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Prefibrotic myelofibrosis: is this diagnosis valid?

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Comment on Wilkins et al, page 60

Prefibrotic myelofibrosis: is this diagnosis valid?

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Wilkins and colleagues evaluate the utility of the World Health Organization (WHO) diagnostic criteria intended to separate cases previously classified as essential thrombocythemia (ET) into 2 groups: “true ET” and “prefibrotic myelofibrosis.” Focusing on bone marrow histology, the authors found substantial variation in classification of cases.

Primarily myelofibrosis (PMF) is a clonal myeloproliferative disorder (MPD) characterized by a proliferation of abnormal megakaryocytes, reactive bone marrow fibrosis, and extramedullary hematopoiesis (see figure). In 2001, the World Health Organization (WHO) published criteria for diagnosing MPDs and included the concept of “prefibrotic myelofibrosis,” which is meant to represent the early stage of PMF.¹ The diagnostic criteria in the WHO classification emphasize bone marrow histology and are based largely on multiple publications from a group that has studied trephine biopsy specimens from patients with MPDs.² This group suggested that many patients diagnosed with ET using the Polycythemia Vera Study Group (PVSG) criteria actually have prefibrotic myelofibrosis, and further suggested that bone marrow biopsies from patients with thrombocythemia can be reliably subdivided into prefibrotic MF and ET and that there is an apparent reduction in life expectancy in the patients with prefibrotic MF compared with true ET.³

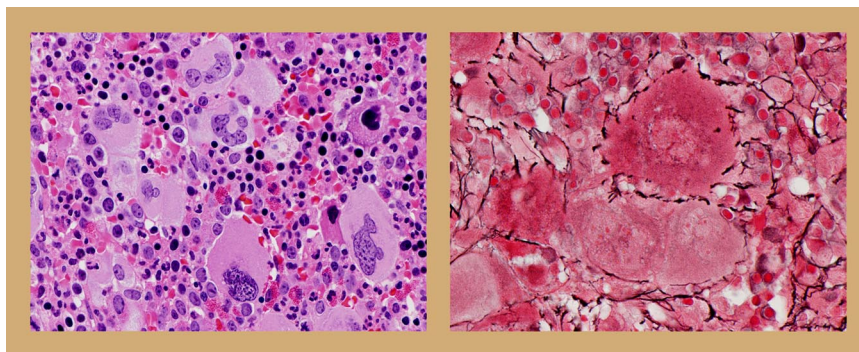
The findings from Wilkins and colleagues are important because diagnosis of prefibrotic myelofibrosis has been controversial and has generated much discussion among those interested in MPDs. Prefibrotic MF is also

included in the revised WHO criteria,⁴ but there is little prospective data available to validate the diagnostic criteria for distinguishing prefibrotic MF from ET.

The current study by Wilkins and colleagues is large and well done. Three experienced hematopathologists independently reviewed bone marrow trephine biopsies from 370 patients with ET that was diagnosed using the PVSG criteria. Biopsies were evaluated for 16 morphologic features reported to be of value in distinguishing prefibrotic MF from ET. Cases were categorized into ET or prefibrotic MF according to the WHO classification. The researchers found substantial interobserver variability in the classification of these cases, even

though there was reasonable agreement about several of the histological features. No difference was found in clinical outcome between those cases classified as ET versus those classified as prefibrotic MF.

The findings of this report suggest that the published histological criteria for distinguishing ET from prefibrotic MF are difficult to apply reproducibly. This does not mean that the criteria are invalid, however. The study could be criticized on the grounds that the investigators were relying on biopsy morphology with limited clinical information (age and sex only), with no laboratory data or blood films to examine. It is possible that evaluating other clinical and laboratory features along with histopathology would have increased the reproducibility of the classification. It is interesting that the rate of transformation to myelofibrosis in the patient population studied was extremely low even though the median follow-up was 68 months. This low rate of progression raises the possibility that the population had a very small number of prefibrotic MF patients, making the detection of the subgroup difficult. As the authors indicate, even experienced hematopathologists may need specialized training to identify prefibrotic MF, but the study addressed the utility of the criteria in a real practice setting.



