Stem Cell Culture

CHAPTER 92

Stem Cell Culture

Shlomo Wientroub
The Division of Orthopaedics
Sackler School of Medicine, Tel Aviv University
Tel Aviv, Israel
and Department of Pediatric Orthopaedics
Dana Children's Hospital
Tel Aviv Medical Center
Tel Aviv 64239, Israel

Dov Zipori
The Department of Molecular Cell Biology
The Weizmann Institute of Science
Rehovot 76100, Israel

Introduction

The Stem Cell Enigma

Pluripotential hemopoietic stem cells are defined as those cells that give rise to blood cells of all lineages. These cells are also capable of self-renewal, namely, they give rise, upon division, to stem cells with identical properties to the mother cell. An additional prerequisite that was later added states that the stem cell should have the ability to repopulate and reconstitute lethally irradiated recipients. There are various criteria that subdivide stem cells into subpopulations. Thus, like the differentiating and the mature blood cell compartments, the stem cell population is exceedingly variable. The first identification of a stem cell was based on a colony formation assay. Colony-forming unit-spleen (CFU-S) cells are those from the bone marrow that upon transplantation seed to the spleen and within 8–12 days form macroscopic clones (Becker *et al.*, 1963) of erythroid, myeloid, and megakaryocytic cells (McCulloch and Till, 1964). Since some of these colonies also contained secondary CFU-S that could be retransplanted, the CFU-S was proclaimed the pluripotential hemopoietic stem cell. Further studies showed, however, that the cells that are most potent in repopulating irradiated animals are in fact poor colony formers since their response to conditions of demand for proliferation is delayed (Jones *et al.*, 1990). In fact, stem cell differentiation is not induced by cytokines but is dictated by intrinsic factors (Mayani *et al.*, 1993) and the progeny of early stem cells are those that respond to cytokines. This was utilized in cloning assays where the existence of stem cells is monitored by their ability to produce progeny that respond to stimulatory cytokines. Thus, the most primitive stem cells are the ones that are the least proliferative in colony assays. Their detection is difficult; therefore, most culture systems that study and enumerate so-called stem cells are in fact cloning assays. There is an obvious discrepancy between the need to culture and monitor stem cells using fast cloning assays and the innate properties of these cells. This should be kept in mind when selecting an assay system and when interpreting the data obtained.

Pluripotential Hemopoietic Stem Cells

Pluripotential stem cells capable of reconstituting irradiated recipients first appear in embryogenesis in the yolk sac (Huang and Auerbach, 1993) and in paraaortic mesoderm (Muller *et al.*, 1994). Later on, the fetal liver becomes hemopoietic and stem cells with repopulating ability are found there as well (Jordan *et al.*, 1995). Eventually the spleen becomes hemopoietically active and fol-

lowing birth, the bone marrow is the major source of stem cells. In adult bone marrow, long-term repopulating puripotent stem cells are rare (about 1/10,000). Various investigators have utilized the fluorescence-activated cell sorter (FACS) technique to purify stem cells (Spangrude *et al.,* 1988; Visser *et al.,* 1984). The purification of stem cells is based on their expression of some markers including Thy-1, Sca-1, c-kit, CD34, sialic acid residues, H-2K, and Ly5, and on the fact that they do not express a host of other markers that characterize more mature cells, a property used in fractionation procedures to discard the latter (reviewed by Morrison *et al.,* 1995). Recent studies show that stem cell populations purified from the bone marrow could be further subdivided according to low expression of "lineage specific" markers such as Mac-1 and CD4 (Morrison and Weissman, 1994). $CD4^+$ cells were also detected in murine fetal liver (Tocci *et al.,* 1995). An additional method for the fractionation of stem cells is the use of rhodamine 123 (Rh123). Stem cells that are less metabolically active are $Rh123^{low}$ and possess an earlier phenotype (Spangrude and Johnson, 1990). Long-term repopulating cells (LTR) that can reconstitute irradiated recipients for one year were found to be characterized by conditional adhesiveness to plastic and were $rhodamine123^{high}$ and $Sca\text{-}1^{high}$ (Trevisan and Iscove, 1995). Although Thy-1 expression has been used for a long time as a cell surface marker only, this molecule apparently plays a regulatory role in stem cell growth (Mayani and Lansdorp, 1994).

The fetal liver stem cell population is highly proliferative and differs from adult stem cells in that some fetal liver stem cells express the AA4 marker (Jordan *et al.,* 1995). A high proliferative state is not a characteristic feature of early bone marrow stem cells and explains their late response to stimuli. It was found that 96% of purified pluripotent stem cells with long-term repopulating potential, such as $Thy\text{-}1^{lo}$, $Sca\text{-}1^+$, and $Lin^{-/lo}$ cells, are not cycling (Morrison and Weissman, 1994). Studies using retroviral tagging of hemopoietic stem cells indicated that the vast majority of stem cells are quiescent, while only very few cells become actively cycling and contribute to the mature pool (Lemischka *et al.,* 1986).

The Stromal Microenvironment and Dependence of Hemopoietic Stem Cells

The initial attempts to propagate hemopoietic cells in culture failed. The various *in vitro* conditions yielded short-term deteriorating cultures ending with differentiated non-proliferating cells. The identification of cytokines, and particularly of colony-stimulating factors, raised hope that stem cell propagation *in vitro* would depend on appropriate growth factor support. This contention is only partially correct. In addition, the *in vivo* environment in which stem cells reside suggests that they might be regulated by a complex control system. Stem cells are found to localize to specialized domains within tissues and intimately interact with a host of cells termed "stroma." Wolf and Trentin (1968) used the CFU-S assay to study the distribution of spleen colonies. They found that tissue stroma induces a characteristic environment that directs differentiation. Thus, whereas the spleen stroma induced preferentially erythropoietic colonies, the bone marrow stroma induced granulocytic colonies, as could be expected from the predominance of erythropoiesis in spleen and granulopoiesis in bone marrow. The term "hemopoietic inductive microenvironment" was thus coined for which the stromal component of the tissue was believed to be responsible. Experiments in which cultured stroma was retransplanted under the kidney capsule confirmed this supposition, since in the site of injection of stroma, a minute hemopoietic island encapsulated with bone formed (Friedenstein *et al.,* 1974). *In vitro* culture models that followed these pioneering studies showed that long-term hemopoiesis can indeed be maintained *in vitro* for prolonged periods, provided that a confluent, well-developed stromal cell population is present (Dexter *et al.,* 1977a). This system is the best alternative available to date to propagate stem cells in culture. We shall therefore begin by describing primary stromal cell cultures and their utilization for short- and long-term support of stem cell growth and continue by presenting cloning assays for stem cells that depend on cytokines rather than on stromal support.

