COMMENTARY

Hunting for hematopoietic transcriptional networks

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Each day an adult human produces roughly 2.5×10^{11} erythrocytes, 1×10^{11} leukocytes, and 1×10^{11} platelets, numbers that can increase 10- to 20-fold in times of heightened demand. Blood cell production, termed hematopoiesis, occurs in the red marrow found mostly in the skull, spine, and proximal ends of the long bones of the body. Within the marrow resides a small number of hematopoietic stem cells (HSCs), the origin of all blood cells, which undergo a series of proliferative and differentiation steps eventuating in the near half a trillion or more cells produced each day. The molecular "wiring diagram" that regulates the production of mature blood cells from HSCs is both cell intrinsic (gene transcription factors and epigenetic changes) and cell extrinsic (growth factors and microenvironmental soluble and cell surface proteins), although whether lineage decisions are stochastic or directed by external factors remains a controversial topic. Much is known about the HSC niche and the cells, hormones, and soluble mediators that affect hematopoiesis, but less well known are the transcription factors that regulate the lineage fate choices made by HSCs and their developmental progeny. The study by Zhu et al. (1) in PNAS has the potential to greatly expand our understanding of this latter aspect of hematopoiesis and provides a roadmap for further discovery.

The Cellular Development of Blood Platelets

Blood platelets derive from the tips of elongated cytoplasmic processes of a large, polyploid marrow cell, termed a megakaryocyte, with each megakaryocyte delivering about 1,000 platelets into the marrow vascular sinusoids. Megakaryocytes, like all marrow cells, are derived from HSCs through a series of expansion and differentiation steps that generate a group of intermediately differentiated cells, including common myeloid progenitors (CMPs), which can ultimately give rise to erythrocytes, granulocytic leukocytes, and megakaryocytes; megakaryocyte-erythroid progenitors (MEPs), which can ultimately give rise to erythrocytes and megakaryocytes; and megakaryocytic progenitors (MkPs), which give rise exclusively to megakaryocytes. Platelets are critical for vascular integrity, both by forming a physical plug (of thousands of platelets) at the site of blood vessel injury and by providing an activated cell surface on which coagulation proteins (*i*) assemble and are activated, stabilizing the platelet plug, and (*ii*) together, initiate vascular healing. Without platelets, we hemorrhage. Platelets are also involved in some of the most important diseases of humans: By clotting on ruptured atherosclerotic plaques, they cause myocardial infarction, stroke, and arterial insufficiency. By binding to circulating tumor cells, platelets promote metastatic spread of cancer. And when platelets are dysfunctional, they can cause pathological clotting and hemorrhage. Thus, a thorough understanding of platelet production—from the HSC to the CMP, the MEP, the MkP, the megakaryocyte, and finally to circulating platelets—may shed light on a number physiological and pathological processes, as well as open doors into novel therapeutic approaches to common human diseases.

Identifying Transcription Factors That Potentially Affect Platelet Development

Over the past few decades, several transcription factors that mediate the development of blood cells from HSCs have been identified, based on the recognition of promoter elements important for the expression of hematopoietic genes [e.g., GATA1 (2)], the rearrangement or mutation of genes found in hematopoietic malignancies [e.g., SCL/TAL 1, AML 1/RUNX (3, 4)] or in congenital disorders of blood cell production [e.g., FLI1 (5)], and in gene-inactivation studies [e.g., MYB (6)]. However, identification of the transcription factors necessary for development of the megakaryocyte/platelet lineage lagged those of erythrocytes and leukocytes because of the rarity of the cells in the marrow (~0.1%) and the lack of the requisite hormones and cytokines needed to stimulate their production. With the cloning and characterization of thrombopoietin (7), large numbers of megakaryocytes, their precursors, and their progeny could be produced and studied. In the study by Zhu et al. (1), a number of transcription factors that are likely involved in megakaryocyte and platelet development are identified

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Author contributions: K.K. wrote the paper.
The author declares no conflict of interest.

See companion article on page E9308.
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Published online September 10, 2018.

through an in silica candidate gene-finding tool, followed by validating loss-of-function and gain-of function assays.

