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Stem Cell Review

Haemopoietic stem cells: their heterogeneity and regulation

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Summary. The maintenance of the various blood cell populations in mammals is achieved by the proliferation and differentiation of precursor cells located primarily in the bone marrow. These precursor cells are all derived from a common haemopoietic stem cell population that is established during embryogenesis and functions for the lifetime of the organism. An overview is given of the various assay systems for haemopoietic stem cells, how these assays have contributed to understanding the considerable heterogeneity within the stem cell compartment, the regulation of stem cells and the development of the haemopoietic system.

Keywords: haemopoiesis, stem cells, growth factors, inhibitors, differentiation, development

The concept of the pluripotential haemopoietic stem cell was introduced by Maximow (1924) who postulated that all blood cells were derived from a single class of progenitor cell. In 1951 it was reported that lethally irradiated mice could be rescued by injecting bone marrow from a syngeneic donor (Lorenz *et al.* 1951) and this observation provided the basis of the experimental evidence for a pluripotential haemopoietic stem cell. The formal demonstration of stem cell function came when it was shown that a single chromosomally marked cell clone could repopulate the haemopoietic system of an irradiated recipient mouse (Ford *et al.* 1956). Lymphomyeloid repopulation has been confirmed by many workers using chromsomally marked donor cells (see, for example Wu *et al.* 1967; Abramson *et al.* 1977) and more recently these findings were reinforced using retroviral gene markers (Dick *et al.* 1985; Jordan & Lemischka 1990; Lemischka *et al.* 1986; Keller & Snodgrass 1990; Szilvassy *et al.* 1989).

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A stem cell in the mouse defined as a spleen colony-forming unit (cfu-s)

In addition to its differentiation potential, a defining feature of a stem cell is its capacity for reproduction (self-renewal) to produce daughter cells that have the same or very similar proliferative and developmental potential as the original parental cell (Lajtha 1979a, 1979b). For many years in extensive studies of haemopoiesis in the mouse, spleen colony-forming units (CFU-S) had been widely regarded as the cells most closely fitting this definition due to their considerable proliferative potential, self-renewal characteristics and capacity for multilineage differentiation Till & McCulloch (1961, 1980). The CFU-S assay is based on the ability of stem cells present in transplanted bone marrow to recue a potentially lethally irradiated recipient by colonizing the ablated haemopoietic tissues. Within the first two weeks post-transplantation, clonogenic cells (operationally defined as CFU-S) seed into the spleen where they produce clonal descendants readily visible as colonies and containing differentiating haemopoietic cells. Only a fraction

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(the 'f number') of the potential spleen colony-forming cells (CFC-S) actually arrive in the spleen and produce a colony (Till *et al.* 1964) and these are defined as CFU-S. The linear relationship between the number of cells injected and the number of colonies produced provides a convenient quantitative assay for these multipotential cells. If the colonies are disaggregated and injected into secondary recipient mice, spleen colonies are produced (Siminovitch *et al.* 1963) demonstrating that, in generating the primary colonies, the CFU-S also produce daughter cells with the properties of CFU-S, i.e. they exhibit self-renewal. Transplantation of a known number of CFU-S present in a primary recipient spleen into secondary recipient mice can be used to determine the proportion of these cells that produce colonies in the secondary spleens. In this way, the seeding factor, or 'f-number' was first determined by (Siminovitch *et al.* 1963) and in a number of subsequent studies by many workers the seeding factor has been reported to be of the order of 0.05–0.1; i.e. only about 5–10% of the injected CFC-S actually produce spleen colonies (see Lord & Schofield 1985). Many workers have reported that, unlike their immediate daughter cells (the lineage-committed progenitors), in the steady state, the CFU-S population is a minimally proliferating population (see, for example, Becker *et al.* 1965; Wright & Lorimore 1987) with the majority of the cells in a G_0 state and this has been widely regarded as a characteristic of haemopoietic stem cells (Lajtha 1979a, 1979b).

Heterogeneity of CFU-S and the identification of a pre-CFU-S

A comprehensive definition of stem cells has been provided by (Potten & Loeffler 1990) who consider them as cells 'capable of (a) proliferation, (b) self-maintenance, (c) the production of a larger number of differentiated, functional progeny, (d) regenerating the tissue after injury and (e) flexibility in the use of these options'. The repopulating potential of stem cells is particularly relevant to haemopoiesis as it is now clear that spleen colony-forming cells are heterogeneous with respect to many biophysical and biological properties and most do not have extensive self-renewal capacity and long-term repopulating potential. In fact, in the early studies of CFU-S self-renewal (Siminovitch *et al.* 1963) it was clearly demonstrated that the distribution of CFU-S per primary colony was very heterogeneous (ranging from 0 to >1000) and that the variation from colony to colony was not due to sampling error alone (Till *et al.* 1964). The heterogeneity was explained by the balance between the two competing properties of stem cells, i.e. self-renewal ('birth') vs. differentiation ('death') such that the variable composition was assumed to reflect the fluctuation in the probability of birth vs. the probability of death. Support for this model was provided by the similarity of the Gaussian distribution curve of CFU-S per colony to the theoretical distribution generated by Monte Carlo statistics. (Vogel *et al.* 1968, 1969) and more recently by *in vitro* studies of stem cell differentiation (Ogawa *et al.* 1983, 1989). It has been demonstrated by many workers that proliferative stress provided by serial transplantation significantly reduces self-renewal capacity suggesting that the stem cell compartment may 'age' and thus have a finite lifespan (Schofield 1978) albeit a lifespan well in excess of the normal physiological requirements of the organism.

In studies of CFU-S self-renewal, not only was there heterogeneity in the number of CFU-S per colony but transplantation of day 14 rather than day 8 colonies produced a greater number of secondary colonies. At the time the interpretation of this finding was that the extra few days allowed greater self-renewal (Lewis & Trobaugh 1964). Histological studies of spleen colonies also revealed differences between colonies scored at different times. Colonies examined 7–9 days post-transplantation generally contained cells of only one lineage – usually erythroid – while colonies examined 12–14 days post-transplantation contained cells of more than one lineage (Curry & Trentin 1967). As the number of spleen colonies generated by normal bone marrow is constant from 8 to 14 days it was assumed that the data reflected cellular evolution within the colonies. However, later research revealed that the majority of early appearing colonies disappear after a few days and that at least half of the colonies scored on day 11–13 are not the same colonies as those scored on day 7–8 (Magli et 1982; Harris *et al.* 1984; Priestley & Wolf 1985; Wolf & Priestly 1986). Although it has been suggested that these early and late appearing colonies reflect kinetic heterogeneity (Blackett 1987) and many experiments have clearly demonstrated kinetic heterogeneity of colony formation by CFU-S (particularly in marrow obtained from mice treated with cytotoxic agents) (see, for example, Hodgson & Bradley 1977; Rosendaal *et al.* 1979; Van Zant 1984; Lord *et al.* 1989), developments in cell separation techniques led to the physical isolation of subsets of cells capable of generating spleen colonies 8 or 12 days post-transplantation (Harris *et al.* 1984; Visser *et al.* 1984). Studies of proliferative status also demonstrated differences as many of the cells producing the early appearing colonies were actively proliferating (Hodgson *et al.* 1982) whereas, as reported by many workers, the late-appearing CFU-S were a minimally

proliferating population with most cells in a G_0 state. Consistent with these observations were the studies documenting differing sensitivities to stimulatory and inhibitory proliferation signals of the CFU-S producing colonies 7, 10 or12 days post-transplantation (Wright *et al.*1985). All these various data are consistent with the 'generation-age hypothesis' (Rosendaal *et al.* 1979) that proposes an age-structured hierarchical CFU-S compartment in which developmentally early or 'young' CFU-S with considerable self-renewal capacity in subsequent divisions produce 'older' CFU-S with decreasing self-renewal capacity and increasing probability of commitment to differentiation.

