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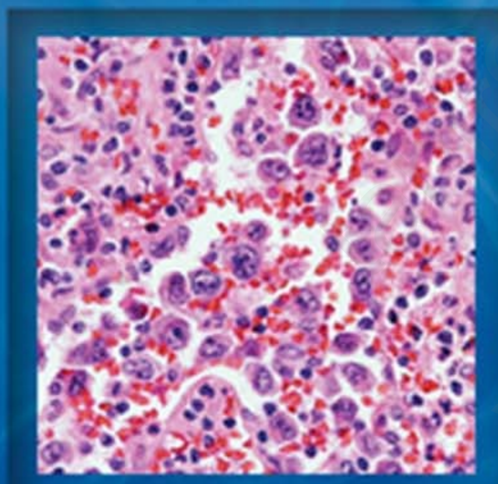
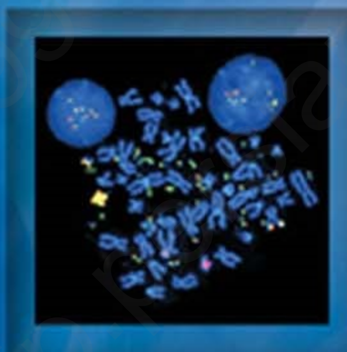
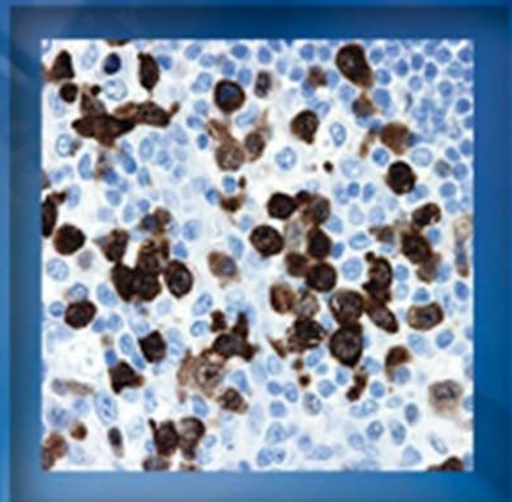

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With love and gratitude
to Marian, Daniel, Louise, and Tyler

—DMK

To Maria, my wife, and to Giulia and Rita, our daughters, for their love and support.
To my parents who by their example have given me the drive and discipline
to tackle all tasks with enthusiasm and determination.

—AO

To Elliott, Jim, Morgan, and Charlie Foucar

—KF

To my wife Tina and my daughters, Dina and Tessa

—LMW

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Preface to the First Edition

Pathologists have traditionally experienced difficulty in diagnosing hematologic neoplasms. One factor that accounts for these difficulties is that malignant lymphoma and leukemia cells often exhibit cytomorphologic features that mimic those of normal differentiating hematolymphoid cells. A second and perhaps more significant factor for many years was the lack of knowledge and understanding of the hematopoietic and immune systems. This prevented pathologists from comprehending the origin and nature of hematologic neoplasms and their relation to the normal cellular components of the hematopoietic and immune systems. The unprecedented explosion of new scientific information concerning the hematopoietic and immune systems that has taken place during the past 20 years, however, has generated the scientific bases for the reliable and reproducible diagnosis and classification of most hematologic neoplasms.

Modern hematopathology began in the early 1970s soon after immunologists discovered that lymphocytes are divisible into two distinct subpopulations: B cells and T cells, which vary according to their differentiation process, anatomic localization, and functional properties and are distinguishable according to their differential expression of various surface membrane and cytoplasmic antigens and receptors. Pathologists soon discovered that many neoplastic lymphoid cells also express B- and T-cell–associated markers, presumptive evidence of their B- or T-cell origin. They also discovered that benign, reactive lymphoid proliferations are polyclonal (i.e., contain mixtures of B and T cells), whereas many non-Hodgkin lymphomas and lymphoid leukemias are monoclonal B-cell proliferations (i.e., contain a predominance of B cells that express only one immunoglobulin light chain class, either κ or λ). These discoveries led to the use of cell marker analysis (the routine classification of lymphoid neoplasms as B- or T-cell derived) as an adjunct to morphologic interpretation in the diagnosis of lymphoid neoplasia, encouraged the correlation of morphologic features with immunologic cell markers, and fostered the development of new terminology and classification schemes.

The second phase of modern hematopathology, the monoclonal antibody era, began in the early 1980s when an array of highly specific monoclonal antibodies that detect B-cell, T-cell, monocyte/macrophage, and myeloid lineage differentiation and subset-associated antigens became commercially available. During the same time, several comparatively inexpensive fluorescent-activated cell sorters that permit rapid, accurate, and objective analysis and sorting of cell populations also became available. In addition, several very sensitive immunohistochemical staining techniques, particularly the avidin–biotin-complex immunoperoxidase and alkaline phosphatase/antialkaline phosphatase methods, were developed, and the reagents were made available in a kit form for use in routine pathology laboratories. The combined commercial availability of sensitive and specific monoclonal antibodies and immunohistochemical reagents and affordable fluorescent-activated cell sorters resulted in the establishment of specialized hematopathology laboratories that routinely perform immunophenotypic analysis. The result has been an enormous collective experience with immunophenotypic analysis in the diagnosis and classification of hematologic neoplasms. This experience has allowed us to document the immunophenotypic profiles exhibited by nearly all the major clinicopathologic categories of hematologic neoplasia and to establish guidelines for their immunodiagnosis, resulting in a substantial improvement in diagnostic accuracy and greatly facilitating our understanding of the relation between malignant hematolymphoid cells and normal cells of the hematopoietic and immune systems.

The third and current phase of modern hematopathology, the molecular biology era, began in the mid-1980s. The development of the Southern blot hybridization technique, the cloning of the immunoglobulin and T-cell receptor genes, and the preparation and dissemination of the DNA probes that detect clonal rearrangements of these genes have provided pathologists with a sensitive, accurate, and objective method for determining the lineage and clonality of lymphoid neoplasms. This approach has allowed for the determination of the lineage of neoplasms that exhibit immature, ambiguous, and anomalous immunophenotypes; the determination of the monoclonal nature of lymphoid proliferations of an uncertain nature; and the detection of the clonal B- and T-cell populations that are undetectable by morphologic examination and/or by immunophenotypic analysis. The availability of many additional DNA probes and the development of DNA amplification techniques such as the polymerase chain reaction now allow us to routinely detect oncogenes, chromosomal translocations, and viral sequences as well, thereby facilitating the investigation of the pathogenesis of hematologic neoplasia.

Unfortunately, this sudden and rapid growth has generated a constantly changing and often confusing and conflicting literature. The rapid turnover of knowledge in hematopathology and the increasing reliance on new scientific techniques by hematopathologists have left many experienced pathologists (unfamiliar with the changing concepts and who are unable to perform these modern diagnostic techniques) uncomfortable in rendering definitive diagnostic opinions concerning hematolymphoid proliferations. Pathologists in training have often been discouraged from studying hematopathology for the same reasons. Furthermore, no single source of information that encompasses the morphologic, immunologic, and molecular aspects of hematolymphoid neoplasia has been available to pathologists (to guide them in daily practice) or to pathologists in training (to assist them in acquiring the basic tenets of knowledge of modern hematopathology) until now.

This book represents the first definitive textbook of modern hematopathology. It is aimed at providing a thorough overview of the morphologic, immunologic, and molecular genetic characteristics of the benign and malignant proliferations derived from the hematopoietic and immune systems. The volume begins with a review of our current understanding of the structural and functional characteristics of the hematopoietic and immune systems, followed by chapters that describe the currently available immunologic markers and their application

in the flow cytometric and immunohistochemical analysis of hematologic neoplasms, the structure and function of the antigen receptor genes and oncogenes and their application in the diagnosis and classification of hematologic neoplasms, and an overview of the role of cytogenetics. Practical guidelines for the organization and operation of a hematopathology laboratory and for the technical evaluation of lymph node biopsies also are provided. The role of fine needle biopsy and imprint cytology in the diagnosis and classification of hematologic neoplasms is discussed next. These background chapters are followed by 21 chapters that describe in detail the benign, reactive lymphoid proliferations that stimulate malignant lymphoma, Hodgkin disease, each major clinicopathologic category of non-Hodgkin lymphoma, and the extranodal lymphoid hyperplasias and malignant lymphomas. This is followed by practical guidelines for the handling and cytochemical and immunohistochemical analysis of bone marrow specimens. The final chapters deal with bone marrow involvement by malignant lymphoma, the acute and chronic lymphoid and myeloid leukemias, the myeloproliferative disorders, histiocytic and dendritic cell proliferations, mast cell disease, and the splenic manifestations of hematolymphoid neoplasia.

This volume is a multiauthored text by necessity. The vast amount of information currently available that concerns the clinical and biologic aspects of the numerous and diverse categories of hematopoietic neoplasia precludes any one individual from successfully preparing a definitive, accurate, and up-to-date reference work on neoplastic hematopathology. For that reason, a sincere effort was made to select for the preparation of each chapter experts who have been closely associated with the growth and development of and who have made significant contributions to that particular aspect of hematopathology. The result is that the list of contributors to this textbook represents a veritable Who's Who in hematopathology. These are the very same investigators who have been largely responsible for the many exciting and important developments that have taken place in hematopathology during the past 20 years. Each expert has responded with excitement and enthusiasm for this project. I am grateful to them for their support of and participation in the preparation of *Neoplastic Hematopathology*.

Daniel M. Knowles, MD

Preface to the Second Edition

Since publication of the first edition in 1992, our knowledge in all facets of hematopathology (i.e., morphology, immunology, and molecular biology) has continued to grow unabated.

In 1992, a lack of consensus on lymphoma classification existed; the Working Formulation was the standard in the United States; and the Kiel classification was the standard in most European countries. However, as our conceptual understanding of newly as well as previously recognized lymphoma entities grew, it became increasingly obvious that both classifications had inherent deficiencies. For example, the Working Formulation categories were broadly defined to accommodate the classification of all lymphomas but did not permit the recognition and distinction of specific disease entities (i.e., mantle cell lymphoma, the low-grade extranodal B-cell lymphomas arising in mucosa-associated lymphoid tissue (MALT), and peripheral T-cell lymphomas). In addition to other shortcomings, the Kiel classification neglected to include extranodal lymphomas and failed to distinguish MALT lymphomas.

A group of 19 expert hematopathologists from the United States, Europe, and Asia, designating themselves the International Lymphoma Study Group (ILSG), began to meet informally in 1991 to exchange ideas and information concerning the lymphomas. In 1993, the ILSG undertook the task to reach consensus on a list of "real" lymphoma entities based upon a combination of clinical, morphologic, immunophenotypic, and molecular genetic characteristics. This consensus list was published in 1994 and designated the Revised European-American Lymphoma (REAL) classification, since it represented a revision of the current European and American lymphoma classifications. Shortly thereafter, the reproducibility and clinical utility of the REAL classification were validated in a multiobserver study of 1,300 cases of non-Hodgkin lymphoma gathered from several institutions around the world.

Since 1995, members of the American and European Hematopathology Societies have been collaborating on a new World Health Organization (WHO) classification of hematologic malignancies. The WHO classification employs an updated version of the REAL classification for the lymphomas and expands the tenets of the REAL classification to codify the myeloid and histiocytic neoplasms. The WHO classification will replace all existing classifications and thus represents the first classification of hematologic malignancies in which true international consensus has been achieved.

By the time the first edition of this book was published, four international white cell differentiation antigen workshops had taken place, and leukocyte antigens CD1 through CDw78 had been defined. Since then, two more workshops have been convened, leading to the further clarification of the structural and functional properties of these antigens and the expanded recognition of distinct leukocyte antigens through CD166. Thus, in the 8-year interval between the publication of the first and second editions of this book, the number of distinct monoclonal antibody–defined leukocyte antigens has doubled.

In 1992, immunophenotypic characterization of lymphoproliferative disorders involving solid tissues was most often performed by immunohistochemical staining of frozen tissue sections. Most monoclonal antibodies commercially available at that time were not immunoreactive in paraffin tissue sections; only a few antigens, principally CD3, CD15, CD20, CD30, CD43, and CD45, were detectable in paraffin tissue sections. However, during the past several years, a concerted effort by many investigators to prepare paraffin-reactive monoclonal antibodies has resulted in an explosion of new antibody reagents capable of detecting most of the additional leukocyte antigens that are critical to immunophenotypic analysis, including CD1a, CD4, CD5, CD8, CD10, and CD79α, in paraffin tissue sections. In addition, several investigators have developed heat-based antigen retrieval techniques capable of "unmasking" heretofore undetectable antigens in paraffin tissue sections. These techniques have further expanded the spectrum of paraffin-reactive monoclonal antibodies as well as enhanced the sensitivity and reproducibility of antigen detection in paraffin tissue sections. Finally, efficient, reliable automated immunohistochemical staining instruments have been introduced and widely accepted. As a consequence, at the present time, unlike in 1992, immunophenotypic characterization of the majority of lymphoproliferative disorders involving solid tissues are performed by the immunohistochemical staining of paraffin tissue sections, and, in many instances, by using automated instrumentation. These advances obviated the special requirements and technical difficulties associated with frozen tissue section immunohistochemistry, which has resulted in a marked expansion of the routine immunophenotypic analysis of hematologic malignancies.

By 1992, molecular characterization of the hematologic malignancies had become an established facet of hematopathology. However, the laborious, time-consuming, and relatively insensitive Southern blot technique restricted such studies to a few specialized laboratories. The introduction of simpler, more rapid, and far more sensitive polymerase chain reaction–based assays has resulted in a marked expansion of studies aimed at deciphering the molecular pathology of the hematologic malignancies. In addition, numerous significant discoveries in basic molecular biology have occurred since 1992. One example is the discovery of the *BCL-6* gene, a transcriptional repressor belonging to the POZ/Zinc finger family of transcriptional factors, which appears to play an important role in germinal center formation. Rearrangements of the *BCL-6* gene preferentially occur in diffuse large B-cell lymphomas where they may be associated with extranodal disease and a better prognosis. Another example is the discovery of the Kaposi sarcoma–associated herpes virus, also referred to as human herpesvirus-8, which is a novel gamma 2-herpesvirus present in virtually all Kaposi sarcoma lesions. This virus also has been found to be highly associated with an uncommonly occurring subset of unusual non-Hodgkin lymphomas referred to as *primary effusion lymphomas*, which appear to originate in the body cavities as an effusion in the absence of an identifiable tumor mass. The combination of these and other scientific discoveries and the ever-widening use of molecular biologic techniques in the study

of hematologic malignancies have contributed significantly to our understanding of the role of molecular genetic lesions in the pathogenesis and the clinical and biologic behavior of hematologic malignancies.

Our enhanced knowledge and understanding of the morphologic, immunologic, and molecular genetic characteristics of the hematologic malignancies and the lesions that simulate them necessitated that this book be updated. That is precisely what we have done. This second edition represents a thorough revision and marked expansion of the first edition to reflect our increased knowledge and current concepts of hematopathology. Each chapter appearing in the first edition has been revised; indeed, nearly all of them have been entirely rewritten. The result is that this book represents the definitive textbook of modern hematopathology.

This book is aimed at providing a thorough overview of the morphologic, immunologic, and molecular genetic characteristics of the benign and malignant proliferations derived from the cellular elements that comprise the hematopoietic and immune systems. The book begins with a review of our current understanding of the structural and functional characteristics of the hematopoietic and immune systems, followed by chapters that describe the currently available immunologic markers and their application in the flow cytometric and immunohistochemical analysis of hematologic neoplasms, the normal histology and immunoarchitecture of the lymphoid organs, the structure and function of the antigen receptor genes and oncogenes and their application in the diagnosis and classification of hematologic neoplasms, and an overview of the role of cytogenetics. Practical guidelines for the organization and operation of a hematopathology laboratory and for the technical evaluation of lymph node biopsies also are provided. The role of fine needle biopsy and imprint cytology in the diagnosis and classification of hematologic neoplasms is discussed next. These background chapters are followed by 23 chapters that describe in detail the benign, reactive lymphoid proliferations that simulate malignant lymphoma, the atypical lymphoproliferative disorders, Hodgkin disease, the current classification of the non-Hodgkin lymphomas and Hodgkin disease, and the clinical significance of these classifications, each major clinicopathologic category of non-Hodgkin lymphoma, and the extranodal lymphoid hyperplasias and malignant lymphomas. This is followed by practical guides for the handling and cytochemical and immunohistochemical analysis of bone marrow specimens. The final chapters deal with bone marrow involvement by malignant lymphoma, acute and chronic lymphoid and myeloid leukemias, myeloproliferative disorders, histiocytic and dendritic cell proliferations, mast cell disease, and the splenic manifestations of hematolymphoid neoplasia.

This book is a multiauthored text by necessity. The vast amount of information concerning the clinical, pathologic, and biologic aspects of the numerous and diverse categories of hematopoietic neoplasia currently available precludes any one individual from successfully preparing a definitive, accurate, and up-to-date reference work on neoplastic hematopathology. For that reason, a sincere effort was made to select for the preparation of each chapter experts who have been closely associated with the growth and development, and who have made significant contributions to that particular aspect of hematopathology. The result is that the list of contributors represents a veritable Who's Who in hematopathology. These are the very same investigators who have been largely responsible for many of the exciting and important developments that have taken place in hematopathology during the past 25 years. Each expert responded with excitement and enthusiasm for this project. I am grateful to each of them for their support and participation in the preparation of the second edition of *Neoplastic Hematopathology*.

Daniel M. Knowles, MD

Preface to the Third Edition

Our understanding of the immunologic and the molecular biologic basis for the clinical and morphologic diversity of the hematologic malignancies has continued to grow unabated during the past decade. Indeed, that growth has accelerated. This, in turn, has helped up to further delineate the morphologic criteria that define distinct clinical pathologic entities. As a result, the 2008 WHO classification represents our most successful effort to date to bring clarity and understanding to the classification of the malignant neoplasms derived from hematopoietic cells. In the 8-year interval between the first and second editions of this textbook, the number of monoclonal antibody–defined leukocyte antigens doubled, reaching CD166. In the ensuing decade since the publication of the second edition, additional leukocyte differentiation antigen workshops have propelled us to CD350. The human genome has been successfully sequenced. Continued molecular technologic advances now allow us to rapidly sequence individual human malignancies. This is allowing us to identify novel therapeutic targets. The introduction of next-generation sequencing into the clinical laboratory over the next several years will usher in the era of personalized medicine. Each individual malignant neoplasm will be sequenced, its unique molecular signature delineated, and a therapeutic strategy tailored to that individual neoplasm will be developed and implemented, hopefully allowing us to cure more individuals who have cancer. The widespread adoption of the new WHO classification, the marked expansion of the monoclonal antibody panel used to characterize hematologic malignancies in daily practice, and the technologic explosion in molecular biology necessitate that this textbook be updated. That is precisely what I have done with the assistance of three of the foremost hematopathologists in the United States today, Drs. Attilio Orazi, Lawrence Weiss, and Kathy Foucar. Together, the four of us have reorganized this textbook, invited the most highly qualified individuals to contribute to this textbook, and have carefully read, edited, and reread every chapter contributed by them. We believe that the result is a completely revised third edition of this classic textbook of modern hematopathology, one that provides a thorough, up-to-date overview of the morphologic, immunologic, and molecular characteristics of the benign and malignant proliferations derived from the hematopoietic and lymphoid systems.

Daniel M. Knowles, MD

Acknowledgments

The opportunity to thank publicly those persons who have contributed substantially to one's professional development and career comes infrequently; therefore, I would like to take this opportunity to do precisely that. I wish to express my sincere appreciation to Dr. Henry Rappaport for his stimulating hematopathology lectures, which I attended as a medical student at the University of Chicago and which sparked my initial interest in hematopathology; to Dr. Henry Rappaport's surgical pathology faculty and house staff at the University of Chicago for inspiring me as a senior medical student to become a surgical pathologist; to Dr. Ralph Williams for introducing me to research during a medical student elective in his laboratory at the University of New Mexico, for assisting me in the preparation of my first abstract and paper, and for guiding me toward academic medicine; to Dr. Donald West King for accepting me into the pathology residency training program at the Columbia University College of Physicians and Surgeons and for providing an extraordinarily flexible training program that was a wonderful, nurturing environment, which allowed me to grow personally and professionally; to Drs. Raffaele Lattes, Nathan Lane, Marianne Wolff, and Karl Perzin for training me in surgical pathology and for establishing a superior standard of diagnostic excellence to which I shall always aspire; to the late Dr. Henry Kunkel, the finest scientific intellect I have ever known, and to his staff at the Rockefeller University for guiding my training in laboratory research; to Dr. Vittorio Defendi, Chairman of Pathology at the New York University, and Dr. Michael Shelanski, Chairman of Pathology at the Columbia University College of Physicians and Surgeons, for their friendship and uncompromising support while I was a member of their departments; to the other members of the International Lymphoma Study Group for making the annual scientific meetings intellectually stimulating and personally rewarding; to Dr. Robert Michaels and Dr. Antonio M. Gotto, former Deans, and Dr. Laurie Glimcher, the present Dean, of the Weill Cornell Medical College, and to Dr. David B. Skinner and Dr. Herbert Pardes, former Presidents, and Dr. Steve Corwin, current Chief Executive Officer, of New York–Presbyterian Hospital, for their generous support of me and the Department of Pathology and Laboratory Medicine; to all the Basic Science and Clinical Department Chairs at the Weill Cornell Medical College for their warm collegiality; and to my faculty for making our department better clinically and academically.

