IDENTIFICATION AND CHARACTERIZATION OF HUMAN HEMATOPOIETIC STEM CELLS USING GENE TRANSFER AND THE NOVEL SCID TRANSPLANTATION ASSAY

by

ANDRE LAROCHELLE

A thesis submitted in conformity with the requirements for the Degree of Doctor of Philosophy
Graduate Department of Molecular and Medical Genetics
University of Toronto

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ABSTRACT

Identification And Characterization Of Human Hematopoietic Stem Cells Using Gene Transfer And The Novel SCID Transplantation Assay

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The absence of preclinical repopulation assays for primitive human hematopoietic cells has hindered the development of stem cell gene therapy. Current human gene therapy trials employ gene transfer methods optimized using surrogate in vitro colony-forming cell (CFC) and longterm culture-initiating cell (LTC-IC) assays and by inference from studies on other mammalian species. In this thesis, we report the identification and characterization of a novel human hematopoietic cell, capable of repopulating the bone marrow (BM) of nonobese diabetic mice with severe combined immunodeficiency (NOD/SCID), namely the SCID-repopulating cell (SRC). Human BM or cord blood (CB) cells were infected with retroviral vectors and subsequently transplanted into NOD/SCID mice. Whereas CFC and LTC-IC were efficiently transduced with retroviruses, SRCs were transduced rarely, establishing that SRC are biologically distinct from most CFC and LTC-IC. Moreover, characterization of the cell surface phenotype indicated that SRC are exclusively CD34⁺CD38⁻, in contrast to CFC and LTC-IC that are mostly found in the CD34⁺CD38⁺ fraction after multiparameter flow sorting, confirming the distinct character of SRC. In combination with various data obtained in the past decade, these analyses
provide the strongest evidence that SRC defines a novel human hematopoietic cell that is more immature than any other cell type detected to date.

The inefficient infection of SRC was consistent with the low level of gene marking reported in primates and human gene therapy trials. This inability to infect the most primitive human repopulating cells, in contrast to most CFC and LTC-IC, highlights the need for appropriate pre-clinical models that will predict the outcomes of human clinical trials. As described in chapter 3 of this thesis, primitive BM cells from β-thalassemia major and sickle cell anemia (SCA) patients can engraft and proliferate in the BM of immune-deficient mice. The BM of transplanted mice contained the entire erythroid lineage from BFU-E to mature erythrocytes expressing human γ-, β- or β⁰-globin as in the original donor. Moreover, human erythroid cells from mice transplanted with SCA bone marrow showed characteristic sickling under reducing conditions in an \textit{in vitro} assay. This system can thus be used to evaluate gene transfer efficiency into primitive human cells, longevity of expression, expression in the appropriate lineage, and correction of the disease phenotype.
This is the last page I write. It is my ultimate chance to acknowledge all of you who helped me get...to the last page...

My first words are intended for my "coach", John "The Great" Dick, who introduced me to the delights and enigmas of this fascinating realm of the hematopoietic stem cell. Yes, we have grown old together... yes, we have learned together... and today, John, I am pleased to say that your faith, your criticisms and your success have greatly influenced my scientific and personal progression. Thank you for your teaching...it will inspire my future career...

My journey in John’s lab was short...but long enough to give me the unique opportunity to discover two extraordinarily different generations of budding scientists.

To the first troop,

Christian...doctor in spite of all...thank you for appeasing the burden of my initial transition among those who could not understand the language I had always spoken. Tsogo...the controversial, the star and the unknown...remember the first bone I dissected? thank you because you have guided my hand. Françoise...scientist, artist, chef and mother...thank you for your warm personality, for all those dinners; unfortunately you had to leave too soon. Josef...the impulsive with a golden heart...I am pleased to acknowledge your inestimable contribution to my...our...work; may your life and career be a reflection of your talent, hard work and generosity.

To the next generation,

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Monica...strong and confident...thank you for taking upon your shoulders the problems of us all, and may the little Mark and Sam of your life continue to bring you love and pride. Barb...physical, active and sensitive...thank you for introducing me to the ABC of retroviruses...thank you for your helping hand from the first days to the last...may you always find pleasure in doing the thousand and one things you like to do...

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December 3rd 1996

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FIRST CHAPTER

INTRODUCTION
The concept of hematopoietic stem cells (HSC) evolved in response to the clinical need for cells capable of protecting humans exposed to lethal doses of irradiation or chemotherapy. The first insight came with the observation that lead shielding of hematopoietic tissues prevented death from otherwise lethal doses of irradiation (Jacobson et al., 1949). Intravenous infusion of syngeneic bone marrow (BM) was also shown to prevent death (Lorenz et al., 1951), but it was unclear whether BM transplantation led to reconstitution via the transfer of cells or "factors". Through the elegant use of mouse strains carrying unique chromosomal markers, Ford et al. showed clearly the ability of BM cells to repopulate all of the hematopoietic tissues of an irradiated recipient (Ford et al., 1956; Micklem et al., 1966). Many investigators quickly recognized that the majority of the different cell types encompassing the hematopoietic system were limited in lifespan and, therefore, must be replaced continuously by differentiation from more immature progenitors. Ultimately, there must be a cell with the potential both to differentiate into all mature cells and to maintain itself that could account for the uninterrupted process of blood cell production (Figure 1-1).

1. Stem cell concepts: the influence of different assays

The pluripotent hematopoietic stem cell is the most primitive source of hematopoietic activity. Measurement of stem cell properties, unlike those of many biological systems, is critically dependent on the nature of the experimental assay utilized to define these properties. Foundational murine transplantation studies have served to provide both conceptual and methodological leads for the identification and characterization of hematopoietic cells with long-term repopulating potential. This section explores the fundamental stem cell concepts defined on the basis of these approaches initially described in mice.

1.1 Defining the stem cell assay

1.1.1 The role of colony assays

In a series of seminal experiments, Till and McCulloch observed that the transfer of BM cells to lethally irradiated mice resulted in the formation of macroscopic colonies of myeloid, erythroid and megakaryocytic cells observable in the spleens of the recipients 7 to 14 days after transplantation (Till and McCulloch, 1961; Wu et al., 1967). Using radiation-induced chromosome aberrations, Becker et al. showed convincingly that each large splenic nodule arose from a single cell, the spleen-colony forming unit (CFU-S) (Till and McCulloch, 1961), capable
Fig. 1-1. Schematic diagram of the organization of the murine hematopoietic system. The figure shows a model for the development of hematopoietic lineages from pluripotent stem cells, Sp. In this model the various lineages (highlighted in gray) are assumed to be derived from restricted stem cells (SL: lymphoid-restricted stem cells; SM: myeloid-restricted stem cells).
of extensive proliferation *in vivo*. Individual CFU-S could also produce more colony-forming cells since transplantation of single spleen colonies into secondary recipients produced multiple secondary colonies (Siminovitch et al., 1963). Taken together, these simple observations suggested that CFU-S must define a unique population of radioprotective cells, the hematopoietic stem cells, present within the BM and capable of extensive proliferation, self-renewal as well as multilineage differentiation *in vivo*.

The identification of a cell capable of clonal differentiation *in vivo* prompted Pluznik and Sachs (Pluznik and Sachs, 1966) and, simultaneously, Bradley and Metcalf (Bradley and Metcalf, 1966) to develop a simple quantitative assay for the growth and differentiation of single cell suspensions of mouse BM *in vitro*. When hematopoietic cells were cultured in a semi-solid soft agar medium, discrete colonies were formed and shown to include cells in multiple stages of differentiation. In line with the properties observed for CFU-S, it was subsequently established that colonies generated *in vitro* could also be initiated by the proliferation of a single colony forming cell/unit (CFC or CFU). This approach quickly led to the recognition that clonal growth, maturation and some functional activities of these cells were dependent on specific regulatory hormones, the colony stimulation factors (CSF), for differentiation along one or more lineages (CFU-B, CFU-T, BFU-E, CFU-G, CFU-M, CFU-GM, CFU-GEMM) (Johnson and Metcalf, 1977; Hara and Ogawa, 1978). However, contrasting with the self-renewal potential of most CFU-S, colonies grown *in vitro* showed no or limited ability to proliferate in secondary cultures. This limitation implied that the most primitive stem cells failed to survive or proliferate in this assay and, therefore, CFC were suggested to define a population of committed progenitors, fed from an earlier, more immature compartment of hematopoietic stem cells.

### 1.1.2 Heterogeneity of stem cells in short-term assays

The claims of the CFU-S to subserve the role of a unitary stem cell able to self-maintain and give rise to all the resident populations within the BM was thrown into doubt by numerous observations of a wide disparity in the ability of individual CFU-S to differentiate and self-renew. It appeared that spleen colonies derived from single CFU-S differed greatly in their content of differentiated cells (Wu et al., 1967). Individual colonies were also heterogeneous in their content of new CFU-S, as determined after secondary transplantations (Siminovitch et al., 1963). Further data substantiating the notion of heterogeneity within the stem cell compartment were obtained when Magli *et al.* observed that colonies appearing in the spleen 8 days after transplantation
arose from a different cell type than those enumerated on day 12 (Magli et al., 1982). The early-appearing colonies were unipotential, had little self-renewal ability and were destined to disappear within 3 days. In contrast, day 12 spleen colonies had extensive self-renewal capacity and contained all the cells of the myelo/erythroid lineage. On the basis of these experimental observations, day 12 CFU-S were recognized as a multipotential and self-maintaining population of HSC, while early-appearing colonies were believed to arise from a more committed hematopoietic cell related to erythroid (BFU-E) or granulocyte-macrophage progenitors (CFU-GM).

### 1.1.3 Long-term repopulation in vivo: the hallmark of stem cell assays

Although day 12 CFU-S appeared to define the ultimate stem cell, failure to demonstrate lymphoid progeny unambiguously within a CFU-S derived clone (Paige et al., 1979) and the inability of CFU-S to provide long-term radioprotection in their lethally irradiated recipients (Pozzi et al., 1973) initially suggested that CFU-S may represent a stem cell population somewhat removed from the most primitive cells. In more recent years, evidence has accumulated indicating that populations of hematopoietic cells providing radioprotective differentiated progeny shortly after transplantation are different from those providing long-term reconstitution (Ploemacher and Brons, 1989; Jones et al., 1989). The most compelling evidence illustrating this difference comes from studies involving serial BM transplantation into lethally irradiated mice. Although serially passaged marrow contained significant numbers of CFU-S and could provide short-term radioprotection, it was incapable of long-term (>1 month) repopulation (Jones et al., 1989).

In agreement with these observations, several recent investigations have also indicated the separability of long-term repopulating cells from the population of CFU-S. In one series of experiments, Jones et al. reported the use of counterflow centrifugal elutriation (CCE) to isolate marrow populations on the basis of their size and density (Jones et al., 1990). The separation isolated a population of large blast cells containing CFU-S and CFC that gave only transient, early radioprotection after transplantation into lethally irradiated mice. In contrast, a population consisting of small, dense lymphoid-like cells comprised virtually no CFU-S or CFC and alone could not protect irradiated recipients. However, when these cells were transplanted together with a source of radioprotective marrow, repopulation could be detected 60 days after transplantation. In parallel, Kiefer et al. were able to obtain separation of long-term repopulating
cells from more mature populations by adherence to plastic tissue vessels (Kiefer et al., 1991). Analogous to the lymphoid-like fraction, the adherent fraction showed a marked depletion in CFU-S and CFC as well as an inefficiency at protecting hosts from radiation aplasia; when transplanted with a source of radioprotective cells, the adherent fraction was able to provide long-term hematopoietic repopulation in the recipients.

The fundamental concept emerging from these studies can be summarized: hematopoietic repopulation is mediated by a succession of cells at various stages of development. Immediately following transplantation, the most mature cells contribute to repopulation. With time, cells at progressively earlier stages of development contribute, with the final stable repopulation being provided by long-lived, multipotential stem cells. Consequently, durable, long-term reconstitution of the hematopoietic system of a recipient animal after transplantation remains the only definitive means of identifying and characterizing the hematopoietic stem cell.

1.1.4 Recipients for long-term repopulation assays

Lethally-irradiated mouse recipients have played an influential role in the development of long-term reconstitution assays. Conditioning renders the animal receptive to donor cell engraftment. However, the lethal hematopoietic aplasia following irradiation has contributed to limit their applicability (see below). Genetically deficient mice [reviewed in (Russell, 1979)] offer an important alternative as recipients of stem cell assays. In these mice, donor cells have a competitive advantage over defective host cells, thereby eliminating the need for lethal irradiation. Since survival does not depend on their receipt of bone marrow graft, it is possible to test limiting numbers of HSC for their potential of differentiation and self-renewal. The use of homozygous W/W<sup>+</sup> and scid/scid mutants have been documented in several transplantation studies.

Mutant alleles at the W locus are responsible for lifelong macrocytic anemia, for sterility in both sexes due to failure of multiplication of primordial cells and for a complete absence of hair coat pigmentation. As originally demonstrated by McCulloch et al., W/W<sup>+</sup> mice have a basic stem cell defect and transplantation of normal BM stem cells easily cures their macrocytic anemia (McCulloch et al., 1964). More recently, the proto-oncogene c-kit encoding a transmembrane tyrosine kinase has been shown to map to the mouse W locus (Chabot et al., 1988).

SCID mice were were first identified accidentally by Bosma et al. in breeding experiments aimed at the development of immunoglobulin (Ig) heavy chain congenic mouse strains (Bosma et
al., 1983). A particular litter arose that lacked the correct Ig allotype and further testing indicated a complete absence of any serum Ig. It was quickly recognized that mice homozygous for the recessive scid mutation fail to generate significant numbers of functional Ig and T cell receptor (TCR) gene rearrangements, resulting in a block in B and T lymphocyte differentiation (Schuler et al., 1986; Schuler et al., 1990). Similar to W/W' mice, SCID animals can be cured of their lymphoid deficiency by grafts of normal marrow (Fulop and Phillips, 1989). All other hematopoietic lineages, including myeloid progenitors and natural killer (NK) cells appear normal. SCID cells are also hypersensitive to ionizing radiation because of a deficient chromosomal double strand break (DSB) repair system (Fulop and Phillips, 1990; Biedermann et al., 1991; Hendrickson et al., 1991). The scid mutation has been mapped to the centromeric end of chromosome 16 (Bosma et al., 1989) to a gene encoding the catalytic subunit of the DNA-dependent protein kinase (DNA-PK) (Kirchgessner et al., 1995; Blunt et al., 1995; Hartley et al., 1995; Peterson et al., 1995). The precise roles of DNA-PK in V(D)J rearrangements and DSB repair remain elusive, although one obvious scenario involves phosphorylation of downstream target molecules in response to DNA lesions (Gottlieb and Jackson, 1994). It has also been suggested that DNA-PK might play a structural role, such as aligning DNA termini to promote their ligation during DNA repair or Ig and TCR rearrangements (Blunt et al., 1995).

1.1.5 Genetic markers in long-term repopulation assays

Although colony assays were shown to involve committed cellular entities, they nevertheless define an elegant tradition of hematopoietic clonal analysis. The early experiment of Micklem et al. stressed the importance of genetic markers to follow the progeny of hematopoietic stem cells after reconstitution of an ablated syngeneic recipient (Micklem et al., 1966). The use of donor versus host genetic differences, including hemoglobin polymorphisms or enzyme isotypes, led to the demonstration that all mature blood cell types in the reconstituted recipient mouse were donor derived (Micklem et al., 1966). Other studies involving reconstitution of genetically anemic W/W' mice with BM cells from different mouse strains with polymorphic hemoglobin and immunoglobulin markers have also suggested the existence of pluripotent stem cell (Fleischman and Mintz, 1984; Fleischman et al., 1982; Mintz et al., 1984). However, the limited resolution (only two possible markers) of the donor versus host marker system does not permit a definition of the developmental potential, self-renewal capability and overall proliferative capacity of individual stem cells in the context of simple transplantation.
experiments. Nor does it permit the enumeration of functioning stem cells and the elucidation of the possible dynamics occurring within the entire stem cell population.

An important refinement to the transplantation system was achieved with the use of X-ray induced random chromosomal abnormalities as markers for individual stem cells and the clones derived from them (Wu et al., 1968; Abramson et al., 1977; Ford et al., 1956; Becker et al., 1963). Developmental potential of a stem cell was inferred from the presence of a given karyotypic abnormality in different cell lineages. Identification of the same marker in all sampled lineages from primary and secondary hosts demonstrated the presence of a lymphoid/myeloid stem cell. Although clonally precise, this approach was still indirect since it was dependent on analysis of marker distribution in mature cells. This strategy suffered from low efficiency as well as marker visibility limited to actively dividing cells. Also, radiation-induced chromosomal translocations could affect the normal differentiation and proliferation of these cells [reviewed in (Phillips, 1991)].

In recent years, several groups have sought to extend in vivo clonal analysis by introducing new genetic information into hematopoietic stem cells via transmissible retroviral vectors (Dick et al., 1985; Keller et al., 1985; Lemischka et al., 1986; Jordan and Lemischka, 1990). Conceptually, these studies are very similar to the early random karyotype marker studies discussed above. The integrated provirus serves as the karyotypic clonal marker (Figure 1-2). However, retroviral marker studies offer many advantages over previous approaches. Firstly, marking with provirus is much more efficient than other means of random marking; it may be expected that all stem cells in a population will be identifiable, providing insight into potential stem cell dynamics in the reconstituted hematopoietic system. Secondly, integrated provirus are visible in the DNA of all cells, not only those in active cell cycle. This facilitates the accurate quantitation of the degree to which a given cell population is repopulated by progeny of single engrafted stem cells. Finally, the genetic alterations to the marked cells is less detrimental than those introduced after X-irradiation. Collectively, the retroviral marker studies have sought to provide a more detailed and quantitative description of stem cell functions. In effect, proviral marker studies, because of their quantitative nature, should provide the same degree of precision as CFU-S studies for the analysis of long-term repopulating stem cells. As discussed below, this approach has provided several important novel insights for the identification and characterization of hematopoietic stem cells.
Fig. 1-2. Use of retroviral integration sites as markers to follow stem cell clones. Each cell in an infected population carries the integrated retrovirus at a unique location. If a single infected stem cell has repopulated the mouse, all the progeny will inherit the same retroviral integration site and the entire hematopoietic system (including myeloid (M) and lymphoid (L) tissues) will be clonal. The integration sites can be detected by digestion of DNA extracted from the mouse bone marrow after reconstitution; the size of the bands is determined by the distance between the restriction enzyme site (A) in the retrovirus and the next adjacent restriction enzyme recognition sequence in the host genome. If all cells from the starting population were present in the mouse after reconstitution, a smear would be obtained after digestion with A, since no one integration site or clone would make up a significant proportion of the total population.
1.2 Organization of the hematopoietic hierarchy

1.2.1 Stem cell commitment and increased cell numbers

Three decades ago, the early observations of heterogeneity and extensive proliferative capacity within the CFU-S population had already laid the premise to the concept of hematopoietic hierarchy (Till and McCulloch, 1961; Siminovitch et al., 1963; Wu et al., 1967). Nakahata and Ogawa also provided evidence for the hierarchical organization of the hematopoietic system with the observation of a unique variety of murine colonies exclusively composed of undifferentiated blast cells (Nakahata and Ogawa, 1982). The progenitors of these blast colonies, the CFU-blasts, appeared to be more primitive than multipotential CFU-GEMM; replating of primary blast colonies revealed a high incidence of secondary blast cell and GEMM colonies, while replating of primary GEMM colonies produced only low frequencies of secondary GEMM colonies. However, the exact hierarchical structure of the hematopoietic system has not been delineated until the introduction of quantitative assays for long-term repopulating HSC.

Although the spleen colony assay permits an approximate quantitation of the short-lived CFU-S in a given hematopoietic tissue, the number of long-term repopulating stem cells cannot be measured unequivocally. With the assumption that single stem cells can permanently reconstitute the hematopoietic tissues of a recipient animal, in vivo limiting dilution approaches were utilized to determine the frequency of pluripotent stem cells within a marrow population. In a first series of experiments, Boggs et al. used the genetically anemic W/Wv mouse to assay stem cell concentrations in normal murine BM. Various doses (10^2-10^7) of nucleated marrow cells were injected intravenously in 150 W/Wv hosts and correction of anemia and erythrocytes’ macrocytosis were used as an end point for limiting dilution calculations (Boggs et al., 1982). Cure was observed in ~5% of animals given 10^2 or 10^3 cells, in 50% given 10^4 cells and virtually all given between 5 X 10^4 and 10^7 cells. To test whether the frequency of cured and noncured mice in the different groups followed single-hit Poisson kinetics, implying cure by a single stem cell, they used the statistical method described by Porter and Berry (Porter and Berry, 1964). This method estimates the likelihood that the data fit single-hit kinetics and calculates the frequency of HSC in the cell suspension. Using this approach, they suggested a frequency of stem cells capable of long-term cure of the anemia on the order of 1-10 in 10^5 BM nucleated cells injected (Boggs et al., 1982; Fleischman and Mintz, 1984). A similar estimation was also obtained in murine fetal liver (Fleischman et al., 1982).
Limited numbers of hematopoietic cells injected into lethally-irradiated mice have also served to confirm the stem cell frequency estimated with anemic W/W^" recipients. Since short-term survival of lethally-irradiated mice rests upon the injection and rapid engraftment of large numbers of progenitor cells, limiting dilution studies have been precluded in these recipients until the development of a competitive repopulation assay for the long-term functional capacity of stem cells (Harrison, 1980). As originally described by Harrison, this approach directly compares long-term proliferative abilities of two populations of donor hematopoietic tissues. One population, the competitor, is a dose of fresh BM cells that serves as a standard for repopulating potential. The second population, the donor, is of unknown HSC content and is measured relative to the repopulating ability of the competitor population. Competitor and donor hematopoietic cells are derived from mice that differ at genes specifying quantitative markers (e.g. enzyme isotypes, immunoglobulin and hemoglobin polymorphisms) to allow determination of the relative percentages of their progeny. Transplanted animals are assayed for the percentages of donor and competitor cell types present in their hematopoietic tissues, and the mean contribution of the unknown population is compared to the competitor over a range of cell doses. The presence of two cell types (donor and competitor) contributing to hematopoietic reconstitution allows the application of binomial statistics for the quantitation of hematopoietic stem cells. Using the binomial formula, in which the stem cell number (n) is inversely proportional to the variance (SD^2) in the percentage (p) of donor cell types between the different recipients [n = p(100-p)/SD^2], the relative number of HSC in the transplanted populations was estimated to 1-10 in 10^5 murine BM cells (Micklem et al., 1987; Harrison et al., 1988), in correlation with the frequency calculated previously (Boggs et al., 1982; Fleischman and Mintz, 1984).

In addition, Szilvassy et al have recently developed a clever modification to the competitive repopulation approach for enumerating long-term repopulating stem cells (Szilvassy et al., 1990). Their experimental system involved transplantation of limiting numbers of donor cells into lethally-irradiated recipients together with a population of competitor marrow cells whose long-term repopulating capacity had been compromised by two previous cycles of marrow transplantation. The compromised cells provided radioprotection during the short interval following irradiation. Applying Poisson statistics to the proportion of recipients containing hematopoietic cells of donor origin at least 5 weeks after transplantation, they could calculate the
occurrence of HSC or "competitive repopulation units (CRU)" in the original donor cell suspension. Their estimate (1 in 10^4) correlates with the numbers obtained by other groups.

Comparison of the frequency of long-term repopulating cells (~1-10/10^5) to the concentration of CFU-S (~220/10^5; (Siminovitch et al., 1963)) and in vitro colony forming cells (~1000/10^5) has suggested a key feature to the structure of the hematopoietic system. It appears that a decision of long-term repopulating HSC to commitment is accompanied by an increase in the number of such committed cells (CFU-S, CFC) and a decrease in their self-renewal capacity. The sum of these observations illustrates the hierarchical nature of the hematopoietic system in which rare (1-10/10^5) pluripotent stem cells can proliferate extensively and generate large populations of more committed cells.

**1.2.2 Stem cell commitment and lineage restriction**

An early notion of a possible hierarchical organization within the stem cell compartment came from the lack of compelling evidence demonstrating lymphoid progeny within a CFU-S derived clone (Wu et al., 1968; Paige et al., 1979). This observation was evoking the possibility of a myelo/erythroid restricted stem cell. A more direct evidence for the existence of myeloid (S_m) and lymphoid (S_i) stem cells was provided by in vivo repopulation studies in which random karyotype markers were detected only in myeloid populations or T lymphocytes, respectively (Abramson et al., 1977).

The concept of lineage restricted stem cells has been controversial. The nature of this polemic, however, nicely illustrates the influences of different assay systems. Indeed, it is argued that the demonstration of the existence of lineage restricted populations of self-renewing stem cells relies solely on the assay utilized. For example, in the CFU-S system, the spleen environment together with the immediate post-irradiation systemic demands may not facilitate lymphoid differentiation. Similarly, when karyotype markers are used, visibility of the chromosomal abnormalities only in T lymphocytes may reflect the long half-life of some T cells and not the continued function of a lymphoid stem cell.

Recent data have nevertheless accumulated to further support the notion of lineage restricted stem cells. Fulop et al. used a limiting-dilution assay to identify and enumerate stem cells capable of restoring lymphoid function (S_i) in immune-deficient SCID mice (Fulop and Phillips, 1989). Since CFU-B are nonexistent in SCID mice, correction of immune-deficiency was inferred from colony formation in the CFU-B cell assay. Following single-hit Poisson kinetics, the
frequency of curing units or lymphoid-restricted stem cells (S\textsubscript{1}) has been estimated to \(-20/10^5\) bone marrow cells. Interestingly, this frequency is 2-20 fold higher than the value of 1-10/10^5 determined for pluripotent stem cells (S\textsubscript{p}), using similar limiting-dilution assays (Boggs et al., 1982; Fleischman et al., 1982). The existence of lymphoid stem cells has also been recently suggested by the absence of B, T and NK lineages in mice homozygous for a germline mutation in the Ikaros DNA-binding domain. In these mutants, however, erythroid and myeloid populations were shown to be unimpaired (Georgopoulos et al., 1994). Finally, short-term studies using retroviral integrants as unique markers have also alluded to lymphoid and myeloid restricted stem cells (see §1.4.1). Taken together, these studies suggest that primitive hematopoietic stem cells exist as a hierarchy of progressively restricted cellular entities.

