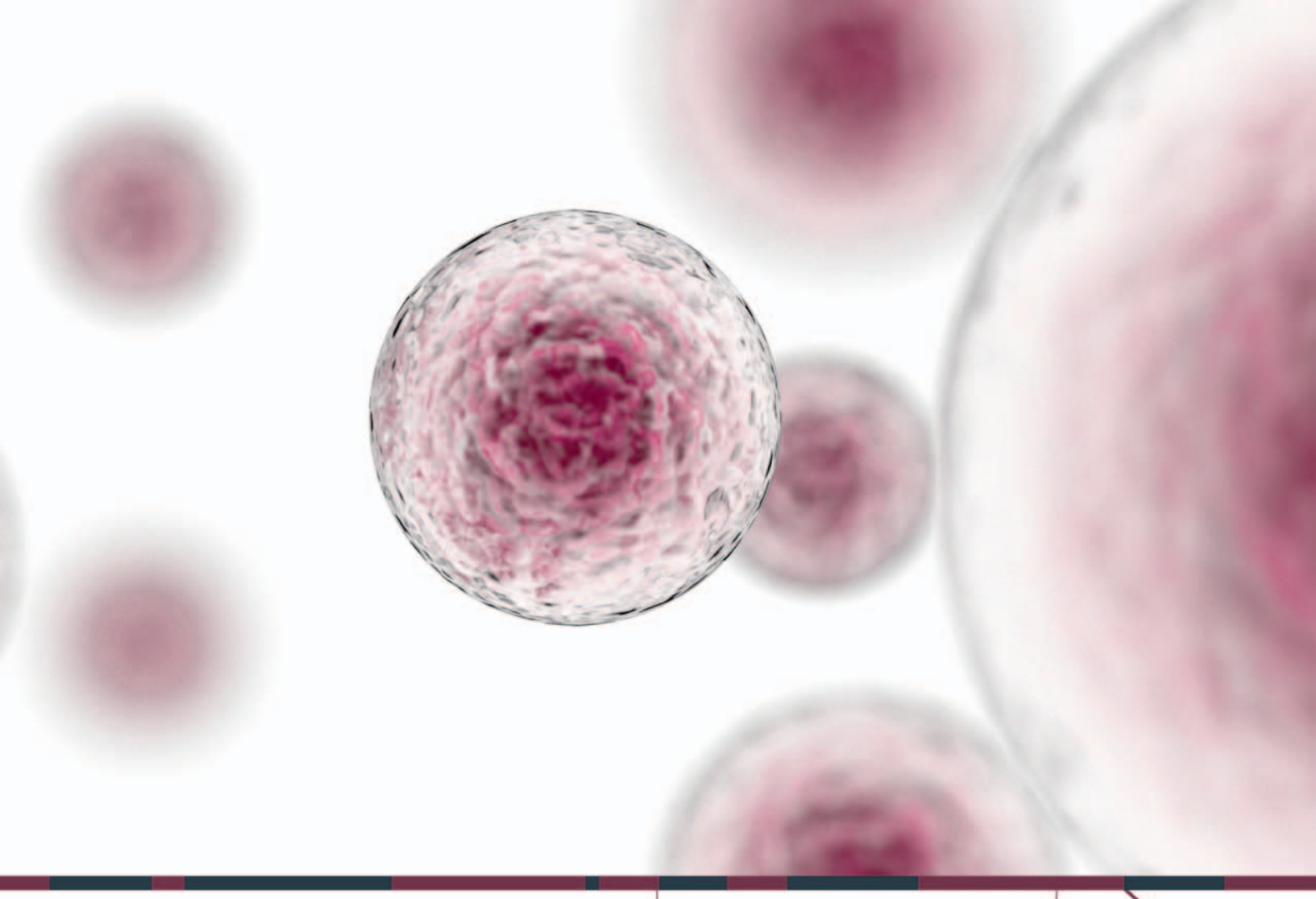

PERINATAL STEM CELLS

Research and Therapy

Edited by
Anthony Atala | Kyle J. Cetrulo
Rouzbeh R. Taghizadeh | Sean V. Murphy | Curtis L. Cetrulo, Jr.

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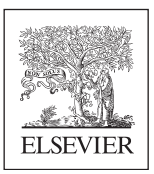

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Preface

The field of regenerative medicine is expanding exponentially, and the use of perinatal tissue—derived products is a major driving force behind this growth. Perinatal tissues are usually discarded at the time of birth and include the amniotic fluid, the placenta, placental membranes, and umbilical cord and blood. As a discarded tissue source, harvesting of stem cells from these sources represents a simple, noninvasive, and safe means for attaining therapeutic cell types.

Perinatal stem cells are a group of cell types that can be derived from postembryonic, perinatal tissues, which include tissues sourced at the time of birth but also encompasses the time period from the 20th week of gestation through the neonatal period. These stem cells have been recognized as a source of cells that are highly multipotent and possess potent immunosuppressive properties. Amniotic fluid—derived stem cells (AFSC) can be isolated from a small amount of the fluid obtained during amniocentesis, a procedure that is already performed in many pregnancies to screen for congenital abnormalities. Mesenchymal and epithelial stem cells can be obtained from the placenta and placental membranes that are discarded after birth. Similarly, mesenchymal, endothelial, and hematopoietic cells can be isolated from the umbilical cord. Finally, the use of noncellular tissues, and cell-derived secreted factors, such as exosomes, has recently been recognized for their therapeutic potential. The advantage of these sources of stem cells is that there are no ethical and legal considerations associated with their collection and use.

Perinatal stem cells have shown the capability to differentiate into functional organ—specific cell types and have also found application in preventing or treating disease through modulation of the immune response. With inflammation playing an important role in disease and injury, regulating this response with cellular therapies could have a major impact on healing and tissue regeneration. As described in this book, perinatal stem cells have found widespread application for the treatment of many diseases, injuries, and disorders. Some examples discussed in this book include the use of perinatal stem cells and tissues for the treatment of congenital diseases, cardiac repair and regeneration, necrotizing enterocolitis, Hirschsprung's disease, stroke, neurodegenerative diseases, hypoxic ischemic injury, acute and chronic kidney diseases, pediatric severe aplastic anemia, wound healing, inflammation- and immune-related conditions, hematopoietic stem cell transplantation, traumatic brain injury, liver diseases, and various other regenerative medicine and tissue engineering applications.

Perinatal Stem Cells: Research and Therapy provides researchers and clinicians with a comprehensive description of the current clinical and preclinical applications of stem cells derived from perinatal sources such as amniotic fluid, placenta and placental membranes, umbilical cord, and Wharton's jelly. The volume is compiled by leading experts in the field and offers the reader a detailed insight into sources of perinatal stem cells and tissues, and their potential for disease treatment. This book has been divided into four major sections, each dealing with commonly applied perinatal stem cell types, and a final section on the clinical and industry perspective on the clinical translation and commercialization of these cells. The contributed chapters cover a wide range of topics, including cell isolation and characterization, preclinical and clinical therapeutic applications, mechanisms of action, clinical translation, and commercialization. These sections include Section I. Amniotic Fluid—Derived Stem Cells, Section II. Placental and Placental Membrane—Derived Stem Cells, Section III. Umbilical Cord—derived cells, and Section IV. Clinical and Industry Perspective.

SECTION I AMNIOTIC FLUID—DERIVED STEM CELLS

In Chapter 1, Sindhu Subramaniam, Eleni Antoniadou, Paolo de Coppi, and Anna David discuss in utero therapy for congenital disorders using amniotic fluid stem cells. In this chapter, the authors will discuss how amniotic fluid stem cells can be used in allogeneic or autologous transplantation to treat congenital diseases in utero.

For Chapter 2, Sveva Bollini, Carolina Balbi, Martina Piccoli, and Michela Pozzobon have contributed a chapter titled "The Amniotic Fluid Stem Cell Secretome: At the Heart of Regeneration." In this chapter, the so-called "paracrine effect" of stem cells and its potential for both cardiac repair and regeneration are discussed.

In Chapter 3, Christopher McCulloh, Yu Zhou, and Gail Besner discuss the transplantation of amniotic fluid–derived neural stem cells. This chapter reviews the origin, derivation, and cellular markers of amniotic fluid–derived neural stem cells and describes their current and potential future therapeutic uses for diseases, such as necrotizing enterocolitis and Hirschsprung's disease, stroke, and neurodegenerative disease.

In Chapter 4, Vivian Guedes, Grant Liska, Nilses Vera, Gautam Rao, Sherwin Mashkouri, Elliot Neal, and Cesar Borlongan highlight the potential of amniotic fluid–derived stem cells as a therapy for stroke, examining the advantages and disadvantages of their use in comparison with other stem cell types.

In Chapter 5, Emily Beck and Jeffrey Jacot discuss approaches to vascular formation by human perinatal stem cells. First, the chapter focuses on the differentiation of these cells into functioning vascular cells and then on approaches where these cells are used to treat ischemic tissues and to develop engineered vascularized tissues.

