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Comment on O'Connor et al, page 4754, and Watkins et al, page e1

Comparative genomics: fishing nets hemostatic catch

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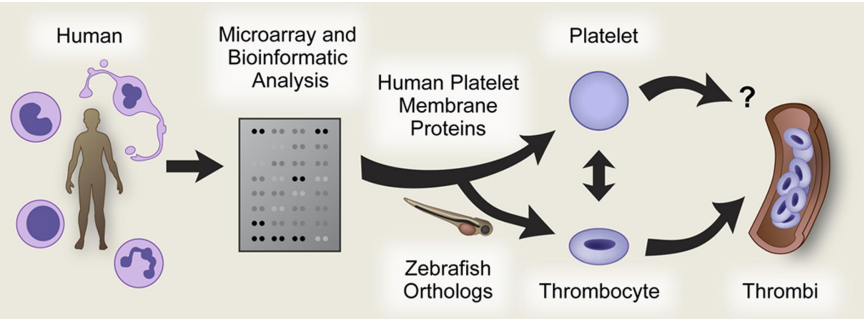
In this issue of *Blood*, Watkins and colleagues report a detailed analysis of gene expression profiles in human blood cells and precursors that identifies genes involved in lineage commitment and cellular responses as well as new candidates with uncharacterized functions. In our new online series, e-*Blood*, O'Connor and colleagues report that a functional screen in zebrafish yields evidence that a subset of candidate gene products—each a platelet membrane protein—have previously unrecognized activities in thrombus formation. Together, these studies provide additional support for genomic surveys as tools for cataloging patterns of expressed genes in human hematopoietic cells and for employing zebrafish as a surrogate system for initial examination of proteins of unknown functions as potential modulators of hemostasis.

Several groups have used transcript profiling to characterize mRNA expression patterns in murine and human blood cells. Although interesting data have emerged, variations in sample representation, purity of starting cell populations, sensitivity, scope of surveyed genes, and rigor of post hoc analysis limits the findings in some cases. Watkins et al[1] performed replicate hybridizations starting with mRNA isolated from positively selected circulating human leukocyte subtypes (granulocytes, monocytes, cytotoxic and helper T cells, B lymphocytes, natural killer cells) separated from the blood of individual donors, and interrogated the samples using whole genome microarrays; model erythroblasts and megakaryocytes differentiated from cord blood hematopoietic progenitor cells[2] were also included (see figure). The resulting individual cell profiles were compiled into an expression atlas and examined for key features and comparisons using bioinformatics approaches. Transcription factors, immunoglobulin superfamily members, and subsets of gene products that may have novel roles in hemostasis and thrombosis were of particular interest, as were comparisons between expressed transcripts in individual human hematopoietic cell types. An additional feature of interest was comparison of the human data sets and expression profiles from analogous murine blood cells, using findings reported by others in a separate study. The percent of total transcripts expressed in both human and mouse cell subtypes ranged from 63% to 75%, and the overlap in transcription factor expression was 49% to 58%. While these comparisons indicate significant similarities in ortholog expression in the 2 species, they also suggest substantial differences that may account for some of the limitations of mouse models intended to reproduce features of human immune and inflammatory diseases.[3]



Fishing for hemostatic proteins using comparative genomics and zebrafish models.Watkins et al compared gene expression patterns in primary human myeloid leukocytes, lymphocytes, and natural killer cells, and in model erythroblastic and megakaryocytic cells cultured from hematopoietic precursors by using whole genome chip microarray technology. Bioinformatics analysis of the resulting atlas of data identified lineage-specific genes, co-expression patterns, similarities and differences in the patterns of expressed genes in analogous murine blood cells, and genes whose protein products are candidates for new functional roles. O'Connor et al used this database to examine a subset of human platelet membrane proteins without established hemostatic functions—identified as candidates by the presence of their mRNA transcripts in megakaryocytic cells in the study by Watkins et al—for activities in experimental thrombosis. The strategy involved identification of orthologs corresponding to human platelet proteins in zebrafish cells, "knockdown" of the zebrafish orthologs using antisense morpholino oligonucleotides, and examination of responses of the morpholino-treated fish in a laser-induced thrombosis model. Reduced expression of 4 candidate proteins resulted in altered thrombus formation. Functional roles of the corresponding human proteins now merit evaluation in relevant models based on this screening strategy.

