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techniques and applications

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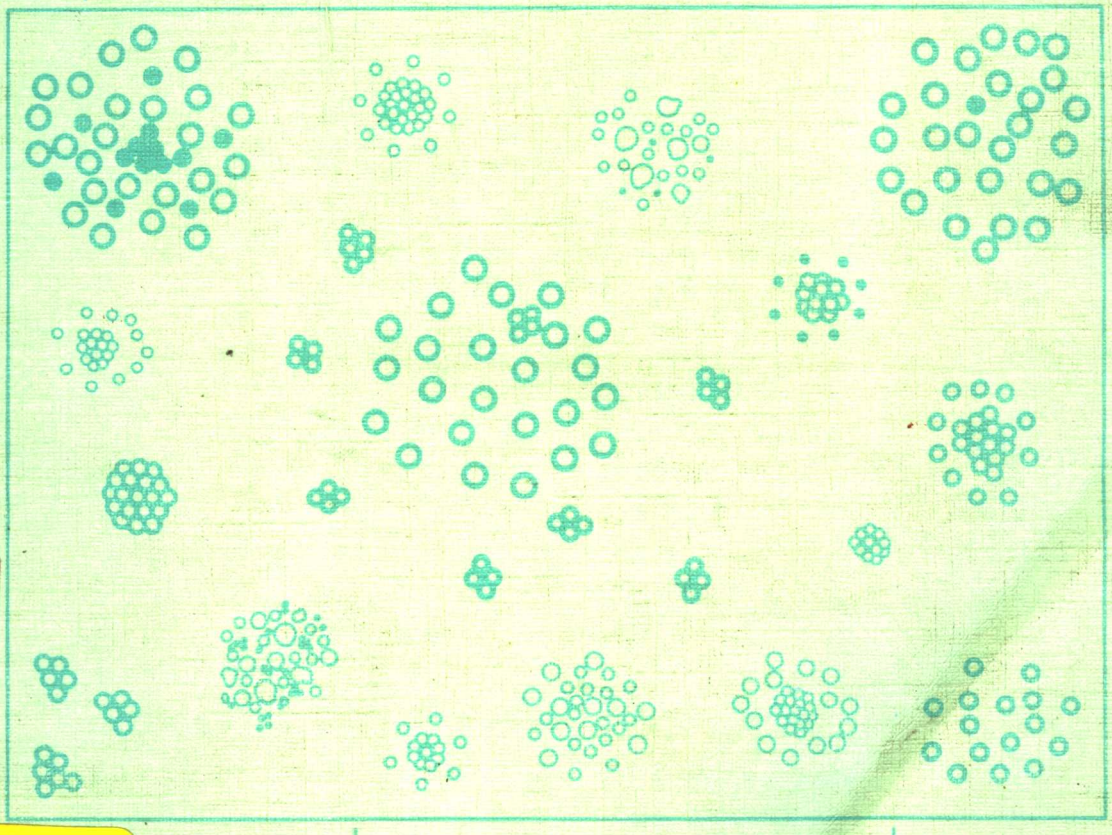

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Preface

Hemopoietic precursor cells can now be cloned in semi-solid medium and, if stimulated by the appropriate specific regulatory molecules – the colony stimulating factors – will proliferate and form colonies of differentiating progeny. These semi-solid culture systems therefore have two quite different uses (*a*) they permit hemopoietic precursor cells to be enumerated and characterized, and (*b*) they permit the colony stimulating factors to be detected and quantified.

This technical manual is extracted from a companion volume containing a full discussion of the biology and nature of the colony stimulating factors (*The Hemopoietic Colony Stimulating Factors*, Metcalf, D., Elsevier, Amsterdam, 1984). This manual contains a special introductory chapter to explain the basic features of hemopoietic populations and their control and then describes in detail the technical methods for culturing and analyzing hemopoietic colonies, their applications and problems. Particular attention has been paid to a description of the use of these cultures to assay for the various colony stimulating factors.