In addition, I wish to thank the numerous physicians with whom I have worked and collaborated during the past 35 years, but especially Drs. Riccardo Dalla-Favera, James Halper, Giorgio Inghirami, Pier Giuseppe-Pelicci, and Chang Yi Wang. I also thank all the fellows, residents, graduate students, technicians, and clerical staff who have worked for me during the past 35 years and who have helped my Hematopathology Laboratory grow and prosper. In this regard, I give special thanks to two of my former fellows: Dr. Ethel Cesarman, now a successful independent research scientist and colleague, whose intelligence and creativity has inspired our hematopathology group for the past 20 years, and Dr. Amy Chadburn, now a successful hematopathologist and former colleague, who oversaw the daily operation of our Hematopathology Laboratory for 20 years; to Angela Murray, our laboratory manager whose intelligence, enthusiasm, devotion, and hard work have been responsible for many of the successes of my laboratory during the past 25 years; to Gina Imperato, my Departmental Administrator, whose extraordinary abilities, dedication, and support have contributed so much to my success as Chairman of Pathology and Laboratory Medicine at the Weill Cornell Medical College; and to my office staff, whose presence makes each workday easier and more pleasant. I also thank Timothy Satterfield of Williams and Wilkins for convincing me to prepare the first edition of this textbook 20 years ago and the staff at Lippincott Williams & Wilkins for being so helpful during the preparation of all three editions of this textbook.

I wish to thank all the contributors to all three editions of this textbook for their time and effort in the preparation of their chapters and for being so tolerant of my editorial recommendations and revisions and my constant nagging to complete their chapters on schedule. This textbook owes its title, *Neoplastic Hematopathology*, to the highly acclaimed "Tutorial on Neoplastic Hematopathology," developed by Dr. Henry Rappaport, which has been successfully conducted annually for more than 40 years under the direction initially of Dr. Henry Rappaport, later Dr. Richard Brunning, and now me. I wish to pay my respects to all of the individuals who have taught in the Tutorial and thereby have contributed to my education and to that of thousands of others. Lastly, but most importantly, I express my deepest and most sincere gratitude to Marian, my wife, who graciously and unhesitatingly supported me while I labored on many evenings and weekends for so many years during the preparation of each edition of this textbook. I could not have completed any of the three editions of this textbook without her support and understanding.

Daniel M. Knowles

Three coeditors have been added for the third edition of this book. Each of these coeditors was influenced by giants in the field of hematopathology such as Drs. Henry Rappaport and Richard Brunning.

In addition, Attilio Orazi wishes to acknowledge that he owes much to those who taught him and influenced him the most, including Franco Rilke with whom he trained and Richard S. Neiman, for his mentoring and friendship. I wish to acknowledge my debt of gratitude to Daniel M. Knowles for inspiring me at many different levels and for offering me the privilege of participating in the creation of the third edition of "his" book. Finally, I wish to thank all of my colleagues in the Division of Hematopathology at Weill Cornell Medical College for their help, insights, and support during the preparation of this book.

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Attilio Orazi, Kathryn Foucar, Lawrence M. Weiss

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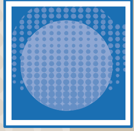

Chapter 1
The Hematopoietic System and Hematopoiesis

Christopher Y. Park • Michael G. Kharas

The hematopoietic system is a wonderfully complex biologic system, composed of numerous cell types that serve a countless number of roles with respect to organismal homeostasis. For example, the hematopoietic system is comprised of the red blood cells (RBCs) that carry oxygen to all the tissues of the body, the platelets that preserve volume by controlling bleeding, and the numerous cells of the innate and cellular immune systems (Fig. 1.1). These mature hematopoietic cells are the products of an intricate system of positive and negative control mechanisms that maintain homeostasis by regulating the ability of hematopoietic stem cells (HSCs) and lineage-committed progenitors to give rise to the cells that replenish postmitotic, terminally differentiated cells. This ability has evolved to allow rapid adaptation to environmental stress, probably best exemplified by the ability of the hematopoietic system to generate large numbers of cells in the setting of acute blood loss and infection. In some situations, this ability is compromised due to acquired genetic or epigenetic alterations, resulting in hematologic malignancies or inherited disorders of inappropriate or ineffective hematopoiesis. Thus, in many ways, disorders of the hematopoietic system can be viewed as a loss of homeostatic control, and understanding the mechanisms that regulate normal hematopoiesis will reveal many insights regarding the molecular pathways underlying the pathogenesis of hematologic disorders.

Because disorders of the hematopoietic system can be viewed as examples of abnormal hematopoiesis, it is imperative for medical professionals and researchers to understand the mechanisms that regulate normal hematopoiesis, which include a large number of cell-intrinsic factors such as transcriptional regulators and cell surface receptors, a variety of growth factors and cytokines, extracellular matrix proteins, and the stromal cells that make up the specialized bone marrow environment that supports HSC self-renewal, survival, and lineage commitment. Such knowledge will provide important clues to mechanisms of disease pathogenesis, provide novel methods for the diagnosis and detection of disease, as well as identify targets for therapy. For example, identifying aberrantly activated self-renewal pathways in hematologic cancers will allow for the development of novel therapies that target disease maintenance pathways. Similarly, elucidation of the cell-intrinsic pathways and extrinsic signals required for HSC self-renewal will allow investigators to develop methods to expand HSCs from autologous sources or induced pluripotent stem cells (iPSCs) induced to differentiate into HSCs *ex vivo*. Thus, patients who previously could not receive a therapeutic bone marrow transplant due to the absence of suitable donors or sufficient numbers of HSCs will have access to this life-saving treatment.

So how does the hematopoietic system maintain homeostasis when it is responsible for the production of more than 1 million mature cells per second over the lifetime of the average human adult? This requires a highly adaptable system of complex feedback mechanisms that maintain HSCs as well as allow them to give rise to appropriate hematopoietic cells in response to physiologic stress. In this chapter we will describe the factors that regulate normal HSC function as well as those that help to give rise to the diversity of cell types that make up this dynamic system. We will discuss the important contributions of the numerous growth factors and cytokines that have long been appreciated to regulate hematopoiesis as well as emerging data that demonstrate that local, systemic, and nervous system inputs play important roles in regulating hematopoiesis. In addition, we will discuss how our understanding of HSC function and differentiation has influenced our view of the pathogenesis of hematologic disorders. Finally, it should be noted that our detailed understanding of hematopoiesis is the result of an enormous effort by a large group of scientists and is also a constantly evolving field. Thus, we apologize in advance to our colleagues for the work we could not cite or discuss due to space limitations.

ORGANIZATION OF THE HEMATOPOIETIC SYSTEM

The hematopoietic system is initiated and maintained by the HSC, which has the ability not only to give rise to all the different cell types of the hematopoietic system, but also to give rise to other HSCs for the lifetime of an organism through a process termed self-renewal. HSCs lie at the top of a hierarchically organized developmental system in which they give rise to progenitors that become increasingly lineage-restricted, or committed (Fig. 1.1). Of note, it is also worth emphasizing proper usage of the term HSC. Usage of this term can be confusing since investigators frequently use this term when referring to heterogeneous populations. As we will discuss later, such populations (e.g., human CD34+ cells, mouse Lin−Sca+c-Kit+ or LSK cells) represent immature hematopoietic populations that comprise a minority of HSCs. Thus, we prefer to use the term "hematopoietic stem/progenitor cells" (HS/PCs) to more accurately reflect the biologic heterogeneity represented in these cell populations. While some committed progenitors do have the ability to self-renew for short periods of time (days to weeks), HSCs are distinguished from progenitors by virtue of their ability to self-renew for long periods of time (months to years). Lineage-committed progenitors, sometimes also referred to as transit-amplifying cells, proliferate more rapidly than HSCs, and as such, they give to numerous progeny that continue to mature to generate terminally differentiated cells. Because of their enormous expansion potential, committed progenitors can give rise to the large number of cells required to reconstitute and maintain the various cellular components of the blood. It is now clear that HSCs give rise to short-term HSCs (defined in the mouse by their ability to give rise to lymphomyeloid reconstituted grafts for <6 months when transplanted into secondary hosts), multipotent progenitors (MPPs) (giving rise to lymphomyeloid cells for <16 weeks), common myeloid progenitors (CMPs), common lymphoid progenitors (CLPs), granulocyte-macrophage progenitors (GMPs), megakaryocyte-erythroid progenitors (MEPs), as well as single lineage restricted progenitors for T, B, natural

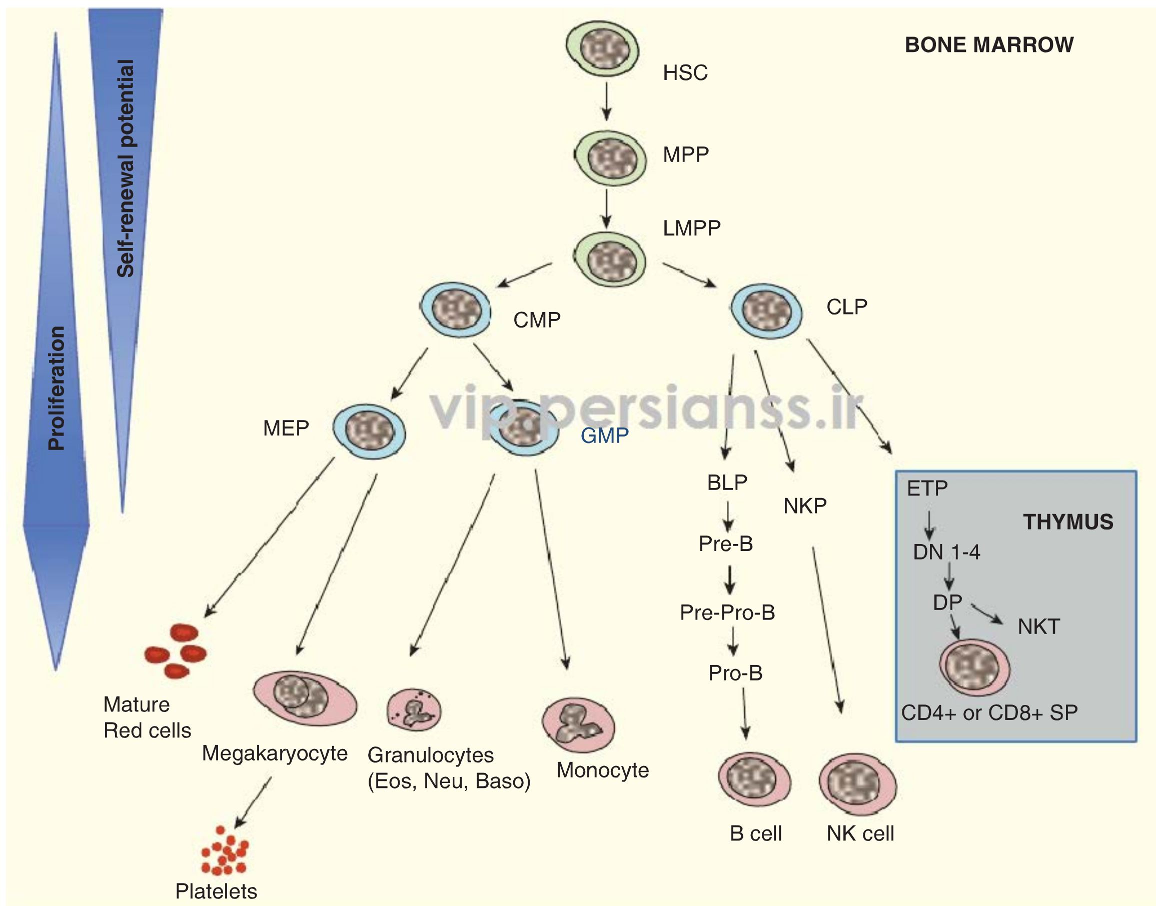

FIGURE 1.1. Hierarchy of hematopoiesis. Hematopoiesis is initiated by the hematopoietic stem cell (HSC), which gives rise to progenitors that exhibit increasing lineage restriction and decreased self-renewal with differentiation, ultimately giving rise to the postmitotic, terminally differentiated hematopoietic cells of the various lineages. While most hematopoietic development occurs in the bone marrow, T-cell development occurs in the thymus, which is seeded by a bone marrow–derived progenitor. MPP, multipotent progenitor; LMPP, lymphoid primed multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage progenitor; BLP, B-cell lymphoid progenitor; NKP, natural killer progenitor; ETP; early thymocyte progenitor; NKT, natural killer/T cells; Eos, eosinophils; Neu, neutrophils; Baso, basophils; NK cells, natural killer cells.

killer (NK), and dendritic cells (Fig. 1.1). While differentiated progenitors exhibit increasingly lineage restricted potential as well as loss of self-renewal capacity, one important exception to this basic rule is the memory lymphocyte, which has the capacity to persist for a lifetime, even after successive rounds of expansion and contraction when rechallenged with antigen.

Much of our knowledge regarding the functional and molecular properties of HSCs is based on studies of the mouse hematopoietic system due to the relative ease of performing transplantation studies using congenic models, which allow discrimination of donor and recipient cells based on differential expression of CD45 alleles (1). Unfortunately, studies with human HSCs have been limited by the lack of experimental models that allowed engraftment of immunologically incompatible human grafts in mouse hosts. Fortunately, our understanding of the organization and molecular mechanisms that regulate the human hematopoietic system has improved tremendously due to the development of experimental models allowing transplantation of human HSCs into immunodeficient mice. These studies demonstrate that while the mouse and human hematopoietic systems share many features, they also exhibit unique properties with respect to the molecular control of differentiation and the hierarchical organization of the hematopoietic systems. Equally important in improving our understanding of hematopoiesis was the development of *in vitro* assays that serve as important complementary techniques to study HSC biology. These *in vitro* assays allow assessment of lineage potential as well as surrogates of self-renewal such as the ability to serially replate and form colonies, as well as the ability to form cells that can reconstitute transplanted recipients after long culture periods; however, these assays are limited by their inability to faithfully recapitulate all the elements of the endogenous hematopoietic microenvironment, and thus all *in vitro* studies must be interpreted with caution and confirmed by *in vivo* studies. In this chapter, we will refer to studies utilizing both mouse and human hematopoietic cells. In most cases, similar findings were observed in both systems, but we will highlight differences when relevant. As our understanding of hematopoiesis continues to evolve, it will be important to be aware of potential differences between humans and other model organisms and to confirm findings using human cells when experimentally possible (2).

IDENTIFICATION OF THE HEMATOPOIETIC STEM CELL

The first efforts to identify HSCs were inspired by the observation that death following radiation exposure is due to hematopoietic failure. This was experimentally confirmed by demonstrating that irradiated mice survive by either shielding the spleen during irradiation or by transplanting spleen or bone marrow cells following exposure to lethal doses (3–5). Later studies showed that radioprotection is not conferred by HSCs, but by more committed PCs that rapidly give rise to mature cells. Formal

demonstration of the existence of HSCs was accomplished nearly a decade later by Till and McCullough, who showed that clonal hematopoietic cells can give rise to all the cells of the various myeloerythroid lineages (granulocytes, macrophages, red cells, megakaryocytes), that some of these cells possess the ability to generate more of themselves (now referred to as self-renewal), and that the spleens of these mice contained cells that could give rise to lymphocytes (6–10). It is interesting to note that while these studies relied on measuring the ability of transplanted cells to form colonies in the spleen (CFU-S) 10 days after transplantation into irradiated hosts, later studies showed that these spleen colony seeding cells actually represent committed progenitors, not HSCs (11–13). Nonetheless, these studies heralded the beginning of the fields of HSC biology and hematopoietic transplantation as it became clear that the ability to replace the hematopoietic systems of irradiated hosts could be therapeutically applied. These studies eventually led to the first successful human allogeneic bone marrow transplant performed by E. Donnell Thomas in 1956. His pioneering work in bone marrow transplantation resulted in his receipt of the Nobel Prize in Physiology or Medicine in 1990.

Although HSCs were shown to exist in the mid-1950s, it was not until the late 1980s that they were prospectively purified from mammals. While experiments using retrospective genetic marking techniques had provided evidence that HSCs existed (8,14), the only way to understand the specific contribution of these cells to transplantation biology, or even to show that self-renewal was limited to a specific cell type, was through their prospective separation. Moreover, limiting dilution analysis of transplanted mouse bone marrow cells determined that HSCs are rare, with early estimated HSC frequencies ranging from 1 in 10,000 to 1 in 100,000 cells in the bone marrow (1,15,16). Early experiments relied on techniques that physically separated cells based on differences in size and density (17,18), but then eventually relied primarily on differences in cell surface protein expression (19), which was only possible due to the advent of new technologies including the ability to generate monoclonal antibodies as well as the ability to identify cells by flow cytometry and separate cells using fluorescence activated cell sorting (FACS) based on the seminal work of Leonard Herzenberg (20,21). Irving Weissman et al. (22) then demonstrated that cells meeting all the functional criteria for HSCs could be isolated from the mouse bone marrow in 1988. This discovery was followed by the isolation of human HSCs, initially using *in vitro* systems, and then followed by *in vivo* experiments utilizing immunodeficient mouse models (discussed below in section “Developing Xenograft transplant models”).

Taking advantage of improved flow cytometry techniques and the discovery of genes differentially expressed in immature hematopoietic cells versus their differentiated progeny, HSC activity has been increasingly purified in both the human and mouse systems. Initial attempts using flow cytometry relied on eliminating cells expressing markers of mature hematopoietic cells (the so-called lineage negative or Lin$^-$ cell fraction) (23–30), and later studies identified other markers enriching HS/PCs including c-Kit (in mouse and humans) (30,31) and CD34 (in humans) (32). After decades of work, the mouse HSC can be purified at a frequency of approximately one in two cells and can initiate long-term hematopoiesis when transplanted into the same mouse strain (see Table 1.1 for frequencies of HSCs) (33,34). While all investigators do not use the same markers to identify HSCs, it is standard to isolate mouse HSCs from lineage low/negative cells (lacking Gr-1, Mac-1, Ter119, CD4, CD8, B220, CD3) that are also c-Kit$^+$, Sca-1$^+$, CD34$^-$, CD150$^+$ (SLAMf150), CD48$^-$. Of note, additional markers have also been shown to enrich for HSCs including rhodamine-low Hoechst-negative, CD49b^{lo}, Flk^{lo}, Esam1$^+$, Endoglin$^+$, and others (35). Unfortunately, most of these markers have been validated for mouse HSCs and many do not similarly purify human HSCs. For example, human HSCs express CD34 and the FLT3 receptor, while mouse HSCs do not (36); similarly, mouse HSCs express CD150, whereas human HSCs do not (37).

Table 1.1 FREQUENCY OF HEMATOPOIETIC STEM CELLS IN MOUSE BONE MARROW

Cell Population	HSC Frequency
Bulk bone marrow cells	<1:20,000
Lin$^-$	~1:2,500
Lin$^-$c-Kit$^+$Sca-1$^+$ (LSK)	~1:25
Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^-$ (LSK34$^-$)	~1:5
Lin$^-$CD34+CD38$^-$Rho^{low}	~1:30
Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^-$CD48$^-$CD150$^+$ (LSK34$^-$)	~1:2

DEVELOPING XENOGRAFT TRANSPLANT MODELS

The ability to perform transplants in histocompatible recipients helped facilitate the identification of HSCs in mouse, but such systems are not possible to study human HSCs *in vivo*. The development of immunodeficient mouse models has allowed xenogeneic transplantation to serve as a surrogate assay for human HSC activity. Such assays were first described in the 1980s with the severe combined immune deficient (SCID) and hu-SCID animal models (38), and further improved with the NOD/SCID, NOD/SCID/β_2 microglobulin null (39) NOD/SCID/IL2R gamma common chain null (NSG) (40–42), RAG2/IL2R gamma common chain null (43,44), and NOD/RAG2/IL2R gamma common chain null mice (45). Using these models, initial estimates of human hematopoietic repopulating activity, termed SCID-repopulating units, was estimated at 1 in 9.3×10^5 mononuclear cells (46), and 1 in 617 CD34$^+$CD38$^-$Lin$^-$ cells (47). Purification of human cells engrafting immunodeficient mice resulted in progressively enriched HSCs characterized by expression of CD34 (42,48), followed by descriptions of CD34$^+$CD38$^-$ (47,49), Lin$^-$CD34$^+$CD38$^-$ (47,50), and Lin$^-$CD34$^+$CD38$^-$CD90$^+$ immunophenotypes (51–53). These findings were followed by the finding that the combination of CD90 and CD45RA markers can distinguish between long-term and short-term engrafting cells (54). Most recently, investigators showed that long-term engrafting HSCs could be further enriched from Lin-CD34$^+$CD38$^-$CD90$^+$CD45RA$^-$ cells based on expression of the integrin CD49f, while CD90$^-$CD49f$^-$ cells could transiently repopulate hosts with lymphomyeloid grafts for up to 16 weeks, consistent with an MPP. Furthermore, they showed that as few as one in two cells present in this highly purified cell population could initiate long-term lymphomyeloid grafts when transplanted into NSG mice lacking T, B, and NK cells (see Table 1.2 for HSC frequencies in human bone marrow populations) (55).

