb group RING finger protein, is a repressor of fetal hemoglobin (HbF). They present strong evidence that the entirety of the effect of BMI1 is exerted by RNA-binding proteins LIN28B, IGF2BP1, and IGF2BP3.

Elevated HbF in adults moderates the symptoms of sickle cell disease and β-thalassemia and is a highly desirable therapeutic goal.[2] The significant discoveries of HbF repressors BCL11A and ZBTB7A (LRF) resulted in encouraging small clinical trials involving gene editing of mobilized patient hematopoietic stem cells ex vivo.[3] However, gene therapies are unlikely to be feasible for the vast patient population in less developed parts of the world because of their complexity and cost. Thus, there remains a strong incentive to develop other approaches, such as small-molecule inhibitors. The feasibility of this approach is illustrated by the rational targeting of components of the nucleosome remodeling and deacetylating complex, through which BCL11A and ZBTB7A work.[4] Qin et al enlarge the field of potential small-molecule targets by identifying polycomb repressive complexes 1/2 (PRC1/2) as indirect inhibitors of *HBG1/2* transcription.

PRC1 and PRC2 are crucial to establishment and maintenance of facultative



RNA binding proteins LIN28B and IGF2BP1/3 link PRC1/2 to HbF repression in adult erythroid cells. cPRC1 and PRC2 components are depicted as differently colored shapes, with the named components that were tested by Qin et al labeled. Silencing of LIN28B and IGF2BP1/3 in HUDEP2 cells or in adult primary cells is depicted by gray type. Depletion of BMI1 and CBX (cPRC1) or EZH2 (PRC2) relieves silencing of these RNA-binding proteins, thereby promoting silencing of BCL11A (gray type) or other events and upregulation of HbF. Professional illustration by Patrick Lane, ScEYEnce Studios.

heterochromatin and have fundamental roles in gene regulation and development.[5] PRC1 catalyzes histone H2A lysine 119 monoubiquitylation (H2AK119ub1) through RING1A/B E3 ligase, whereas PRC2 catalyzes histone H3 lysine 27 trimethylation (H3K27me3) through EZH1/2 methyltransferase activity. PRC1 also contains 1 of 6 polychrome group RING finger proteins (PCGF1-6). These are considered canonical (cPRC1) or noncanonical (ncPRC1), depending on whether they contain a CBX protein that recognizes H3K27me3-marked chromatin, making the complex dependent on PRC2 (cPRC1), or instead contain RYBP or YAF1, and bind chromatin independent of PRC2 (ncPRC1). Modestly elevated *HBG* mRNA was observed after depleting PRC2 subunit EZH2 in primary adult erythroblasts.[6] Recently, a pharmacologic inhibitor of PRC2 component EED (FTX-6058) was reported to reduce BCL11A and robustly elevate HbF and is in clinical trials.[7] However, our understanding of PRC1/2 involvement in *HBG* silencing is incomplete.

Qin et al performed a domain-focused screen targeting human E3 ligases in umbilical cord erythroid progenitor-derived HUDEP2 cells, which have low basal expression of HbF. BMI1 (PCGF4, a PRC1 component) was a strong hit with BMI1-edited cells among the highest HbF expressors. BMI1 reduction in HUDEP2 cells and in primary adult erythroblasts robustly activated transcription of the fetal globin genes *HBG1* and *HBG2*, HbF protein, and the percentage of cells with HbF. Thus, BMI1 is revealed as a repressor of HbF in adult erythroid cells.

RNA sequencing confirmed *HBG1/2* activation in BMI1-depleted HUDEP2 cells. Interestingly, IGF2BP1 and LIN28B were upregulated in these cells. Overexpression of these RNA-binding proteins had been shown to increase HbF in adult erythroid cells, and interference with BCL11A mRNA translation or turnover was proposed to explain the result.[8,9] In the current studies, increased LIN28B and IGF2BP1 correlated with reduced BCL11A protein after BMI1 depletion, and overexpression of BCL11A largely restored *HBG* silencing. Moreover, combinatorial depletion of BMI1, LIN28B, and IGF2BP1 lowered HbF to essentially basal levels, supporting that upregulation of LIN28B and IGF2BP1 was sufficient to account for HbF activation by BMI1 loss. Unexpectedly, RNA sequencing of primary adult erythroblasts after BMI1 depletion revealed LIN28B was unchanged but IGF2BP1 and IGF2BP3 were upregulated. Overexpression of either IGF2BP1 or IGF2BP3 increased *HBG* expression, supporting that in BMI1-depleted primary cells, these 2 proteins drive HbF production.