It is hoped that this description of the technical procedures will assist the newcomer to this field and help him avoid many of the problems encountered by earlier workers. The techniques are both simple and elegant but, as with all biological procedures involving tissue culture, problems can arise that can usually be avoided by attention to detail and common sense. The book is dedicated to my technical assistants whose diligence and care have made possible most of our contributions to this field.

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November 1983

Contents

1 Introduction

Since the introduction of the first cloning techniques for the growth of granulocyte-macrophage colonies in semi-solid medium (Bradley and Metcalf, 1966; Ichikawa et al., 1966), the range of available techniques has been increased so that at present it is possible to grow clonally in vitro all the major subsets of hemopoietic precursor cells both from the mouse and man.

If performed using adequate techniques, these relatively simple clonal culture systems not only are highly efficient in detecting and quantifying precursor cells but, since the proliferation of these cells is accompanied by normal maturation, they allow an analysis of many aspects of the biology of blood cell formation in a manner that relates each maturing clonal population to its initiating individual precursor cell. These techniques therefore have revolutionized cellular hematology both at the laboratory and clinical level by providing versatile procedures for identifying and characterizing the cellular abnormalities in many blood diseases.

Of equal importance, the introduction of these techniques led to the discovery of a

family of specific regulatory proteins – the colony stimulating factors (CSFs) – that control the production and differentiation of hemopoietic cells. From the earliest work on the growth of hemopoietic colonies it became obvious that hemopoietic cells cannot proliferate unless stimulated by the appropriate CSF and, as these studies have progressed, it has become evident that the CSFs not only control cell division but also differentiation commitment and the level of functional activity of hemopoietic populations.

It is the purpose of this handbook to provide for the beginner a description of: (*a*) the principles involved in the clonal culture of hemopoietic cells, (*b*) specific procedures for the culture of the major subsets of hemopoietic cells, (*c*) methods for scoring and analyzing hemopoietic colonies, (*d*) procedures for assaying the various hemopoietic regulators or colony stimulating factors, and (*e*) guidelines for the purification of these factors. These technical sections have been reproduced from an accompanying volume (Metcalf, 1984) which contains a full description of the biochemistry, mechanisms of action and biological significance of the colony stimulating factors.

To provide a sufficient background of information for this technical manual, it is necessary to describe briefly the various hemopoietic populations able to be cloned in vitro and the colony simulating factors acting on these cells.

I THE HEMOPOIETIC CELLS

The production of the mature cells appearing in the peripheral blood occurs continuously in normal adult life and is achieved by the proliferative activity of a series of precursor populations located mainly in the bone marrow, with smaller numbers (less than 1% of the total) in the spleen. The production of lymphoid cells occurs in a more diverse series of locations – T-lymphocytes in the thymus, with subsequent additional proliferation in the spleen and lymphoid tissues, and B-lymphocytes in the bone marrow with again additional proliferation in the spleen and lymphoid tissues.

The precursor hemopoietic populations can be visualized as comprising three overlapping compartments, each of larger size than the preceding (Figure 1).

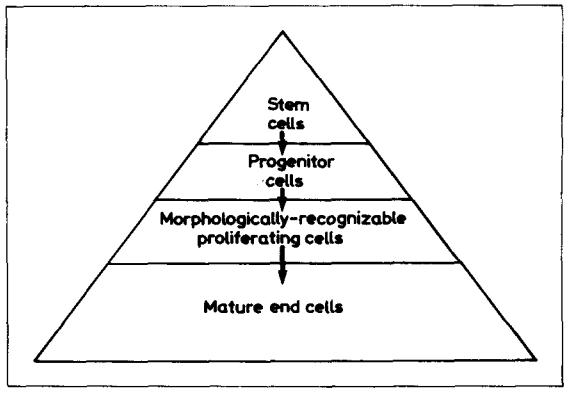


Figure 1. The precursor hemopoietic cells can be separated into three compartments – stem cells, progenitor cells and immature, morphologically recognizable cells – each compartment is larger in size than that preceding.