In Chapter 6, Valentina Villani, Astgik Petrosyan, Roger De Filippo, and Stefano Da Sacco discuss the derivation, characterization, and plasticity of amniotic fluid stem cells and renal progenitors with a specific focus on their application in kidney repair and regeneration. Moreover, the chapter discusses potential mechanisms of action, recent results in renal regenerative medicine and therapy, and the novel challenges in our understanding of their biology and therapeutic potential for treatment of acute and chronic kidney diseases.

SECTION II PLACENTAL AND PLACENTAL MEMBRANE–DERIVED STEM CELLS

In Chapter 7, Georges Makhoul, Kashif Khan, and Renzo Cecere summarize the cardiomyogenic potential of placenta-derived stem cells addressed in vitro and in animal model studies, and highlight placental stem cells as an appealing therapy that could mitigate ventricular remodeling and promote cardiac healing.

In Chapter 8, Juliette Peltzer and Jean-Jacques Lataillade describe some sources of variability impacting perinatal mesenchymal stromal cell products and proposed in vitro tests attempting to predict in vivo efficiency. The authors also address what remains to be done to allow a fair comparison between the most likely efficient mesenchymal stromal cells (MSCs) sources and up-to-date pharmacological treatments.

In Chapter 9, Jatin Patel and Kiarash Khosrotehrani review recent findings regarding the prospective isolation of large quantities of fetal endothelial progenitors as well as pure preparations of fetal or maternal mesenchymal stromal cells from the same placenta. The authors also review the cell identification of endothelial progenitors and mesenchymal stem cells and the establishment of better guidelines in disseminating populations and their functional capacity.

In Chapter 10, Xiaofan Zhu and coauthors present a clinical pilot study designed to determine the feasibility and safety of transplantation of autologous perinatal stem cells in pediatric patients with pediatric severe aplastic anemia. The authors demonstrate that cotransplantation of perinatal hematopoietic stem cells and umbilical cord mesenchymal stem cells results in an excellent therapeutic effect and could become a better treatment of choice for pediatric severe aplastic anemia.

In Chapter 11, Marta Magatti, Anna Cargnoni, Antonietta R. Silini, and Ornella Parolini comprehensively review in vitro and in vivo studies of the immunomodulatory properties of human amniotic epithelial cell (hAECs) and amniotic mesenchymal stromal cells (hAMSCs) and discuss differences between the two cell populations.

In Chapter 12, Jeremy Lim and Thomas Koob review application of placental tissue matrices to promote healing. The authors highlight that the preservation of intact stem cells, their endogenous signals, and their associated stem cell niches allows nonviable placental tissue allografts to be preserved to deliver a diverse set of bioactive signals that modulate cellular microenvironments at the site of repair to promote healing.

In Chapter 13, Cristina Ivan, Aleksander Skardal, and Sean Murphy provide a detailed overview of the previous, current, and future applications of perinatal-derived materials and cells for the treatment of wounds. This includes the application of various forms of amnion membrane products and use of placental mesenchymal cells, amnion epithelial cells, and stem cells derived from the amniotic fluid.

In Chapter 14, Joanna James, Susan McGlashan, and Lawrence Chamley provide an overview of stem cells derived from the placental villi. The authors provide a summary of our knowledge of the characteristics of these two villous stem cell populations, their role in the placenta, and their potential therapeutic applications in a range of organs.

In Chapter 15, Ramon Coronado describes how cells produce extracellular vesicles, how these vesicles are currently classified, and how their contents have been showing strong and broad therapeutic and immunoregulatory effects.

The chapter also provides examples of microRNAs known to be produced by amniotic mesenchymal stem cells exosomes and their current uses to treat immune-related conditions.

SECTION III UMBILICAL CORD–DERIVED CELLS

In Chapter 16, Christopher Porada, Daniel Almeida-Porada, and Graça Almeida-Porada discuss the use of expanded umbilical cord blood as an alternate graft source for hematopoietic stem cell transplantation. This chapter reviews the adoption of umbilical cord blood as an alternate source of hematopoietic stem cells, approaches to ex vivo expansion, and clinical applications.

In Chapter 17, Richard Haspel and Karen Ballen review the advantages of using cord blood as the donor source for hematopoietic stem cell transplantation for hematologic malignancies and the current research addressing the major disadvantage of low hematopoietic stem cell content.

In Chapter 18, Akshita Kumar and Charles Cox Jr highlight the potential of stem cells derived from the umbilical cord due to their lower immunologic effects and better prolific properties that are required to restore damaged brain tissue after moderate to severe injuries. The authors review the potential of autologous human cord blood as a treatment for traumatic brain injury in children.

In Chapter 19, Robert Briddell and coauthors describe the heterogeneous population of perinatal stem cells isolated using enzymatic digestion from the complete umbilical cord tissue. The authors review the phenotypic and functional characterization of these cells and their use in a model of hypoxic ischemic injury.

In Chapter 20, Rita Anzalone, Radka Opatrilova, Peter Kruzliak, Aldo Gerbino, and Giampiero La Rocca provide insights on the developmental history of Wharton's jelly cells, as well as the recent developments for their use in both "replacement type" or "supportive" regenerative medicine applications in key diseases, coupling their hidden differentiative program to their frank immunomodulatory phenotype.

In Chapter 21, Marcin Majka, Maciej Sułkowski, and Bogna Badyra summarize the current progress with Wharton's jelly mesenchymal stem cells usage in cardiovascular diseases, specifically covering regeneration of ischemic cardiovascular damage using Wharton's jelly as an unlimited source of therapeutic stem cells, as future clinical applications.

In Chapter 22, Timothy Ganey, H. Thomas Temple, and Wendy Weston explore the umbilical cord stem cells, or cord blood, and address potential for modulation in directing durable phenotypic derivatives for regenerative applications, including the potential for effecting future phenotype with focused paracrine fractions.

SECTION IV CLINICAL AND INDUSTRY PERSPECTIVE

In Chapter 23, Toshio Miki provides an overview of the therapeutic properties of human amniotic epithelial cells (hAECs) for the clinical cell therapy applications and summarizes preclinical studies with various disease models. Proposed applications of these cells include an alternative for hepatocyte transplantation, a promising cell replacement therapy for various liver diseases, and congenital liver metabolism disorders.

In Chapter 24, Frances Verter reviews two topics that can each stand alone, but which are intertwined with each other. The first topic is the history of advanced cell therapy with perinatal stem cells over the decade 2005–15. The second topic is a survey of private cord blood banks that provide perinatal cell storage for family use. The review of the first decade of regenerative medicine with perinatal cells shows there is growing use of these cells, but so far most of the research is taking place in only a handful of countries. A review of clinical trials that use all sources of MSCs for regenerative medicine indicates that there remain unexplored opportunities for perinatal MSCs.

In Chapter 25, Steven Leung and coauthors provide a review of processing technology and techniques for perinatal stem cells banking and clinical applications. The most common processing, isolation, and cryopreservation protocols for different cell types are outlined. Several commercial products for stem cell processing and applications are discussed, and the main steps for stem cell procurement, processing, culturing and passaging, and cryopreservation are detailed.

In Chapter 26, Noa Sher and Racheli Ofir outline the main considerations and challenges involved in product development of cell therapies derived from the maternal and fetal components of the placenta. The authors share their experience in clinical development of placenta-derived cell therapies, which includes discussions of preclinical research and development, manufacturing, and quality assurance, as well as clinical and regulatory considerations.

In Chapter 27, John Wetherell highlights how patents and strategies, which use stem cells, play an essential role in laying the foundation and securing financing needed to nurture the growth of stem cell companies. The chapter covers

some of the unique challenges arising from the complexity of the research, ethical considerations, manufacturing, and regulations involved in the path to commercialization.

In Chapter 28, James Sherley discusses the need for better assays to predict the effectiveness of tissue stem cell treatments, specifically the means to determine the dose of distributed stem cells (DSCs) used for cell therapies. In this chapter, the author also highlights new approaches to solving the more basic distributed stem cell counting problem with the potential to enable therapeutic distributed stem cell dose determination for the first time.

Chapter 28

Dose Determination for Stem Cell Medicine: A Need Whose Time Has Come

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Chapter Outline

INTRODUCTION

The importance of dose in medical practice is a well-established conceptual and operating principle. In fact, the significance of dose in the application of chemical and biological agents to treat illness and ailment extends beyond curative disciplines such as medicine to disciplines such as toxicology that are concerned with how chemical agents induce illness and disorders instead of with how they cause or accelerate healing. Toxicologists as a group often recount the history of King Mithradates VI, who legend holds took small amounts of poison to build up resistance against future fatal higher doses that might be used against him by his mother, who preferred his brother as the next heir [1]. Mithradates strategy illustrates two essential biological aspects of dose: one, usually a reduced dose gives reduced effects of an agent and two, differences in dose can elicit differences in biological processes and responses in the human body.

