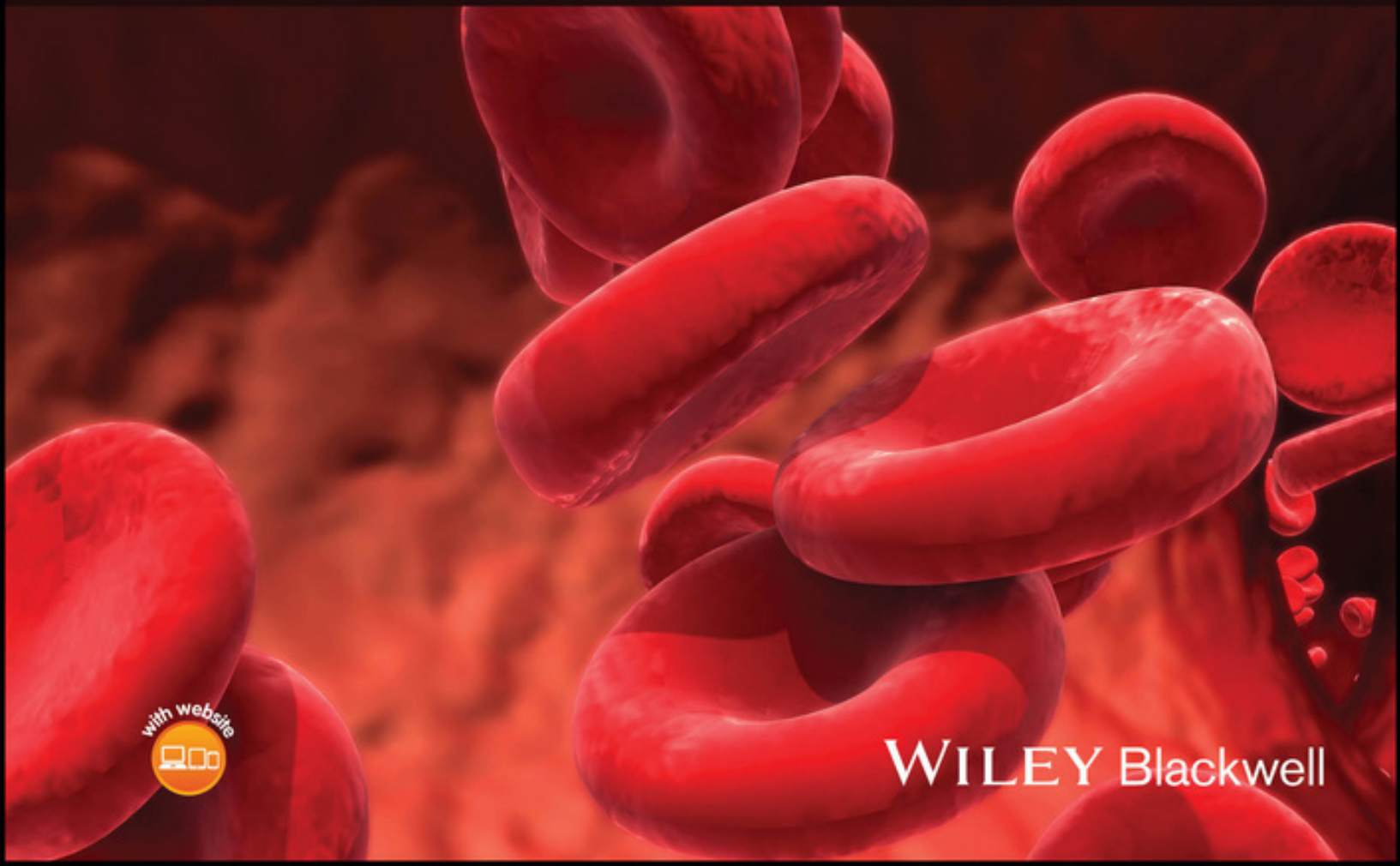
Eighth Edition

Edited by Adam J **Mead** • Michael A **Laffan**
Graham P **Collins** • Deborah **Hay**
Consulting Editor A Victor **Hoffbrand**

Hoffbrand's Postgraduate Haematology



WILEY Blackwell

Hoffbrand's Postgraduate Haematology

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Eighth Edition

WILEY Blackwell

This edition first published 2025

Edition History
William Heinemann Ltd (1e, 1972), Butterworth Ltd (2e, 1981; 3e, 1989), Butterworth-Heinmann Ltd (4e, 1999), John Wiley & Sons Ltd (5e, 2005; 6e, 2011; 7e, 2016)

Registered Offices
John Wiley & Sons, Inc., 111 River Street, Hoboken, NJ 07030, USA
John Wiley & Sons Ltd, New Era House, 8 Oldlands Way, Bognor Regis, West Sussex, PO22 9NQ, UK

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Wiley also publishes its books in a variety of electronic formats and by print-on-demand. Some content that appears in standard print versions of this book may not be available in other formats.

Library of Congress Cataloging-in-Publication Data
Names: Mead, Adam, editor. | Laffan, Michael A., editor. | Collins, Graham (Hematologist) editor. | Hay, Deborah, 1977- editor. | Hoffbrand, A. V., editor.
Title: Hoffbrand's postgraduate haematology / edited by Adam Mead, Michael Laffan, Graham P. Collins, Deborah Hay ; consulting editor, A. Victor Hoffbrand.
Other titles: Postgraduate haematology
Description: Eighth edition. | Hoboken, NJ : Wiley-Blackwell, 2025. | Preceded by Postgraduate haematology / edited by A. Victor Hoffbrand, Douglas R. Higgs, David Keeling, Atul B. Mehta. Seventh edition. 2016. | Includes bibliographical references and index.
Identifiers: LCCN 2024048152 (print) | LCCN 2024048153 (ebook) | ISBN 9781119706618 (cloth) | ISBN 9781119706663 (adobe pdf) | ISBN 9781119706670 (epub) | ISBN 9781119706687 (obook)
Subjects: MESH: Blood Physiological Phenomena | Hematologic Diseases | Leukemia–blood | Thrombosis–blood
Classification: LCC RC280.H47 (print) | LCC RC280.H47 (ebook) | NLM WH 100 | DDC 616.99/418–dc23/eng/20241206
LC record available at https://lccn.loc.gov/2024048152
LC ebook record available at https://lccn.loc.gov/2024048153

Cover design: Wiley
Cover image: © Design Cells/Adobe Stock Photos

Set in 9.5/12pt MinionPro by Straive, Pondicherry, India

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About the editors

A Victor Hoffbrand qualified in medicine in 1959 at the University of Oxford. After 10 years research at the Royal Postgraduate Medical School, he was appointed in 1974 as professor of Haematology at the Royal Free School of Medicine where he was to spend the rest of his career.