Stem cell or multipotential cell compartment

This is a population of cells each with the ability to generate committed precursor cells of more than one lineage and with a capacity for self-renewal. These cells were originally identified (Till and McCulloch, 1961) by their capacity to form hemopoietic colonies in the spleen of lethally irradiated syngeneic recipients – hence their name of colony-forming unit – spleen (CFU-S). Stem cells have been purified to homogeneity by fluorescence-activated cell sorting and have the morphology of medium-sized, undifferentiated cells with a round nucleus and no identifying cytoplasmic morphology (Goldschneider et al., 1980). More recent studies have shown that stem cells are heterogeneous in nature, with a varying capacity for self-generation and a varying capacity to generate committed cells. The most mature stem cells have little self-generative capacity and may only be bipotential or tripotential, in contrast to the capacity of the most ancestral cells in the stem cell population to form cells of all lineages – erythroid cells, granulocyte-macrophages, eosinophils, megakaryocytes, mast cells, T- and B-lymphocytes, NK cells and possibly some endothelial cells.

Two broad classes of CFU-S are recognized – the more ancestral (CFU-S-I) form 14 day spleen colonies and have extensive self-rene-

wal capacity, while the more mature stem cells with less proliferative potential and a more restricted differentiation potential (CFU-S-II) form 7 day spleen colonies.

Both types of CFU-S can be grown clonally in vitro. CFU-S-I form small blast cell colonies (Keller and Phillips, 1982; Nakahata and Ogawa, 1982) while CFU-S-II form large multipotential or mixed erythroid colonies and carry the alternative name of multipotential colony-forming cells (Multi-CFC) (Johnson and Metcalf, 1977; Metcalf et al., 1979). Whereas blast cell colonies typically exhibit a delayed onset in growth and often contain only 100–200 cells, the multipotential or mixed colonies can be extremely large and spectacular in appearance, containing 10 000–20 000 cells within 7 days of culture (Figure 17D, Chapter 3).

Progenitor cell compartment

The progenitor cells are the immediate progeny of stem cells but differ in two respects; firstly they have a restricted or possibly no capacity for self-renewal, and secondly the cells are committed irreversibly to a single lineage of hemopoietic differentiation (or two, in the case of many granulocyte-macrophage progenitor cells). All of these cells can be grown clonally in semi-solid cultures – hence the alternative name of colony-forming cells (CFC). These are the cells responsible for forming the vast majority of colonies grown in most cultures of hemopoietic cells. Table 1 lists the original papers describing the clonal culture of the various progenitor cells, using mouse or human cells.

Progenitor cells also have been purified by fluorescence-activated cell sorting and identified as undifferentiated blast-like cells with a round nucleus and of a somewhat larger size with more cytoplasmic basophilia than stem cells (Nicola et al., 1980; Visser and Bol, 1981). Progenitor cells in the different lineages are usually identified by a prefix to indicate the lineage, for example GM-CFC for granulocyte-macrophage colony-forming or progenitor cells. In the erythroid lineage the progenitor cells carry the names BFU-E for the more ancestral, and CFU-E for the more mature progenitor cells. The various progenitor cells are illustrated in Figure 2, which depicts the lineages and gives the terminology used for the various progenitor cells.

Progenitor cells are usually, but not always, more numerous than their parent stem cells and this compartment provides the first ampli-