Assays to Measure Hematopoietic Lineage Potential and Self-Renewal

Despite the early adoption of human bone marrow transplantation as a therapeutic modality, our understanding of the molecular mechanisms that regulate HS/PC function were severely hampered by the lack of experimental models to study human cells as well as the use of time-consuming and resource-intensive transplantation studies. Investigations of HS/PCs were greatly aided by the development of *in vitro* culturing techniques in the 1970s including the Whitlock-Witte culture and its variants, which used a combination of recombinant cytokines, semisolid support media including methylcellulose, and/or bone marrow

Table 1.2 **FREQUENCY OF HEMATOPOIETIC STEM CELLS IN HUMAN BONE MARROW**

Cell Population	HSC Frequency	Assay System	Reference
Bulk bone marrow cells CD34^{+}	1/1e6	SCID SCID/beige/XID NOD/SCID NOD/SCIDIL2Rgamma null (NOG) mice	Wang et al. (46) Dick et al. (48) Shultz et al. (42)
Lin^{-}CD34^{+}CD90^{-}	N/A	*In vitro* SCID-hu mice	McCune et al. (38) Murray et al. (52)
Lin^{-}CD34^{+}CD38^{-}	~1:600	NOD/SCID	Bhatia et al. (47) Hogan (55a)
Lin^{-}CD34^{+}CD38^{-}Rho^{low}	~1:30	SCID	McKenzie et al. (55b)
Lin^{-}CD34^{+}CD38^{-} CD90^{+}CD45RA^{-}	~1:10	NSG	Majeti et al. (54)
Lin^{-}CD34^{+}CD38^{-} CD90^{+}CD45RA^{-}CD49fSPlo	~1:2	NSG	Notta et al. (55) Science et al. 2011

or fetal liver–derived stromal support cells, to allow for growth of hematopoietic colonies *in vitro* (56). Following culturing, the resulting cells could be assessed for reconstitution potential in transplantation assays, thereby allowing incorporation of an *in vivo* readout into these experiments. While *in vitro* assays are very convenient systems to characterize alterations in hematopoiesis and to support conclusions based on *in vivo* functional evaluation of HS/PCs, it should be stressed that these assays are primarily assays of PC function and that true HSC function can only be determined by *in vivo* transplantation assays, which are described below.

HSCs can be cultured long-term when cocultured with preestablished stromal layers, and the physical association of HSCs with stromal cells is presumed to be required to recapitulate HSC interactions with the bone marrow microenvironment (57). This method has been modified to assess the ability of hematopoietic cells to migrate and grow beneath the established stromal layer to form “cobblestone-area forming cells” (CAFCs) (58–60), which are presumed to recapitulate the interactions between HSCs and their specialized niche since CAFC frequency at different time points following culture initiation correlates well with different colony-forming activities using other assays including CFU (in culture), and CFU-S on day 12 (CFU-S-12), and marrow-repopulating activity. A similar *in vitro* culturing technique involves growing HSCs on stromal layers followed by the assessment of PC colony-forming ability, otherwise known as the long-term culture-initiating cell (LTC-IC) assay. Instead of scoring CAFC, the LTC-IC determines the presence of committed progenitors by replacing the culture medium after 5 to 6 weeks of culture with a semisolid medium such as methylcellulose and scoring for colonies arising 7 to 14 days later (61,62). This assay was used initially to assess human HSC growth *in vitro* (61), but was also useful to measure mouse HSC frequency and was modified to support lymphomyeloid progenitors (62). While the CAFC and LTC-IC assays are the only *in vitro* assays that have the ability to measure HS/PC frequencies as verified by long-term *in vivo* reconstitution experiments and can provide a basis for comparisons with long-term repopulating activity in radiosensitivity (63), or cytotoxicity (64) assays, their utility as predictors of HSC activity under all conditions is not clear, and in some cases, the two assays may generate conflicting results; this appears to depend on the specific stromal line used and the accessory cells (65–68). On balance, it is likely that these assays reflect the activity of immature hematopoietic cells, but they do not allow direct assessment of HSCs.

Colony-forming cell (CFC) assays, first described in the 1960s (69,70), and later modified for human hematopoiesis (71,72), evaluate the growth of individual HS/PC clones in semisolid agar—or defined methylcellulose-based culture media containing cytokines that support the growth of hematopoietic progenitors that can give rise to the erythroid, myeloid, lymphoid, and megakaryocytic lineages (73). The semisolid nature of the media prevents the migration of PCs, and thus the colonies are clonal, arising from single cells. These colonies represent the progeny from progenitors that can give rise to single, dual, or multiple lineages including erythroid-restricted burst-forming units-erythroid (BFU-E), which are presumed to arise from more immature cells than colony-forming units-erythroid (CFU-E), since they produce larger numbers of mature progeny. Additional types of colonies that describe the types of hematopoietic cells produced include megakaryocyte-restricted (CFU-Mk), colony-forming units-granulocytes (CFU-G), and colony-forming units-granulocytes/macrophages (CFU-GM). Some colonies contain granulocytes, erythrocytes, macrophages, and/or megakaryocytes (CFU-GEMM or CFU-mixed). These are usually measured 12 days after initiation of the culture and are presumed to arise from a primitive progenitor. Generation of B- and T-lymphocyte CFCs is more difficult than for the myeloid lineages and therefore typically requires specialized coculture systems (74,75), although a commercially available methylcellulose-based colony assay to measure pre-B cells is available. As the cellular composition of individual colonies can be determined on the basis of colony morphology, by cytologic evaluation of cells in individual colonies, or by flow cytometric evaluation of individual colonies, the lineage potential of individual HS/PCs can be readily assessed. As colonies are largely derived from PCs, serial replating assays using the progeny of cells generated in methylcellulose or liquid cultures can be used to reinitiate similar cultures sequentially. HSC-enriched populations can give rise to colonies during serial replatings in methylcellulose, and therefore gains and losses of HSC self-renewal can be estimated in this manner.

When the soft agar and methylcellulose colony assays were first developed, the concept that factor(s) could increase colony formation was postulated and they were named colony stimulating factors (CSFs) (69,70,76). Over the next two decades, the purification, cloning, and characterization of four different CSFs was accomplished including M-CSF, GM-CSF, G-CSF, and multi-CSF, with most of the cloning of the CSFs completed in the 1980s (IL-3). Both GM-CSF and G-CSF were found to be robust promoters of neutrophil development and activation, as well as mobilization of HSC/progenitors. Many laboratories over the years have utilized these factors for understanding myeloid development. Most importantly, these factors have become extraordinary therapies for treating neutropenic patients and mobilizing HSCs into the peripheral blood as a superior source of blood donor cells (77–80). Indeed, because of the ability to generate mobilized peripheral blood HS/PC grafts, the use of traditional bone marrow grafts for transplantation is now dwindling.

While myeloid and lymphoid cells develop from HSCs and MPPs, they require different external cues to execute their differentiation programs, and therefore different culture conditions are required to efficiently generate them *in vitro*. Development of lymphocytes *in vitro* can be achieved with specialized coculture systems (74,75). However, similar to the initial transplantation studies in which CFU-S were initially mistaken as initiated by HSCs (6), the cellular origins of colonies generated in these assays is not uniform. Colony types can be incorporated into a hierarchical scheme of hematopoiesis based on their lineage output, with multilineage colonies presumed to arise from the most immature cells and lineage-restricted colonies presumed to arise from lineage-restricted progenitors (81). However, since even the most highly purified HSCs can give rise to both multilineage and restricted colony

types when cultured in methylcellulose, the colony assay is not necessarily reflective of *in vivo* potential. Moreover, these assays cannot efficiently promote significant self-renewal of HSCs since they cannot faithfully recapitulate all of the features of the bone marrow microenvironment. The relationships between distinct HS/PC populations identified based on cell surface marker expression and the CFU initiating cells has been determined for a subset of mouse erythroid progenitors (82), but these correlations have not been rigorously characterized for most mouse and human PC populations. Because of difficulties in defining human HSCs functionally using the serial transplantation assay, identification of candidate human progenitor populations has largely relied on *in vitro* assays. Using *in vitro* culturing conditions such as methylcellulose colony assays and stromal coculture models, investigators were able to identify candidate CLPs (83,84) as well as multiple myeloid progenitor populations including the CMP, GMP, and MEP (85). Finally, additional caution is required regarding identification of specific HS/PC populations using cell surface markers in genetically manipulated models since surface marker profiles may be altered under such conditions, thereby providing misleading conclusions from sorted cells.

Experimental demonstration of HSC activity requires long-term repopulation or reconstitution assays. This typically involves transplantation of cells into irradiated or otherwise compromised hosts, and in mice these experiments are aided by the presence of different alleles of the hematopoietic marker CD45, in which CD45.1 and CD45.2 represent donor and recipient alleles. Following transplantation, donor engraftment levels can be assessed by flow cytometry using the CD45 congenic marker as well as lineage markers, with long-term HSCs defined by their ability to give rise to myeloid lineage cells for at least 16 weeks posttransplant. Lymphoid cells are not considered good indicators of ongoing HSC contributions to the hematopoiesis since they can persist for long periods of time after their generation, especially through the generation of memory lymphocytes. In some cases, transplantation is performed in the presence of competitor bone marrow cells derived from mice with the same CD45 allele as the host, allowing the donor and competitor HSCs to be compared with respect to engraftment capacity (15,16), but it does not necessarily allow for direct quantification of HSC frequency or number. In order to calculate the frequency of competitive repopulating units (CRUs), the limiting dilution assay is used. In these experiments, test cells are titrated, transplanted into congenic hosts, and then the percentage of hosts that fail to engraft donor cells is determined. With a minimum of three different doses tested in which positive and negative recipients are present, a best-line fit can be generated by Poisson statistics to calculate the number of CRUs, or HSCs (86,87).

While all of the previously described assays can assess the ability of candidate HSC populations to reconstitute hosts, the most stringent test of stem cell activity is the serial transplantation assay (14). In this gold standard assay, cells from primary recipients are transplanted successively into irradiated recipients for up to a maximum of five to seven rounds (due to the finite self-renewal of normal HSCs) (88,89). However, it should be noted that transplantation itself stresses HSCs, with the process of transplantation being sufficient to induce HSC cell cycling that can last for at least 4 months after transplant (90). Indeed, recent studies suggest that transplantation into irradiated hosts is sufficient to affect HSC function by promoting a myeloid lineage bias (91). Thus, alternative methods to engraft donor cells without irradiation may become a more common strategy to evaluate HSC function, as both genetic mouse models (e.g., those mutant in c-Kit such as W/W^v) (92,93) and antibody-mediated conditioning regimens (94) also allow engraftment of transplanted HSCs. One caveat that warrants mentioning is that these assays, while assessing the presence of self-renewing cells, also assume that the self-renewing cells mature normally. This may, in fact, not be the case for mutant cells in which downstream progenitors gain aberrant self-renewal or fail to mature normally (95). In such cases, readouts may be misleading and therefore more detailed evaluation of specific HS/PC populations may be required to determine the specific developmental stage at which functional defects manifest. Finally, it should be noted that noncompetitive transplants most closely mimic the clinical situation, and that competitive transplants may, in fact, yield misleading negative results, especially if the number of cells tested is small, or if test, transplanted HSCs are outcompeted, even though they were functional under normal, steady-state hematopoiesis prior to being harvested for transplantation studies (96).

Development of the Hematopoietic System

While the major site of hematopoiesis in the adult is the bone marrow, the major sites of hematopoiesis during embryonic development are in the yolk sac followed by the fetal liver. Thus, it is likely that factors that regulate HSCs are developmental stage and cell context dependent. Investigation of the factors that regulate HSCs in each site of hematopoiesis is likely to provide insight into the cell-intrinsic and cell-extrinsic factors that regulate HSC function. Some of the earliest discoveries in developmental hematopoiesis originated from studies performed in vertebrate model organisms including zebrafish, *Xenopus*, and chicken. For more thorough reviews on these developmental systems please refer to Refs. (97–100). Our understanding of early mammalian hematopoiesis is largely drawn from investigations in mice. Recent studies of *in vitro* directed hematopoietic differentiation from embryonic stem cells (ESCs) and iPSCs have also been an important source of information regarding the earliest commitment to the hematopoietic lineage, and this approach holds great promise as a tractable system for generating large numbers of HSCs *ex vivo*.

During mammalian embryonic development, blood cells arise in two phases (Fig. 1.2). During the first phase, also known as embryonic hematopoiesis, primitive erythrocytes and myeloid cells are generated in the yolk sac. This phase is followed by definitive hematopoiesis, which is defined by the emergence of definitive HSCs (dHSCs), which are capable of giving rise to all mature adult blood cell types (erythroid, myeloid, and lymphoid) as well as reconstituting multilineage hematopoiesis when transplanted into adult recipients. Thus, during embryonic hematopoiesis, committed blood precursors appear prior to dHSCs. This phenomenon is thought to reflect the needs of the developing embryo, since embryonic hematopoiesis allows the embryo to address its short-term need for proper oxygenation, while definitive hematopoiesis allows the organism to establish and maintain lifelong hematopoiesis. While this model makes evolutionary sense, it is not clear whether embryonic and definitive (adult) hematopoiesis occur independently or are initiated simultaneously from a common ancestor. While both models have been proposed, the latter would require one to hypothesize that the common ancestor is not detectable using standard transplantation assays since there is no experimental evidence of a multilineage HSC during early development.

The first evidence of blood formation in the mouse occurs in the yolk sac between E7.0 and 7.5 during mouse development (mouse gestation is ~21 days, with gestational day denoted with a preceding “E”) as the extraembryonic mesoderm ingresses through the posterior primitive streak (101). While hematopoietic cells were first identified in the yolk sac in the late 19th century, it was not until the early 1970s that investigators were able to demonstrate the presence of hematopoiesis in the yolk sac using the novel *in vitro* hematopoietic assays developed at that time (102). Later, more stringent assays requiring regeneration of CFU-S *in vivo* were utilized to show that the yolk sac

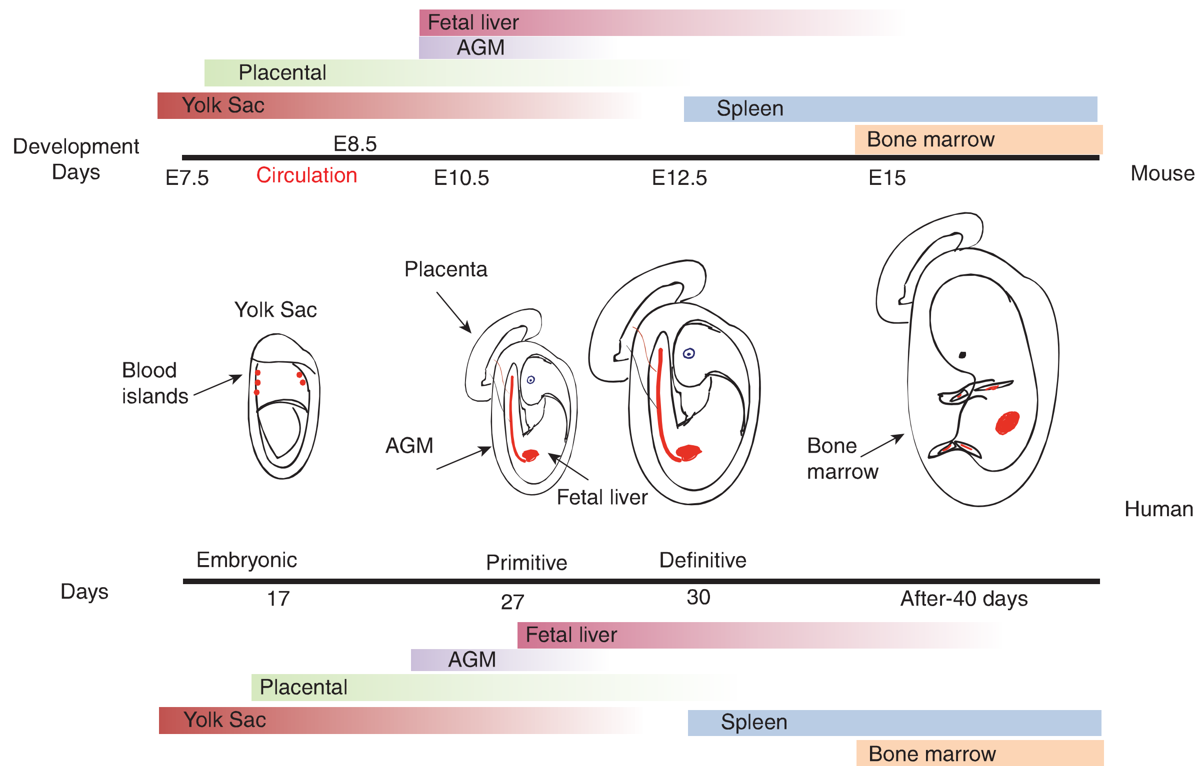

FIGURE 1.2. Developmental hematopoiesis in mouse and humans. The major sites of hematopoiesis shift during embryonic and fetal development with hematopoiesis starting in the yolk sac, then shifting to the aorto-gonado-mesonephros (AGM), then to the fetal liver, and then to the bone marrow. The *arrows* indicate the various sites of hematopoiesis based on the developmental stage. In the mouse, the developmental stage is designated based on the day postconception (E). *Colored boxes* indicate the different waves of hematopoiesis.

lacks CFU-S prior to E9.5 and that dHSCs are absent prior to E11.5 (103,104).

dHSCs form in the aorto-gonado-mesonephros (AGM) region, where they can be generated autonomously (103,104), and explants of AGM are able to initiate and expand dHSCs at E10.5 and E11.5, respectively (105). It is likely that the AGM is the major source of dHSCs since they cannot be initiated or expanded from other hematopoietic tissues (106,107), while AGM derived and AGM explants can be cultured in the presence of appropriate growth factors to give rise to a 150-fold expansion of dHSCs (108,109).

While the first blood cells arise from the wall of the aorta of the developing embryo, typified by the generation of RBCs and macrophages, the site of hematopoiesis shifts to the fetal liver later during development as dHSCs from the AGM region, placenta, and yolk sac (105,110) colonize the liver at E12.5 to establish definitive hematopoiesis in a process that likely requires β1 integrins (111,112). Once in the liver, dHSCs expand through the action of molecules that promote proliferation such as angiopoietin-like factors and the Sox17 transcription factor (113,114). At this point, HSCs can be detected experimentally using standard transplantation assays in adult animals.

The relationship between embryonic sites of hematopoiesis and adult hematopoiesis remains a source of controversy. While the presence of yolk sac hematopoiesis and AGM generation of dHSC activity strongly suggest that adult mammalian hematopoiesis originates in the embryo, this does not exclude the possibility that the yolk sac directly contributes to adult hematopoiesis and that embryonic ancestors of dHSCs are not detectable simply due to their inability to engraft adult recipients. Using such assays, dHSCs have also been identified outside of the AGM in the embryo (99,115).

The importance of the circulation in inducing early hematopoiesis has been the subject of intense investigation in recent years. The initial indication of a functional link between the vascular system and hematopoiesis was shown in mouse mutant models. *Ncx-1* null mice fail to develop beating hearts but do develop erythrocytes and CFU-Cs in the yolk sac normally; however, their body lacks these progenitors, indicating that AGM hematopoietic activity depends on colonization by yolk sac cells (116). Similarly, *Rac1* mutant embryos exhibit impaired cell migration from the yolk sac to the body proper, which results in a similar phenotype (117). Together, these studies showed dissociation between the establishment of hematopoiesis in the yolk sac and the AGM, and they also showed that yolk sac–derived precursors colonize the AGM region.

The importance of mechanical stress generated by blood flow on endothelial cell function has been long appreciated (118). Endothelial cells respond to shear stress via expression of mechanoreceptors, resulting in the induction of specific genes, and one of the major products induced by such stress is nitric oxide (NO) (119). These observations led a number of groups to hypothesize that the physical forces generated by blood flow may induce hematopoietic cell formation from the endothelium lining the AGM region via NO signaling. Validation of this hypothesis involved experiments in which shear stress was recreated *in vitro*, resulting in both ESCs and AGM-derived cells up-regulating *Runx1* and *cellular myeloblastosis viral oncogene homolog* (*c-Myb*) with an accompanying increase in CFU-C production (120). These effects could be suppressed by addition of inhibitors of NO signaling (120,121). Additionally, evaluation of "silent-heart" zebrafish mutants that lack blood flow revealed that the number of Runx1$^+$ and CD41$^+$ HS/PCs was markedly reduced (121,122). The dependence of this phenotype on NO production was confirmed by treating with an NO donor, s-nitroso-*N*-acetylpenicillamine, which rescued the phenotype. Finally, mice lacking *nitric oxide synthase 3* (*Nos3*) demonstrated defects in AGM hematopoietic development, confirming these studies (121).