Given the fundamental importance of dose in the development and practice of medicine, it might seem untenable for a medical discipline to exist and progress without dose determination at even the most elementary level. Yet, in the case of stem cell medicine, just such an untenable state of affairs has maintained for the entirety of the discipline, which spans more than half a century. This long-standing deficiency in stem cell medical practice reflects unique problems presented by the use of transplanted or targeted distributed stem cells (DSCs) in situ as medicines. In this chapter, these problems, unique to DSCs, will be examined. From the examination, it will be possible to fashion prospective approaches to solve

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this long-standing medical dilemma. Such strategies, currently underway, will be illuminated. Others are envisioned based on theoretical considerations of the unique properties of potentially therapeutic DSCs. However, although both types of potential solutions would be significant advances over the current dose-lacking, nonquantitative state of stem cell medical practice, it still seems likely that none will achieve the accuracy and precision enjoyed with dose determinations for chemical and molecular therapies. DSCs are rare, physically indistinct, and difficult to produce in high yield. These properties conspire to make determination of their clinical dose an inherently uncertain process.

THE IMPORTANCE OF TREATMENT DOSE IN MODERN MEDICINE

Determination of treatment dose is so basic in medical practice that it is largely taken for granted. The study and knowledge of how medicines interact with the human body constitute the field of pharmacology, which includes the subdomains of pharmacokinetics and pharmacodynamics. The former is the study of how the body manages and alters medicines, including their absorption, distribution, metabolism, clearance, and excretion; and the latter is the study of how the body responds to medicines, including a myriad of molecular, biochemical, cellular, physiological, physical, emotional, and cognitive reactions that vary with time and can lead to healing and cures or injury and harm.

The degree of all the effects noted for pharmacokinetics and pharmacodynamics depends to a large extent on how much of a medicine is administered, i.e., its dose. Dose–response relationships are essential elements for the discovery and development of new drugs. Among the best recognized are the relationship between dose and drug efficacy and the relationship between dose and drug safety (Fig. 28.1). Drugs can have complex dose–response mathematical forms for efficacy or safety. Depending on the mechanisms involved, they can be typical monotonic hyperbolic responses, sigmoidal, and even parabolic. The most common forms are simple monotonic isotherms that often reflect a key, molecular interaction or metabolic process that is rate determining for either the therapeutic effect or toxicity, as the case may be. As illustrated by the simple response curves of this type shown in Fig. 28.1, an important aspect of drug development is balancing the dose–response for a therapeutic effect against the respective dose–response for detrimental side effects of a drug to achieve the highest overall benefit for patients.

Dose is also the essential metric for all comparative analyses in drug development and drug administration. This need is derivative of the dose–response principle itself, and it is most readily appreciated in the case of addressing person-to-person variability in dose–response. In the simplest interpretation, the dose–response curve describes how an examined effect of a medicine changes corresponding to changes in the concentration of the medicine in the body. However, a drug's concentration in the body after administration depends on many other factors other than the amount of the drug administered. Important intervening factors include body size, body mass index, metabolic rate, and excretion rate, which all vary among individuals. Because drug concentration in the body can vary for many different reasons, drug response may vary for reasons other than differences in the amount of drug administered. If the administered dose were not known, it would be difficult to recognize and define other determinants of drug response. In fact, to further investigate and define dose–response modifying factors, it can become important to know not only the administered dose of a medicine but also its concentration in the body (see Fig. 28.2).

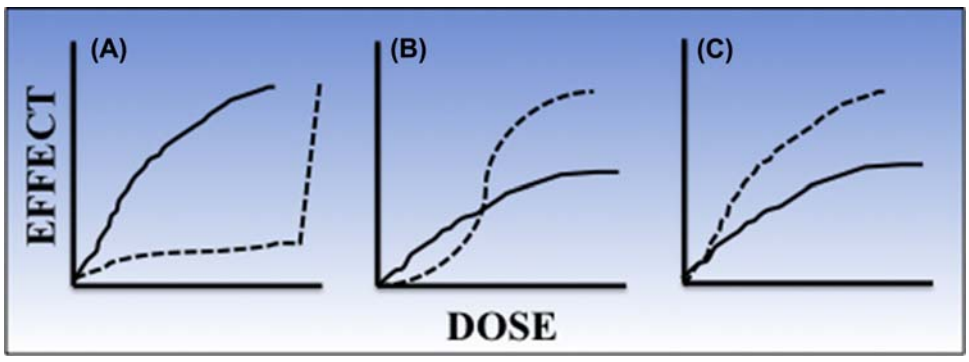

FIGURE 28.1 Examples of dose–response relationships between drug efficacy and drug toxicity. (A) Example of a well-tolerated efficacious drug. (B) Example of a less efficacious drug with a narrower therapeutic window when the beneficial effects of the drug outweigh the adverse effects. (C) Example of a drug whose toxicity is too intolerable to provide benefit above its detrimental effects. *Solid line*, drug's efficacious effect; *Dotted line*, drug's toxicity effect.

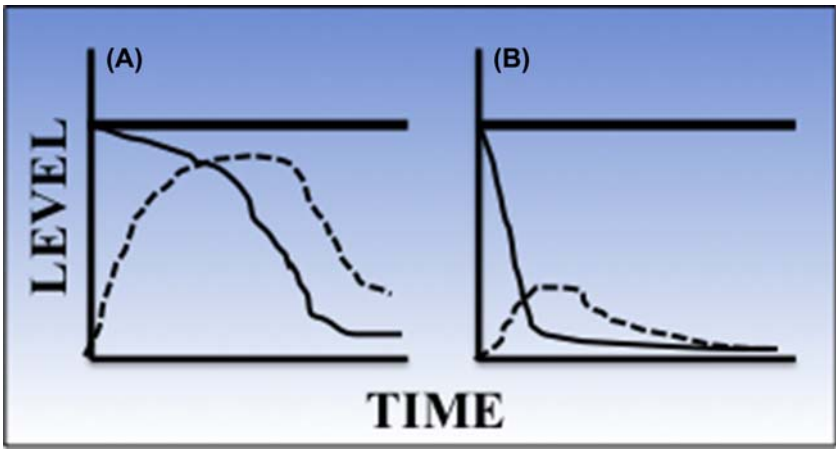

FIGURE 28.2 Dose–response can depend on other constitutional or external factors. In the example shown, subject (A) and subject (B) both received the same dose of drug (indicated by the level of the *bold horizontal lines*). Whereas subject (A's) rate of catabolism of the drug is close to the average rate observed for many people, subject (B) has a much higher rate of catabolism. The higher rate could be an intrinsic variation or it might be induced by other external factors (e.g., interactions with other drugs or dietary factors). Because of subject (B's) higher rate of drug catabolism, the level of drug measured in subject (B's) tissues declines more rapidly (*thin solid lines*). As a result, although subject (B) received the same dose of drug as subject (A), the tissue level achieved by subject (B) is much less (*thin dotted lines*).

Based on the relationships describe above, it is evident that dose is an important factor for clinical trial comparisons made between different individuals, for different clinical features (e.g., gender), between different medicines, between different routes of administration, between different points in time, between different treatment arms of clinical trials, between different clinical trials, and, of course, between different amounts of drug. From this explicit recognition of how integral dose is to all of medicine and medical research, it is understandable that it is largely taken for granted. But this unnoted practice extends to an even deeper level that is very relevant to the problem of DSC dosing.

Consider absolute dose versus relative dose. In the later discussion of current dosing strategies for stem cell therapies, relative dosing is one approach that will be reviewed for its deployment to circumvent the problem of unknown DSC dose. It is rare in modern medicine for a drug to be administered without an accurate measure of the number of active medicine molecules or biomolecules. Knowing the number of active molecules is an expected and obligatory, intrinsic quality control standard. Once a drug preparation has been certified quantitatively, thereafter it might be administered after precise dilution or division based on the starting chemical or molecular amount. However, it would rarely, if ever, be doled out to research subjects or patients without knowing the quantified number in the starting preparation. So, in modern medicine, not only relative dosing is taken for granted, but also absolute dosing, by a metric indicating the number of active molecules, is taken for granted even more so. Yet, presently, in stem cell medicine, neither absolute dosing nor relative dosing is the rule.

THE DISTRIBUTED STEM CELL COUNTING PROBLEM

The formidable difficulty of determining DSC dose is due to one basic technical problem that arises from a number of interacting, complex features of the biological uniqueness of DSCs. That technical challenge is counting them. To begin developing metrics for the dose of DSCs in putative therapeutic preparations, they must be countable. Currently they are not. To count a specific type of tissue cell, one of two conditions must occur. First, if the cell type has unique features that can be detected with high sensitivity and, more importantly, with a high degree of specificity, the cell type can be counted. There are many examples of such cell types in human tissues. Highly specific features of morphology, biochemistry, molecular expression, or different combinations of the three allow some cell types to be identified definitively and counted both in situ and after isolation. Examples include erythrocytes, hepatocytes, and cardiomyocytes. The same cell types also share the second condition that makes tissue cell types countable after isolation and abundance. Because of their initial large numbers in the body, many cell types can be readily isolated at such high fractions that essentially every cell counted is one of the isolated types. So, no special means of identification is required.