To find direct targets of BMI1, the authors performed CUT&RUN (cleavage under targets and release using nuclease) to localize BMI1 in HUDEP2 cells and in primary adult erythroblasts, and profiled H2AK119ub1 and H3K27me3 in control and BMI1-depleted cells. Notably, there was no enrichment of BMI1 or H2AK119ub1/H3K7me3 at the β-globin locus, consistent with BMI1 acting indirectly to repress *HBG1/2*. H2AK119ub1 and H3K27me3 overlapped with BMI1 at the CpG islands near the LIN28B and IGF2BP1 promoters in HUDEP2 cells and were reduced on BMI1 loss. The same was true for IGF2BP3 in primary cells, indicating that BMI1 maintains repression of chromatin at these loci in the respective cell systems.

To distinguish whether cPRC1 or ncPRC1 was involved in HbF repression, the authors performed multiplex CRISPR targeting. Indeed, combined depletion of cPRC1 subunits CBX2/4/8 in HUDEP2 cells activated LIN28B and IGF2BP1 and induced a fetal erythroid gene expression pattern, like BMI1 depletion. The authors propose a model in which cPRC1/BMI1/CBX represses LIN28B and IGF2BP1 through H3K27me3 recognition, invoking PRC2 involvement (see figure). Supporting PRC2 participation, after EZH2 depletion in HUDEP2 cells, there was significant *HBG1/2* upregulation, and modest upregulation of IGF2BP1 and LIN28B. Furthermore, in differentiating primary erythroid cells, the EZH2 inhibitor, EPZ-6438, reduced bulk H3K27me3 and upregulated IGF2BP1/3 mRNA and protein. Thus, in both cellular backgrounds, cPRC1 and PRC2 repress HbF through developmental silencing of LIN28B, IGF2BP1, and/or IGF2BP3.

PRCs propagate repressive marks through cell division, prompting the authors to examine the effects of interfering with their activity on HbF induction over time. HUDEP2 cells were induced to differentiate after treatment with either the EZH2 inhibitor EPZ-6438 or the experimental HbF inducer pomalidomide for 3 days or were grown absent the drug for an additional 5 days before induction. Strikingly, HbF induction by EPZ-6438 was sustained even when cells underwent 5 additional days of growth in the absence of the drug before differentiation, but this was not the case for pomalidomide. This potential epigenetic effect has implications for possible future applications of specific therapeutic polycomb group-targeted HbF inducers.

Overall, these new studies provide significant insight into how BMI1 and PRC1/2 function in hemoglobin switching and enlarge the armamentarium of potential therapeutic targets to increase

HbF. The data point to BCL11A as a link between BMI1 and HbF repression through BMI1 targets LIN28B and IGF2BP1/3: how these RNA-binding proteins affect BCL11A still has open questions. Epigenetic manipulation by PCR1/2 emerges as a viable therapeutic option to increase HbF. The observation that the effects of EPZ-6438 seem to be sustained after removal of the drug is promising and encouraging.

Conflict-of-interest disclosure: The author declares no competing financial interests. ■