Bone marrow biopsy from patient with early primary myelofibrosis. (Left) The marrow is hypercellular with clusters of abnormal megakaryocytes. (Right) Reticulin stain highlights reticulin fibrosis with reticulin surrounding megakaryocytes.

This paper will undoubtedly stimulate additional discussion regarding the classification of the MPDs. It provides further insight into our understanding of the classification MPDs at a time when our knowledge is being enhanced and diagnostic criteria revised by the recent and ongoing discoveries of JAK2 and other mutations^{5,6} in these neoplasms.

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

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dently associated with an elevated tricuspid regurgitant jet velocity, an indicator of pulmonary hypertension. Finally, the erythrocyte glutamine–glutamate ratio was also decreased in patients with SCD and inversely correlated with the severity of pulmonary hypertension and markers of hemolysis. The authors conclude that decreased glutathione and glutamine contribute to alterations in the erythrocyte redox environment, compromised erythrocyte integrity, and NO bioavailability, which may be implicated in the pathogenesis of pulmonary hypertension associated with SCD.

The findings of this study are novel and potentially clinically significant. However, several questions still remain. The authors' conclusions are mostly supported by statistical associations that suggest, but do not conclusively establish, a direct link between the proposed pathways and the pathogenesis of pulmonary hypertension in SCD. As such, the mechanisms by which decreased glutathione and glutamine contribute to SCD-related vasculopathy need to be explored. For example, are these alterations a direct cause of oxidant stress to the erythrocyte or a marker of accumulation of redox active heme and iron from lysed red blood cells? Furthermore, the direct implications of these pathways to morbidity and mortality in these patients will have to be addressed in larger prospective studies. We hope that the study by Morris and colleagues will stimulate further research that will ultimately lead to new therapeutic options for patients with SCD and pulmonary hypertension.

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

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● ● ● RED CELLS

Comment on Morris et al, page 402

Amino acids and the erythrocyte under stress?

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In this issue of *Blood*, Morris and colleagues show that erythrocyte glutathione and glutamine levels are low in patients with sickle cell disease (SCD) and that these markers are linked to markers of hemolysis and pulmonary hypertension.

Pulmonary hypertension is a major threat to the well-being of adults with sickle cell disease (SCD) that is linked epidemiologically and mechanistically to hemolysis.¹ As a result of hemolysis, hemoglobin is released into plasma, where it reacts with and destroys nitric oxide (NO).² Hemolysis also releases erythrocyte arginase into plasma, depleting L-arginine, the substrate for NO synthesis.³ Together, these factors contribute to a state of decreased NO bioavailability and resistance to NO-dependent vasodilation.² In addition, the accumulation of redox active heme and iron from lysed red blood cells can contribute to the generation of reactive oxygen species that could further impair NO-dependent vascular function.⁴ Increased reactive oxygen species generated by sickle erythrocytes can also compromise the integrity of the red blood cell.

As Morris and colleagues extensively review, the glutathione buffering system is critical for maintaining cellular redox bal-

ance. In the lung, glutathione is a major antioxidant, and excessive pulmonary production of oxidants leads to alterations in glutathione levels. Interestingly, in patients with idiopathic pulmonary arterial hypertension, glutathione content in the bronchoalveolar lavage fluid is increased, likely as an adaptive response to increased oxidants resulting from lung inflammation.⁵ In this context, Morris et al's study adds another pathway to the list of potential contributors to the pathogenesis of SCD related vasculopathy. The authors hypothesized that abnormal glutathione and glutamine metabolism may play a role in hemolysis and pulmonary hypertension in SCD. They show that total plasma and erythrocyte glutathione levels are significantly decreased in patients with SCD. Patients with pulmonary hypertension had significantly lower erythrocyte glutamine levels; these levels inversely correlated with the severity of pulmonary hypertension and were indepen-

Comment on Gardiner et al, page 165

Braking platelet activation: deactivating the receptors

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Glycoprotein (GP) VI and Fc receptor FcγRIIa are 2 ITAM (immunoreceptor tyrosine-activation motif)-bearing platelet surface receptors. In this issue of *Blood*, Gardiner and colleagues show that activation of either receptor results in simultaneous proteolytic cleavage of GPVI ectodomain and FcγRIIa cytoplasmic tail, providing distinct mechanisms for their down-regulation on platelet activation.