Developments in cell sorting techniques led to further demonstration of stem cell heterogeneity and an important development was the use of rhodamine-123, a supravital, cationic, fluorescent dye with a relatively high affinity for mitochondrial membranes. Day-12 CFU-S that stain poorly ($Rho123^{dull}$) were shown to have a greater marrow repopulating ability than brightly staining ($Rho123^{bright}$) day-12 CFU-S (Bertoncello *et al.* 1989) and only about half of the $Rho123^{dull}$ day-12 CFU-S had radioprotective ability (Mulder & Visser 1987). Interestingly, the weak staining of stem cells with rhodamine-123 seems to be due not only to their smaller and less active mitochondria but also to the expression of the MDR-1 gene (Chaudhary & Roninson 1991). The MDR-1 gene encodes a membrane protein (Pgp), which is an efflux pump that efficiently pumps out the dye. This finding might also explain the earlier observation (Baines & Visser 1983) that stem cells are poorly stained with the fluorescent dye Hoechst 33342. Using a Rhodamine-123 sorting protocol, Ploemacher & Brons (1988, 1989), separated cells forming spleen colonies from cells responsible for reconstitution of the bone marrow stem cell compartment following irradiation. These experiments demonstrated for the first time, and without the application of negative selection by the use of cytotoxic agents, that marrow repopulating cells can be separated from day-12 CFU-S and should be considered as pre-CFU-S.

Using a combination of negative and positive selection with immunomagnetic beads and fluorescence-activated cell sorting that did not include rhodamine-123, coenrichment of day-12 CFU-S and cells providing 30-day radioprotection has been reported (Spangrude *et al.* 1988). These authors have demonstrated that <100 marrow cells, isolated by their lack of lineage specific markers and the expression of the Thy-1 and Sca-1 antigens, would produce long-term repopulation of all lymphomyeloid lineages in mice, although only a subset undergoes long-term self-renewal (Smith *et al.* 1991). The long-term self-renewing cell has been purified by sorting cells on the basis of multiple antigenic properties (Morrison & Weissman 1994).

Long-term repopulation of the mouse as a measure of stem cell function

That pre-CFU-S are the major marrow repopulating cells has received support from experiments demonstrating that 5-fluorouracil, a drug that preferentially spares pre-CFU-S (Hodgson & Bradley 1979), also spares cells responsible for long-term myeloid and lymphoid repopulating in a competitive repopulation assay (Lerner & Harrison 1990). Furthermore, it has been well established that the repopulating abilities of transplanted haemopoietic cells are all less than expected from assays of their CFU-S content (Micklem *et al.* 1975; Harrison & Astle 1982; Kretchmar & Conover 1970) and estimates of pluripotential stem cell numbers are 10–100 times lower than predicted from CFU-S concentrations (Boggs *et al.* 1982; Micklem *et al.* 1987; Harrison *et al.* 1988; Nakano *et al.* 1989). In radiation chimeras where the donor and host cells are readily distinguished, the numbers and dispersion of repopulating clones can be estimated using binomial statistics. Using isoenzyme markers, Micklem *et al.* (1987) calculated the frequency of pluripotential stem cells in the donor bone marrow to be about 1 in 40 000. A similar figure (approximately 1 per 10^5 normal mouse bone marrow cells) was obtained by transplanting a mixture of marrow cells of different isoenzyme types and measuring the proportions of the two types of differentiated cells to determine the stem cell number (Harrison *et al.* 1988). Using this approach, the number of stem cells depends inversely of the variability in the percentage of donor cell types between the different recipients. A similar competitive repopulation strategy has been described in which limiting numbers of male 'test' cells are transplanted into lethally irradiated female syngeneic recipients together with syngeneic female marrow cells whose long-term repopulating ability had been compromised by previous transplantation. Using Poisson statistics, the proportion of assay recipients whose repopulated haemopoietic tissues contain >5% cells of test origin 5 weeks or more post-transplantation is used to calculate the frequency of 'Competitive Repopulating Units' (CRU), i.e. the number of stem cells capable of long-term repopulation (Szilvassy *et al.* 1990). The incidence of this cell (approximately 1 per 10^5 marrow cells) is comparable to the frequency estimated in the other studies and the lymphoid and myeloid lineages seem to diverge at this pre-CFU-S stage.

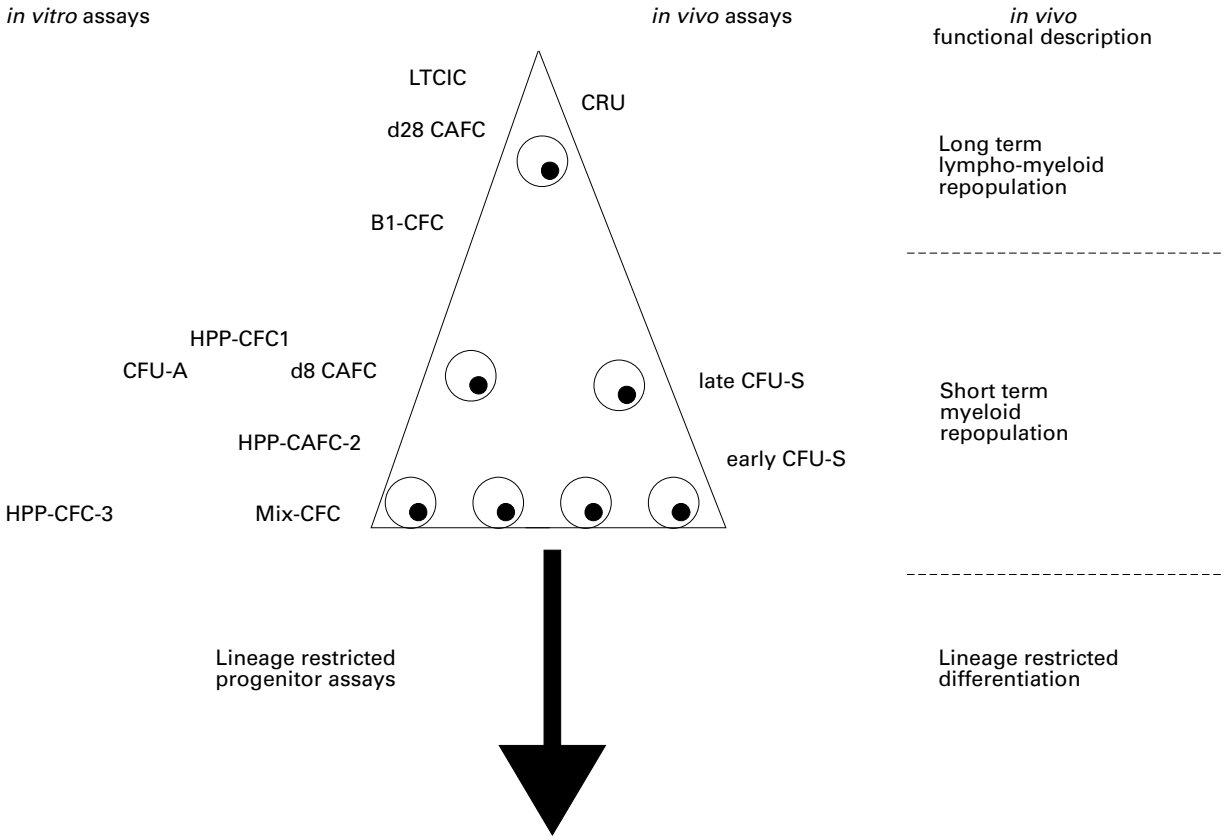

Figure 1. A schematic representation of the stem cell hierarchy and interrelationships of the operationally defined assays discussed in the text. The most ancestral stem cells heading the hierarchy have the greatest self-renewal and long-term repopulation capacities. The cells recognized by assays downstream of these most ancestral cells demonstrate increasing Rhodamine-123 staining, increasing sensitivity to 5-fluoruracil, increasing probability of being triggered into proliferation associated with decreasing proliferative capacity and decreasing self-renewal and long-term repopulating capacities.

Current interpretation of the assays for CFU-S and haemopoietic repopulation

An interpretation of information currently available is that the stem cell compartment is a developmentally structured continuum (see Figure 1). In this continuum the most primitive members have the greater long-term repopulating ability and are the most resistant to proliferation and differentiation stimuli. In subsequent divisions, cells become detectable as CFU-S with decreasing self-renewal capacity and increasing probability of becoming committed to the various haemopoietic lineages. In the steady state, day-12 CFU-S are more primitive than day-7/8 CFU-S but are not the most primitive stem cells. Those cells with the greatest long-term repopulating ability, as assessed by transplantation into lethally irradiated recipients, reside in the pre-CFU-S compartment. A developmentally structured stem cell compartment may have important functional significance *in vivo*, since transplantation experiments have demonstrated two phases of bone marrow engraftment; an initial but transient engraftment (essential for survival following the conditioning irradiation) followed by a delayed but long-term reconstitution of the haemopoietic system. These two phases can probably be attributed, respectively, to the later and earlier members of the continuum, with the later members being detected by spleen colony formation and the earliest members only by their long-term repopulating capacity (Jones *et al.* 1989, 1990).