Cultured Stromal Cells

Primary Stromal Cultures and Fibroblast Colony-Forming Units

Bone marrow cells seeded *in vitro* in medium supplemented with serum tend to survive for a limited period of time. While the addition of growth factors may somewhat prolong the process, nonetheless, the vast majority of hemopoietic cells die or differentiate in the absence of added growth factors and the cultures contain primarily adherent cell layers at the third week of incubation (Zipori and Bol, 1979). An examination of such fixed and stained stromal cell layers indicates that they are primarily composed of colonies of a fibroblast-like morphology and scattered macrophages. A subpopulation of fibroblastoid cells was found to be capable of transferring the hemopoietic environment upon subcapsular kidney transplantation (Friedenstein *et al.,* 1974). The cells forming these clones of fibroblastoid cells were called CFU-F (Klein *et al.,* 1984). Under modified culture conditions, quite different colony morphology has been observed. In methylcellulose-plasma clot cultures supplemented with human plasma and with conditioned medium of phytohemagglutinin (PHA) stimulated mononuclear cells, an extensively branched variant of fibroblastoid colony that also tends to accumulate fat appears. It remains to be seen whether

these 'reticulo-fibroblastoid' colonies are derived from a progenitor (CFU-RF) (Aye *et al.,* 1992) distinct form CFU-F. Thus, the fibroblastoid compartment of primary cultures is heterogeneous. Some of this heterogeneity has been documented through morphological examinations of long-term cultures (Dexter, 1979). It is also evidenced by the distinct properties of the cell lines that have been derived form these cultures which differ markedly in their capacity to induce hemopoiesis (Zipori *et al.,* 1985). However, clonal analysis of primary murine stroma suggested the opposite. It was found that all the primary stromal clones had a similar capacity to support the growth of both lymphoid and myeloid cell lines (Deryugina *et al.,* 1994). These primary stromal clones were derived by stimulation with the conditioned medium of the same specific stromal cell line which may by itself induce selection, as evidenced in the experiments utilizing conditioned media that induces the formation of CFU-RF colonies.

Human CFU-F bear an antigen recognized by the antibody STRO-1. Stromal precursors were isolated from human bone marrow using the STRO-1 antibody and their growth requirements have been defined under serum-free culture conditions. Human CFU-F are dependent for growth in serum-free medium on platelet-derived growth factor (PDGF), epidermal growth factor (EGF), L-ascorbate and dexamethasone (Gronthos and Simmons, 1995). These purified stromal cells had an osteogenic potential as evidenced by deposition of hydroxyapatite (Gronthos *et al.,* 1994). This characteristic was originally observed when studying primary stromal cultures transplanted *in vivo* (Friedenstein *et al.,* 1987) and bone formation *in vivo* induced by clonal populations of stromal cells (Benayahu *et al.,* 1989, 1991, 1992, 1994; Fried *et al.,* 1993).

Stromal Cell Lines

The cells in fibroblastoid colonies and their precursors have limited growth potential but can, under certain conditions, be adapted for long-term *in vitro* growth. Various laboratories have raised cell lines with different properties, either from primary cultures of stromal cells or from Dexter or Whitlock-Witte bone marrow cultures (Dexter *et al.,* 1977a; Whitlock and Witte 1982, and reviewed by Deryugina and Müller-Sieburg, 1993). The methods used varied considerably. The MBA cell line series (Zipori *et al.,* 1985) was derived by maintaining primary cultures for prolonged periods without transfer. When signs of overgrowth were observed, the cultures were split by scraping with a rubber policeman to avoid selection of cells that do not require cell contact for survival. The resulting cell lines developed an ability to survive at confluence for many months of incubation. Other cell lines prepared by repeated subculturing of cells transformed by SV40 tend to overgrow and detach upon confluence (Novotny *et al.,* 1990). Stromal cell lines vary considerably in their properties and only a few are capable of supporting long-term hemopoiesis (Zipori and Lee, 1988). The molecular basis for these differences has not been established. It is not clear whether the cells that support hemopoiesis express a function missing in the non-supportive cells or whether the latter possess an inhibitory activity absent in the former.

Stromal cell lines have been derived from various organs such as the spleen (Gimble *et al.,* 1989), thymus (Tamir *et al.,* 1987) and from the yolk sac (Yoder *et al.,* 1994), which is the primary site of hemopoiesis in the embryo. Like the bone marrow stromal cell lines, some of the above were also found to induce hemopoiesis. The yolk sac cell lines were prepared from endoderm and mesoderm cell layers and harbored properties of their tissues of origin. These cell lines supported proliferation of hemopoietic progenitor cells. Certain thymus stroma lines, as well as cell lines from nonhemopoietic organs (e.g., lung), could also induce hemopoiesis in culture. It is therefore possible that cells with this phenotype are common in the organism and that the characteristic feature of hemopoietic organs is the frequency, rather than the mere presence, of these cells. Their presence in various tissues may explain the mechanism of extramedullary hemopoiesis following bone marrow damage.

The dogmatic concept that stromal cells are distinct from hemopoietic progenitors has to be reevaluated in view of observations of a common progenitor for stromal and hemopoietic cells. Stromal cell lines from dog bone marrow were found to give rise to $CD34^{+}$c-kit^{+} cells that further differentiate to either CFU-F or CFU-GM (Huss *et al.,* 1995). The clonal relationship between the fibroblastoid and hemopoietic descendants has not been established, but nonetheless, the results imply that in the dog, some stromal cells may give rise to hemopoietic cells.

Stroma-Supported Hemopoietic Cultures

Hematons

Multicellular complexes made of intimately associated stromal cells and hemopoietic cells have been observed in dissociated mouse and human bone marrow. These structures were found in normal human bone marrow, but were absent in bone marrow aspirates of chronic and acute myeloid leukemia patients (Blazsek *et al.,* 1990). This finding suggested that the structures, termed hematons (Blazsek *et al.,* 1995), may have a physiological function which is missing in certain hemopoietic diseases. The hematon is made of a web of endothelial cells, fibroblasts, preadipocytes, and macrophages packed with myeloid, erythroid, and megakaryocytic cells. Analysis of stem cell populations showed that the hematon contains cells with marrow-repopulating ability (MRA), CFU-S forming colonies at day 8 following injection (CFU-S-8) and CFU-S-12, high proliferative potential-colony forming cells (HPP-CFC) as well as less primitive progenitors. *In vitro*

culture of hematons is followed by spread of the stromal component followed by migration of progenitor cells. The number of the latter tends to decline with time. Thus, hematons may represent a functional unit of the bone marrow microenvironment, which features the interactions between stem cells and stroma. These interactions are commonly studied by *in vitro* models in which dissociated bone marrow suspensions are used to reconstruct structures that may relate to the hematon and the bone marrow microenvironment *in vivo*.