He has published over 700 peer-reviewed scientific papers and chapters in the fields of megaloblastic anaemia, iron chelation, the biochemical, immunological, and molecular biological aspects of leukaemia and lymphoma and stem cell transplantation. He has authored nine editions of *Hoffbrand's Essential Haematology*, five of *The Color Atlas of Clinical Hematology*, four of *Haematology at a Glance* and has edited seven editions of *Postgraduate Haematology*. He authored the haematology section of the *British National Formulary* (1971–1999) and was Chairman of the Editorial Board of the *British Journal of Haematology* from 1990 to 2000. With Robin Foa, he edited *Reviews in Clinical and Experimental Haematology* (2000–2005) and also co-edited with Malcolm Brenner seven editions of *Recent Advances of Haematology*. In 2023, he authored *The Folate Story: A Vitamin Under the Microscope*.

He is a past-president of the British Society for Haematology and was one of the two UK founding councillors of the European Haematology Association. He has lectured in over 40 countries and been visiting professor in Melbourne, Toronto, Rawalpindi, Chandigarh and South Africa. He has advised the Ministries of Health in Kuwait, Cyprus, Hong Kong and Lebanon on their haematology services.

He has been awarded Honorary DSc degrees from Queen Mary (2012) and University College (2019), London. In 2018, he received the Sultan Bin Khalifa International Thalassemia Award, the President's Medal of the Royal College of Physicians and the Wallace H. Coulter Award for Lifetime Achievement, the American Society of Hematology. In 2019, he was awarded the British Society for Haematology Lifetime Achievement Award. He was elected F. Med. Sci. in 2000 and from 2012 to 2013 he was a fellow commoner at The Queen's College, Oxford.

Adam J Mead is a Professor of Haematology in the University of Oxford. Professor Mead is the Clinical Lead for Myeloproliferative Neoplasms (MPN) at Oxford University Hospitals NHS Foundation Trust and has been the Principal or Chief Investigator of over 50 clinical trials in MPN and chronic myeloid leukaemia (CML). He chaired the National Cancer Research Institute MPN subgroup from 2016 to 2022. Professor Mead leads a research group at the MRC Weatherall Institute of Molecular Medicine. The overarching focus of his research group is to discover novel and rational therapeutic strategies to address the current unmet need in persons with MPN, fuelled by a better understanding of the biological principles underlying disease development and progression. He has published over 150 peer-reviewed scientific papers and was elected F. Med. Sci in 2024.

Professor Graham P Collins is an associate professor of Haematology at Oxford University and lead of the Haematology and Lymphoma service at Oxford University Hospitals NHS Foundation Trust. He is deputy chair of the National Cancer Research Institute (NCRI) lymphoma clinical study group, past chair of the Hodgkin and T-cell lymphoma study groups and clinical expert on the National CAR-T Clinical Panel for lymphoma. His academic interests are in Hodgkin and high-grade non-Hodgkin lymphoma, and he is chief investigator on a number of investigator-initiated and commercial clinical trials. He has worked with the National Institute for Health and Care Excellence on guidelines for non-Hodgkin lymphoma and has also co-authored British Society of Haematology guidelines on Hodgkin lymphoma and T-cell lymphoma. Professor Collins is founder and co-course director of the national Lymphoma Management Course and is a supervisor for a number of PhD and MD students. He speaks widely at national and international congresses on a variety of lymphoma-related topics.

Deborah Hay qualified in medicine from the University of Oxford and completed postgraduate training in London, Glasgow and Oxford. After a period of research training in red cell haematology at the Weatherall Institute of Molecular Medicine, she was appointed as the clinical tutor for Laboratory Medicine and Pathology at Oxford University Medical School, and subsequently as deputy director of the graduate-entry medical course. Clinically, her time is divided between consultant practice in diagnostic laboratory haematology and haematopathology in Oxford.

Professor Michael A Laffan is a visiting professor of Haemostasis and Thrombosis at Imperial College London. He was previously consultant haematologist at the Imperial College Healthcare NHS Trust and director of the Hammersmith Hospital Haemophilia Centre. He received his medical degree from the University of Oxford and later trained in Haematology at the Hammersmith and Royal Free Hospitals in London before joining the Royal Postgraduate Medical School, later Imperial College Medical School, as senior lecturer and honorary consultant in 1992.

Professor Laffan is the author of numerous guidelines and contributions to textbooks and is an editor of Practical Haematology. He chaired the British Society for Haematology Task Force on Haemostasis and Thrombosis for six years and the United Kingdom Haemophilia Centre Doctors' Organisation (UKHCDO) von Willebrand disease working party until 2020. He is currently chair of the BSH Guidelines Executive and has been a senior examiner for the RCPath, chair of the North London Training committee and training programme director.

His research interests have been on the structure–function relationship of von Willebrand factor, with a focus on the role of glycosylation, the role of von Willebrand factor in angiogenesis and the mechanisms and regulation of thrombin generation. He was a lead investigator on the BRIDGE study of genetics in bleeding disorders, precursor to the 100k genomes project and on gene therapy trials for haemophilia.

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Preface to the eighth edition by Adam J Mead

It is a huge honour to take on the role of Editor-in-Chief of *Postgraduate Haematology* for its eighth edition. The book has been an important text in haematology for over five decades, with the fourth edition providing essential reading during my postgraduate exams in haematology 20 years ago. It was a particular pleasure therefore to edit the book alongside Professor Victor Hoffbrand, who edited the first seven editions since the book's inception in 1972. For the eighth edition, we have renamed the book *Hoffbrand's Postgraduate Haematology* in honour of Victor's incredible contribution to the text over so many years. We have also established a new editorial team with a broad range of expertise across different areas in haematology. We have extensively updated the book for the new edition which now includes 57 chapters written by international experts in their respective fields, with chapters divided across 6 themes. Haematology remains at the forefront of the application of advances in molecular biology to the management of human disease, and reflecting this, we have introduced a number of entirely new chapters covering important advances in clinical applications of gene editing and therapy, application of next-generation sequencing in haematology and cancer immunotherapy. Additional new chapters are focussed on clonal haematopoesis, macrophages, dendritic cells and histiocytic disorders, obstetric haematology and consultative haemostasis and thrombosis.

In this digital age, I firmly believe that *Hoffbrand's Postgraduate Haematology* will remain a crucial text for those undergoing postgraduate training in haematology, as well as practising clinicians and scientists working in the field. Faced with the challenge of keeping up to date with an increasingly complex landscape across the wonderfully diverse areas encompassed by clinical and laboratory haematology, we hope that *Hoffbrand's Postgraduate Haematology* will provide a useful resource of authoritative information.

It is an enormous collaborative effort putting together a 57-chapter textbook, and I am immensely grateful to my co-editors, chapter authors and the team at Wiley for their help and patience along the way.

Preface to the eighth edition by A Victor Hoffbrand

Postgraduate Haematology began life 52 years ago as *Haematology: Tutorials in Postgraduate Medicine*. The authors were all members of the Department of Haematology at the Royal Postgraduate Medical School (RPMS), Hammersmith Hospital. John Dacie, Head of the Department, was first invited to edit the book but with other major book writing commitments, *Practical Haematology* and *The Haemolytic Anaemias*, he suggested Mitchell Lewis and me as editors.