Inherent to their specific function in the body, DSCs are present at very low tissue fractions and to date, exclusive morphological, biochemical, or molecular expression properties have not been defined for them. They are unique in their tissue cell renewal function but not in their general cellular attributes investigated to date. Existing at cell fractions

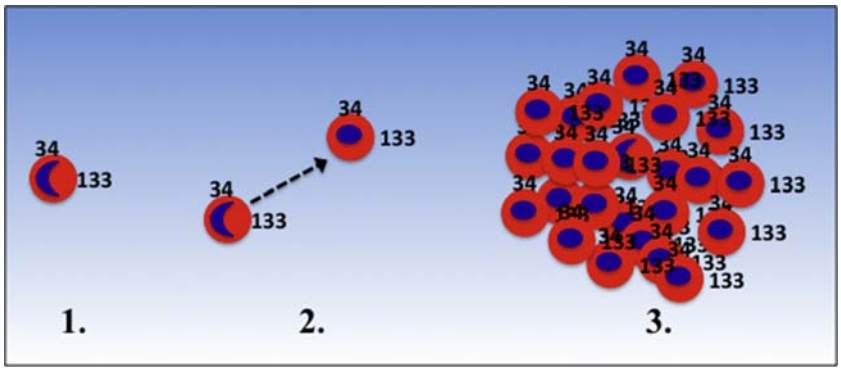

FIGURE 28.3 Illustration of the distributed stem cell counting problem using the hematopoietic system as an example. (1) For illustration purposes, a single hematopoietic stem cell (HSC) is shown with a *crescent-shaped* nucleus. The "stem cell biomarkers" CD34 and CD133 are expressed on the plasma membrane of the HSC. (2) By asymmetric self-renewal division, HSCs produce lineage-committed progeny cells (denoted with *ovoid* nuclei) whose further division and differentiation give rise to many diverse mature cell lineages that compose the hematopoietic system. The earliest of these committed progenitor cells (CPCs) also express plasma membrane CD34 [11] and CD133. (3) Whether in in vivo settings or in ex vivo isolates, because CPCs divide extensively before their differentiation into $CD34^-$ and $CD133^-$ derivatives, their presence obscures specific detection of HSCs. Moreover, because both types of cells express these biomarkers, they cannot be used to specifically identify and count even isolated single HSCs.

generally less than one per thousand tissue cells, isolating them to counting purity has been prohibitive for achieving dose determination. Attempts at achieving the first condition needed for counting have been a major cause for confusion regarding the current state of DSC dose determination. Given the earlier discussion in this chapter, it may seem surprising that many generally knowledgeable stem cell scientists think that DSCs can be counted. This fairly common misunderstanding is due to the existence of "stem cell biomarkers," especially in studies for hematopoietic stem cells (HSCs).

The confusion arises because surface proteins such as CD34, CD133, and CD90 are expressed preferentially on the plasma membrane of HSCs compared with both more mature hematopoietic cells and nonhematopoietic tissue cells. These "stem cell biomarkers" can be used to isolate cell populations enriched for HSCs compared with whole tissue sources, but there is a major problem that prevents their use to count HSCs, which is often overlooked. The same biomarkers are expressed on the plasma membrane of committed progenitor cells (CPCs), which are the progeny cells of HSCs that do not have the long-term hematopoiesis renewal properties that define their parent HSCs. Compared with HSCs, CPCs are abundant, whether in the body where they reside in the same locations as HSCs or in isolated enriched cell preparation. So, currently available "stem cell biomarkers" are actually "stem cell and committed progenitor cell biomarkers." Quantitatively, they are only CPC biomarkers because committed progenitors typically outnumber HSCs by as much as 1000 to 1. Moreover, even if an HSC existed in its purest state, as a single cell, expression of the current biomarkers would not delineate between a lone HSC and a lone committed progenitor (Fig. 28.3).

The scarcity and nonspecific biomarker problems are not unique to the hematopoietic system. Similar challenges apply to DSCs in other organs and tissues. In many cases the "stem cell biomarkers" described for other DSCs are also hematopoietic markers (e.g., CD133, prominin). However, there are others like Lgr5 that appear to be tissue-specific. However, these biomarkers often identify cells whose tissue fractions are too high to all be DSCs as dictated by predictions for rarer fractions of DSCs [2]. Thus far, the author is not aware of any case of a morphological or molecular expression biomarker that has sufficient specificity to quantify DSCs for dose determinations.

PSEUDODOSING, THE PRACTICAL RESPONSE TO THE DISTRIBUTED STEM CELL COUNTING PROBLEM

Despite the lack of a means for quantifying DSC dose, stem cell medicine has not proceeded without a dosing basis. Indeed, in keeping with the general importance of dose in medical principle and practice as described earlier, scientists and physicians have developed surrogate dosing metrics that stand in for the desired correct principle, which is DSC-specific dose. As illustrated in the following discussion, it would be more appropriate to refer to these surrogates as *pseudodosing* because the essential principles for treatment or research, DSCs, are not actually counted in the dose determination.

In the case of HSC transplant therapies, the main pseudodosing metric is $CD34^+$ phenotype [3–10]. HSC transplant is the only approved stem cell therapy in routine clinical practice, and as such, it is also the stem cell therapy with the longest and most extensive clinical experience. A portion of HSC transplant treatment preparations is stained with fluorescently

conjugated or fluorescently detected anti-CD34 antibodies, and positive cells are scored by epifluorescence microscopy or flow cytometry. As noted before, the vast majority of cells detected with this biomarker are CPCs, not HSCs. However, despite this limitation for determining absolute HSC number, the $CD34^+$ cell count has proven to be a clinically useful predictor of clinical outcome in many HSC transplantation settings [3–6].

Several studies have demonstrated dose–response relationships between $CD34^+$ cell count and HSC engraftment success. Importantly, these studies have focused on establishing a universally critical level of administered $CD34^+$ hematopoietic cells required to insure a high probability of effective restoration of hematopoiesis [3–6]. However, over time, it has become clear that differences in $CD34^+$ cells from different sources do not always reliably predict clinical outcome [11]. This issue became clearer in evaluations of HSC transplant therapies for which two independent sources of umbilical cord blood were combined to achieve a sufficient number of $CD34^+$ cells to insure therapeutic engraftment. In this setting, the number of $CD34^+$ cells in respective combined cord blood samples was not a predictor of which source's descendant cells would dominate in the recipient patient [8]. Given the parent–progeny relationship of HSCs and hematopoietic CPCs, the existence of a consistent relationship was a plausible hypothesis but not one likely to prove correct with closer scrutiny. Beyond the disruptions of the relationship due to cell isolation procedures, many physiological factors impact the rates at which HSCs divide to yield committed hematopoietic cells or to self-renew [12], guaranteeing unpredictable interindividual and constitutional variability in the relationship [11]. These factors may not be evident when an optimal or saturating level of HSCs is administered. However, with combined umbilical cord blood samples, although the total number of HSCs may be sufficient for successful engraftment, individual populations remain rate determining for their own persistence. The results obtained in this limiting condition may manifest the reality that although $CD34^+$ cells are certainly related in some quantitative manner to HSCs, their relationship is not a directly proportional one [11].

Other types of commonly transplanted stem cells—mainly mesenchymal stem cells isolated from a variety of tissues [13]—lack single markers with the degree of stem/progenitor specificity shown by hematopoietic cell biomarkers. Combinations of surface biomarkers are generally used to characterize their preparations, with none having sufficient specificity to quantify the stem cell fraction of these rather heterogeneous cell populations. In these cases, the total nucleated cell (TNC) count, and/or the related mononuclear cell (MNC) count, is used as the pseudodosing metric for relating differences in dose. TNC is also often used to complement the $CD34^+$ cell fraction for HSC transplant therapies. Unlike the $CD34^+$ phenotype, its relationship to DSC fraction is unreliable [3,4,8]. Although widely used as if they were meaningful quantitative metrics for DSCs, neither of these measurements provides an effective means of determining the absolute dose of the crucial principles in stem cell transplantation medicine, which are the therapeutic DSCs [11] (Fig. 28.4).

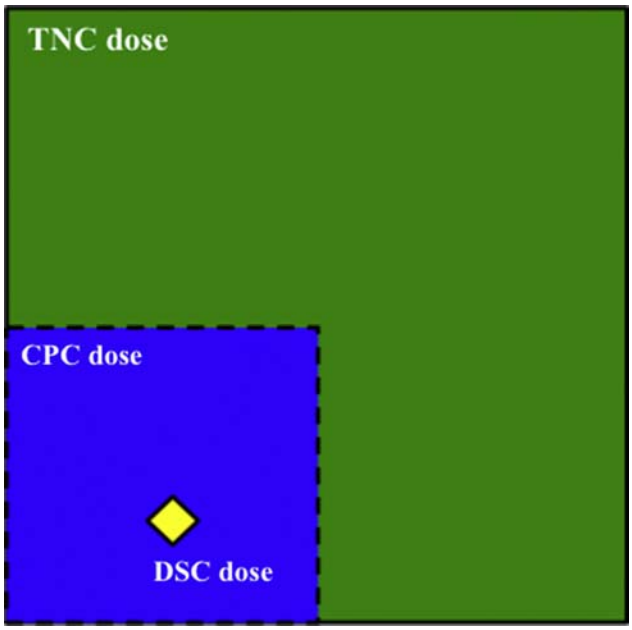

FIGURE 28.4 Conceptualization of distributed stem cell (DSC) pseudodosing. The diagram illustrates two common forms of pseudodosing of DSCs. Total nucleated cell (TNC) count enumerates all the intact nucleated cells in a treatment sample. It includes relatively infrequent DSCs (*yellow diamond compartment*), lineage-committed progenitor cells (CPCs; *blue compartment*), and committed differentiating cells (*green compartment*). Many external and constitutional factors intervene to prevent TNC dose from having a consistent relationship to DSC dose. Depending on the relative proportions of CPCs and committed differentiating cells, TNC dose may provide a consistent indication of CPC dose (*dotted line* and proportional dimensions of *blue compartment*). Unlike TNC dose, the $CD34^+$ cell CPC dose may serve as a semiquantitative surrogate for DSC dose.