The interaction between the platelet and subendothelial matrix, a process mediated by platelet receptors GPVI for collagen and GPIb-IX-V for von Willebrand factor, is an initial event in thrombus formation on vessel injury. Although we know a lot about the events following receptor activation, our understanding is limited with respect to the mechanisms that down-regulate these responses once activated. Glycoprotein VI (GPVI) is a member of the immunoglobulin (Ig) receptor family with 2 extracellular Ig domains; it is noncovalently linked with an Fc receptor γ-chain (FcRγ) dimer, an association essential for GPVI surface expression. The cytoplasmic portion of FcRγ has an ITAM that is phosphorylated by Src family kinases on GPVI activation, culminating in the cleavage of the extracellular domain of GPVI and down-regulation of receptor function. The ITAM-dependent mechanisms are essential to this process. Also present on platelet surface is the IgG receptor FcγRIIa, which has 2 extracellular Ig domains and, importantly, an ITAM in the cytoplasmic domain. Ligation of this receptor by immune complexes induces platelet activation.

Gardiner and colleagues have explored the interplay of the 2 ITAM-bearing receptors to address whether signaling through FcγRIIa would also induce the cleavage of the GPVI ectodomain that was previously noted with GPVI ligation.¹ They demonstrate that antibody ligation of platelet FcγRIIa results in the shedding of GPVI ectodomain mediated by a metalloproteinase, as well as cleavage of FcγRII intracellular domain by calpain. This parallel but differential cleavage of the 2 receptors was also observed on activation of GPVI, providing evidence of simultaneous down-regulation by distinct proteolytic cleavage of

both receptors on ligation of either ITAM-bearing receptor. These observations extend our insights of the mechanisms that terminate signaling through these receptors in the context of hemostasis and thrombosis.

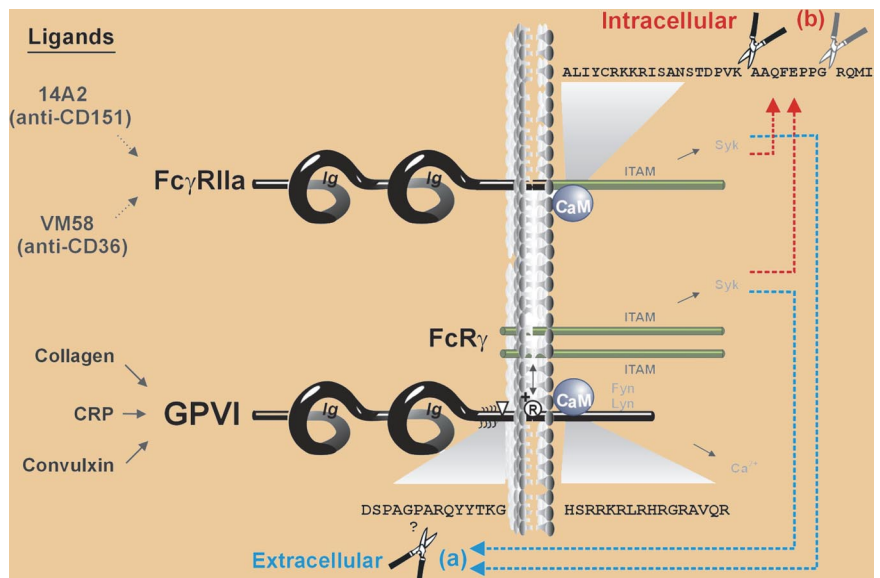
From the clinical perspective, there are important implications. Platelet FcγRIIa mediates the intense platelet activation that accompanies heparin-induced thrombocytopenia (HIT) induced by platelet binding of the immune complexes. Gardiner et al go on to demonstrate in vitro down-regulation of GPVI and FcγRIIa in platelets exposed to IgG from HIT patients. This predicts blunted collagen-induced platelet activation in HIT patients, a finding of considerable interest and in need of further supporting evidence. Also relevant in this context are the previous observations that GPVI antibody depletes platelet GPVI in vivo and decreases collagen responses