The chapters of this first edition were all written by distinguished colleagues in Dacie's Haematology Department including Ted Gordon-Smith (haemolytic and aplastic anaemias), David Galton (leukaemias, myeloma and lymphomas), John Goldman (leucocytes), Leon Szur (myeloproliferative diseases), Bob Pitney (coagulation), and Sheila Worlledge (blood transfusion). They were based on the lectures we gave in the annual Diploma in Clinical Pathology Course at the RPMS.

The book was not intended as a reference to me but as an easily read text for learning about the blood diseases – their pathogenesis, clinical and laboratory features, relevant diagnostic tests, treatment and prognosis. A suggested reading list was given at the end of each chapter but references in the text were avoided. The title *Postgraduate Haematology* was adopted for the second and subsequent editions published by William Heinemann, Butterworth Heinemann, Arnold, Oxford University Press, Blackwell and now Wiley-Blackwell.

With the vast increase in knowledge of blood and its diseases, the book has inevitably increased in size but all editions have followed the same formula. *Postgraduate Haematology* is intended to provide a comprehensive, well-illustrated text, suitable for higher examinations and to the level of consultant haematologist.

Inevitably, a wider range of editors and authors have been needed since the first edition. Edward Tuddenham joined the original two editors to cover the coagulation section for editions four to six; both Daniel Catovsky (editions five and six) and Anthony Green (edition six) were editors for the neoplastic diseases. The editors of the seventh edition included Douglas Higgs, David Keeling and Atul Mehta. The authors, experts in their respective fields, are from around the world, especially from the UK, Europe and North America.

It has been my privilege to edit the first seven editions of *Postgraduate Haematology*. I am delighted to have been replaced as 'editor-in-chief' for the eighth edition by a younger colleague, the talented Oxford haematologist Adam Mead and to welcome his distinguished team of co-editors. They can be just proud of this superb new edition. Finally, I thank the publisher Wiley-Blackwell for adding my name to the book's title.

Victor Hoffbrand, London, 2024

Preface to the first edition

In this book the authors combine an account of the physiological and biochemical basis of haematological processes with descriptions of the clinical and laboratory features and management of blood disorders. Within this framework, each author has dealt with the individual subjects as they thought appropriate. Because this book is intended to provide a foundation for the study of haematology and is not intended to be a reference book, it reflects, to some extent, the views of the individual authors rather than providing comprehensive detail and a full bibliography. For these the reader is referred to the selected reading given at the end of each chapter. It is hoped that the book will prove a particular value to students taking either the primary or the final part of the examination for membership of the Royal College of Pathologists and the Diplomas of Clinical Pathology. It should also prove useful to physicians wishing to gain special knowledge of haematology and to technicians taking the Advanced Diploma in Haematology of the Institute of Medical Laboratory Technology, or the Higher National Certificate in Medical Laboratory subjects.

We acknowledge kind permission from the editors and publishers of the *British Journal of Haematology*, the *Journal of the Royal College of Physicians of London* and the *Quarterly Journal of Medicine* to reproduce Figures 4.1, 4.5, 4.10, 4.11, 4.12, 9.4 and 9.10, the publishers of *Progress in Haematology* for Figure 7.2 and many other publishers who, together with the authors, have been acknowledged in the text. We are particularly grateful to Professor J. V. Dacie for providing material which formed the basis of many of the original illustrations in Chapters 4–8. We are greatly indebted to Mrs T. Charalambos, Mrs J. Cope and Mrs D. Haysome for secretarial assistance and to Mrs P. Schilling and the Department of Medical Illustration for photomicrography, art work and general photography.

Finally, we are grateful for the invaluable help and for bearance we have received from Mr R. Emery and William Heinemann Medical Books.

London, 1972
A. Victor Hoffbrand
Michael Laffan

About the companion website

This book is accompanied by an instructor companion website:

www.wiley.com/go/mead/HPH8e

The website includes the following:

- Bonus chapter 58
- PDFs of the tables
- PowerPoints of the figures

PART I

General principles of haematology

CHAPTER 1

Human stem cells and haemopoiesis

1

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Introduction

The regenerative potential of tissues in the human body has fascinated researchers for many years, perhaps sparked by the ancient Greek myth describing Prometheus's fate, punished by Zeus to have a vulture feed off his liver during the day, only for the liver to be regenerated and the process repeated daily. Although the ancient Greek myth at that time was unlikely to be based on knowledge on the regenerative potential of the liver, we now know that most tissues in the human body contain regenerative potential to replace cells lost due to their normal limited lifespan, stress, disease or injuries, and that this process depends on tissue-specific stem cells. Of these, the stem cells responsible for the production and maintenance of mature blood cells, the haemopoietic stem cell, remains the most well characterised stem cell system to date. The routine clinical use of bone marrow transplantation to rescue diseased haemopoiesis highlights the impressive power of stem cell therapy, facilitated by the culmination of decades of biological and clinical research. Although representing the only curable treatment option for several disease conditions, it still is not possible to offer this treatment to all patients in need. As such, more research towards both the biology and clinical application of human haemopoietic stem cells are required to further improve our understanding and clinical application of haemopoietic stem cell therapies.

Human haemopoietic stem cells and the haemopoietic differentiation hierarchy

Blood is one of the most regenerative tissues in the body, where every minute millions of mature blood cells need to be replaced in a normal healthy individual due to their finite lifespan. In contrast to solid tissues, blood cells are dispersed throughout the body. Although the majority of mature blood cells are found in peripheral blood and haemopoietic organs such as the bone marrow, spleen, thymus and lymph nodes, mature blood cell infiltration in other organs and tissues are also important for prevention of infections and response to injury. With the exception of long-lived memory B and T cells, and some tissue-resident macrophages, mature blood cells have a short lifespan, ranging from just a few days for mature granulocytes, monocytes and platelets to 120 days for red blood cells (Figure 1.1). Ensuring the continuous replacement of lost cells is critical to maintain normal homeostasis, as insufficient blood cell production results in cytopenia and increased risk for infection, and if not corrected threatens the ability to sustain life. The process of blood cell production, haemopoiesis, results in the daily generation of 10^{11}–10^{12} mature blood cells in an adult healthy individual, numbers which can increase in response to acute stress such as large blood loss, infection or disease conditions.

Haemopoiesis is a tightly regulated cellular process that depend on the lifelong pool of haemopoietic stem cells (HSCs) defined by their ability to self-renew and potential to generate

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).