Table 1

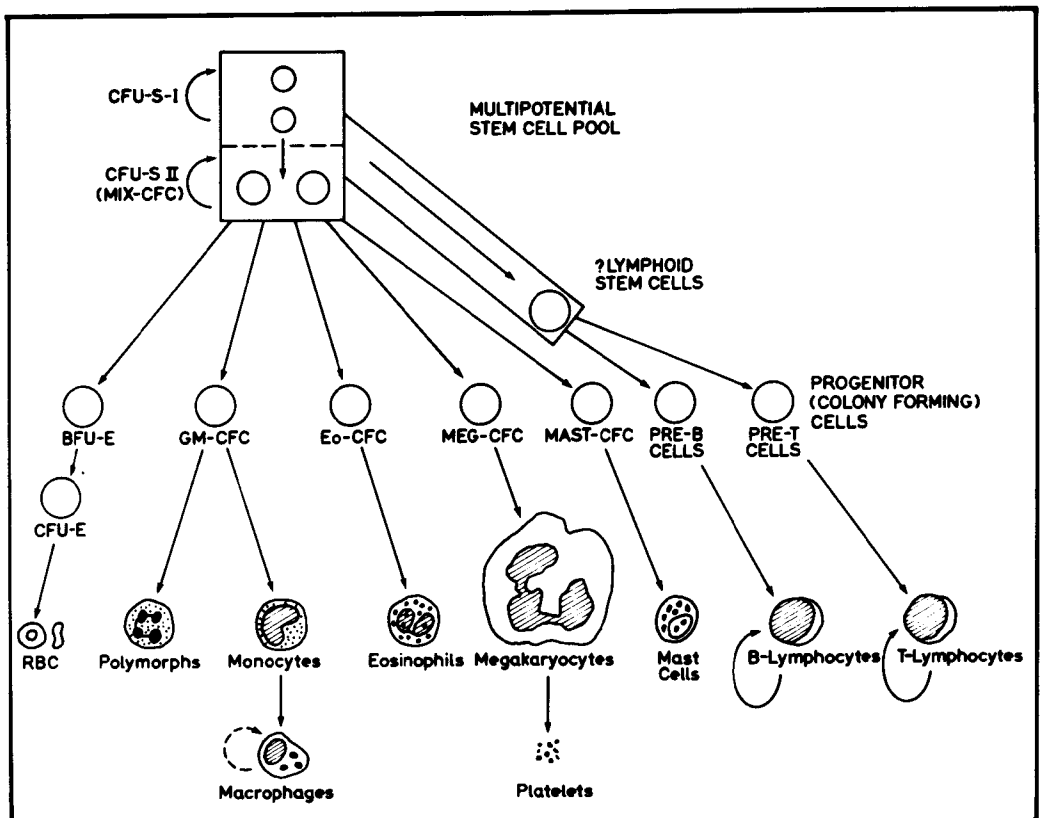
Initial papers describing the various clonal culture techniques for hemopoietic cells

Cell type	Species	Reference
Granulocyte-macrophage progenitors	Mouse	Bradley and Metcalf, 1966 Ichikawa et al., 1966
Granulocyte-macrophage progenitors	Human	Pike and Robinson, 1970
Eosinophil progenitors	Human	Chervenick and Boggs, 1971
Erythroid progenitors (CFU-E)	Mouse	Stephenson et al., 1971
Erythroid progenitors (BFU-E)	Mouse	Axelrad et al., 1973
Erythroid progenitors (CFU-E)	Human	Tepperman et al., 1974
Megakaryocyte progenitors	Mouse	Metcalf et al., 1975
B-lymphocytes	Mouse	Metcalf et al., 1975a
T-lymphocytes	Human	Rozenszajn et al., 1975
Multipotential progenitors	Mouse	Johnson and Metcalf, 1977
Multipotential progenitors	Human	Fauser and Messner, 1978
Megakaryocyte progenitors	Human	Vainchenker et al., 1979
Eosinophil	Mouse	Johnson and Metcalf, 1980
Mast cells	Human	McCarthy et al., 1980
B-lymphocytes	Human	Izaguirre et al., 1980
Mast Cells	Mouse	Schrader et al., 1981
T-lymphocytes	Mouse	Rozenszajn et al., 1982
Multipotential stem cells	Mouse	Nakahata and Ogawa, 1982 Keller and Phillips, 1982
Multipotential stem cells	Human	Nakahata and Ogawa, 1982a

fication step in the initial formation of mature blood cells.

Purified progenitor cells have a very high cloning efficiency (approaching 100% well-prepared semi-solid cultures) and the presumption is that such cultures are equally efficient in detecting progenitor cells in cultures of unfractionated marrow, spleen or blood cells.