in immune thrombocytopenic purpura (ITP) patients^{2,3} and in mouse models,^{4,5} and that GPVI antibody has an antithrombotic effect.⁵ Proteolytic cleavage of GPVI is the mechanism in at least some of these studies. Moreover, for both receptors studied by Gardiner et al, calmodulin is a major intermediary player and calmodulin inhibitors induce their cleavage, suggesting a potential role for these agents as antithrombotic strategies to down-regulate platelet receptor function, provided that their other effects on hematopoietic cells are not a limitation.

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

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Scheme showing ITAM-mediated proteolytic pathways for irreversible inactivation of platelet receptors.

Comment on Le Guyader et al, page 132

A photographic fishing expedition with granie

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In this issue of *Blood*, Le Guyader and colleagues use a variety of elegant techniques during zebrafish development to identify a unique population of neutrophils derived from myeloid progenitors originating from primitive embryonic macrophages.

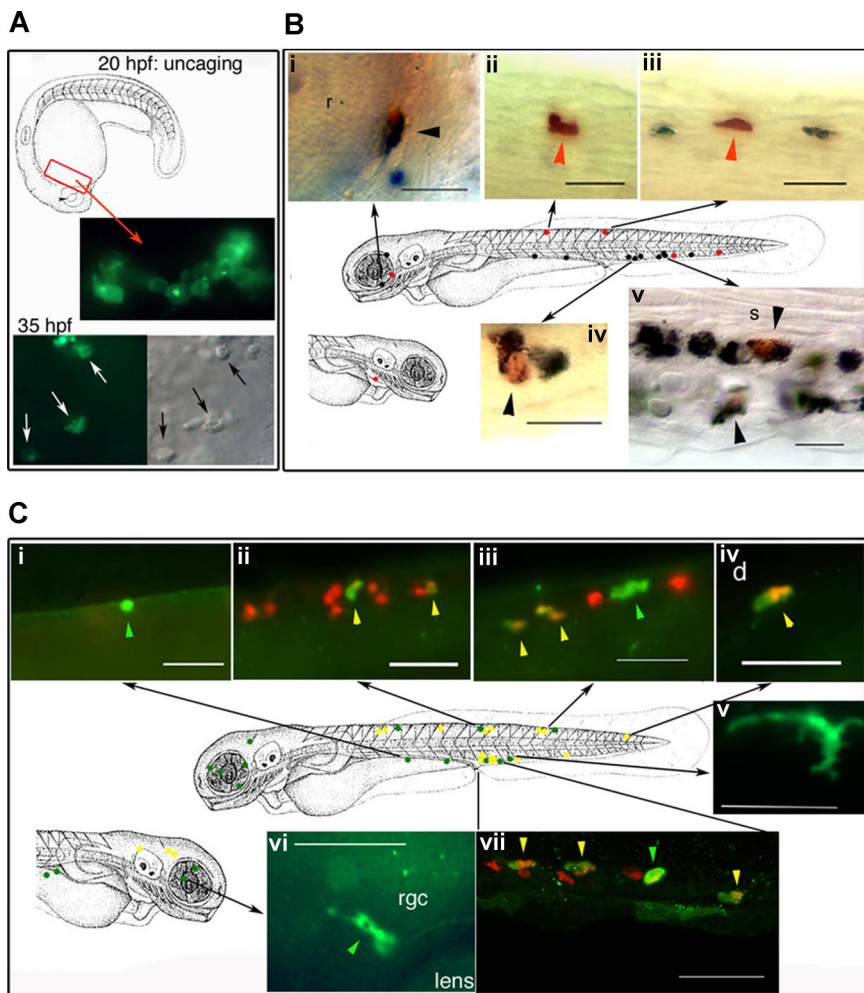
Zebrafish, the tropical freshwater *Danio rerio*, has been an exceptional vertebrate model for investigating organogenesis and in particular the molecular regulation, differentiation, and development of vertebrate hematopoiesis.^{1,2} There are several advantages to using zebrafish as a model of developmental