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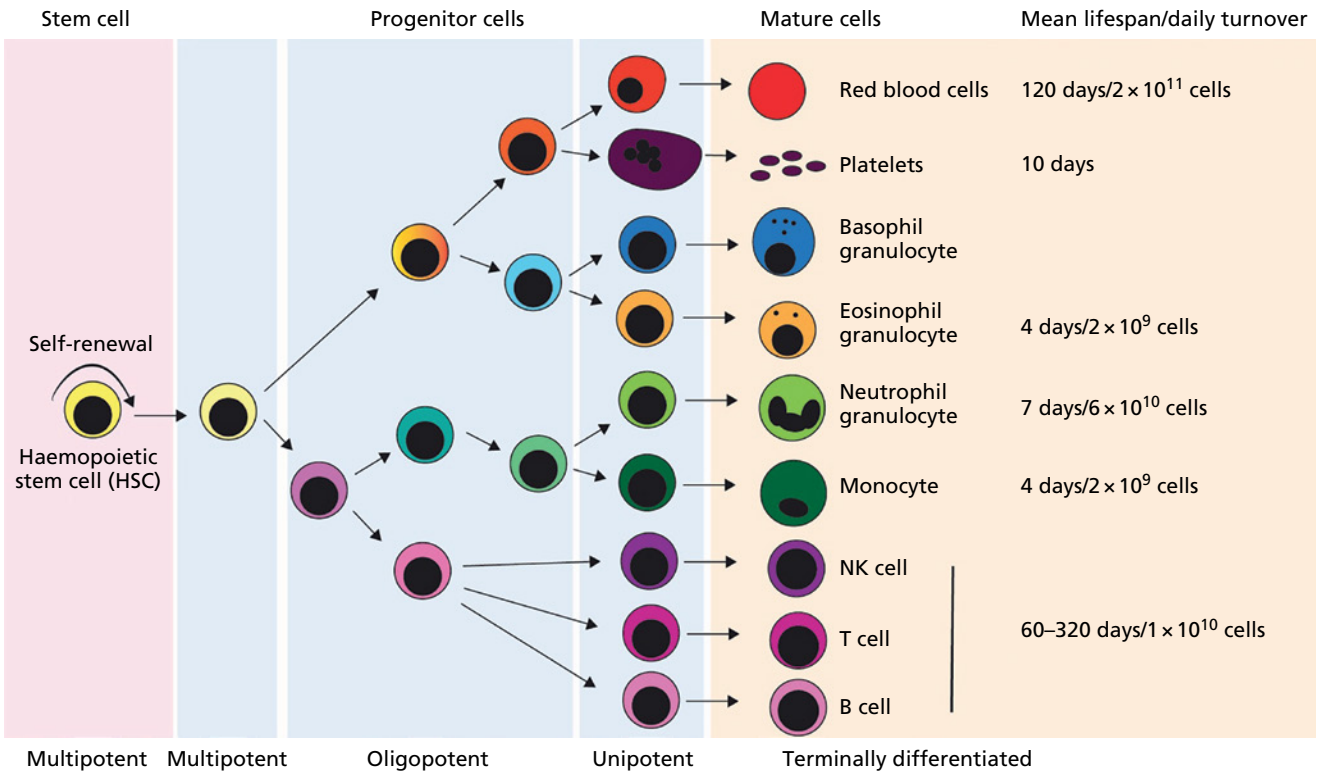


Figure 1.1 The human haemopoietic hierarchy. The human haemopoietic hierarchy has a unidirectional differentiation path (arrows going from left to right only) with the haemopoietic stem cell at the apex and the mature blood cells at the end. Due to the short lifespan and daily turnover of mature blood cells, continuous replenishment of lost cells is facilitated by haemopoietic stem cells through the generation of series of haemopoietic progenitor cells which gradually lose lineage potential. This process of active haemopoiesis is dependent on a limited pool of quiescent and long-lived haemopoietic stem cells in the bone marrow with life-long self-renewal potential and potential to generate all mature blood cells.

all blood cell lineages (multipotent). In adults, this process takes place in the bone marrow, the major site of residency for HSCs after birth. HSCs represent an infrequent population of cells in the bone marrow, and studies to understand how such a rare population of cells can generate trillions of cells on a daily basis has been at the centre of research since their existence was proposed. Studies in model organisms and on material from human subjects have demonstrated that the impressive production of blood cells is achieved through a unidirectional cellular hierarchy where HSCs are located at the apex and give rise to the mature blood cells through the generation of series of intermediate progenitor cell stages. In contrast to HSCs, the majority of these intermediate progenitor cells are short-lived as they lack self-renewal potential and therefore depend on continuous replenishment from the HSCs. The generated progenitor cells are highly proliferative and as they differentiate through the haemopoietic hierarchy, they gradually lose potential to generate one or more lineages. Although the cellular differentiation pathway from human HSCs is still under investigation, studies have suggested that HSCs first give rise to progenitor cells that carry the same potential as HSCs (multipotent) but have lost or reduced ability for self-renewal and therefore will not be sustained for an extended period of time. The multipotent progenitor then gives rise to oligo-potent progenitors that has lost potential for one or more blood cell lineages, which ultimately gives rise to unipotent progenitors restricted towards one blood cell lineage. In both mouse and human bone marrow, many of these intermediate progenitor cell stages have been identified suggesting that the first lineage-restriction that occurs after a multipotent progenitor cell results in division between progenitors with potential to generate lymphoid cells (B, T and NK cells), neutrophils and monocytes/macrophages and progenitors with potential to generate erythroid, megakaryocyte/platelets, eosinophils and basophils. Identification and characterisation of these stages is important not just for understanding the underlying cell biology, but also since these intermediate stages often are perturbed in diseases and haematologic malignancies, they remain relevant for understanding how haemopoietic dysregulation leads to clinical

disease. Although the haemopoietic hierarchy often is displayed as discrete stages of cellular intermediates (Figure 1.1), it is probable that a more gradual continuum of lineage restriction exists, rather than the binary distinct stages typically displayed in figures in the literature.

Human haemopoietic stem cell characteristics

HSCs, similar to stem cells responsible for the regeneration of other tissues, are defined functionally by their ability to self-renew, meaning that they can persist for the duration of life through generation of daughter HSCs, and their capacity for multipotent differentiation, meaning they have the potential to generate all mature haemopoietic cell types. In light of the dependence of blood production on HSCs it is critical that the number of HSCs and their activity is tightly controlled, both under steady-state conditions and under stress conditions. In response to signals from HSCs themselves (intrinsic) and the surrounding environment (extrinsic), HSCs can undergo different fates that regulate the number of HSCs in the body (Figure 1.2). In response to proliferative signals HSCs undergo cell division, where an asymmetric division generates two daughter cells, one which is an exact HSC copy of the parental HSC, endowed with both self-renewal and multipotency, and a differentiated progeny which has lost self-renewal. Such a cell division would result in neither gain or loss of HSCs and therefore preserve the number of HSCs in the bone marrow. Alternatively, HSCs can undergo two different types of symmetric cell divisions. If a HSC undergo a symmetrical self-renewal division, two new daughter HSCs will be generated from the parental HSC, both with self-renewal and multi-potency, resulting in an expansion of HSC numbers. However, if a parental HSC undergo a symmetric differentiation division, two differentiated daughter cells without self-renewal will be generated, resulting in HSC loss. In addition to cell division, HSCs can also undergo apoptosis which similar to symmetric differentiation division results in HSC loss.