Given that pseudodosing is so widely used in current stem cell medicine practice, it is instructive to consider its impact on the quality of stem cell medicine and stem cell biomedical research [11]. It is possible to evaluate internally consistent dose–response relationships based on $CD34^+$ and TNC counts if enough source material is available. Sufficient cells might be obtained by pooling tissue cell samples from multiple donors. However, generally, such research strategies are not possible because source tissue is usually scarce and only adequate for treating a single recipient. This usual situation is further constrained by the well-known difficulty of expanding many types of potentially therapeutic DSCs by in vitro cell culture [14]. So, clinical trials are not conducted with evaluated patients all getting transplanted cells from a common batch of cells. In fact, many clinical trials are based on autologous cell transplant, so that each study patient receives a different preparation of stem cells [13]. In the case of trials based on allogeneic transplant, the challenges of identifying sufficient numbers of matched donors result in a similar cell-limited situation. Therefore, in the typical stem cell transplantation clinical trial today, how the amount of the crucial tested principle—transferred DSCs—relates to any clinical outcome is entirely indeterminate.

Because the crucial denominator for all clinical trial comparisons, which is the variance in effective DSC dose, goes unmeasured in pseudodosed clinical studies, the observed variance in clinical outcome cannot be related to properties of DSCs. Even in the event that every patient benefits greatly from a cell transplant treatment, the cause may not be DSCs presumed present in the transplanted preparations. Formally, other cell types in complex transplant cell populations or even noncellular factors might be responsible [11]. Such a misattribution might be recognized if the variance in DSC number were poorly correlated with clinical outcome, but pseudodosing cannot provide such discrimination. Pseudodosing confounds every clinical trial analysis in this manner, including comparisons of different patients, different numbers of transplanted cells, different treatment regimens, different clinical trials, and on and on.

Pseudodosing precludes other important capabilities that promote higher quality clinical trials and medical treatment. If it were possible to monitor DSC number and function specifically, then it would be possible to detect agents and conditions that alter DSC viability and function to beneficial or injurious effects. For example, the author has suggested that some immunosuppression agents used for allogeneic DSC transplant therapies may reduce DSC self-renewal and thereby compromise rates of DSC engraftment [15]. The capability to monitor reagent and treatment effects on DSCs in vitro could be especially beneficial for gene therapy and gene-editing stem cell medicine. For lifelong cures, both gene therapy and gene-editing manipulations must occur in long-lived, renewing DSCs [16]. However, if the required gene modifications are not efficient or if they injure the targeted therapeutic DSCs, the effectiveness of these treatments could suffer dramatically. The ability to monitor the targeted DSCs during these procedures would enable optimization and ensure a sufficient number of transplanted modified DSCs for more efficacious therapies.

THE CURRENT STATE OF THE ART FOR ESTIMATING DISTRIBUTED STEM CELL NUMBER

The field of stem cell biology has been dominated by the same principle for defining DSCs since its beginning. In the early days of stem cell research, this future guiding principle was implicit, but with time, it has grown to be dogmatic. It is a very important principle, but as is often the case when ideas become dogmatic, it may now limit creativity toward discovery of a solution to the DSC counting problem.

The current obligatory requirement for defining a cell as a DSC is its ability to repopulate its complete respective tissue cell lineage continuously for a period of time, approximating the lifespan of its animal host when it is returned to its normal in vivo environment or an environment that adequately mimics its own natural environment. The gold standard for this principle of definition in both mice and humans is the ability of isolated cell populations containing HSCs to rescue recipients, who have been rendered unviable because of complete elimination of their endogenous HSCs, by restoring the lifelong production of hematopoietic cells. In the case of mice, this remarkable feat can be shown possible by a single HSC [17]. Furthermore, in cross-species experiments, in which human cells are transferred into immune-deficient mice, human HSCs can be detected by virtue of their long-term production of the complete repertoire of human hematopoietic cell lineages. Although tissue lineage repopulation analyses are definitive for the detection of DSCs, they are not quantitative, and as will be considered next, their technical aspects are not conducive for development of quantitative methods that incorporate the now dogmatic requirement, which they meet.

The In Vivo Spleen Colony-Forming Unit Assay for Mouse Hematopoietic Stem Cells

The current persistent lack of a means for quantitative determination of therapeutic DSC dose belies more than a half-century of effort in the field of stem cell research to quantify DSC number. In fact, one of the earliest experiments that

demonstrated the existence of mammalian HSCs initially appeared also to be a quantitative method for subsequent study of DSCs. Mouse HSCs were first detected and defined by their ability to form complex differentiated hematopoietic cell colonies in the spleens of lethally irradiated recipient mice [18,19]. The spleen colony-forming unit (CFU-S) was a metric that related the number of complex hematopoietic cell colonies that a tissue cell sample could produce in the spleen of a recipient mouse. The HSC origin of the CFU-S was confirmed by showing that, after their isolation, a high fraction of these colonies could reconstitute the hematopoietic system of mice whose endogenous HSCs were destroyed by radiation. This ability was taken to indicate the association of CFU-S with HSCs that presumably divided asymmetrically to produce the CFU-S.

Despite its tremendous experimental and scientific advance, the CFU-S assay was destined never to become a quantitative tool for studying HSCs. It would remain inherently semiquantitative, and its technical shortcomings would preclude its use as a routine method for even estimating HSC number. The essential problem was that although the number of CFU-S produced was proportional to the number of HSCs in a sample, the two numbers were not numerically equivalent, and the process had mathematically stochastic characteristics [19–21]. There are many variable physiological factors that determine whether an injected HSC is successful in producing a complex colony in a recipient's spleen [12]. As a result, although the CFU-S assay had good specificity, it had low sensitivity. However, achieving good specificity required expert training in the morphological evaluation of spleen colonies. CPCs and other later precursors in the hematopoietic developmental lineage also produced spleen colonies. Although the latter colony types were smaller and more monotonous in morphological features, they were also more abundant than HSC-derived CFU-S, making CFU-S miscalls a significant source of error. The use of animals is always a source of uncontrollable variability in cell determination assays. Despite efforts to control the diet and environment of test mice, many other unknown physiological and constitutional factors lead to animal–animal variability that can be only somewhat addressed by statistical measures, which generally require larger numbers of animals for an assay that takes at least 10 days to complete.

The In Vitro Colony-Forming Unit Assay for Hematopoietic Stem Cells

The emergence of technologies for culturing hematopoietic cells and the discovery of specific molecular markers for many differentiated hematopoietic cell lineages allowed the CFU-S assay to be translated into in vitro cell culture assays [17,22]. The resulting colony-forming unit (CFU) culture assays evaluate in vitro colonies with molecular probes and automated enumeration platforms that reduce assay times [23]. However, assigning colony types still requires expert training, and the problems with sensitivity, specificity, and general reliability persist. Although less expensive and faster than CFU-S analyses, the continuing semiquantitative nature and variability of in vitro CFU analyses precluded the in vitro method from gaining general use for laboratory research. Today, several companies provide the CFU assay as a contract service to cell therapy and pharmaceutical companies. Although the assay is quantitatively inadequate for determining HSC dose, if their effects are sufficiently robust, it can detect agents and conditions that alter HSC function [23].

The Immune-Deficient Mouse Repopulation Assay for Hematopoietic Stem Cells

At present, an adaptation of the in vivo tissue repopulation assay is viewed as the best method for relative comparisons of different HSC-containing preparations [17,22], whether of mouse or of human origin. Before transplantation of evaluated cell populations, they are serially diluted in microtiter plates to achieve a high and statistically confident number of wells with single cells based on Poisson statistics theory. Under the assumption of a high plating efficiency, this "limiting dilution" approach can be used to develop an approximation of the fraction of single cells that are able to confer hematopoietic repopulation, which transforms into an estimate of relative HSC number. Although widely considered as the best method for estimating relative HSC number, this method also fails to provide a confident absolute stem cell number. Attempts to shore up the statistical aspect of limiting dilution by using fluorescence-activated cell sorting to confirm isolation of single cells do not fair significantly better. The interposition of an animal readout compromises both approaches. Even if the in vitro isolation does not harm HSCs, achieving repopulation by single HSCs is not a high efficiency event. Improvements in repopulation efficiency have been achieved by mixing isolated HSCs with HSC-depleted support cells. However, this progress has not been sufficient to overcome the basic shortcomings of animal-based assays. For example, with animal assays, it is not possible to distinguish between differences in HSC number and differences in HSC quality.