vertebrate hematopoiesis: (1) zebrafish fertilize a large number of eggs externally that can be readily collected and investigated; (2) zebrafish embryos are transparent, facilitating direct observation of organogenesis; (3) zebrafish embryonic development is rapid; and (4) a variety of technologies, including

morpholino-induced gene knock-down, RNA in situ hybridization, unique methods of mutagenesis, and the study of transgenic zebrafish have provided critical insights into molecular regulation of vertebrate hematopoiesis.¹ The zebrafish model has allowed the use of “forward genetics” to identify new and sometimes unanticipated genes that regulate developmental vertebrate hematopoiesis, identify the genetic basis of a few human hematopoietic disorders, provide insights into the genetic basis of myelodysplastic syndromes and leukemic transformation, and find potential novel targets for therapeutic interventions.

Hematopoiesis was originally thought to arise only from the intermediate cell mass (ICM) or caudal hematopoietic tissue (CHT) located in the posterior region between the notochord endoderm in primitive zebrafish. However, researchers have recently identified a second site of embryonic “primitive” hematopoiesis that originates in the anterior-lateral mesoderm (rostral blood island [RBI]) and eventually migrates to the kidney, which serves as the location of “definitive” hematopoiesis during the remainder of the zebrafish’s development and lifespan.^{1,2} In contrast, in mammals, primitive hematopoiesis originates from mesoderm outside the embryo that migrates onto the embryonic yolk sac to develop blood islands; during the course of further development, it migrates to the aorta-gonad-mesonephros (AGM) region, fetal liver, and finally the bone marrow for “definitive” hematopoiesis.¹⁻³

Macrophages have been identified as the first leukocytes to be derived during developmental myelopoiesis in vertebrate embryos, originate from the RBI, before dispersing throughout the embryonic mesenchyme prior to blood circulation in the developing zebrafish. Neutrophilic granulocytes appear next in primitive hematopoiesis and are identified in blood circulation and connective tissue (mesenchyme) by 48 hours after fertilization (hpf). Neutrophil development during mammalian hematopoiesis was thought to originate from primitive hematopoietic cells similar to macrophages but not from macrophages themselves. In this issue, Le Guyader and coworkers have identified a unique subset of neutrophilic granulocytes that originate from primitive macrophages from the RBI giving rise to precursor primitive myeloid progenitor



In vivo cell labeling with a photoactivatable cell tracer demonstrates the double potential of the primitive (rostral) myeloid progenitors. See the complete figure in the article beginning on page 132.

cells. Furthermore, these neutrophilic granulocytes arising from primitive macrophages have unique properties, including expression of PU.1 and L-plastin, migrating toward invading microbes without phagocytosis, and are dispersed into mesenchyme and epidermis but not the blood circulation. Using a fluorescein dye to detect peroxidase activity of activated granulocytes by an ultraviolet laser, Le Guyader et al show, through cell tracing of sudan black (SB)-stained granulocytes, a population of neutrophilic granulocytes that were derived from myeloid progenitors originating from primitive macrophages of the RBI (see figure).

These novel findings of a unique population of neutrophils derived from primitive macrophages are contrary to the dogma of mammalian myelopoiesis and provide direction for future critical investigations. Does this unique population exist in the human neonate, providing a partial source for their immaturity in phagocytic immunity?⁴ What are the mo-

lecular mechanisms regulating this developmental process, and can they be exploited for ex vivo human myelopoiesis? What, if any, is the role of this population of neutrophils in tissue injury, repair, and defense in humans? Le Guyader et al have shed a new light on the origin and regulation of developmental vertebrate myelopoiesis.