Hemopoietic Colony Assays Supported by Primary Stromal Cells

Two Phase Assays Demonstrating Production of Novel Stromal Modulators

Cytokine-induced proliferation and differentiation of bone marrow cells has been studied in sparse cell, liquid or semisolid cultures. The latter have the advantage of allowing clonal analysis (Bradley and Metcalf, 1966; Pluznik and Sachs, 1965). Similar studies were carried out using adherent stromal layers onto which hemopoietic cells were seeded. The use of methylcellulose cultures and cultures in which the stromal compartment was separated from the hemopoietic cells by an agar barrier showed that the stroma itself, in the absence of added factors, secretes hemopoietic modulators, some of which appeared to be of a novel nature. Primary stroma secreted activities that induced the formation of small hemopoietic colonies. The incidence and size of these colonies markedly increased when colony-stimulating activity was exogenously added or endogenously induced, indicating that the stroma produced a synergistic activity (Zipori and Sasson, 1981). It was further proposed that this activity acts on early stem cells. Such a molecule, steel factor (SLF), was, in fact, later isolated (Anderson *et al.,* 1990; Flanagan and Leder, 1990; Huang *et al.,* 1990; Zsebo *et al.,* 1990) and shown to be a stroma-derived synergistic cytokine that acts on early stem cells.

Despite the years that have elapsed since the first demonstration of stromal effects on hemopoiesis, the question of whether stroma produces molecules other than those that have already been identified, and are responsible for induction of stem cell attraction, attachment, renewal and differentiation, is still unresolved. A modification of the original two phase cultures involves the use of microporous membranes to separate the stroma from the hemopoietic cells (Burroughs *et al.,* 1994). Relatively early stem cells have been maintained in such cultures supported by either human stroma or mouse and human cell lines. The diffusable factors that may account for this phenomenon have not yet been identified. Production of a stem cell chemotactic factor, of unknown nature, has also been observed (Cherry *et al.,* 1994).

Direct seeding of hemopoietic cells onto the stroma resulted in the formation of colonies within the stroma. It has been shown that there is a limited number of sites per unit area of primary stromal layers that can accommodate the formation of these colonies. These stroma-induced colonies were totally independent and unaffected by the addition of exogenous cytokines (Zipori and Sasson, 1981).

Cobblestone Area Formation Assay

The colonies formed by directly seeding bone marrow cells onto the stroma have been termed "cobblestone areas." They are identified in phase contrast microscopy by their flattened and dark appearance, due to the fact that the cells are actually positioned underneath the stroma. These cells are relatively immature and proliferative. As they mature, they migrate to the surface of the stromal layer, forming colonies of more mature cells that have bright microscopic appearance. The phenomenon of cobblestone area formation was used by Ploemacher *et al.* (1991) for the development of an assay system that monitors the stem cell population. Since the production of more differentiated descendants correlated with the presence and incidence of the cobblestone areas, it was anticipated that early stem cells account for this phenomenon. The assay is a limiting dilution analysis of colony formation by bone marrow cells seeded onto irradiated stroma in microtiter well plates. The cells forming the cobblestone areas (CAFC) are enriched following treatment with 5FU. CAFC that are WGA^{low} and $Rh123^{dull}$ are highly migratory and spread by forming secondary colonies. CAFCs are also found in the $Rh123^{bright}$ and WGA^{bright} population, but form large non-subcolonizing areas (Ploemacher, 1994). Indeed, the CAFC population is highly variable. CAFC that form cobblestone areas 10 days following seeding (CAFC-10) seem to be equivalent to CFU-S-12, while late cobblestone area formers (CAFC-28) contain among them long-term repopulating stem cells. The differences between the various CAFC cells is well demonstrated by their differential adhesion to fibronectin. Whereas most CAFC-10 did not adhere to fibronectin, the majority of CAFC-28/35 did (van der Sluijs *et al.,* 1994). The population of CAFC therefore represents a variety of stem cell types also detectable by *in vivo* assays and was proposed as an *in vitro* substitute for the cumbersome *in vivo* methods.

Stroma-Supported Long-Term Bone Marrow Cultures and Long-Term Initiating Cells

Dexter Culture

Long-term cultures of hemopoietic cells were first successfully carried out by Dexter *et al.* (1977a). The features of these cell cultures that allowed longevity were preformation of a stromal cell layer or the seeding of a relatively high initial cell inoculum combined with careful selection of serum (only some commercial horse serum batches are adequate, although addition of hydrocortisone

was found to increase the incidence of suitable sera), incubation at 33°C and careful monitoring of the culture conditions (Dexter *et al.,* 1984). During the first three weeks of culture, the adherent layer is formed and hemopoietic precursor cells migrate underneath this layer forming cobblestone areas. In the adherent phase of the culture, stromal cells described as blanket cells and adipocytes are common. In addition, macrophages may be found either underneath blanket cells, on top of them or in the inter-stromal cell space. Cobblestone areas of myeloid cells are associated with the blanket cells. As cells differentiate, they migrate to the liquid phase of the culture and are removed with the weekly medium change. These cultures can be maintained for periods of four months or more, continuously producing new hemopoietic stem cells, including stem cells endowed with long-term repopulating ability. This is therefore an *in vitro* model system that most closely approximates the hemopoietic microenvironment of the bone marrow.

The cells that give rise to long-term hemopoiesis have been termed long-term culture-initiating cells (LTC-IC) (Sutherland *et al.,* 1989). Their incidence is determined by limiting dilution analysis in a manner that is similar to the quantitation of CAFC. The two assays may in fact be monitoring the same populations. The early stem cells that initiate the culture or their immediate progeny are not proliferative. This is due to differentiation restraint imposed by the stroma (Zipori and Sasson, 1980). On the other hand, weekly refeeding perturbates the culture, triggering proliferation and differentiation. This implies that some inhibitors produced by the stroma itself are depleted. In addition, the stroma is induced to express cytokine genes such as colony-stimulating factor genes which may be responsible for the triggering of hemopoiesis. Indeed, addition of interleukin (IL)-1β of exogenous source triggers proliferation and differentiation (Cashman *et al.,* 1985).