Even if it were possible to overcome the fundamental flaws and technical challenges to quantifying HSC number by mouse repopulation assays, the approach would still be too expensive and take too long (several months) to serve as an efficient and effective means for routine determination of therapeutic stem cell dose. In addition, the methods described so far are only applicable to HSCs. Although HSC transplantation is the clear leader for approved stem cell transplantation

therapy, in clinical trials and private stem cell clinics the volume of HSC use is now rivaled by mesenchymal stem cells from several tissue sources (e.g., adipose, umbilical cord, amniotic fluid) and other organ-specific DSCs (e.g., cardiac, muscle satellite, corneal) [13,24].

In Vitro Sphere Culture Assays for Nonhematopoietic Stem Cells

There are no well-established methods for quantifying the DSC-specific number in tissue cell preparations from nonhematopoietic tissues. More than a decade ago, stem cell scientists were excited by the prospect that it might be possible to quantify DSCs from other types of tissues by a conceptually similar colony formation assay. A new seemingly general property of DSCs was described that pertained to their growth under nonadherent culture conditions. Unlike many types of adherent, nontransformed, differentiated cells, DSCs did not undergo apoptosis when unable to adhere to a culture substrate. They not only survived in suspension but also formed cell clusters called spheres [25]. In many ways, spheres were like HSC in vitro CFUs.

Although DSC-derived spheres were unable to grow in the matrices used to stabilize HSC CFU, they propagated well in suspension culture. The concept of a single DSC for each sphere held the promise of a simple and convenient way not only to define the presence of DSCs but also to quantify them. But with continued experimentation and investigation, this appealing idea was quickly proven overly optimistic. The sphere hypothesis for DSC counting went the way of an earlier hope for simple enumeration of the numbers of epidermal and epithelial DSCs in adherent cell cultures. Beginning with the seminal studies of Barrandon and Green [26], it had been known for a long time that when mammalian tissues are dissociated and plated under conditions that lead to formation of adherent colonies, three main phenotypically different colonies are observed. Called paraclones, meroclones, and holoclones by Barrandon and Green [26] for skin keratinocyte cultures, the colonies are progressively larger and more complex in cellular phenotypes. More importantly, when isolated, only holoclones can give rise to all three colony types and insure the long-term propagation of cell cultures or renewal of transplanted cells. The lasting interpretation of these observations is that paraclones and meroclones are, respectively, derived from the division of cells near the end and the middle of the tissue's developmental lineage (Fig. 28.5), whereas holoclones are produced by DSCs.

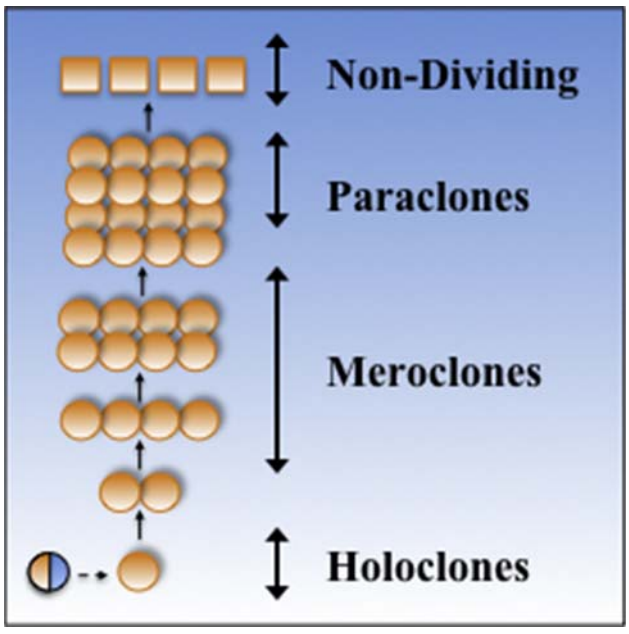

FIGURE 28.5 Relationship between adherent tissue cell colony types and the cell kinetics architecture of mammalian organs and tissues. The diagram schematizes the basic cell kinetics hierarchy of many mammalian tissues. Asymmetrically self-renewing (*dotted arrow*) distributed stem cells (DSCs; *bivalent circle*) divide to replace themselves and produce lineage-committed progenitor cells (CPCs; *early monovalent circles*), which divide to produce progressive generations of transiently amplifying, differentiating cells (*later monovalent circles*). After a finite number of generations, which is tissue-specific, a final generation of post-mitotic, terminally arrested mature cells is produced (*squares*). These mature functional cells expire within a time period that is only a small fraction of a mammal's lifespan. They are removed by apoptosis or physical wear, being continuously replenished by the asymmetric stem cell kinetics of the unit. Approximate cell kinetics ranges (*double-headed arrows*) are diagrammed for tissue cells responsible for indicated adherent cell colony types that emerge in culture from dissociated organs and tissues. Note that both DSCs and early CPCs can produce apparent holoclones based on colony size and morphology. However, only DSCs can produce true holoclones, which can also be serially propagated in culture.

Given this understanding of the relationship between in vitro colony formation kinetics and in vivo tissue cell kinetics, it should be possible to quantify DSCs by counting the number of holoclones produced. Yet, this method did not emerge from adherent cell studies nor from tissue cell sphere research, around 15 years later beginning in the early 2000's. Why not? There are both political and technical reasons. The politics of science certainly did not help, but ultimately it was technical causes that guaranteed the fate of these methods. In their times, neither methods could meet the standard of tissue cell repopulation, as unlike for HSCs, convenient animal repopulation models for other DSC types were largely unavailable. They continue to be challenging to develop and to evaluate.

The technical barriers included those that destined HSC colony formation to inadequacy, as well as other hurdles specific to colonies in dishes or spheres in suspension cultures. All colony formation assays have uncertainties regarding the clonality of colonies and colony formation efficiency. The efficiency problem is further magnified by the need to determine DSC number in complex cell populations, which have many cell types undergoing cell death. As always, specificity confounding by early committed progenitors is a problem. In culture, a secondary evaluation is required to insure that a large complex colony is due to a DSC, as opposed to an early generation committed progenitor. Finally, specific to adherent and sphere cultures, in which cells are not physically constrained, intercolony cell migration can confound equivalency between colony number and DSC fraction, i.e., colonies and spheres may develop from more than a single DSC. This feature was found to be particularly problematic in sphere cultures [25].

A VISION FOR AN EFFECTIVE MEANS TO DETERMINE DISTRIBUTED STEM CELL DOSE

Now, after reviewing the challenging history of attempts to quantify DSCs in complex cell preparations for research or medicine (Fig. 28.6), it becomes easier to consider, in general terms, the categories of strategies that could work. With the historically basic technical deficiencies clearly conceptualized, solving the problem becomes simply a matter of envisioning and devising technical approaches to overcome the now recognized deficiencies. There are three main identifiable categories of technical deficiency: (1) indistinct morphological and molecular properties; (2) scarcity; and (3) biomarker specificity, which is closely related to categories 1 and 2.

The fact that it has not been possible to distinguish many types of DSCs from their lineage–CPC progeny, based on morphological or molecular expression differences, is a difficult deficiency to address in any practical manner. It is biologically reasonable to expect that such differences must exist, but past and current imaging and molecular characterization techniques have been unable to discover them. In the future, either more discriminating imaging technologies or specialized reporter probes may remedy this current deficiency, rendering DSCs, from at least selected tissues, quantifiable.

A Morphomolecular Vision for Therapeutic Distributed Stem Cell Dose Determination

An early example of the possibility of future morphomolecular detection methods can be found in recent research with one described DSC type that has been defined as morphologically distinct from its progeny cells. Gostjeva and Thilly [27] discovered "metakaryotic" DSCs that are very abundant in embryonic and fetal mammalian tissues but become a rare tissue

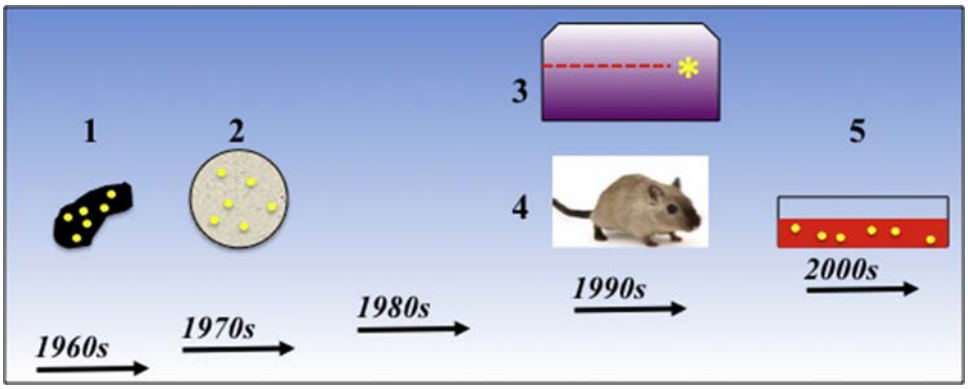

FIGURE 28.6 The historical timeline for developments in the estimation of distributed stem cell (DSC)-specific number. (1) Mouse spleen colony-forming units (CFU-S) in irradiated mice. (2) In vitro colony-forming units (CFU) in semisolid medium cultures. (3) Flow cytometry analyses with stem/progenitor-specific molecular expression biomarkers. (4) Evaluation of human hematopoietic stem cells by reconstitution of human hematopoiesis in immune-deficient mice. (5) Sphere culture.