In a parallel study, O'Connor et al used information from the multiple cell, whole genome expression atlas,[1] and from a previous examination of expression patterns in human megakaryocytic cells differentiated in vitro,[3] focusing on a subset of genes encoding putative transmembrane proteins in megakaryocytes and platelets that might have uncharted roles in thrombus formation.[4] They documented expression of transcripts and protein products of candidate genes in primary human platelets from blood, adding evidence for use of examination of the platelet transcriptome and proteome,[5,6] and also identified orthologs

of the candidate genes in zebrafish (*Dario rerio*).[4] The strategy here was to attempt to predict functions of the human proteins using a "reverse genetic" screen in which expression of the fish orthologs was "knocked down" and the phenotype, or lack of it, was determined in an induced thrombus model.[4] Zebrafish have been touted as unique surrogate systems for reverse genetic analysis, and their potential use in specific studies of hemostasis is supported by similarities between blood cells and humoral coagulation pathways in humans and this fish species.[4,7,8]

The fishing expedition looking for new functional proteins was a success, and O'Connor et al netted 4 factors with roles in laser-induced thrombosis (2 promoters, 2 modulators) in the surrogate zebrafish system. The phenotypes presumably indicate activities of the fish orthologs when they are expressed on circulating thrombocytes, but a caveat is that knockdown by morpholino antisense technology, which was the approach used, is not cell specific. Thus, one or more of the proteins could also have activities on endothelial cells or other cell types.[4,8] Nevertheless, the results of the reverse genetic screen are enticing enough to merit evaluation of the candidate proteins in human platelets—where their functions remain in question—and, again with appropriate caveats, in mouse knockout models.

Conflict-of-interest disclosure: The authors declare no competing financial interests. ■

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Comment on French et al, page 4512

Old drug, new lessons

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In this issue of *Blood*, French and colleagues use several genome-wide approaches and report that acquired genetic variation has a stronger impact on methotrexate polyglutamate accumulation in acute lymphoblastic leukemia cells than inherited genetic variation.

This paper by French et al is among the first to combine high-throughput analyses of malignant cells for acquired genetic variation (mRNA expression and DNA copy number variation) and of normal cells to detect inherited genetic variation (DNA single nucleotide polymorphisms and DNA copy number variation) in the same patients.[1] A relatively large cohort of children with acute lymphoblastic leukemia (ALL) was tested, and methotrexate polyglutamate (MTXPG) accumulation was determined ex vivo in samples obtained 42 to 44 hours after the patients had been treated with single-agent MTX at 1 g/m^2 given intravenously over 4 or 24 hours. Acquired genetic variation was assessed in leukemic cells obtained at diagnosis while inherited genetic variation was studied in DNA extracted from whole blood sampled after the patients achieved complete remission.

This study is important for several reasons. First, it demonstrates the importance of characterizing malignant cells themselves in order to predict or explain their sensitivity to a particular drug. Apparently, the biology of malignant cells is more important in this respect than inherited factors, which, for instance, influence pharmacokinetics. Second, it identifies novel genes, especially on chromosomes 10 and 18, which seem important in explaining variation in MTXPG accumulation. Third, the paper contains a wealth of information on the relevance of individual chromosomes and genes regarding MTX accumulation. Finally, it shows that combining information on acquired and inherited genetic variation can be useful, since this analysis identified 7 genes that had the strongest impact on MTXPG accumulation.

Inevitably, the study also has some weaknesses, and several questions remain to be answered. While 248 patients were eligible for the study, actual characterization was limited to 145 patients for mRNA expression, 82 patients for leukemia cell DNA copy number variation, and 144 patients for inherited DNA genotyping. The authors demonstrate that this did not result in a statistically significant selection bias, but some impact of the subset of patients available for each assay type cannot be excluded. Moreover, patient numbers limited the power of the study to detect smaller but still relevant correlations between genetic variation and MTXPG levels. An open question is whether the genes that explained variation in MTXPG accumulation have a causal role in determining the clinical response to MTX treatment. After all, this was a correlative study and moreover, MTXPG levels are a surrogate for sensitivity or resistance to MTX, although other studies demonstrated a correlation between MTXPG levels and both in vitro[2] and in vivo[3] efficacy of MTX. It also would be interesting to know what the study results would have been in case of a higher dose of MTX (eg, 5 g/m^2), a dose now being used in many protocols. French et al indeed report that MTXPG levels differed between the 4- and 24-hour infusion schedules, and they cite literature that reported that gain-of-chromosome 21 was associated with increased MTXPG accumulation, but only in the case of treatment with MTX at 180 mg/m^2 given orally over 36 hours and not in the case of 1 g/m^2 given as a 24-hour infusion.[4] Similarly, the findings explain up to two-thirds of the variation in MTXPG levels, but what about the remaining one-third? Finally, the authors do not provide data on toxicity of MTX. The current analysis might suggest that characterization of inherited genetic variation is of limited value. However, such variation is more