The Dexter type long-term culture thus supports survival, limited renewal and differentiation of stem cells. Under the conditions described above, the macrophage/granulocytic series is the major component of the cultures, although megakaryocytes are also found in low frequency. Modification in the culture conditions may cause a shift in the lineage representation. Anemic mouse serum induced erythropoiesis probably through the activity of a combination of cytokines (Dexter *et al.,* 1981). As shown in the next section, a modification involving the use of fetal calf serum rather than horse serum resulted in the induction of lymphopoiesis of the B lineage.

WHITLOCK-WITTE CULTURE

In long-term cultures initiated with fetal calf serum, small regular cells accumulate in colonies within the stroma, on the surface of the stroma and in the liquid phase. These cells have been characterized as being mostly pro- and pre-B cells, although B cells could be monitored too (Whitlock and Witte, 1982). Similar production of B lineage cells has been documented when Dexter cultures are switched to fetal calf serum, as may be anticipated from the presence of stem cells and lymphoid progenitors in these cultures. IL-7 accounts for some, but not all, of the pre-B cell growth in these cultures.

Stromal Cell Line Supported Hemopoietic Cultures

MODIFICATION OF THE DEXTER AND WHITLOCK-WITTE CULTURES

The long-term bone marrow cultures, usually performed in large flasks, have been miniaturized in various laboratories for the purpose of frequency analysis of stem cells as described above for CAFC and LTC-IC. Further modifications include the use of stromal cell lines and the design of tridimensional cultures. Long-term cultures of both myeloid and lymphoid cells were performed using clonal populations of stromal cell lines. Particular cell lines were found to support both myeloid and lymphoid differentiation depending on the conditions applied (Zipori and Lee, 1988).

The interactions of stromal cells with hemopoietic progenitors do not require matching of histocompatibility antigens (Dexter *et al.,* 1977b) and thus, human cells may be propagated on mouse stroma (Otsuka *et al.,* 1992). This has recently permitted the development of a culture system for long-term production of human B cell precursors. $CD34^+$ cells were isolated from cord blood and were seeded with a mouse stromal cell line. The majority of the cells that eventually accumulated in those cultures were CD10, CD19, and CD38 positive and did not express membrane IgM. They did express low levels of cytoplasmic μ chain. These cells proliferated for a period exceeding four months (Rawlings *et al.,* 1995).

THYMOCYTE CULTURE

The Dexter and Whitlock-Witte culture systems provide the conditions to study predominantly myeloid or pre-B lymphoid cells, respectively. Variants of these basic culture systems can be adapted for the study of erythropoiesis (Dexter *et al.,* 1981). All of these use primary stromal cells but were modified so that the stromal cell component is provided by a stromal cell line. Long-term bone marrow cultures supported by the 14F1.1 stromal cell line induced long-term hemopoiesis, including maintenance of CFU-S and production of pre-B cells (Zipori and Lee, 1988). The same cell line was also used for propagation of T cell precursor cells. Thymocytes seeded *in vitro* die within a few days of culture. Upon seeding with the 14F1.1 stromal cell line, the incidence of CD4 and CD8 cells gradually declined, while immature cells with thymic repopulating ability selectively survived and their numbers increased (Tamir *et al.,* 1990).

MYELOGENOUS LEUKEMIA CULTURE

Leukemia cells have been a convenient tool for the study of stromal interactions with hemopoietic cells. Stro-

mal cells were found to differentially affect leukemia cell growth in a lineage and differentiation stage-dependent manner. This has led to the identification of restrictin-P/stromal activin A as a specific killer molecule for plasmacytoma cells (Brosh *et al.,* 1995; Sternberg *et al.,* 1995). The myelogenous leukemia cell line M1 interacted with stroma in a fashion reminiscent of stem cells, i.e., forming characteristic cobblestone areas. In co-cultures of M1 cells and the stromal cell line 14F1.1, multinucleated cells with osteoclast properties accumulated (Benayahu *et al.,* 1994). Osteoclast differentiation of progenitors from long-term bone marrow cultures could also be achieved in the absence of stroma using a cytokine combination (Liggett *et al.,* 1994).

One major complication of all stromal cell-supported cultures is the use of serum. As indicated above for the Dexter and Whithlock-Witte culture systems, the nature of the serum used dictates the cell lineage that predominates. However, the study of the interactions between stromal cells and target hemopoietic cells is aimed to simulate the conditions that prevail in the hemopoietic microenvironment. Needless to say, serum is not a major bone marrow microenvironmetal component under normal conditions, but becomes a dominant factor for tissue cells following injury (Dvorak, 1986). In serum-free co-cultures with stromal cells, M1 cells proliferated extensively while maintaining an undifferentiated phenotype and the cell growth exceeded that observed in serum-supplemented cultures (Peled *et al.,* 1996). Such an inhibitory effect of FCS was reported in cultures of human cord blood progenitors (De-Bruyn *et al.,* 1994). The mode of growth of M1 cells with the stroma resembled that of hemopoietic stem cells. The M1 cells formed characteristic cobblestone areas and proliferated primarily within the stromal layer. Part of the growth-promoting effect of the stroma was due to synergy between M-CSF and TGFβ produced by the stroma (Peled *et al.,* 1996). Notably, neither of the latter have previously been known as growth factors for M1 cells. Thus, the use of serum-free co-cultures of the M1 myeloblast with the stroma revealed new growth requirements.

Three-Dimensional Cultures

Wang and Wu (1992) designed a perfusion bioreactor for long-term maintenance of bone marrow cells. The rationale behind building such a system was that it allows the seeding of cells in porous collagen microsphere carriers and thus provides a tridimensional structure for the development of a hemopoietic-like organ *ex vivo.* Since Dexter cultures are made of multilayers of stromal cells engulfing hemopoietic cells, they also are tridimensional to some extent, yet the porous collagen matrix provides a tridimensional structure of much larger volume. By contrast to flask long-term cultures, in the bioreactor system, hemopoietic cells of erythroid, granulocytic, megakaryocytic, and lymphoid lineages were monitored concomitantly. Erythroid, myeloid, and lymphoid cells at various stages of differentiation and maturation were recovered from the bioreactor for eight weeks while megakaryocytes were not detectable after three weeks of culture (Wang *et al.,* 1995). Although the presence of hemopoietic stem cells was not monitored, it is unlikely that hemopoiesis would last two months in the absence of such cells. This system seems to be a superior *ex vivo* model of hemopoiesis; however, it is by far more complex compared to flask cultures and does not allow the study of variables that is possible with flask and microtiter cultures.