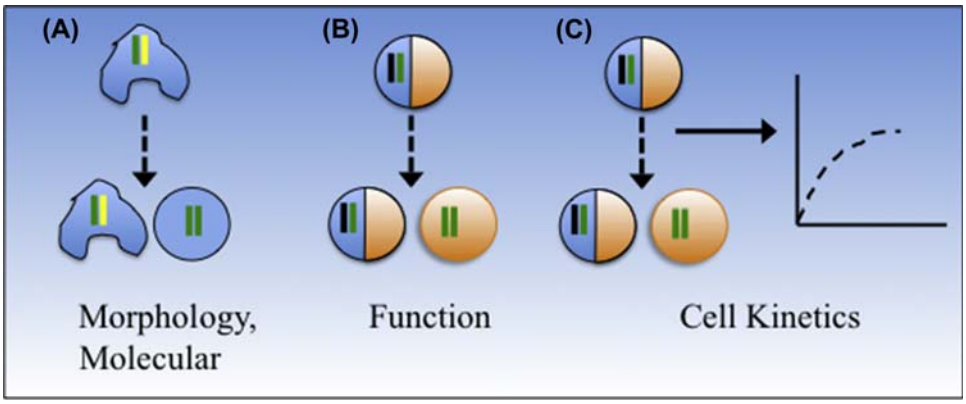

FIGURE 28.7 The vision for strategies to achieve specific counting of distributed stem cells (DSCs) for the determination of DSC dose. Shown are three currently investigated approaches to achieving specific counting of DSCs. (A) Some types of DSCs, such as recently identified metakaryotic stem cells, have both morphological and molecular properties that distinguish them from their lineage-committed progeny cells and other differentiated cells. For metakaryotic stem cells that undergo asymmetric self-renewal (indicated by *dotted arrows*) with amitotic divisions, these properties include bell-shaped nuclei that have a DNA:RNA hybrid genome (*green*, DNA strand; *yellow*, RNA strand). Asymmetrically produced committed progenitor cells (CPCs) of amitotic metakaryotic DSC divisions resume mitotic divisions with spherical nuclei and double-stranded DNA. (B) Many DSCs do not have the distinctive physical and molecular features of metakaryotic DSCs. However, they share the unique function of asymmetric self-renewal, and many have been shown to have tightly associated nonrandom chromosome segregation (*black*, oldest immortal DNA strands). Already molecular biomarkers have been defined that are specifically associated with these DSC-specific functions. Such biomarkers should identify asymmetrically self-renewing DSCs but not their lineage-committed progeny and other cell types that lack these special functions. (C) The most general approach to counting DSCs specifically is based on the use of computational analyses to detect the cell kinetics signature of DSCs when they are rate determining for the total cell output of cultures. Although this method does not visualize DSCs directly, it can quantify their number and other cell kinetics factors distinctly from those of CPCs and differentiated cells with great precision.

cell species in postnatal tissues. These DSCs were initially identified by virtue of the "bell" morphology of their nuclei after a specialized staining procedure [27]. The bell morphology was later shown to manifest amitotic cell divisions, which could either be symmetric (producing progeny cells with two bell nuclei), or asymmetric (producing one cell with a bell nucleus and one with a typical spherical nucleus) [27] (see Fig. 28.7). The different nuclear forms manifest different molecular DNA properties that could be distinguished with well-studied DNA-intercalating dyes. Specific dye fluorescence studies indicate that although the spherical nuclei contain double-stranded DNA, the bell nuclei contain a DNA:RNA hybrid genome [28].

The unique morphomolecular features of metakaryotic DSCs can be used to identify and quantify them in situ and in vitro in cell culture [27–29]. Thus far, these cells have not been isolated and evaluated for the dogmatic standard of in vivo tissue repopulation capability. However, a very elegant demonstration of their asymmetric self-renewal in vitro with vector production of progressively more mature differentiated cells has been reported [29]. Metakaryotic DSCs have been detected in many different mammalian tissues, but their potential for stem cell transplantation therapies has not been investigated to date. Like many previously described postnatal DSCs, their asymmetric self-renewal is a barrier to their expansion to therapeutic numbers [14,29]. However, if they were found to have therapeutic potential, they would serve as an ideal model for the vision of morphomolecular dose determination.

A Purity Vision for Therapeutic Distributed Stem Cell Dose Determination

In concept, the solution to the scarcity problem for DSC quantification is quite simple. The answer is purity. If a treatment sample is known to contain therapeutic DSCs and also to be homogenous in composition, then a simple TNC count is equivalent to DSC dose. The challenge, of course, is achieving routine production of such highly enriched treatment preparations.

Although new strategies are on the horizon, the ability to biomanufacture therapeutic DSCs to high degrees of purity is still a formidable challenge in stem cell medicine. Unfortunately, too many stem cell biomanufacturing scientists and engineers often adopt the same tendencies as described for stem cell biomarker discovery scientists. Rather than address the related respective problems of heterogeneous cell types and poor specificity, they often ignore them [11]. For example, in all reported cases of cultures and reactors expanded for $CD34^+$ cells, the vast majority of the cells produced are not DSCs. This attitude is even more prevalent in studies and trials with mesenchymal stem cells that have even less specific

biomarkers, but whose associated non-stem cells expand at higher rates and for longer periods. Research to manufacture consistent cell populations with majority DSC fractions would reduce the need for specific biomarkers to estimate DSC dose.

A Biomarker Specificity Vision for Therapeutic Distributed Stem Cell Dose Determination

In many ways, of the three visions presented here for achieving therapeutic DSC dose determination, the third basis, improving biomarker specificity for DSCs, would bring the greatest benefit to stem cell biomedicine. The availability of high-specificity biomarkers to replace existing misnomer "stem cell biomarkers" would, with time, end much of the DSC identifying ambiguity that presently continues to undermine the quality of stem cell research and the effectiveness of stem cell medicine. For example, high-specificity biomarkers are crucial tools needed to optimize the bioprocess engineering that is necessary for effective DSC biomanufacturing.

Biomarkers with greater specificity for DSCs could take the form of newly identified physical or molecular properties like those known for metakaryotic DSCs; they could be improved molecular expression markers such as CD34 and CD133; or they could be measures of DSC tissue cell output that are more reliable and more quantitative than CFU assays. With colleagues, the author attempted to solve the DSC counting problem by taking a fundamentally different approach to discovery of highly specific molecular expression biomarkers. Earlier investigators tried to identify DSC-specific biomarkers by first attempting to fractionate DSCs—assayed by their in vivo tissue repopulation activity—away from other tissue cell types to achieve pure populations. By design, once "pure" populations were in hand, molecular methods would be deployed to identify cellular biomarkers associated with DSC preparations that were not found in DSC-depleted fractions or at least reduced in mature, differentiated cell populations. However, in practice, this idealized approach did not yield biomarkers with the desired high specificity for DSCs. Achieving the necessary degree of purity with the available cell fractionation methods proved quite impossible. In addition, even initially highly enriched DSC populations soon contaminated themselves by their own natural programming to divide and produce CPCs. As a result, as noted earlier in the chapter, all currently available tissue "stem cell biomarkers" also identify lineage-CPCs, which do not have the essential stemness property of asymmetric self-renewal. Because, in the best case scenario, CPCs may outnumber DSCs by more than 50 to 1, currently available biomarkers are useless for identifying or counting therapeutic DSCs.

The biomarker discovery approach taken by the author and coworkers immediately solves the progenitor cell fractionation problem by not requiring DSC fractionation at all and focusing instead on their special functions not shared with CPCs. These two DSC-unique properties are asymmetric self-renewal and non-random chromosome segregation. When DSCs undergo asymmetric self-renewal divisions, there is a tightly associated change in how they segregate newly replicated chromosomes. In other typical cell divisions, newly replicated chromosomes segregate randomly, in terms of their age, between new sister cells. Both new sister cells get roughly an equal number of chromosomes that contain the oldest DNA strands inherited from their parent cell. However, asymmetric self-renewal divisions end with the DSC sister getting only and all the chromosomes that have the oldest DNA strands, which are often referred to as the immortal DNA strands. Therefore, chromosome segregation is non-random; and more importantly, the stem cell sister is identifiable by having the full set of chromosomes with immortal DNA strands. These immortal chromosomes may function to reduce DSC mutation rates and/or to maintain their genetic program for stemness functions [2,30,31].

To circumvent the inherent pitfall of insufficient purity encountered by earlier investigators, the author's research group used genetically engineered immortalized cell lines that underwent associated asymmetric self-renewal and non-random chromosome segregation, conditionally, under experimental control [31]. These specialized cell lines provided homogenous cell populations undergoing these highly DSC-specific processes that could be compared with homogeneous control cell populations that were not [32,33]. To the extent that asymmetric self-renewal and non-random chromosome segregation were the exclusive providence of adult DSCs, this experimental model was predicted to yield biomarkers that would provide sufficient specificity for identifying and counting DSCs for the first time. Early evaluations support this conclusion [33], but the full range of applications of the new biomarkers is still under investigation.