1.2.3 Cell cycle status of HSC: quiescence correlates with "primitiveness"

Analysis of the cell cycle status of different populations of hematopoietic cells contributed to provide important insights into the organization of the hematopoietic hierarchy. Although stem cells are at the origin of the most proliferative cellular system, it is generally held that, in the steady state of hematopoiesis, the majority of stem cells exist in a growth-arrested, albeit viable and reversible, G\textsubscript{0} state. It was reasoned that this dormancy state confers to stem cells time to repair DNA damage, thus allowing maintenance of the genetic integrity of the stem cell populations (Lajtha, 1979). The concept of cell cycle dormancy of stem cells has been supported by a number of observations. For example, exposure of mouse BM cells to tritiated thymidine (\textsuperscript{3}HTdR) did not reduce the number of immature multipotential progenitors (CFU-GEMM) formed \textit{in vitro}, suggesting that the majority of these cells are not synthesizing DNA at any one time (Hara and Ogawa, 1978; Becker et al., 1965). In contrast, parallel assessment of proliferative states of unipotential erythroid progenitors (BFU-E) revealed a high proportion of these cells in active DNA synthetic phase (Hara and Ogawa, 1977). Further evidence was obtained when Hodgson and Bradley brought together CFU-S assay and cell cycle-specific cytotoxic agents (such as hydroxyurea, 5-fluorouracil and bromodeoxyuridine) that preferentially eliminate proliferating cells while sparing the non-dividing cells found in the G\textsubscript{0} phase of the cell cycle. At doses that eradicated early-appearing more committed spleen colonies, these cytotoxic drugs were able to spare late-appearing more immature colonies (Rosendaal et al., 1976; Hodgson and Bradley, 1979; Hodgson et al., 1982; Hodgson and Bradley, 1984).
These investigations suggest that the tendency of hematopoietic cells to reside in $G_0$ is directly correlated with their degree of "primitiveness". Thus, cell cycling status defines an important marker of progressive commitment in the hematopoietic hierarchy. As discussed hereafter, the mechanisms governing the establishment of this hierarchy are believed to be stochastic or inductive in nature.

### 1.3 Hematopoietic stem cell regulation

A priori, self-renewal and commitment to terminal differentiation represent opposite outcomes, and normal hematopoiesis requires a regulated balance between these two outcomes. In its essence, therefore, the key to understanding normal and abnormal hematopoietic development is a comprehension of the mechanisms governing the decision to commit to a pathway of differentiation or to self-renew.

#### 1.3.1 The stochastic model

The CFU-S assay has been instrumental to provide insights into the mechanics of commitment versus self-renewal because all members of a CFU-S derived clone could be physically isolated. Retransplantation of spleen colonies revealed a variable content of secondary colony forming cells in individual primary colonies (Siminovitch et al., 1963). This observation provided the important understanding that the frequency of self-renewal decision at each cell division is not fixed. The random distribution of colony numbers in secondary recipients was rationalized by the stochastic model proposed by Till and McCulloch in which the "birth and death" process, used to define self-renewal and differentiation respectively, must occur at random during colony growth. The model was initially confirmed using a computer simulation based on generation of random numbers that correlated with experimental observations of CFU-S distributions in individual colonies (Till et al., 1964). Subsequently, Nakahata et al. took advantage of the replating capacity of immature progenitor cells (CFU-blasts) to provide a validation of the stochastic model *in vitro* (Nakahata et al., 1982). They related the production of secondary blast cell colonies to a self-renewal process while generation of secondary multilineage colonies was associated to a differentiation process. The random distribution of both types of colonies generated by individual blast cell colonies *in vitro* were reminiscent of the random distribution of CFU-S observed *in vivo* by Till *et al.* and, therefore, consistent with the stochastic mechanisms of stem functions (Till et al., 1964).
The stochastic model also postulates that the genetic events responsible for commitment or self-renewal of HSC are already in place, prior to stimulation by external factors; the presence of growth factors only supports survival and proliferation of the stem cells. In this model, the apparent induction of differentiation by a growth factor is interpreted as a consequence of proliferation and maturation of a specific population of progenitors that are supported by that particular factor and concomitant death of the progenitors that are not supported by the same factor. This concept has been well demonstrated in an immature murine hematopoietic cell line, FDC-Pmix. These cells exhibit a predominant blast cell morphology when maintained in the presence of IL-3, while, in its absence, the cells die from apoptosis. When exposed to fetal calf serum, FDC-Pmix cells differentiate into all myeloid lineages (Spooncer et al., 1986). Furthermore, replacement of IL-3 with GM-CSF leads to short-term differentiation and apoptosis in absence of proliferation. However, when the onset of apoptosis is delayed by the expression of the bcl-2 oncogene, the cells are capable of differentiation in the absence of growth factors (Fairbairn et al., 1993), demonstrating that commitment to cell differentiation does not require the presence of growth factors in this system. In a recent experiment, Dubart et al. have also provided support to the concept of genetic predetermination postulated by the stochastic model. Indeed, they found that erythropoietin (EPO) stimulation of murine pluripotent progenitors, forced to constitutively express a normal EPO receptor, induced a proliferative response via the EPO signalling pathway without preferential erythroid cell differentiation, suggesting the inability of EPO to influence the commitment of pluripotent progenitors (Dubart et al., 1994).

1.3.2 The deterministic model

A deterministic model was also proposed as an alternative mechanism to regulate the decision making of a stem cell to either self-renew or differentiate. The deterministic view was originally formulated by Curry et al. following sequential cytochemical examination of colonies expanding in the the spleen and BM of transplanted mice (Curry and Trentin, 1967; Wolf and Trentin, 1968). Referred to as the "hematopoietic inductive microenvironment (HIM)" model, it envisions lineage-specific microenvironmental niches and humoral factors that direct the differentiation of uncommitted stem cells. However, the observation by Magli et al. of the transient nature of early spleen colonies (see §1.1.2) (Magli et al., 1982) presented a need for reinterpretation of the experimental data on which the HIM was established. Also, the observation that a single hematopoietic blast cell colony revealed multilineage differentiation
upon replating (see §1.2.1) (Nakahata et al., 1982) suggests that microenvironmental influences are not obligatory for stem cell differentiation.

1.3.3 Role of cytokines in the survival and proliferation of HSC

Whereas self-renewal and differentiation of stem cells and progenitors appear stochastically regulated, survival and proliferation of these cells is, however, tightly regulated by a complex network of cytokines. It is thought that cytokines prevent cells from apoptotic death (Koury, 1992). Although initial concepts of cytokine action were relatively simplistic and monolithic, as represented by a now anachronistic nomenclature emphasizing specific lineage actions (eg G-CSF), it has become appreciated that cytokines typically manifest a spectrum of effects crossing boundaries between the specific lineages. Despite the significant redundancy among cytokines, three distinct classes have been defined: (1) late-acting, (2) intermediate-acting, and (3) early-acting factors [reviewed in (Ogawa, 1993)].

Most of the late-acting factors show a dominant action in a specific lineage and support proliferation and maturation of committed progenitors. They include erythropoietin (EPO), M-CSF, interleukin-5 (IL-5), G-CSF and thrombopoietin (TPO) that show a prominent activity in erythroid, monocyte/macrophage, eosinophil, neutrophil and megakaryocyte/platelet lineages, respectively. While the effect of individual regulators prevails on a specific cell type, they are usually not restricted to cells of a single lineage. For example, G-CSF also serves as a synergistic factor for primitive dormant progenitors (see below). Similarly, IL-5 can stimulate the terminal differentiation of murine B (Harriman et al., 1988) cells and cytotoxic T cells (Takatsu et al., 1987). Also, in presence of erythropoietin, TPO has recently been shown to enhance erythroid burst formation (Kobayashi et al., 1995; Papayannopoulou et al., 1996). Even erythropoietin, possibly the most restricted regulator, is also believed to possess an activity on megakaryocyte precursors (Ishibashi et al., 1987).

Using serial observations (mapping) of the development of murine blast cell colonies, Ogawa and colleagues characterized the functions of several cytokines that appear to regulate the proliferation kinetics of primitive hematopoietic progenitors and developed a model of cytokine regulation of early hematopoiesis [reviewed in (Ogawa, 1993)]. In this way, IL-3, IL-4 and GM-CSF have been classified as intermediate-acting lineage non-specific factors. Similarly, IL-6, G-
CSF, IL-11, IL-12, leukemia inhibitory factor (LIF), flt3/flk2 ligand (FL) and stem cell factor (SCF), alternatively known as mast cell growth factor (MGF), c-kit ligand (KL) or steel factor (SF), have been categorized in the early-acting family of synergistic factors required for the entry of the dormant blast progenitors into the cell cycle.

IL-3 and IL-4 have not been tagged as early-acting regulators because of their property to support the proliferation of CFU-blast progenitors only after their exit of the G₀ phase of the cell cycle. The first indication suggesting this mode of action came from studies of the effects of murine IL-3 (Suda et al., 1985) and IL-4 (Kishi et al., 1989) on blast cell colony formation by populations of murine progenitors enriched in G₀ cells by 5-fluorouracil (5-FU) pre-treatment. For example, the daily observation of blast colony growth in the presence of IL-3 revealed a sequential and continuous development of these colonies in vitro, suggesting that CFU-blasts emerge from a dormant cell-cycle state at varying periods of time. When the addition of IL-3 was delayed to day 7 of incubation, the earlier-appearing blast cell colonies were eliminated. However, the delayed addition did not appear to alter the proliferative characteristics of late-appearing blast cell colonies which revealed identical doubling rates with those in cultures receiving IL-3 at day 0. Based on these observations, Suda et al. proposed that IL-3 does not trigger cell cycling of dormant CFU-blasts, but rather provides an appropriate milieu for progenitor proliferation after the dormant cells have begun to divide (Suda et al., 1985). Furthermore, a subsequent investigation (Koike et al., 1986) demonstrated the inability of IL-3 to support by itself the terminal stages of hematopoiesis. Indeed, the responsiveness of murine CFU-blasts to IL-3 was shown to diminish as the cells differentiate and become more committed.

While CFU-GM was originally identified as a lineage-specific factor regulating only progenitors in the granulocyte/macrophage lineages, subsequent studies showed a lack of lineage specificity of GM-CSF. For example, Metcalf et al. and Koike et al. observed that murine GM-CSF supports the proliferation of CFU-blast progenitors, suggesting an overlap in the cellular targets of IL-3 and GM-CSF (Metcalf et al., 1980; Koike et al., 1987). This functional overlap could well be explained by the common β-subunit shared by the GM-CSF and IL-3 receptors (Kitamura et al., 1991). In addition to their action on multilineage CFU-blast progenitor cells, distinct hematopoietic effects have also been clearly documented for IL-3 and GM-CSF. For
example, whereas GM-CSF can provide support for myeloid progenitors (Kaplan et al., 1989), IL-3, on the other hand, can mediate a response on erythroid and megakaryocytic progenitors (Ganser et al., 1990). Furthermore, these two cytokines can effectively synergize with late-acting growth factors in the production of more mature cells. Interestingly, their complementary biological effects and cross-competition for receptor binding have recently prompted the development of PIXY321, a synthetic hybrid protein consisting of the active domains of GM-CSF and IL-3 coupled by a flexible amino acid linker sequence allowing the binding domains to fold into their native conformation (Curtis et al., 1991). The early results of in vivo and in vitro investigations suggest that PIXY321 elicits the biological effects of both its component cytokines and represents a novel means of delivering two independent but interactive cytokines in combination [reviewed in (Vadhan-Raj, 1994)].

Evidence has been provided for the involvement of IL-6 in the triggering of cell divisions of dormant hematopoietic progenitors. Ikebuchi et al. reported that IL-6 acts synergistically with IL-3 in support of the proliferation of murine multipotential progenitors in culture (Ikebuchi et al., 1987). In this study, post-5-FU murine spleen cells were plated in semisolid cultures in presence of IL-3 and/or IL-6, and the emergence of new blast cell colonies as well as their subsequent proliferation were recorded daily. The average number of days required for colonies to reach 100 cells was found to be significantly reduced in cultures containing IL-3 and IL-6 as compared to those including only one factor, suggesting that a combination of IL-3 and IL-6 significantly shortens the duration of the G0 phase of primitive progenitors. The synergistic effect of IL-6 was confirmed using CFU-S assays performed on suspension cultures of murine BM cells harvested after 5-FU treatment and cultured 6 days in the presence of IL-3 and/or IL-6 (Bodine et al., 1989). The combination of IL-3 and IL-6 increased the number of CFU-S in culture 5-10 fold over the number obtained with IL-3 alone. The role of IL-6 was also assessed for stem cell function as measured by competitive repopulation assay (Bodine et al., 1989). When BM cells from two distinguishable donors were cultured in the presence of IL-3 alone (donor 1) or IL-3/IL-6 (donor 2), the contribution of the second donor was 2-fold more important than the contribution of the first donor, indicating a synergistic effect of IL-6 for the proliferation of hematopoietic cells with long-term repopulating potential. On the basis of these studies, IL-6 was also tested for its ability to increase the efficiency of retroviral-mediated gene transfer into primitive stem cells (Bodine et al., 1989). While only 12% of mice reconstituted with cells
infected in the presence of IL-3 alone showed long-term expression of the transferred gene, up to 55% of mice transplanted with cells infected in the presence of both IL-3 and IL-6 maintained gene expression over extended periods of time. Together with more recent studies performed on purified populations of immature hematopoietic cells (Leary et al., 1992), these investigations strongly imply a synergistic role of IL-3 and IL-6 for entry into the cell cycle of dormant primitive hematopoietic cells.

Mapping studies of blast cell colony formation have shown that G-CSF (Ikebuchi et al., 1988), IL-11 (Musashi et al., 1991), SCF (Tsuji et al., 1991), LIF (Leary et al., 1990), IL-12 (Hirayama et al., 1993) and FL (Hirayama et al., 1995) also act synergistically with IL-3 to support the proliferation of dormant murine hematopoietic progenitors. A possible synergistic role of IL-1 appears controversial (Leary et al., 1988; Muench et al., 1993). Most of these early-acting factors also interact with other intermediate-acting factors including GM-CSF (Tsuji et al., 1992) and IL-4 (Rennick et al., 1989). Molecular studies have provided some explanations for the functional similarities observed among these synergistic factors. For instance, IL-6 and G-CSF share great structural homology, suggesting a common ancestral gene (Hirano et al., 1986). Homology between IL-12 and IL-6 and its receptor have also been reported (Merberg et al., 1992), whereas LIF, IL-11 and IL-6 receptors use a common signal transducing protein, gp130 (Yin et al., 1993; Gearing et al., 1992). However, among these related synergistic factors, SCF and KL present unique characteristics. Namely, SCF and FL can interact with other early-acting factors, including IL-6, IL-11 G-CSF, to support the formation of multipotential blast cells and multilineage colonies while other do not appear to have this capacity. Both cytokines have also been shown to interact with late-acting factors, in particular EPO (Hirayama et al., 1995; Dai et al., 1991). Interestingly, FL, by itself and in synergy with SCF or IL-7, can support the proliferation of B cell progenitors (Hirayama et al., 1995).

It appears that cytokines are also required for primitive hematopoietic cells to survive in the dormancy state. Bodine et al. examined the effects of 10 different growth factors alone and in combination on both CFU-S and on long-term repopulating stem cell survival after 6 days in liquid suspension culture (Bodine et al., 1991). They proposed IL-3, G-CSF and IL-4 as single survival factors for CFU-S in vitro, whereas IL-3 or IL-4 could support only long-term repopulating cells. Using highly purified populations of murine G0 cells, Itoh et al. and Katayama et al. also presented evidence for the role of IL-3 and G-CSF (Itoh et al., 1992) or IL-3 and IL-4
Katayama et al., 1993) in supporting survival of dormant primitive progenitors (Katayama et al., 1993). SCF was also demonstrated as a survival factor for dormant cells (Katayama et al., 1993).

The impressive proliferative potential of the hematopoietic progenitors in response to growth factors is opposed by the action of negative regulators, including the transforming growth factor-β (TGF-β), the tumor necrosis factor-α (TNF-α), the macrophage inflammatory protein-1α (MIP-1α), interferon-γ (IFN-γ), the pentapeptide pyroGlu-Glu-Asp-Cys-Lys (pEEDCK) and the tetrapeptide acetyl-N-Ser-Asp-Lys-Pro (AcSDKP). In general, interferons (Raefsky et al., 1985) and TNF-α (Akahane et al., 1987) appear to be lineage nonspecific inhibitory factors affecting progenitors throughout a wide range of developmental stages. In contrast the inhibitory effect of TGF-β appears to be directed at the early stages of hematopoiesis (Kishi et al., 1989; Ohta et al., 1987). Similarly, MIP-1α was reported to inhibit proliferation of primitive hematopoietic progenitors (Broxmeyer et al., 1990). Whether or not TGF-β and MIP-1α play a physiological role in negative regulation of stem cells in G0 is not clear at this time. However, an interesting observation in this regard is the report that antisense TGF-β sequence (Hatzfeld et al., 1991) or TGF-β antiserum (Abrams et al., 1981) significantly enhances the frequency of multilineage colony formation in culture. Regulation of stem cells therefore appears to be a complex integration of intracellular signaling induced by both positive and negative signals, resulting in a single readout of either active or inactive cell cycling.

Taken together, the in vivo and in vitro analyses presented here emphasize the functional importance of growth factors, alone or in combination, for the survival and proliferation of hematopoietic cells rather than their ability to direct self-renewal and differentiation. Primarily, they pinpoint the dominant effect of IL-3 in synergy with IL-6 and other early-acting factors, such as SCF, for the survival and proliferation of primitive hematopoietic cells. The results of such investigations have served as an important premise for the definition of cytokines acting on long-term repopulating human hematopoietic cells. However, as illustrated in chapter 2, human stem cell studies based on extrapolation from progenitor assays or by inference from investigations on other species suggest that there may be species-specific characteristics of primitive cells in their susceptibility to various cytokines.
1.4 HSC properties analyzed with retroviral markers

Most models of HSC behavior are based on extrapolation from in vitro colony assays or short-term CFU-S studies, which are amenable to precise manipulation. Recently, retroviral marking studies have provided several noteworthy insights into the behavior of individual stem cells and the entire reconstituted hematopoietic system that spread beyond the understanding gained from colony assays.

1.4.1 Short-term studies

Initial applications of the retroviral marking strategy (Dick et al., 1985; Keller et al., 1985; Lemischka et al., 1986; Snodgrass and Keller, 1987) yielded results that extended earlier classical studies (Wu et al., 1968; Abramson et al., 1977). The observation of lymphoid and myeloid cells harboring the same proviral integrant 10-20 weeks after transplantation confirmed the existence of pluripotent stem cells capable of contributing to all cell lineages. In addition, the ability to accurately quantitate the extent to which a given tissue was repopulated by progeny of a marked stem cell made possible a rigorous demonstration of the ability of a single stem cell to reconstitute the entire murine hematopoietic system (Dick et al., 1985). This remarkable capability had been suggested previously by limiting-dilution (Boggs et al., 1982; Harrison et al., 1988) and allozyme marker studies using indirect binomial statistics (Micklem et al., 1987; Micklem et al., 1983).

Molecular analysis of the spectra of proviral integration sites found in each fractionated cell population also provided evidence for the existence of lineage restricted stem cells, as previously proposed (see §1.2.2) (Wu et al., 1968; Abramson et al., 1977). Discrete proviral integrants present only in the BM but absent from the spleen or the thymus suggested the existence of a cell restricted to myeloid differentiation (S_m) (Dick et al., 1985; Lemischka et al., 1986). Similarly, the concept of a lymphoid restricted stem cell (S_l) was supported by the identification of single junction fragments only in the thymus or in both the thymus and spleen after reconstitution (Dick et al., 1985; Lemischka et al., 1986).

Models postulating the existence of distinct lymphoid and myeloid stem cells are often viewed with caution [reviewed in (Lemischka, 1992)]. The concept of a "quantitatively restricted" stem cell is favored by some investigators (Lemischka, 1992). This notion originated from the observation of a given retroviral integrant showing more prevalence in some lineages than in others. This unequal representation of a discrete proviral marker in different myeloid and
lymphoid lineages implied that HSC may be skewed in their ability to contribute to mature cell types, favoring some over others. An extension of this reasoning argues that stem cell clones whose progeny are not visible in all myeloid and lymphoid lineages may not be intrinsically restricted in potential, but, rather, may represent extreme examples of skewed contribution by an unrestricted pluripotent stem cell.

Earlier, classical studies had proposed a clonally successive organization of the hematopoietic system (Kay, 1965; Micklem et al., 1983; Mintz et al., 1984; Micklem et al., 1987). The basic feature of all clonal succession models reveals that only a small subset of the available stem cell compartment is hematopoietically active at any given time. One corollary of such models may be that stem cells have a finite proliferative potential (Hayflick, 1965) and are destined to extinction after their recruitment for hematopoietic reconstitution; alternatively, stem cells that have ceased to contribute may simply return to a quiescent state. In line with the former prediction, recent studies have hinted at the existence of a mitotic clock limiting the proliferative potential of HSC [reviewed in (Lansdorp, 1995)]. It was suggested that purified HSC, similar to all somatic cells studied, may lose telomeric DNA upon each cell division, resulting in cell cycle exit and cellular senescence. In contrast, *in vivo* systems utilizing allophenic intrastrain chimaeric mice have favored the conclusion that actively cycling stem cells can return to a quiescent state over time and, upon transplantation into secondary allophenic recipients, can be activated again for hematopoietic function (Van Zant et al., 1983; Van Zant et al., 1991; Van Zant et al., 1992).

Several observations derived from short-term retroviral marking studies have been interpreted as evidence for clonal succession models of hematopoiesis. Indeed, retransplantation of marked BM from primary to secondary hosts have revealed novel integrants not readily detectable in the mature tissues obtained from the original animals (Lemischka et al., 1986). These novel proviral markers suggested the existence of dormant, non-contributing stem cells in the primary recipients that have subsequently been recruited for reconstitution of the secondary animals. Similarly, additional investigations examined the fate of marked stem cell clones in single hosts at different points in time (Lemischka et al., 1986; Snodgrass and Keller, 1987). In these studies, dramatic clonal fluctuations could be detected over the time course of the experiments (1-5 months). These fluctuations were categorized as increases, decreases or lineage shifts in the contributory activity of individual stem cell clones and provided support for clonal succession models of stem cell utilization.
1.4.2 Long-term studies

In an effort to gain further insight into the clonal dynamics of reconstituted hematopoiesis and stem cell behavior, the initial gene marking strategy was modified to encompass longer time intervals (Capel et al., 1989) and to permit a continuous analysis of multiple lineages at many points in time (Jordan and Lernischka, 1990). Thus, a complete, near lifelong picture of stem cell clonal behavior following transplantation has emerged.

In a first investigation (Capel et al., 1989), virally marked BM of fetal liver was transplanted into anemic (W/Wv) recipients. Animals were sampled at 3 months, and later at 9-11 months. Identical proviral markers observed at these two time points suggested that individual stem cell clones can persist for long time periods as major contributors to hematopoietic cell populations. Of particular significance was the maintenance of the same integrant in myeloid tissues. Due to the short half-lives of mature myeloid cells, the continued presence of a distinct integrant in these cells was a notably valid indicator of continuous stem cell function and, by definition, an indirect evidence of self-renewal potential.

A key feature of the experimental design of a subsequent investigation (Jordan and Lernischka, 1990) was the ability to sample the major peripheral blood lineages at 6-8 weeks intervals for periods up to 16 months. A large number of marked clones were analyzed sequentially, allowing several important conclusions to be drawn in respect to individual stem cell function. When viewed over long time intervals, hematopoiesis is often oligo or monoclonal in stem cell origin. That is, a small number of pluripotent stem cell clones function continuously and stably to repopulate all mature cell populations. Thus, at least in theory, it appears that individual stem cells present at the time of engraftment are both necessary and sufficient for permanent hematopoiesis. In addition to stable pluripotent stem cell activity, these long-term studies also described a variety of lineage restrictions and clonal fluctuations. Interestingly, nearly all of these behaviors occurred in the first 20 weeks post-engraftment, suggesting that they indeed reflect a system not yet functioning at equilibrium rather than intrinsically distinct classes of stem cells. As the time frame of the short-term marking experiments was also only 20 weeks, many investigators argue that models favoring clonal succession and lineage restricted stem cells must be reinterpreted in the light of these findings.

In essence, these studies introduce the concept of a reconstituted hematopoiesis comprising two distinct phases. A first interval is characterized by fluctuations and unequal,
lineage restricted contributions by individual stem cells. In a second, much longer time interval, hematopoietic activity becomes clonally stable, the reconstituted cells being derived exclusively from a single or a few pluripotent stem cells. Therefore, a key insight gained from retroviral marker studies in the mouse indicates that stem cell developmental potential as well as proliferative capacity can be evaluated only as a function of time.

1.4.3 A model of stem cell behavior

The temporal description of murine stem cell behavior can be synthesized into an overall model of reconstituted hematopoiesis [reviewed in (Lemischka, 1992)]. In this model, all lineage restricted contributions, clonal fluctuations as well as clonal stability result from commitment versus self-renewal decisions occurring during the expansion of a pluripotent stem cell clone. The temporal distribution of fluctuations and clonal stability is most consistent with a stochastic component mediating the decision to self-renew or commit to differentiation. Thus, stem cell clones choosing predominantly to self-renew in the initial phase after engraftment appear as minimal contributors to mature tissues. Subsequent stochastic mechanisms operating on the expanded pool of self-renewed target cells give rise to equal and stable contribution. Similarly, clones choosing to commit to differentiation early following engraftment may be destined to extinction at later time points. Alternatively, a balance of gradual self-renewal and commitment decisions may initially yield lineage restricted contribution as well as fluctuation. Over time, as the size of the stem cell clone increases, contribution to all lineages would equilibrate and stabilize.

While supporting stochastic mechanisms at the level of self-renewal versus commitment, the retroviral studies suggest that the outcome of such decisions may also be dependent on specific systemic demands. Indeed, since short-lived host myeloid cells turn-over occurs quickly following irradiation, there is an initial need for these cell populations. Consistently, retroviral marking studies have shown that lineage restricted contribution by pluripotent clones always takes place first in the myeloid compartment; subsequently, contribution expands to include lymphoid populations. The opposite situation has not been documented. Thus, \textit{in vivo} lineage-specific commitment decisions may occur in a relatively ordered fashion in spite of a stochastic mechanism mediating the decision to commit or self-renew.
1.5 HSC properties measured by competitive repopulation

Additional insights into long-term stem cell function were provided by means of competitive repopulation of murine bone marrow. The competitive repopulation assay (Harrison, 1980) offers the advantage of inferring stem cell developmental properties without the often damaging procedures used to introduce clonal markers. While retroviral marking studies quantitatively follow the progeny of single, uniquely marked stem cells, competitive repopulation directly measures the function of entire populations of pluripotent stem cells. Thus, it can be viewed as a complementary technique to clonal analysis.