The same type of heterogeneity evident in stem cells is seen also in progenitor cells. Within a single lineage, the individual progenitor cells exhibit considerable variation in size (in part cell cycle-related), density, adherence and membrane markers and can generate in a 7 day culture period in vitro progeny ranging in total numbers from 50 to 20 000 cells. Within any one lineage, progenitor cells can differ widely in responsiveness to stimulation by the various colony stimulating factors and, in the case of GM-CFC, can vary in differentiation


CFU-S-I
CFU-S II
(MIX-CFC)
MULTIPOTENTIAL
STEM CELL POOL
?LYMPHOID
STEM CELLS
PROGENITOR
(COLONY FORMING)
CELLS
BFU-E
GM-CFC
Eo-CFC
MEG-CFC
MAST-CFC
PRE-B
CELLS
PRE-T
CELLS
CFU-E
RBC
Polymorphs
Monocytes
Eosinophils
Megakaryocytes
Mast
Cells
B-Lymphocytes
T-Lymphocytes
Macrophages
Platelets

potential with some forming only granulocytic progeny, some only macrophage progeny and some forming progeny of both lineages.

Since hardly any two colonies grown in vitro are exactly identical in size and cellular composition at any one point in time (Figure 3), it is likely that in some lineages future work will document the existence of definable subsets of progenitor cells within each lineage, each possibly programmed to generate mature end cells with slightly differing functional capacities.

Stem and progenitor cells may be genetically programmed to have certain restrictions in the total number of progeny they are able to produce. However it is important to appreciate that the *actual* number of progeny they produce within this broad restriction is not fixed. According to the culture conditions used and to the type and concentration of proliferative stimuli applied to the cells, a cell may produce a few mature progeny or a very large number. This gives great flexibility to the hemopoietic populations in responding to changing demands for the production of mature end cells.

All cultures of normal hemopoietic cells develop larger numbers of small clones than clones of colony size. By convention, clones of subcolony size are termed "clusters" and cell separation studies have shown that the cells forming such clusters are the progeny of colony-forming cells and are separable from colony-forming cells. In the case of erythropoietic cells, these cluster-forming cells have the name CFU-E but correspond in nature to the cluster-forming cells in other lineages.

In cultures of T- and B-lymphocytes, most of the cells forming colonies in vitro do not correspond in differentiation status to hemopoietic progenitor cells. Many mature lymphocytes retain a potential for extensive proliferation and, as a consequence, most colonies are in