Although cell division is required for the progeny generation, cell divisions are infrequently performed by HSCs during steady-state conditions. Instead, most HSCs remain dormant in a quiescent state, referred to as G_0 in the cell cycle. Quiescence is a hallmark feature of HSCs in steady-state conditions, defined

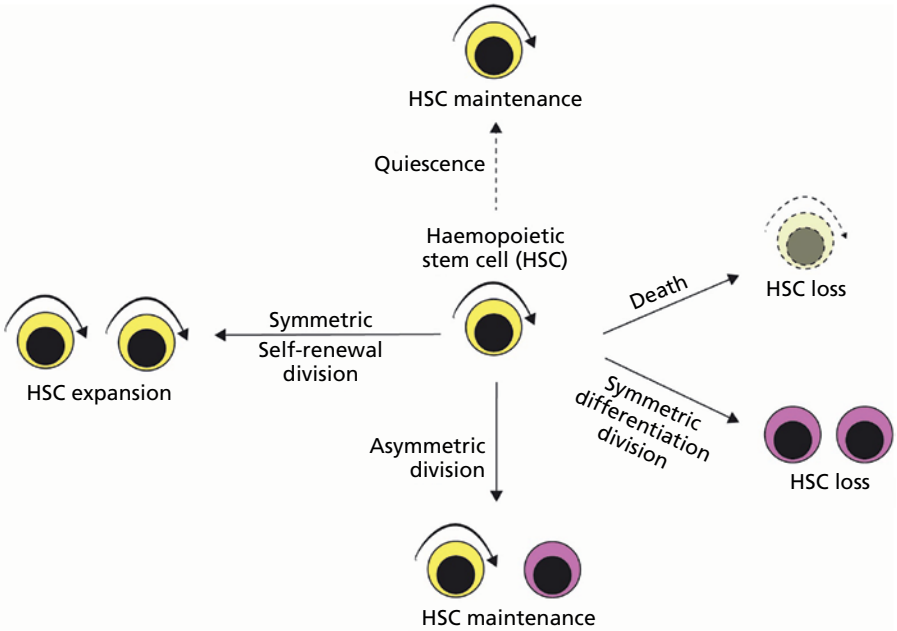


Figure 1.2 Haemopoietic stem cell fate options. Controlling the number of haemopoietic stem cells is critical for preserving normal blood cell production. During steady-state, the majority of haemopoietic stem cells are highly quiescent, protecting the haemopoietic stem cells from the potential harmful effects actively dividing cells are exposed to and preserving the haemopoietic stem cell pool. Upon stimulation, haemopoietic stem cells can be activated and undergo cell division. Symmetric division can either result in haemopoietic stem cell expansion or loss, dependent on the generated progeny being haemopoietic stem cells or differentiated progenitor cells. Asymmetric division, where cell division generated one daughter haemopoietic stem cell and one differentiated progenitor cell, preserve the haemopoietic stem cell pool. Upon exposure to harmful effects, lack of appropriate stimulation or haemopoietic stem cell exhaustion, haemopoietic stem cells can undergo programmed cell death. The fate of haemopoietic stem cells is regulated by the complex interplay between extrinsic (environmental) and intrinsic (inside haemopoietic stem cells) factors.

by infrequent cell cycle divisions and low metabolic activity. This is a critical cellular mechanisms that maintain HSC functional integrity by protecting against HSC exhaustion, harmful substances and DNA damage that can result in leukemic transformation. Quiescence is therefore a critical mechanism for preserving the HSC pool. In light of the high degree of cellular dormancy associated with HSCs, it is proposed that primitive multipotent progenitor cells to a large degree actively contribute to steady-state haemopoietic. Although these progenitors depend on generation from HSCs, they can sustain blood production for long but finite periods of time, reducing the need for a continuously active HSC pool. However, under stress-conditions, also the HSCs are activated and proliferate until normal blood production is restored.

Assays for human haemopoiesis

The existence of HSCs was already proposed in the early 1900s where a common progenitor was proposed to explain the heterogeneity among different blood cells. However, the experimental proof for HSCs was not established until after the post-World War II era when the impact of large-dose irradiation was explored and the lethal effect of irradiation was found to be rescued by bone marrow transplantation. In part, this led to the clinical use of haemopoietic stem cell transplantation which is widely used in the clinic today, but also sparked the development of experimental assays that led to the identification of HSCs. These were spearheaded by two Canadian researchers, James Till and Ernest McCulloch, who first developed an assay that demonstrated the existence of cells with ability to self-renew and produce cells of different blood lineages. These initial studies were performed in mice which after receiving a lethal dose of irradiation were transplanted with bone marrow harvested from a non-irradiated mouse. Upon termination of the mice following transplantation, they noticed distinct nodules in the spleen resembling colonies where an individual colony was demonstrated to originate from a single cell (clonal), contain progeny of multiple blood cell lineages (multipotent) and could be serially transplanted into new irradiated recipients (self-renew). Cells that contained this potential were referred to as colony forming unit (CFU) spleen and formed the birth of stem cell biology and development of additional assays that allowed for the identification and characterisation of HSCs in mouse and man.