In general, comparison of random marking studies with competitive repopulation analyses reveal concordant results. Both approaches suggest that long-term investigation is necessary for accurate measurement of primitive stem cell function (Jordan and Lemischka, 1990; Harrison, 1980). Also, clonal succession or sequential stem cell activation models are not supported by either approach (Jordan and Lemischka, 1990; Harrison et al., 1988). Finally, the in vivo function of intrinsically lineage restricted stem cells is not demonstrable unequivocally by either experimental procedure (Jordan and Lemischka, 1990; Micklem et al., 1987).

In spite of a general agreement, several important disparities emerge with these two approaches. In particular, competitive repopulation suggests a direct relationship between numbers of engrafting functional stem cells and absolute input cell numbers (Harrison et al., 1993), implying that all stem cells are active at any time point. In contrast, retroviral marker studies suggest that long-term hematopoiesis derives from selection and dominance of few initially engrafted stem cells in a manner largely independent of input cell counts (Jordan and Lemischka, 1990; Capel et al., 1989). A possible explanation for this discrepancy may be found in the assumption, inherent in statistical interpretation of competitive repopulation data, that all pluripotent stem cells exert equal hematopoietic function after transplantation. Alternatively, it can be argued that clonal dominance may reflect the artefactual inability of the retroviral marking assay to follow the fate of large numbers of stem cell clones rather than dominance by one or few clones (Dick et al., 1985; Lemischka et al., 1986; Keller et al., 1985). Here again, the influence of the assay is well illustrated. Perhaps, competitive repopulation assays used in conjunction with clonal analyses could provide valuable information.
1.6 Summary

The experimental strategies underlying our current understanding of the biological features of hematopoietic stem cells have been presented in this section and emphasize several important issues. In particular, the remarkable ability of hematopoietic stem cells to reconstitute and maintain a functional hematopoietic system over extended periods of time in lethally-irradiated or genetically defective hosts represents a hallmark of stem cell behavior. Thus, although short-term colony assays were instrumental in defining fundamental stem cell concepts of hematopoiesis, long-term in vivo reconstitution remains the only relevant assay for these cells. This endowment of HSC to sustain long-term and functional hematopoiesis has been related to their dual ability to either differentiate to produce mature progeny of all myeloid and lymphoid blood cell lineages or self-renew to replace the cells that became progressively committed to differentiation. The majority of HSC, however, are resting in G0 and do not contribute to the daily production of mature blood cells. The mechanisms governing the decision of a stem cell to enter cell cycle and commit to differentiate or self-renew can be described in a model of stem cell regulation that incorporates stochastic and non-stochastic components. Competitive repopulation assays and clonal analysis have served to provide novel, sometimes controversial, insights into stem cell function. For instance, the concept of lineage restricted stem cells, originally defined in karyotype marker studies, was thrown into doubt when retroviral integrants were followed for extended periods of time in vivo. Also, while classical studies hypothesized a clonal succession model of hematopoiesis, competitive repopulation and viral marking approaches rather present evidence of clonal stability. Similarly, the notion of clonal dominance remains controversial and clearly illustrates the critical influence of different assays in defining stem cell properties. Taken together, these observations present a blood forming system organized in an irreversible hierarchy including rare, non-cycling pluripotent stem cells, a larger population of more restricted progenitor cells and a vast pool of mature cells with defined functions (Figure 1-1).

2. In search of the human hematopoietic stem cell

Although studies in the mouse were instrumental in defining fundamental concepts of hematopoietic stem cells, parallel investigations on human long-term repopulating cells have been impeded by the absence of in vivo assays equivalent to those developed for murine stem cells.
Indeed, human studies have relied for the most part on *in vitro* assays of progenitor cell behavior and on the results of clinical bone marrow transplantations.

Evidence for pluripotent stem cells in man is derived from studies of BM mononuclear cells in chronic myelogenous leukemia (CML). Whang *et al.* have obtained evidence that the Philadelphia chromosome characteristic of this disease is present in erythroblasts, granulocytes and megakaryocytes, indicating that these classes of cells are descended from a single leukemic stem cell (Whang *et al.*, 1963). In subsequent studies of glucose-6-phosphate dehydrogenase (G6PD) female patients, only one isoenzyme was found in erythrocytes, granulocytes and platelets although both were present in non-hematopoietic cells (Fialkow *et al.*, 1977), providing evidence for the existence of a cell common for these three lineages. In addition, the presence of transplantable lympho-myeloid stem cells in normal adult marrow was suggested by recent studies of women transplanted with marrow from normal female donors heterozygous for restriction enzyme polymorphisms at the X-chromosome-linked PGK or HPRT loci. Analysis of the circulating blood in these individuals indicated the presence of granulocytes and T cells originating from a common, donor-derived precursor (Turhan *et al.*, 1989). Finally, *in vitro* exposure of human marrow cells to agents such as 4-hydroperoxy cyclophosphamide (4-HC) at doses that kill ~90% of cells detectable by *in vitro* colony assays has been found to spare the ability of the same marrow to serve as protective autograft (Yeager *et al.*, 1986), suggesting little overlap between human clonogenic progenitors and reconstituting cells.

Although the presence of human hematopoietic reconstituting cells has been confirmed in normal and leukemic adult bone marrow, peripheral blood (PB) from adults and umbilical cord blood (CB) from newborns have also been used as alternative sources of hematopoietic cells with long-term repopulating capacity. Concentration of hematopoietic stem/progenitor cells in circulating adult blood has been estimated to 0.1-0.001 of that found in BM (36) and, consequently, the use of this cell type for transplantation is dependent on multiple lengthy apheresis procedures. However, it has been known for some time that primitive hematopoietic cell numbers increase in PB of patients during the regeneration phase following myelo-suppressive chemotherapy, as a result of stem cell movement (or mobilization) out of the BM (Abrams *et al.*, 1981). Immature cells were also found to be mobilized in the circulating blood by infusion of cytokines such as G-CSF, GM-CSF and SCF alone or in combination with cytoreductive agents [reviewed in (Janssen, 1993)]. In the past decade, mobilized peripheral
blood stem cells (PBSC) have been used for autologous transplantations in humans. Most often, this form of therapy has been applied for patients diagnosed with lymphomas, leukemias, breast cancers or other solid tumors. Concerns have been expressed however that adult PBSC may have only a finite capacity for maintaining hematopoiesis (Brito-Babapulle et al., 1989). The use of human umbilical CB for hematopoietic reconstitution was suggested by E.A. Boyse who observed that lethally-irradiated mice could be rescued and reconstituted with neonatal mouse blood [reviewed in (Broxmeyer et al., 1992)]. Ontologically, hematopoietic stem cells proceed from yolk sac and probably from paraortic regions to liver and from liver to marrow, spleen and thymus, by sequential colonization via the bloodstream [reviewed in (Zon, 1995)]. Vestige at birth of fetal blood, umbilical CB has also been shown to contain high numbers of primitive hematopoietic cells, including multipotent, erythroid, granulocyte-macrophage and megakaryocyte progenitor cells (Broxmeyer et al., 1989). Moreover, hematopoietic progenitor cells from human CB could be maintained for many weeks in long-term culture systems (see §2.2), suggesting their production from more immature cells (Salahuddin et al., 1981). Ultimately, the presence of reconstituting hematopoietic cells in CB has been established for the first time by Gluckman et al., who reported successful engraftment of a child suffering from Fanconi’s anemia, after transplantation with cells collected at birth from CB of an HLA identical sister (Gluckman et al., 1989).

Current interest in gene therapy protocols to permanently correct, or at least modify, the phenotype of particular blood cell types highlights the importance of defining an assay for human long-term repopulating stem cells. As discussed before, studies in the mouse have established that retroviral vectors can efficiently transfer and express new genes into primitive pluripotent stem cells capable of reconstituting all lymphoid and myeloid tissues of recipient mice (Dick et al., 1985; Lemischka et al., 1986; Keller et al., 1985). However, the efficiency of gene transfer into hematopoietic stem cells was much lower when similar protocols were applied to large mammals (Bodine et al., 1993; Schuening et al., 1991) and in human gene therapy trials (Dunbar et al., 1995; Kohn et al., 1995), suggesting the presence of species-specific characteristics of stem cells in their susceptibility to retrovirus infection (see chapter 2). Moreover, it is well established that pathological states in the mouse do not always recapitulate human diseases. For example, development of mouse models for β-thalassemia by insertional disruption results in very different cellular phenotypes between the two species because of the ten-fold difference in effectiveness of
the human and mouse β-minor genes [reviewed in (Smithies, 1993)]. Hence, there are subtle differences between the two species that dramatically affect the nature of the disease that is induced in experimental models.

The previous observations underscore the need for assays that permit direct identification and characterization of human long-term reconstituting cells. These assays should also offer sufficient sensitivity and specificity to permit isolation and distinction of human repopulating cells from closely related but derivative progenitor populations. Since in vivo transplantation studies are impractical in man, alternative approaches have been explored for the development of human specific stem cell assays. The first approach to study primitive human hematopoietic cells was stimulated and based on the observations by Dexter and colleagues that CFU-S could be maintained in cultures of mouse BM for many weeks under conditions that allow the rapid establishment of a supportive feeder layer. Hematopoietic cells maintaining these so-called long-term bone marrow cultures (LTBMC) or Dexter cultures appear to have characteristics of very primitive cells. The second approach is based on the recognition of the potential use of immune-deficient xenogeneic hosts to create in vivo models of human hematopoietic reconstitution. In this section, both procedures will be described and assessed for their relevance in human stem cell studies. Given the importance of stem cell purification in each approach, strategies currently employed for enrichment of primitive hematopoietic cells will also be presented.

### 2.1 Stem cell enrichment strategies

Many attempts to purify stem cell populations have used a combination of approaches based on the physical and biological properties and the immunophenotype of the target cells.

#### 2.1.1 Physical properties

Early work on murine BM used a variety of density gradients and filtration through glass-wool columns to separate subpopulations of cells. These experiments revealed that the transplantable hematopoietic stem cells co-purified with lymphocytes (Cudkowicz et al., 1964; Morrison, 1967) and led to the idea that pluripotent stem cells are morphologically indistinguishable from lymphocytes (vanBekkum et al., 1971). Density gradient separations, such as Ficoll® and Percoll® gradients, are still commonly used as a pre-enrichment step in stem cell purification protocols.
2.1.2 Biological properties

As discussed before (§1.2.3), the majority of stem cells rest in the quiescent phase of the cell cycle. By combining the stimulating activities of hematopoietic growth factors and the cytotoxic effect of an antimetabolite agent, Berardi et al. exploited this property of stem cells to isolate a population of primitive human hematopoietic cells (Berardi et al., 1995). Based on the assumption that IL-3 and SCF do not trigger cell cycling of dormant human stem cells, they selectively induced the proliferation of and subsequent death of committed progenitors by incubating BM mononuclear cells in the presence of IL-3, SCF and high concentrations (200µg/ml) of 5-FU. The surviving cells resembled small lymphocytes and had the phenotypic and functional attributes of hematopoietic stem cells. However, this approach presents the disadvantage of requiring physical purification of the isolated cells subsequent to the antimetabolite treatment. Moreover, it is not clear what proportion of stem cells survived the selection procedure since in vivo treatment with pegylated SCF followed by 5-FU have been previously shown to result in complete BM failure in the mouse (Molineux et al., 1994).

Primitive hematopoietic cells can also be distinguished from their progeny by their different adhesive abilities to bind to cultured stromal layers (see §2.2.1) and to tissue culture plastic [reviewed in (Scott and Gordon, 1995)]. Plastic adherence has been used to separate transplantable long-term marrow repopulating cells from more committed progenitor cells (Bearpark and Gordon, 1989). Moreover, primitive plastic-adherent cells have been shown to exist in adult BM (Gordon, 1994), as well as umbilical CB (Gordon et al., 1995) and PB collected for autologous transplantation (Scott et al., 1995).

2.1.3 Immunophenotype

Immunization of mice with a human leukemic cell line, KG1a, has led to the generation of the first monoclonal antibody (My10) that recognize the sialomucin CD34 antigen, a highly O-glycosylated cell surface glycoprotein. Several other antibodies recognizing the same glycoprotein on different epitopes have been produced subsequently (12.8, 8G12, IMU133, QBEND10, B13C5, etc.). Approximately 1-3% of bone marrow and cord blood cells express the CD34 antigen. The functional significance of this glycoprotein has been difficult to delineate, but it may be involved in intercellular adhesion, as suggested by structural characteristics of the molecule, by the localization of its gene on the long arm of chromosome 1 among several genes coding for adherence molecules, and by the elegant demonstration that CD34, expressed on
endothelial cells, can bind to the lymphocyte adhesion protein, L-selectin (He et al., 1992; Simmons et al., 1992). Additional data have also suggested that two serine residues in the cytoplasmic portion of the molecule are phosphorylated in response to protein kinase C activation of hematopoietic cell lines, consistent with a potential function of this antigen in cell activation (Fackler et al., 1992). Along these lines, protein kinase C treatment of hematopoietic cell lines upregulates the surface expression of CD34. Finally, more insights into CD34 function have recently been obtained with the development of CD34-deficient mice (Cheng et al., 1996). In these mice, the colony-forming activity of both embryonic and adult hematopoietic progenitors derived from their bone marrow and spleen was significantly reduced. Together, these data are consistent with a role of CD34 in the regulation of hematopoiesis, perhaps via unspecified cell signaling or adhesion pathways.

It has been recognized for some time that the expression of CD34 diminishes as hematopoietic cells differentiate, a result consistent with the potential role of this sialomucin in early hematopoietic development [reviewed in (Stella et al., 1995)]. A number of evidence has been presented that relate CD34 expression and hematopoietic "primitiveness" [reviewed in (Bernstein et al., 1994)]. Indeed, the CD34\(^+\) population has been shown to contain virtually all the lymphoid and myeloid progenitor cells that will form colonies when grown in semisolid cultures, as well as a small subset of cells that can initiate and maintain long-term cultures (see §2.2). More importantly, it includes cells capable of long-term engraftment, as shown by stable reconstitution of lymphopoiesis and myelopoiesis in lethally-irradiated mice (Krause et al., 1994), baboons (Andrews et al., 1992; Xu et al., 1995) and myeloablated patients (Dunbar et al., 1995). As discussed later, xenotransplantation studies in fetal sheep (Srour et al., 1992) and immune-deficient mice (Lapidot et al., 1994) have also demonstrated the capacity of human CD34\(^+\) cells to generate all hematolymphoid lineages. Hence, it is the expression of the CD34 marker antigen that has dominated attempts to isolate, purify and characterize human hematopoietic stem cells by a variety of immunological means. Much of this work has been done using the fluorescence-activated cell sorter (FACS) but, recently, several companies have developed equipment for cell purification using antibody-coated magnetic beads, avidin-coated beads or antibody bound to tissue culture plastic flasks.

Heterogeneity within the CD34\(^+\) subset was quickly recognized when CD34\(^+\) cells depleted of committed colony-forming progenitors were shown to provide long-term engraftment
in vivo. For instance, Robertson et al. demonstrated a delayed in vivo hematopoietic reconstitution following infusion of marrow cells depleted of CD33-expressing myeloid progenitors (Robertson et al., 1992). The delayed engraftment observed with this population suggested that the initial wave of hematopoietic reconstitution must be supported by the CD34^-CD33^+ colony-forming subset, whereas the long-term engraftment was provided by the CD34^-CD33^- population. Similarly, the cell-cycle dependent drug 4-HC has been used to eliminate acute myelocytic leukemic (AML) progenitors while sparing hematopoietic cells with long-term repopulating ability (Yeager et al., 1986). Other studies using antibodies against antigens associated with T or B cells to purge acute lymphocytic leukemic (ALL) cells from marrow before autologous infusion have provided evidence for the absence of these antigens on the subset of the CD34^- population responsible for long-term engraftment (Martin et al., 1985). Because of this apparent heterogeneity within the CD34^- population, many groups have further characterized these cells according to the presence or absence of additional cell surface antigens.

CD34^- cells which express high levels of antigen (CD34^bright) are considered to be more primitive than the CD34^null cells. An earlier subset of this CD34^bright population has been identified based on their lack of expression of a number of surface markers associated with commitment to the various hematopoietic lineages (Lin^-), including erythrocyte (e.g. Glyco A), T cell (e.g. CD2, CD3), B cell (e.g. CD19), NK cell (e.g. CD56), monocyte (e.g. CD14, CD16), granulocyte (e.g. CD24) and megakaryocyte (e.g. CD66b) lineages. Human HSC from adult BM have also been described as HLA-DR^- (Srour et al., 1991), whereas stem cells from fetal BM (Huang and Terstappen, 1994) and CB (Traycoff et al., 1994) appear as HLA-DR^+. Finally, analyses of the expression of CD38 (Terstappen et al., 1991), Thy-1 (Craig et al., 1993), CD71 (Mayani et al., 1993), the isoforms of CD45 (Lansdorp et al., 1990) and the uptake of rhodamine-123 (Rh-123) (Udomsakdi et al., 1991) have resulted in a consensus stem cell phenotype which is CD34^bright, Lin^-, CD38^-, Thy-1^+, CD71^low^, CD45RO^+, CD45RA^-, Rh-123^null^. This subset comprises less than 10% of the total CD34^- cells.

2.2 Long-term bone marrow culture

2.2.1 The hematopoietic microenvironment

During normal hematopoiesis, the different myeloid and lymphoid cells develop within the cavities or trabecular regions of the bones and, most often, they are not released into the peripheral blood until they reach maturity. Thus, egress of the developing hematopoietic cells is a
highly regulated process governed by interactions with the microenvironment (ME) (or stroma) with which cells are found to be in close contact. The specificity of the hematopoietic ME is well illustrated by BM transplantation into irradiated recipients. Intravenously injected hematopoietic cells can home to the BM and, lodging in the appropriate ME, they can proliferate and reconstitute (engraft) hematopoiesis of the host.

Within the bone marrow, the hematopoietic ME is composed of stromal cells and extracellular matrix (ECM) molecules. The bone marrow stroma in vivo is difficult to analyze because of the very dense cell packing in this tissue and, consequently, the origin and the precise role of the ME remain uncertain. For instance, the origin of the BM stromal cells is not clear, and the question of whether stromal and hematopoietic cells have a stem cell in common has not yet been resolved (Huang and Terstappen, 1992; Huang and Terstappen, 1994). However, various experimental systems have served to provide evidence that intimate contacts with the stromal cells (cell-cell interactions) and the ECM (cell-matrix interactions) are probably essential for pluripotent stem cells to express their properties.

Cell-cell interactions

Some of the most original and compelling evidence for the role of cell-cell interactions in hematopoietic regulation comes from studies of the Sl/Sld (steel) mutant mice [reviewed in (Russell, 1979)]. These mice are severely anemic even though they have competent stem cells. In contrast to the W/Wv mice (see §1.1.4), attempts to cure the anemia with BM transplantation have been uniformly unsuccessful, suggesting that the anomaly in these mice could be attributed to a non-transplantable stromal element unable to provide appropriate signals. Complementary defects in the Sl/Sld and W/Wv mutant mice can now be traced to abnormalities to the steel factor and its receptor c-kit, respectively. Since the steel factor is most abundant as a membrane-bound form in BM stromal cells, the W and Sl mouse models provide a clear example of receptor-ligand interactions between hematopoietic cells and the stromal cells of the ME. Given the organized architecture of the ME, it has been hypothesized that specific positional effects are probably critical for facilitating binding of SCF and other factors to their respective receptors.

The introduction of LTBMC has also greatly facilitated analysis of the ME interactions, although it represents only a gross approximation of the in vivo complexity (see §2.2.2). Important insights into the nature of the stromal cells have been obtained in LTBMC, as defined by morphological criteria, enzyme reactions and antibody staining of the cytoskeleton or cell
surface markers. Fibroblast-like cells represent a major component of the adhesive layer in LTBMC. The importance of these cells in stromal interactions is controversial. However, the demonstration by Roberts et al. that fixed NIH3T3 fibroblasts can stimulate proliferation and differentiation of early murine multipotent progenitors strongly suggest that these cells execute some form of critical cell-cell interactions (Roberts et al., 1987). The heterogeneous population of stromal cells has also been shown to include reticular cells, adipocytes, macrophages and endothelial cells [reviewed in (Chabannon and Torok-Storb, 1992)]. Lymphocytes, although long considered a nonessential part of the ME, are present in the adherent layer of LTBMC (Berneman et al., 1989). Some T cells may be long-lived peripheral blood lymphocytes contaminating the initial BM sample; however, very primitive T and B cells also appear to be present in LTBMC (Fulop and Phillips, 1989; Dorshkind and Phillips, 1982). Finally, osteoblasts have been detected in some mouse (Takahashi et al., 1988) and primate cultures (Takahashi et al., 1987), but there is no evidence that they exert a direct regulatory role in hematopoiesis.

Another way to help dissecting the elaborate cell-cell interactions within the ME comes from studies of simplified interactions between stromal cells and hematopoietic cells, using stromal cell lines generated mainly from murine (eg. MS-5, derived from newborn spleens) but also human (eg. SV-MSC, derived from normal donors and patients with hematological diseases) BM stromal cells [reviewed in (Deryugina and Muller-Sieburg, 1993)]. Although the relationship between these immortalized cell lines and the stromal cells found in vivo is not clearly defined, they have already been used in different assay systems to purify and characterize early hematopoietic cells. Recent data suggest that stromal cell lines may become important in clinical settings, as a means to maintain or expand hematopoietic stem cells ex vivo, or as a support to improve gene transfer protocols.

Cell-matrix interactions

Extracellular matrix molecules of seven distinct families, including collagens, proteoglycans, fibronectin, tenascin, thrombospondin, laminin and hemonectin, have been shown to be involved in different biological functions such as cell adhesion, anti-adhesion, binding and presentation of various cytokines and regulation of cell growth [reviewed in (Klein, 1995)]. Two proteins, proteoglycans and fibronectin, deserve a particular interest because of the substantial body of information recently acquired that underscores their importance in the regulation of hematopoiesis.
Proteoglycans consist of a polypeptide backbone (core proteins) with covalently linked glycosaminoglycan (GAG) side chains. These GAG side chains are very long, unbranched and composed of different repeating disaccharide units. According to the their different disaccharide units, five major proteoglycan classes can be distinguished: heparan sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate and hyaluronate proteoglycans [reviewed in (Kjellen and Lindahl, 1991)]. Most studies on the role of proteoglycans have concentrated on the nature and function of the GAG chains; the involvement of the various core proteins is less well documented. Although receptors on hematopoietic cells specific for interactions with proteoglycans have not yet been identified, studies involving long-term bone marrow cultures have suggested an adhesive role for proteoglycans. Indeed, myelopoiesis in LTBMC is only sustained in the presence of hydrocortisone and this hormone is known to influence the sulfatation pattern of GAG (Siczkowski et al., 1992). A decreased adhesion of hematopoietic progenitor cells to the adherent stromal layer is observed in hydrocortisone-deficient cultures which can be correlated to the modulated sulfatation pattern of the proteoglycans. In sharp contrast, perlecan, a defined member of the heparan sulfate proteoglycans (Klein et al., 1995), presents an anti-adhesive effect on hematopoietic cells. This was shown in vitro with the repulsion of the human K562 hematopoietic cell line from an area of a plastic dish coated with perlecan. Another important role of proteoglycans appear to be the binding and presentation of cytokines to hematopoietic cells. Gordon et al. showed that GAG isolated from BM were capable of binding exogenous GM-CSF (Gordon et al., 1987). Further work by Roberts et al. identified heparan sulfate as the components responsible for binding of GM-CSF and IL-3 and their biologically active presentation of hematopoietic progenitors (Klein et al., 1995). Thus, it is believed that proteoglycans could serve to compartmentalize various growth factors in the BM and, in combination with their adhesive and anti-adhesive capacities, they may provide sites of intermittent stimulating contacts for either induction or inhibition of hematopoietic cell growth or differentiation.

Fibronectin (FN), a ubiquitous ECM molecule, consists of two similar polypeptide chains joined by disulfide bonds (Figure 1-3). Recent work has demonstrated that multipotent progenitor cells and day 12 CFU-S, adhere to fibronectin (Williams et al., 1991; Verfaillie et al., 1991) through a variety of membrane-bound cell adhesion receptors. According to structural and functional similarities, these adhesion molecules have been grouped into various families,
including the integrins, cadherins, selectins, the immunoglobulin superfamily and syndecans, and several non-classified adhesion receptors also exist [reviewed in (Ruoslahti et al., 1994)]. The most intensively studied matrix receptors are members of the integrin family. Integrins are heterodimers consisting of an α and β subunit. Fifteen different α chains and at least seven different β chains have been characterized so far. Integrins including the β1 or β3 subunit mainly serve in cell-matrix interactions and it is known that enriched human progenitor cells, characterized by the CD34 antigen, do express α4β1 (also called very-late antigen-4, VLA-4) and α5β1 (VLA-5) integrins (Teixido et al., 1992). The modular FN molecule of the ECM contains several attachment sites for these cellular receptors (Figure 1-3). The central domain contains the amino acid sequence Arg-Gly-Asp-Ser (RGDS) that binds to a complementary site in the VLA-5 integrin. An RGDS-independent cell adhesion site (termed IIICS) is located in the COOH-terminal 35kDa fragment (FN35). This fragment harbours the sequence Leu-Asp-Val (LVD) known to interact with the VLA-4 integrin (Guan and Hynes, 1990). Moreover, two regions of the heparin-binding site II, termed FN-C/H I and FN-C/H II, can interact with the VLA-4 integrin or with membrane-bound proteoglycans (Haugen et al., 1990).