Clonogenic *In Vitro* Stem Cell Assays

High Proliferative Potential Colony-Forming Cells

Bradley and Metcalf (1966) and Pluznik and Sachs (1965) were the first to show that hemopoietic cells seeded in semisolid agar cultures supplemented with appropriate factors formed macroscopic colonies. This colony assay system was the basis for the identification of a variety of hemopoietic cytokines, including CSFs, synergistic factors and various inhibitors. The physical and biological properties of the cells forming colonies in culture indicated that they were often unipotential committed progenitors. Yet, it was found that some *in vitro* colony-forming cells are bipotential (such as CFU-GM) and others would be trilineal. Rare cells in mouse bone marrow (about 1/2400) are defined as HPP-CFC since they form giant colonies containing over 50,000 cells. This colony formation, like most other clonogenic hemopoietic assay, requires growth factor stimulation provided, in this case by a combination of cytokines, typically M-CSF and a mixture of other factors secreted by specific cell lines (Bradley and Hodgson, 1979; McNiece *et al.,* 1986). The HPP-CFC population is heterogeneous. Whereas some of these cells (termed HPP-CFC-1) are relatively resistant to 5FU, are multipotential and can give rise to CFU-S (properties which are common to those of early stem cells), others (termed HPP-CFC-2 and 3) are produced from HPP-CFC-1 and are sensitive to 5FU, as expected of more differentiated cell types. As in the case of early stem cells, selection for HPP-CFC requires elimination of mature cells using lineage-restricted markers. HPP-CFC are found among the Rh123 dull population, yet only a fraction HPP-CFC conforms to the pluripotent stem cell criteria detailed above.

Blast Colony-Forming Cells

Blast cells are immature hemopoietic cells characterized by a round shape, a relatively large nucleus with prominent nucleoli and no obvious signs of differentiation in either the nucleus shape or in the cytoplasmic contents. Hemopoietic cells cultured *in vitro* form such colonies. The colonies that appear late and remain as blast colonies

on day 16 in culture have been shown to be derived from quiescent progenitors. Indeed, blast colony-forming cells are enriched by 5FU treatment. Some of the cells accumulating in the blast colonies are multipotential. Like HPP-CFC, these colony formers are stimulated by growth factors. Whereas IL-3, IL-4, and GM-CSF stimulate the growth of blast cells that are already cycling, other cytokines including IL-6, G-CSF, and IL-11, trigger cell cycling of dormant blast colony-forming cells and thus synergistically augment colony formation when added to the former group of cytokines (Ikebuchi *et al.,* 1987; Kishi *et al.,* 1989; Ogawa, 1993; Suda *et al.,* 1985).

CFU-A

These colony-forming cells are quite abundant in the bone marrow (1/500 cells) and as may be expected from this high frequency, the CFU-A assay measures a population that is more differentiated than the cells forming blast colonies and the HPP-CFC-1 cells. Yet, like the latter, CFU-A form larger colonies than those produced by most committed progenitors, and the formation of the colonies is dependent on combinations of cytokines. Conditioned media of specific cell lines (like mouse lung fibroblasts, L929) usually serve as a source of stimulating activity (Pragnell *et al.,* 1988).

Cytokines in Stem Cell Cultures

Long-Term Bone Marrow Cultures

As mentioned above, long-term stroma supported cultures are undoubtedly the best system available to date for maintenance of hemopoietic stem cells *in vitro.* The stromal factors, whether cytokines, ECM components or others, that are required for long-term hemopoiesis have not yet been identified. Stromal cells have been shown to express and secrete a variety of cytokines, but no clear correlation between the cells' ability to support hemopoiesis and the expression of a specific cytokine was noted. Addition of antibodies to TGFβ to long-term murine bone marrow cultures caused an increase in the production of granulocytes, macrophages and megakaryocytes, as well as an increase in number of CFU-S (Waegell *et al.,* 1994). It appears, therefore, that TGFβ, produced in long-term cultures, attenuates proliferation of stem cells, and its removal augments proliferation coupled with differentiation. Similarly, the maintenance of LTC-IC was extended from five to eight weeks by a combination of interleukin-3 and macrophage inhibitory protein (MIP)-1α (Verfaillie *et al.,* 1994), which seems to attenuate differentiation, allowing longer maintenance of precursors. IL-3 alone, expressed by stromal cells (Zipori and Lee, 1988) or added to long-term cultures (Verfaillie *et al.,* 1994), caused exhaustion of hemopoiesis.

Attempts to Maintain Cells in Cytokine Combinations

HPP-CFC-1 were shown to be stimulated *in vitro* by a combination of M-CSF and additional undefined factors in cell line-conditioned media. A detailed study of cytokine mixtures showed that these cells respond to a combination of the cytokines M-CSF, IL-3, and IL-1 and the same mixture supplemented with either SLF, IL-6, or GM-CSF (Kriegler *et al.,* 1994). Conditioned media from specific cell lines (like mouse lung fibroblasts, L929) usually serve as a source of stimulating activity for CFU-A. Sca1+ cells from the bone marrow of mice treated with 5FU were seeded in a combination of IL-11, IL-3, IL-6, and SLF. This combination enhanced the recovery of long-term repopulating ability and dramatically increased the number of progenitors produced by the stem cell population (Neben *et al.,* 1994). The *in vitro* death, by apoptosis, of human CD34+HLA-DR-cKit+ cells was found to be reduced by SLF (Brandt *et al.,* 1994). A combination of SLF with GM-CSF or IL-3 induced the proliferation of purified human bone marrow $CD34^+$ cells for a few weeks in culture while stimulating production of mature progeny (Steen *et al.,* 1994). The use of such cytokine combinations in culture is suggested to be useful for transplantation of purified stem cell populations following expansion *in vitro.* However, this type of manipulation causes a differentiation drift, and the cytokine combination mentioned cannot be said to induce renewal. In fact, Peters *et al.* (1995) showed that bone marrow cells transplanted following *in vitro* expansion failed to establish a graft in an efficiency similar to that of control unexpanded cells.

Several cytokines were reported to negatively influence the accumulation of stem cells and progenitors in culture. In human cord blood cultures stimulated by SLF, IL-3, IL-6, and erythropoietin (Epo), MIP-1α reduced the expansion of the CD34+ population without affecting other cells, while TGFβ inhibited various stages of differentiation. Differential inhibitory effects of MIP-1α and TGFβ were observed with Thy-1^{low} Lin^- cells (Keller *et al.,* 1994). Preferential inhibition of a specific population was also documented for TNFα and β (Mayani *et al.,* 1995). The inhibition of HPP-CFC, which were Sca1+Lin− by TNFα, was mediated through the 55 and the 75-kDa TNF receptors, while the bidirectional effects on growth of more mature progenitors were mediated through the 55 kDa receptor only (Jacobsen *et al.,* 1994; Rusten *et al.,* 1994). Again, none of these various cytokines, either by themselves or in combination with others, have thus far been found to mimic the activity of stromal cells, and further research is required to unravel the minimal requirements for stem cell renewal *in vitro.*

References

Anderson, D. M., Lyman, S. D., Baird, A., Wignall, J. M., Eisenman, J., Rauch, C., March, C. J., Boswell, H. S., Gimpel, S. D., Cosman,

D., and Williams, D. E. (1990). Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. *Cell* **63,** 235–243.