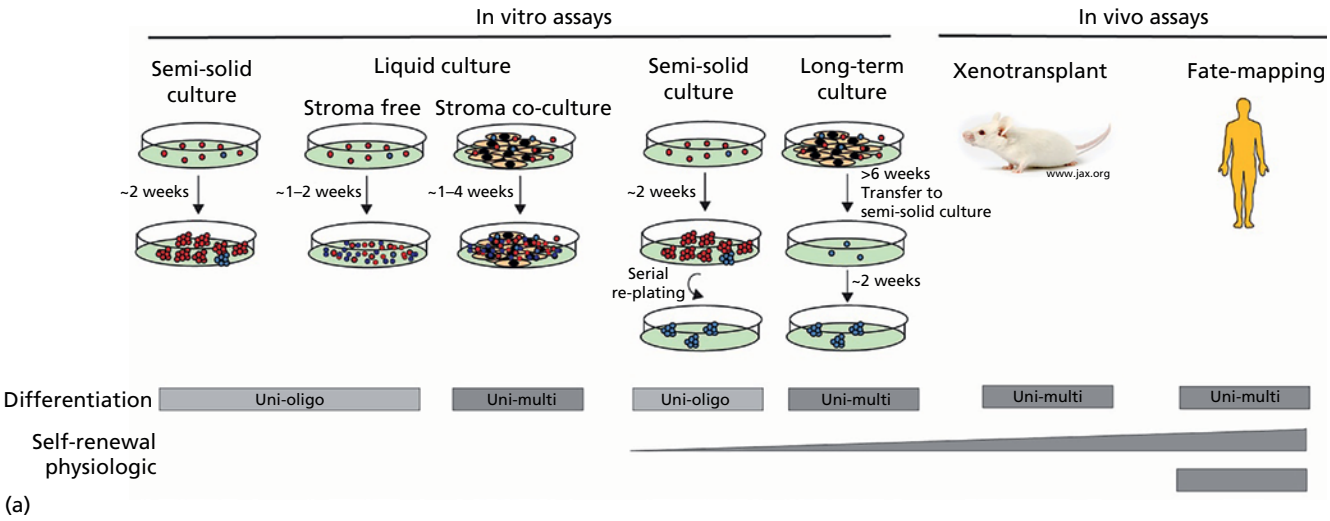
Short-term in vitro assays

In vitro assays (Figure 1.3a) have been instrumental for both identification of distinct haemopoietic stem and progenitor cell populations and the cytokines, as well as other growth factors, that regulate their ability to generate different mature blood cell lineages. Culture of haemopoietic cells in a semi-solid medium composed of methylcellulose supplemented with haemopoietic growth factors supports the proliferation and subsequent differentiation of haemopoietic stem or progenitor cells following short-term culture. Similar to the colonies observed in the spleen (CFU-spleen) of irradiated mice following transplantation of non-irradiated bone marrow, as the proliferation from stem and progenitor cells are physically restricted within the semisolid medium this results in the generation of multiple distinct colonies, where each colony originates from one single stem or progenitor cell. The characteristic colonies can be both classified into different blood cell lineages based on morphology and size, and enumerated in order to quantify the number colony forming cells (CFCs), representing primitive haemopoietic stem and progenitor cells, within the investigated population of cells. Although first developed in the 1960s, this assay is still widely used and even frequently applied in clinical laboratories to enumerate the functional integrity of haemopoietic stem and progenitor cells in cell preparations used for clinical transplantations. This assay was also central for the identification and characterisation of the impact of different haemopoietic growth factors on proliferation and differentiation from progenitor cells. In fact, many haemopoietic growth factors carry names that reflect this, including the cytokines granulocyte colony-stimulating factor (G-CSF), granulocyte-monocyte colony-stimulating factor (GM-CSF) and monocyte colony-stimulating factor (M-CSF).

Culture of haemopoietic stem and progenitor cells in short-term liquid cultures can also be applied to evaluate their potential towards one or more lineages. These liquid culture assays come in two flavours, with or without supporting stromal cells. Although several stromal-free conditions can support the generation of myeloid and erythroid cells, the generation of lymphoid cells appears more dependent on stromal-cell support. For human stem and progenitor cells, differentiation towards B cells can be supported by OP9 stromal cells that were derived from the calvaria of newborn mice lacking M-CSF expression, whereas in order for the OP9 stromal cells to support differentiation towards the T cell lineage, transduction of the OP9 stromal cells with the Notch ligands Delta-like 1 or Delta-like 4 is required. Although culture in semi-solid medium allows quantification of single progenitors within a population, qualitative evaluation of lineage-potentials are primarily obtained from liquid cultures seeded with bulk populations. However, quantitative assessment can also be achieved by either plating cells by limiting dilution or through single-cell plating.

Long-term in vitro assays

Although useful for evaluating lineage-potentials, short-term cultures in liquid or semi-solid media are limited in their ability to evaluate self-renewal potential, and as such does not allow assessment of HSCs or long-lived progenitor cells. Instead, self-renewal potential can be assessed either by serial-replating of



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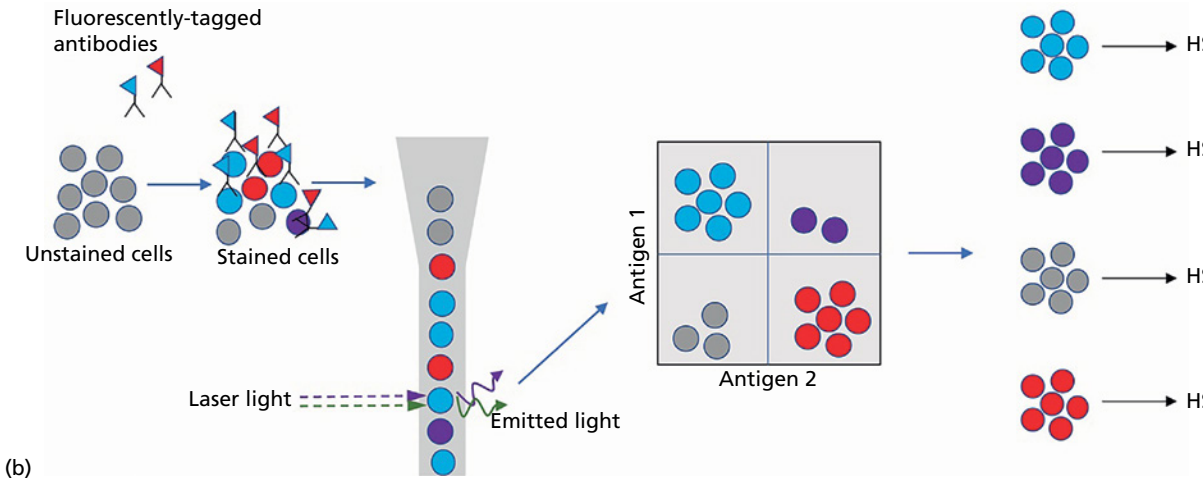

(b)

Figure 1.3 Human haemopoietic stem and progenitor cell assays. (a) Overview of haemopoietic stem and progenitor cell assays where differentiation potential towards one or more blood cell lineages are explored (unipotent, oligopotent or multipotent) *in vitro* and *in vivo*. The ability to assess self-renewal potential is indicated where the scale indicates the relative longevity of the assay. Although in vitro assays and xenotransplantation are important for addressing the potential of haemopoietic cells, fate-mapping cells in human individuals is the only assay that provides information on behaviour of the cells under physiologic conditions. (b) Flow cytometry allows high-throughput analysis of protein expression on single cells within complex tissues by staining with fluorescently-tagged antibodies that bind to specific antigens expressed on the cell surface. Following exposure to specific laser light that excites the fluorescent marker, emitted light will be detected, where the level of emitted light is proportional to the number of molecules (antigens) expressed on the cell surface and bound the different fluorescently-tagged antibodies. As a result, different cells can be purified based on their cell surface expression patterns and then compared for their ability to read out in haemopoietic stem cell assays.

colonies generated in semi-solid medium, or by extended culture in liquid medium. Serial replating of colonies in semi-solid medium has in particular been applied to investigate self-renewal potential of leukemic stem cells which in contrast to HSCs, have reduced ability to differentiate and in some leukaemia can preserve their stem-like activity in the culture following several passages. Following serial replating, progenitor cells that lack self-renewal will not generate colonies that can be replated, whereas colonies derived from HSCs or leukemic stem cells can go through additional passages.

