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HEMATOPOIESIS

Comment on Ebert et al, page 4620, and Flygare et al, page 4627

RPS19: pleads guilty in DBA case?

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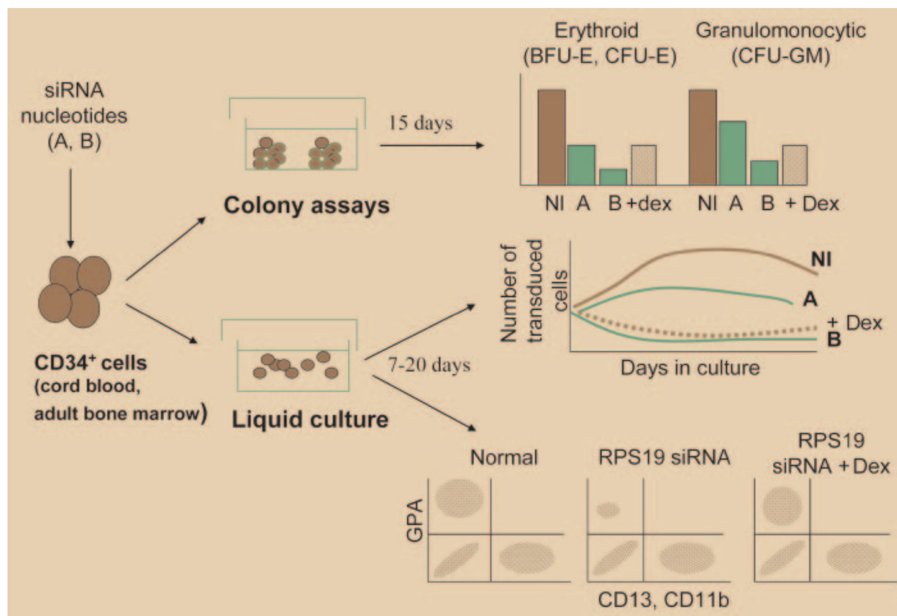
In order to establish a causal link between RPS19 gene mutations and DBA physiopathology, 2 very elegant studies report that reducing RPS19 levels by siRNA in normal $CD34^+$ cells leads to a major impairment in the hematopoietic process.

Almost every available strategy in experimental hematology has been tried in attempts to unravel the molecular defect or defects underlying Diamond-Blackfan anemia (DBA).[1] With the identification of heterozygous mutations in the ribosomal protein S19 (*RPS19*) gene, which encodes a 16-kDa ribosomal protein,[2] in 25% of DBA patients, the challenge is now to prove the causal link between RPS19 haploinsufficiency and erythroid alterations characterizing DBA. Unfortunately, C57BL/6J mice heterozygous for the *RPS19* deletion are not anemic,[3] limiting experimental approaches to the use of human primary cells. The good news is that in vitro, human erythroid (erythroid burst-forming unit [BFU-E] and erythroid colony-forming unit [CFU-E]) progenitors will proliferate and complete differentiation up to the reticulocyte stage, and granulocytic (granulocyte macrophage–colony-forming unit [CFU-GM]) progenitors will do so to the polymorphonuclear stage with specific cytokines. The bad news is that proliferation and differentiation are coupled in hematopoiesis, making it very tricky to tell which pathway is guilty and to evaluate differentiation if proliferation is inhibited. We know that in patients with DBA, the progression of CFU-E into hemoglobinized erythroblasts is preferentially affected, independently of the status of the *RPS19* gene.[4]



Presentation of the experimental strategy used to analyze the consequences of reducing RPS19 levels by introducing siRNA in human $CD34^+$ cells. Erythroid and granulocytic colonies were scored in semisolid assays. Cell proliferation was determined by counting cells in liquid culture, and differentiation by analyzing cell phenotype by flow cytometry or cytologic analysis. A indicates 50% RPS19 levels; B, less than 50% RPS19 levels; and Dex, dexamethasone. Results are adapted schematically from the original figures in the papers by Flygare et al, page 4627 and Ebert et al, page 4620.

Direct loss- and gain-of-function experiments now yield important information on the cellular functions of the *RPS19* gene: Hamaguchi and colleagues[5] recently found that forced expression of RPS19 in cells from patients with DBA partially rescued the erythroid defect, and in this issue of *Blood*, Flygare and colleagues and Ebert and colleagues report a major impairment in the development of $CD34^+$ cells in both erythroid and granulocytic lineages after reducing RPS19 levels by siRNA. Different oligonucleotides targeting endogenous *RPS19* RNA were expressed in primary fetal or adult $CD34^+$ cells via retroviral or lentiviral vectors, decreasing the RPS19 protein to various levels. In both studies, this led to a dramatic drop not only in the number of erythroid colonies generated by transduced $CD34^+$ cells, but also in the output of marrow-derived CFU-GMs, which was of the same order of magnitude. This drop in both CFU-GMs and BFU-Es was explained by a major defect in progenitor cell proliferation, proportional to the level of the RPS19 protein; this proliferation defect was also observed in leukemic cell lines, and the nonhematopoietic 293 cells when expressing the RPS19 siRNAs. Reintroducing the *RPS19* cDNA in siRNA-treated cells rescued the defect. If $CD34^+$ cells do not proliferate, a putative alteration along their erythroid differentiation process would be masked. Flygare and colleagues tried to address this point by comparing expression of erythroid (glycophorin A)– and granulocytic-specific antigens in cells that escaped the blockade, and showed that terminal erythroid differentiation is more severely compromised than granulocytic maturation. Interestingly, as shown by Ebert and colleagues, adding dexamethasone (to which 65% of the patients respond in vivo) to siRNA-treated cells did not rescue the proliferation defect, but boosted erythroid differentiation through an RPS19-independent mechanism.