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

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elin cascade in FL, diffuse large B-cell lymphoma (DLBCL), and mantle cell lymphoma (MCL), there was no inhibition of PKC ζ activity in DLBCL and MCL; there was only selective inhibition in FL, for reasons not yet understood. Another novel finding in Leseux et al's study is that RTX-mediated inhibition of PKC ζ results in inhibition of the MAPK pathway and leads to inhibition of the mTOR pathway that is, in part, regulated by MAPK. Hence, the authors have established the link between RTX and-PKC ζ /Raf-1/mTOR dysregulation, resulting in inhibition of cell growth. Leseux et al's novel findings identify a pivotal proximal kinase in the mechanism by which RTX inhibits survival pathways by inhibiting PKC ζ activity and establish PKC ζ and mTOR as targets for therapeutic intervention.

The study by Leseux et al raises several intriguing questions that deserve attention. For instance, the selective activity in FL, and not in other lymphomas, supports the phenotypic and genetic heterogeneity of these diseases and their response to rituximab. Hence, different therapeutic approaches are needed to treat these various malignancies. PKC ζ is highly activated in FL, but it is not clear what regulates its hyperactivation, whether constitutively or by autocrine/paracrine loops. PKC ζ activation depends on the phosphatidylinositol (PI)-3, 4, 5-triphosphate (PIP³), which is mainly induced by PI-3 kinase. 3'-PI-dependent protein kinase 1, which binds with high affinity to PIP³, phosphorylates and activates PKC ζ .³ Leseux et al show that the role of PKC ζ in the inhibition of Raf-1 signaling does not involve Raf-1 kinase inhibitor protein (RKIP). They find that, unlike in Burkitt lymphoma,² RTX does not induce RKIP overexpression in FL. RKIP has been identified as a member of the phosphatidylethanolamine binding protein (PEBP) family and shown to bind Raf-1 and inhibit MEK binding to Raf-1⁴ and inhibit downstream Raf-1-induced transformation and AP-1-dependent transcription. Corbit et al⁵ reported that PKC ζ phosphorylates RKIP at Ser153, resulting in the dissociation of RKIP from Raf-1. Thus, inhibition of PKC ζ by RTX should result in the inhibition of phosphorylation of RKIP and prevent RKIP dissociation from Raf-1, thereby inhibiting Raf-1 signaling. The failure of RTX to up-regulate RKIP expression in FL in Leseux et al's findings, however, does not rule out the possibility that RTX inhibited

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Comment on Leseux et al, page 285

The rituximab-PKC ζ /Raf-1/mTOR connection

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In this issue of *Blood*, Leseux and colleagues report rituximab-mediated inhibition of PKC ζ activity in follicular lymphoma (FL) resulting in the inhibition of Raf-1 and mTOR signaling pathways and leading to inhibition of tumor cell proliferation.

Rituximab (RTX; chimeric anti-CD20 mAb), the first antibody approved by the Food and Drug Administration (FDA) for cancer treatment, is used in the treatment of B-cell non-Hodgkin lymphoma (B-NHL) alone, or in combination with chemotherapy, with significant clinical responses. While antibody-dependent cellular toxicity, complement-dependent toxicity, and apoptosis have been proposed as underlying mechanisms of RTX activity in vivo, the exact mechanisms of responsiveness and unresponsiveness of tumor cells remain unknown. Several reports have investigated the potential ways by which RTX mediates its inhibitory effect on cell proliferation, induction of apoptosis, and chemo-immunosensitization.^{1,2} These studies demonstrated RTX's ability to

induce mobilization of CD20 into lipid rafts, activate the sphingomyelin pathway, and inhibit Src kinases and multiple cell survival signaling pathways (such as the Raf-1/NF- κ B/p38MAPK/AKT). However, these studies did not identify the proximal targets involved following RTX triggering.

The study by Leseux and colleagues in this issue of *Blood* identifies a key player in cell signaling by RTX, PKC ζ , which leads to inhibition of the Raf-1/mTOR pathways. PKC ζ 's involvement was suspected based on previous findings that RTX activates the sphingomyelin cycle and augments ceramide, which was reported to target PKC ζ . The authors demonstrate that RTX inhibits the activity of the atypical PKC subfamily isoform, PKC ζ . In addition, while RTX triggers the sphingomy-

phosphoRKiP via inhibition of PKC ζ activity. The findings on the role of mTOR inhibition by RTX and the demonstration of synergy achieved by combination of RTX and rapamycin is of paramount clinical significance.