In long-term culture assays, such as the long-term culture initiating-cell assay, the ability of cells to sustain CFC activity is assessed after an initial stromal cell co-culture period of 6 weeks or longer. Following such long culture, the only cells that can

sustain ability to generate either myeloid or erythroid colonies upon transfer to semi-solid CFC culture are the cells that are endowed with extensive self-renewal potential. Cells that lack self-renewal potential, such as short-lived progenitor cells, are not able to sustain this long period of stromal cell co-culture, and therefore not able to produce colonies when transferred to the semi-solid medium. Three-dimensional scaffold cultures have also been applied to support long-term differentiation cultures.

Xenotransplantation assay

In vitro assays have been essential for the identification and characterisation of candidate human haemopoietic stem and progenitor cells, as well as providing a system where manipulations of extrinsic (cytokines, stromal cells and extracellular matrix proteins) and intrinsic (transcription factors, cytokine receptors and DNA integrity) factors can be investigated under controlled experimental settings to evaluate their impact on haemopoietic functional potential. However, mimicking the complex three-dimensional environment where HSCs reside in the human bone marrow is challenging to recapitulate with *in vitro* conditions. In particular, maintaining the quiescent nature of HSCs in adult BM has so far been unsuccessful *in vitro*. Furthermore, no single *in vitro* assays, neither after short- and long-term culture, have been demonstrated to fully address the potential to generate all blood cell lineages (multipotency). As a result, the development of an *in vivo* assay that allows assessment also of human haemopoietic stem and progenitor cells has been important.

Delineation of the haemopoietic hierarchy has been explored in mouse models, where single HSC transplantations have conclusively established the existence of truly multipotent HSCs with long-term self-renewal potential by the ability of a single HSC to reconstitute all mature lineages of the haemopoietic system, and can sustain this ability following serial transplantation. An important factor for allowing such experiments in mice is the ability to transplant donor cells that do not elicit an immunological rejection response by the recipient cells following transplantation. This has been possible through the use of mice on the same genetic background whilst still allowing the ability to distinguish between donor- and recipient-derived cells. Establishing such an *in vivo* assay for human cells, however, required further development, as it is not possible to perform similar controlled experiments in human recipients, and transplantation into wild-type mice will result in the immediate rejection of human cells due to species incompatibility (xeno-rejection). Realising the importance of an *in vivo* assay that enables investigation of human haemopoiesis, much efforts have been focused towards the development of immune-compromised mouse models that would not reject transplanted human cells.

Initial studies exploring the possibility of engrafting human haemopoietic cells in mice were performed using severe combined immune-deficient (*Scid*) mice that lack B and T cells. These studies demonstrated that human cells indeed could engraft in immunocompromised mice but it was not possible to demonstrate whether engraftment originated from multipotent human HSCs, as only lymphoid cells were observed in these early studies. Only by combining transplantation of human cells with infusion of human haemopoietic growth factors, including interleukin 3 (IL-3), GM-CSF and stem cell factor (SCF) was the long-term generation of lymphoid and myeloid cells observed, supporting that also multipotent and long-lived human stem or progenitor cells can engraft mice. However, as Scid mice were not fully immunocompromised, with spontaneous emergence of both mouse B and T cells, the immunocompromised mouse models have gone through series of genetic modifications to further enhance their ability to support long-term *in vivo* studies of human cells.

By crossing Scid mice to nonobese diabetic (NOD) mice to generate NOD/Scid mice, the rejection mediated by the mouse innate immune system was inhibited as the protein Sirpa expressed on NOD/Scid myeloid cells could bind to CD47 expressed on human cells, thereby preventing phagocytosis of human cells. Additionally, the NOD/Scid mice have been further modified to ablate expression of the common γ chain receptor, critical for transmitting signals from haemopoietic growth factors required for lymphoid differentiation. In addition to being more immunocompromised than the NOD/Scid mice, as NOD/Scid/common γ chain knockout (NSG) mice lack B, T and NK cells, the NSG mouse model also allows for more long-term studies as the risk for spontaneous lymphoma development is dramatically reduced in comparison to what seen in ageing NOD/Scid mice. Both NOD/Scid and NSG mice support long-term multipotent differentiation from transplanted human HSCs, including development of mature myeloid, lymphoid and erythroid cells. However, as several mouse haemopoietic growth factors are not cross-reactive to human cells, several critical components for supporting maintenance, propagation and differentiation of human haemopoietic cells are lacking. As a result, even newer generations of mice have been developed where factors supporting human haemopoietic growth and differentiation are expressed in the mouse bone marrow environment, including haemopoietic growth factors, as well as human leukocyte antigens (HLA) critical for allowing generation of mature T lymphocytes.

Fate-mapping

The use of immunocompromised mice has enabled *in vivo* studies of human HSC and progenitor cells, even down to the single cell level, and as such represent an essential tool for the characterisation of human haemopoiesis. However, this still is an assay where human cells are transferred into a stressed environment which lack several factors critical for supporting human haemopoiesis due to species incompatibility. As a

result, xenotransplantation does not necessarily reflect the behaviour of human cells under normal physiological conditions in the human bone marrow. Studies on mouse haemopoiesis using elegant genetically-modified mouse models have highlighted differences in the contribution of mouse haemopoietic stem and progenitor cells to active blood production under normal unperturbed conditions (steady-state) as compared to following stress, such as after transplantation. These studies, where the fate of different haemopoietic stem and progenitor cells are tracked over time (fate-mapping), have until recently been challenging to apply to studies of human haemopoiesis as the they depend on genetic modification of the mouse genome to generate suitable mouse strains for this purpose. However, with the advances in next-generation DNA sequencing, fate-mapping approaches can now be applied to study human haemopoiesis. Although X chromosome inactivation provided some insights, the advancement of DNA sequencing has allowed the ability to track the fate of single HSCs and long-lived progenitor cells within an individual, as will be explained in a separate section later.

Identification of human haemopoietic stem cells

As HSCs are rare in adult bone marrow, where as few as only one in a million bone marrow cells in human bone marrow represent a transplantable HSC, further enrichment is required in order to separate these cells from other cells in the bone marrow. In addition to the assays for evaluation of the functional behaviour of human haemopoietic cells, the development and application of fluorescently tagged antigen-specific antibodies combined with fluorescent activated cell sorting (FACS), has allowed the prospective identification and purification of haemopoietic stem and progenitor cells with different functional characteristics (Figure 1.3b). As haemopoietic cells in bone marrow and peripheral blood already are in single-cell suspension and easily accessible for collection from healthy and diseased individuals, these are particularly amenable to FACS. By staining haemopoietic cells with fluorescently tagged monoclonal antibodies recognising and specifically binding cell surface antigens, the protein expression on thousands of single cells can be analysed per second and viable cells with different expression patterns can be extracted for investigation in different haemopoietic assays. In initial studies, only a few cell surface markers could be investigated simultaneously, but with development of new fluorescent colours that can be tagged to antibodies and separated from one another based on the wavelength of emitted light following excitation of focused laser lights, current technology allows for the simultaneous analysis of more than 20 cell surface markers on single cells.