Assays for stem cells *in vitro*

A variety of *in vitro* assays that detect cells from within the stem cell continuum have been reported, although there is still some uncertainty as to how and where many of these cells fit in the continuum and what contribution, if any, they make to normal steady-state haemopoiesis. The assays are conveniently considered in two classes, quantitative clonogenic assays and assays of cells that initiate haemopoietic proliferation and differentiation on preformed stromal cultures.

Clonogenic assays for the Stem Cell compartment in vitro. There is considerable literature documenting that appropriate growth factor conditions will stimulate primitive progenitor cells from within the continuum of the stem cell compartment to produce clonal descendants in viscous media, usually agar or methyl cellulose, where the progeny are readily identified as a colony. These clonogenic assays can be grouped into three classes based on the characteristics of the cells produced in the colonies (Muller-Sieburg *et al*. 1991): mixed-lineage colony-forming cells (Mix-CFC) high proliferative potential colony-forming cells (HPP-CFC) and blast colony-forming cells (Bl-CFC).

Mixed-lineage colony-forming cells (Mix-CFC). Mixed lineage colony formation in agar cultures was first described by (Johnson & Metcalf 1977) reporting a system in which 'red coloured' colonies were generated from mouse foetal liver cells composed of up to 5000 cells that were mainly erythroblasts at varying stages of differentiation. The interesting feature was that approximately half of all colonies exhibited myelomonocytic and occasionally megakaryocytic differentiation in addition to the erythroid cells. Subsequent investigations demonstrated these Mix-CFC in adult mice with an approximate incidence of $150/10^5$ marrow cells and that in normal bone marrow they were minimally proliferating cells like the CFU-S (Metcalf *et al*. 1978; Johnson 1980). These colonies contain cells with various capacities for secondary colony formation and, although rather infrequently, CFU-S. A more primitive Mix-CFC has also been described (Humphries *et al.* 1979a) with an incidence in the range $5–10/10^5$ normal bone marrow cells. Colonies derived from these clonogenic cells contained sinngle lineage-restricted cells capable of secondary colony formation and subsequent experiments indicated that each colony contained about one CFU-S (Humphries *et al.* 1979b). As with all *in vitro* systems, it is often difficult to decide whether limited self-renewal is an intrinsic property of the cell (i.e. the clonogenic cells is an 'old' member of the stem cell compartment) or whether the cell culture conditions are inappropriate for providing self-renewal signals. Different culture conditions have been described that generate large colonies composed of normoblasts, macrophages and often megakaryocytes produced by a clonogenic cell with an incidence of only $3–4/10^5$ marrow cells and with proliferative and biophysical characteristics in common with CFU-S (Hara & Ogawa 1978). Unfortunately, the self-renewal of these cells has not been measured 'because of the low plating efficiency'. Overall, it seems that the Mix-CFC is representative of the 'older' part of the stem cell compartment overlapping significantly with the clonogenic cell that generates early appearing spleen colonies *in vivo* (i.e. the day 7/8 CFU-S).

Blast colony-forming cells (Bl-CFC). An *in vitro* clonal assay for a cell that produces blast cells but does not drive them to differentiation and is ancestral to Mix-CFC has been described (Nakahata & Ogawa 1982). The incidence of the clonogenic cell is approximately $4/10^5$ in normal marrow and the colonies contain progenitor cells and produce secondary multilineage colonies on replating. Most Bl-CFC-derived colonies usually contain CFU-S (up to 16 per colony). The frequency in bone marrow and the properties of the Bl-CFC suggest that they may be pre-CFU-S in nature.

High proliferative potential colony-forming cells (HPP-CFC). HPP-CFC able to generate large colonies in agar culture (>0.5 mm in diameter) were first reported as primitive macrophage progenitors (Bradley & Hodgson 1979). More recently, HPP-CFC (usually assayed in marrow from 5-FU-treated mice to obtain sufficient enrichment for their detection) have been defined further and three classes have emerged; HPP-CFC-1, -2, and -3 in order of primitiveness (McNiece *et al*. 1986, 1987, 1990). HPP-CFC-1 generated up to six day-13 CFU-S per developing colony and also cells with short-term repopulating ability (McNiece *et al*. 1987; Bartelmez *et al*. 1989). The development of the HPP-CFC-derived colonies requires a mixture of growth regulatory factors with specific and different combinations associated with the three classes The properties ascribed to the HPP-CFC series suggest that this compartment falls into and may well mostly include the CFU-S populations detected by *in vivo* assay. The relationship of HPP-CFC to Bl-CFC is not yet entirely clear, but it is possible that the most primitive HPP-CFC may have properties in common with the Bl-CFC.

A robust assay for an HPP-CFC-like cell that has an incidence of $150–200$ per 10^5 cell in normal marrow (i.e. does not require enrichment by cytotoxic treatment to detect significant numbers) detects a clonogenic cell that has been operationally defined as CFU-A. The colonies are easily scored because they are >2 mm diameter and extensive characterization of the clonogenic cell (Lorimore *et al*. 1990) is consistent with the CFU-A assay detecting that part of the multipotential stem cell compartment that is also detected by the *in vivo* day 12-CFU-S assay and the *in vitro* HPP-CFC-1 assay.

Clonogenic assays for human haemopoietic stem cells

The haemopoietic stem cell compartment in humans has been less well characterized than in the mouse. Much of the success with the mouse system can be attributed to manipulations of haemopoiesis *in vivo* using techniques that are not applicable to the human situation. However, *in vitro* colony-forming assays similar to those described for stem cells in the mouse have been developed for the study of human stem cells.

Mixed lineage colony forming cells have been described by many workers. The first report operationally defined such a cell as a CFU-GEMM as it generated clonal progeny in which granulocytic, erythroid, monocytic and megakaryocytic lineages were readily identified (Fauser & Messner 1979a). The clonogenic cell is present at a frequency of approximately $1/10^4$ normal mononuclear marrow cells and (similar to the mouse Mix-CFC) most of the clonogenic cells are proliferatively quiescent in normal marrow but in samples obtained during early regeneration after bone marrow transplantation are actively proliferating (Fauser & Messner 1979b).

A clonogenic assay that detects a cell capable of generating a large (>0.5 mm in diameter) colony of macrophages and progenitors of granulocytes, erythrocytes and megakaryocytes after 28 days in culture has been described (McNiece *et al*. 1989). The clonogenic cell is present in normal marrow at a frequency of $2/10^5$ and at a 7-fold higher concentration in bone marrow treated with 5-fluorouracil. The characteristics of these clonogenic cells, i.e. multifactor responsive cells producing large colonies after prolonged time in culture and showing relative resistance to the cytoxic effect of 5-fluorouracil, is consistent with the assay detecting cells equivalent to the HPP-CFC in the mouse.

Using a technique based on the mouse Bl-CFC assay, Rowley *et al.* (1987) described the culture from normal human bone marrow of colonies of blast cells with uniform morphological appearance. The colonies usually contained 200–500 cells and occasionally up to 1000 cells and developed at varying times during 28 days of culture; the later the development of the colony the more primitive the clonogenic cell. The incidence of the clonogenic cell producing colonies scored on day 14 was 3–4/5 $x10^4$ but less than 1 cell in 5 $x10^4$ produced colonies scored on days 21–28. The late-appearing colonies had a high secondary plating efficiency with an average of 79% of colonies (range 50–100%) producing secondary colonies. The secondary clonogenic cells were were predominantly lineage-restricted (erythroid or granulocyte/macrophage colonies) but approximately 1 in 3 produced CFU-GEMM (1–16 per colony) and 1 in 8 produced up to 3 secondary blast colonies *i.e* exhibited self-renewal. A similar Bl-CFC assay has been described by Leary & Ogawa (1987) and Leary *et al*. (1992).