The in vitro defects fit with the broad action expected from a ribosomal gene in which deletion is embryonically lethal,[2] but their severity and extension exceed what is seen in patients, even though compensatory mechanisms may exist in vivo. Of note, Ebert and

colleagues used a large-scale microarray analysis of CD34+ erythroid differentiation to show that the expression of ribosomal genes (including *RPS19*) and erythroid-specific genes varies independently. Is there a specific crossroad between the RPS19 and the erythroid differentiation pathway, and, if so, when in the hematopoietic hierarchy does it occur? Does it involve an extraribosomal role for RPS19 or RPS19 partners encoded by the additional genetic loci recently involved in DBA?[6] The invariant clinical picture and in vitro defects in patients with DBA with an intact *RPS19* gene strongly argues that RPS19 should not be the only one to blame. ■

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● ● ● HEMATOPOIESIS

Comment on Wang et al, page 4598

Simplifying hESC culture

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Human embryonic stem cells are a valuable resource for research and cell replacement therapy but are notoriously cumbersome to culture. Bhatia and colleagues show that an increased dose of basic fibroblast growth factor eliminates the need for feeder layer coculture.

Human embryonic stem cells (hESCs) represent an important tool for research and a potential resource for cell-replacement therapies, but they are difficult to cultivate. Typically, hESCs are cocultured with a supportive feeder cell layer of murine fibroblasts that provides factors that maintain self-renewal divisions and inhibit the otherwise spontaneous tendency of the cells to differentiate. The cumbersome and finicky conditions for growing hESCs have hindered wider exploitation of these cells by the research community, and significant advances in cell manufacturing and bioprocess engineering are needed before these cells are ready for clinical applications. In this issue of *Blood*, Bhatia and colleagues have taken an important first step toward simplifying hESC culture by showing that high doses of basic fibroblast growth factor (bFGF) are adequate to maintain hESCs under feeder-free and serum-free growth conditions.

Mouse and human ESCs share many important properties, yet for years scientists have been perplexed by the different cytokine requirements for propagating the 2 cell types. Mouse ESCs can be grown in the absence of feeder cells if the media is supplemented with leukemia inhibitory factor (LIF), which maintains symmetrical self-renewal divisions and acts as a potent inhibitor of ESC differentiation by activating signal transducers and activators of transcription 3 (STAT3) signal transduction. In contrast, LIF does not prevent differentiation of hESCs, despite the fact that the LIF receptor and the STAT3 signaling pathway are intact.[1,2] Human ESCs can be propagated in the absence of feeders as long as they are plated on a preformed extracellular matrix (provided by matrigel [BD Biosciences] or laminin), provided with media conditioned by growth on feeder fibroblasts, and supplemented with bFGF (typically at doses $<$10 ng/mL[3]). The advantages of feeder-free culture have prompted enormous interest in identifying the "LIF-like" components in fibroblast conditioned medium (CM). Surprisingly, Bhatia and colleagues have now shown that simply increasing the dose of bFGF added to a simple serum-free culture media allows researchers to maintain hESCs in the absence of feeders or CM. Cells cultured in this manner retain pluripotency and can be differentiated into hemogenic-endothelial precursors, thereby facilitating in vitro studies of human hematopoietic development. Furthermore, Bhatia et al show that incubation of CM from feeders with a neutralizing antibody against bFGF abrogates the capacity of CM to support hESCs, suggesting that bFGF is an essential factor produced by feeder cells, although direct quantification of the amount of bFGF in CM would have helped determine whether all of the critical activities of CM can be accounted for by bFGF. Recently, the observation that high-dose bFGF (40 ng/mL) can sustain hESCs has been independently reported by 2 other groups, thereby corroborating this important insight.[4,5]

Although bFGF appears to function as an LIF equivalent in hESC culture, there are probably other active constituents of CM. Thomson's group[5] report that feeders may serve in part to neutralize or antagonize ill-defined inducers of differentiation that are contributed by serum or various commercially available cocktails of "serum-replacement." Other elements currently employed in hESC culture also remain a mystery, especially the complex and variable composition of matrigel (a basement matrix prepared from a murine sarcoma tumor line) and the proprietary constituents of various serum replacements. The derivation of new hESC lines free of contamination with animal products, to minimize risks associated with animal pathogen transmission, is a worthy goal for hESC research. Conditions for growth of hESCs in entirely chemically defined media have yet to be discovered, but identification of bFGF as an essential component is an important first step. ■

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