REFERENCES

1. Qin K, Lan X, Huang P, et al. Molecular basis of polycomb group protein–mediated fetal hemoglobin repression. *Blood*. 2023; 141(22):2756-2770.
2. Vinjamur DS, Bauer DE, Orkin SH. Recent progress in understanding and manipulating haemoglobin switching for the haemoglobinopathies. *Br J Haematol*. 2018; 180(5):630-643.
3. Demirci S, Leonard A, Essawi K, Tisdale JF. CRISPR-Cas9 to induce fetal hemoglobin for the treatment of sickle cell disease. *Mol Ther Methods Clin Dev*. 2021;23: 276-285.
4. Sher F, Hossain M, Seruggia D, et al. Rational targeting of a NuRD subcomplex guided by comprehensive in situ mutagenesis. *Nat Genet*. 2019;51(7):1149-1159.
5. Blackledge NP, Klose RJ. The molecular principles of gene regulation by polycomb repressive complexes. *Nat Rev Mol Cell Biol*. 2021;22(12):815-833.
6. Xu J, Bauer DE, Kerenyi MA, et al. Corepressor-dependent silencing of fetal hemoglobin expression by BCL11A. *Proc Natl Acad Sci U S A*. 2013;110(16): 6518-6523.
7. Stuart B, Bruno P, Polioudakis D, et al. P1498 inhibition of polycomb repressive complex 2 through EED induces fetal hemoglobin in healthy and sickle cell disease models. *HemaSphere*. 2022;6:1380-1381.
8. de Vasconcellos JF, Tumburu L, Byrnes C, et al. IGF2BP1 overexpression causes fetal-like hemoglobin expression patterns in cultured human adult erythroblasts. *Proc Natl Acad Sci U S A*. 2017;114(28):E5664-E5672.
9. Lee YT, de Vasconcellos JF, Yuan J, et al. LIN28B-mediated expression of fetal hemoglobin and production of fetal-like erythrocytes from adult human erythroblasts ex vivo. *Blood*. 2013;122(6):1034-1041.

https://doi.org/10.1182/blood.2023020345

TRANSPLANTATION

Comment on Socié et al, page 2771

Novel biomarker-based probability engine

Yoshihiro Inamoto | National Cancer Center Hospital

In this issue of *Blood*, Socié et al[1] reported the prognostic value of 21 blood biomarkers in predicting the treatment response of steroid-refractory or steroid-dependent acute graft-versus-host disease (GVHD) using samples collected in the phase 3 REACH2 trial (ruxolitinib vs best available treatment; NCT02913261).[2] The authors identified several baseline clinical factors and biomarkers at baseline and at day 14 after treatment that predicted probabilities of treatment response.

Acute GVHD remains a major limitation of allogeneic hematopoietic cell transplantation. Systemic, high-dose glucocorticoids are the recommended first-line treatment for patients with more than mild acute GVHD. The goal of therapy is to control the acute manifestations of GVHD with its potential for causing life-threatening organ damage and to taper the dose of glucocorticoids as soon as clinically possible. Approximately 60% of patients respond to initial treatment with glucocorticoids,[3,4] but the remaining patients (with either steroid-refractory or steroid-dependent GVHD) require second-line systemic treatment. No clearly superior second-line systemic treatment for steroid-refractory or steroid-dependent acute GVHD has been identified, despite many clinical trials. The recently reported REACH2 randomized trial showed that ruxolitinib was more effective than the investigator's best choice of other therapies.[2] The overall response rates at day 28 were 62% with ruxolitinib and 39% with best available therapies. However, we still do not know which patients are more likely to respond to second-line systemic treatment. Identification of biomarkers and clinical factors that predict treatment response to the second-line systemic treatment would further guide treatment decisions.

Using samples collected in the REACH2 trial, Socié et al succeeded in identifying biomarker profiles and clinical factors that predict treatment response at day 28 after second-line systemic treatment. A total of 295 patients at baseline, and 242 patients at day 14 after second-line systemic treatment, were included in the analysis, with half of the patients treated with ruxolitinib and the remaining patients treated with best available therapies. A total of 29 biomarkers (proteins or immune cell subsets) were measured, and 8 biomarkers were excluded because baseline values exceeded limitations of quantitation or because of lack of change over time, leaving 21 biomarkers for the final analysis. Notably, the authors generated acute GVHD-, immune-, and inflammatory-related principal components to account for complex interactions between multiple biomarkers, and the principal components were used as variables together with other important clinical variables (see figure). Important to note is that these principal components were determined without referring to the data on treatment response. The authors found that treatment with ruxolitinib, skin involvement, reduced-intensity conditioning, and younger patient age were associated with treatment response. Lower levels of several biomarker-derived immune or GVHD-related principal components both at baseline and at day 14 were associated with treatment response. A notable finding is that change in biomarkers from baseline over time was not useful. Treatment with ruxolitinib was the strongest factor associated with treatment response. The bias-corrected areas under receiver operating characteristic values were reasonably high (0.73 for the baseline model and 0.80 for the day-14 model), showing the potential of this approach and the need for continued refinement.

The Mount Sinai Acute GVHD International Consortium (MAGIC) biomarkers (ST2 and REG3α) have been validated in several studies of initial systemic