A clonogenic assay in which multifactor stimulation of normal human marrow produces macroscopic (>1 mm in diameter) multilineage colonies based on the CFU-A assay for mouse stem cells has been described (Holyoake *et al.* 1993). This human CFU-A has an approximate incidence of 25 per 10^4 cells and most of the cells are proliferatively quiescent in normal marrow. About half the colonies contained cells that generated secondary colonies (mainly lineage-restricted) and 3% of the primary colonies contained CFU-A which replated to give large mixed lineage colonies. Thus CFU-A are cells of a primitive nature with high proliferative potential and limited self-renewal in the assay conditions which, like many of the clonogenic assays for members of the stem cell compartment, are developed on the basis of driving differentiation and may not reveal the full self-renewal potential of the cells.

The interaction of stem cells with the haemopoietic stromal microenvironment

In vivo, stem cell replication and commitment to differentiation is intimately associated with the stromal cells of haemopoietic tissues and in the mouse multipotential and lineage-restricted clonogenic cells are not uniformaly distributed throughout the bones. The mouse femoral bone marrow can be fractionated longitudinally to provide a population close to the longitudinal axis of the bone (axial cells) and a second population closer to the endosteal surface of the bone (marginal cells). By varying the fractionation, different ratios of axial to marginal cells are obtained and this has enabled a cross-sectional distribution of various cell types to be obtained (Lord 1990). These distributions indicate a well defined spatial organization of the various cell types with, for example, a 2–3 fold increase in the concentration of CFU-S from the axis to the bone surface (Lord *et al.* 1975) and differences in the production, and response to, proliferation signals (Lord & Wright 1984) with significant proliferation of CFU-S with limited self-renewal capacity in the sub-endosteal region of the marrow (Lord & Schofield 1980). An important conclusion from these studies is that in addition to a temporal organization of stem cells there is also a spatial organization of the stem cell compartment, an organization requiring an appropriately structured microenvironment.

Stem cells identified by the initiation of stroma-associated haemopoiesis

In the 1970s, Dexter *et al.* (1977a, 1977b) and Toksoz *et al.* (1980) described a culture system of mouse bone marrow that maintained a CFU-S population for many months. One important aspect of this culture system was that the maintenance of stem cells was dependent on the generation of an adherent multilayer of stromal cells. In the early 1980s the culture system was adapted for human marrow and these long-term bone marrow cultures have proved valuable in dissecting the structure and regulation of the haemopoietic system (Wright & Greenberger 1984). When this long-term culture system was introduced, the CFU-S was regarded as the haemopietic stem cell but, as described above, the pre-CFU-S population is mainly responsible for long-term marrow repopulating activity. These developments led to experiments demonstrating that (reminiscent of the transplantation studies) pre-CFU-S were more effective than CFU-S in seeding into a preformed bone marrow-derived stromal layer depleted of haemopoietic cells by irradiation and subsequently generating haemopoietic progeny (Ploemacher *et al.* 1989; van der Sluijs *et al.* 1990). This demonstration was refined to establish a limiting dilution assay for stem cells that were operationally defined as cobblestone area-forming cells (CAFC) on the basis of the morphological appearance of the clonal progeny of the seeded cell. Assessment of cobblestone areas at different times after seeding provided a relatively convenient *in vitro* system to define the stem cell hierarchy: Day 12 CFU-S correlated with day-8 CAFC and pre-CFU-S with day 28 CAFC. These most primitive CAFC have an incidence of approximately 6/10^5 normal marrow cells (Ploemacher *et al.* 1989). It seems likely that this population has significant overlap with the primitive pluripotent stem cells identified as competitive repopulation units (CRU) present at a frequency of 1/10^5 normal marrow cells (Szilvassy *et al.* 1990). A similar approach (Sutherland *et al.* 1989) identified long-term culture initiating cells (LTCIC) in human bone marrow at a frequency of 6/10^4 normal marrow cells and more recently self-renewal of LTCIC *in vitro* and their expansion in defined medium has been demonstrated (Petzer *et al.* 1996).

A xenografting approach to studying human stem cells

Much of our understanding of the hierarchical organization and regulation of the haemopoietic system is derived from mouse models and, until recently, studies of human stem cells have been restricted by the absence of *in vivo* assays to measure their repopulation capacity. The development of methods to transplant human haemopoietic cells into immune-deficient mice is providing the basis for human stem cell assays. Two different approaches have been taken to measure the repopulation capacity of human stem cells. The simplest has been to inject human haemopoietic cells intravenously into sublethally irradiated immune-deficient (beige/nude/x-linked immunodeficiency (bg/nu/xid), severe combined immunodeficiency (SCID) or non-obese diabetic/SCID (NOD/SCID)) mice (Kamel-Reid & Dick 1988). The alternative approach has been to implant human foetal organs under the renal capsule or bone fragments subcutaneously to provide a suitable haemopoietic environment to support stem cell proliferation and differentiation (McCune *et al.* 1988; Kaneshima *et al.* 1994).

The injection of human haemopoietic cells into irradiated immune-deficient mice results in a modest maintenance of human haemopoietic cells. The production of human cells is dramatically increased if transplanted mice are regularly infused with a mixture of human haemopoietic growth factors (Lapidot *et al.* 1992), if the recipient mice are injected with growth factor (IL-3)-secreting stromal cells (Nolta *et al.* 1996) or if immune deficient (SCID) mice transgenic for human human growth factors (IL-3, GM-CSF and SCF) are used (Bock *et al.* 1995). However, these systems are far from ideal as the recipient mice possess significant nonspecific immune responses including natural killer (NK) cell activity and large cell doses ($>10^7$) are required to overcome host resistance. This limitation makes it difficult to perform limiting dilution assays for the primitive repopulating cells. The approach has been significantly improved by the development of NOD/SCID mice (Shultz *et al.* 1995) which lack B and T lymphocytes because of the SCID mutation and have diminished NK activity, defects in complement-mediated reactions and macrophage functions because of the NOD-associated defects. Using the intravenous transplant approach, the human cells produced in the mice have been shown to be derived from a $CD34^+$ $CD38^-$ cell fraction (see below) and probably from a pluripotential cell ancestral to the *in vitro* colony-forming cells and the long-term culture initiating cells (Larochelle *et al.* 1995; Nolta *et al.* 1996).

The generation of a human microenvironment capable of sustaining human haemopoiesis in a mouse is a highly attractive model for studying human stem cells. Normal T-lymphocytes can be generated by coimplantation of foetal thymus and liver and myeloid cells and B-lymphocytes can be generated from bone fragments. However. in both cases the number of cells produced is modest

and sustained for only a few weeks (Kyoizumi *et al*. 1992, 1993). A recent development of this system is the implantation of foetal bone, thymus and spleen fragments together (Fraser *et al*. 1995) which results in multilineage haemopoiesis for at least 36 weeks.

It is not yet proven that the stem cells assayed in these mice are identical to those repopulating a human recipient but the models do provide an *in vivo* approach to the study of human stem cells. Although at an early stage, rapid progress is being made in the characterization of these cells and in the development of a quantitative assay for repopulating stem cells. The approach is also proving valuable in studies of leukaemic cells (Uckun *et al*. 1993, 1995; Lapidot *et al*. 1994; Sirard *et al*. 1996; Kamel-Reid *et al*. 1989, 1991; Dick 1996).

Antigenic characteristics of human stem cells

A considerable variety of cell surface antigens have been identified that are expressed by mature haemopoietic cells but it has proven more difficult to identify antigens restricted to stem cells and/or their immediate progeny – the lineage-restricted progenitor cells. An important development was the development of the CD34 monoclonal antibody raised against the KG1 leukaemic cell line (Civin *et al*. 1984). The antibody recognizes a 115 kDa antigen present on 1%-4% of bone marrow cells. Cells that are stimulated in the *in vitro* clonogenic assays including those for stem cells are present in this $CD34^+$ fraction of marrow and CD34 antibodies are used routinely for enrichment of clonogenic cells. The function of the CD34 is not fully understood but is implicated in signal transductions as the cDNA (Simmons *et al*. 1992) predicts a membrane protein with nine potential N-linked and numerous O-linked glycosylation sites in its extracellular domain. There are two consensus protein kinase C phosphorylation sites and one potential tyrosine kinase phosphorylation site in the cytoplasmic portion. The identification of splice variants (Suda *et al*. 1992) differing in the cytoplasmic domain may indicate that that different carboxyterminals may be associated with different signal transduction pathways. However, because CD34 is also expressed on vascular endothelial and stromal cells and binds leukocyte L-selectin *in vitro* (Baumheuter *et al*. 1993) it may function as an adhesion molecule. Considerable effort is being expended to identify physiologically relevant ligands.