The continued high fatality rates in NHL patients, despite recent advances, illustrate the need for innovative approaches. mTOR inhibitors are good candidates for intervention, and several inhibitors that show promise are currently in clinical trials. However, the effects of mTOR inhibitors in cancer cells are largely unknown, and better understanding of the mTOR pathway can lead to more rational therapies. Since patients can develop resistance to RTX treatment and also resist mTOR inhibitors, Leseux et al's examination of FL cell lines that have developed resistance to these agents will be useful.

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

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● ● ● CLINICAL TRIALS AND OBSERVATIONS

Comment on Raza et al, page 86

“Fairest of them all” for myelodysplastic syndromes

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Several therapeutic options are now available for patients with low-risk myelodysplastic syndrome (MDS).

Raza and colleagues report that lenalidomide reduced red cell (RBC) transfusion requirements $\geq 50\%$ in 43% of 214 patients with non-deletion 5q, primarily low-risk, myelodysplastic syndrome (MDS); 26% (95% CI, 20%-32%) became transfusion independent for a median of 41 weeks. The results, although not as dramatic as those noted with lenalidomide in similar patients with deletion 5q, are perhaps more significant, since most MDS patients do not have deletion 5q.

Medicine is fundamentally concerned with choosing between different therapeutic options. Several exist for patients like those treated by Raza et al. While epoetin or darbepoetin, possibly with granulocyte-colony stimulating factor, may produce prolonged transfusion independence (TI) in two-thirds of patients who require fewer than 2 units of red blood cells (RBCs) monthly and have

serum erythropoietin (EPO) levels below 500,¹ only 35% of Raza et al's patients had such low transfusion requirements, and probably even fewer had such low EPO levels. A similarly small proportion is likely to respond to immunosuppressive therapy.² A third option is a clinical trial. However, within the past 3 years, the Food and Drug Administration has approved lenalidomide, azacitidine, and decitabine for use in MDS, and many patients might prefer these options. Thus, practically speaking, physicians seeing patients with RBC transfusion-dependent low-risk non-deletion 5q MDS patients must decide which of these 3 drugs most effectively reduces the need for transfusions, which are typically these patients' principal clinical problem. However, attempts to assess which drug is the “fairest of them all” are complicated by the variability in the design, as well as the reports, of the

relevant trials. For example, the literature suggests that azacitidine produced TI more frequently (45%; 95% CI, 32%-57%) and for as long as lenalidomide.³ But while a need for more than 4 units of red cells in the 8 weeks preceding lenalidomide (the median requirement) predicted a lower subsequent rate of TI, the azacitidine literature does not note pretransfusion requirements, so the nominally higher rate of TI might merely reflect the frequent inclusion of patients with low transfusion requirements. The effects of the 3 drugs on RBC transfusion needs may be similarly confounded by the much higher proportion of high-risk (International Prognostic Scoring System [IPSS] int-2 or high) patients in the azacitidine³ and decitabine⁴ studies, reports of which do not analyze rates of TI by IPSS score. On the other hand, the latter 2 trials included a randomized, supportive-care-only group, thus allowing the occasional “spontaneous” response and “toxicity” due to disease rather than treatment to be taken into account.

My first choice would be lenalidomide, simply because median time to TI was 5 weeks, compared with 3 to 6 months for azacitidine or decitabine. But the practicing physician can be forgiven for wishing for a trial randomizing among lenalidomide, azacitidine, and decitabine to address the issues noted above. Such a trial is unlikely to be conducted, and perhaps there are greater priorities. Nonetheless, much as the queen in “Snow White” needed a mirror to determine the “fairest of them all,” perhaps we need a mirror in the form of standardized reporting requirements from single-arm clinical trials in this population.

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

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