The intimate relationship between the vascular and hematopoietic systems has been appreciated for many years,

leading some to propose that the endothelial and hematopoietic systems may arise from a common, bipotent progenitor called the hemangioblast. Indeed, early blood islands first appear as clusters of cells immediately surrounded by an outer endothelial lining (123,124). These blood islands eventually give rise to endothelial networks filled with blood, and these areas of blood formation encircle the yolk sac in a single, beltlike structure (125). While there was no experimental evidence of a hemangioblast in the developing embryo for more than a century, *in vitro* ESC blast colony assays finally provided the first evidence for such a cell (126–128). Subsequently, equivalent cells were identified in the mouse embryo within the posterior primitive streak, and these cells were also shown to give rise to smooth muscle cells, providing evidence for a tripotent progenitor (129). The difficulties in identifying a hemangioblast earlier during development is likely due to the fact that by the time the yolk sac is colonized by hemangioblasts, they have already committed to the endothelial and hematopoietic lineages.

While these studies provide evidence for a hemangioblast-like precursor in developing embryos, it is not clear whether endothelial cells can give rise to hematopoietic cells later during embryogenesis or to the adult hematopoietic system. Evidence for such hemogenic endothelium in the developing embryo has been supported by live imaging studies in which it was demonstrated that aortic endothelium in zebrafish (130,131), or AGM region cells in mouse (132) give rise to dHSCs directly. But what is the relationship between the hemangioblast and hemogenic endothelium? A recent study proposed that FLK1$^+$ hemangioblasts generate hemogenic endothelium first, when they generate hematopoietic cells (133). While these studies demonstrate the presence of hemogenic endothelium, it is important to note that only a small subset of endothelial cells have this capacity. In the mouse yolk sac, it is estimated that <2% of total endothelial cells at E8.5 (134,135) and within the AGM at E10.5 exhibit this potential (136).

While hemogenic endothelium can generate multilineage HS/PCs in the yolk sac, AGM, and placenta, evidence for hemogenic endothelium in the fetal liver and bone marrow is lacking. While such cells may possess such potential, it is likely that the fates of hemogenic endothelium progeny may depend on their site of origin or developmental stage. For example, hemogenic endothelial cells within the yolk sac can give rise to MPPs *in vitro* (137), but they have limited capacity to do so when transplanted *in vivo* (103). In contrast, the multilineage precursors (dHSCs) from the AGM can repopulate neonatal and adult recipients. Overall, these observations support a model in which HSCs are born in the yolk sac and AGM and then migrate to subsequent sites of definitive hematopoiesis (i.e., the fetal liver and bone marrow).

In the mouse, HSCs leave the liver to colonize the bones by E17.5 and through the first 2 weeks of postnatal life. They are actively recruited by the chemokine CXCL12/SDF-1 through interactions with CXCR4 expressed on HSCs (138). In humans, liver hematopoiesis is established at approximately 23 days of gestation (139). Additional factors promote localization of HSC to the bone marrow in concert with CXCR4 such as prostaglandin E2 and Robo4 (140,141) or through effects independent of CXCR4 including c-Kit, the calcium-sensing receptor, and the transcription factor Egr1 (142–144). Once entering the specialized bone marrow niche in the bone marrow, HSCs remain in the niche through integrin-dependent interactions (145,146). However, a small percentage of HSCs flux out of the bone marrow into the circulation before returning to the bone marrow niche even during homeostatic hematopoiesis (147,148), which explains why the cellular composition of the adult bone marrow appears similar regardless of the bone marrow site sampled.

Late during embryonic development (e.g., third trimester in humans), hematopoiesis slowly shifts to the bone marrow and coincident with this transition, numerous other changes occur in the hematopoietic system. HSC expansion becomes attenuated and self-renewal decreases such that at the time of birth, the human hematopoietic system is maximally proliferative, allowing for expansion of the blood-forming capability of the maturing young human.

Characteristics of Hematopoietic Stem Cells

We now know that in addition to their properties of self-renewal and multilineage differentiation, HSCs exhibit other characteristics that distinguish them from lineage-committed progenitors and other hematopoietic cells. One of the most frequently described features of adult HSCs is their relative quiescence. Approximately 5% of total bone marrow HSCs are actively cycling (defined as S, G2, or M phases) at any given time during adult life as demonstrated by stains that distinguish the cell cycle status as well as pulse-labeling experiments that allow investigators to estimate the frequency at which HSCs undergo cell division (149–151). Interestingly, this characteristic of HSCs is established as rapidly as 4 weeks after birth, even though HSCs in the mouse fetal liver actively cycle with 95% to 100% of HSCs cycling with a cell cycle transit time of 10 to 14 hours (150,152). Using markers of cell division such as BrdU retention and histone 2B-green fluorescent protein dilution, bone marrow HSCs exhibit heterogeneity in their degree of quiescence (153,154) and appear to be composed of cells (5% to 10% of HSCs) that can be readily recruited into cell cycle as well as those that are relatively resistant to cycling. Estimations of division times suggest that dormant HSCs divide only once every 145 days or more and are enriched for long-term reconstitution potential. This has led to the hypothesis that the relatively dormant pool of HSCs may represent a reservoir of HSC activity only called upon to cycle during stress hematopoiesis. Interestingly, human HSCs appear to enter the cell cycle on average only once every 40 weeks (155), indicating a significant species difference in HSC cell cycling.

The importance of HSC quiescence is perhaps best exemplified by the poor performance of HSCs in the S-G2-M phases of the cell cycle to reconstitute recipients in transplantation studies, suggesting that cycling itself severely compromises adult HSC self-renewal (152,156). Other examples linking cell cycling to impaired HSC self-renewal are observed in mice lacking *p21* (157), *Gfi1* (158,159), and the thrombopoietin (TPO) receptor, *c-Mpl* (160). HSC self-renewal must also be maintained in other situations when HSCs undergo rapid expansion following bone marrow insult in the form of chemo-, radio-, or immunoablation (161,162), a point supported by the ability of single HSCs to long-term reconstitute the hematopoietic system of lethally irradiated recipients (163). While these studies indicate that cell cycling is a physiologic state in which HSCs may exhibit diminished self-renewal capacity, they also indicate that cycling does not, in and of itself, confer loss of self-renewal to HSCs. There is some suggestion that specific phases of the cell cycle may be particularly critical for maintaining HSC function since while fetal and adult HSCs divide at different frequencies, once they enter into the G1 phase of the cell cycle, both exhibit the same slow cell cycle transit rates compared to their more differentiated progeny (152). In addition, as noted previously, fetal liver and bone marrow HSCs possess the ability to undergo increased proliferation and expansion without exhaustion following marrow injury. Thus, HSC decisions to enter the cell cycle and the consequences of cell cycle entry appear to be context dependent, and likely depend on both cell-intrinsic and cell-extrinsic factors.

Due to the high turnover of the blood and the longevity of HSCs, it is important for HSCs to maintain themselves by avoiding senescence and maintaining a proper metabolic state. Consistent with this notion, HSCs exhibit elevated telomerase activity compared to committed progenitors and mature cells

(164). In addition, telomere erosion is associated with the decreased functional engraftment observed in aging HSCs or during serial transplantation of HSCs (90,165,166). Mice deficient in telomerase activity through deletion of the telomerase RNA component (Terc) exhibit progressive shortening of telomeres, reduced life span, decreased stress responsiveness, decreased hematopoietic cell proliferation, genetic instability, and increased spontaneous malignancies (167–169). *Terc* null mice showed decreased replating ability of CFU-GMs, reduced proliferative capacity of bone marrow cells, and reduced long-term reconstitution capacity and decreased serial replating capacity (90,170,171). Together, these data strongly suggest that telomere maintenance is critical for HSC maintenance.

HSCs also exhibit a unique metabolic state due to their relative quiescence as well as their presence in a hypoxic bone marrow microenvironment (172). Indeed, it is well established that HSCs cultured under hypoxic conditions exhibit better colony-forming and transplantation properties (173–175). However, as hypoxia also induces HSC quiescence (176,177), it is not clear whether the effects of hypoxia are independent of cell cycle kinetics/status. Moreover, mice lacking key metabolic enzymes including Lkb1 (178–180) and FoxO3 (181,182) experience significant HSC loss, indicating that HSCs are susceptible to cellular stressors, in particular, reactive oxygen species (ROS). Indeed, HSCs with lower levels of ROS retain higher reconstitution ability than those with higher levels of ROS, as assessed by staining with the ROS-sensitive substrate dihydro-dichlorofluorescein diacetate (183). Direct measurement of HSC mitochondrial activity has shown that HSCs have low mitochondrial activity and increased glycolysis; however, these changes in mitochondrial activity are thought to reflect the hypoxic environment (184). Such changes in mitochondrial metabolism have raised interest in the role of autophagy in HSC function. Consistent with this notion, LSK cells from mice deficient in *Atg7*, an essential component of the autophagosome, failed to reconstitute the hematopoietic system in lethally irradiated hosts (185). The loss of *Pten*, which induces constitutive phosphatidylinositol 3-kinase (PI3K)/Akt signaling, leads to HSC exhaustion (186,187), which can be restored by loss of p53 or $p16^{Ink4a}$ (188). Thus, activation of HSC bioenergetic signaling through the PI3K/Akt/mTOR pathway is associated with increased HSC proliferation and exhaustion (189). In contrast, the loss of Akt signaling in *Akt1/Akt2* double knockout HSCs results in increased HSC quiescence and impaired differentiation, presumably by reducing ROS (190). Unfortunately, these data do not distinguish between mutational effects on HSC proliferation and loss of self-renewal, and thus whether or not HSC self-renewal depends on cell cycle status remains an open question.

Recent evidence suggests that HSCs possess unique DNA damage responses and DNA repair mechanisms that make them more stress tolerant. HSCs possess the ability to efflux drugs via ATP-dependent transporters such as the breast cancer resistance protein (Bcrp1, or Abcg2), presumably preventing HSCs from accumulating toxic molecules (191). HSCs have also developed mechanisms to cope with DNA damage. DNA double-stranded breaks that occur during the G_0 phase of the cell cycle in mice are p53 dependent (192) and immediately repaired by nonhomologous end-joining ability (193), which is more error prone than homologous recombination. Given the quiescent nature of HSCs, this is thought to lead to increased retention of mutations in HSCs (194). Paradoxically, this phenomenon is predicted to contribute to HSC senescence, apoptosis, and leukemic transformation since the acquired genetic changes through this DNA repair mechanism would be propagated to their progeny, thereby serving as the altered genetic context in which transforming events could occur (195–197). Interestingly, the DNA damage response appears to differ in human HSCs, which are sensitized to apoptosis after irradiation, leading to loss of damaged HSCs and improving maintenance of genomic integrity (198).

Regulators of HSC Function

While the determinants of HSC self-renewal are not completely understood, numerous cellular and molecular pathways, both cell-intrinsic and cell-extrinsic, have been implicated in HSC function. Cell-intrinsic pathways include cell cycle regulators, conserved developmental pathways, signal transduction pathways, epigenetic regulators, and RNA and posttranscriptional regulators. Because the number of factors that regulate HSC function is rather large, in this section we can only present some of the major pathways that regulate HSC function. We have attempted to provide a summary of experimentally validated HSC regulators (Table 1.3), but for a more complete discussion of these factors, we point the reader to a number of excellent reviews on this subject (35,199,200). Cell-extrinsic regulators of HSC function will be discussed within the broader context of the bone marrow microenvironment and its associated factors.

Cell Cycle Regulators

P53 and P21

Mice lacking *p53* show significant increase in HSC number as assessed by competitive reconstitution experiments using *p53*$^{-/-}$ bone marrow cells or purified LSK cells, as well as by cell surface immunophenotype (204,219). However, when more purified *p53*$^{-/-}$ HSCs were transplanted (Lin$^-$CD41$^-$ CD150$^+$CD48$^-$), they showed lower levels of engraftment, consistent with reduced HSC function (219). This reduction in HSC potential is associated with relative loss of HSC quiescence (205). As cycling HSCs have been shown to possess decreased long-term reconstitution potential (220), it is possible that the increase in *p53*$^{-/-}$ HSC number and concomitant loss of engraftment capability may be due to increased cycling.

P21 is a cyclin-dependent kinase (Cdk) inhibitor that acts by binding to and inhibiting cyclin E/Cdk2 and cyclin D/Cdk4/6 complexes. Consistent with its negative regulatory role in cell cycle progression, mice deficient in p21 exhibit increased frequency of cycling HSC/progenitors as well as increased CFU frequency *in vitro* (157). However, when *p21* null HSC self-renewal was assessed through serial bone marrow transplants and multiple 5-fluorouracil (5-FU) treatments, they displayed an early exhaustion phenotype (157). While this led investigators to conclude that p21 is required to maintain HSC quiescence and hence self-renewal, more recent work using more highly purified HSCs (LSKCD48$^-$CD150$^+$) showed no differences in HSCs quiescence in *p21* null HSC (154). Another study confirmed a limited role for p21 in maintaining HSC quiescence during steady-state hematopoiesis, with *p21*-deficient HSCs showing a deficiency only in the context of competitive transplants using irradiated bone marrow cells (221).

Retinoblastoma

The retinoblastoma (Rb) family of transcriptional repressors, including *pRb*, *p107*, and *p130*, restricts cell cycle entry by repressing E2F-dependent transcription of positive cell cycle regulators including E-type cyclins. Rb proteins are regulated through phosphorylation events that alter their biologic activity, with hypophosphorylated Rb inhibiting entry into the G1 phase. Upon phosphorylation by cyclin dependent kinases such as the cyclin D-Cdk4/6 complex, Rb proteins are partially inactivated and cells are allowed to progress through G1. Subsequent phosphorylation by cyclin E-Cdk2 further inactivates Rb-mediated inhibition of E2F, resulting in G1 exit and entry into the S phase. In HSCs, a role for Rb in cell cycle regulation did not become evident until all family members were deleted, as *p130*-deficient mice did not exhibit a hematopoietic phenotype (222), *p107*-deficient mice only exhibited a mild myeloid hyperplasia (223), and *pRb*-deficient mice did not exhibit any defects in HSC self-renewal, although they did

Table 1.3 INTRINSIC CELLULAR REGULATORS OF HSCs

Category	HSC Phenotype	Genes	Details	Reference
Cell cycle	Defect	Rb	Cell intrinsic/extrinsic effect	Walkley et al. (201) Walkley and Orkin (202)
		p21	Loss of quiescence/no phenotype	Cheng et al. (223a)
		p57	Quiescence and self-renewal	Zou et al. (203) Matsumoto et al. (223b)
	Improved	p53	Improved engraftment/loss of quiescence	TeKippe et al. (204) Dumble et al. (223c) Liu et al. (205)
		p27	Increased engraftment	Cheng et al. (223d.)
Conserved Developmental Pathways				
Notch pathway	None	Numb/NumbL, Notch1/2, Jagged1 (extrinsic)	Normal	Wilson et al. (223e)
TGF-β pathway	None	Tgfb2, Tgfbr1, Smad5	Tgfbr1 unresponsive to Tgfb1, mainly *in vitro* expansion defects otherwise normal	Langer et al. (223f) Larsson et al. (223g) Singbrant et al. (223h)
	Defect	Smad4, Tgfbr2 (extrinsic)	Defects in engraftment	Karlsson et al. (206)
Wnt/β-catenin pathway	Defect	Ctnnb1	Normal/defect depending on Mx1 vs. Vav-Cre insensitive to GS3b inhibitor expansion	Cobas et al. (207) Zhao et al. (208) Huang et al. (209)
	Defect	Wnt3a (extrinsic)	Defect in serial transplants (fetal liver)	Luis et al. (210)
Hedgehog pathway	None	Smo	Normal HSC	Gao et al. (211) Hofmann et al. (212)
	Improved	Gli	Increased engraftment	Merchant et al. (213)
Transcription and lineage transcription factors	Defect	Bmi1	Decreased engraftment	Park et al. (214) Iwama et al. (223i)
		Pbx1	Decreased developmental HSC	DiMartino et al. (223j) Boyarsky et al. (223k)
		c-Myc	Increased in phenotypic HSC with decreased engraftment and development	Baena et al. (223l) Laurenti et al. (223m) Wilson et al. (223n)
		Gfi1/Gfi1b	Myeloid differentiation defects alteration in HSC	Hock et al. (158) Khandanpour et al. (223o)
		Scl/Tal1	Increase in number of HS/PC differentiation	Mikkola et al. (223p) Curtis et al. (223q)
		IRF2	Defect in HSC	Sato et al. (223r)
Signaling pathways	Defect	FoxOs (FoxO1, FoxO3a, FoxO4)	Loss of quiescence	Tothova et al. (182) Miyamoto et al. (181)
		Pten	Increased cycling and exhaustion	
PI3K/AKT and metabolic sensing		mTORC1/mTORC2	Loss of self renewal	
		Lkb1	Loss of HSC	Gurumurthy et al. (180) Gan et al. (179) Nakada et al. (178)
Other signaling regulators		STAT5	Decreased engraftment	Li et al. (223s) Wang et al. (223t) Bradley et al. (223u) Bunting et al. (223v)
	Improved	Lnk	Increased engraftment	Takaki et al. (223w)
Epigenetic regulators	Defect	Dnmt1	Decreased lymphoid engraftment	Broske et al. (215)
	Improved	Dnmt3a	Improved engraftment	Challen et al. (216)
	Improved	Tet2	Increased engraftment and self renewal	Quivoron et al. (223x) Ko et al. (217) Moran-Crusio et al. (223y)
RNA-related binding proteins	Defect	Msi2	Decreased self renewal	Kharas et al. (293)
		Ott1/Spen	HSC defects and engraftment and differentiation	Ito et al. (40) Hope et al. (294) de Andres-Aguayo et al. (218)
		HuR (Elav1)	Loss of HS/PC	Xiao et al., Niu et al. (223z,224) Ghosh et al. (224a)

exhibit a non–cell autonomous myeloid expansion (201,202). However, conditional loss of all three family members resulted in a lethal, cell-intrinsic myeloproliferative disorder that was associated with an increase in HSC number and proliferation, but markedly decreased self-renewal, as assessed by transplantation (224b). These studies indicate that Rb family members serve overlapping and redundant roles in maintaining HSC function.

Conserved Developmental Pathways

Notch

The Notch family consists of cell surface proteins that interact with members of the Jagged and Delta family of ligands on adjacent cells, including Jagged 1 and 2 as well as Delta 1, 3, and 4 (225). Upon Jagged or Delta binding, Notch is first

cleaved in its extracellular domain by the TNF-α-converting enzyme, followed by cleavage of the intracellular domain by a γ-secretase complex. The cleaved intracellular domain is then competent for translocation into the nucleus, where it activates transcription by interacting with coactivators such as recombination signal binding protein for immunoglobulin kappa J region (RBP-jk, also known as CBF-1) and Mastermind-like (Maml) to induce transcription of targets such as *Hes1*, *Hes5*, *Hey1*, and *Notch1* itself (226,227). The critical role of this pathway in embryonic HSC development has been shown in mice lacking *Notch1*, *Jagged1*, and *Cbf-1* (228–230). Although the Notch1/2 receptors are expressed on HSCs, and Jagged/Delta are expressed on niche cells, the role for the Notch pathway in adult hematopoiesis remains controversial. Studies that have inhibited Notch signaling with a dominant negative Maml or deletion of CSL/RBP-Jk have produced conflicting results (231,232). In favor of a more limited role, mice that lack Notch1/2, Numb/L, or Jagged1 have normal HSC function even in stress-related settings. Additional studies suggest that HSCs are able to respond to Notch signaling, as *in vitro* stimulation can support expansion and supraphysiologic levels results in the loss of self-renewal *in vivo* (233–236).