Although human stem cells are enriched in the $CD34^+$ fraction of bone marrow it is a heterogeneous population of multipotential and lineage-restricted cells. Many workers have used the various stem cell assays and panels of antibodies to provide an immunological phenotype of stem cells and to use the information to obtain highly enriched populations of stem cells. Antigens expressed by the progeny of stem cells in one or more of the various haemopoietic lineages have been useful in depleting these cells as part of the purification strategies. Commonly used are antibodies directed against CD38, originally described as a T-lineage marker with unknown function but expressed by most haemopoietic cells that express lineage markers (Terhorst *et al*. 1981; Sieff *et al*. 1982) and detectable on aproximately 95% of $CD34^+$ marrow cells. Enrichment of more than 1000-fold of cells with stem cell characteristics is readily achieved by positive selection for $CD34^+$ and negative selection against $CD38^+$ cells. However, such preparations of $CD34^+$ $CD38^-$ cells are heterogeneous for differentiation and self-renewal capacities.

What we have learned from the various assay systems

A large number of *in vitro* assay systems have been described using a range of growth factor combinations which detect a series of overlapping clonogenic cell populations broadly defined in the mouse haemopoietic system by the *in vivo* CFU-S assay (See Figure 1). It is likely that this population of cells may be largely responsible for continued long-term haematopoiesis *in vivo* in normal steady state conditions. However, in conditions of haemopoietic stress such as post radio- and/or chemotherapy and after transplantation into lethally irradiated recipients, the more primitive pluripotential stem cells (pre-CFU-S) play a crucial role in the long term repopulation of the haemopoietic system. Thus, it is the pre-CFU-S cells that are the targets for gene therapy and this is giving considerable impetus to current studies of the further characterization of these cells including attempts to expand them *ex vivo* (see later) and the development of transduction vectors (Dunbar & Young 1996).

The control of haemopoietic stem cells

Factors regulating the haemopoietic stem cell can be envisaged as acting at a number of different levels as outlined in Figure 2. These include:

- Regulation of proliferation in the absence of differentiation. Such proliferative regulation could in principal involve either stimulation or inhibition of proliferation.
- Regulation of differentiation;
- Regulation of cell viability and suppression/induction of apoptosis.

In practice, many of these activities and their associated

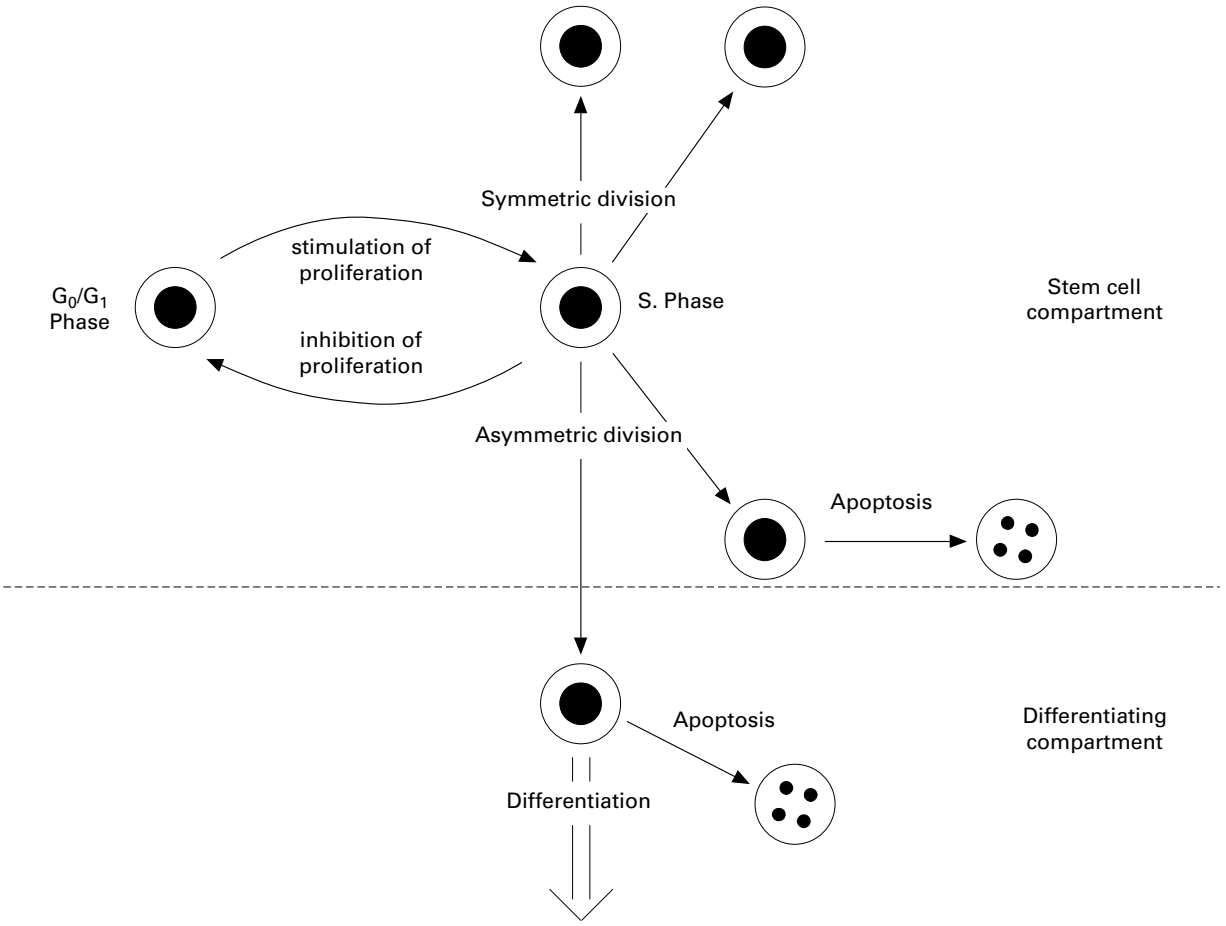

Figure 2. A schematic representation of factors potentially regulating the proliferation and commitment to differentiation of haemopoietic stem cells.

factors overlap and as is outlined below, the pleiotropic nature of many factors means that they can regulate a range of stem cell functions depending on the context in which they are required to work. The stem cell regulatory factors will be dealt below under the two broad headings of Proliferation and Inducers of differentiation.

Stem cell proliferation

A number of studies have indicated that the majority of cells within the stem cell compartment are quiescent, residing in the Go phase of the cell cycle (see Lajtha 1979a, 1979b; Wright & Pragnell 1992; Graham & Pragnell 1992). The reason for this extensive dormancy is unclear, however, it may relate to the requirements for the maintenance of the genetic purity of the essentially immortal stem cell pool. Spontaneous mutations in differentiating haemopoietic cells will often be of little consequence as the mutation bearing cell is programmed to die as a normal consequence of differentiation. In contrast, mutations accumulated by the stem cell may be regarded as permanent given the presumed immortality of this self renewing population (although see below for a discussion on this point). This means that the stem cells are likely to require specific protective mechanisms to avoid such accumulation of mutations and thus the notion of quiescence as a period of 'genetic housekeeping' has been proposed to explain the extensive dormancy of the stem cells (Lajtha 1979b). Intriguingly, the stem cell population in colonic epithelial crypts is a continuously proliferating population which does not display a high sensitivity to mutagens or transformation (Potten & Loeffler 1990). It may be therefore, that other explanations for the cell cycle dormancy of stem cells require to be put forward.

The dormancy of stem cells is rapidly reversible and following cytotoxic chemical insult or radiation insult to the haemopoietic system, the normally quiescent stem

cell rapidly enters cell cycle. These cycling progenitors then serve to replenish the crippled mature cellular system and, having done so, return to the resting quiescent state. It has been inferred from these observations that the stem cell must be open to both positive and negative proliferative control and as outlined below, a great deal is now known about putative inhibitors and stimulators of haemopoietic stem cell proliferation. A cautionary note must however, be introduced into this discussion of stem cell proliferative regulators and that is, that whilst evidence for inhibitors and stimulators of proliferation exists, there is no *a priori* reason to assume that the proliferation of the stem cell could not be regulated entirely on the basis of the presence or absence of stimulators or equally of inhibitors. It may be therefore that *in vivo*, whilst both stimulators and inhibitors of stem cell proliferation are known to be expressed, they may play roles more appropriate to pathological than to physiological circumstances. This point is discussed in more detail below.