In addition to the adhesion mediating effect, the interaction of FN with the VLA-5 receptor can also contribute to negative regulation of hematopoiesis. This was indicated by a study of Sugahara et al. who showed a striking inhibition of GM-CSF- or SCF-induced proliferation of a myeloid cell line (MO7E) by FN, ultimately leading to cell death (Sugahara et al., 1994). Thus, FN also seems to be involved in the growth control of hematopoietic cells. Another interesting observation of FN action on hematopoietic cells was reported by Moritz et al. (Moritz et al., 1994). These authors showed that retroviral-mediated gene transfer into committed progenitors, and to a lesser degree in multipotent progenitor cells, was significantly increased when these cells adhered to the FN35 fragment containing the IIICS, FN-C/H I and II cell attachment domains. However, FN fragments lacking the IIICS binding domain did not show this effect. An additional FN fragment (CH-296, see Figure 1-3) containing all binding sites described before, has recently been synthesized (Kimizuka et al., 1991). As discussed in chapter 2, human hematopoietic progenitors bound to this fragment can be maintained for at least 3 days in vitro and, interestingly, high levels of gene transfer can be achieved in these cells. However, no convincing evidence of gene marking into long-term repopulating cells could be provided. Nevertheless, these results indicate that hematopoietic progenitor cell adhesion to specific ECM
**Fig. 1-3. Schematic representation of fibronectin.** Fibronectin (FN) consists in two polypeptide chains (A and B) joined at the carboxy termini by two disulfide bonds. Each chain is composed of a series of type I (rectangles), type II (circles), and type III (squares) homogeneous repeats. Some of the known functional binding domains have been indicated, they include: 1) Type III repeat containing the RGDS cell adhesion recognition sequence (hatched squares); 2) High affinity heparin binding-binding sequences located within the heparin II domain (gray squares) (FN-C/H I and FN-C/H II); 3) Alternatively splices, non-type III connecting segment (IIICS), which contains the second cell adhesion sequence represented by the CS-1 peptide (black square). Fragments of FN corresponding to FN35 and CH-296 (see text) are represented by solid arrows.
molecules can alter survival and retroviral infection efficiency. Ultimately, this may aid to develop improved gene transfer protocols for somatic gene therapy of hematopoietic cells.

2.2.2 The long-term culture-initiating cell (LTC-IC)

Attempts to develop procedures which could mimic the normal BM microenvironment resulted in the development of long-term bone marrow culture (LTBMC). Culture conditions have been defined for mouse (Dexter et al., 1977) and the need for an assay specific for human hematopoietic stem cells prompted the adaptation of murine LTBMC for use as a means to detect primitive human hematopoietic cells (Gartner and Kaplan, 1980). In this assay, formation of an adherent stromal cell layer which produces and deposits an ECM meshwork is a prerequisite for the development of hematopoietic cells. These adherent stromal cells are generated from the light density BM mononuclear fraction obtained after Percoll® or Ficoll® gradient centrifugation of BM aspirates. The different radiosensitivity of stromal cells and hematopoietic cells offers the opportunity to suppress the latter while maintaining the former in culture. The adherent stromal layer is formed between day 5 and day 20 of the LTBMC and, in close contact with the hematopoietic cells, it can provide all the necessary stimuli for growth of hematopoietic cells without addition of exogenous growth factors. Hence, these cultures are thought to mirror hematopoiesis in vivo.

In association with the feeder layer, hematopoietic cells can proliferate and differentiate over several months in culture, releasing into the culture medium their clonogenic and mature cell progeny, mainly granulocytes and macrophages. The ongoing production of these CFU and mature cells is the result of the differentiation and proliferation of very primitive cells and, in recognition of their method of detection, these cells have been called long-term culture-initiating cells or LTC-IC. They represent the most immature cells that can be assayed in vitro. The presence of these LTC-IC can be detected by assaying for the presence of CFU in cultures maintained for a minimum of 5 weeks. Beyond this time point, any colony-forming cells initially present in the culture should have disappeared through differentiation or death (Eaves et al., 1986; Andrews et al., 1986), and those detected will be the result of differentiation by LTC-IC (Winton and Colenda, 1987).

2.2.3 Is the LTC-IC the pluripotent hematopoietic stem cell?

The original introduction of an adherent layer to murine liquid BM cultures by Dexter and colleagues represented a significant improvement by maintaining granulopoiesis and CFU-S
proliferation for several months (Dexter et al., 1977). This time scale was the first indication for the initial input and maintenance of a very primitive murine hematopoietic cell that might be more primitive than any known progenitors detectable in the semi-solid culture system.

Several lines of evidence have since accumulated which suggest that LTBMC can support primitive hematopoietic cells of human origin that are earlier in ontogeny than colony-forming cells. As with murine cultures, it has been quickly recognized that short-lived clonogenic cells in human LTBMC were in a constant state of turnover and differentiation, implying their production from more primitive cells capable of extensive proliferation and long-term maintenance (Cashman et al., 1985). The continued retrieval of progenitor cells from human LTBMC was further investigated using BM cells treated with 4-HC, a cell-cycle specific drug, to remove malignant cells before autologous transplantation. The 4-HC treated marrow was shown to be depleted of CFU but retained its marrow-repopulating ability, as revealed after autologous transplantation. Similar to repopulating cells, LTC-IC were also resistant to 4-HC killing, as indicated by production of CFC by the treated marrow maintained in LTBM for 2-6 weeks (Winton and Colenda, 1987).

In a subsequent investigation, Sutherland et al. provided a direct quantitation of LTC-IC in BM suspensions as well as an assessment of their proliferative and differentiative potential by placing limiting numbers of hematopoietic cells on feeder layers pre-established in microtiter wells (Sutherland et al., 1990). After 5 weeks of culture, the contents of each well were assayed for CFU and the frequency of negative wells was used to calculate, by Poisson statistics, the frequency of LTC-IC in the starting BM population. Using such limiting dilution assays, the concentration of LTC-IC in normal BM was estimated to be ~5/10^5 nucleated cells, a frequency similar to that found for murine HSC (see §1.2.1) but lower than the frequency of most cells conventionally identified as colony-forming cells. The proliferative capacity exhibited by individual LTC-IC cultured under seemingly identical conditions was found to be highly variable. Values for the number of clonogenic cells produced per LTC-IC in 5-week-old cultures ranged from 1 to 30, with an average of 4. The majority of the clonogenic progenitors derived from a single LTC-IC were exclusively of the granulocyte-macrophage lineage (CFU-GM). However, a fraction of the LTC-IC produce CFU of more than one lineage, usually CFU-GM in combination with erythroid (BFU-E) and mixed-lineage progenitors (CFU-GEMM), suggesting a multipotential character for some LTC-IC.
The cell surface phenotype of LTC-IC has been used for their partial purification from human bone marrow and their separation from cell types detectable as CFC (Sutherland et al., 1989; Udomsakdi et al., 1992). LTC-IC show low forward and side light scatter properties, reflecting small size and low granularity, respectively, and like clonogenic cells, they strongly express the CD34 antigen. However, they differ from most CFU in that they are largely negative for HLA-DR (Sutherland et al., 1989), CD71 (Mayani et al., 1993) or CD33 (Andrews et al., 1986), and they show weak retention of Rh-123 (Udomsakdi et al., 1991). Moreover, when CD34⁺ cells were sorted on the basis of Thy-1 expression, most clonogenic cells were recovered in the CD34⁺Thy-1⁻ fraction, whereas the majority of LTC-IC were found in the CD34⁺Thy-1⁺ fraction (Craig et al., 1993). Similarly, Lansdorp et al. also showed specific expression of CD45RO isoform on the subset of CD34⁺ cells corresponding to LTC-IC; CFU were CD34⁺CD45RO⁻ (Lansdorp et al., 1990). Although LTC-IC appear to be largely separable from CFC via a number of surface markers, because of the much lower frequency of LTC-IC (as compared to CFC) and the heterogeneity within the CFC population, cell suspensions containing a higher absolute number of LTC-IC than CFC were, for several years, not readily obtainable, even after extensive LTC-IC purification. Only when CD38 staining of normal BM cells was combined with CD34 marking was the situation achieved (Sauvageau et al., 1994). Whereas 4-29% of LTC-IC were CD34⁺CD38⁻, corresponding to 1500-2000-fold enrichment, very few (0.5-2.5%) CD34⁺CD38⁻ had a CFC activity (Sauvageau et al., 1994). Thus, isolation of a CD34⁺CD33⁻HLA-DR⁻CD71⁻Rh-123dull Thy-1⁺CD45RO⁺CD38⁻ fraction of marrow frequently yields a suspension in which >10% of the cells will read out in a LTC-IC assay whereas <1% of the cells will produce detectable colonies within 2 weeks of culture in semi-solid medium even in the presence of combinations of growth factors including SCF, IL-3 and IL-6 (Sauvageau et al., 1994).

A recent investigation combining competitive repopulation assay and retroviral gene marking indicates that individual repopulating stem cells can be maintained and proliferate in LTBMC initiated with mouse BM (Fraser et al., 1992). In this study, marrow cells from male mice pretreated with 5-FU were infected with neomycin-resistant recombinant retroviruses and then used to initiate LTBMC on irradiated adherent marrow feeder layers. Four weeks later, LTBMC cells were harvested and injected into lethally irradiated female recipients together with compromised female BM cells. Analysis of the reconstituted mice revealed the consistent
presence of marked progeny in repopulated tissues for up to 7 months. A high proportion (50%) of all clones detected were represented in both lymphoid and myeloid populations at analysis. In some instances, this result occurred even when limiting numbers of cells were injected, thus providing evidence of totipotent stem cell maintenance in LTBMC. Moreover, the repeated demonstration of the same retroviral insertion fragments in hematopoietic tissues of different mice injected with cells from the same LTBMC further show that at least some of the pluripotent cells in 5-FU treated marrow undergo clonal expansion in vitro with preservation of their long-term lymphoid- and myeloid-repopulating ability. In humans, although marrow grown in LTBMC has been successfully used as hematopoietic support in myeloablative therapy for patients with leukemia (9), autologous studies of human stem cells combining retroviral marking, long-term maintenance on stromal support and in vivo transplantation have not been performed.

Together, these experiments indicate that LTC-IC are primitive hematopoietic cells from which clonogenic cells are derived and many features of LTC-IC, including resistance to 4-HC killing, immunophenotype, frequency, high proliferative capacity, multilineage differentiation capacity and ability for in vivo reconstitution, suggest they may be closely related to repopulating stem cells. However, definitive assimilation of LTC-IC of human origin to pluripotent HSC has been controversial and eagerly awaits additional evidence. Indeed, human BM adaptation of Dexter cultures presents limitations similar to those found in colony assays, suggesting that pluripotent stem cells are not surviving or proliferating in these cultures. Whereas murine BM cultures have been sustained for more than one year, human LTBMC decay steadily from culture initiation and last only 5-12 weeks (Gartner and Kaplan, 1980). Also, the limiting dilution techniques developed by Sutherland et al. have confirmed that the number of primitive cells capable of sustaining progenitor production begins to decline by 1 week in Dexter cultures (Sutherland et al., 1993). Another limit of this experimental system comes from the unequal character of the hematopoiesis observed, since the production of granulocytes and macrophages outweighs the output of erythrocytes, megakaryocytes and lymphocytes. Consistently, Harrison et al. found that hematopoietic cells cultured in LTBMC repeatedly showed less capacity than fresh BM to repopulate erythropoiesis in irradiated recipients (Harrison et al., 1987). Finally, in a recent investigation, Ponchio et al. used a procedure involving a prolonged exposure of BM cells to high specific activity $^3$H-TdR to achieve a readily detectable differential survival of cycling versus noncycling LTC-IC (Ponchio et al., 1995). Using this procedure, they showed that most
circulating human LTC-IC were normally quiescent but, in contrast to repopulating stem cells, an important proportion of LTC-IC in normal BM were also proliferating and the absolute number of cycling LTC-IC could be increased by incubation of the cells for 72 hours in a medium containing a simple cytokine cocktail (SCF, IL-3 and G-CSF). Therefore, these observations underscore the need to further investigate the relationship between human LTC-IC and the pluripotent hematopoietic stem cells to assess the relevance of the LTBMSC as an assay for the most primitive repopulating cells.

2.3 SCID transplantation assay

A second approach to developing an assay for long-term repopulating hematopoietic cells of human origin is based on the recognition of the potential use of immunodeficient xenogeneic hosts to create in vivo models of human hematopoietic reconstitution. This approach makes use of SCID mice and repopulation of either human tissue implants or murine hematopoietic tissues themselves are now being widely investigated in this regard. As described in this section, the chimeric animals produced by each method have shown to acquire divergent biological attributes and present different experimental applications.

2.3.1 Engraftment of SCID mice by IP injection of blood leukocytes

Adult human peripheral blood leukocytes (PBL) were used as a source of hematopoietic cells for transplantation into immune-deficient mice [reviewed in (Tary-Lehmann et al., 1995)]. The majority of leukocytes in the peripheral blood inoculum are fully differentiated, mature cells, encompassing all cellular elements, mainly B and T lymphocytes, required for a functional immune system. Mosier et al. found that human lymphocytes could engraft SCID mice only when injected intraperitoneally (IP); intravenous (IV) injection was ineffective (Mosier et al., 1988). During the first three weeks following IP injection of the PBL, most injected cell types, including lymphocytes, can be detected within the peritoneal cavity of these so-called hu-PBL-SCID chimeras (Hoffmann-Fezer et al., 1992). During this period, the human immune system is functional and immune response can be induced in the chimeric mice. After approximately one month, human leukocytes appear in other organs (thymus, lungs, liver and spleen), all bearing B-cell or T-cell markers; no human macrophages or other hematopoietic lineages are detectable.

B cells occur in low numbers, are oligoclonal (Saxon et al., 1991) and frequently are not detectable by flow cytometry (Tary-Lehmann and Saxon, 1992). Nevertheless, substantial levels of human immunoglobulins can be detected in the peripheral circulation, including spontaneously
produced antibodies to recall foreign antigens such as tetanus toxoid, diphtheria toxin or purified protein derivative (Abedi et al., 1992). T cells constitute the majority (96-100%) of the human cells found in these mice. However, T cells are uniformly single positive (CD4+ or CD8+) and, in addition, express HLA-DR and CD45RO, which defines them as mature, activated/memory T cells (Hoffmann-Fezer et al., 1993; Duchosal et al., 1992). Although hu-PBL-SCID mice do not develop classical symptoms of graft-versus-host disease (GVHD), the anti-mouse-reactive human T cells have recently been shown to be stimulated and clonally expanded in the chimeras, making the anti-mouse reactivity the primary stimulus for long-term hu-PBL-SCID chimerism (Tary-Lehmann et al., 1994). At the peak of chimerism, 1-2 months after grafting, the numbers of human T cells recovered from thymus, lungs, liver and spleen can exceed the number injected by 10-fold, with potentially larger numbers residing in organs that cannot readily be studied. Subsequently, the number of human lymphocytes steadily declines, usually becoming undetectable by the fifth month.

Thus, this model does not live up to the expectations of providing a stable, functional human immune system in a mouse. The absence of maturing T cells and multiple lineages four weeks after injection suggest that human HSC that copurify in the PBL fraction, may not successfully colonize the murine host. This notion is supported by the observation that IP injection of cell suspensions richer in human stem cells than adult peripheral blood does not lead to lymphopoiesis in the mice. Indeed, injection of CB or splenocytes also results in only mature activated T cells repopulating the primary lymphatic tissues (Alegre et al., 1994; Reinhardt et al., 1994). Furthermore, IP injection of human BM to adult (Tary-Lehmann and Saxon, 1992) or newborn mice (Pflumio et al., 1993) does not lead to any long-term engraftment of human cells, suggesting that this route of transplantation is incompatible with human stem cell repopulation. Another limitation of this model comes from the development of only few B cell clones resulting in a small repertoire of immunoglobulin isoforms. In addition, naive T cells, which are thought to be long-lived (Mclean and Michie, 1995), and which constitute up to 50% of T cells in adult peripheral blood, have been found to disappear 3-4 weeks after transplantation, suggesting that SCID mice do not provide the appropriate microenvironment for supporting the survival of all T cells. Finally, cells derived from Epstein-Barr virus (EBV) seropositive donors frequently develop lymphoproliferative disorders when transplanted IP in SCID mice (Pflumio et al., 1993; Mosier et al., 1988; Cannon et al., 1990). It is possible that the outgrowth of EBV-infected cells is caused
by a deficiency in human T-cell surveillance, since a similar lymphoproliferative disorder develops when the EBV-specific T-cell immunity is impaired in immunosuppressed individuals who undergo an organ transplant (Gaston et al., 1982).

Nevertheless, the functional immune system observed during the first few weeks following PBL injection may serve to develop multiple applications of this system. For example, antigen-specific human B cells can be activated in the chimeras and subsequently fuse with myeloma cells (Carlsson et al., 1992), as is required for monoclonal antibody production. Thus, it is believed that human antibodies could be produced that are directed against new antigens for which man cannot be immunized because of their toxicity. The function of human lymphocytes against tumors in vivo has also been studied in this system. After transfer into the mouse, anti-lymphoma T-cell clones stimulated in vitro were shown to have anti-tumor activity in vivo (Malkovska et al., 1992). Administration of an antibody against tumor-associated antigens seemed to increase lymphokine-activated (LAK) and natural killer (NK) function against the tumor in hu-PBL-SCID mice (Takahashi et al., 1993). Moreover, human lymphocytes in chimeras, although nonfunctional 3 weeks after grafting, remain susceptible to infection by lymphotropic microorganisms (Mosier et al., 1993). Thus, when chimeric mice are infected, direct microorganism-mediated pathology can be studied in vivo. Finally, effector functions of human T cells that have been primed either in the human donor or in vitro can be tested by adoptive transfer into hu-PBL-SCID mice. For instance, to assess the efficacy of HIV vaccination in humans, PBL of vaccinated donors were injected into HIV-infected hu-PBL-SCID mice. Since such PBL inhibited HIV replication in the chimeras, it was elegantly demonstrated that protective immunity had been induced in the vaccinated donors (Mosier et al., 1993).

2.3.2 Engraftment of SCID mice by implantation of human fetal tissues

An alternative approach to the transplantation of human hematopoietic cells into SCID mice, the SCID-hu model, is based on the concept that the human microenvironment is required for successful engraftment. The original "construction" of such SCID mice involved intravenous injection of fetal liver cells, as a source of stem cells, in mice previously implanted by fetal thymus and lymph nodes under the renal capsule, providing the necessary human microenvironmental elements (McCune et al., 1988). In this system, 4 to 11 weeks after transplantation, a transient wave of human mature CD4+ and CD8+ cells was detected in the peripheral blood at levels of about 10%; no human lymphoid cells were found in the SCID thymus. High levels of IgG and
mature B cells were also detected in the peripheral circulation of mice transplanted with fetal lymph nodes. Based on different HLA donor phenotyping of the fetal liver and thymus, it was deduced that the peripheral T cells originate from the fetal liver donor (McCune et al., 1988). Since in the absence of fetal thymus the fetal liver cells do not generate human lymphoid elements, the authors concluded that the mature T cells derived from T cell progenitors which homed to, and differentiated through the engrafted human thymus.

In an effort to extend the duration of lymphopoiesis observed in these mice, a derivative of the human fetal implant model was created by providing the fetal liver cells as an intact piece of the fetal organ implanted contiguous to the fetal thymus. The resulting organ structure ("Thy/Liv") represents a fusion product that contains both the hematopoietic compartment of the fetal liver and the functional compartments of the thymus (Namikawa et al., 1990). For 6-12 months post-implantation, the human fetal tissues in these SCID-hu mice demonstrated continued, functional T lymphopoiesis, production of human B cells, and maintenance of colony-forming units of the myeloid and erythroid lineages (Namikawa et al., 1990). This striking improvement was rationalized by the presence of stromal elements present in the intact Thy/Liv structure that allowed maintainance of the fetal liver hematopoietic stem cells, implying that the transient engraftment seen in the previous system was due to a loss of the supportive stromal environment during IV injection. However, this Thy/Liv organ system sustains only low levels of human T cells in the periphery and circulating human cells of other lineages are undetectable. Therefore, its primary application is in the analysis of human T-lymphoid differentiation, function and pathology within the context of the organ system itself. This system also provided an in vivo model for the analysis of human infectious diseases. In early experiments, human thymus in the SCID-hu mouse was shown to be permissive for HIV infection (Namikawa et al., 1988). More recent studies have now shown that such infection is associated with suppression of human thymopoiesis (Bonyhadi et al., 1993).

To construct small animal model with a multilineage hematopoietic compartment more similar to that of normal human BM, human fetal long bone fragments were implanted into the subcutaneous space of the SCID mouse (Kyoizumi et al., 1992). Post-implantation, the bones were vascularized, the stromal microenvironment persisted, and active multilineage human hematopoiesis could be maintained within the bone for periods of time as long as 5 months. This small animal model has provided a useful preclinical model for the evaluation of human
hematopoiesis. Thus, the bone grafts have been used as recipients for transfer of a rare population of human fetal BM (CD34<sup>+</sup>Thy-1<sup>-</sup>Lin<sup>-</sup>) that was shown to differentiate into human T, B and myeloid cells (Baum et al., 1992). Additionally, BM cells from various subtypes of acute myeloid leukemia patients were demonstrated to be propagated in this SCID-hu mouse (Namikawa et al., 1993). Finally, expansion of myeloid and erythroid cells could be observed when appropriate growth factors (IL-3, IL-6, G-CSF and EPO) were exogenously provided (Kyoizumi et al., 1993), and the model could be used to assess in vivo the damage to the hematopoietic progenitors by radiation (Kyoizumi et al., 1994), chemical compounds and viral infection.

A further improvement to the SCID-hu model involved subcutaneous implantation of fetal bone, thymus and spleen fragments adjacent to each other (SCID-hu BTS). The BM of SCID-hu BTS grafts was found to maintain B cells, assayable progenitors (CFU-GM) and cells with an immature phenotype (CD34<sup>+</sup>Thy-1<sup>-</sup>Lin<sup>-</sup>) for at least 20 weeks post-transplant. In addition, early progenitors within the BM grafts were capable of migrating to the human thymus and undergoing differentiation through immature CD4<sup>+</sup>CD8<sup>-</sup> double-positive T cells and produce mature T cells with a CD4<sup>+</sup>CD8<sup>-</sup> or CD8<sup>-</sup>CD4<sup>+</sup> phenotype. The implanted tissues were also directly injected with partially purified fetal liver or cord blood CD34<sup>+</sup>Thy-1<sup>-</sup>Lin<sup>-</sup> cells to assess the ability of the BTS system to provide an environment where several lineages might differentiate from a common stem cell pool. Although multilineage hematopoiesis was observed in individual grafts when analyzed 8 weeks post-reconstitution, one cannot ascertain if the engraftment is coming from a single stem cell or a population of cells each committed to a different lineage. Finally, CD34<sup>+</sup> Lin<sup>-</sup> cells reisolated from the BTS organ were retransplanted into a secondary mouse and shown to repopulate to a similar extent as the primary fragment. While suggesting self-renewal, this data may also be explained by the repopulation of the secondary BTS organ with a cell that was only maintained in the first mouse, rather than by a cell that self-renewed in the primary mouse. This question can only be approached by transplanting single cells or by gene marking (see below). Hence, the SCID-hu BTS model permits measurement of all characteristics assigned to stem cells. However, since the presence of human cells is always restricted to the transplanted organs, without spread to the peripheral circulation, and since human hematopoiesis declines with time, these models do not adequately reproduce a dynamic system to study human hematopoiesis. Moreover, the total cellularity within the bone
marrow implants is extremely low, limiting the possible analyses which could be performed. Finally, the need for surgical intervention and dependence on fetal tissues that are difficult to procure represent important issues that may limit the wide applicability of this model.

2.3.3 Engraftment of SCID mice by intravenous injection of BM or CB cells

The third system that was developed uses a strategy closely modeled on conventional BM transplantation and murine long-term reconstitution assays for stem cell function. In the initial model, large numbers ($\geq 10 \times 10^6$) of human BM cells were injected intravenously into SCID or beige/nude/xd (bnx) mice previously conditioned with sub-lethal doses of radiation (Kamel-Reid and Dick, 1988). Human hematopoietic cells were found to migrate to the murine BM and spleen and give rise to a sustained pool of macrophage progenitors for several weeks. In this system, the cells responsible for initiating human engraftment after transplantation into SCID mice have been functionally termed the SCID-repopulating cells (SRC) (Larochelle et al., 1995). In contrast to the studies using fetal implants in SCID mice, these experiments indicated that the murine microenvironment could support normal human hematopoiesis. This conclusion is strongly supported by in vitro studies which show that murine stromal cell lines allow the maintenance and proliferation of immature human progenitor cells (Issaad et al., 1993; Baum et al., 1992; Otsuka et al., 1992; Sutherland et al., 1991). However, the low levels of human cell engraftment and the absence of multilineage differentiation obtained in the initial experiments suggested the inability of murine stromal elements to support a complete growth proliferation of SRC. A recent investigation revealed that regular treatment of engrafted SCID mice with a cocktail of SCF, PIM321 and EPO increased the levels of engraftment by 10-fold compared to untreated mice, and multiple hematopoietic lineages including B cells, immature CD34$^+$ cells, mature erythrocytes and multilineage or committed myeloid/erythroid progenitor cells were detected in the murine marrow for over 4 months (Lapidot et al., 1992).

It was quickly recognized that the dependence on large quantities of cytokines and the requirement for high cell doses, precluding engraftment with limiting dilution of whole BM or with CD34$^+$ cells, represented major limitations to this transplantation system. One approach to override the need of cytokines for engraftment was developed by Nolta et al. who co-transplanted human hematopoietic cells together with IL-3 secreting stromal cells, which produced sustained levels circulating levels of human IL-3 for at least 4 months in the mice (Nolta et al., 1994). Long-term multilineage engraftment was specifically seen in the stroma-
transplanted animals, but an average of only 6% of the hematopoietic cells removed from the mice were of human origin. In addition, Bock et al. generated SCID-transgenic mice expressing the genes for human IL-3, GM-CSF and SCF to provide human specific factors for the transplanted BM cells (Bock et al., 1995). Although multilineage engraftment was observed for up to 6 months in transgenic mice, twice as long as non-transgenic littermates, human DNA accounted for only 0.5-1.5% of the total DNA extracted from peripheral blood; levels of engraftment in the mouse BM were not provided.

As discussed before, cord blood has recently been defined as a major source of long-term repopulating hematopoietic cells. Interestingly, lower numbers of transplanted CB cells were found to be required to achieve high levels of multilineage human hematopoiesis in the murine BM (Vormoor et al., 1994). Moreover, high levels of SRC proliferation could be attained without any cytokine treatment in SCID mice. Similar results were obtained when fetal BM or liver cells were injected intravenously (Kollmann et al., 1994). Although the exact mechanism is unknown, it is hypothesized that the primitive fetal liver or CB cells intrinsically have a higher in vivo proliferative capacity, respond differently to the murine environment or contain accessory cells that provide the necessary growth factors.