Aye, M. T., Hashemi, S., Leclair, B., Zeibdawi, A., Trudel, E., Halpenny, M., Fuller, V., and Cheng, G. (1992). Expression of stem cell factor and c-kit mRNA in cultured endothelial cells, monocytes and cloned human bone marrow stromal cells (CFU-RF). *Exp. Hematol.* **20,** 523–527.

Becker, A. J., McCulloch, E. A., and Till, J. E. (1963). Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature* **197,** 452–454.

Benayahu, D., Gurevitch, O., Zipori, D., and Wientroub, S. (1994). Bone formation by marrow osteogenic cells (MBA-15) is not accompanied by osteoclastogenesis and generation of hematopoietic supportive microenvironment. *J. Bone Miner. Res.* **9,** 1107–1114.

Benayahu, D., Fried, A., Zipori, D., and Wientroub, S. (1991). Subpopulations of marrow stromal cells share a variety of osteoblastic markers. *Calcif. Tissue Int.* **49,** 202–207.

Benayahu, D., Horowitz, M., Zipori, D., and Wientroub, S. (1992). Hemopoietic functions of marrow-derived osteogenic cells. *Calcif. Tissue Int.* **51,** 195–201.

Benayahu, D., Kletter, Y., Zipori, D., and Wientroub, S. (1989). Bone marrow-derived stromal cell line expressing osteoblastic phenotype *in vitro* and osteogenic capacity *in vivo*. *J. Cell Physiol.* **140,** 1–7.

Benayahu, D., Peled, A., and Zipori, D. (1994). Myeloblastic cell line expresses osteoclastic properties following co-culture with marrow stromal adipocytes. *J. Cell Biochem.* **56,** 374–384.

Blazsek, I., Liu, X. H., Anjo, A., Quittet, P., Comisso, M., Kim, T. B., and Misset, J. L. (1995). The hematon, a morphogenetic functional complex in mammalian bone marrow, involves erythroblastic islands and granulocytic cobblestones. *Exp. Hematol.* **23,** 309–319.

Blazsek, I., Misset, J. L., Benavides, M., Comisso, M., Ribaud, P., and Mathe, G. (1990). Hematon, a multicellular functional unit in normal human bone marrow: structural organization, hemopoietic activity, and its relationship to myelodysplasia and myeloid leukemias. *Exp. Hematol.* **18,** 259–265.

Bradley, T. R., and Hodgson, G. S. (1979). Detection of primitive macrophage progenitor cells in mouse bone marrow. *Blood* **54,** 1446–1450.

Bradley, T. R., and Metcalf, D. (1966). The growth of mouse bone marrow cells *in vitro*. *Aust. J. Exp. Biol. Med. Sci.* **44,** 287–300.

Brandt, J. E., Bhalla, K., and Hoffman, R. (1994). Effects of interleukin-3 and c-kit ligand on the survival of various classes of human hematopoietic progenitor cells. *Blood* **83,** 1507–1514.

Brosh, N., Stenberg, D., Honigwachs-Sha'anani, J., Lee, B-C., Shav-Tal, Y., Tzehoval, E., Shulman, L. M., Toledo, J., Hacham, Y., Carmi, P., Jiang, W., Sasse, J., Horn, F., Burstein, Y., and Zipori, D. (1995). The plasmacytoma growth inhibitor restrictin-P is an antagonist of interleukin-6 and interleukin-11: identification as a stroma derived activin A. *J. Biol. Chem.*, **270,** 29594–29600.

Burroughs, J., Gupta, P., Blazar, B. R., and Verfaillie, C. M. (1994). Diffusible factors from the murine cell line M2-10B4 support human *in vitro* hematopoiesis. *Exp. Hematol.* **22,** 1095–1101.

Cashman, J., Eaves, A. C., and Eaves, C. J. (1985). Regulated proliferation of primitive hematopoietic progenitor cells in long-term human marrow cultures. *Blood* **66,** 1002–1005.

Cherry, Yasumizu, R., Toki, J., Asou, H., Nishino, T., Komatsu, Y., and Ikehara, S. (1994). Production of hematopoietic stem cell-chemotactic factor by bone marrow stromal cells. *Blood* **83,** 964–971.

De-Bruyn, C., Delforge, A., Bron, D., Ley, P., de-Hemptinne, D., and Stryckmans, P. (1994). Modulation of human cord blood progenitor cell growth by recombinant human interleukin 3 (IL-3), IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and stem cell factor (SCF) in serum-supplemented and serum-free medium. *Stem Cells* **12,** 616–625.

Deryugina, E. I., and Müller-Sieburg, C. E. (1993). Stromal cells in long-term cultures: keys to the elucidation of hematopoietic development? *Crit. Rev. Immunol.* **13,** 115–150.

Deryugina, E. I., Ratnikov, B. I., Bourdon, M. A., and Müller-Sieburg, C. E. (1994). Clonal analysis of primary marrow stroma: functional homogeneity in support of lymphoid and myeloid cell lines and identification of positive and negative regulators. *Exp. Hematol.* **22,** 910–918.

Dexter, T. M. (1979). Haemopoiesis in long-term bone marrow cultures: A review. *Acta Haematol.* **62,** 299–305.

Dexter, T. M., Allen, T. D., and Lajtha, L. G. (1977a). Conditions controlling the proliferation of haemopoietic stem cells *in vitro*. *J. Cell Physiol.* **91,** 335–344.

Dexter, T. M., Moore, M. A., and Sheridan, A. P. (1977b). Maintenance of hemopoietic stem cells and production of differentiated progeny in allogeneic and semiallogeneic bone marrow chimeras *in vitro*. *J. Exp. Med.* **145,** 1612–1616.

Dexter, T. M., Spooncer, E., Simmons, P., and Allen, T. D. (1984). Long-term marrow culture: An overview of techniques and experience. *In* "Long-term bone marrow culture" (D. G. Wright and J. S. Greenberg, ed.), pp. 57–96, Alan R. Liss, New York.