Inhibitors of stem cell proliferation

To date a number of candidate inhibitory molecules have been characterized ranging from small peptides to proteins and varying in their mode of action. Much interest has recently focused on such factors given their potential clinical role in cancer therapy and their possible involvement in the pathogenesis of a number of leukaemias.

The tetrapeptide. Frindel & Guigon (1977) have described a dialysable low molecular weight inhibitory activity which was initially derived from foetal calf bone marrow and which is active in preserving the quiescent status of haemopoietic stem cells. The peptide has been purified and sequenced revealing the sequence AcSDKP (Lenfant *et al.* 1989). Data base searching with this sequence demonstrates it to be present in a number of larger proteins including Thymosin$-\beta 4$ (Tβ4), Tumour Necrosis Factor$-\alpha$ (TNF-α) and Rat Phenylalanine hydroxylase. Of these, the most promising precursor is Tβ4 which carries the AcSDKP sequence at its amino terminus and from which the tetrapeptide can be released through the action of the endoproteinase AspN (Grillon *et al.* 1990).

Within the stem cell compartment the inhibitory activities of the tetrapeptide appear to be largely confined to the transiently engrafting stem cell population as typified by the cell detected in the CFU-S d12 assay (Bonnet *et al.* 1995) however, other studies have suggested effects on more primitive elements of the stem cell compartment. Activities on CFU-S have been demonstrated both *in vitro* and *in vivo* and an *in vivo* role in controlling regenerative haemopoiesis has been inferred from studies on alterations in tetrapeptide levels following cytotoxic drug treatment (Frindel *et al.* 1992). More recent data suggests that the tetrapeptide may act primarily by blocking the response of stem cells to proliferative stimulatory molecules (Robinson *et al.* 1993) and indeed results, from our own laboratories, suggest the tetrapeptide to be active in blocking the proliferative effects of interleukin 11. Intriguingly, Tβ4 is also active in inhibiting stem cell proliferation (Bonnet *et al.* 1996), however, this activity locates to regions of the molecule that are carboxy terminal to the tetrapeptide sequence and may therefore involve alternative mechanisms.

In addition to the stem cell inhibitory properties of the tetrapeptide, this molecule is active in protecting stem cells from radiation induced damage *in vivo* (Watanabe *et al.* 1996) and also in hyperthermic purging protocols *in vitro* (Wierenga & Konings 1996). The mode of action of the inhibitor in these contexts remains to be elucidated.

Recently studies into the basis for the very short half life of the tetrapeptide in serum (Grillon *et al.* 1993) have identified AcSDKP as a specific and natural substrate for the metaloproteinase Angiotensin Converting Enzyme (Rousseau *et al.* 1995).

The pentapeptide. A second peptide inhibitor of stem cell proliferation has been identified from rodent bone marrow and human leukocytes (Laerum & Maurer 1973). This has been purified to homogeneity revealing the sequence pEEDCK (Paukovits & Laerum 1982). This peptide appears to act through a protease sensitive membrane receptor (Paukovits & Paukovits 1975) and is active in inhibiting murine and human myelomonocytic-restricted progenitor cells, the granulocyte/macrophage colony-forming cells (GM-CFC) as well as CFU-S and Long-term culture initiating cells (Paukovits *et al*. 1990, 1991, 1993). Inhibitory activity is observed at 10^{-13} M however, the dose response is biphasic and inhibitory properties are lost at higher concentrations. This is likely to be due in large part to the ability of the pentapeptide to form a dimeric variant through generation of disulphide bonds involving the cysteine residues on two apposing monomers. Interestingly this dimer is inactive as an inhibitor but is active as a stimulator of murine and human GM-CFC proliferation and the response to this stimulatory variant is also biphasic with maximal activity being seen at 10^{-9} M (Frostad *et al.* 1993). This tendency to dimerise has hampered preclinical studies with the pentapeptide, however, recently stable monomeric and dimeric variants of this molecule have been generated.

These have now been used to great effect in preclinical trials with the monomer acting to protect the stem cell from cytotoxic damage and the dimer being used following cytotoxic treatment to stimulate recovery of the damaged mature system (Paukovits *et al.* 1993).

Transforming Growth Factor-beta (TGF-β). TGF-β is a member of a large family of related peptides which include activin, inhibin, the bone morphogenetic proteins and decapentaplegic (Sporn *et al*. 1987; Sporn & Roberts 1992). Members of this family play important roles in development and in the control of adult tissue function (Mummery *et al.* 1993). TGF-β is a homodimer in its mature form consisting of two chains of 112 amino acids. Each chain is derived from a larger precursor the remnants of which remain non covalently bound to the mature homodimer following secretion from the cell. This complex of mature homodimer and precursor fragment is referred to as latent TGF-β and removal of the precursor segments is required for activation of the mature TGF-β protein. This activation therefore represents an important mechanism controlling the level of TGF-β activity and may involve the protease plasmin. Recently a physiological role for plasmin in TGF-β activation has been inferred from studies on mice transgenic for the plasminogen activation blocking agent apolipoprotein (Grainger *et al.* 1994), in which activation of latent TGF-β is seen to be blocked. In addition to plasmin, a number of other activating mechanisms are available, most of which however, are likely to be non physiological (Brown *et al.* 1990).

Analysis of the inhibitory properties of TGF-β suggest that it will inhibit proliferation of cells throughout the stem cell compartment, however, this in some cases is dependant on the relative growth factor combination in which the cells are cultured (Jacobsen *et al*. 1991a; Keller *et al.* 1994). For example, TGF-β will inhibit human GM-CFC stimulated by IL3 but will stimulate in a biphasic manner similar cells stimulated by GM-CSF. Thus TGF-β must be regarded as a bidirectional regulator of stem cell proliferation demonstrating both stimulatory and inhibitory properties depending on the cellular and cytokine context.

In addition to its activity as an exogenous regulator, data from antibody and antisense studies have revealed a role for endogenous autocrine TGF-β in regulating the proliferation of haemopoietic stem cells (Hatzfeld *et al.* 1991; Li *et al*. 1994; Soma *et al*. 1996). Thus antibodies or antisense oliogonucleotides to TGF-β serve to enhance the proliferation of stem cells in a variety of assay systems. Furthermore studies on Long-term Bone Marrow Cultures have demonstrated constitutive expression of TGF-β in the stromal layers which remains unaltered following media change (Otsuka *et al.* 1991). This suggests, again, that endogenous TGF-β may be active in regulating the proliferation of haemopoietic cells in this system. In addition it has been demonstrated that TGF-β levels in mice are unaffected by sub lethal irradiation and subsequent haemopoietic regeneration (Chang *et al.* 1995). This observation, and the data from the LTBMC studies suggests TGF-β to be more likely to play a role as a regulator of HSC proliferation under steady state than under regenerative conditions.

One feature complicating our understanding of the role of TGF-β as a haemopoietic stem cell inhibitor is the known ability of TGF-β to induce apoptosis in this and other cell types depending on concentration and time of exposure (Jacobsen *et al*. 1995; Veiby *et al*. 1996). It may be therefore that in certain circumstances, what has been registered as inhibition of proliferation is in fact induction of apoptosis. Indeed a mechanistic explanation for the induction of apoptosis by TGF-β may be provided by the demonstration of cytokine receptor down-regulation by TGF-β (Jacobsen *et al.* 1991b). It is known that impaired cytokine signalling may predispose primitive haemopoietic cells to undergo apoptosis (Williams *et al.* 1990) and thus the reduction in receptor numbers mediated by TGF-β may contribute to this effect.

Recently mice with a homozygous null mutation in the TGF-β1 gene have been generated. These mice show a range of pathologies ranging from inflammatory abnormalities to aberrations in cardiac development which under appropriate circumstances are perinatally lethal (Schull *et al*. 1992; Kulkarni *et al*. 1993; Letterio *et al*. 1994). Embryo lethality due to deficiencies in haemopoietic and vascular development has also been reported (Dickson *et al.* 1995). There is no data however, to suggest that HSCs from these TGF-β1 null mice display aberrant proliferative regulation thus TGF-β1 must play, at best, a dispensable role in the regulation of HSC proliferation *in vivo*.