TGF-β Family

The transforming growth factor-β (TGF) family of cytokines is large, consisting of 3 mammalian isoforms of TGF-β, approximately 20 isoforms of bone morphogenic protein (BMP), and activin. These family members play important roles in diverse developmental processes (237,238). In the bone marrow, both stromal cells and immature hematopoietic cells secrete TGF-β, suggesting that TGF-β may be part of a feedback loop that regulates HSC function (239). Early studies of TGF-β function showed that TGF-β1 and 3 exert antiproliferative effects on HS/PCs *in vitro* (240). Indeed, *ex vivo* treatment of LSK34$^-$ cells with TGF-β results in attenuation of mitogen-mediated Akt and Src activation, with attenuation of Akt signaling resulting in increased activation and nuclear accumulation of FoxO3a, which then negatively regulates ROS levels and maintains HSC quiescence *in vivo* (181,241). However, *in vivo* studies using mouse models deficient in components of the TGF-β signaling pathway suggest a more complex role of TGF-β in HSC biology. Deletion of *Tgf-β1* results in multiple developmental defects with most mice dying during embryonic development, and those that do survive develop an autoinflammatory condition leading to a wasting disease (242–244). Deletion of *TβRI* results in a similar autoimmune phenotype, but there is no evidence of alterations in HSC number, cell cycle distribution, or self-renewal. Similarly, deletion of downstream signaling components Smad1/Smad5 also results in normal HSC function. In contrast, when *TgfbR2* or *Smad4*, a downstream component of the TGF-β signaling pathway, is deleted, HSCs exhibit self-renewal defects during competitive transplantation assays (245). In the *Smad4*-deleted cells there was decreased expression of c-Myc and Notch-1 (206). One potential mechanism for TGF-β's effects may be its downstream target p57Kip2, a cell cycle inhibitor protein that has also shown to be essential for maintaining quiescence in HSCs (203,246).

WNT/β-Catenin

The Wnt proteins are soluble glycoproteins that exert a wide range of effects on both immature and mature hematopoietic cells (247). Originally identified as regulators of *Drosophila* development and named for the *Wingless* phenotype, these proteins activate this cell-signaling pathway by interacting with a cell surface receptor complex consisting of Frizzled (Fz) and its two coreceptors; Low Density Lipoprotein Receptor-related Protein 5/6 (Lrp5/6). In the absence of Wnt stimulation, the transcription factor β-catenin remains inactivated, destabilized, and is degraded when it is sequestered in a cytoplasmic multimeric protein complex that consists of adenomatous polyposis coli (Apc), Axin1, and the kinases glycogen synthase kinase 3β (Gsk3β) and casein kinase 1 (Ck1). Sequential phosphorylation by these proteins allows for β-Trcp E3 ubiquitin ligase to recognize β-catenin and target it for proteasomal-mediated elimination. Without nuclear β-catenin, Wnt-responsive genes are bound and repressed by the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors. However, in the presence of Wnts, the Fz/Lrp5/6 protein complex recruits another scaffold called Dishevelled (Dvl) to the plasma membrane, resulting in the dissociation of the β-catenin-Axin complex, thus liberating β-catenin to translocate to the nucleus, bind to the TCF/LEF proteins, and activate Wnt-target genes such as *Cyclin D1* and *c-Myc*.

Although still controversial, Wnt protein has been shown to regulate HSC function in a number of contexts. *In vitro* treatment of LSK cells with Wnt5a induces expansion of *B-cell lymphoma gene-2* (*bcl2*)-transgenic LSK cells (247). Ectopic expression of a constitutively active form of β-catenin results in similar findings, with inhibition of the pathway by Axin1 overexpression decreasing HSC growth. A greater role for β-catenin in regulating hematopoietic differentiation was supported by experiments in which expression of a constitutively active β-catenin in lineage-committed myeloid and lymphoid progenitors conferred multilineage potential to these cells (248). The controversy concerning the role of β-catenin signaling stems from studies that failed to demonstrate a phenotype in mice lacking β-catenin (207,249,250). Additionally, overexpression of constitutively activated β-catenin resulted in a loss of HSCs (251,252). In contrast, experiments in which β-catenin was deleted or in which the canonical Wnt signaling inhibitor *Dickkopf1* (*Dkk*) was expressed by osteoblasts revealed impaired HSC self-renewal in reconstitution assays (208,253). Similarly, *Wnt3a*$^{-/-}$ fetal liver HSCs showed a loss in self-renewal potential (210). The role of Wnt signaling has also been investigated through a series of gain-of-function studies. Treatment of mice with the GSK3 inhibitor CHIR-911 increased the reconstitution potential of both mouse and human HS/PCs in NOD/SCID mice (254). Activation of the Wnt/β-catenin pathway by inhibiting GSK3β and adding rapamycin to block growth factor signaling results in the maintenance of LT-HSCs in an *in vitro* culture system using both mouse and human HSCs (209). Moreover, deletion of *β-catenin* reverses this maintenance effect with the combined inhibitor treatment. Finally, HSCs from *Apc*^{min} mice carrying a mutated *Apc* gene with a premature stop codon predicted to exhibit increased Wnt signaling displayed increased reconstitution potential in competitive transplantations (255). In sum, it is clear that there is a role for β-catenin in HSC self-renewal and that the concentration and levels of activation dictate if there is a gain or a loss of self-renewal.

Hedgehog Pathway

The Hedgehog (Hh) family comprises three secreted proteins including Sonic hedgehog (Shh), Indian Hedgehog (Ihh), and Desert hedgehog (Dhh), which regulate numerous development processes by binding to receptors including Patched (Ptch1), which associates with and negatively regulates another cell surface protein, smoothened (Smo). Following Hh binding to Ptch1, Smo is released from Ptch1, allowing Smo to activate the glioblastoma (Gli) transcription factors, Gli1 and Gli2, which activate transcription of key target genes including *Cyclin D1*, *Cyclin E*, *c-Myc*, *platelet derived growth factor*, *Ptch1*, and *vascular endothelial growth factor* (*Vegf*) (254).

Several studies support a role for the Hh pathway in promoting HSC self-renewal. Ihh expression on human stromal cells promotes colony formation by human CD34$^+$ cells (256), and Shh has been shown to promote proliferation of human CD34$^+$ cord blood cells (257,258). Studies in mice have produced less consistent results, as fetal liver cells from Vav-Cre-driven deletion of *Smo* resulted in reduced reconstitution and colony formation in serially passaged colonies (259,260). Given this finding, it was unexpected to find increased numbers of HSCs in *Gli*1-deficient mice (213). In mice heterozygous for *Ptch1*, LSK numbers were increased, but they showed increased cycling and increased short-term reconstitution, consistent with progenitor expansion, but decreased reconstitution potential, supporting decreased HSC frequencies (254). Such a defect in long-term reconstitution was not observed when *Ptch1*$^{+/-}$ fetal liver cells were transplanted, although the significance of this finding to adult HSCs is not entirely clear (259). When *Smo* was conditionally deleted in adult mice using *Mx-Cre*, no changes in hematopoiesis were observed, either in the steady-state or under stress hematopoiesis as assessed by transplantation or 5-FU treatment (211,212); however, this may indicate that Hh signaling is only required during early development and dispensable during adult hematopoiesis. Additional studies utilizing appropriately timed deletions and highly purified HSCs will be required to clarify the role of the Hh pathway in HSC function.

Signal Transduction Pathways

PI3K Signaling Pathway

The PI3K pathway is an important signaling pathway that regulates cell proliferation, growth, and survival in numerous cellular contexts by integrating numerous upstream signals including growth factors, nutrients, and growth status (261). The pathway includes downstream regulators such as Akt and the mammalian target of rapamycin-1 (mTOR) kinase as well as negative regulators including phosphatase and tensin homolog (PTEN) and promyelocytic leukemia, which negatively regulate Akt activation. The mTOR kinase associates with two separate protein complexes mammalian target of rapamycin complex (mTORC1)/regulatory associated protein of MTOR, complex-1 (Raptor), and mTORC2/Rictor, which are nonredundant, regulated differently, and play distinct roles in HSCs. For example, tuberous sclerosis complex 1/2 can inhibit activation of mTorc1 and deletion of mTORC1/Raptor results in a loss of HSC regenerative capacity while loss of mTORC2 results in minimal alterations in HSC transplant capacity (262,263). Activated Akt also inhibits the activity of the Forkhead box protein O (FoxO) family of transcription factors, which are critical mediators of oxidative stress, by leading to their nuclear exclusion. In HSCs, activation of this pathway leads to dramatic phenotypes. Activation of the pathways through conditional deletion of *Pten* or activation with a constitutive AKT in adult HSC results in a rapid T-cell acute lymphoblastic leukemia (T-ALL) and myeloproliferative disorder (186,187,189, 190) which then quickly progresses to acute myeloid leukemia (AML) in the *Pten*-deficient animals (186). Additionally, analysis of HSCs (LSKFCD48$^-$) in *Pten*-deficient mice showed that there was a threefold increase in cycling HSCs, suggesting that Pten acts to limit HSCs proliferation (186). While the mice initially experienced an increase in HSCs, over time the absolute number of HSCs actually decreased. Consistent with a loss of HSC reconstitution potential, *Pten*$^{-/-}$ HSCs transplanted into recipient mice did not persist. Rapamycin, an inhibitor of mTOR, eliminated the leukemia and rescued the HSC phenotype. Similarly, loss of mTORC1 could delay development of the leukemia and the loss of mTORC2 could reverse the effect on both the HSCs as well as T-ALL progression (262,263). Thus, the downstream targets of the PI3K axis play a complex role in both normal HSC and leukemia biology.

The FoxO family of transcription factors integrates growth and stress signals to modulate metabolism, proliferation, and survival. In *Caenorhabditis elegans,* activation of the homologous factor DAF-16 can extend the lifespan as much as 50%. HSCs from mice deficient in *FoxO3a* differentiate normally but exhibit a marked stem cell exhaustion phenotype when challenged with serial transplants or with 5-FU treatment (181). Interestingly, when mice deficient in multiple family members including *FoxO1*, *FoxO3a*, and *FoxO4* are evaluated, they show a more severe hematopoietic defect, with triple null mice exhibiting early loss of HSCs in the bone marrow and a concomitant increase in extramedullary hematopoiesis associated with a myeloid expansion (182). Both *FoxO*-deficient mice show increased HSC cycling and higher levels of ROS in HSCs (181,182). Treatment of mice with the antioxidant N-acetyl L-cysteine (NAC) reversed many of the observed hematopoietic phenotypes. The deletion of FoxO family members extends the latency and maintains the leukemia stem cell (LSC), which provides additional evidence for FoxO's role in stem cell self-renewal (264). These data add additional complexity to the previously discussed proleukemic activity and negative regulatory function of Akt.

Lkb1

Lkb1 is an evolutionarily conserved regulator of cellular energy metabolism in eukaryotic cells and functions as the major upstream kinase that phosphorylates AMP-activated protein kinase (AMPK) and 12 other AMPK-related kinases (265–267). Several groups recently generated *Lkb1*-deficient mice and demonstrated that deletion of *Lkb1* results in severe pancytopenia and subsequent death (178–180). Evaluation of HSCs from these mice reveals that HSCs exhibit increased cell cycling, decreased survival, and eventual exhaustion. Transplantation of HSCs showed a marked reduction in reconstitution potential. The cell cycling and survival effects appeared to be specific for HSCs and was not observed in downstream progenitors, indicating a specific functional role of Lkb1 in HSCs, likely reflective of their unique metabolic state.

CDC42

The *cell division cycle 42* (Cdc42) protein is a GTPase associated with cell polarity (268–270). Recent studies indicate that Cdc42 expression is increased in HSCs during normal aging and required for HSC maintenance through its ability to regulate HSC polarity. Using mice deficient in *p50RhoGAP*, a selective inhibitor of *Cdc42* (*Cdc42GAP*$^{-/-}$ mice), the authors confirmed that the previously described aging phenotype (271) is also present in HSCs. Furthermore, they showed that Cdc42 is not asymmetrically distributed, but that it is diffusely distributed in the cell body of *Cdc42GAP*$^{-/-}$ HSCs. They then showed that pharmacologic inhibition of Cdc42 activity using a novel Cdc42 specific inhibitor, CASIN, reduced Cdc42 activity in aged HSC to levels comparable to young HSC, restored their polarity, and reversed age-related functional HSC phenotypes, including their decreased ability to reconstitute recipients in competitive transplants, myeloid lineage skewing, and cell polarity defects (272). Moreover, the "rejuvenation" phenotype was long lasting, persisting even through a second round of bone marrow transplantation. These data provide evidence supporting the notion that maintaining cell polarity is critical for HSC function, and provide a rationale for pharmacologic intervention to prevent HSC aging phenotypes. Whether or not such interventions may serve as preventative interventions for age-associated hematologic diseases including malignancies such as AML and the myelodysplastic syndromes (MDS) remains to be seen.

Epigenetic Regulators

Bmi1

B lymphoma Mo-MLV insertion region 1 homolog (Bmi1) is a member of the Polycomb Group gene family that functions in multimeric protein complexes to repress gene expression through addition of epigenetic marks at genetic loci. Bmi1 controls cell proliferation by repressing the *Ink4/Arf* locus, which encodes two structurally distinct proteins, the CDK-inhibitor $p16^{Ink4a}$ and the tumor suppressor gene $p19^{ARF}$ (273). Mice lacking *Bmi1* exhibit severe hematopoietic defects early in life with bone marrow failure due to a loss of HSCs (214,274). Consistent with loss of self-renewal capacity, HSCs transplanted from $Bmi1^{-/-}$ bone marrow transiently reconstitute recipients. As expected, *Bmi-1* loss results in increased $p16^{Ink4a}$ and $p19^{ARF}$ protein expression, and retroviral reconstitution of immature hematopoietic cells with $p16^{Ink4a}$ and $p19^{ARF}$ is sufficient to induce growth arrest and cell death, respectively, *in vitro*. Deletion of $p16^{Ink4a}$ or $p19^{ARF}$ restores many of the defects observed in the $Bmi\text{-}1^{-/-}$ bone marrow but combined deletion only partially rescued the loss of HSCs, indicating that Bmi-1 may exert effects on HSCs independently of its cell cycle targets (275).

DNA Methyltransferase

While evaluation of somatically mutated genes in hematologic malignancies has identified many genes that regulate HSC function, recent data also suggest that epigenetic factors play a major role in determining HSC function. Deletion of the maintenance DNA methylation enzymes results in marked pancytopenia and death due to loss of immature LSK cells (215). Hypomorphs of *Dnmt1* exhibit attenuated, but significant hematopoietic phenotypes, with reduced numbers of HS/PCs, decreased HSC self-renewal, and alterations in lineage potential with loss of lymphoid potential with concomitant aberrant expression of myeloid-erythroid genes in LSK cells. Loss-of-function mutants of the maintenance DNA methyltransferase *Dnmt3a* exhibit decreased HSC differentiation as well as HSC expansion. Examination of methylomes demonstrated that *Dnmt3a* null HSCs up-regulated HSC multipotency genes and down-regulated differentiation factors. In addition, their progeny exhibited global hypomethylation and incomplete repression of HSC-specific genes (216). Thus, the epigenome is highly dynamic during even the earliest stages of hematopoiesis and widespread loss of DNA methylation is a necessary step in lineage commitment.

RNA and Posttranscription Levels of Regulation

Approximately 2% of the genome is transcribed and encodes for proteins while approximately 40% of the genome is actively transcribed but not translated. The role for this vast number of non–protein-encoding RNAs that includes piwiRNAs, microRNAs, small nucleolar RNA (snoRNAs), and large noncoding RNAs remains largely unknown and provides an exciting direction for investigation. These various RNAs have vastly different functions from silencing genes to acting as transcriptional scaffolds for epigenetic regulators. Furthermore, RNA-binding proteins (RBPs) may functionally interact with these RNAs, and even compete for the same binding complementary seed sequences thereby modifying RNA function. In this chapter we will discuss microRNAs and select RBPs to highlight this emerging field.

microRNAs

MicroRNAs are small, 22 to 25 nucleotides long of RNA that do not encode for proteins, but are able to exert significant inhibitory effects by binding to the 3′UTRs of target mRNAs and inducing message degradation or inhibiting protein translation (276). Because miRNAs recognize their targets through perfect or imperfect base-pair complementary via a 6 to 8 nt “seed sequence,” they can bind to numerous mRNA targets simultaneous. It is estimated that on average, each miRNA can bind to approximately 300 mRNA targets. Since there are over 1,000 unique miRNAs in humans, miRNAs are predicted to target more than 30% of transcripts in human cells (277). Recent studies have demonstrated that a number of miRNAs can regulate HSC function. miR-125 family members have been shown to regulate HSC self-renewal by blocking apoptosis (278–280). One additional effect of this block in apoptosis is skewing in HSC lineage bias since lymphoid-biased HSCs preferentially exhibited decreased apoptosis when miR-125b was ectopically expressed in mouse bone marrow cells (278). miR-29a was also shown to regulate self-renewal of hematopoietic progenitors, as expression of miR-29a was sufficient to induce aberrant self-renewal of mouse committed progenitors, but target genes for this miRNA were not definitively identified (281). Enforced expression of miR-155, a miRNA highly expressed in HSCs, results in the development of a hematologic disorder resembling a myeloproliferative neoplasm (MPN) (282). Other groups have shown that miRNAs are differentially regulated during hematopoietic development and that numerous miRNAs are differentially expressed in HSCs (279,283,284). miR-126 is also highly expressed in HSCs (285), and recent studies have shown that miR-126 regulates HSC self-renewal and expansion, but not cell cycling, in both mouse and human HSC by modulating activity of the PI3K pathway (286). Together, these data demonstrate that multiple miRNAs regulate HSC function and that their misexpression in hematologic malignancies makes them potential therapeutic targets.

RNA Binding Proteins

RBPs have been shown to regulate hematopoiesis and to be dysregulated in hematologic disorders. RBPs can function in a variety of biologic roles including the regulation of mRNA splicing, polyadenylation, mRNA export, mRNA stability, and translational control (287). Some of the best-studied RBPs are downstream mediators of the PI3K/mTOR pathway and include several elongation and initiation factors (eIFs such as eIF4G and eIF4E) that allow for specific sets of transcripts to be translated and to increase cell proliferation and growth (288). mTOR phosphorylates and blocks 4E-BP1, an inhibitor of EIF4G/E, thus resulting in translation initiation of target mRNAs (289). This pathway was shown to be critical in HSCs and leukemia-initiating cells because mTOR inhibitors could reverse the effects of constitutive PI3K/Akt activity (186,189).

Another set of RBPs have been more recently shown to be important for hematopoietic cells include splicing factors SF3B1, serine/arginine-rich splicing factor 2 (SRSF2), U2 small nuclear RNA auxiliary factor 1 (U2AF35), zinc finger (CCCH type), RNA-binding motif and serine/arginine rich-2 (ZRSR2), SF3A1, PRP40 pre-mRNA processing factor 40 homolog B (PRP40B), SF1, U2AF1, and U2AF65—all found to be commonly mutated or highly expressed in hematologic malignancies and disorders (290). Investigations of how these splicing factors alter hematopoietic development and their role in disease is only in its initial stages. Other RBPs control several aspects of RNA processing important for cell fate determination. For example, ELAVL1 (HuR) controls stability and export from the nucleus to the cytoplasm, and postnatal deletion of HuR reduces hematopoietic PC function and results in decreased engraftment in transplantation assays. Another RBP, Lin28b, possesses pleiotropic functions in HSCs and ESCs through its regulation of the processing of the Let7 family of miRNAs; however, it can also bind to mRNAs to alter their stability (291). Overexpression of Lin28b can reprogram adult

HS/PCs and increase the development of B-1a, marginal zone B cells, gamma/delta (γ/δ) T cells, and NK/T cells (292). Additionally, RBPs can regulate self-renewal. For example, Msi2 overexpression can increase HSC numbers, and deletion results in reduced self-renewal and engraftment of HSCs and MPPs (218,293,294). How expression of these RBPs is controlled and what pathways they regulate remain to be determined, but the pathways they activate are clearly multifunctional and have wide-ranging effects on metabolism, cell cycle control, and self-renewal (295).

Extrinsic Factors

Bone Marrow and the HSC Niche

HSCs depend on extrinsic signals for their function and the bone marrow provides a unique microenvironment to support adult hematopoiesis. Our understanding of the critical components that regulate hematopoiesis has expanded significantly in the past decade. As part of this discussion, we would like to review the anatomic composition of the bone marrow as well as the different specialized microenvironments within the bone marrow that control hematopoiesis.

Cortical bone is mainly composed of mineralized, calcified connective tissue, and the bony cavity is occupied by a 3D matrix of cancellous or spongy trabecular bone. Within the inner portions of the bone, or the medullary region, hematopoietic cells reside in a specialized microenvironment called the bone marrow, which is fed and penetrated by the vasculature via a nutrient artery and periosteal artery; the former connects to the central artery running along the bone while radial arteries connect to the latter (Fig. 1.3A) (296). The vasculature of the bone marrow follows a path from the arterioles to the capillaries, eventually leading into a plexus of venous sinuses, and the sinusoids or small vessels then drain into the longitudinal vein, followed by the nutrient vein that connects it to the systemic circulation (Fig. 1.3A). Thus, the bone marrow vasculature is organized similar to other peripheral tissues. While these architectural features of the bone are easy to identify morphologically, the areas of the bone marrow that maintain HSCs are less defined.