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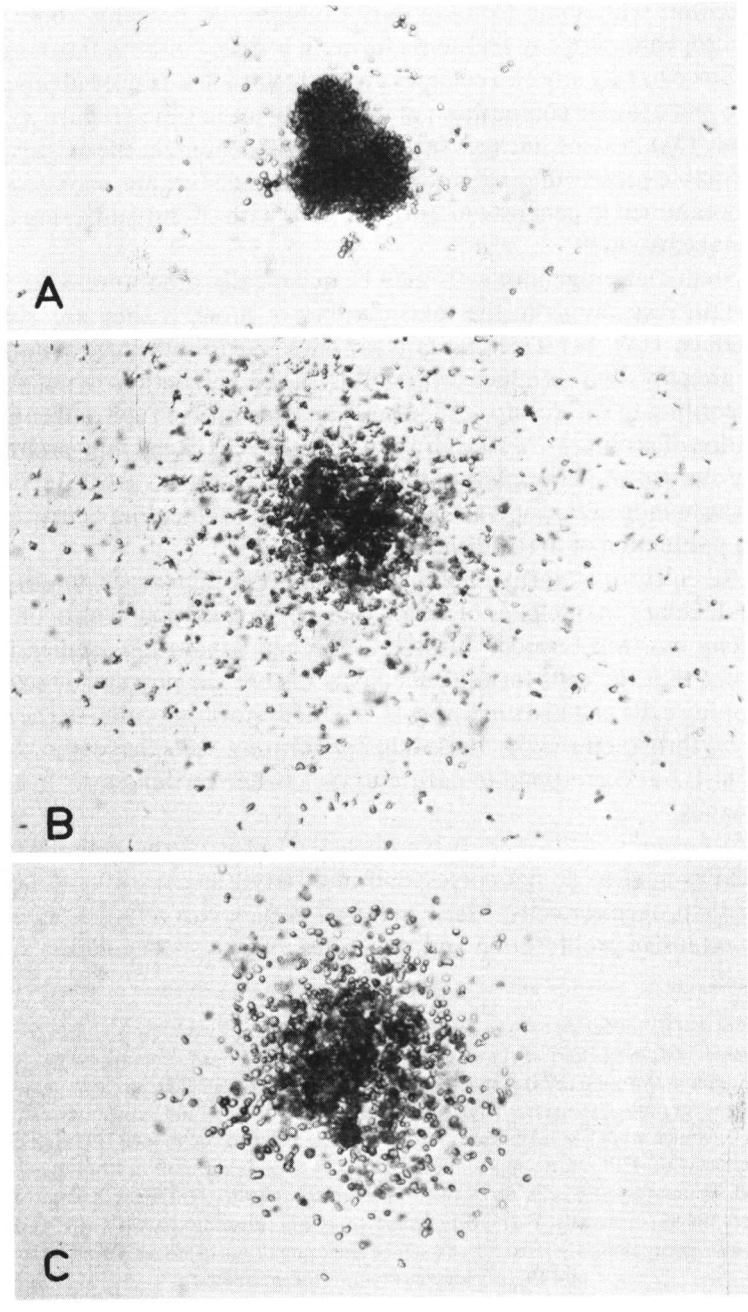
Figure 2. The precursor cells in each hemopoietic lineage. Two subsets of self-generating stem cells exist (CFU-S-I and CFU-S-II). These give rise to committed progenitor cells (colony-forming cells) restricted to one differentiation lineage – or two in the case of many granulocyte-macrophage progenitor cells. All stem and progenitor cells can form colonies in semi-solid cultures. CFU-S-I form blast cell colonies, CFU-S-II (Mix-CFC or Multi-CFC) form mixed colonies usually containing some erythroid cells. All other colony-forming cells form colonies of restricted differentiation lineage. Some macrophages, mast cells, T- and B-lymphocytes retain substantial proliferative capacity and also form colonies in vitro. The existence and exact origin of stem cells common to T- and B-lymphocytes are unresolved questions.


A
B
C

fact produced by relatively differentiated T- or B-lymphocytes, possibly corresponding to "memory cells" in immunological terminology. However it is likely that, amongst such colony-forming cells, there is also a minor population of genuine progenitor cells corresponding closely in differentiation status to other hemopoietic progenitor cells.

Two mature hemopoietic cell types, the macrophage and mast cell, are now recognized to retain a previously unsuspected capacity for extended proliferation and can generate colonies in vitro under certain culture conditions.

Morphologically recognizable proliferating cell compartment

The immediate progeny of progenitor cells are the immature but morphologically identifiable hemopoietic cells in the various lineages (e.g. erythroblasts, myelocytes). This large population of cells represents the second major amplification step in hemopoiesis and, while the capacity of these cells for proliferation is relatively restricted, they are responsible for generating the cells that pass through the familiar maturation sequences leading to the formation of the mature cells that enter the peripheral blood, e.g. myeloblast – myelocyte – metamyelocyte – polymorph.

It is not yet clear whether all cells of this compartment can proliferate clonally in vitro. Some almost certainly are able to generate clusters of progeny cells and it is likely that the initial proliferation of many of these cells in vitro is often missed because of the routine practice of scoring cultures after a relatively long incubation period of 7 – 14 days.

II RELATIVE FREQUENCIES OF HEMOPOIETIC STEM AND PROGENITOR CELLS

As shall be discussed in the following chapters, the proper performance of clonal cultures requires that individual colonies grow as

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Figure 3. Three colonies present in a 7 day culture of mouse bone marrow cells. Note the varying size and shape of the colonies despite the fact that all have been formed by progenitor cells of the granulocyte-macrophage lineage. A, granulocytic colony; B, granulocyte-macrophage colony, and C, macrophage colony.

clearly discrete and separate cell populations, to permit the accurate enumeration of clonogenic cells. To prepare cultures containing suitable numbers of clonogenic cells, it is necessary to know the frequency of such cells in populations like the bone marrow or spleen.