Macrophage inflammatory protein-1 alpha (MIP-1α). MIP-1α is a member of the chemokine family of inflammatory mediators which is defined on the basis of sequence homology and on the presence of a conserved 4 cysteine motif (Schall 1991). The family can be further subdivided on the specific distribution of cysteines within the conserved motif, with the α−chemokines having an amino acid inserted between the first two cysteines (. . .CXC. . .C.C. . .) and the β-chemokines being characterized by juxtaposition of the first two cysteine residues (. . . .CC. . . .C. . . .C. . . .). Members of the α-chemokine family include Interleukin-8, platelet factor-4 and

β-thromboglobulin, and members of the β-chemokine family include MIP-1α, MIP-1β, RANTES and the Monocyte Chemoattractant proteins 1–5.

During a search for inhibitors of day 12 CFU-S proliferation (Wright *et al*. 1985; Wright & Lorimore 1987; Pragnell *et al*. 1988), we purified an inhibitory activity from the murine bone marrow macrophage cell line J774.2 and sequencing revealed it to be identical to MIP-1α (Graham *et al*. 1990). This protein is active in inhibiting short-term myeloid repopulating stem cells (Broxmeyer *et al*. 1990; Keller *et al*. 1994; Mayani *et al.* 1995) and appears to have little detectable effect on very primitive members of the stem cell compartment (Quesniaux *et al*. 1993; Soma *et al*. 1996). In contrast, and in common with TGF-β, MIP-1α acts to stimulate the proliferation of more mature haemopoietic progenitor cells, again depending on the nature of the costimulus (Broxmeyer *et al*. 1990; Clements *et al*. 1992). MIP-1α is active *in vivo* in inhibiting stem cell proliferation and pre clinical studies from our own and other laboratories have revealed that 10–20μg of recombinant MIP-1α administered to a mouse is sufficient to suppress the proliferation of CFU-S and to protect them from the effects of cell cycle specific cytotoxic agents (Dunlop *et al*. 1992; Lord *et al*. 1992). Recent results suggest that the protective effects of MIP-1α may not be restricted to cell cycle specific agents (Gilmore *et al.* 1996; Parker *et al*. unpublished) and this is likely to be advantageous in a clinical context in which typically, combinations of differently acting cytotoxic agents are used in routine therapy. A further therapeutic role for MIP-1α may lie in its ability to mobilize leukocytes peripheral circulation (Lord *et al.* 1995), a property it shares with IL8 (Laterveer *et al.* 1996). However, it is clear that MIP-1α is not as effective in this regard as G-CSF and may therfore not be a preferred therapeutic agent in this context.

Recently mice with a homozygous null mutation in MIP1α have been generated (Cook *et al.* 1995). These mice are viable but demonstrate altered responses to a number of viral pathogens such a Coxsackie virus and Influenza virus. No proliferative abnormalities have been noted in the stem cells of these mice indicating that in common with TGF-β1, MIP-1α may play a limited or dispensable role in the physiological control of HSC proliferation.

Other inhibitory molecules. It is important to point out that the above is not an exhaustive list of stem cell inhibitory molecules. Other inhibitors exist which include Interferon-γ (Snoeck *et al.* 1994) and TNF-α (Zhang *et al.* 1995) and evidence has been presented suggesting IFNγ to be a selective inhibitor of very primitive haemopoietic stem cells. In common with TGF-β and MIP-1α, both IFNγ and TNF-α are bidirectional proliferative regulators and show proliferative enhancing effects on more committed haemopoietic progenitor cells.

Stimulators of stem cell proliferation

There is now an almost bewildering array of factors which can act to stimulate the proliferation of haemopoietic stem cells, however, there are few factors which can do this in isolation. Typically, combinations of positively acting factors synergise to induce proliferation and in some cases differentiation of primitive cells (although see below for a discussion on this point). In this context it is interesting to note that a functional distinction can be made between the different types of regulators. Thus for example, SCF, IL3 or GM-CSF can maintain the viability of dormant primitive haemopoietic cells, but will not induce their proliferation. Proliferative induction requires a synergising factor such as IL6 or IL11 which will induce the viable dormant cells to proliferate (Leary *et al*. 1989; Bodine *et al*. 1991; Katayama *et al*. 1993). This proliferative stimulus frequently involves a shortening of the G1 phase of the cell cycle (Tanaka *et al.* 1995). Amongst synergising factors, SCF and FL appear to be unique in their ability to synergise with a wide variety of factors to support the proliferation of a number of different components of the stem cell compartment.

Much interest has recently focused on the ability of synergising growth factor combinations to expand stem cell numbers following *in vitro* (*ex vivo*) culture of stem cells (Verfaillie 1993). The hope from such studies is that limited numbers of stem cells could, through the *ex vivo* expansion process yield sufficient stem cells for transplantation. What is unclear at present is whether expansion of stem cell numbers during such synergistic cytokine treatment *in vitro*, is a result of stimulation of proliferation (self renewal) or alternatively is a consequence of expansion through differentiation from more primitive precursors. In our own studies (Holyoake *et al.* 1996), the combination of SCF and IL11 appears optimal for expansion of transiently engrafting stem cells as detected in the CFU-A assay and some 50 fold enhancement of CFU-A numbers is achieved in this growth factor combination following culture for 7 days. It is not clear from our studies however, whether these expanded cell numbers have resulted from self renewal of existing CFU-A stem cells or whether as alluded to above, the expansion in numbers is a consequence of differentiation from more primitive components of the stem cell compartment. Indeed, whilst it is controversial, there is much evidence suggesting that the very primitive long-term

repopulating stem cell pool is compromised following ex vivo expansion (Henschler *et al*. 1994; Peters *et al*. 1996). This clearly could be explained on the basis of the inability of the chosen growth factors to support these cells but equally may indicate that these very primitive cells have been induced to differentiate. It is clear that a resolution of these questions is required before *ex vivo* expanded stem cells can be reliably used therapeutically.

These results also raise important biological questions which are central to our understanding of haemopoietic stem cell biology. The *ex vivo* expansion models may recapitulate to some extent, the expansion *in vivo* of stem cell numbers that is likely to be seen under extreme pathological conditions. Notionally, the self renewal properties of stem cells have been put forward to argue that there will be a limit to the losses in this primitive cellular population following such expansions. However, it has not yet been unequivocally demonstrated that self renewal without attendant differentiation or phenotypic alteration can occur with HSCs *in vivo* or even *in vitro*. Most current data suggests in fact that despite evidence of telomerase expression in HSCs (Morrison *et al.* 1996a) the stem cell pool can age (Morrison *et al.* 1996b) and that even at its most primitive end, it has a finite lifespan and that serial transplantation as a proliferative stress sequentially reduces the repopulation potential of stem cells (see above). In the absence of evidence of self renewal therefore it may be necessary to redefine the roles of proliferative stimulators in controlling stem cell proliferation. Thus whilst as mentioned above, synergising growth factor combinations such as SCF and IL11 conspire to increase stem cell numbers, it is unlikely that this is a direct consequence of stimulation of self renewal. It may be therefore that there are no factors currently identified that have the ability to act as 'mitogens' for stem cells without incurring attendant differentiation or phenotypic change. This does not mean however, that in the presence of blockers of differentiation, such synergising factors may allow self renewal. Perhaps the best example of such a factor is Leukaemia Inhibitory factor (Smith *et al.* 1988) which acts to suppress the *in vitro* differentiation of embryonic stem cells (Robertson 1987) thus allowing them to be maintained as a totipotent self renewing population which can be genetically manipulated in this state prior to induction of differentiation *in vitro* or *in vivo*. It is worthy of note that LIF will not work in this context in the haemopoietic system, however, identification of similar HSC active factors would have profound implications for our ability to manipulate haemopoietic stem cells for both experimental and therapeutic purposes. As yet no such factors capable of restricting differentiation have been identified.

Inducers of stem cell differentiation

Upon identification and characterization of the classical colony stimulating factors (Metcalf 1984, 1988), much emphasis was placed upon the ability of these factors to instruct the processes of lineage specific differentiation. Thus it was envisaged that G-CSF and the other CSFs may act as 'master switches' triggering the transcription of genes in stem or progenitor cells, that are required for differentiation along specific lineages. More recently, it has become clear that in common with many other 'growth factors', the CSFs may act primarily to maintain the viability of the progenitor cells and may therefore be supportive or permissive rather than instructive in the differentiation process (Williams *et al*. 1990; Chaouchi *et al*. 1996; Ritchie *et al*. 1996). This viability promoting effect appears to correlate directly with the ability of many of these factors to block apoptosis indeed IL3 range of haemopoietic cells (Rinaudo *et al.* 1995) and this may be suggestive of the mode of action of this regulatory factor. This then leaves open the question – what is the specific trigger for differentiation?. It may be that the complex actions of the CSFs involves simultaneous blocking of apoptosis and active induction of differentiation. In contrast it may be that if the CSFs act essentially as 'happiness factors', maintaining the viability of the stem/progenitor cells, then differentiation may proceed spontaneously from the protected progenitor cell. The lineage specificity of differentiation in this model will be dictated by the lineage specificity of the supportive growth factor used.