Dexter, T. M., Testa, N. G., Allen, T. D., Rutherford, T., and Scolnick, E. (1981). Molecular and cell biologic aspects of erythropoiesis in long-term bone marrow cultures. *Blood* **58,** 699–707.

Dvorak, H. F. (1986). Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N. Engl. J. Med.* **315,** 1650–1659.

Flanagan, J. G., and Leder, P. (1990). The kit ligand: a cell surface molecule altered in steel mutant fibroblasts. *Cell* **63,** 185–194.

Fried, A., Benayahu, D., and Wientroub, S. (1993). Marrow stroma-derived osteogenic clonal cell lines: Putative stages in osteoblastic differentiation. *J. Cell. Physiol.* **155,** 472–482.

Friedenstein, A. J., Chailakhjan, R. K., Latsinik, N. V., Panasyuk, A. F., and Keliss-Borok, I. V. (1974). Stromal cells responsible for transferring the microenvironment of hematopoietic tissues. *Transplantion* **17,** 331–340.

Friedenstein, A. J., Chailakhyan, R. K., and Gerasimov, U. V. (1987). Bone marrow osteogenic stem cells: *in vitro* cultivation and transplantation in diffusion chambers. *Cell Tissue Kinet.* **20,** 263–272.

Gimble, J. M., Pietrangeli, C., Henley, A., Dorheim, M. A., Silver, J., Namen, A., Takeichi, M., Goridis, C., and Kincade, P. W. (1989). Characterization of murine bone marrow and spleen-derived stromal cells: analysis of leukocyte marker and growth factor mRNA transcript levels. *Blood* **74,** 303–311.

Gronthos, S., Graves, S. E., Ohta, S., and Simmons, P. J. (1994). The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors. *Blood* **84,** 4164–4173.

Gronthos, S., and Simmons, P. J. (1995). The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions *in vitro*. *Blood* **85,** 929–940.

Huang, E., Nocka, K., Beier, D. R., Chu, T. Y., Buck, J., Lahm, H. W., Wellner, D., Leder, P., and Besmer, P. (1990). The hematopoietic growth factor KL is encoded by the Sl locus and is the ligand of the c-kit receptor, the gene product of the W locus. *Cell* **63,** 225–233.

Huang, H., and Auerbach, R. (1993). Identification and characterization of hematopoietic stem cells from the yolk sac of the early mouse embryo. *Proc. Natl. Acad. Sci. USA* **90,** 10110–10114.

Huss, R., Hong, D. S., McSweeney, P. A., Hoy, C. A., and Deeg, H. J. (1995). Differentiation of canine bone marrow cells with hemopoietic characteristics from an adherent stromal cell precursor. *Proc. Natl. Acad. Sci. USA* **92,** 748–752.

Ikebuchi, K., Wong, G. G., Clark, S. C., Ihle, J. N., Hirai, Y., and Ogawa, M. (1987). Interleukin 6 enhancement of interleukin 3-dependent proliferation of multipotential hemopoietic progenitors. *Proc. Natl. Acad. Sci. USA* **84,** 9035–9039.

Jacobsen, F. W., Rothe, M., Rusten, L., Goeddel, D. V., Smeland, E. B., Veiby, O. P., Slordal, L., and Jacobsen, S. E. (1994). Role of the 75-kDa tumor necrosis factor receptor: inhibition of early hematopoiesis. *Proc. Natl. Acad. Sci. USA* **91,** 10695–10699.

Jones, R. J., Wagner, J. E., Celano, P., Zicha, M. S., and Sharkis, S. J.

(1990). Separation of pluripotent haematopoietic stem cells from spleen colony-forming cells. *Nature* **347,** 188–189.

Jordan, C. T., Astle, C. M., Zawadzki, J., Mackarehtscian, K., Lemischka, I. R., and Harrison, D. E. (1995). Long-term repopulating abilities of enriched fetal liver stem cells measured by competitive repopulation. *Exp. Hematol.,* **23,** 1011–1015.

Keller, J. R., Bartelmez, S. H., Sitnicka, E., Ruscetti, F. W., Ortiz, M., Gooya, J. M., and Jacobsen, S. E. (1994). Distinct and overlapping direct effects of macrophage inflammatory protein-1 alpha and transforming growth factor beta on hematopoietic progenitor/stem cell growth. *Blood* **84,** 2175–2181.

Kishi, K., Ihle, J. N., Urdal, D. L., and Ogawa, M. (1989). Murine B-cell stimulatory factor-1 (BSF-1)/interleukin-4 (IL-4) is a multilineage colony-stimulating factor that acts directly on primitive hemopoietic progenitors. *J. Cell Physiol.* **139,** 463–468.

Klein, A. K., Rosenblatt, L. S., Stitzel, K. A., Greenberg, B., and Woo, L. (1984). *In vitro* radiation response studies on bone marrow fibroblasts (CFU-F) obtained from normal and chronically irradiated dogs. *Leuk. Res.* **8,** 473–481.

Kriegler, A. B., Verschoor, S. M., Bernardo, D., and Bertoncello, I. (1994). The relationship between different high proliferative potential colony-forming cells in mouse bone marrow. *Exp. Hematol.* **22,** 432–440.

Lemischka, I. R., Raulet, D. H., and Mulligan, R. C. (1986). Developmental potential and dynamic behavior of hematopoietic stem cells. *Cell* **45,** 917–927.

Liggett, W. J., Lian, J. B., Greenberger, J. S., and Glowacki, J. (1994). Osteocalcin promotes differentiation of osteoclast progenitors from murine long-term bone marrow cultures. *J. Cell Biochem.* **55,** 190–199.

Mayani, H., Dragowska, W., and Lansdorp, P. M. (1993). Lineage commitment in human hemopoiesis involves asymmetric cell division of multipotent progenitors and does not appear to be influenced by cytokines. *J. Cell Physiol.* **157,** 579–586.

Mayani, H., and Lansdorp, P. M. (1994). Thy-1 expression is linked to functional properties of primitive hematopoietic progenitor cells from human umbilical cord blood. *Blood* **83,** 2410–2417.

Mayani, H., Little, M. T., Dragowska, W., Thornbury, G., and Lansdorp, P. M. (1995). Differential effects of the hematopoietic inhibitors MIP-1 alpha, TGF-beta, and TNF-alpha on cytokine-induced proliferation of subpopulations of $CD34^+$ cells purified from cord blood and fetal liver. *Exp. Hematol.* **23,** 422–427.

McCulloch, E. A., and Till, J. E. (1964). Proliferation of hemopoietic colony-forming cells transplanted into irradiated mice. *Rad. Res.* **22,** 383–397.