The described biologically engineered molecular expression study defined a wide array of cellular factors that are specifically expressed in cells undergoing asymmetric self-renewal and non-random chromosome segregation [32–35]. Some of these factors even identify the set of oldest chromosomes in DSCs directly [33–35]. Using this new class of specific DSC biomarkers, the author with coworkers very recently published the first micrographs of *definitive* mammalian eukaryotic DSCs in culture [35]. Because some of the new DSC biomarker genes are expressed exclusively in asymmetrically self-renewing DSCs, it may also be possible to develop either multiplex qRTPCR or RNAseq signatures for detecting them and quantifying their number in research samples, cell cultures, patient biopsies, and therapeutic treatment samples. Some of the new DSC biomarker genes may have DSC-specific promoters, which could be used to develop

DSC-specific reporters and expression constructs. Although perhaps not needed for general DSC dose determination, such new tools would have important applications for targeting expression of gene therapy and gene-editing constructs to long-lived DSCs. Such targeting is essential for the success of these new gene and gene-editing therapies, which also fall under the rubric of stem cell medicine [16].

A Second Biomarker Specificity Vision for Therapeutic Distributed Stem Cell Dose Determination

The most recently reported new method for specific quantification of DSCs is also related to the cells' unique, and by definition exclusive, property of asymmetric self-renewal but in an operationally different manner. Instead of keying on the DSC molecular signature of asymmetric self-renewal, the second method is based on detecting their cell kinetics signature.

In the earlier work, the author and coworkers formulated the hypothesis that at a sufficiently low starting number of dissociated tissue cells, the rate of total cell output in serially passaged cultures is related to the number of explanted DSCs and the frequency of their asymmetric self-renewal divisions [36]. Initially, the hypothesis was investigated as the basis for developing a new approach for ex vivo expansion of DSCs. By maintaining explanted tissues under conditions that favored symmetric divisions by DSCs, it was possible to increase their numbers to significantly higher cell fractions and promote their clonal expansion as well [37–40].

The success of the DSC expansion studies supported the underlying cell kinetics model for DSC kinetics in culture (see Fig. 28.5). Using computational approaches, it is possible to use the cell kinetics model to deduce specific DSC kinetics parameters from simple total viable cell count data from serially passaged cultures containing even rare DSCs. Among the accessible parameters is DSC number. Conceptually, the new method is the same as previously failed CFU strategies. It infers information about asymmetrically self-renewing DSCs by reading aspects of the complex array of mature tissue cells that DSCs produce. However, the new computational approach also differs in several key respects. Unlike CFU assays, the method is precise, quantitative, and most importantly, can distinguish the number of DSCs from the number of CPCs [41].

At this time, experience with the asymmetric self-renewal biomarkers and the newer DSC kinetics modeling approach is limited. Although these have been validated for only a few types of DSCs, the universal nature of the unique properties that they manifest makes it likely that they will have applications for determining the dose of DSCs in many different human and animal tissues. Because of their distinct detection formats, future integration of the two approaches may provide even higher degrees of sensitivity and specificity.

The two new approaches represent a new generation of DSC quantification tools that now has the potential to provide determination of therapeutic DSC dose. However, each is not without its own shortcomings and limitations. For example, the cell kinetics approach requires that cultured DSCs cycle asymmetrically with sufficient frequency to limit the proliferation of serially passaged cultures, and biomarkers specific for asymmetric self-renewal may be unable to detect DSCs when they are dividing symmetrically. So, even though the new strategies have the potential to be significant advances, they still lack the facility of direct detection as in the special case of metakaryotic DSCs' intrinsic biomarkers. Continued research, leveraging the advances of the two new approaches, holds the promise that in the future similar, but universal, intrinsic biomarkers will be identified for DSCs that will make therapeutic DSC dose determination a routine clinical practice.

CONCLUSIONS

After the detailed consideration of this chapter, the importance of knowing the therapeutic DSC dose for stem cell transplant medicine becomes self-evident. Comparison with the importance of dose in pharmaceutical medical research and medical practice makes any other competing position on the issue immediately untenable. In fact, given the greater chemical complexity of cells compared with individual molecules, whether comparing small drugs or large biomolecules, the argument could be made that knowing the dose is even more crucial for stem cell therapy.

Despite such obvious and acceptable ideas, for more than a half-century, stem cell research and stem cell medicine have operated without a means to quantify DSC number and/or dose, respectively. This situation has persisted because the unique and inherent properties of DSCs, which are responsible for their ability to provide their essential function in organs and tissues, have also constituted a formidable, multifaceted barrier to the development of methods for their quantification.

The past failures of many varied assaults on the DSC counting problem have left DSC-related fields in various states of chagrinned awareness, denial, resignation, and unfortunately, the ignorance that such attitudes can foster. Many investigators who are well aware of the problem have become so habitual in applying the pervasive usage "stem/progenitor" to refer to stem cells that they apply it even when conceptually "stem cell" alone would be valid. Worse, many also often

misapply the descriptor "stem cell biomarker" conceptually, experimentally, and therapeutically [11]. So, the elusive nature of DSCs, defying detection and quantification, has engendered skittish fields that are uncertain and unconfident about how to approach, discuss, and manipulate the essential principles of their work, DSCs.

Solving such a challenging problem requires a deeper understanding of the nature of the problem, in particular, its essential cause(s). For a long-running scientific or technical problem, having a good grasp of its history and the history of previously attempted solutions is also an important element for success in developing new ideas that can converge for formulation of an optimal solution. Sometimes, an approach that did not work can never work. At other times, an approach that was originally unsuccessful can later succeed after the incorporation of additional knowledge and/or better technology. However, sometimes, a completely different strategy must be recognized and tried for any chance for success.

Within the past several years, two new methods emerged that appear to have the ability to finally address the long unmet need for determining the dose of therapeutic DSCs. Design features of both methods address the major shortcomings of previous failed approaches. Both have a detection basis that depends on a defining and exclusive property of DSCs, asymmetric self-renewal. For full acceptance by stem cell research and stem cell medicine communities, it is certain that both of these methods will need to meet the continuing dogmatic requirement of long-term in vivo tissue cell repopulation. Initial studies indicate that the recently described biomarkers identified by their specific association with asymmetric self-renewal are likely to meet this standard. Although many of these biomarkers are intracellular proteins, several are plasma membrane proteins. One of these, the cytokine receptor CXCR6, is able to immunoprecipitate human cancer stem cells and human HSCs, which promote in vivo tumor formation [42] and in vivo hematopoiesis ([43]; unpublished data), respectively. Because the DSC kinetics method does not image the quantified DSCs or provide a means for their physical isolation, evaluating the quantified DSCs in this case for long-term tissue cell repopulation activity will be more challenging. However, by integrating negative-selection procedures with statistical correlation analyses, it should be possible to establish a high degree of confidence that this more general method also meets the high standard of quantifying cells that have long-term in vivo repopulation activity, as well as asymmetric self-renewal.

The ability to quantify DSCs is predicted to have major impacts in stem cell science and derivative stem cell medicine. Quantification will enable many new applications in stem cell medicine beyond dose determination. For example, the ability to specifically determine the number of DSCs in small tissue biopsies could open up a wealth of new diagnostic and prognostic clinical tests and metrics. Changes in DSC number (and function) might indicate or predict normal or abnormal development, presage a number of disease processes, provide a physiological indicator of age and aging rate, and provide information about drug effects. For drug development and environmental sciences, the ability to quantify DSCs could empower new assays for evaluating the effects of drug candidates [29] and environmental toxicants [23] on DSCs. Tissue cell renewal, which is the main function of DSCs, is essential for human postnatal development, health, aging, disease, and healing. Finally being able to reveal DSCs with quantitative confidence will mean finally really knowing them for the first time.

LIST OF ACRONYMS AND ABBREVIATIONS

CFU Colony-forming unit
CFU-S Spleen colony-forming unit
DSC Distributed stem cell
HSC Hematopoietic stem cell
MNC Mononuclear cells
TNC Total nucleated cells

GLOSSARY

Asymmetric cell kinetics Rates of changes in the number of lineage-related cell types produced by asymmetrically self-renewing, distributed stem cells.

Asymmetric self-renewal The process by which distributed stem cells maintain a constant number in renewing mammalian tissues while simultaneously producing lineage-committed progeny cells.

Biomarker A cell feature or constituent that can be used to identify and quantify a particular cell type with a degree of sensitivity and specificity.

Cell kinetics Processes by which cells effect changes in their number.

Distributed stem cells Asymmetrically self-renewing stem cells that are responsible for homeostatic tissue cell renewal in postnatal tissues and possibly late fetal tissues, including "tissue stem cells," "somatic stem cells," "adult stem cells," germinal stem cells, etc.

Dose The quantified amount of a medicine administered to a patient.

Engraftment The process by which transplanted DSCs take up residence in recipient tissues and establish long-term, stable repopulation of their cognate mature tissue cells.

Stemness The property of DSCs to employ asymmetric self-renewal for long-term, homeostatic tissue cell renewal.

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PERINATAL STEM CELLS

Research and Therapy

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