The high cell doses required to ensure detectable levels of engraftment in SCID or bnx mice may be due, at least in part, to their residual antigen-nonspecific immunity, including NK cell activity, hemolytic complements and functional mature macrophages. For this reason, human cell engraftment was assessed in several strains of immune-deficient mice created by homologous recombination [reviewed in (Dick, 1996)]. Surprisingly, human cell engraftment was negligible in Rag-2, Rag-2/β-2 microglobulin, Rag-2/ζ-chain, and perforin knock-out mice. In contrast, high levels of human cells were obtained in a novel immunodeficient mouse strain created by backcrossing the scid mutation 10 generations onto the NOD/Lt strain background (Shultz et al., 1995). The resulting mouse (NOD/LtSz-scid/scid or simply NOD/SCID) lacks functional lymphoid cells, shows little or no serum immunoglobulins with age and has reduced non-specific immunity. As illustrated in chapter 2 and 3 of this thesis, these mice show high levels of engraftment after transplantation of normal human hematopoietic cells and, more importantly, they enable engraftment with lower cell doses and purified CD34+ cells. Interestingly, NOD/SCID mice transplanted with high doses of human BM or CB cells result in similar levels of engraftment with or without the exogenous addition of growth factors. Preliminary evidence
indicates that the BM microenvironment of NOD/SCID mice provides a better support for human hematopoiesis as compared to regular SCID mice, suggesting that immunodeficiency of the recipient mouse is not the only parameter involved in human cell engraftment.

Unfortunately, long-term repopulation of NOD/SCID mice with human SRC is constrained by the high incidence of spontaneous thymic lymphomas which results in a mean lifespan of only 8.5 months under specific pathogen-free conditions (Shultz et al., 1995). These thymic lymphomas are characterized by activation and subsequent genomic reintegrations of Emv30, an endogenous murine ecotropic retrovirus unique to the NOD genome (Serreze et al., 1995). Recently, a stock of Emv30null NOD/SCID mice was generated to test the role of this endogenous retrovirus in thymomagenesis (Serreze et al., 1995). Although thymic lymphomas still initiated in Emv30null NOD/SCID animal, their rate of progression was significantly retarded as compared to the Emv30+ segregants. Interestingly, the elimination of Emv30 did not abrogate the enhanced ability of NOD/SCID mice to support the growth of human hematopoietic cells. Hence, the Emv30null NOD/SCID mice may represent a further improved recipient for long-term hematopoietic reconstitution.

Several lines of evidence have accumulated using the IV transplant system that lend credence to the assumption of human stem cell engraftment in the microenvironment of immune-deficient mice. First, in contrast to BM or CB cells, mature normal peripheral blood cells injected intravenously do not engraft immune-deficient mice (Lubin et al., 1991; Mosier et al., 1988). Second, examination of the kinetics of engraftment indicated that only 0.1-1% of the original injected number of CD34+Thy-1b, CFC or LTC-IC were present 2 days after transplantation. Over the next four weeks their number increased exponentially to almost the numbers originally injected and since these progenitor cells have a very low self-renewal capacity, an earlier cell type must be responsible for this large increase (Kamel-Reid and Dick, 1988; Cashman et al., 1994). Thirdly, with specific cytokine treatment, human progenitors of all types as well as mature erythrocytes, T cells, B cells, and myeloid cells could be detected in animals for at least 4 months after transplantation. Moreover, mice in which growth factor treatment was delayed for 1-3 months after transplant showed the same multilineage differentiation as mice in which cytokine treatment was started immediately after transplantation (Lapidot et al., 1992). This experiment supported the hypothesis that primitive human cells can home to the murine BM, be maintained by this microenvironment for several months and still remain responsive to human growth factor
treatment. Further evidence came from the identification of LTC-IC and very immature CD34<sup>+</sup>Thy-1<sup>+</sup> and CD34<sup>+</sup>CD38<sup>-</sup> cells in the BM of transplanted animals (Cashman et al., 1994). Finally, purification experiments performed with human acute myeloid leukemia (AML) cells (Lapidot et al., 1994) or normal BM (chapter 2) on the basis of cell-surface marker expression revealed that CD34<sup>+</sup> and not CD34<sup>-</sup> cells engraft NOD/SCID mice. As presented in chapter 2, further purification of the CD34<sup>+</sup> fraction indicated that all the SRC activity could be found in the CD34<sup>+</sup>CD38<sup>-</sup> fraction and as few as 500 CD34<sup>+</sup>CD38<sup>-</sup> cells could engraft NOD/SCID mice; however, no SRC activity was detected in the CD34<sup>+</sup>CD38<sup>-</sup> fraction.

**2.4 Summary**

The observations outlined in this section underscore the importance of defining an assay that will specifically measure the long-term repopulating ability of human hematopoietic stem cells. For the most part, *in vitro* assays have served to identify the currently known physical, biological and immunophenotypic properties of primitive human hematopoietic cells. In turn, these properties have provided the basis for a variety of stem cell purification strategies. Long-term culture-initiating cells appear to define the most immature human hematopoietic cells that can be assayed *in vitro*, but their assimilation to the human pluripotent stem cell remains highly controversial. Alternatively, various routes of transplantation of human hematopoietic cells into immune-deficient mice have been used to model human hematopoiesis *in vivo*. Systems based on IV injection of human BM or CB inoculum appear promising to assay the reconstitutive ability of pluripotent stem cells. Although a substantial body of information suggests that an immature cell type with multilineage capacity can home and proliferate in the BM of immune-deficient mice, the exact nature of the SCID-repopulating cells (SRC) has not been characterized and the relationship between this cell type and other cellular entities (e.g. LTC-IC) of the human hematopoietic hierarchy is not yet known. As elegantly shown in the mouse (Dick et al., 1985; Lemischka et al., 1986; Jordan et al., 1990; Keller et al., 1985) and discussed in the next section, the ability to transfer new genes into hematopoietic cells provides one powerful approach to characterize and resolve lineage relationships between various cells in the hematopoietic hierarchy.
3. Genetic manipulation of human hematopoietic stem cells

The ability to introduce clinically relevant genes into human hematopoietic cells offers an important new approach for the treatment of many inherited or acquired blood-related diseases (Bordignon et al., 1995; Kohn et al., 1995; Blaese et al., 1995). To achieve permanent correction, the target cell for gene transfer must be the elusive human hematopoietic stem cell since mature cells and committed progenitors do not have the proliferative capacity to reconstitute the entire hematopoietic system. However, stem cells are rare (1/10^4-10^5) and, therefore, any method to transfer genes into these cells must be very efficient. Also, progress in defining optimal protocols for gene transfer into rare human HSC has been hindered by the absence of pre-clinical models that assay the repopulating capacity of primitive human hematopoietic cells. Instead, current gene transfer methods employed in gene therapy clinical trials have been optimized using in vitro assays for CFC and LTC-IC. The relevance of these methods for stem cell gene transfer is, however, unclear because the relationship between these cells and the in vivo repopulating human stem cell is not established.

The early experiments in the mouse had revealed that the insertion of a new genetic sequence into a chromosome also provides a means of marking the DNA of the progeny of an individual stem cell clone, allowing the determination of the developmental potential of an individual stem cell (see §1.1.5). As summarized in this section and detailed in chapter 2 of this thesis, gene marking of human hematopoietic cells has been used to demonstrate the primitive nature of the cells initiating human hematopoiesis in immune-deficient mice by defining their relationship to human CFC and LTC-IC. Hence, this in vivo model provides a novel tool to evaluate gene therapy vectors and methods targeted to the correction of defective human repopulating cells. The implications of the SCID transplantation assay for evaluating human gene therapy protocols will be highlighted in this section and further discussed in chapter 3 of this thesis, in the context of two in vivo models of stem cell diseases developed by transplantation of BM cells from β-thalassemia and sickle cell anemia (SCA) patients into immune-deficient mice.

3.1 Methods of gene transfer

The aim of any methods of gene transfer to be incorporated into daily clinical practice is to achieve an easy, safe, non-toxic delivery of unlimited size DNA to a specified target tissue. On arrival at the tissue, the DNA should be rapidly and efficiently endocytosed across the lipid
Fig. 1-4. Moloney Murine Leukemia Virus (MoMuLV) and derivative gene transfer vectors. A. Structure of the proviral form of a MoMuLV and pattern of transcription products. The essential cis-acting sequences are outlined: PBS= tRNA primer binding site; ψ= packaging signal sequence; PPP= polypurine tract; sd= splice donor; sa= splice acceptor. B. The second generation PA317-AmmA12 55/6 amphotropic packaging cell line. Structure of the expression plasmid construct for MoMuLV gag-pol-env proteins (top) and structure of the PGKmADA provirus (bottom). C. The third generation PG13-LN GaLV packaging cell line. Structure of the expression plasmid constructs for MoMuLV gag-pol (top) and GaLV env proteins (middle), and structure of the pLN provirus (bottom).
bilayer, and directed to the host cell nucleus, regardless of the mitotic status. Sequences encoded by the foreign DNA interacting with regulatory host factors should achieve long-term expression. *En route* to these goals, several fundamental scientific issues must still be addressed, including a more detailed understanding of endocytosis, nuclear transfer and regulated long-term gene expression in transduced cells. At present approaches using viruses, primarily retroviruses, have evolved as clear favorite for gene delivery, with more than 80% of approved gene therapy trials using this approach. However, some limitations of viral vectors have also provided an impetus to the development of alternative noninfectious gene transfer technologies for *in vivo* delivery of expression vectors. The merits and limitations of each method are presented in this section.

### 3.1.1 Viral approaches

**Vectors based on viruses of the Retroviridae family**

The *Retroviridae* family of viruses has been divided into three subfamilies, based primarily on pathogenicity: *Oncovirinae*, including retroviruses (RV) such as Moloney Murine Leukemia Virus (MoMuLV); *Lentivirinae*, such as Human Immunodeficiency Virus types 1 and 2 (HIV-1, HIV-2); and *Spumavirinae*, such as Human Foamy Virus (HFV). Members of each subfamily are now used to derive retroviral gene transfer vectors, with an emphasis on constructs developed from MoMuLV.

Retroviruses infecting mammalian cells have evolved sophisticated and specific mechanisms for cell attachment, penetration, survival and replication. Hence, gene transfer vectors based on the natural life cycle of highly infectious RV offers several important advantages for delivering genes into various cell types, including hematopoietic cells [reviewed in (Vile and Russell, 1995)]. Retroviruses carry their genetic information in the form of RNA; however, once the virus infects a cell, the RNA genome is reverse-transcribed into a DNA form, which stably and efficiently integrates into the genomic DNA of the infected cell. The integrated DNA form is called a provirus and the gene order within this provirus is always maintained (LTR-gag-pol-env-LTR; see Figure 1-4A). As well as offering stable and efficient biological integration, which markedly reduces the possibility of gene rearrangement, retroviral vectors present the advantage of single- or low-copy-number transfer of the gene of interest. Moreover, infection of mammalian cells with RV is known to be nontoxic to these cells. Currently, most retroviral vectors are derived from murine or avian retroviruses. Retroviral constructs based on MoMuLV are the most extensively used. This is because the biology of this RV is considerably well understood, the
vector systems based on MoMuLV give relatively high titers of recombinant virus, and because of the availability of retroviral variants, including amphotropic and xenotropic viruses, that efficiently infect human cells, in contrast to ecotropic MoMuLV that infect only rodent cells.

Retroviral vectors are derivatives of wild-type, replication-competent retroviruses from which part of the retroviral coding information (gag, pol and env) has been discarded, leaving ~8 kb available for insertion of a new gene of interest (Figure 1-4B and 1-4C). However, to ensure normal life cycle of these altered retroviruses, essential cis-acting sequences must be retained within the retroviral genome and the products of the deleted gag, pol and env genes must be provided in trans. The necessary cis-acting sequences include the packaging signal (ψ-signal) that permits encapsidation of the vector RNA into virions; the primer binding site (PBS), terminal repeat (R) sequences and a purine-rich sequence upstream of the 3'-LTR that direct the process of reverse transcription; and finally specific sequences near the ends of the LTR essential for the stable and efficient integration into the host cell chromosome (Figure 1-4A). Retroviral packaging cell lines (Miller, 1990) have been generated to provide the viral "helper" functions that have been deleted from the vector genome, namely gag, pol and env proteins. These helper functions are stably expressed in the packaging cells from one or more helper plasmids whose RNA transcripts are not efficiently packaged into viral particles because they lack the packaging signal sequence. When the vector genome containing the gene of interest is transfected into such packaging cells, the viral gag, pol and env proteins recognize and package the vector RNA genome into viral particles that are released into the culture supernatant. These recombinant retroviruses can be used to infect a specific population of target cells. In principle, since no genetic information (gag, pol, env) for virus production is transferred from the packaging cells, the resulting replication-defective viruses are unable to spread to other cells to perpetuate an infection.

The possibility of generating replication-competent wild-type viruses within the packaging cell lines is often quoted as a major drawback of retrovirus-based gene transfer systems. Indeed, replication-competent RV were frequently encountered with early packaging cells typified by the ψ-2 (Mann et al., 1983) and ψ-am (Cone and Mulligan, 1984) cell lines. The retroviral helper functions were provided by a retroviral genome carrying a unique deletion in the ψ packaging signal. The ψ' RNA transcripts were shown to be co-packaged with ψ' vector transcripts at low frequency (0.1%) into budding RV and replication-competent particles most
frequently arose by recombination of the co-packaged transcripts during reverse transcription. The spread of replication-competent viruses is highly undesirable because it can lead to insertional activation of cellular proto-oncogenes or inactivation of tumor suppressor genes resulting in tumor formation. This was illustrated by a recent investigation in which replication-competent-contaminated vector stocks were used to infect primate BM cells that were subsequently transplanted into monkeys. Three out of ten animals became viremic and developed aggressive T cell lymphomas (Donahue et al., 1992). However, the titers of replication-competent RV used in this study were exceptionally high, and in another study, monkeys exposed to replicating MoMuLV showed no signs of malignancy even after seven years of follow-up (Cornetta et al., 1991).

Several strategies have been used to develop packaging cell lines unable to generate and transfer wild-type viruses to target cells. The best way to reduce the risk of replication-competent RV is to provide the helper functions on more extensively deleted helper plasmids having minimal sequence homology with the vector genome or, better still, to split them onto multiple helper plasmids. Second and third generation packaging cell lines have been developed according to these principles and are considerably safer due to the increased number of helper-vector recombination events required to generate a wild-type vector genome. For instance, the second generation packaging line PA317 (Miller and Buttimore, 1986), used to generate amphotropic RV for the gene marking studies described in chapter 2 of this thesis, was developed using the plasmid pPAM3 which lacks not only the Ψ sequence but also the 3'-LTR and polypurine tract that have been replaced by an SV40 polyadenylation signal sequence (Figure 1-4B). Two recombination events between helper and vector sequences are therefore necessary to generate replication-competent RV and this double recombination has proven to be a very low frequency event. The third generation packaging cells, such as PG13 (Miller et al., 1991) also discussed in chapter 2, have further reduced the risk of replication-competent RV by separating the gag, pol transcription unit from the env transcription unit on different plasmids and by using packaging constructs with minimal areas of homology with the vector genome (Figure 1-4C). However, as shown by Scadden et al., even third generation cell lines may package and transfer endogenous murine viral genomes (Scadden et al., 1990).

For maximum efficiency of gene delivery, a retroviral vector should be available at high titer, a condition not always met by the currently available packaging cell lines. Under optimal
conditions, the most widely used retroviral producing cell lines can generate $10^6$-to-$10^7$ infectious vector particles/ml of tissue culture supernatant, but most gene transfer applications to date have employed retroviral producers giving titers below $10^6$/ml. Retroviral instability is an important limitation of RV vectors that contributes to the low titers observed. Loss of infectivity is significant during continued incubation at 37°C ($t_K \sim 8$ hrs), after each freeze-thaw cycle ($\sim 50\%$) and after virus concentration by centrifugation. This instability has been attributed to dissociation of gp70 envelope protein from the virus surface. Viral stability is enhanced at 32°C compared to 37°C due to decreased SU dissociation and the titers of retroviral stocks can be increased 10-fold by harvesting from producer cells cultured at 32°C (Kotani et al., 1994). Alternatively, MoMuLV-based pseudotype vectors incorporating the Vesicular Stomatitis Virus (VSV) G protein in place of their natural envelope protein were shown to be more stable and allow efficient concentration to very high titer by high-speed ultracentrifugation (Burns et al., 1993).

Several reports have shown that infection with retroviruses of the Oncovirinae subfamily is inhibited in non-replicating (stationary-phase) cells (Harel et al., 1981; Fritsch and Temin, 1977). Miller et al. have further documented that block to infection could not be relieved by stimulating stationary cells to divide at times from 6 hours to 10 days after infection, suggesting that an unintegrated retrovirus intermediate could not persist and subsequently be integrated after release of the block to replication. It was concluded that, for successful retroviral infection, the infected cells must be replicating at the time of infection (Miller et al., 1990). This requirement for active cell division represents an important drawback of MoMuLV-based gene transfer vectors. As discussed in chapter 2, human hematopoietic stem cells, for instance, cannot readily be stimulated to proliferate in vitro and are therefore difficult to transduce with murine retroviral vectors.

**Vectors based on other viruses**

A number of different infectious agents that infect human cells, including adenoviruses (Ads), adeno-associated viruses (AAV), and herpes simplex viruses (HSV) have been modified to generate efficient vehicles for gene delivery [reviewed in (Kremer and Perricaudet, 1995)]. Other viruses, not discussed here, have also been considered for specific applications, such as immunization, where it is desirable to transiently express large amounts of gene products. These include vectors derived from vaccinia viruses, polioviruses and several other RNA viruses.
The most recognized advantage of adenoviral vector-mediated gene transfer is related to the lack of pathogenicity of the parental adenovirus. Indeed, adenovirus serotypes modified for vector production (Ad2 and Ad5) do not induce tumor formation in rodents and they appear to cause only minor respiratory problems in man. In fact, many adenoviral infections remain subclinical and only result in antibody formation. Although respiratory epithelial cells represent the primary target for adenoviruses *in vivo*, human wild type adenoviruses infect virtually all cell types *in vitro* and *in vivo*. Interestingly, dividing and quiescent cells are equally susceptible to adenoviral infection, rendering these vectors putatively attractive for the non-cycling hematopoietic stem cells. Adenoviruses show considerable stability and are thus amenable to purification and concentration. Consequently, the rarity of HSC could also benefit from the high titers (up to $10^{13}$ viral particles/ml) routinely obtained with these vectors. Furthermore, these double stranded DNA viruses can accept relatively large foreign nucleic acid inserts, with a theoretical limit established at 7-8 kb (Bett et al., 1993).

The inability of adenoviruses to integrate into the host genome is sometimes perceived as a benefit because of the resulting impossibility to activate dormant oncogenes or interrupt tumor suppressor genes. However, this same benefit may also limit potential uses of adenovirus-mediated gene transfer. For instance, since adenoviruses remain as a non-replicating extrachromosomal entity after infection, copies of their genome will be lost or reduced during each subsequent cell division. Clearly, this scenario would not permit long-term treatment of genetic defects using cell types such as the hematopoietic stem cells that require multiple divisions for long-term reconstitution. The limited duration of expression of the transgene (~8 weeks) in adult animals is another consequence of the non-integrated state of adenoviral genome in their host cell. Therefore, repeated administration would be prescribed for gene therapy purposes. Yet, multiple injections of adenoviruses have been shown to induce severe immune responses to capsid polypeptides. Also, overexpression of the transferred gene in infected cells may target these cells for destruction by a cytotoxic T-cell response. Globally, although adenovirus-based vectors present appealing characteristics for stem cell gene transfer, their immunogenicity and limited persistence of expression have restrained their utilization in the treatment of hematopoietic diseases.

Adeno-associated viruses are unique among animal viruses in that they normally require coinfection with an unrelated helper virus (such as adenoviruses) for productive infection. Their
linear single-stranded DNA genome includes the coding regions rep and cap, involved in replication during coinfection and polypeptide capsid synthesis, respectively. Both rep+ and rep vectors have been constructed. The repAAV constructs have the advantage of a high transduction frequency, but their utilization is somewhat cumbersome since they cannot be rescued by adenovirus challenge alone; they also require wild type AAV coinfection (McLaughlin et al., 1988). In contrast to adenoviruses, both AAV vector types integrate in their host genome after infection. Integration appears less efficient than retroviral integration and also less precise, as tandem viral genomes with slight deletions, substitutions or rearrangements are often observed (Muzycka, 1992). The rep constructs appear to integrate randomly, raising the same concerns as retrovirus-mediated gene transfer with respect to potential cell transformation (Muzycka, 1992). On the contrary, rep-AAV vectors present a preferred site of integration at 19q13.4 of the human genome where it can exist as a latent infection for the lifetime of the cell (Samulski et al., 1991). However, because this region has been implicated in chromosomal rearrangements associated with B cell leukemias (McKeithan et al., 1987), it is unclear, from the standpoint of safety, that such site-specific integration would offer any advantages over random integration. Finally, viral stocks of 10⁸-10⁹ infectious units/ml are sometimes obtainable, but difficult to prepare and easily contaminated with wild type AAV and helper adenoviruses. The complete separation of helper viruses from AAV vectors is currently problematic and this complication has become the primary drawback of AAV-based approaches.

In spite of major limitations, vectors based on adeno-associated viruses also possess advantageous features for gene transfer into long-term repopulating stem cells [reviewed in (Kremer and Perricaudet, 1995)]. Similar to adenovirus-based vectors, AAV constructs present the benefit of safety since parental viruses have not been associated with any clinical symptoms in any host and are not known as tumorigenic infectious agents (Berns, 1990). The wide tropism of AAV is often quoted as another advantage for vector production. Indeed, although natural tropism of AAV is presumably the respiratory and gastro-intestinal tract, all human cells tested in vitro, including hematopoietic cells, have been successfully transduced. Interestingly, previous studies have shown that cellular proliferation is not a prerequisite for efficient AAV transduction (Podsakoff et al., 1994). For instance, transduction efficiencies of >70% in CD34⁺-enriched cells have been obtained using a variety of AAV vectors. In this study, efficient gene transfer occurred with or without prior cytokine stimulation of target cells. Similarly, Miller et al. reported
transgene expression in 20-40% of hematopoietic colonies following transduction with a recombinant AAV vector encoding human γ-globin (Miller et al., 1994). Using AAV vectors containing the Neo<sup>R</sup> gene, Zhou et al. described transduction efficiencies of 33-75% in human CB progenitors in the absence of cytokine prestimulation (Zhou et al., 1994). Finally, Walsh et al. described the use of an AAV vector encoding the Fanconi anemia complementation group C gene to infect CD34<sup>+</sup> cells isolated from a patient with this disorder (Walsh et al., 1994). In this study, transduction efficiency was up to 60% and transduced cells could be engrafted into SCID-hu murine model (Walsh et al., 1994). Thus, because of high transduction efficiencies in the absence of cytokine stimulation, AAV may be promising for gene transfer into primitive hematopoietic cells and are currently undergoing intensive evaluation for in vivo efficacy (Podsakoff et al., 1994).

The potential of herpesviruses as gene transfer vectors lies in their ability to carry large foreign DNA inserts and their ability to establish lifelong latent infections in which the virus genome exists as a stable episome with no apparent effect on host cells [reviewed in (Efstathiou and Minson, 1995)]. Epstein Barr viruses and other members of the γ-herpesvirus subfamily can establish latent infection in dividing cells. In that respect, this group of herpesviruses has the potential for gene delivery to stem cells and their differentiated progeny. However, in part because of their strong association with lymphoproliferative diseases, no experimental or clinical evidence currently exists to support that claim. Herpes simplex viruses (HSV) and other members of the α-herpesvirus group, cannot maintain latent infection in dividing cells and these viruses rather persist by establishing latent infection in long-lived cells, such as sensory neurons. HSV has developed as a unique gene transfer strategy for cells of the central nervous system, although their inherent toxicity has complicated their specific application in gene therapy. Because of their natural tropism for neuronal cells, there is no report of HSV-based vectors for hematopoietic cells.

3.1.2 Non-viral approaches

The ultimate goal of non-viral methods of gene delivery is to imitate features of viruses whilst avoiding their inherent limitations [reviewed in (Schofield and Caskey, 1995)]. Most of these approaches are still in their infancy and their relevance for stem cell gene transfer is currently not apparent. As discussed below, major limitations include a low delivery efficacy as well as short-term expression of the transferred sequence.
Particle bombardment has recently been described as a novel means of gene transfer both *in vivo* and *in vitro* (Yang et al., 1990). In this physical method, plasmid DNA is coated onto the surface of metallic beads and the resulting particles are directly introduced into tissues using a "gene gun" to accelerate the particles to a high velocity. Analysis has shown that once inside the cell, the foreign DNA does not integrate into the host cell genome, and exists as a relatively unstable episome. Levels of expression usually peak within 3 days and rapidly decline to between 1-5% of peak levels after 1 week (Cheng et al., 1993). The requirement for a surgical procedure has also been cited as a major limitation of this technology. This would suggest a limited application of this approach for gene therapy.

Another non-viral approach receiving attention involves direct injection of pure closed circular plasmid DNA or RNA into a desired tissue. Using direct skeletal muscle injection, Wolff *et al.* showed significant levels of expression of the reporter gene constructs within mouse skeletal muscle cells (Wolff *et al.*, 1990). Southern blot DNA analysis suggested that the DNA exists as a non-replicating closed circular episome. This method of direct plasmid DNA injection is simple, inexpensive, and non-toxic procedure when compared to viral delivery. The potential to carry large DNA constructs is also advantageous. However, very few cell types possess the cellular characteristics that permit direct DNA penetration across the cell membrane. Furthermore, the levels and persistence of gene expression do not appear sufficient for gene therapy purposes.