McNiece, I. K., Bradley, T. R., Kriegler, A. B., and Hodgson, G. S. (1986). Subpopulations of mouse bone marrow high-proliferative-potential colony-forming cells. *Exp. Hematol.* **14,** 856–860.

Morrison, S. J., Uchida, N., and Weissman, I. L. (1995). The biology of hematopoietic stem cells. *Annu. Rev. Cell Dev. Biol.* 35–71.

Morrison, S. J., and Weissman, I. L. (1994). The long-term repopulating subset of hemopoietic stem cells is deterministic and isolatable by phenotype. *Immunity* **1,** 661–673.

Muller, A. M., Medvinsky, A., Strouboulis, J., Grosveld, F., and Dzierzak, E. (1994). Development of hematopoietic stem cell activity in the mouse embryo. *Immunity* **1,** 291–301.

Neben, S., Donaldson, D., Sieff, C., Mauch, P., Bodine, D., Ferrara, J., Yetz-Aldape, J., and Turner, K. (1994). Synergistic effects of interleukin-11 with other growth factors on the expansion of murine hematopoietic progenitors and maintenance of stem cells in liquid culture. *Exp. Hematol.* **22,** 353–359.

Novotny, J. R., Duehrsen, U., Welch, K., Layton, J. E., Cebon, J. S., and Boyd, A. W. (1990). Cloned stromal cell lines derived from human Whitlock/Witte-type long-term bone marrow cultures. *Exp. Hematol.* **18,** 775–784.

Ogawa, M. (1993). Differentiation and proliferation of hematopoietic stem cells. *Blood* **81,** 2844–2853.

Otsuka, T., Satoh, H., Ogo, T., Bairy, O., Gluck, U., Zipori, D., Nakano, T., Okamura, S., and Niho, Y. (1992). Long-term survival of human myeloid progenitor cells induced by a mouse bone marrow stromal cell line. *Int. J. Cell Cloning* **10,** 153–160.

Peled, A., Lee, B.-C., Sternberg, D., Toledo, J., Aracil, M., and Zipori, D. (1996). Interactions between leukemia cells and bone marrow stromal cells: stroma-supported growth versus serum dependence and the role of TGFβ and M-CSF. *Exp. Hematol.,* **24,** 728–787.

Peters, S. O., Kittler, E. L. W., Ramshaw, H. S., and Quesenberry, P. J. (1995). Murine marrow cells expanded in culture with IL-3, IL-6, IL-11, and SCF acquire an engraftment defect in normal hosts. *Exp. Hematol.* **23,** 461–469.

Ploemacher, R. E. (1994). Cobblestone area-forming cell (CAFC) assay. In: Culture of Hematopoietic Cells (R. I. Freshney, I. B. Pragnell, M. G. Freshney, ed.), Wiley-Liss, New York, pp. 1–21.

Ploemacher, R. E., Van-der Sluijs, J. P., Van-Beurden, C. A., Baert, M. R., and Chan, P. L. (1991). Use of limiting-dilution type long-term marrow cultures in frequency analysis of marrow-repopulating and spleen colony-forming hematopoietic stem cells in the mouse. *Blood* **78,** 2527–2533.

Pluznik, D. H., and Sachs, L. (1965). The cloning of normal "mast" cells in tissue culture. *J. Cell. Comp. Physiol.* **66,** 319–324.

Pragnell, I. B., Wright, E. G., Lorimore, S. A., Adam, J., Rosendaal, M., DeLamarter, J. F., Freshney, M., Eckmann, L., Sproul, A., and Wilkie, N. (1988). The effect of stem cell proliferation regulators demonstrated with an *in vitro* assay. *Blood* **72,** 196–201.

Rawlings, D. J., Quan, S. G., Kato, R. M., and Witte, O. N. (1995). Long-term culture system for selective growth of human B-cell progenitors. *Proc. Natl. Acad. Sci. USA* **92,** 1570–1574.

Rusten, L. S., Jacobsen, F. W., Lesslauer, W., Loetscher, H., Smeland, E. B., and Jacobsen, S. E. (1994). Bifunctional effects of tumor necrosis factor alpha (TNF alpha) on the growth of mature and primitive human hematopoietic progenitor cells: involvement of p55 and p75 TNF receptors. *Blood* **83,** 3152–3159.

Spangrude, G. J., Heimfeld, S., and Weissman, I. L. (1988). Purification and characterization of mouse hematopoietic stem cells. *Science* **241,** 58–62.

Spangrude, G. J., and Johnson, G. R. (1990). Resting and activated subsets of mouse multipotent hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA* **87,** 7433–7437.

Steen, R., Morkrid, L., Tjonnfjord, G. E., and Egeland, T. (1994). c-kit ligand combined with GM-CSF and/or IL-3 can expand CD34+ hematopoietic progenitor subsets for several weeks *in vitro. Stem Cells* **12,** 214–224.

Sternberg, D., Honigwachs-Sha'anani, J., Brosh, N., Malik, Z., Burstein, Y., and Zipori, D. (1995). Restrictin-P/stromal activin A, kills its target cells via an apoptotic mechanism. *Growth Factors,* **12,** 277–287.

Suda, T., Suda, J., Ogawa, M., and Ihle, J. N. (1985). Permissive role of interleukin 3 (IL-3) in proliferation and differentiation of multipotential hemopoietic progenitors in culture. *J. Cell Physiol.* **124,** 182–190.

Sutherland, H. J., Eaves, C. J., Eaves, A. C., Dragowska, W., and Lansdorp, P. M. (1989). Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis *in vitro. Blood* **74,** 1563–1570.

Tamir, M., Eren, R., Globerson, A., Kedar, E., Epstein, E., Trainin, N., and Zipori, D. (1990). Selective accumulation of lymphocyte precursor cells mediated by stromal cells of hemopoietic origin. *Exp. Hematol.* **18,** 322–340.

Tamir, M., Rozenszajn, L. A., Malik, Z., and Zipori, D. (1987). Thymus-derived stromal cell lines. *Int. J. Cell Cloning* **5,** 289–301.

Tocci, A., Rezzoug, F., Wahbi, K., and Touraine, J. L. (1995). Fetal liver generates low CD4 hematopoietic cells in murine stromal cultures. *Blood* **85,** 1463–1471.

Trevisan, M., and Iscove, N. N. (1995). Phenotypic analysis of murine long-term hemopoietic reconstituting cells quantitated competitively *in vivo* and comparison with more advanced colony-forming progeny. *J. Exp. Med.* **181,** 93–103.