The “niche” is a general term used to describe the presumed local environment required to support HSC self-renewal and differentiation, but the cellular and molecular components that make up the niche are not completely understood. Schofield (11) first hypothesized the concept of a “niche” in the late 1970s and suggested that stromal components in the bone marrow regulate hematopoiesis. The niche is thought to provide HSCs with the factors required for their maintenance and activation through cell-cell interactions, extracellular matrix proteins, and other secreted regulators that modulate hematopoiesis during steady-state and stress conditions. The concept

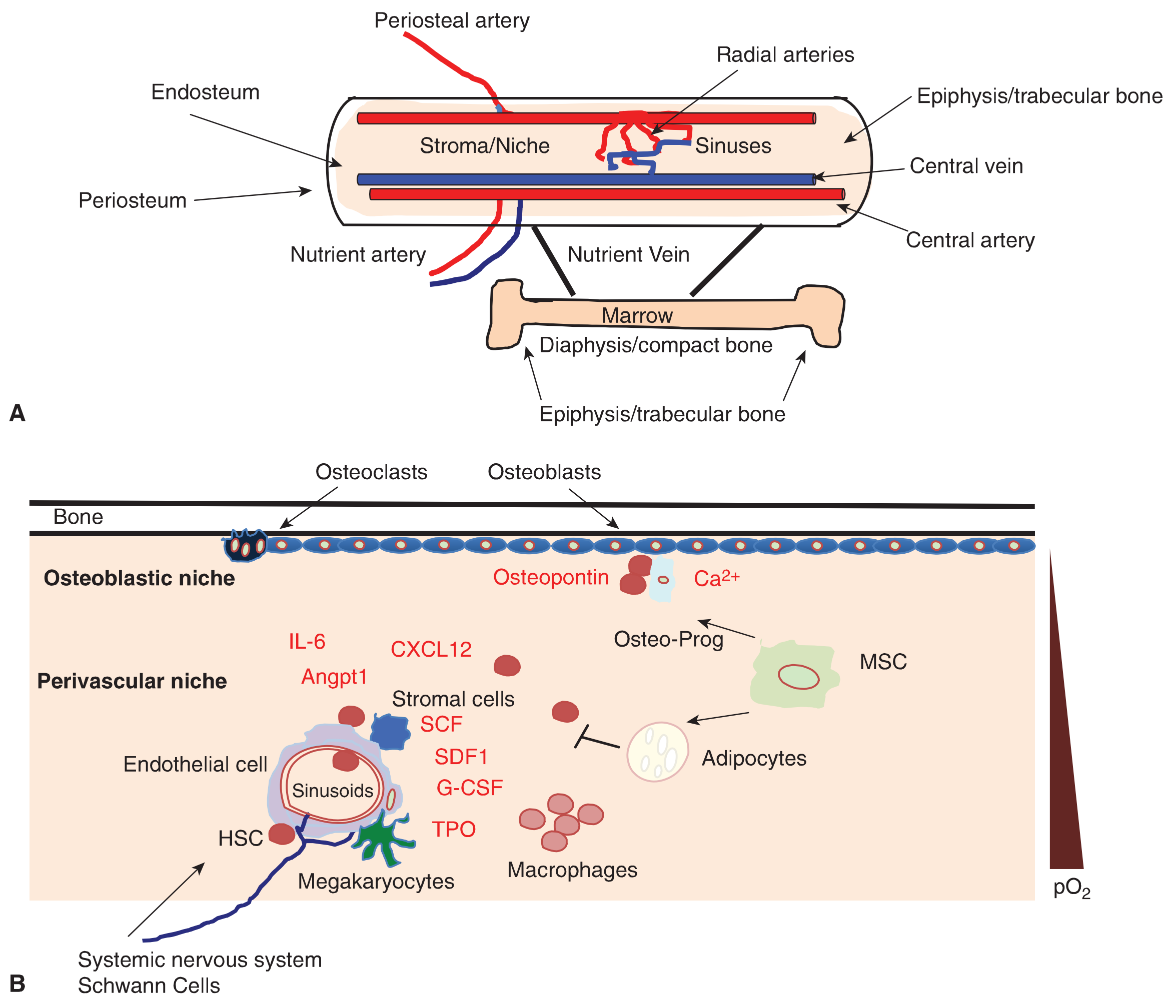

FIGURE 1.3. Hematopoietic niche in the bone marrow. A: Schematic of the physical structure and vasculature of the bone marrow. **B:** The hematopoietic “niche” contains numerous cell types that maintain HSCs through direct physical interactions or through the elaboration of secreted factors. Cytokines and regulators are in *red*. Oste-Prog, osteoblast progenitor; MSC; mesenchymal stem cell; HSC, hematopoietic stem cell.

of an architecturally definable HSC niche was supported by studies of other tissue systems, in particular in the gonads of other model systems including *Drosophila* and *Caenorhabditis elegans*, where interactions of germ cells with niche cells was demonstrated (297–299). We now know that several hematopoietic and nonhematopoietic cell types contribute to the HSC niche. Perhaps the best-described niche component is the endosteal cell layer that covers the trabecular bone and is made up of several cell types including osteoblasts, osteoclasts, and reticular cells (296). The location of specific cell types within the marrow has been investigated since as early as 1938 when mature hematopoietic cells were observed to be localized further away from the endosteum (300). While this led to a model that held that immature hematopoietic cells reside near the endosteum and move away as they differentiate, this model has been challenged by recent findings demonstrating the presence of endosteal and vascular HSC niches, which will be discussed in greater detail below.

What are these stromal components of the bone marrow that modulate hematopoiesis? These hematopoietic and nonhematopoietic components are collectively referred to as the "stroma" and have been described over the years as composed of a variety of cell types including mesenchymal stromal cells, osteoblasts, adipocytes, reticular stromal cells, neural cell types, endothelial cells, macrophages, and megakaryocytes (301,302). Unfortunately, as most of these stromal components have been described on the basis of morphologic, immunophenotypic, and/or functional differences definitive experimental evidence of their function and tissue/lineage origin have been frequently lacking. For example, reticular stromal cells are classified based on their appearance, but it is not clear from which lineage they are derived, and methods to isolate them have not been developed (303). Advances in identifying cell surface markers that mark specific stromal cell populations, improved animal genetic models of hematopoiesis, and *in vivo* imaging techniques have improved our understanding of the niche and its complexity. Currently, one of the prevailing models of the hematopoietic niche posits that there are two separate, distinct regions: the osteoblastic and perivascular niches. This is based on the observation that morphologically immature hematopoietic cells are enriched in these two areas of the bone marrow. However, this simplified scheme has been complicated by *in situ* live imaging studies suggesting that these niches might not be anatomically separate as once thought, especially in the calvarium, where direct live imaging studies of HSC interactions in the bone marrow are technically possible (304,305), and demonstrate that the blood vessels are often physically located in proximity to the endosteum. Nevertheless, cells physically associated with, or close to, the trabecular bone are considered to be part of the endosteal or osteoblastic niche, which includes osteoblasts and osteoclasts. Cells that are more closely associated with vascular sinusoids include endothelial cells, macrophages, mesenchymal stromal cells, and megakaryocytes.

The endosteal niche is thought to represent a relative hypoxic environment compared to the vascular niche (173,306). The overall partial pressure (pO_2) is approximately 55 mm Hg and the saturation of O_2 is close to 90% in the bone marrow, but these measurements do not account for the microenvironmental variation in local oxygen concentration, since the pO_2 drops rapidly as the distance from the capillary bed increases (307). HSCs have been shown to be closely associated with the areas that are poorly perfused (173,306,308). This continuum of oxygenation levels between the niches is thought to be functionally important for maintaining HSC quiescence, numbers, and function, as hypoxia limits the production of reactive oxygen radicals that inhibit HSC function (309). Mice with a mutant $Vegfa^{\delta/\delta}$ gene lacking the hypoxia inducible factor (HIF) binding element showed reduced HSC function and VEGFR1 expression is dependent on HIF1 (310,311). Cells within the vascular niche respond to VEGF signaling by up-regulating CXCL12. Consistent with the importance of a hypoxic HSC niche, HSCs grown under hypoxic conditions *in vitro* exhibit improved growth, reduced cell cycling, and increased engraftment capability when transplanted into mice (312,313). Mice lacking *HIF1α* exhibit reduced quiescence in HSCs and decreased engraftment in transplants (314). Besides an effect on HSCs, deletion of HIF members in mice leads to a block in B-cell development and alters macrophage activation (315). Given the difference in oxygenation at the endosteal and vascular niches, the two likely serve different functions with respect to HSC maintenance (316) (see Fig. 1.3B for details on the different cells of the niche).

Mesenchymal Stromal Populations

Many of the nonhematopoietic components of the bone marrow are derived from mesenchymal stem cells (MSCs). Like HSCs, MSCs exhibit long-term self-renewal and possess multilineage potential, being able to differentiate into mesenchymal stromal cells, CXCL12-abundant reticular (CAR) cells, and adipocytes. Since MSCs and their progeny include cell types that have each been shown to have the capacity to maintain and regulate hematopoiesis, many investigators have developed methods to study their function. Of note, MSCs are thought to have broad therapeutic potential as a cellular regenerative therapy with the promise of efficacy in multiple organ systems and utility in various diseases [reviewed in (317,318)]. Perivascular MSCs contribute to the maintenance of HSC numbers and function and are characterized by Nestin positivity in mice and CD146 expression in humans (303,319). Current models hold that mesenchymal stromal cells are located at the sinusoids, tethering HSCs to the niche and providing migration and growth signals such as Angpt1, stem cell factor (SCF), and CXCL12 (303). Another subpopulation of MSCs, CXCL12 abundant reticular cells (CARs), are also critical for HSC maintenance, as depletion leads to a reduction in HSC number (320). These cells can signal through CXCR4 and are located between endothelial and osteoblastic cells (320). These CXCL12 expressing mesenchymal stromal cells can also retain and modulate the migration of HSC out of the marrow and into the circulation (320).

Systemic Nervous System

Sensory and autonomic fibers are found along the arterioles in the bone marrow and associate with a variety of cell types in both the osteoblastic and perivascular niches; thus, the nervous system can mediate direct effects on hematopoiesis in response to acute stressors and normal oscillations in circadian rhythms. These actions are thought to rely on the hypothalamic suprachiasmatic nucleus of the brain, which can stimulate the local secretion of catecholamines such as noradrenaline in the bone marrow during normal circadian cycles, and this activates β-adrenergic receptors (β2 and β3) on osteoblasts and perivascular mesenchymal stromal cells (303). Thus, normal oscillations in the circadian rhythms might provide a mechanism for controlling the migratory turnover of HSCs and even hematopoietic cells from the niche into the periphery (321–324). After stimulation of G-CSF or GM-CSF, human hematopoietic PCs up-regulate the expression of dopaminergic receptors resulting in the increased sensitivity to neurotransmitters that then alters the migration, proliferation, and engraftment into immunodeficient mice (325). Glial fibrillary acidic protein–positive Schwann cells wrapping around nerve fibers in the bone marrow provide signals to HSCs such as TGFβ, which is important for maintaining HSC quiescence and dormancy as previously discussed (245). They do so by activating the large reservoir of latent and inactive TGFβ produced by numerous cells present in the bone marrow, thereby maintaining HSCs (245).

Osteoblasts

MSCs can differentiate into osteoblasts, which are located in close physical proximity to HSCs. However, the functionally relevant osteoblasts that support HSC function may not represent the most mature osteoblasts that synthesize bone matrix, but may instead be composed predominantly of osteolineage PCs (301). Nonetheless, homing assays showed that the HSCs lodge in the bone marrow in close proximity to N-cadherin and Flamingo (Fmi) expressing osteoblasts and that cotransplantation of osteoblasts with HSCs improved overall engraftment (326–329).

Osteoblasts are thought to maintain HSCs through cell-cell contacts as well as their elaboration of secreted products. Initial reports of N-cadherins playing an important role in both HSCs and osteoblasts has been tempered by more recent reports utilizing a set of mouse lines that delete them within the osteoblastic niche and within HSCs (330–332). In contrast, preosteoblasts express N-cadherin and signal through noncanonical Wnt signaling via the interactions of Flamingo (Fmi) and Fz receptors that maintain HSC quiescence and maintenance within the niche. Thus, these conflicting results might be explained by this alternative signaling mechanism (329). Osteoblasts can also support hematopoietic cells by secreting multiple factors including CXCL12, SCF, IL-6, GM-CSF, Angpt1, and others. It remains unclear if specific factors secreted by osteoblasts are required for normal hematopoietic development. However, loss of osteopontin secreted from osteoblasts results in reduced HSC number and decreased migration away from the endosteum (333,334). Moreover, mice deficient in osteopontin demonstrated an increase in hematopoietic progenitors while osteopontin treatment resulted in reduced cycling of HSCs *in vitro* (333,334). Taken together, these studies suggest an inhibitory role for osteopontin on HS/PC.

Recent studies suggest that osteoblastic niche cells may directly contribute to the pathogenesis of hematologic disorders. Mice lacking Dicer, an enzyme required for microRNA biogenesis, specifically in the osteoblast lineage exhibited a reduction in HSCs and altered differentiation with ineffective hematopoiesis and morphologic evidence of dysplasia; some of the mice exhibited a progressive phenotype with the development of AML (335). Consistent with the concept that specific subsets of osteoblasts are critical for proper HSC homeostatic control, deletion of *Dicer* in more committed osteoblasts yielded no phenotype, indicating that only a specific subset of osteolineage cell types has significant ability to regulate hematopoiesis. Overall, these studies underscore the importance of proper interactions between the niche and the hematopoietic cells and suggest that modulation of niche function may become a therapeutically relevant strategy for the treatment of bone marrow–based disease in the future.

Adipocytes and Mature Hematopoietic Cells

Some established stromal cell lines have the ability to maintain limited self-renewal of HSCs, and as described previously, these may be used to assess HS/PC activity in *in vitro* experimental models such as LTC-IC and CAFC assays. One commonly used stromal line, OP9, is derived from the osteopetrotic mouse lacking CSF-1 (*op/op*) and can be differentiated into adipocytes under certain conditions. The supportive function of these stromal cells for HSCs becomes severely compromised once they show evidence of adipocytic differentiation, suggesting that adipocytes may negatively regulate HSCs (336,337). In support of this concept, vertebral bodies of the tail in mice contain higher proportions of adipocytes and decreased frequencies of HS/PCs compared to the thoracic portion of the vertebral column (336). Additionally, genetic or pharmacologic inhibition of adipocytic differentiation led to increased numbers of immunophenotypically defined HSCs as well as their reconstitution capacity upon bone marrow transplant (336). Taken together, these data provide evidence for a negative role of adipocytes in HSC maintenance (336). As bone marrow adipocyte content increases with age, this raises the intriguing possibility that adipocytes may contribute to age-related changes in the hematopoietic system.

Hematopoietic cells that reside in the marrow may also contribute to the maintenance of HSCs. For example, deleting macrophages expressing CD169 or M-CSFR leads to increased mobilization of HSCs, supporting the notion that macrophages provide negative signals for HSC retention (338). While these signals could emanate directly from macrophages, it could also be mediated by other niche cell types such as osteoblasts, Nestin$^+$ mesenchymal stromal cells, or sympathetic nerves. These studies also suggest that G-CSF's ability to stimulate macrophages relieves the negative feedback inhibition on HSCs and mobilizes them into blood. When the immune system becomes activated due to an infection, inflammatory signals from hematopoietic cells from both the innate and adaptive immune system can directly alter the self-renewal and differentiation potential of HSCs in the bone marrow (339). Proinflammatory cytokines such as IL-1, IL-6, IL-8, TNF, and type 1 and II interferons (IFNs) have pleiotropic effects on hematopoietic development (Table 1.4). For example, lipopolysaccharide (LPS) stimulation can result in TNF activation which inhibits HSC self-renewal. (340). During chronic infections, sustained levels of IFNγ can deplete HSCs by inducing increased HSC cycling and differentiation (341). Type 1 IFNs such as IFNα can increase Ly-6A/E (Sca-1) levels on hematopoietic cells, thereby confounding attempts to identify or prospectively separate HS/PCs based on immunophenotype in the mouse system (342).

Osteoclasts

Osteoclasts are derived from the monocyte lineage and are critical for resorption of the bone matrix. While these cells are activated in response to bone injury as well as in response to aberrant growth factor secretion in hematologic malignancies such as multiple myeloma (343), they are also required to create the complex meshwork and cavities within the bone marrow space during normal bone turnover and growth. Based on their essential role as bone remodelers, many predicted that osteoclasts would be an important component or regulator of the niche. However, osteoprotegerin-deficient mice exhibiting osteoporosis due to increased osteoclastogenesis and bone resorption showed a reduction in HS/PC mobilization. Also, mutant mice exhibiting defects in osteoclast development such as *op/op* mice, r*eceptor activator of nuclear factor κappa B ligand, (Rankl)*-deficient mice, and *c-Fos*-deficient mice demonstrated comparable or increased HS/PC mobilization with no apparent increase in HSC numbers (344). These results indicate that osteoclasts inhibit HSC maintenance and mobilization. Recent studies have attempted to link bone remodeling to HSC expansion (345,346). During the process of mineralizing bone, Ca^{2+} is increased near the endosteal surface, and this increase may signal to the calcium sensing receptors present on HSCs which are required for HSC self-renewal and engraftment (143).

Perivascular Niche Cells

Evidence for a perivascular HSC niche originated from studies of normal development and the observation that early hematopoietic cells arise from hemangioblasts and hemogenic endothelium. Supporting the absence of a requirement for an endosteal niche for HSC maintenance, HSCs reside in the yolk sac, AGM, or fetal liver in the developing embryo where no such niche is present. Imaging studies have shown that HSCs physically associate with endothelial cells and osteoblasts

Table 1.4 CYTOKINES REGULATING HEMATOPOIESIS

Cytokines (Ligand)	Cognate Receptor	Sources	Target Cells	Biologic Activity
SCF/KitL	c-Kit	Perivascular niche cells/ osteoblast cells	HSCs and progenitors	Increased proliferation/differentiation
TPO	MPL	Liver and stromal cells from bone marrow	HSC/HS/PC/Megakaryocytes	Promotes megakaryocyte and HSC proliferation and development
FLT3L	FLT3R	Stromal components	Myeloid progenitors and progenitor B cells	Progenitors proliferation and early maintains B-cell development and survival
M-CSF	M-CSFR	Stromal cell and macrophages	Monocytes, HSCs, myeloid progenitors	Alters myeloid differentiation and DCs
G-CSF	G-CSFR	Stromal cell and macrophages	Granulocytes, myeloid progenitors	Myeloid differentiation and DCs
GM-CSF	GM-CSFR	Stroma cells, endothelial cells, macrophages	Granulocytes, macrophages progenitors and erythroid progenitors	Increase proliferation
EPO	EPOR	Kidneys and endothelial cells	Erythroid progenitors	Maintains erythroid and RBC production
IL-3	IL-3R	T-lymphocytes, mast cells, and NK cells	Myeloid cells and lymphoid cells	Stimulates growth and survival
IL-4	IL-4R	Basophils, T cells	Monocytes, mast cells, and T cells	Commit monocytes toward DC lineage, TH2 skewing of T cells
IL-5	IL-5R	T cells	Eosinophils, B cells	Activation, proliferation
IL-6	IL-6R	Stromal cells, endothelial cells, T cells	Progenitors, myeloid progenitors	Activation, proliferation
IL-7	IL-7R	Stromal cells	Progenitor B and T cells	Proliferation and survival
IL-11	IL-11R	Stromal cells	Megakaryocytes	Megakaryocyte development
IL-13	IL-13R	NK, TH2-skewed T cells	Macrophages	Activation
IL-15	IL-15R	T cells	NK cells	Development of NK cells, stimulation of CD4 T cells
IFNγ	IFNγR	Macrophages, T cells, NK cells	T cells, macrophages	TH1-T-cells, macrophages, B-cell activation and development
IFNα	IFNαR	Plasmacytoid DCs	M1 macrophages, HSCs	Activation and development after infections

(304,347,348). Providing a supportive role for endothelial cells in HSC maintenance, hematopoietic cells can be maintained *in vitro* on endothelial-derived cell lines, and embryonic sources of endothelial cells can support adult HSCs (314). When HSCs are grown on transformed endothelial cells, they can maintain their ability to self-renew by up-regulating Notch ligands (314). Recent studies provide the best evidence for the importance of the perivascular niche. While it is known that osteoblasts, MSCs, and endothelial cells all express SCF, selective deletion of SCF in Nestin$^+$ or osteoblasts showed no changes in HSCs numbers or activity; however, when SCF was deleted from perivascular, leptin receptor expressing cells or endothelial cells, the HSC compartment was greatly reduced (349). These studies indicate that one of the critical growth factors for HSC maintenance is expressed in cells that reside in the perivascular niche. While Leptin receptor expressing perivascular cells are similar to MSCs, they are unable to differentiate into osteoblasts, and thus it remains unclear how these stromal populations are related to the perivascular MSCs mentioned above (349). It should be noted, though, that in addition to SCF, endothelial cells secrete a variety of other cytokines known to regulate hematopoiesis as summarized in the Table 1.4.