For example, in the mouse, the approximate cell frequencies in the bone marrow are: stem cells, $200/10^5$ cells; colony-forming cells, $300/10^5$ cells; cluster-forming cells, $1200/10^5$ cells; immature morphologically recognizable cells, $12\,000/10^5$ cells. Frequencies of stem and progenitor cells are lower in the spleen and blood. For example, the frequences of CFU-S are: marrow $200/10^5$ cells; spleen, $2/10^5$ cells and blood, $0.1/10^5$ cells and progenitor cell levels occur in similar relative frequencies. The approximate relative frequencies of the different stem and progenitor subsets in mouse bone marrow are shown to scale diagrammatically in Figure 4.

As shall be discussed in a following chapter, most culture systems permit the growth of colonies of more than one lineage in the same culture dish. Since techniques have yet to be developed for separating the various subsets of progenitor cells in pure form, the identification and enumeration of a particular subset of colonies requires methods for staining and typing the colonies grown in each culture dish. These problems and techniques will be discussed at some length in the following chapters.

III THE RELEVANCE OF IN VITRO STUDIES

From the above brief outline it can be seen that it is now possible to detect all the major types of hemopoietic stem and progenitor cells by their ability to generate colonies of cells in semi-solid culture. From the observed frequency of such cells and their observed capacity to generate various numbers of progeny cells, it can be calculated that if such cells behaved in vivo as they do in vitro, they could account for the required daily production of mature blood cells. Since the culture systems can be demonstrated to have a high cloning or detection efficiency for progenitor cells, it is likely that satisfactory systems now exist to reproduce in vitro the cellular proliferation occurring in vivo during hemopoiesis.

Apart from their ability to support what appears to be the correct level of proliferative activity of hemopoietic precursor cells, the semi-

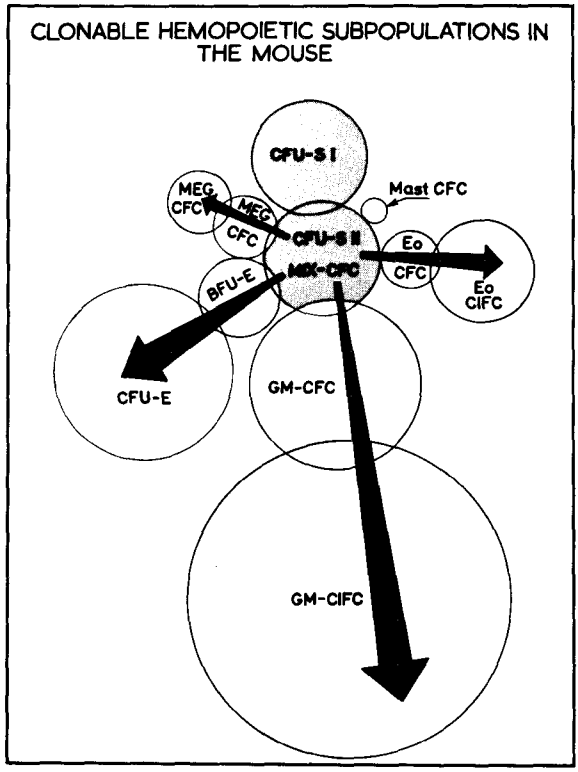

Figure 4. Scale drawing showing the relative frequencies of various stem and progenitor cells (CFC, colony-forming; CIFC, cluster-forming) in adult mouse bone marrow.

solid culture systems possess an additional property of great importance in that they support the maturation of hemopoietic cells within developing colonies. Thus, in a developing erythroid colony, cells pass eventually through their full maturation sequence to form non-nucleated erythrocytes and in granulocytic colonies the colony cells finally mature to readily recognizable polymorphs. Application of stringent tests for maturation has suggested that the maturation achieved in vitro may be slightly incomplete but, for most purposes, colony cells are able to achieve a normal enough sequence of maturation within a time interval expected from in vivo studies.

A wide range of studies has documented that hemopoietic colonies are clones formed by a single cell and in many cases cultures can be