Polycationic lipids can be mixed with polyanionic DNA or RNA to spontaneously form liposome structures. These colloidal particles are thought to fuse with the target cell membrane and thereby mediate delivery of the nucleic acids directly into the cytoplasm, bypassing the lysosomal degradation pathway (Felgner and Ringold, 1989). Several lipid preparations have been formulated for this application, including mixtures of dioleoyl phosphatidylethanolamine (DOPE) with DOTMA (lipofectin), DOSPA (lipofectamin), DDAB (lipofectace), DOGS (transfectam), DOTAP, DMRIE, and DC-cholesterol (Felgner *et al.*, 1995). This method is considered as an attractive alternative to viral transfer methods due to the absence of DNA size constraints, lower immunogenicity, minimal systemic toxicity and easier bulk preparation. Perhaps the most intensively studied tissue for cationic liposome delivery *in vivo* is the arterial vessel wall (Nabel *et al.*, 1990). DNA-liposome catheter delivery systems are feasible, but are limited by low transfection efficiencies (<1%) and toxicity increasing with concentrations,
regardless of the vector DNA-liposome system used. The low efficacy of transfection predicts the inadequacy of this method for gene delivery into rare pluripotent stem cells.

Gene transfer can also be performed in a receptor-mediated fashion by conjugating the DNA to be delivered with a cell-specific carrier molecule which is the ligand for a surface receptor. This molecular conjugate is formed by modifying the ligand with a DNA-binding agent, such as poly-L-lysine. Transferrin-polylysine/DNA has been used for binding transferrin receptors (Wagner et al., 1992), but most research has targeted the hepatocyte-specific asialoglycoprotein receptor (Wu et al., 1989). Because of their inbuilt tissue targeting signal, these complexes are easily and inexpensively delivered by peripheral venous injection. Two further significant advantages are the low immunogenicity of the conjugates, and the ability to transfer large fragments of DNA (up to 48 kb). However, following binding to their specific receptors, the complexes undergo endocytosis and DNA becomes trapped within intracellular vesicles; it is at this stage that this otherwise elegant system encounters significant limitation. Cytoplasmic liposomes fuse with the DNA-containing vesicles, forming endosomes. The DNA is largely degraded by the action of liposomal nucleases within the acidified endosome. Despite the use of chloroquine to inhibit endosomal acidification (Tietz et al., 1990) or the addition of adenoviruses or individual viral proteins (e.g., hemagglutinin) to enhance endosome membrane disruption (Cristiano et al., 1993; Wagner et al., 1992), the in vivo efficiency of gene expression remains relatively low and rapidly declines with time. Although these particular structures appear cumbersome for gene transfer into stem cells, the concept of receptor-mediated delivery is nevertheless subject of intense investigation in the context of retroviral vectors (see §2.1.2, chapter 4).

Delivery of active transgenes to the host cell nucleus could be enhanced if shuttling through the endosome were avoided. Kaneda et al. have developed such a system by combining the advantages of liposome carrying capacity for large DNA fragments with the fusion properties of hemagglutinin virus of Japan (HVJ; also called Sendai virus) (Kaneda et al., 1989). Following attachment to the plasma membrane at the cell surface, HVJ fuse to the membrane and release their viral contents at neutral pH directly into the host cell cytoplasm, bypassing endosome formation. The HVJ-liposome has been used for gene delivery to vascular walls, liver and kidney [reviewed in (Schofield and Caskey, 1995)]. It is efficient, easy to perform and theoretically has an unlimited DNA fragment size capacity. However, following peripheral vein injection, HVJ-
liposomes present little organ uptake. Other disadvantages are concerns regarding the use of intact HVJ, despite assurances of its non-pathogenicity in humans, and the short duration of expression of delivered transgenes. Indeed, the delivered sequence is not integrated into the host genomic DNA, and exists as an unstable episome. However, the ability of this system to co-deliver DNA with any protein may eventually permit integration by the co-delivery of DNA recombinase enzymes within the HVJ-liposome.

3.2 Gene transfer as a tool for marking human hematopoietic cells

The concept of marking hematopoietic cells to study their biological behavior in vivo has evolved from tracking cells with specific chromosomal markers (see §1.1.5), to the current practice of genetically tagging cells using the readily identifiable retroviral vectors described before. This technology has already shown its utility by improving our understanding of the developmental potential of individual murine hematopoietic stem cells (see §1.1.5). As discussed in this section, this retrovirus-based marking strategy can now be extended to characterize the biological features of human hematopoietic cells, including the pluripotent hematopoietic stem cells.

3.2.1 Clinical gene marking studies

In the first clinical application of the gene marking technology, Rosenberg et al. retrovirally marked tumor infiltrating lymphocytes (TIL) with the Neo<sup>R</sup> gene and followed the fate of vector-marked TIL after infusion into patients with malignant melanoma (Rosenberg et al., 1990). Gene-marked TIL could consistently be detected in the bloodstream of patients for 3 weeks, with frequencies dropping from 1 in 300 cells at day 3 to less than 1 in 10,000 cells in later samples. Small numbers of gene-marked TIL were also found in tumor samples as long as 9 weeks after infusion. Thus, for the first time, it was demonstrated that an exogenous gene could be safely transferred into a patient and subsequently detected in cells taken back out of the patient. This investigation laid the foundation for the following gene marking and gene therapy clinical trials.

Brenner et al. used a gene marking strategy in cancer patients undergoing autologous bone marrow transplantation (ABMT) to determine whether contaminating tumor cells in the transplant contributed to tumor relapse and to establish if the transplant was responsible for long-term hematopoietic reconstitution (Brenner et al., 1993). Bone marrow cells were harvested from 20 patients with AML or neuroblastoma who had completed consolidation chemotherapy and
had no evidence of residual disease. Approximately 30% of the harvested BM was genetically marked by infection with a retroviral vector containing the neomycin resistance marker gene. The patients were treated with myeloablative chemotherapy, and BM was infused. Two patients with AML were shown to relapse, both with genetically marked cells, implying that the "remission" BM did contain tumor cells that contributed to relapse. These gene-marked cells contributed for only 0.1–1% of the total BM cells and approximately 5% of progenitors in vivo. Several lines of evidence suggest that this contribution to marrow reconstitution included long-lived multipotent stem cells. For instance, gene-marked marrow progenitor cells were detected for as long as 18 months after ABMT. Also, by 6 months after transplantation, multilineage CFU-GEMM colonies which contained and expressed the marker gene began to appear, although in a minority of patients. Finally, detection of the marker gene in T cells and B cells for at least 18 months is consistent with the transfection of primitive hematopoietic cells with multilineage capacity. In a similar marking study, Deisseroth et al. determined that autologous BM used for transplantation in patients with CML following intensive therapy also contained cells that contributed to relapse (Deisseroth et al., 1994). In both investigations, the efficiency of gene transfer into CFC derived from patients was slightly higher than predicted from large animal models (Bodine et al., 1993; Schuening et al., 1991). One explanation is that BM was harvested during the marrow recovery phase that follows multiple cycles of intensive marrow-ablative chemotherapy. During this recovery period there is a profound proliferation of early marrow progenitor cells that may favor integration of the viral genome (Wieder et al., 1991). However, similar to the findings of previous studies, the frequency of vector-containing cells in the total BM of these patients was up to 100-fold lower than the frequency specifically observed in the CFC isolated from the total BM. The explanation for this dichotomy is unknown. Potentially, the presence of the transferred gene may interfere with mature hematopoietic cell production. Some reports have suggested that the NeoR gene may impair hematopoietic cell function and induce an immune response (Valera et al., 1994; von Melchner and Housman, 1988).

Recently, Dunbar et al. described 11 cancer patients who were transplanted with both CD34+–enriched peripheral blood cells transduced with a retroviral vector containing the NeoR marker gene, and with CD34+–enriched BM cells transduced with a second distinguishable NeoR-containing vector (Dunbar et al., 1995). The marker gene was detectable in multiple lineages, including granulocytes, B cells and T cells, and persisted in three of nine evaluable patients for
greater than 18 months post-transplantation. Marked cells originated from both peripheral blood and BM repopulating cells. In comparison to the previous clinical gene marking studies (Deisseroth et al., 1994; Brenner et al., 1993), the steady state levels of marking of the total BM cells from the patients were even lower with only 0.001% to 0.0001% of the cells positive for the marker gene. The younger age of patients, the absence of cytokines during infection and the lower amount of induction chemotherapy that patients received have been proposed to explain the higher levels of transduction reported in the previous experiments. Along these lines, Cornetta et al. recently reported low long-term gene transfer rates in adult patients that were treated with heavy doses of chemotherapy prior to transplantation of genetically marked autologous bone marrow cells (Cornetta et al., 1996).

3.2.2 Human gene marking studies in immune-deficient mouse models

Although the above clinical gene marking studies have facilitated advances in cancer therapy, autologous BM transplantation, and stem cell biology, they have also confirmed the relative inability of retroviral vectors to efficiently transduce human repopulating cells and the need to evaluate different experimental conditions to augment stem cell transducibility. With the development of novel in vivo assays for primitive human hematopoietic cells based on IV injection into immune-deficient mice (see §2.3.3), the same marking strategy used in a clinical setting can now be applied to characterize repopulating hematopoietic cells of human origin in the context of a small animal model more easily amenable to various experimental conditions impossible to test in humans.

Similar to what was done in the mouse (see §1.1.5), gene marking has been utilized in this thesis (chapter 2) to validate the use of the SCID transplantation model as an assay for human hematopoietic cells with long-term reconstitution capacity. Using methods previously optimized for gene transfer into clonogenic cells (Dick et al., 1991), human BM and CB cells were infected with retroviral vectors containing the NeoR or murine ADA marker gene. While a high proportion (up to 100%) of CFC and LTC-IC in the donor inoculum were readily transduced, these gene-marked cells and their progeny made only minor contributions to the total number of human multilineage cells present in engrafted NOD/SCID mice, indicating that only a small proportion of SRC were transduced. This result is consistent with the low levels of gene marking of repopulating human hematopoietic cells observed in the clinical investigations outlined before. Since mice contained high numbers of unmarked human progenitors and mature cells, unmarked
SRC must have initiated the graft. Hence, we conclude that most CFC and LTC-IC are incapable of long-term engraftment of NOD/SCID mice and are therefore biologically distinct from SRC. The differential infection of SRC and CFC/LTC-IC thus provides new insights into the organization of the human hematopoietic system and highlights the need for appropriate assays that predict outcomes in human clinical trials.

Exciting evidence documenting the pluripotent character of primitive human hematopoietic cells capable of repopulating immune-deficient mice has recently been obtained using the bnx mouse transplantation model (Nolta et al., 1996). Nolta et al. took advantage of the individual genetic markers generated by random integration of RV into their target cells to follow uniquely marked stem cells and their progeny in vivo. Human CD34+ cells were transduced with NeoR-containing retroviral vectors in a stromal culture system and used for long-term engraftment into immune-deficient bnx mice. Although engraftment in this model is relatively low (<10%), gene-marked human lymphoid and myeloid populations were recovered from the marrow of the mice 7-11 months after transplantation. Gene-marked myeloid and T cells clones were expanded ex vivo and, using inverse PCR, common proviral integration sites were seen in myeloid and lymphoid populations, confirming gene transfer and engraftment of bnx mice with a cell capable of multilineage differentiation. Interestingly, the frequency of gene transfer into this pluripotent bnx-repopulating cell was low, consistent with the observations described in chapter 2 of this thesis using the NOD/SCID transplantation system. It will now be interesting to determine if this bnx-repopulating cell can sustain the production of high numbers of hematopoietic cells of all lineages and if it is endowed with self-renewal capacity following transplantation into secondary recipients.

### 3.3 Gene transfer as a tool for curing human diseases

Notwithstanding the limitations associated with the currently available gene transfer vectors and methods resulting in low transduction efficiency, a plethora of clinical protocols with therapeutic objectives have already been approved throughout the world. This section will focus on curative applications of gene transfer into primitive human hematopoietic cells. The results of transduction of these cells obtained to date represent a useful paradigm for understanding progress and limitations in gene therapy as a whole.
3.3.1 Scope and limitations of human gene therapy

No human disease has yet been cured by gene transfer. However, several studies have demonstrated that therapeutic genes transferred to humans by various means can evoke biological responses that are relevant to the gene product and to the specific disease state of the recipient. The majority of these studies are open to patients with advanced malignancy, in whom the risk-to-benefit ratio is most appropriate.

Generation of tumor vaccines by genetic modification of tumor cells from BM

In the majority of protocols aimed at cancer treatment, the malignant disease is treated either (1) by modifying the tumor so as to make it more immunogenic, for example, by using cytokine genes, adhesion molecules, or allogeneic HLA molecules; or (2) by modification of the tumor to make it less malignant, for example, by correcting mutant p53; or (3) by transferring prodrug metabolizing enzymes, for example, thymidine kinase to sensitize tumors to gancyclovir [reviewed in (Hanania et al., 1995)].

The first approach, referred to as tumor vaccines generation, has been applied to primitive BM cells from cancer patients. It is based on the frequent failure of the immune system to eradicate malignant disease as a result of poor immunogenicity of most tumors (Rosenberg, 1992). In an attempt to enhance immune recognition, investigators have transduced tumor cells with cytokine genes, with allogeneic MHC molecules (Nabel et al., 1993) or with B7 (Chen et al., 1992), a costimulatory molecule that activates cytotoxic T cells after engaging their surface CD28 or CTLA4 ligands. In several different murine model systems, injection of transduced neoplastic cells in doses that would normally establish a tumor results instead in the recruitment of immune system effector cells and eradication of the injected tumor cells [reviewed in (Colombo and Forni, 1994)]. Often, the animal is then resistant to challenges by further local injections of nontransduced parental tumor. Thus, the transduced tumor cells appear to behave as a vaccine. This principle has been incorporated into more than 14 cancer vaccine trials [reviewed in (Hanania et al., 1995)].

Gene transfer to modulate immunocyte function

A smaller number of protocols propose to modify the immune system to enhance its antitumor activity. The anti-tumor activity of marrow-derived immunocytes can be genetically enhanced by increasing the levels of cytotoxic cytokines (e.g. tumor necrosis factor) that they produce at local tumor sites. This approach is being evaluated in studies using tumor infiltrating
lymphocytes (TIL). Unfortunately, it has proved difficult to induce TIL to secrete high levels of cytokines, and evidence to support the belief that reinfused human TIL selectively home to tumor sites is limited (Rosenberg, 1992).

**Modifying the drug sensitivity of progenitor cells**

The capacity to render HSC resistant to one or more cytotoxic drugs has raised the possibility to protect normal host tissues from the toxic effects of chemotherapy. This might enable patients to resist the myelosuppressive effects of cytotoxic drugs, allowing more prolonged or more intensive chemotherapy and, potentially, increasing cure rates. Also, drug-resistance could behave as a dominant selectable marker, and co-transfection with a therapeutic gene could provide a mechanism to positively select gene-modified HSC to a level where therapeutic benefit could be obtained, despite low-efficiency gene transfer (Sorrentino et al., 1992).

The product of the MDR1 gene functions as a drug efflux pump and confers resistance to a wide variety of naturally occurring chemotherapeutic agents (Pastan and Gottesman, 1991). At least 3 trials propose transfer of the MDR1 gene to BM or peripheral blood stem cells from adult cancer patients in the setting of ABMT. However, these applications face several potential pitfalls. The low stem cell transduction efficiency observed in current clinical protocols remains the major drawback as it predicts that drug-induced myelosuppression will not be reduced in the absence of dramatic in vivo selection. Also, there is the risk of transferring drug resistance genes to tumor cells that contaminate the marrow graft, posing the possibility of a drug-resistant relapse.

**Correction of single gene defects**

With the accumulation of more safety data from previous clinical trials, the focus of gene therapy has begun to shift. An increasing number of approved protocols are designed to treat patients suffering from nonmalignant diseases caused by classical, single-gene defects. Severe combined immunodeficiency disease (SCID) resulting from ADA deficiency has been the focus for the initial attempts of gene therapy, because transduced T-lymphoid cells are expected to have a selective survival advantage in vivo. Culver et al. performed monthly infusions of mature lymphocytes genetically modified with an ADA-containing virus and tests of cellular immunity indicated an improvement that was coincident with increases in the serum levels of the ADA protein (Culver et al., 1991). In an attempt to produce a permanent cure of the disease, Kohn et al. treated neonates by ADA retroviral-mediated transduction of the CD34+ cells from their
umbilical CB followed by autologous transplantation (Kohn et al., 1995). The continued presence and expression for 18 months demonstrated that CB cells had been genetically modified with retroviral vectors and could engraft in neonates for gene therapy. However, while 4-6% of CFC derived from patients were infected, only 0.01% of the patient total BM cells were shown to contain the transferred gene. Hence, the aspiration that this small proportion of ADA gene-transduced cells would prove to have a selective advantage in vivo, and would therefore substantially increase in number, has not yet been realized.

Protocols to transduce stem cells with the glucocerebrosidase gene are also well advanced to treat patients with Gaucher’s disease (Bahnson et al., 1994). Other protocols to treat Fanconi anemia, X-linked immunodeficiencies and other lysosomal storage disorders are all in varying stages of development. Finally, sickle cell anemia (SCA) and β-thalassemia although originally considered as ideal candidates for gene therapy were quickly recognized to present special problems for therapy. Beyond the challenge of stem cell transduction, these defects also require that the newly introduced globin genes be expressed only in erythroid cells and be precisely regulated in order to produce a functional hemoglobin molecule.

3.3.2 Development of pre-clinical models of stem cell gene therapy

From the various clinical trials described before, it appears that the story of human gene therapy for stem cell disease to date has largely centered on efforts to circumvent the inability of available vectors and methods of gene transfer to permit efficient gene delivery into rare and quiescent stem cells. Because of the absence of an appropriate assay for human HSC, it has been difficult to design pre-clinical experiments that could permit a conclusive evaluation of the gene transfer efficiency and safety into human stem cells and that could assess conditions essential to increase their susceptibility to retrovirus-mediated gene transfer. This problem has placed major constraints on the development of efficient therapeutic protocols. The problem is nicely illustrated in the case of β-thalassemia and sickle cell anemia that have not been able to negotiate the crucial passage from basic science to clinical trials because of the absence of efficient gene transfer methods and vectors and because of the lack of an appropriate pre-clinical model where safety and efficiency of these methods could be tested.

Globin gene organization and regulation of transcription

β-thalassemia and SCA syndromes are common inherited anemias that occur because of mutations affecting the synthesis of hemoglobin (Hb). Hemoglobin is a tetrameric protein
composed of two dimeric polypeptide units encoded by two different gene families found on two separate chromosomes (Figure 1-5). The α-globin gene cluster, located on chromosome 16, includes the duplicated α genes (α1, α2) and the ζ-gene (Figure 1-5). The cluster of β-like genes is located on the short arm of chromosome 11 and encompasses nearly 100 kb of DNA. The cluster contains five functional genes (ε, γ, δ, and β) that are arranged on the chromosome with an order that corresponds to their developmental stage of expression (Figure 1-5). During normally developing erythropoiesis, six distinct hemoglobin species are present in the transition from intrauterine to adult life. Hemoglobin A (Hb A), defined by an α2β2 structure, is the predominant Hb found in normal adult red cells. Adult erythrocytes also contain two minor hemoglobins, Hb A2 and Hb F, characterized by their α2δ2 and α2γ2 subunit composition, respectively. Although a minor hemoglobin in adult red cells, Hb F is the predominant Hb in fetal red blood cells during the latter two trimesters of gestation. Earlier in gestation, embryonic hemoglobins with differing globin chain composition (ζ2ε2, α2ε2 and ζ2γ2) are found in yolk sac-derived macrocytic red cells. This process of coordinated Hb expression is known as hemoglobin switching.

The human β-globin locus is located in active or open chromatin in cells of the erythroid lineage [reviewed in (Dillon and Grosveld, 1993)]. In contrast, the chromatin structure of the locus is closed in non-erythroid tissues and the β-globin is completely inactive. There are several types of regulatory elements within the β-globin cluster that interact to direct erythroid specific gene expression and to coordinate the switches in Hb phenotype during development. These include the cis-acting globin promoters and enhancers 3’ of the expressed genes, and a distant locus control region (LCR) that flanks the cluster of expressed genes (Figure 1-5). These distant regulatory elements are associated with DNase I hypersensitive sites (HS); four sites (5’ HS 1-4) are located several kilobases 5’ of the ε-globin gene, and one site (3’ HS 1) is mapped 3’ to the β-globin gene (Figure 1-5) (Tuan et al., 1989; Forrester et al., 1986). The LCR directs copy number-dependent, position-independent expression of a linked human β-globin gene in transgenic mice, suggesting its role as the primary regulator of chromatin structure in the locus (Grosveld et al., 1987). This copy number-dependent expression activity of the LCR in transgenic mice has been mapped to the 200-300 bp cores of 5’-HS2, 5’-HS3 and 5’-HS4. More recently, the LCR activity has more specifically been shown to be the culmination of at least two
Fig. 1-5. Chromosomal organization of the human β-globin gene cluster. The solid boxes indicate functional globin genes, whereas the open box indicates a pseudogene. The larger downward arrows mark the developmentally stable, erythroid-specific hypersensitive sites that constitute the locus-control region (LCR), flanking the cluster. Hypersensitive sites are also found over the promoters of the expressed genes (smaller downward arrows). The location of the enhancers in the cluster are marked by letters. The scale of the depicted chromosomal segments is in kilobases of DNA (kb).
separable functions including an activity located in 5’-HS3 that dominantly opens and remodels chromatin structure and a recessive enhancer activity residing in 5’-SH2 (Ellis et al., 1996).

**Globin pathophysiology**

β-thalassemia and SCA syndromes result from mutations in the β-globin gene. Homozygous β-thalassemia is characterized by absent (β⁰-thalassemia) or reduced (β'⁰-thalassemia) synthesis of the β-subunit of adult hemoglobin (Hb A). The consequent excess unbound α-globin chains precipitate in red cell precursors in the BM, resulting in decreased red blood cell membrane flexibility, ineffective erythropoiesis, and accelerated red cell destruction. Sickle cell disease is caused by a unique amino acid substitution (⁶Glu→Val) in the human β-globin peptide, producing hemoglobin molecules (HbS) with lower solubility upon deoxygenation. The erythrocytes become rigid and deformed (sickled) as a result of the intracellular polymerization of the deoxygenated hemoglobin S and become trapped in the microcirculation causing local hypoxia with consequent tissue damage.

**Treatment of hemoglobinopathies: the importance of pre-clinical models of gene therapy**

Although the molecular basis of these conditions is well established, no definitive treatment is currently available. Patients with severe phenotypes rely on regular erythrocyte transfusions that can be associated with life-threatening iron overload despite intensive chelation (Wolfe et al., 1985). Long-term transfusion may also result in the development of anti-erythrocyte antibodies making subsequent transfusions difficult or, in some instances, impossible (Rebulla and Modell, 1991). Successful BM transplantations have been reported, but the availability of related HLA-matched donors limits the general applicability of this therapy to only 25% of patients (Lucarelli et al., 1990; Ferster et al., 1992). Recent work has focused on the pharmacologic manipulation of fetal hemoglobin. Underpinning these efforts is the premise that increased γ-globin gene transcription and fetal hemoglobin synthesis will lead to more effective erythropoiesis and/or decreased hemolysis in patients with β-thalassemia and sickle disease (Perrine et al., 1993). These treatments are, however, potentially toxic with unknown long-term complications. Hence, the development of methodologies and models that will permit efficient, safe and stable transfer of human globin genes into HSC is eagerly awaited for the treatment of hemoglobinopathies.
Initial gene transfer methodologies for hemoglobinopathies were evaluated in 3T3 fibroblasts, utilizing a 3 kb fragment of human genomic β-globin into an ecotropic retroviral vector in both orientations (Cone et al., 1987). In these experiments, the level of expression was only 0.01% compared to endogenous murine β-globin expression. Similar studies using an amphotropic vector containing a Neo\textsuperscript{R} gene with a γ/β-globin hybrid were used to infect murine erythroleukemia (MEL) cells, resulting in human β-globin expression at 10% of the endogenous induced expression (Karlsson et al., 1987). However, in both instances, viral titer was low and the provirus rearranged in some of the clones tested. Subsequently, constructs containing portions of the 5’ untranslated region of the β-globin gene and of its second intron in the reverse orientation were developed, but they were found to interfere with the generation of full-length transcripts and to yield low titer recombinant virus (Miller et al., 1988). It was shown that the 5’ region could be removed, but the intron 2 was required for expression. Examination of retrovirally transduced β-globin in human hematopoietic cells also showed a low frequency (0.04%) as a consequence of the low viral titer (5 X 10\textsuperscript{4} cfu/ml) (Bender et al., 1988). The first \textit{in vivo} experiment described a recombinant retroviral construct encoding a human β-globin gene that was used to infect murine hematopoietic cells and reconstitute transplanted mice (Dzierzak et al., 1988). Expression was limited primarily to the erythroid lineage in the transplanted animals and persisted for 4 to 9 months at levels ranging from 0.4% to 4% of the endogenous mouse β-globin mRNA level. However, the infection rate was low, with only 18 of 104 animals reconstituted with infected BM. Confirmatory experiments in several laboratories also demonstrated β-globin retroviral transfer in murine hematopoietic cells (Bender et al., 1989; Karlsson et al., 1988). Long-term expression in all lineages from secondary recipient animals indicated that pluripotent HSC rather than committed progenitor cells were infected. Despite this improved transduction frequency in pluripotent BM cells, globin expression still ranged from 1% to 5% of the endogenous level.

It was not until the discovery of the LCR regulatory elements flanking the β-globin gene cluster that a new approach to the design of retroviral vectors for hemoglobinopathies was suggested (Grosveld et al., 1987). Individual LCR fragments were included within a marked β-globin/Neo\textsuperscript{R} gene cassette and used to generate recombinant amphotropic virions infectious for MEL cells (Novak et al., 1990). Constructs incorporating an HS2 fragment had titers of 10\textsuperscript{4}-10\textsuperscript{5}
and resulted in high level expression in a few clones, but with extreme expression variability (10% to 310%). A more recent report described the use of a 36-bp sequence encompassing the NFE-2 transcription factor binding site within the HS2 region linked to human β-globin (Chang et al., 1992). The level of β-globin expression increased marginally from 6% to 12% with the addition of the enhancer element. However, viral titers were low, and introduction of multiple copies of the 36-bp fragment promoted gross proviral rearrangements. In addition, Plavec et al. reported the generation of an ecotropic retrovirus containing an LCR cassette with truncated HS 4, 3, 2 and 1 sites linked to a human β-globin. This vector yielded 60% to 70% expression in MEL cells compared with endogenous murine globin expression. Transfer into murine hematopoietic progenitors and subsequent transplantation into lethally-irradiated recipients resulted in human β-globin expression, suggesting that inclusion of LCR may support high level β-globin gene expression in murine hematopoietic stem cells (Plavec et al., 1993). Leboulch et al. have recently identified potential structures responsible for proviral instability and low titer of these β-globin/LCR retroviral vectors (Leboulch et al., 1994). These included an A/T-rich segment in the second intron of the human β-globin gene, and several complementary/reverse polyadenylation signals and splice sites. Elimination of these sequences by extensive mutagenesis of the transduced β-globin gene rendered proviral transmission stable upon infection of cell lines and murine BM marrow repopulating cells, and increased viral titer 10-fold without significantly perturbing expression of the transduced β-globin gene. Hence, development of human β-globin/LCR-containing vectors that can infect primitive human hematopoietic cells may serve as an important tool for gene therapy of human hemoglobinopathies.