Megakaryocytes

TPO is well known for its ability to promote megakaryocytic differentiation and platelet production by binding to its receptor, myeloproliferative leukemia (MPL) virus protooncogene, present on HS/PCs, and thus mice deficient in TPO have dramatically reduced megakaryocytes and platelets (350,351); however, TPO is also an important regulator of HSC cell cycle progression. In fact, mouse HSCs can be distinguished on the basis of c-Mpl expression and separated into quiescent (LSK34$^-$MPL$^+$) and proliferative (LSK34$^+$MPL$^+$) forms (352). Megakaryocytes are mostly associated with sinusoids and may act as a sink for TPO in the stroma (352). Besides its ability to bind to c-Mpl on megakaryocytes, TPO can initiate signals in HSCs and mediate quiescence (160,352). *c-Mpl*-deficient mice have reduced plasma cells, indicating that megakaryocytes can regulate other cell types within the niche, raising the prospect that megakaryocytes may indirectly regulate HSC function (350).

Migration, Trafficking, and Development from Secreted and Other Niche Components

One of the remarkable characteristics of the hematopoietic system is that at any given moment in time, samples taken from different sites of hematopoiesis are similar with respect to cellular composition. This is possible because HSCs are highly mobile cells, constantly exiting and reentering the bone marrow after traveling through the circulatory system, seeding sites conducive for hematopoiesis. But why should HSCs traffic normally? A number of models have been proposed. First, given the importance of an organism to be able to maintain hematopoietic output, migration of HSCs to multiple sites of hematopoiesis ensures that hematopoietic cell production is not reduced, even when a single hematopoietic site is injured. Second, HSC mobilization may allow for more rapid responses to immune challenge since the lymphatic circulation can be used by migrating HSCs and progenitors to migrate to peripheral tissues and secondary lymphoid organs where they can rapidly give rise to inflammatory cells locally through a process partially regulated by the sphingosine 1 phosphate receptor (147). Regardless of the evolutionary and biologic explanations for HSC trafficking, it is this property that allows clinical HSC transplantation possible, as HSC can migrate back to the bone marrow after a simple infusion of cells into the peripheral blood.

The earliest evidence for HSC migration was observed in rat parabiosis experiments, which involves connecting the circulation of one rat to that of another. When one of the rats was lethally irradiated, the hematopoietic cells from its non-irradiated partner reconstituted its damaged immune system

(353). These studies were followed by experiments in mice in which the peripheral blood of 200 donors was transplanted into irradiated recipients and shown to be able to reconstitute the recipient's immune system (354). More recent mouse parabiosis experiments using genetically marked animals have shown that HSCs constitutively circulate in the peripheral blood and can repopulate unconditioned bone marrow (355). While it had been assumed for some time that myeloablative conditioning may be required to eliminate HSCs from their niches, it appears that approximately 0.1% to 1% of all HSC niches are unoccupied at any given moment in time and therefore can be engrafted by circulating HSCs (356). Moreover, HSC egress from the bone marrow does not depend on alterations in HSC cell cycle status (148), indicating that this process is likely regulated. The mechanisms underlying this process are poorly understood.

As noted earlier, G-CSF is a widely used agent for mobilizing HS/PCs in the clinic. Although it is known that HSC mobilization following administration can occur as early as 5 days after treatment, the exact mechanism underlying this effect remains unclear since the target cell of G-CSF's action in the bone marrow has not been clearly identified (357,358). One would postulate that G-CSF directly binds to HS/PCs to drive mobilization, but it has long been thought to not require expression of the G-CSF receptor on the HS/PCs themselves (359). However, more recent studies demonstrated that functional G-CSF receptors are expressed on the surface of human and mouse HSCs (360). Besides this potential direct effect of G-CSF on HSCs, monocytic cells that express high levels of G-CSF receptor have been shown to be sufficient to drive mobilization, likely through the release of proteases that process CXCL12, c-Kit, or VCAM (309,361). However, this model has not been formally proven because it is unclear which proteases are essential for mobilization, as deletion of neutrophil serine proteases and metalloproteinase inhibitors had no effect on mobilization (362–364). Besides proteases, other factors may facilitate mobilization such as the stimulation of complement receptors C3a or C3 allowing for HS/PCs to become sensitive to G-CSF stimulation (365).

Despite these advances in our understanding of HSC migration, currently employed strategies to mobilize HSCs for autologous bone marrow transplants do not always succeed in collecting sufficient numbers of HSCs, and thus other strategies will be required to optimize such protocols. Secreted factors such as chemokines provide signals to hematopoietic cells to migrate, lodge, and engraft in the bone marrow, and therefore their manipulation may be useful. CXCR4 and its ligand CXCL12 are critical players that regulate HSC migration to the bone marrow. HSCs express CXCR4 and cells from the niche can provide CXCL12, which activates the Rac/Rho family of GTPases, to signal HSCs to migrate toward the endosteal niche (366). An antagonist to CXCR4 (AMD3100) has shown efficacy in inducing proliferation and mobilizing HS/PCs in mice, nonhuman primates, and in humans in a variety of clinical settings (367–369). Another clinically relevant factor that may increase HS/PC mobilization is parathyroid hormone (PTH). PTH initially was thought to act on HS/PCs by inducing signals from osteoblasts, but recent data suggest that this occurs, instead, through interactions with T cells (234,370–372). Other cytokines and factors known to be capable of mobilizing HSCs from the marrow include SCF, MIP-1α, Grob, IL-1, IL-3, IL-6, IL-7, IL-8, IL-1, and IL-12 (373–381).

Selectins are adhesion molecules expressed on endothelial cells that are critical for mediating recruitment of mature leukocytes to inflamed sites (382,383). Consistent with a similar function in mediating HS/PC migration to the marrow, homing was compromised in the absence of either E- or P-selectins (384). Integrins also play an important role in HSC migration, as inhibition of $\alpha 4$ integrins or VCAM-1 function results in markedly reduced HS/PC homing (385). Integrins including CD49d/CD29 ($\alpha 4\beta 1$ or VLA-4), $\alpha 4\beta 7$, and CD49b ($\alpha 2$) have also been shown to regulate HSC homing to the bone marrow (386–390). Within the marrow, LFA-1 and VLA-4 promote the migration of HSCs toward osteoblasts (388,391).

Factors secreted by the niche such as BMP4 and angiopoietin-1 (Ang1) have been shown to be important in maintaining HSCs *in vitro* (392–394). Ang1 is also expressed by osteoblasts and binds to HSCs (394). Many products expressed from MSCs and osteoblasts have been shown to regulate HSC function. Tissue inhibitor of metalloproteinase-3 was shown to regulate HSC quiescence and fate determination as well as normal bone formation and maintenance (395,396). Agrin, a proteoglycan typically associated with the neuromuscular junction, supports hematopoietic progenitor proliferation, presumably by interacting with its receptor, α-dystroglycan, which is expressed on HSCs (397). However, transplantation of agrin-deficient HSCs demonstrated no defect in engraftment or reconstitution of the immune system.

Stem Cell Factor

SCF is produced by multiple cell types in the bone marrow niche, including osteoblasts, endothelial cells, and perivascular cells, and engages c-Kit on the surface of HSCs to initiate cell signals. SCF exists in two forms, membrane-bound and soluble, generated by proteolytic cleavage of SCF by a number of proteases including MMPs (398–400). The importance of this pathway in HSC maintenance has been demonstrated through a number of approaches. Mice with mutations in SCF (e.g. *Sl/Sld*, steel dickie mutants) or in c-Kit (e.g. *W/Wv*, white spotting mutant) exhibit reduced numbers of HSCs and decreased HSC function (401,402). Blocking SCF:c-Kit interactions with an antibody results in loss of HSCs (94). The importance of membrane-bound SCF was demonstrated by analyzing HSC function in mice lacking this form. These mice showed decreased HSC numbers and reduced ability to support HSCs following transplant (403). More recently, studies utilizing tissue-specific Cre-recombinase indicate that the predominant functional source of SCF in the bone marrow is the endothelial cells and/or the perivascular cells surrounding them, but not in osteoblastic lineage cells (349).

The Niche in Hematologic Disorders

Given the importance of the niche in regulating HSC homeostasis, it is not difficult to imagine that an altered niche may allow for the survival and maintenance of abnormal cells in hematopoietic disorders and malignancies. Emerging evidence indicates that many regulators of normal HSCs in the niche are co-opted by LSCs (404,405). Cytokines such as IL-6, IL-3, G-CSF, and GM-CSF increase proliferation of leukemic cells. CXCR4 is frequently increased on AML blasts, and anti-CD44 monoclonal antibody treatment decreases leukemic blast homing and engraftment (406,407). An attractive theory is that the hypoxic and low perfusion environment within the niche may provide a protection from classical chemotherapy (408). Antiangiogenic inhibitors also could be used as a therapy to prevent VEGF signaling to drive leukemia. Overall, developing inhibitors to disrupt the HSC and LSC interactions with the niche components could provide an additional therapeutic strategy for treating leukemias.

Hematopoietic Development

Hematopoietic Progenitors

The classical hematopoietic hierarchy held that MPPs differentiate into a CMP or a CLP, establishing the myeloid and lymphoid arms of the hematopoietic system (27,28,409). More recently this model has been amended to include another PC that reflects the recognition that there is also a hierarchy of multipotent progenitors. In humans this cell has been described

as the multilymphoid progenitor (MLP), which has the potential to differentiate into the GMP or CLP; in mouse, they are referred to as early lymphoid progenitor or lymphoid primed multipotent progenitor (82,410–413). Markers for the MLP are CD34pos CD38neg Thy1lo CD45RApos. This more recent concept incorporates the idea that the lymphoid/myeloid switch occurs in progenitors downstream of the MPP and also loses its ability to differentiate into the megakaryocyte/erythroid lineages (414). Evidence for the existence of this progenitor comes from single-cell assays from sorted populations, lineage tracing, and myeloid potential being retained in thymocyte precursors (415,416). Although the mouse system is better defined, confusion remains regarding the exact lineage order and overall plasticity of human progenitors.

Transcriptional Factor Network

Extrinsic signals activate a set of lineage-specifying transcriptional modulators in HS/PCs. Extensive transcriptional profiling of functionally distinct populations within the hematopoietic hierarchy as well as numerous loss- and gain-of-function mouse models have revealed a set of critical regulators that dictate hematopoietic developmental fates (417,418). As one would expect, alterations in regulators of hematopoietic differentiation and self-renewal can lead to hematopoietic disorders and malignancies. Due to the large number and complex regulation of transcription factors and other modulators, this chapter will only highlight a small set.

One of the most critical transcriptional factors is Runx1, shown to be required for embryonic and fetal hematopoiesis, but not for adult hematopoiesis (314). However, Runx1 plays an important role in mediating proper megakaryocytic, B-cell, and T-cell development (314). One critical target of Runx1 is *Pu.1*, which can act as a master regulator of hematopoiesis by itself (419). As a member of the E-twenty six (ETS) family, deletion of this transcription factor leads to a block in the generation of CMPs and CLPs (420,421). PU.1 is expressed during many stages of hematopoietic development, but can also regulate a critical myeloid PC fate decision since high levels of expression skew progenitors toward the macrophage lineage while lower levels favor granulocyte differentiation (420,422). Consistent with its important role as a driver of myeloid differentiation, loss of *Pu.1* and its gene regulatory regions induces AML in mice (420).

Myeloid differentiation and commitment is also regulated by the transcription factor CEBPα, which is commonly deleted in AML and a critical driver of the CMP to GMP transition (423,424). Its potent activity as an inducer of myeloid fates is perhaps best illustrated by its ability to reprogram mature B or T cells into macrophages (425).

Another lineage program can be dictated through overexpression of GATA-1, which redirects CLPs into the megakaryocytic and erythroid lineages (425). Additional transcription factors such as growth factor independent 1 (GFI1) and interferon regulatory factor 8 (IRF8) can modulate the differentiation fate of myelocytes and granulocytes. Gfi-1 is required for terminal neutrophil differentiation, and deletion of *Irf8* results in fewer myeloid cells with an additional skewing toward granulocytes at the expense of macrophages (236,426,427). This complex interplay of transcriptional regulators is compounded by the general feature that the same set of transcription factors or gene expression programs tends to be reused to regulate cell fate in both immature and mature progenitors as well as in later stages of differentiation (417). Although there are many examples of this phenomenon, PU.1 has been shown to be important in HSC self-renewal, development of CMPs, and then again in the development of different dendritic cell (DC) subtypes (428,429).

Cytokines can coordinate transcriptional programs, which can then dictate a specific cell fate. Specific cytokines through signaling pathways can be connected to individual transcription factors. For example, when Mafb/c-Maf transcription factors were deleted, these mice demonstrated increased monocytic development and proliferation *in vitro* in the presence of only M-CSF and not other myeloid-specific cytokines. Interestingly, this cytokine specific proliferation was robust and did not result in transformation to leukemia (430). Additionally, this model illustrates the importance of understanding the role of transcription factors in the context of specific extrinsic signals.

Myelopoiesis

Monocytes and Macrophages

The innate immune system functions mainly through myeloid lineage cells that migrate throughout the body and provide the first line of defense for a variety of pathogens. The mononuclear phagocytic system (MPS) is comprised of a diverse set of cell types expressing different cell surface markers, levels of phagocytic and antigen-presenting activity, and gene expression profiles and includes monocytes, macrophages, and DCs (see Fig. 1.4 for the hierarchy of the MPS) (431). The MPS is essential for mediating the inflammatory response and interacting with the adaptive immune system.

Monocytes arise from GMPs and progress through several morphologically defined differentiation stages downstream of the GMP including the monoblast, promonocyte, and mature monocyte (432). Increased nuclear folding, the appearance of cytoplasmic granules, and an increasing cytoplasm to nuclear ratio characterize the basic developmental stages of monocytes. However, the appearance of mature monocytes can be quite heterogeneous based on their activation state. After developing in the bone marrow, monocytes migrate into the peripheral blood, where they represent about 10% of the total white blood cells in humans and circulate for as short as 3 hours to 3 days (433). Monocyte development depends on M-CSF, as mice lacking *Csf-1R* (M-CSFR) exhibit dramatically reduced monocytes (434–436). Although mature monocytes enter the periphery and have limited proliferation potential, they can undergo further differentiation into tissue-resident macrophages or inflammatory DCs, and thus monocytes can restore the pool of macrophages in specific tissues. While monocytes and macrophages are both capable of phagocytosing pathogens, producing cytokines to interact with the adaptive immune system, and presenting antigen via class II MHC to clear infections (432), they are less effective in doing so compared to macrophages or DCs.

While monocytes and their terminally differentiated counterparts share these activities, it is not clear that all monocytes serve similar physiologic functions. “Classical monocytes” that are involved in an infection are characterized by a Gr1/Ly6C^{high} cell surface phenotype and differentiate into DCs that secrete TNFα, ROS, and NOS (437). Some of these cells can also be described as myeloid suppressor cells that have the ability to inhibit immune cells recruited to clear tumor cells or respond to chronic infections (438). Nonclassical monocytes include Gr1/Ly6C^{low} cells, which are smaller, and express CX3CR1 and LFA-1, but lack CCR2 and L-selection expression and differentiate into the M2 subtype of macrophages (439). GM-CSF and IL-4 can drive differentiation toward the DC lineage, and M-CSF commits monocytes toward the macrophage lineage (440). As in mice, humans have a larger subset of monocytes with increased phagocytic activity and a smaller subset with the ability to expand rapidly. The larger subset is characterized by a CD14$^+$CD16$^-$ immunophenotype and expresses similar chemokines to the mouse equivalents, consistent with the cells defined as “classical monocytes” (437).

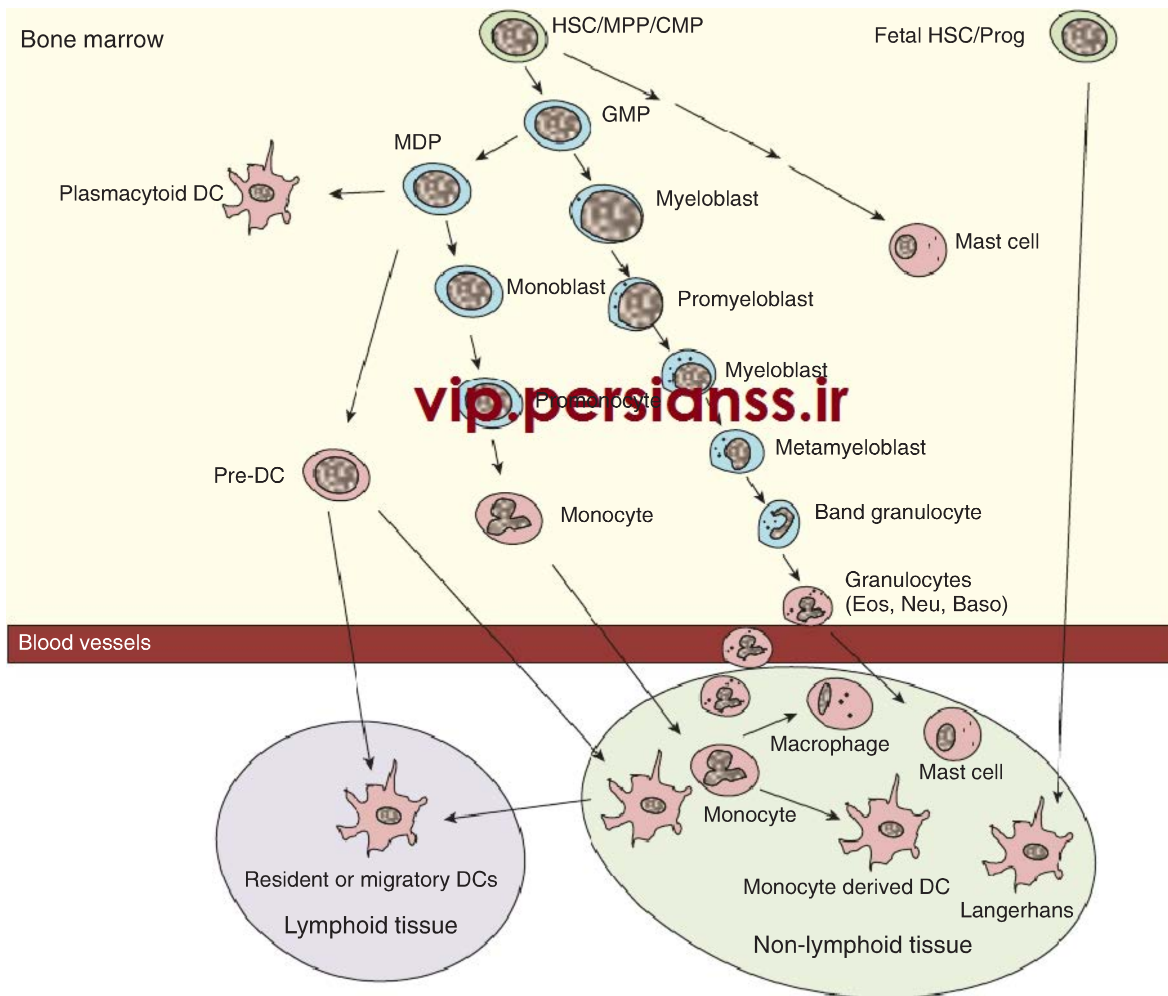

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FIGURE 1.4. Hierarchy of the myeloid developmental program. Myeloid development can also be described in terms of cell surface marker antigens as well as the cytologic features of differentiating precursors. Once monocytes leave the bone marrow and enter peripheral sites, they differentiate into macrophages. Some myeloid lineages such as DCs may arise from more than one developmental pathway while others such as Langerhans cells can differentiate rapidly from immature hematopoietic cells without generating large numbers of identifiable intermediates. HSC, hematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; Prog, progenitor; GMP, granulocyte monocyte progenitor; MDP, monocyte plasmacytoid progenitor cell; DC, dendritic cell; Pre-DC, pre-dendritic cell; Eos, eosinophils; Neu, neutrophils; Baso, basophils. *Arrows* indicate order of differentiation and lineage relationship. Granules (*small black circles*) and changes in nuclear morphology are represented during maturation stages.

Once monocytes enter peripheral tissue, their designation depends on their tissue of residence. For example, microglia are macrophages of the nervous system, and Kupffer cells are macrophages found lining the sinusoids in the liver. Similar to monocytes, it is now recognized that macrophages can be divided into classes based on their function. These classes include the M1, M2, and tumor-associated macrophages (441–443). These macrophages are polarized based on the cytokines they secrete as well as their ability to induce specific T-helper responses (444,445). M1 macrophages show increased antigen-presenting ability and are activated by LPS or IFNγ, whereas M2 macrophages are closely associated with responses to parasites, wounds, tissue repair, and other anti-inflammatory responses (446,447). The M2 macrophages can be activated by IL-4 or IL-13. It should be emphasized that these different macrophage effector functions are not necessarily fixed and that the transition and conversion between these two types of macrophages may occur depending on the persistence of antigens or changes in the local cytokine environment. Additionally, tumor-associated macrophages, although phenotypically similar to M2 type macrophages, are functionally distinct. These macrophages suppress the immune response and can promote tumorigenesis (297,312).