By combining FACS with the haemopoietic assays described above, it has been possible to enrich cells with HSC properties based on their cell surface expression pattern. In addition to enriching human HSCs by exclusion of known markers expressed on mature blood cells (so-called lineage markers), expression of the cell surface glycoprotein CD34, expressed by approximately 5% of the cells in adult human bone marrow, allows for purification of human HSCs. Although studies have reported that some human HSC activity can be found also outside of the $CD34^+$ cells, it is proposed that at least 99% of human HSCs are confined within the $CD34^+$ compartment. However, as the majority of the $CD34^+$ cells represent non-HSCs, including multipotent and oligopotent progenitor cells, additional markers have been identified to further separate HSCs from $CD34^+$ non-HSCs. The culmination of this work has led to the current phenotype allowing for the highest enrichment of human HSCs from both cord blood and adult bone marrow to encompass cells that lack expression of lineage markers ($Lineage^-$), are positive for CD34 and CD90, and negative for CD38 and CD45RA. By further sub-fractionating $Lineage^-CD34^+CD38^-CD90^+CD45RA^-$ cord blood cells-based expression of CD49f and ability to exclude the triarylmethane dye Rhodamine, a further enrichment can be achieved, where 14–28% of $Lineage^-CD34^+CD38^-CD90^+CD45RA^-CD49f^+Rhodamine^-$ cord blood cells were able to long-term reconstitute myeloid and lymphoid lineages following single cell transplantation into NSG mice. To date, this represent the most stringent and highest enrichment of human HSCs that have been demonstrated experimentally through single cell transplantation, where the inclusion of additional candidate markers, such as EPCR, could allow even further enrichment of bona fide human HSCs.

The application of cell surface molecules to enrich for human HSCs will be important for further functional characterisation of human HSCs. Although previously viewed as a relatively homogenous population, the mouse HSC compartment has recently been shown to include multiple HSC subsets, all endowed with long-term self-renewal potential but with different preferences in their contribution towards mature blood cell lineages. Single mouse HSC transplantation experiments have revealed that all mouse HSC subsets actively contribute to the platelet lineage, but with the exception of HSCs contributing to all mature blood cell lineages, these studies have demonstrated that the adult mouse bone marrow contains a high frequency of HSCs which lack contribution towards one or more blood cell lineages. HSCs exclusively contributing to mature platelets were the only uni-lineage HSC detected in these studies. Platelets generated from megakaryocytes are critical for preserving normal body function and life through their ability to prevent life-threatening bleeding. Mature megakaryocytes undergo multiple rounds of endomitosis, increasing ploidy and cytoplasm to ultimately allow platelet formation (see Chapter 40 for

more details on platelet formation and function). Although platelets are the second most abundant cell in blood (after red blood cells), they have the shortest lifespan and critically dependent on efficient production from HSCs. Interestingly, both in human and mouse haemopoiesis alternative differentiation pathways for the generation of mature platelets from haemopoietic stem and progenitor cells have been proposed, including so-called emergency pathways which bypass intermediate multipotent progenitor cell stages and allow the generation of megakaryocyte progenitor cells directly from HSCs. The identification of human HSC subsets generating platelets through alternative and potentially faster differentiation pathways has clinical relevance as these represent candidate pathways that can be exploited therapeutically to expedite platelet production in patients with life-threatening platelet deficiencies.

The ability to enrich human HSCs has not only been important for allowing cellular, molecular and functional characterisation of human HSCs, but has also been applied in clinical practice. Allogeneic bone marrow transplantation, where the donor bone marrow originates from a person that has a similar but not fully identical HLA match to the recipient, carries the risk of promoting a graft-versus-host-disease (GvHD) mediated by the donor T cells included in the transplant which mediate an allogeneic immune reaction against the recipient's own cells. In the worst case, such a reaction can have deadly outcome. In an effort to minimise the severity of the GvHD reaction, identifying a way to reduce the number of T cells included in the transplant without impacting the number of HSCs required for the reconstitution of recipient haemopoiesis was explored. Towards this, CD34 enrichment is sometimes used for this purpose, allowing the simultaneous enrichment of HSCs and depletion of T cells from whole bone marrow, as well as peripheral blood following donor-conditioning with a HSC mobilising agent that recruits $CD34^+$ cells from the bone marrow into peripheral blood.

Development of haemopoietic stem cells

Although the bone marrow is the primary site of HSCs and blood cell production in the adult, the emergence, expansion and differentiation of HSCs takes place at different sites and time points during embryonic development. Studies in mice and human embryos have shown a conserved appearance of the first haemopoietic cells, which are first detected in the human extra-embryonic yolk sac already 16–18.5 days post-conception. However, these cells lack bona fide HSC activity as the direct isolation of cells capable of reconstituting all blood lineages so far has not been possible from these first haemopoietic cells found in the developing yolk sac and are referred to as primitive haemopoiesis. Instead, the first HSCs and definitive haemopoiesis originate from within the human embryo around 19–27 days post-conception, where emerging haemopoietic cells can be found as budding off from endothelial cells lining the dorsal aorta in the aorta-gonad mesonephros region. Upon establishment of circulation, these cells migrate to and seed the fetal liver, which represent the major site of HSCs and haemopoietic differentiation during the embryonic period. In contrast to the highly quiescent HSCs in adult bone marrow, HSCs are during embryonic development highly proliferative and to a large degree undergo symmetric self-renewal divisions resulting in the rapid expansion of the number of HSCs in the embryo. This is critical, as the pool of HSCs required to sustain haemopoiesis through adult life are formed during this period. Shortly before birth, HSCs migrate from the fetal liver and other embryonic haemopoietic sites to seed the bone marrow which now takes over as the primary haemopoietic organ. In the mouse, bone marrow HSCs still remain in a proliferative phase for a period of around 3–4 weeks after birth, whereupon they enter the quiescent phase of the cell cycle and remain largely dormant unless stimulated to exit quiescence following exposure to stress.

In light of the challenges with recovery of sufficient HSCs for clinical application, including bone marrow transplantation, the mechanisms regulating the distinct proliferative states and symmetrical self-renewal divisions during embryonic and adult haemopoiesis has been a major focus. Uncovering the conditions and regulatory mechanisms that allow for rapid HSC expansion without loss of multipotency and self-renewal potential represents a key to unlocking the ability to expand HSCs for clinical use. Recent techniques have been developed which allow the *ex vivo* expansion of human and mouse HSC and now awaits clinical validation. Several transcription factors have been implicated towards this and this remains a major interest for translational biologists. Although the site of HSC and active haemopoiesis does not change following birth, ageing is associated with altered HSC performance and output. Both in mice and humans, the number of phenotypic HSCs expands with age. However, they appear to loose both proliferative potential and capacity for multi-lineage differentiation. In particular lymphoid potential is reduced with ageing, resulting in skewed myeloid differentiation from aged HSCs. Similarly, the frequency of single mouse HSCs capable of reconstituting mouse haemopoiesis declines with age.

Regulation of haemopoietic stem cells

The preservation of the normal HSC pool in the adult bone marrow, including maintaining their predominant quiescent state during steady-state haemopoiesis and the activation of