Animal models for sickle cell disease have recently been realized in several laboratories by the generation of transgenic mouse lines expressing human HbS or derivative forms of this mutant (Fabry et al., 1995). In addition, two cases of β*-thalassemia have been described as a result of spontaneous deletion (Skow et al., 1983) or targeted insertional disruption (Shehee et al., 1993). More recently, targeted deletion of both murine β-globin genes in embryonic stem cells have also been used to generate mouse models for β⁰-thalassemia. However, none of these murine models permit an evaluation of human gene therapy protocols directly on the defective human long-term reconstituting cells. As presented in chapter 3, BM cells from pediatric patients
with β-thalassemia and SCA can be transplanted into sublethally irradiated SCID and NOD/SCID mice to develop in vivo pre-clinical models for defective human long-term repopulating cells (Larochelle et al., 1995). Levels of engraftment similar to those observed with normal BM and CB cells were seen. High numbers of human multipotential and committed progenitors of all the erythroid and myeloid lineages were maintained in the SCID mouse BM for at least two months post-transplantation. FACS analysis of highly engrafted EPO-treated mice showed the presence of CD34⁺CD38⁻ immature human cells as well as glycophorinA⁺CD45⁻ and glycophorinA⁺CD45⁺ human erythroid lineage cells in the murine BM. These data suggest that immature hematopoietic cells had engrafted the murine BM and were able to differentiate along the erythroid lineage in response to EPO. The results of several procedures including RNase protection assay, RT-PCR, the functional metabisulfite-based sickling test and staining with human-specific globin monoclonal antibodies, confirmed that human erythroid cells and human BFU-E recovered from the marrow of engrafted mice expressed the original disease phenotype of the donor. This system can thus be used to evaluate gene transfer efficiency into primitive human cells, longevity of expression, expression in the appropriate lineage, and correction of the disease phenotype. The same approach should also be applicable to other single gene defects such as Gaucher's disease and severe combined immunodeficiency disease.

3.4 Summary

Gene transfer into human hematopoietic stem cells represents an area that has attracted particular attention, both because of the biological insights that can be gained and because of the wide range of pathologies that may be corrected in these cells and their progeny. Whilst the potential of gene transfer into stem cells is considerable, current applications have been restricted by the limitations of the viral and non-viral vectors currently available. In support of the non-viral systems is a practical argument favoring their relative simplicity, greater flexibility in the size and sequence of DNA molecules that can be delivered as well as their relative safety; however, their inability to efficiently and stably transfer genetic material into rare populations of cells have restrained their applicability. Although a variety of infectious agents have been described as vehicles for gene transfer, retroviral-based vectors remain the preferred system for gene delivery, mainly because of their ability to infect and stably integrate into a large number of cells. However, because of their requirement for cells in active division, these vectors are not able, as yet, to produce the desired targeted and efficient gene transfer into primitive human repopulating
cells. This difficulty, previously suggested from marking studies in large animal models, is well illustrated in the results of the early clinical trials and also, as discussed in chapter 2, in the context of the SCID transplantation assay for primitive human hematopoietic cells. In this model, the poor infection of primitive human SRC, in contrast to CFC/LTCIC highlights the need for appropriate assays that predict the outcomes of human clinical trials. Transplantation of human BM cells from hemoglobinopathy patients into SCID mice provides such an assay that allows evaluation of gene transfer vectors and methods targeted at the correction of the most primitive human repopulating cells.

The work described in this thesis builds upon the value and problems of gene transfer discussed above, as a means to identify and characterize human hematopoietic stem cells (gene marking), and to treat diseases associated with alterations in their genetic make-up (gene therapy). Along these lines, the following objectives will be presented: (1) Establish the feasibility of efficient gene delivery to human repopulating cells using the SCID transplantation assay; (2) Identify and characterize the SCID-repopulating cells (SRC) using a gene marking strategy; (3) Develop animal models to evaluate gene therapy protocols for human hemoglobinopathies. Objectives 1 and 2 are discussed in chapter 2; the third objective is presented in chapter 3.
4. References


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SECOND CHAPTER

IDENTIFICATION OF PRIMITIVE HUMAN HEMATOPOIETIC CELLS CAPABLE OF REPOPULATING NOD/SCID MOUSE BONE MARROW: IMPLICATIONS FOR GENE THERAPY


AL and JV made equal contribution to this work.

Figures 2-3b and 2-4b were done by HH

Figure 2-5 was done by JCYW and MB
1. Abstract

Progress to develop methods for introducing genes into hematopoietic stem cells has been hindered by the absence of pre-clinical models that assay the repopulating capacity of primitive human hematopoietic cells. Consequently, current human gene therapy trials employ gene transfer methods optimized using surrogate in vitro colony-forming cell (CFC) and longterm culture-initiating cell (LTC-IC) assays and by inference from studies on other mammalian species. We report the identification and characterization of a novel human hematopoietic cell capable of repopulating the bone marrow of NOD/SCID mice (SCID-repopulating cell, SRC) that is more primitive than most LTC-IC and CFC. Both CFC and LTC-IC were efficiently transduced with retroviruses (up to 80% and 70%, respectively); however, these gene-marked progenitors and their progeny made minor, if any, contributions to the total number of human multilineage cells present in transplanted NOD/SCID mice, establishing that SRC are biologically distinct from most CFC and LTC-IC. By contrast, SRC were transduced only rarely, a result consistent with the low level of gene marking observed in human gene therapy trials. Characterization of the cell surface phenotype indicated that SRC were exclusively CD34+CD38- and could be highly purified in this cell fraction. Thus, the in vivo SRC assay allows both characterization of the developmental program of the most primitive human hematopoietic cells and optimization of vectors and transduction methods specific for these cells prior to their use in human clinical trials.
2. Introduction

Permanent correction of human disorders affecting the hematopoietic system requires that genes are introduced into pluripotent stem cells because only these cells can initiate long-term reconstitution of the entire hematopoietic system (Anderson, 1992). Studies in the mouse have established that retroviral vectors can efficiently transfer and express new genes into primitive pluripotent stem cells capable of reconstituting all lymphoid and myeloid tissues of recipient mice (Dick et al., 1985). However, the efficiency of gene transfer into hematopoietic stem cells was much lower when similar protocols were applied to large mammals (Bodine et al., 1993; Schuening et al., 1991) and in human gene therapy trials (Dunbar et al., 1995; Kohn et al., 1995; Deisseroth et al., 1994; Brenner et al., 1993), suggesting that there may be species-specific characteristics of stem cells in their susceptibility to retrovirus infection. Because of the absence of in vivo assays that measure the repopulating capacity of human stem cells, current gene transfer methods employed in human clinical trials have been optimized using in vitro CFC assays (Dick et al., 1991; Hock and Miller, 1986) that detect committed, multipotential, and blast progenitor cell types, and LTC-IC assays that detect a cell capable of maintaining production of CFC for at least 5 weeks on a layer of stromal cells (Moritz et al., 1994; Eaves et al., 1992; Hughes et al., 1989). However, the relevance of these methods for stem cell gene transfer is unclear because the relationship between these progenitor cells and the in vivo repopulating human stem cell is not established.

The engraftment of normal human hematopoietic cells in immune-deficient mice provides a system to develop an assay that measures the repopulating capacity of human stem cells (Vormoor et al., 1994; Baum et al., 1992; Lapidot et al., 1992; Nolta et al., 1994). We have previously reported that intravenous (IV) injection of human bone marrow (BM) (Lapidot et al., 1992) or cord blood (CB) (Vormoor et al., 1994) into SCID or NOD/SCID (Shultz et al., 1995) mice results in the engraftment of a small number of primitive cells that proliferate and differentiate in the murine BM producing large numbers of LTC-IC, CFC, immature CD34+Thy1+, CD34+CD38- cells and mature myeloid, erythroid, and lymphoid cells (Vormoor
et al., 1994; Lapidot et al., 1992; Larochelle et al., 1995; Cashman et al., 1994). The primitive cells that initiate the graft were operationally defined as SCID-repopulating cells (SRC) (Larochelle et al., 1995; 41). However, SRC have not been characterized and the relationship between SRC and in vitro progenitors such as LTC-IC and CFC is not known, nor is it known whether LTC-IC and CFC themselves have SRC activity. We have used retrovirus gene marking of human hematopoietic cells and cell purification based on surface marker expression to characterize SRC and to determine its relationship with LTC-IC and CFC. Both of these methods demonstrated that most LTC-IC and CFC were incapable of engrafting NOD/SCID mice providing strong evidence that SRC is a more primitive and distinct cell population. SRC were highly enriched in the CD34+CD38- cell fraction; no SRC were found in any other cell fraction. While CFC and LTC-IC could be very efficiently transduced with retroviruses using either coculture over retrovirus packaging cell lines or supernatant infection with the carboxy terminal fragment of fibronectin (CH-296) (Kimizuka et al., 1991), SRC were rarely transduced. Hence, in contrast to the in vitro assays, the SRC assay provided a more accurate prediction of the low levels of gene marking recently reported from human gene therapy trials (Dunbar et al., 1995; Kohn et al., 1995; Deisseroth et al., 1994; Brenner et al., 1993).
3. Methods

3.1 Retroviral infection.

Ficoll-separated human cord blood or bone marrow cells were prestimulated for 24 hrs in suspension cultures, containing IMDM, 10% FCS, huIL3 (50 ng/ml), huIL6 (20 ng/ml) and huSCF (50 ng/ml) (Immunex Corp., Seattle, WA). Cells were then co-cultured with irradiated (1500 cGy $^{135}$Cs) retrovirus packaging cells PG13LNC8 [neomycin phosphotransferase (NEO) vector] or AmmAl2 55/6 (PGK-murine ADA vector) in the same medium as above for 24 to 48 hours at a cell density of $1 \times 10^6$ cells/ml. The titer of the NEO virus was $10^5$ CFU/ml while the ADA virus had a titer of $10^7$ CFU/ml. Regular marker rescue tests for helper virus were negative. After co-culture, human cells were recovered by collecting the culture medium and vigorously washing the fibroblast layer twice with PBS. This cell fraction is referred to as the non-adherent fraction. In most experiments, cell dissociation buffer (CDB) (enzyme free/EDTA based; GIBCO BRL, Gaithersburg, MD) was used according to the manufacturers instructions to improve recovery of human cells adhering to the fibroblast cell lines. For supernatant infections on CH296, human CB or BM cells were first enriched for CD34$^+$ cells by negative selection using a cocktail of lineage antibodies (Roath et al., 1994) and the StemSep device as described by the manufacturers (Stem Cell Technologies Inc., Vancouver, BC). The CD34$^+$ enriched cells (described as Lin$^-$) were then prestimulated as described above and co-cultured ($10^6$ cells/ml) on 35mm petri dishes (Sarstedt, St. Laurent, QC) coated with the carboxy-terminal (CH-296) fragment of fibronectin (supplied by courtesy of Takara Shuzo Co. Ltd, Japan) using the mADA virus supernatant in the presence of cytokines (as above). Viral supernatant was replaced with fresh virus-containing media (IMDM + 5% FCS + cytokines) every 12 hours for 48 hours.

3.2 Transplantation of human CB or BM cells into immune-deficient mice

Sublethally irradiated (400 cGy) 8 week-old C.B17-scid/scid (SCID) and NOD/LtSz-scid/scid (NOD/SCID) mice were transplanted according to our standard protocol (Vormoor et al., 1994; Lapidot et al., 1992; Larochelle et al., 1995) with 30 to $50 \times 10^6$ nonadherent human
CB or BM cells alone or with a mixture of $8 \times 10^6$ nonadherent cells and $3$ to $6 \times 10^6$ adherent cells recovered after co-culture. The transplanted mice were treated with either $6.8 \ \mu g$ PIXY321 plus $10 \ \mu g$ huSCF (Immunex Corp.) or $7 \ \mu g$ each of huIL-3 and huGM-CSF plus $10 \ \mu g$ huSCF (Amgen) every other day. The animal experiments were approved by the Animal Care Committee of the Hospital for Sick Children and the Ontario Cancer Institute. The mice were sacrificed 4-8 weeks after transplantation.

3.3 Analysis of human cell engraftment

High molecular weight DNA was isolated from the BM of transplanted mice and the Southern was blotted using standard procedures. EcoRI digests of genomic DNA (1μg) were loaded into each lane and the blot was hybridized with a human chromosome 17-specific α-satellite probe (p17H8) which detects a characteristic 2.7 kb fragment. To quantify the human cells present in these tissues, the intensity of the 2.7-kb band was compared to human/mouse DNA mixtures (0, 0.1, 1, 10, 50% human DNA) as previously described (Vormoor et al., 1994; Lapidot et al., 1992). To determine whether human progenitors were present in the BM of engrafted mice, mononuclear cells were plated in methylcellulose cultures as described under conditions selective for the growth of human progenitors (Vormoor et al., 1994; Lapidot et al., 1992; Moritz et al., 1994). The different myeloid and erythroid progenitors were scored at 14 days. The total number of human progenitors present in the total BM of the mice was calculated by multiplying the number of progenitors per cells plated by the total mononuclear cell count from the four long bones multiplied by 5 because these bones represent 20% of the total BM in the mouse. In some experiments, the BM of engrafted mice was analyzed by two or three colour flow cytometric analysis using the FACScan. For erythrocyte lysis, mouse and human bone marrow samples were diluted with 14 volumes of a NH₄Cl lysis buffer ($10^{-4}$ M EDTA, $10^{-3}$ M KHCO₃, 0.17 M NH₄Cl in water (pH7.3)) and after gentle mixing kept at room temperature for 3 - 5 min. Thereafter, bone marrow cells and single cell suspensions of lungs were washed in phosphate-buffered saline (PBS) (containing 0.02% NaN₃) and incubated for 30 min on ice with saturating amounts of human specific monoclonal antibodies. Data acquisition and analysis was
performed with LYSYS II-software. Dead cells were gated out by their staining with propidium iodide (PI) and their orthogonal light scattering properties. In each experiment, cells from a non-transplanted mouse were stained with the same antibodies as a negative control. IgG1 isotype controls conjugated to PE, FITC and PerCP were also included.

### 3.4 Analysis of retroviral infection

To determine the proportion of infected CFC and LTC-IC, a small fraction of the recovered human cells was plated in methyl cellulose assays and in long-term marrow cultures (LTMC), respectively, as previously described (Vormoor et al., 1994; Lapidot et al., 1992; Moritz et al., 1994). The LTMC were harvested after 5 weeks and plated in methylcellulose to assay for CFC. ADA gene transfer was measured by PCR or isoenzyme analysis of individual colonies (Lim et al., 1989). The efficiency of gene transfer into primitive human cells capable of repopulating immune-deficient mice was measured by PCR analysis of CFC derived from engrafted mice and by PCR analysis of the total mononuclear cells present in the BM of engrafted mice. ADA PCR was carried out on individual colonies picked from methylcellulose cultures and from DNA extracted from the BM of transplanted mice. Both PCR primers were within the murine ADA cDNA present in the ADA vector: mADA1-sense primer: 5'-CCC AGA CAC CCG CAT TCA ACA AAC C-3'; mADA2-antisense primer: 5'-AGC GCA CTT CCA CAT AGA CCA CGC C-3'. A fragment of 299bp is produced; genomic mouse ADA sequences are not amplified as 3 introns separate the primers. The cycling conditions were 95°C for 30 seconds, 65°C for 30 seconds and 72°C for 60 seconds for a total of 40 cycles using a capillary thermocycler (Corbett Research). A human-specific internal amplification control was performed for each colony using primers in the dystrophin gene: 7632-sense primer: 5'-AAT TCA CAG AGC TTG CCA TGC TG-3'; 6975-antisense primer: 5'-TGC CTC CCA GAT CTG AGT CCT GTA-3'. A 230bp fragment is produced. The cycling conditions were the same as for ADA. For quantification of gene-marked human cells in the total BM of transplanted mice, 0.5μg DNA was used for each ADA PCR reaction and 29 (Figure 2-3D) or 25 (Figure 2-4D) cycles of amplification were used to ensure linearity. Each PCR amplification was repeated 3 times. The
primers used for the internal dystrophin amplification control were: 7632-sense primer; 9918-antisense primer: 5'-ACC ACC CTT CTT CAT CTC CTG ACA-3'. A 423bp product is generated. This primer pair amplifies both mouse and human sequences. The intensity of the signal in the samples was compared to DNA mixtures of a cell line with 1 integrated ADA provirus and an uninfected cell line. The value obtained from the PCR analysis was divided by the level of human cells present in the BM of the mouse to determine the proportion of gene marked human cells.

3.5 Cell sorting

Ficoll-separated human CB cells were stained as previously described. Cells with low forward and side scatter characteristics were sorted into CD34+ and CD34- by fluorescence activated cell sorting (FACStarPLUS, BD) (Figure 2-5A). In other experiments, CB cells were first enriched for CD34+ cells using the StemSep device and then sorted by FACS into a CD34+CD38- fraction and a CD34+CD38+ fraction (Figure 2-5B).

3.6 Inverse PCR

Inverse PCR was modified from Ochman et al. (Ochman et al., 1988) using primers located in the U3 region of the retrovirus long-terminal repeat (LTR) sequences (Jonsson et al., 1996). Following digestion and ligation, this method yields a common retrovirus fragment derived from internal sequences adjacent to the 3' LTR and an integration fragment in the genomic DNA adjacent to the 5' LTR. 3μg of genomic DNA were isolated from the BM of engrafted mice and digested with 20U Rsal that cuts at the 3' end of MoMuLV LTR. The digestion was done in 100μl PCR buffer (10mM tris-HCl pH 8.3, 50mM KCl, 2mM MgCl2, 0.1% gelatin) in presence of 10μg RNase A. The reaction was heat-inactivated at 65°C for 30 min. and left at room temperature for 1 hour to allow DNA re-annealing. A third (1 μg) of the digested DNA was ligated at 20°C in 100μl PCR buffer containing 5U T4 DNA ligase (Boehringer Mannheim, Laval, Québec) and 1μl 10mM ATP. 200ng of the ligated product were amplified by 40 cycles of PCR (1 min. 94°C, 2 min. 49°C, 3 min. 72°C) using 20 pmoles of primers U3A (5'-TCCATGCCTTGCAAAATGGC-3') and U3B (5'-
ATGACCTGTCCTATT-3'). An aliquot of each PCR reaction was diluted 1:1000 and submitted to a 40 cycle nested PCR at the same conditions as above. The nested primers were U3C (5'-CTTGCCAAACCTACAGGT-3') and U3D (5'-CTCGCTCTGTTCG-3').
4. Results
4.1 Survival of SRC during *in vitro* infection

Human BM or CB cells were infected with retroviral vectors by incubating cells with human cytokines for 24 hours prior to 1 or 2 days of co-culture over PG13LNc8 (NEO vector) (Miller et al., 1991) or AmmA12 55/6 (murine ADA vector) (Moritz et al., 1993) retrovirus packaging cell lines. Murine (Bodine et al., 1991) and human (Moritz et al., 1993) studies have shown that higher levels of gene transfer into stem and progenitor cells, respectively, were obtained by co-culture compared to supernatant infection. To determine whether SRC survived these *in vitro* manipulations, human CB or BM cells were transplanted into SCID or NOD/SCID mice and the extent of human cell engraftment in the murine BM was measured by DNA analysis after 30-60 days. NOD/SCID mice were examined because they have additional defects in non-adaptive immunity (Shultz et al., 1995) as compared to SCID mice and our preliminary data showed higher human cell engraftment in NOD/SCID mice when injected with equivalent cell doses (Larochelle et al., 1995). Reduced numbers of human cells were observed in the BM of both SCID and NOD/SCID mice transplanted with human cells that were co-cultured for 2 days as compared to 1 day (Figure 2-1) However, after 2 days of co-culture, the levels of engraftment were higher in NOD/SCID mice (Figure 2-1B) as compared to SCID mice (Figure 2-1A). The summary of human cell engraftment from 52 independent experiments (n=79 mice) demonstrates that SRC can be found after *in vitro* infection but there is a time-dependent loss of human SRC, probably due to differentiation and/or adherence to retrovirus packaging cells (Figure 2-1C). Quantitative analysis of human BM or CB cultured on murine packaging cells or human stroma suggest a 5 fold loss of SRC in 7 days of culture (Gan et al, in preparation). These results also indicate that NOD/SCID mice are superior recipients compared to SCID mice because fewer human SRC are required to achieve high levels of engraftment.

To determine whether SRC were adhering to the retrovirus packaging cells, the fibroblast monolayer was treated with cell dissociation buffer (CDB) (Moritz et al., 1993) to release hematopoietic cells prior to transplantation into NOD/SCID mice. Six mice transplanted with
Fig. 2-1. **Comparison of human cell engraftment** in the BM of SCID (A) versus NOD/SCID (B) mice transplanted with human hematopoietic cells that were co-cultured for 1 or 2 days with NEO or ADA retrovirus packaging cell lines in the presence of IL-3, IL-6, and stem cell factor (SCF). Following co-culture, the human CB or BM cells were transplanted into SCID or NOD/SCID mice and after 4 to 8 weeks, human cell engraftment in the BM was estimated by Southern blot. C. Summary of the DNA analysis of the BM of 79 SCID and NOD/SCID mice transplanted with cells from 52 different donors. For comparison, SCID and NOD/SCID mice injected with 10 to 20 x 10⁶ unmanipulated CB cells are also included. The horizontal line indicates the mean level of human cells.
Time of coculture (days)
only 3 to 4 x 10^6 adherent cells were engrafted to equal or higher levels (1 - 50%, mean: 27%), compared to 11 mice transplanted with 35 - 50 x 10^6 non-adherent cells (0 - 50%, mean: 15%), indicating that SRC do adhere to fibroblasts which support their maintenance for at least 2 days. This observation is consistent with the finding that murine stem cells adhere to the stromal cells and extracellular matrix of long-term marrow cultures (Williams et al., 1991; Harrison et al., 1987).

4.2 Multilineage differentiation of SRC after in vitro retroviral infection

Flow cytometry and progenitor assays were used to determine whether SRC recovered after 1 or 2 days of co-culture maintained their capacity to differentiate into multiple hematopoietic lineages after transplantation into NOD/SCID mice. The analysis of a representative mouse indicates that immature (CD34+/CD38-), myeloid (CD13+), erythroid (glycophorin A+), and B-lymphoid cells (CD19+) were present (Figure 2-2). Multilineage human hematopoiesis was seen in 12 of 12 engrafted mice analyzed; the myeloid lineage ranged from 0.2 to 12%, the B cell lineage from 0.5 to 64%, and the CD34+ cells ranged from 0 to 5.8% of the total mononuclear cells found in the bone marrow of these 12 mice. High numbers of multilineage human CFC, including BFU-E, CFU-G, CFU-M, CFU-GM and CFU-GEMM, were also found in engrafted mice (see legend to Figure 2-3 and 2-4 and data not shown). These results indicate that the SRC recovered following in vitro culture and infection repopulate NOD/SCID mice generating multiple hematopoietic lineages, similar to what was observed with SCID mice transplanted with unmanipulated CB cells (Vormoor et al., 1994).

4.3 Comparison of the efficiency of retrovirus-mediated gene transfer into SRC, CFC, and LTC-IC

The recent results of human clinical trials utilizing transplantation of genetically manipulated CB cells indicate that primitive human repopulating cells are poorly infected with retroviruses following supernatant infection (Dunbar et al., 1995; Kohn et al., 1995). Using the co-culture gene transfer method described above, we evaluated the efficiency of retroviral gene
**Fig. 2-2.** Cell surface marker analysis of a representative highly engrafted NOD/SCID mouse using flow cytometry. BM cells from a NOD/SCID mouse transplanted with a mixture of $4 \times 10^6$ adherent and $8 \times 10^6$ non-adherent ADA-transduced human CB cells were analyzed by three-color flow cytometry 6 weeks later as described. The percentage of cells with a particular cell surface phenotype in the total ungated population is given in each quadrant. A. Cells were stained with an anti-CD45-PerCP antibody and compared to cells stained with a mouse isotype IgG1-PerCP control antibody. Dead cells were excluded by their intense staining with propidium iodide (PI). The viable cells were gated according to their CD45 expression (horizontal line). This mouse (CBADA43.1) contained 58.8% CD45$^+$ human cells. B. Detection of immature CD34$^+$CD38$^-$ human cells in the BM of a transplanted NOD/SCID mouse. The CD45$^+$ gated cells were stained with anti-CD34-FITC and anti-CD38-PE antibodies. C. Detection of B cells by staining with anti-CD19-FITC antibody. D. Detection of myeloid cells by staining with anti-CD13-PE antibody. E. Detection of erythroid cells by staining with anti-glycophorin A-FITC antibody.
transfer into SRC and also systematically compared the efficiency of gene transfer into CFC and LTC-IC from the same sample. A representative experiment (CBADA28) is shown in Figure 2-3. PCR analysis of individual CFC recovered before transplantation indicated that 32% (6 of 19) were positive for the ADA vector (Figure 2-3A). In addition, LTC-IC were efficiently transduced: 48% (11 of 23) of the CFC derived from 5 week long-term cultures expressed the transferred murine ADA cDNA as determined by ADA isoenzyme assay (Figure 2-3B). High levels of human cells (20%) were found in the BM of a NOD/SCID mouse (CBADA28.2) 6 weeks after transplantation with these transduced cells. Human CFC (136 human CFC/2 x 10^5 cells) were recovered from the murine BM; however, none (0 of 30) of these colonies contained the ADA vector (Figure 2-3C, upper panel). Moreover, PCR analysis of the total DNA from the BM of this transplanted mouse (Figure 2-3C, lower panel, lane 8) indicated that only 2% of the human cells in this mouse contained the ADA vector. The results from 23 independent experiments with CB cells and 2 with BM cells are shown in Figure 2-3D. Overall, a high proportion of the human CFC (41%, range 5 to 80%; n= 25) and LTC-IC (29%, range 0 to 77%; n=12) were transduced using these methods. From the calculated frequency of CFC and LTC-IC in normal BM and CB, we estimated that at least 10^5 transduced CFC and 10^3 transduced LTC-IC were injected into each NOD/SCID mouse. However, none of the CFC (10 to 30 CFC tested per mouse) derived from the transplanted mice were transduced and <0.01% gene-marked human cells were detected in 9 of 16 highly engrafted mice (Figure 2-3C, lower panel); somewhat higher levels (≤2%) were detected in the BM of the other 7 engrafted mice. Similarly, the BM from highly engrafted mice transplanted with NEO vector infected CB cells (n=14) had undetectable or low levels (≤ 0.1%) of gene-marked human cells in their BM (data not shown).