Dendritic Cells

Historically, DCs were originally recognized as a unique cell type based on their "veiled" appearance characterized by their irregular shape and pseudopods. It is now clear that these cells can arise from diverse developmental pathways and serve critical roles in linking the innate and adaptive immune responses. Immature DCs are generally less spindle-shaped, while mature DCs contain increased lamellipodia (432,443,448). DCs are considered part of the MPS system and can be classified into four major groups based on their cellular derivation and function including the conventional or classical DCs (cDCs), plasmacytoid DCs, Langerhans DCs, and monocyte-derived DCs (mDCs) (449). A particular challenge in the DC field has been characterizing these different subsets of DC since markers expressed on the different DC populations rapidly change after activation. Moreover, DCs are rare and studies using expansion approaches to study their biology alter their fate and/or marker profile, making it especially challenging definitive conclusions about specific subtypes.

Pre-DC progenitors can develop into macrophages and cDCs, but these progenitors are not considered monocytes (450,451). These DCs can be either migratory or resident in lymph nodes or tissues (448,452,453). The migratory DCs can be further

subdivided into interstitial DCs (CD11b$^+$) or the CD11b$^-$ CD103$^+$ integrinαEβ7 DCs (454–456). Initially, migratory cDCs reside within the tissue where they exhibit a high phagocytic capacity. However, upon activation by antigenic challenge, they increase MHCII expression and migrate to lymph nodes where they present antigens to T cells. Unlike the migratory DCs, the lymphoid tissue–resident DCs are found within all secondary lymphoid organs and can be additionally subclassified based on CD4 and CD8 expression (457,458). Lymph node–resident DCs are presumed to be necessary for the surveillance of antigens circulating in the blood and entering the lymphatic system.

mDCs, described earlier in this section, are mainly resident in nonlymphoid organs (421,459,460). The mDCs are similar to cDCs in their ability to act as potent antigen-presenting cells. Also, the mDCs express markers CD11c, MHCII, CD209a, MAC3 and are negative for LyGC.

The search to identify the source of cells that produce high-level type 1 IFNs eventually led to the discovery of plasmacytoid pDCs in 1999 (461,462). This subset of DCs originates in the bone marrow and arises from a similar pre-DC progenitor that also produces cDCs (463,464). pDCs are generally long-lived and are usually found in the lymphatic system, where they can directly recognize viruses using toll-like receptors 7 and 9 (TLR7/9) and express CD45RA with low-level expression of CD11b/c or MHCII (465). Several markers can be used to identify pDCs including IL-T7 and BDCA-2 in humans and Siglec-H Bst2 in mice. Since pDCs can produce high concentrations of type 1 IFNs after viral infections, they are thought to be critical for triggering the adaptive immune response in response to infection.

Langerhans DCs are found mainly in the skin but can also migrate to regional lymph nodes to provide T-cell help and activate the adaptive immune system in the presence of a pathogen (466,467). These cells are derived from mononuclear cells during development, mainly from the fetal liver, and a minority from the yolk sac (468,469). Langerhans DCs are characterized by high Fcγ expression. Deletion of *M-Csfr* in mice leads to a loss of Langerhans cells (468). Remarkably, and unlike many hematopoietic cells, Langerhans cells have the ability to renew and can survive for long periods in the dermis. They are also known to express c-type lectins and are characterized by Birbeck granules (rod-shaped organelles), which can be identified by electron microscopy (470). Because these cells are rare, they have been difficult to isolate and study. However, novel mouse models that utilize a Langerin promoter specifically mark Langerhans cells *in vivo* and will provide key tools to further our understanding of this interesting DC population (471,472).

Granulocytes

Neutrophils, basophils, mast cells, and eosinophils are myeloid lineage cells that are collectively known as granulocytes based on the presence of cytoplasmic granules following staining by special cytologic and histologic stains. There are five morphologically defined stages of development that occur stepwise in the bone marrow: myeloblast, promyelocyte, myelocyte, metamyelocyte, and band form. Large myeloblasts containing nuclei with open chromatin, high nuclear to cytoplasmic ratios, and few cytoplasmic granules, give rise to promyelocytes characterized by a slightly decreased nuclear to cytoplasmic ratio and primary granules. As development continues, the cell becomes smaller, exhibits increased granulation, and undergoes increased packing and folding of the nucleus until it becomes the mature granulocyte (Fig. 1.4).

Neutrophils

Neutrophils, also known as polymorphonuclear leukocytes, develop in the bone marrow and are the most common cell in the peripheral blood (473). These cells comprise 70% of the blood in humans, but only approximately 20% in mice, and are characterized by a multilobed nucleus with neutral pink cytoplasmic staining. Neutrophils are potent first responders and effectors of the innate immune system that are produced in the bone marrow at a constant daily rate of 10^{11} cells with a life span that varies from as short as 6 to 8 hours to 5 days (474–476). The morphologic development of neutrophils has been well-characterized downstream of the GMP. The primary azurophilic or myeloperoxidase-positive granules are the largest in the neutrophils. Secondary granules contain lactoferrin, and the third class of granules containing gelatinases and metalloproteinases arises during the band neutrophil stage (477).

Mature neutrophils can become captured through selectins expressed on endothelial cells lining the blood vessels and then adhere to them via integrin interactions (478–480). Migration across the endothelial cells and into the tissue is mediated by signals from bacterially derived factors and chemokines such as IL-8 that are released by other immune cells. Higher levels of IL-8 expression can lead to degranulation, while G-CSF can rapidly expand the population. Neutrophils can be attracted and activated through the interactions of other cells in the innate immune system including macrophages and DCs. The ability of neutrophils to phagocytize pathogens is further enhanced by their ability to use neutrophil extracellular traps, including fibers with proteases and chromatin that can bind bacteria (305,481,482). Neutrophils rely on NADPH oxidases to create enough ROS to destroy phagocytized bacteria. Neutrophils can also be engulfed by macrophages, which can serve as a mechanism to remove toxins or pathogens from the local environment.

Mast Cells and Basophils

Mast cells play important roles in both the innate immune and allergic responses. Although the developmental pathway for mast cells has not been settled, earlier studies suggesting that mast cells are derived from GMPs have been challenged by more recent work suggesting that the CMP or MPP has the potential to differentiate into a common, bipotential precursor that can give rise to basophils and mast cells (429,483). This is perhaps not a surprising finding since mast cells and basophils have similar appearances and have some overlapping functions. Mast cells are mainly found within tissues where they express the IgE receptor (FcεRI). Binding of this receptor initiates a rapid strong inflammatory response characterized by a release of inflammatory mediators including proteases, histamines, and lipids.

Mast cells remain immature in the periphery and terminally differentiate once they migrate to and reside in tissues. Critical growth and differentiation factors for mast cells include SCF in humans and IL-3 and SCF in mice (484). Mice lacking the SCF receptor c-Kit/CD117 are almost devoid of mast cells (485). β7 integrin expressed on mast cells was required for proper homing to the small intestine, but not required for any other tissue (486). β7 expression can distinguish the fate of the bipotent mast cell/basophil precursor with high expression associated with cells differentiating toward the mast cell lineage (487).

Like mast cells, basophils are involved in allergic responses and express the FcεRI receptor (488). Although fewer in number than mast cells and comprising only 0.5% of all immune cells, basophils account for the majority of IL-4 that is produced *in vivo* (489). Basophil granules appear bluish-black after staining with basophilic aniline dyes. Basophils are characterized by the surface marker phenotype c-Kit$^-$, FcεRI$^+$, CD11b$^+$, IL-3R^{hi} (490). Basophils play a role in the defense against certain parasites, ticks, and in allergy models involving the skin (488). Most importantly, they can control the initiation of the Th2-dependent response through the secretion of IL-4 and other factors (491).

Eosinophils

The eosinophil is a key cellular component of the innate immune response to parasites and microbial infections and also actively coordinates the adaptive immune system (217). Eosinophils differentiate from the GMP, and like mast cells and other hematopoietic cells, they develop in the bone marrow and migrate to the periphery to become tissue-resident. These cells, named for their characteristic strong staining of their secondary granules by the acidic dye eosin, can release proteins such as major basic protein, toxins, and peroxidases after activation to stimulate other immune cells or to destroy parasites. Eosinophils can also contribute to T-cell-mediated responses through their expression of costimulatory molecules, cytokines, and presenting antigen via the MHCII. Cytokines including IL-3 and GM-CSF are able to stimulate eosinophils, but only IL-5 remains a specific regulator of activation (492). The critical role of IL-5 in eosinophil development was demonstrated in mice deficient in IL-5/eotaxin, which exhibited a near absence of eosinophils. These mice also exhibited reduced IL-13 production and defective TH2 responses (493).

Erythroid and Megakaryocyte Development

Erythropoiesis

The human bone marrow has the remarkable capacity to produce approximately 2 million RBCs per second. RBCs, only 6 to 8 μm in size, are characterized by a distinctive concave shape and circulate in the peripheral blood with a half-life of about 120 days while carrying oxygen bound to hemoglobin to tissues throughout the body. As described previously, human RBCs first arise in the yolk sac from hematopoietic progenitors as early as gestational day 19 and continue as early erythrocytes for as long as 9 weeks (103,494). Definitive erythropoiesis originates from the AGM then switches to the fetal liver followed by the bone marrow beginning between the fifth and sixth weeks of gestation. In addition to being produced in different anatomic sites during development, erythroid cells exhibit a number of other important differences compared with other hematopoietic cells, perhaps best exemplified by alterations in the types of hemoglobins produced due to the action of a set of transcription factors (495). Primitive erythroblasts are characterized by predominant expression of ($\zeta_2\varepsilon_2$) and ($\alpha_2\varepsilon_2$) hemoglobins and begin to express the ζ, ε, α, γ globin and minimal amounts of the β globin during the transition to definitive erythropoiesis. As fetal and adult erythropoiesis replaces embryonic erythropoiesis, ε hemoglobin is silenced and α, γ hemoglobins are highly expressed, eventually being replaced at birth by β globin (496). Adult erythrocytes are also generally smaller than fetal erythrocytes.

Erythroid development has been defined in a number of ways, including morphologic, functional, and now immunophenotypic. It has been appreciated for some time that erythroid development is characterized by stereotypic morphologic changes. After erythroid progenitors commit to the lineage, maturation is accompanied by condensation of the nucleus, decreasing in overall size and followed by the process of enucleation, which can take about 2 weeks to complete *in vitro*. Several rounds of division, increased ribosomal assembly, and synthesis of hemoglobin characterize the first cells during erythroid development named proerythroblasts. The basophilic erythroblast, polychromatic erythroblast, and orthochromatic erythroblast are sequential stages named according to their Wright-Giemsa–stained appearance that are associated with increasingly condensed nuclear chromatin and decreased size. The latter stages are defined by a more acidic cytoplasm, condensed chromatin, and the extrusion of the pyknotic nuclei. The nucleus becomes quickly phagocytized by macrophages that are usually closely associated with erythroid progenitors (497,498). This immature RBC or reticulocyte is retained in the bone marrow for several days before it migrates into the periphery and fully matures into a RBC.

Erythroid intermediates have also been classified based on their activity in colony assays. BFU-E colonies arise from early, proliferative erythroid progenitors that rise to grapelike clusters of erythroid cells. These cells are developmentally upstream of progenitors that give rise to CFU-E, which are smaller colonies composed of more mature RBCs. While these progenitor types allow one to functionally identify erythroid progenitors with specific activity, unfortunately they do not allow their prospective separation.

Most recently, flow cytometric and FACS technology have allowed the identification of prospectively isolatable populations including the MEP, as previously discussed. Downstream of the MEP, flow cytometric markers can be used to capture a continuum of specific developmental changes characterized by increasing glycophorin-A expression and expression of specific blood antigens (A, B, O, and Rh). Transferrin receptor (CD71) expression is higher in earlier stages and then becomes reduced in the later stages of erythroid development. These trends can also be observed in mouse erythropoiesis, and combined with an additional mouse erythroid lineage marker Ter119, immature erythroid progenitors can be distinguished from proerythroblasts.

Like other hematopoietic lineages, cytokines play a major role in regulating the proliferation and survival of progenitors representing various stages of erythroid development. The most important cytokines that stimulate erythropoiesis are erythropoietin (EPO) and GM-CSF, whereas SCF, FLT3, and IL-3 can provide additional proliferative signals. EPO, predominantly synthesized by the renal peritubular cells in the kidneys, provides homeostatic and systemic control of red cell output in the bone marrow, as hypoxia sensed in the kidney via HIF signaling stimulates the secretion of EPO that stimulates blood production (499). EPO/EPOR stimulation of early erythroid progenitors leads to activation of JAK/STAT signaling, and in combination with the other cytokines activates PI3K signaling to allow expansion of erythroid progenitors (500).

The commitment and development of the erythroid lineage requires evolutionarily conserved transcription factors including GATA1/2, FOG-1, erythroid Krüppel-like factor (EKLF) (501,502). For example, deletion of *Eklf* blocks globin expression and loss of *Gata1* blocks differentiation at the proerythroblast stage (503,504). The importance of GATA1 in erythroid differentiation is exemplified by patients that harbor familial mutations in GATA1, who exhibit severe anemia and thrombocytopenia (505). Due to the wide variety of diseases characterized by defects in erythroid development, understanding the relationship among the erythroid transcription factors, globin genes, and epigenetic regulators is an important and exciting area of investigation and may provide exciting opportunities for novel therapies incorporating cellular engineering technologies.

Megakaryopoiesis

Platelets were first described as megakaryocyte derivatives during the early 20th century (506). Because megakaryocyte generate platelets, they are critical for maintaining hemostasis and thrombosis (507,508). Although megakaryocytes make up only a small fraction of all bone marrow cells, in humans they possess the capacity to produce platelets at a rate of approximately 1 million per second with one megakaryocyte being able to produce 1 to 3 thousand platelets; this results in approximately 1 trillion platelets in circulation at any given time (509,510). Besides their primary function as cellular clotting factors in response to tissue injury, platelets serve additional roles in both the innate and adaptive immune system. For example, platelets express P-selectin and costimulatory

receptors such as CD40L and TLRs, which can participate in recruiting leukocytes to sites of injury and promoting the inflammatory response (511).

During development, megakaryocytes are first detected in the embryonic yolk sac, similar to erythroid cells. In the adult bone marrow, megakaryocytes are thought to arise from more than one developmental pathway, differentiating directly from HSCs/MPPs and bypassing the CMP (82), or from classically defined bipotent MEPs (424,508). TPO promotes the differentiation and expansion of megakaryocytes and leads to an increase in platelets.

After commitment to the megakaryocytic lineage, megakaryocytic progenitors undergo a series of rounds of DNA replication without cell division (endomitosis) in order to generate the unique morphology characteristic of mature megakaryocytes (512). This differentiation process can take approximately 6 to 10 days (512). In the early stages of this process, megakaryoblasts undergo rounds of proliferation, are 15 μm in size and 4N in DNA content, and exhibit basophilic staining. The promegakaryocyte stage is easily identified by increased numbers of granules that represent dense proteins in organelles specific for platelet production and contain peroxisomes (α, β, and γ) (513). In the final stage, megakaryocytes begin to produce platelets, are as large as 150 μM in size, and can be associated with as much as 64N ploidy. Interestingly, ploidy does not always correlate with the maturation and development of megakaryocytes (514). Terminally differentiated megakaryocytes exhibit increased nuclear folding and significantly increased size and generate lobulated projections. Such mature megakaryocytes, located in close proximity to marrow sinusoids and bone, rapidly shed platelets directly into the circulation via their projections that can extend through the sinusoidal endothelium, and the platelets can last up to 9 days in the circulation (515). Once the megakaryocytes stop producing platelets, they are denuded, become senescent, and undergo apoptosis and subsequent clearance by macrophage engulfment (516). Besides their distinct morphology, megakaryocytes express a variety of markers including CD31, CD41, CD42b and CD61, Gly Ib/IIB, and factors V and VII, with later stages expressing the von Willebrand factor (513,517). Platelets largely express the same markers present on mature megakaryocytes (517).

As discussed previously, the major cytokine that regulates megakaryopoiesis is TPO through interactions with its cognate receptor MPL. Deletion of this receptor in mice reduces platelet production dramatically, although not completely, suggesting there is a minor non-TPO-dependent pathway for platelet production present (350,455,518). TPO is mainly produced in the liver and bone marrow stromal cells. Platelets have the ability to bind soluble TPO in the periphery, effectively regulating overall levels in the blood and providing a negative feedback loop for TPO production (156,519–521). Other cytokines, while not necessary for platelet production, can stimulate megakaryocyte development including SCF, IL-3, IL-11, and GM-CSF (522). In addition, like other cell types, SDF1 (CXCL12) can cause increased migration and force megakaryocytes out of their niche in the bone marrow to provide local sources of platelets in other tissues such as the spleen (100,523).

Similar to erythropoiesis, megakaryocyte development relies on a set of key transcription factors. Deletion of GATA1 and FOG in megakaryocytes resulted in reduced megakaryocytes and platelet production (524,525). Mice deficient in NF-E2 exhibit a dramatic developmental block near the final stage of megakaryopoiesis before platelets are produced and result in the development of dysplastic megakaryocytes and thrombocytopenia (526).

Lymphopoiesis

While the exact ontogeny and marker expression of the lymphoid progenitor is somewhat controversial (see Hematopoietic Progenitor section), subsequent steps of development are more clearly defined. Both mature B cells and T cells possess the potential to be long-lived and maintained in a quiescent state like long-term HSCs. These seemingly dormant cells can rapidly proliferate following stimulation through their immune regulatory receptors.

B-Cell Development

B cells are central mediators of the adaptive immune system through their roles as immunoglobulin producers. They were originally named based on their discovery in the bursa of fabricius, a specialized organ found only in birds. In humans, B-cell development initially occurs in the fetal liver at 5 to 7 weeks gestation but eventually moves to the bone marrow when HSCs migrate there (527–529). B-cell development has been rigorously studied in an attempt to understand how they produce the impressive antibody diversity that is essential for effective adaptive immunity. The first committed B-cell progenitor is the pro-B cell, which is characterized by heavy chain rearrangement. This developmental stage is followed by the pre-B cell in which light chain rearrangements occur, and this stage is critical since improperly rearranged B-cell receptors cause the developing B cells to quickly undergo apoptosis. Productive B-cell antibody rearrangement results in $\mathrm{IgM}^{\mathrm{high}}$ and $\mathrm{IgD}^{\mathrm{low}}$ expressing immature B cells that then exit the bone marrow to enter the periphery and secondary lymphoid organs. The maturation of the B cells then continues after stimulation within these lymphoid organs. The early committed B-cell progenitors express CD10, then gains in CD19 expression following expression of V-PreB. Once the B cell matures, it also expresses CD10, CD19/CD22, CD24, and CD40. Overall, this complex developmental process is generally characterized by proliferative bursts followed by resting stages, marker alterations, and sensitivity to stromal interactions.

Specification of B cells requires a defined set of transcription factors including paired box protein 5 (Pax5), early B-cell factor 1, surrogate light chain (VpreB), Rag1 and Rag2, terminal deoxytidyl transferase (TdT), and others. Besides the role for these transcription factors, cytokines can also sustain B-cell development. Stromal cells provide important signals including TGFβ, CXCL12, FLT3L, and IL-7. Most importantly, IL-7 in mice sustains B-cell development *in vitro*, but only partially stimulates human B-cell precursors. Deletion of the common γ-chain of the IL-7 receptor results in the depletion of B cells, T cells, and NK cells in mice, but SCID patients with disruption of IL-7 are only deficient in T cells. Consequently, this reduced reliance on IL-7 has made it more challenging to study human B-cell development.

A more comprehensive description of B-cell development and function will be presented in Chapter 2.

T-Cell Development

T cells make up another major component of the adaptive immune system. These cells are named based on their origin and development in the thymus. T cells interact with components of both the adaptive and innate immune system and confer the ability to rapidly stimulate the cellular immune response against specific pathogens. While cytotoxic T-cell populations directly clear infections, regulatory T cells act as suppressors of the immune system, providing a way to control aberrant activation of the immune response and peripheral tolerance. Early T-cell lineage progenitor (ETPs) are initially established in the bone marrow from lymphoid progenitors. In mice, these T-cell precursors are classically defined by their surface immunophenotype, $\mathrm{Lin}^{\mathrm{low}}$, CD44, $\mathrm{CD117}^{+}$, and $\mathrm{CD35}^{-}$. In humans, ETPs express $\mathrm{CD34}^{\mathrm{high}}$, $\mathrm{CD33}^{\mathrm{low}}$, $\mathrm{CD38}^{\mathrm{low}}$, CD44, and IL-7Ra. These cells, destined to become T cells, then leave the bone marrow to enter the thymus. In humans, pre-T cells