Evidence for such spontaneous differentiation has been presented recently, most notably using the murine FDCPmix cell line (Fairbairn *et al.* 1993). This line is dependent on IL3 for proliferation and survival. In the absence of this factor, the cells die by apoptosis. Introduction of the anti-apoptotic bcl-2 gene into these cells significantly delays the time to onset of apoptosis and intriguingly, this maintenance of survival is accompanied by a spontaneous differentiation of the cells along a range of haemopoietic lineages which this line typically is competent to generate. In this model it seems that IL3 acts to maintain the viability of the cells and, in addition, to block differentiation either directly or alternatively through enforced self renewal. Maintenance of viability of this cell line without a direct or indirect differentiation block therefore allows the cells to proceed at random down a range of lineages.

Some studies have confirmed the ability of apoptotic blockers to allow differentiation albeit limited (Rodel & Link 1996), however, other studies have failed to

demonstrate such effects (Lin *et al.* 1996a). Studies, again using bcl2, in neuronal cells have suggested that spontaneous differentiation may take place in the presence of blockers of apoptosis (Sato *et al.* 1994) and it may turn out that this effect is very general and that the processes of differentiation are spontaneous and require supportive rather than instructive factors for completion. The impetus for differentiation in this model may be given by endogenous expression of transcription factors within the non committed stem or progenitor cells but, this remains to be demonstrated.

A further example of spontaneous differentiation can be seen when murine Embryonal Stem (ES) cells (Robertson 1987) are studied *in vitro.* These cells are totipotent murine cells derived from the d3.5 blastocyst. ES cells can contribute to all embryological tissues and can in fact be used to generate viable mice in conjunction with tetraploid embryonic cells (Nagy *et al.* 1990, 1993). These cells are the basis for current transgenic technologies. *In vitro*, ES cells are primed to differentiate and have to be actively maintained in the undifferentiated state by exogenous addition of the differentiation inhibiting agent, LIF (Smith *et al.* 1988). Removal of this factor is sufficient to allow the differentiative process to proceed and it does so in the absence of added factors. Typically such spontaneous differentiation will result in the generation of viable and functional haemopoietic stem cells and cardiac myocytes (Doetschman *et al.* 1985). Thus in this system differentiation can occur spontaneously supporting the suggestion that such mechanisms may act *in vivo* to generate mature haemopoietic cells.

Development of the haemopoietic system

Until recently, it was assumed that the development of the haemopoietic system in mammals followed an ordered pattern, emerging initially in the yolk sac and subsequently proceeding to the foetal liver and eventually to the bone marrow and spleen which are maintained as the major adult organs of haemopoiesis (Moore & Metcalf 1970). This picture has conflicted with that seen in birds or amphibians in which adult or definitive haemopoiesis has been demonstrated to be derived from intraembryonic pools of emerging stem cells and not from yolk sac derived cells (Chen & Turpen 1995). It is assumed that in these species yolk sac haemopoiesis is transient and may play a distinctive role in early development which is redundant in later development.

More recently, the role of the yolk sac as the source of definitive haemopoiesis in mammals has been called into question. For example, in the mouse, whilst day 8 CFU-S and committed lymphoid progenitors are detectable in the developing yolk sac at early time points (d8) there is no strong evidence of pluripotent long-term repopulating stem cells being present in the yolk sac at these time points. Such cells are not detectable in the yolk sac until late day10/early day11 of development (Dzierzak & Medvinsky 1995). This has prompted a number of researchers to examine possible early intraembryonic sources of definitive haemopoiesis and indeed evidence for a range of nonyolk sac derived primitive haemopoietic progenitors in the developing murine embryo has recently been put forward (Godin *et al.* 1993; Medvinski *et al.* 1993). The intraembryonic location of these progenitors has been identified as the region analogous to the avian paraaortic splanchnopleura which has also been labelled the AGM region to indicate the presence of the developing aorta, gonads and mesonephros. This site has been shown to be the richest site of CFU-S in the early embryo and in addition to be the site of initial emergence of long-term repopulating pluripotent haemopoietic stem cells (Muller *et al.* 1994). The pluripotent stem cells arising in this region carry many of the cell surface markers typical of adult haemopoietic stem cells including CD34 and c-kit. Also some evidence of CD44, mac-1 and AA4.1 cell surface marking has been presented (Sanchez *et al.* 1996).

One problem complicating these studies has been the fact that embryonic circulation is active at these early time points and thus it is possible that whilst the earliest detectable pluripotent stem cells are seen in the AGM region, they may still arise from the yolk sac, migrate to this position and undergo a numerical expansion. It has however, been demonstrated using organ culture of isolated yolk sac and AGM/paraaortic splanchnopleura, that the yolk sac has no independent ability to generate pluripotent stem cells and that these cells arise autonomously in the AGM/paraaortic splanchnopleural region (Cumano *et al.* 1996; Medvinski & Dzierzak 1996). This therefore strongly suggests that this region is the source of definitive adult haemopoiesis and that, as has been shown for avian and amphibian systems, the yolk sac is a source of committed transient haemopoietic progenitors. It is likely that the long-term repopulating stem cells identified in the yolk sac at late d10 of murine development have migrated to this location from the intraembryonic source.

In addition to the emerging cellular biological studies, techniques of transgenesis have allowed an initial dissection of the genes involved in developmental haemopoiesis. To date a large number of genes have been identified as being important in the processes of developmental haemopoiesis and a number of these are listed in Table 1.

Table 1. Genes of importance in developmental haemopoiesis as identified by gene targeting

Gene	Reference	Main observation
ALL1	(Fidanza *et al.* 1996)	
AML1	(Okuda T. *et al.* 1996)	Block in definitive haemopoiesis
β1-integrin	(Hirsch *et al.* 1996)	Block in HSC migration during development
CD34	(Cheng *et al.* 1996)	Developmental delay and functional impairment of HSCs
Epo Receptor	(Lin *et al.* 1996b)	Block in definitive haemopoiesis
Flk1	(Shalaby *et al.* 1995)	Absence of blood islands
GATA1	(Pevny *et al.* 1995)	Block in erythroid development
GATA2	(Tsai *et al.* 1994)	Early block in haemopoietic development
GATA3	(Ting *et al.* 1996)	Aberrant foetal liver haemopoiesis and T cell development
Ikaros	(Georgopoulos *et al.* 1994)	Block in lymphoid development
Myb	(Mucenski *et al.* 1991)	Block in haemopoietic migration to the foetal liver
PU1	(Scott *et al.* 1994; McKercher *et al.* 1996)	Block in B cell and macrophage development
Rbtn2	(Warren *et al.* 1994)	Block in erythroid development
Scl	(Shidvasani *et al.* 1995; Robb *et al.* 1995; Robb *et al.* 1996; Porcher *et al.* 1996)	Block in very early haemopoiesis
SDF	(Nagasawa *et al.* 1996)	Block in B lymphoid development and foetal liver–one marrow movement of haemopoietic cells
TGF	(Kulkarni *et al.* 1993; Letterio *et al.* 1994; Schull *et al.* 1992; Dickson *et al.* 1995)	Defects in yolk sac haemopoiesis and vasculogenesis
TGF–β1 Receptor II	(Oshima *et al.* 1996)	Defects in yolk sac haemopoiesis and vasculogenesis

Much of our current information on the nature of the genes involved in developmental haemopoiesis has come from studies on genes involved in leukaemias or in adult haemopoietic cells. The technical difficulties associated with studies on developmental haemopoiesis have precluded direct attempts at identifying genes of importance in this context. However, the recent identification of the precise embryological regions involved in HSC generation may now facilitate a more focused examination of the genes of importance to these processes. In addition much emphasis is currently being placed on the use of differentiating Embryonal Stem cells as an *in vitro* model of developmental haemopoiesis which may be more open to molecular analysis than any other model currently available.

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