With the advent of microarray technology, numerous investigators have published gene-expression data from variably purified hematopoietic stem, progenitor, and mature cell populations. While any given microarray-expression experiment is subject to a myriad of technical issues that could limit interpretation (cell purity, state of differentiation, method of production, level of cell stimulation during purification, use of transformed cell lines instead of purified normal cells, etc.), the use of bioinformatics to assemble and analyze the data from thousands of such experiments could provide far more robust results. Several years ago, Weissman and colleagues (8) reported on such an approach, termed Gene Expression Commons (GEXC; https://gexc.riken.jp/), which now contains the data from over 10,000 microarray analyses of murine hematopoietic cells. Prior analysis of hematopoietic differentiation suggests that lineage determination is characterized by a binary branching hierarchy: a progenitor with two possible cell fates divides and produces daughter cells that remain at the same stage of development (bipotent), differentiate into one or the other, or both differentiate into unipotent progeny. For example, the MEP can divide and remain as two MEPs or differentiate into progenitor cells committed exclusively to the megakaryocyte lineage (i.e., MkP), the erythroid lineage (i.e., EP), or some combination in a "yin and yang" outcome. It is felt that such lineage fate choices are directed by the transcription factor composition of the parent cell (MEP in this example); a rise in erythroid transcription factors or fall in megakaryocyte transcription factors tilts the cell toward becoming an EP, while the opposite transcription factor complement favors the daughter cells developing into MkPs. Hence, Zhu et al. (1) queried the GEXC database with the search phrase "inactive in MEP and EP, while active in MkP" to identify candidate transcription factors that drive megakaryocyte development. Using this strategy, the expression patterns of the 60 highest-scoring genes were analyzed using real-time PCR assays in purified MEPs, MkPs, and EPs. The expression patterns of many of the 60 candidate genes were found as expected, that is, high in MkP and low in MEP and EP, and were then tested in loss-of-function (CRISPR) and then in gain-of-function (lentiviral-mediated expression) assays in a mixed population of primitive, marrow-derived hematopoietic cells after optimizing cell transduction methods. The cellular outcomes of these experiments were followed by cell surface phenotype (flow cytometry), assessment of known patterns of erythroid and megakaryocyte gene expression (real-time PCR), and in vitro culture (colony-forming assays). Since the test cell population included very primitive hematopoietic cells, assessment of the types of progenitors that expanded or contracted after genetic manipulation could also allow assessment of the stage of differentiation at which the various transcription factors exerted their influence. As a two-way control on the veracity of experimental results, a transcription factor known from several prior studies to be critical for megakaryocyte development, Fli1, was identified in the GEXC screen, and its experimental reduction and overexpression led to substantial reductions and increases, respectively, in megakaryocyte output in tissue culture experiments. Based on these in silica and in vitro experiments, 10 genes (*MZF1*, *GSX2*, *HOXC6*, *HDAC11*, *HES7*, *FOXB1*, *MXD3*, *HOXA9*, *NFATC1*, and *PCGF2*) were found to drive megakaryocyte development. Obviously, verification of their precise role in hematopoiesis awaits their genetic manipulation in animals or their association with either congenital or acquired reductions or overproduction of platelets in humans.

Regulating the Regulators: Identifying Transcriptional Networks

In a second set of experiments, Zhu et al. (1) tested whether the identified transcription factors are affected by epigenetic changes in megakaryocyte development, by assessing the expression patterns of the enzymes that regulate histone acetylation and by using histone deacetylase (HDAC) inhibitors to begin to better understand the network of processes that regulate megakaryocyte production.

Together, the findings reported by Zhu et al. could add substantially to the wiring diagram of megakaryocyte development and provide new methods for regulatory network discovery and verification.

Moreover, given the widespread use of such agents in cancer patients, such experiments could lay the groundwork for pharmacological manipulation of these genes for therapeutic benefit. Zhu et al. (1) found that six of the HDACs (HDACs 1, 2, 3, 7, 9, and 10) were expressed at higher levels in MEPs and EPs than in MkPs, suggesting that histone modifications could also play an important role in megakaryocyte lineage fate determination. To test this hypothesis Zhu et al. then utilized a series of HDAC inhibitors that differentially affect different groups of HDACs and found that GSX2, MXD3, HOXC6, and HES7 were profoundly up-regulated and that PCGF2, FOXB1, and MZF1 were modestly up-regulated, suggesting that these genes are affected by epigenetic changes. Together, the findings reported by Zhu et al. could add substantially to the wiring diagram of megakaryocyte development and provide new methods for regulatory network discovery and verification.

In addition to expanding our understanding of megakaryocyte development, particularly noteworthy in the Zhu et al. (1) study is the use of GEXC to identify candidate genes. Granted, of the 60 highest-scoring genes revealed by probing the GEXC database, only 10 survived the loss-of-function and gain-of-function verification testing. But this commentator suspects that most investigators would settle for such a yield in any gene-finding exercise. In fact, a one in six yield could be considered prima facie evidence that GEXC is a robust gene-finding tool. Also described in the report by Zhu et al. is an extremely effective (>90%) primary cell transduction methodology, using highly concentrated lentiviral vector stocks, retronectin, an optimized multiplicity of infection, and multiple rounds of cell transduction. Many experimental hematopoiesis strategies are foiled by low transduction efficiency.

Finally, while identification of a number of candidate transcriptional regulators of megakaryocyte development is noteworthy, it should not be construed as evidence that the rise and fall of networks of transcriptional regulators that drive the yin and yang decisions HPCs make during development are necessarily also cell intrinsic. A longstanding debate in hematopoietic lineage fate determination is whether the levels of transcription factors that drive one or another lineage rise and fall by stochastic probability, or whether external signals can influence which transcriptional program predominates in any given cell. For example, of the transcription factors recognized in the Zhu et al. (1) paper to drive megakaryocyte development, several are known to be influenced by megakaryocytic cytokines that exist in the hematopoietic microenvironment. For example, thrombopoietin regulates the

nuclear localization of HOXA9 (9), MZF1 is regulated by GM-CSF (10), another hematopoietic growth factor, and HES7 is regulated by the Notch signaling pathway (11), which is activated by cell surface contact with Delta ligand-bearing cells and by the Wnt pathway (12), another extracellular signal-responsive process. Thus, while the report by Zhu et al. (1) provides many candidate, cell-intrinsic transcription factors to better understand platelet production and a method that can be applied to other, important developmental questions, the work does not necessarily imply that networks of hematopoietic transcriptional regulators are cell intrinsic instead of being regulated by elements of the hematopoietic niche.

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