These results demonstrate that while high numbers of CFC and LTC-IC are readily transduced, they contribute little to the repopulation of NOD/SCID mice following transplantation, indicating that most CFC and LTC-IC are biologically distinct from SRC. Moreover, these results establish that only a small proportion of SRC, if any, can be transduced with retroviral vectors using co-culture with packaging cell lines.
Fig. 2-3. Comparison of the gene transfer efficiency into human CFC, LTC-IC and SRC.

Human mononuclear CB cells (150 to 300 x 10^6) were infected with an ADA retroviral vector, collected, and the extent of gene transfer into CFC, LTC-IC, and SRC was determined. A representative experiment is shown (CBADA28). A. PCR analysis for the presence of the ADA vector in 19 individual CFC picked from methylcellulose cultures; 6 of 19 colonies (9 are shown) contained the transferred cDNA. For each colony, one half was used for the ADA PCR and the other half as a control to ensure the presence of amplifiable DNA. The control PCR was specific for the human Duchenne muscular dystrophy gene (DMD). Only colonies positive for DMD sequences were taken into account to calculate the ADA gene transfer efficiency. For each PCR, four control reactions were included: MC, methyl cellulose control; R, reagent control; +, DNA from AmmA12 55/6 cells; -, human DNA. B. ADA isoenzyme analysis for the presence of the murine ADA vector expression in 23 individual CFC (11 are shown) picked from methylcellulose cultures derived from 5 week LTC. The positive control (C) is murine peripheral blood {2121}. The murine ADA enzyme was expressed in 48% (11 of 23) of the human CFC and by definition in the LTC-IC that initiated the LTC. C. PCR analysis for the presence of the ADA vector in 30 individual CFC (10 are shown, upper panel) derived from a NOD/SCID mouse (CBADA28.2) 6 weeks after transplantation with transduced CB cells. This mouse contained a total of 6252 BFU-E, 8082 CFU-G, 26382 CFU-M, 152 CFU-GM progenitors in the humeri, femurs, and tibias. None of the 30 colonies tested were positive for the transferred gene. PCR analysis for the presence of the transduced ADA vector in the BM of 16 mice transplanted with 15 different donor samples (lower panel). Mouse CBADA28.2 corresponds to lane 8. Lane 15 and 16 are mice transplanted with BM cells. D. Summary of the efficiency of ADA gene transfer into human CFC and LTC-IC present in the donor inoculum, and in the CFC and total human cells derived from transplanted NOD/SCID mice. The results from 25 independent experiments are presented. The efficiency of gene transfer into total human cells present in mouse BM was quantified and the horizontal line indicates the mean gene transfer efficiency.
a) CFC from 5 week stromal cultures

b) CFC from 5 week stromal cultures

c) Human CFC derived from NOD/SCID mice

BM from NOD/SCID mice ADA dilutions (%)

- 299 bp
- 230 bp
- 299 bp
- 423 bp
I CFC cells

Derived from NOD/SCID mice

Gene transfer efficiency (%)

CFC  LTC-IC  Human CFC  Total human cells

Derived from NOD/SCID mice
4.4 Gene transfer into SRC

In four subsequent experiments, the number of SRC co-cultured with retrovirus packaging cells and transplanted per recipient mouse was increased 3 to 5 times to determine whether transplantation of more transduced SRC would result in the detection of marked CFC derived from NOD/SCID mice. In the experiment shown in Figure 2-4, high levels of gene transfer were obtained into CFC [18 of 19 colonies positive by PCR (Figure 4A); 20 of 20 by ADA isoenzyme assay (not shown)] and LTC-IC [23 of 23 by ADA isoenzyme assay (Figure 2-4B)]. Interestingly, 65% of human CFC derived from the transplanted NOD/SCID mouse contained the ADA vector (Figure 2-4C, upper panel). PCR analysis of the total BM from this mouse (43.1) showed that ~17% of the human cells present in the murine BM contained transduced sequences (Figure 2-4C, lower panel). In 2 of 3 other experiments where mice were transplanted with large numbers of SRC, transduced human CFC and gene-marked cells were also detected in the murine BM, although at lower levels (Figure 2-4D). The presence of high numbers of transduced CFC and the 10 to 200 fold higher level of gene-marked human cells in mouse 43.1, compared to the mice described in Figure 2-3, make it unlikely that these gene-marked cells arose simply as a consequence of the transplantation and engraftment of 3 to 5 times more transduced cells. Rather, these data suggest that this mouse was engrafted with one or more transduced SRC with extensive proliferative capacity that produced large numbers of gene-marked CFC and mature cells. Inverse PCR was used to determine the number of transduced clones that were present in the BM of mouse 43.1 (Figure 2-4E) (Ochman et al., 1988). Three different integration sites were detected, indicating this mouse was indeed repopulated with only 1 to 3 transduced SRC (1 SRC could have more than 1 integration site).

4.5 Characterization of the cell surface phenotype of SRC

Since the gene marking experiments indicated that the SRC is a primitive and novel cell type, we characterized the cell surface phenotype of SRC by sorting human CB and BM cells into various fractions based on surface expression of CD34 and CD38 antigens and determining which fraction was able to repopulate NOD/SCID mice. CD34 is a cell-surface marker normally
Fig.2-4. Comparison of the efficiency of gene transfer into human CFC, LTC-IC and SRC when large numbers of CB cells (300 to 500 x 10^6) were co-cultured with retrovirus packaging cells and transplanted per mouse. A representative experiment is shown (CBADA43). The experimental details are similar to Figure 3 except that after infection, the total number of adherent cells were transplanted into one mouse. A. PCR analysis for the presence of the ADA vector in 19 individual CFC (10 are shown) picked from methylcellulose cultures; 18 of 19 colonies (95%) contained the vector. B. ADA isoenzyme analysis for the presence of the murine ADA vector expression in 20 individual CFC (11 are shown) picked from methylcellulose cultures derived from 5 week stromal cultures. The murine ADA enzyme was expressed in 20 of 20 (100%) of the CFC derived from the LTC. C, murine peripheral blood. T, peripheral blood from a transgenic mouse expressing the human ADA gene. C. PCR analysis for the presence of the ADA vector in 20 individual CFC (10 are shown, upper panel) derived from a NOD/SCID mouse 6 weeks after transplantation with transduced CB cells (mouse CBADA43.1); 65% of the colonies tested (13 of 20) were positive for the transferred gene. This mouse contained 60359 BFU-E, 21653 CFU-G, 12654 CFU-M, 947 CFU-GM, 203 CFU-GEMM human progenitors in all the humeri, femurs, and tibias. PCR analysis for the presence of the transduced ADA vector in the BM of 4 mice transplanted with 4 different donor samples (lower panel). D. Summary of the efficiency of ADA gene transfer into human CFC, LTC-IC, and CFC and total human cells derived from transplanted NOD/SCID mice. The results from 4 independent experiments are presented. The level of human cell engraftment for each mouse was: CBADA41.1, 40% (●); CBADA42.1, 1% (○); CBADA43.1, 60% (●); CBADA44.1, 30% (□). (E) Inverse PCR analysis of the retrovirus integration sites present in the BM of mouse 43.1. The stars indicate unique retrovirus integration sites within genomic DNA adjacent to the 5' LTR present in the gene marked cells from the BM of mouse 43.1. Lane 1-reagent control, lane 2-mouse DNA, lane 3-human DNA, lane 4 -mouse mADA retrovirus packaging cell line DNA, lane 5-BM DNA from mouse 43.1 The 183bp band is derived from the internal retroviral sequences adjacent to the 3' LTR.
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- **299 bp**
- **230 bp**

### b

- **HuADA**
- **MuADA**

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- **299 bp**
- **230 bp**

- BM from NOD/SCID mice

- ADA dilutions (%)

- **R**

- **299 bp**

- **423 bp**
d

Gene transfer efficiency (%)

< 1  
1  
10  
100

CFC   LTC-IC  Human CFC  Total human
       cells

Derived from NOD/SCID mice


e

M  1  2  3  4  5

183 bp
expressed on a small population of bone marrow cells (≈1.0%) (Terstappen et al., 1991; Civin et al., 1984) that includes progenitor cells and human repopulating cells. Expression of CD38 on CD34+ cells is an important marker for lineage commitment and the phenotype CD34+CD38- (~0.01% of total BM) appears to define an immature cell in normal bone marrow (Terstappen et al., 1991). CB cells were first separated into CD34+ and CD34- fractions and transplanted into NOD/SCID mice. High levels of human cells (Figure 2-5A) and progenitors (data not shown) were observed in the BM of mice transplanted with as few as 5 x 10^4 CD34+ cells, comparable to levels seen in mice transplanted with unseparated CB cells. In contrast, mice transplanted with 2.6 x 10^6 CD34- cells were not engrafted. CD34+ CB cells were further purified based on CD38 expression and transplanted into mice. As few as 500 CD34+CD38- cells were sufficient to engraft NOD/SCID mice, while 10^5 CD34+CD38+ cells did not result in detectable engraftment (Figure 2-5B). These results demonstrate that SRC are exclusively CD34+CD38+, in contrast to CFC and LTC-IC which are also found in the CD34+CD38+ fraction (Craig et al., 1993; Huang and Terstappen, 1994; Terstappen et al., 1991; Rusten et al., 1994; Hao et al., 1995). The purified SRC were able to repopulate mice with multiple hematopoietic lineages similar to that shown in Figure 2-2 (Bhatia et al, in preparation). These cell fractionation results are consistent with the gene marking studies and provide further independent evidence that SRC are distinct from most of these in vitro progenitors. Since the frequency of SRC in CB is 1 per 8 x 10^5 mononuclear cells (Wang and Dick, in preparation), and there is at least 1 SRC in 500 CD34+CD38- cells, SRC were enriched by 1600 fold in the CD34+CD38- cell fraction.

4.6 Evaluation of gene transfer into cells enriched for SRC using the CH-296 fibronectin fragment

Since co-culture over retrovirus packaging cell lines is unacceptable for human clinical trials, we have evaluated the efficiency of gene transfer into SRC using supernatant infection methods. We have recently demonstrated an increased transduction of murine reconstituting hematopoietic stem cells, CFC, and LTC-IC, utilizing retroviral supernatant infection in combination with the carboxy-terminal fragment of fibronectin (CH-296) (Kimizuka et al., 1991)
Fig 2-5. Engraftment of NOD/SCID mice with human cord blood cells fractionated according to CD34 and CD38 expression A. Mice were transplanted with the indicated number of purified (CD34+ or CD34−, 70% and 100% pure, respectively) or unsorted cord blood cells and, 4 weeks later, human cell engraftment was estimated by Southern blot. B. Cells fractionated into CD34+CD38− (98% pure) and CD34+CD38+ (98% pure) were transplanted into NOD/SCID mice at the indicated cell numbers with (*) or without 10^5 CD34− cells.
as compared to supernatant alone (Moritz et al., 1994). This increase appears to be due, at least in part, to direct viral adhesion to CH296 and co-localization of viruses and target cells. We initiated several experiments to evaluate the survival of SRC using this method and to determine whether the SRC could be transduced (Table 1). Human BM or CB cells were enriched for both CD34+ and CD34+CD38- cells (Lin-) by negative selection using a cocktail of antibodies directed against lineage antigens using the StemSep device (Roath et al., 1994). The Lin- cells were prestimulated for 24 hours with the same human cytokines used for co-culture followed by incubation on CH296 with virus supernatant for a further 48 hours. The level of human cell engraftment in NOD/SCID mice (BMADA3, CBADA50-52) was consistently much higher after in vitro infection on CH296 suggesting that more SRC survived this treatment than co-culture on packaging cell lines. Similar levels of gene transfer were detected in human CFC with CH296 (42%, n=4) compared to co-culture. However, similar to the data obtained by co-culture on retrovirus packaging cell lines, no gene-marked human CFC were recovered from these highly engrafted mice and only low levels (mean 1.4%, n=3) of transduced human cells were found in the murine BM (Table 1). In additional experiments (CBADA54-57), we evaluated the effect of increased duration of prestimulation and changes in cytokine combinations. Removal of cytokine stimulation resulted in lower transduction of CFC, while increased duration of prestimulation and/or culture with CH296 generally resulted in higher levels of gene transfer into CFC. However, similar to our observations with co-culture, there was a rapid loss of SRC if cells were cultured for more than 3 days. These experiments demonstrate the usefulness of the SRC assay in the optimization of gene transfer methods, allowing evaluation of both gene transfer efficiency and survival of primitive cells.
Table 2-1 Efficiency of gene transfer into human hematopoietic cells after in vitro infection on the fibronectin fragment CH296

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For each experiment, one NOD/SCID mouse was transplanted with 0.07-15 X 10^6 Lin+ bone marrow (BM) or cord blood (CB) cells infected on CH296 with retroviral supernatant (murine ADA vector). CD34+ cells averaged 0.5 ± 0.3% in CB and 2% in BM prior to purification and increased to an average of 58 ± 14% in the lin+ fraction. CD34+CD38- cells averaged 0.1 ± 0.1% in CB and 0.15% in BM prior to purification and increased to an average of 13 ± 8% in the lin+ fraction.

* 10-20 colonies were tested. N.A. Not available.
5. Discussion

5.1 SRC are primitive hematopoietic cells biologically distinct from most CFC and LTC-IC

The differential susceptibility to retroviral infection of SRC and CFC/LTC-IC, the inability of gene-marked CFC and LTC-IC to engraft NOD/SCID mice, and the purification of SRC based on surface marker expression reported here identify SRC as a novel primitive cell type and provide new insights into the organization and developmental program of the human hematopoietic system. The NOD/SCID mice were injected with large numbers of transduced CFC and LTC-IC (up to 80% and 70% respectively), yet these gene-marked progenitors and their progeny did not contribute significantly to the human graft, indicating that most CFC and LTC-IC are incapable of engrafting NOD/SCID mice. Since all these mice contained high numbers of unmarked human progenitors and mature cells of multiple lineages, the graft must have been initiated from unmarked SRC. It is also possible that the unmarked LTC-IC (<30%) had different properties than the marked LTC-IC and also participated in engraftment. Most mice shown in Figure 2-3 did not contain any gene marked cells. In the remaining mice small numbers of marked cells were detectable by PCR in the murine BM and yet no marked CFC were detected. It is probable that these gene marked cells, derived from a small number of transduced SRC, were diluted by the progeny of co-transplanted non-transduced SRC, resulting in small proportions of marked CFC that were below our limit of detection (10 to 30 colonies tested per mouse). Alternatively, the gene-marked cells may be derived from a small number of transduced CFC or LTC-IC that produced limited numbers of differentiated progeny but that were no longer able to produce CFC.

The cell surface characterization of SRC provides additional independent support that SRC are distinct from most LTC-IC and CFC. The SRC were found exclusively in the CD34^+ CD38^- fraction after cell purification. By contrast, CFC are highly enriched in the CD34^+ CD38^+ fraction and a large number of LTC-IC are also present in this fraction. Although 5 week stromal LTC-IC are approximately 30-fold enriched in the CD34^+ CD38^- fraction
(frequency of 1 in 20) compared to the CD34+CD38+ fraction (data not shown and Craig et al., 1993; Huang and Terstappen, 1994; Terstappen et al., 1991; Rusten et al., 1994; Hao et al., 1995), we transplanted ~100 times more CD34+CD38+ cells into mice compared to CD34+CD38- cells. Hence the mice transplanted with CD34+CD38+ cells received very large numbers of LTC-IC and yet were not engrafted confirming that SRC are distinct from CD34+CD38+ LTC-IC. These cell fractionation experiments together with the gene marking indicate that the LTC-IC assay detects a heterogeneous population of cells. In vitro experiments also suggest that the LTC-IC assay detects a heterogeneous population of cells and those with the highest proliferative capacity (Extended-LTC-IC, ELTC-IC) persist after longer times in culture and are from the CD34+CD38- fraction (Hao et al., 1995). Therefore, it is possible that some primitive LTC-IC in the CD34+CD38- fraction are unable to be infected with a retrovirus and may have SRC activity; in other words the same cell could read out in both assays. The results presented here, together with quantitative analysis recently performed on CD34+CD38- cells (Bhatia et al, in preparation), demonstrate that SRC are enriched by at least 1600 fold (frequency of 1 in 500) in the CD34+CD38- fraction. Hence this assay can be used to develop better cell purification methods to obtain more homogenous cell populations. The identification of SRC as biologically distinct from most CFC and LTC-IC is also supported by kinetic experiments we recently performed, where only 0.1\% of the total injected number of CFC and LTC-IC were detected in the BM of NOD/SCID mice 2 days after transplantation; over the next 4 weeks, their number increased by 100 to 1000 fold, implying their production from a more primitive cell (Cashman et al., 1994).

Comparison of the level of gene marked cells and CFC in the mice shown in Figure 2-3 with mouse 43.1 (Figure 2-4D and Figure 2-4E) suggests that there may be heterogeneity in the proliferative capacity of SRC. Mouse 43.1 was repopulated with 1 to 3 transduced SRC that were able to proliferate to a much greater extent, producing 65\% marked CFC and 17\% marked BM cells. In contrast, the transduced SRC detected in the mice shown in Figure 2-3 produced no marked CFC and only 0.1 to 2\% marked BM cells. Taken together, these results establish that
SRC represent a novel cell population with extensive proliferative and differentiative potential and are the most primitive human hematopoietic cells detected to date. The SRC assay can be used now in the development of purification strategies enabling comparison with human leukemic stem cells identified using similar approaches (Lapidot et al., 1994; Sirard et al., 1996). The pure populations can be used in cellular and molecular studies to identify the specific cytokines and genes that regulate their developmental program using recently developed methods to create cDNA libraries from small cell numbers (Brady et al., 1995).

5.2 Implications for gene therapy

The two most important conclusions of our study for gene therapy are that primitive SRC can be infected with retrovirus vectors but that the transduction efficiency is very low. If we assume that the mice which contained some gene marked human cells shown in Figure 2-3D were engrafted with ~1 transduced SRC, it is possible to calculate the efficiency of gene transfer by using estimates of the total number of SRC that were transplanted into each mouse. Limiting dilution experiments suggest that SRC are present in CB cells at a frequency of 1 SRC per 8.5 x 10^5 cells (Wang and Dick, in preparation). By assuming that all SRC were recovered from the adherent fraction and engraft the mice, we calculate that 55 to 150 SRC were transplanted into each mouse shown in Figure 2-3. This is likely an overestimate because some SRC are lost during co-culture (Figure 2-1). Nevertheless, by this rationale approximately 0.7 to 2% (1 of 150 to 1 of 55) SRC were transduced. Moreover, similarly low efficiency of SRC transduction also was observed during the supernatant infection of Lin^- cells using CH296, despite better in vitro survival of SRC and the higher ratio of virus to target cells due to enrichment of SRC.

The low efficiency of gene transfer into SRC that we observed, contrasts with the high transduction efficiencies obtained into progenitors detected by in vitro assays (Moritz et al., 1994; Hughes et al., 1989), and is consistent with the low level of gene marking reported in primates (Bodine et al., 1993), in dogs (Schuening et al., 1991), and in human clinical trials (Dunbar et al., 1995; Kohn et al., 1995; Deisseroth et al., 1994; Brenner et al., 1993), providing further evidence that SRC are related to the human repopulating stem cell. The human trials have
shown that the level of detection of gene marked cells in the blood is very low <0.1% although the levels of marked CFC can be as high as 20%. The gene transfer data we report here are also somewhat different from those reported earlier using the bg/nu/xid transplant model where a significant percentage of gene-marked human progenitors were detected in transplanted mice (Dick et al., 1991; Nolta et al., 1994), although the total number of gene marked cells present in the BM was very low. It is somewhat difficult to compare human cell engraftment in these two animal models since the engrafting cells undergo much greater proliferation in NOD/SCID mice producing higher numbers of all lineages of human cells compared to bg/nu/xid mice. Hence, it is possible that different types of progenitors can engraft these different strains of mice. In an elegant study, Nolta et al have recently shown that the bg/nu/xid mice can be engrafted rarely with a transduced pluripotent stem cell with capacity to produce T-lymphoid and myeloid cells, although more commonly these mice contain transduced CFC that persist long-term (Nolta et al., 1996). Interestingly, the transduction efficiency of the pluripotent stem cell they observed was very low, consistent with the data we report here for SRC.

The NOD/SCID model should make it possible to investigate both the mechanisms underlying poor retroviral infection of primitive human hematopoietic cells and the conditions that permit maintenance of these cells during in vitro culture, allowing optimization of infection conditions prior to proceeding to human clinical trials. For example, the absence of appropriate viral receptors or of culture conditions that stimulate the proliferation of primitive cells are two possible mechanisms for poor retroviral infection that can now be directly tested. Our data demonstrate that the use of co-culture infection, especially beyond 3 days (unpublished), may be deleterious to the engraftment potential of SRC. Moreover, we show that co-culture on packaging cells can be effectively replaced with the use of CH296 infection protocols permitting better maintenance of SRC. We are currently examining optimization of infection of SRC from cord blood, mobilized peripheral blood, and bone marrow using recombinant CH296. Since animal models for human hematopoietic genetic diseases can be created by transplantation of human cells into NOD/SCID mice (Larochele et al., 1995), the means are now available to
assess the efficiency of gene transfer into primitive target cells, the longevity and lineage specificity of gene expression, and the correction of cellular defects.
6. References


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THIRD CHAPTER

ENGRAFTMENT OF IMMUNE-DEFICIENT MICE WITH PRIMITIVE HEMATOPOIETIC CELLS FROM β-THALASSEMIA AND SICKLE CELL ANEMIA PATIENTS:
IMPLICATIONS FOR EVALUATING HUMAN GENE THERAPY PROTOCOLS


Figure 3-5 was done by TF
Figure 3-6 (a-f) was done by QL
1. Abstract

Permanent correction of genetic deficiencies of the hematopoietic system requires gene transfer into stem cells and long-term lineage specific expression after autologous transplantation. However, progress to develop gene therapy protocols has been hampered by the absence of in vivo assays that detect genetically deficient human hematopoietic stem cells and their diseased differentiated progeny. The establishment of systems to transplant human cells into immune-deficient SCID mice provides such an assay. We report that primitive bone marrow cells from β-thalassemia major and sickle cell anemia patients engraft immune-deficient mice, giving rise to high levels of human erythroid and myeloid cells in response to treatment with human cytokines. The bone marrow of transplanted mice contained the entire erythroid lineage from BFU-E to mature erythrocytes expressing human γ, β or β5-globin. Moreover, human erythroid cells from mice transplanted with sickle cell anemia bone marrow showed characteristic sickling under reducing conditions in an in vitro assay. This model provides a powerful in vivo system that can be used to evaluate the efficiency of globin gene transfer into primitive human hematopoietic cells, lineage-specific expression in mature erythrocytes, and ultimately correction of the cellular defect found in the erythroid lineage.
2. Introduction

The ability to introduce new genes into hematopoietic cells provides a novel approach to correct human genetic diseases (Morgan and Anderson, 1993). To achieve permanent correction, the target for gene transfer must be the pluripotent stem cell because mature cells and committed progenitors do not have the proliferative capacity to reconstitute the entire hematopoietic system. Most studies, over the past 10 years, have used highly infectious retrovirus vectors to introduce genes into stem cells (Mulligan, 1993). Methods for retrovirus-mediated gene transfer were first developed using mouse bone marrow because of the availability of repopulation assays for pluripotent stem cells. These studies showed that retroviruses could efficiently transduce pluripotent stem cells that completely reconstituted the entire hematopoietic system of the mouse (Dick et al., 1985). Surprisingly, the same methods of retrovirus infection were much less efficient for canine (Carter et al., 1992; Schuening et al., 1991) or primate (Bodine et al., 1993; vanBeusechem et al., 1992) stem cells where the level of genetically marked cells after reconstitution were generally lower than 1%. The early experience in human clinical trials (Kohn et al., 1995) also suggests that gene transfer into normal human stem cells is inefficient. Therefore, significant species differences exist in the efficiency of retrovirus infection, pointing out the importance of extensive pre-clinical evaluation of vectors and conditions specific for human stem cell gene transfer.

The most significant difficulty in developing and evaluating gene transfer protocols for human cells is the lack of a relevant in vivo re-population assay for human hematopoietic stem cells from patients with genetic deficiencies. Stem cells can only be assayed by their ability to proliferate and differentiate into all blood lineages after transplantation into another recipient (Till and McCulloch, 1980; Phillips, 1991). In vitro cultures are not capable of measuring human stem cell activity. Most of the currently employed gene transfer methods used in the human clinical trials have been optimized using the human in vitro progenitor and LTC-IC assays (Moritz et al., 1993), however stem cells do not share the same biological properties (e.g. proliferative and self-renewal capacity, response to growth factors, cell surface markers, etc.) as progenitors (Till and McCulloch, 1980; Phillips, 1991) so these methods may have little relevance for stem cell gene transfer.
Recently, progress has been made to develop an *in vivo* re-population assay for normal human stem cells by transplanting normal human bone marrow or cord blood cells into severe combined immune-deficient (SCID) or beige/nude/xid mice (Vormoor et al., 1994; Lapidot et al., 1992; Kamel-Reid and Dick, 1988; McCune et al., 1988; Nolta et al., 1994; Kyoizumi et al., 1992; Kollmann et al., 1994). High levels of committed and multilineage myelo-erythroid progenitors, as well as mature human myeloid, erythroid, and B cells develop in the murine bone marrow and are maintained for at least 4 months. We have recently found that the human 'SCID-repopulating-cell' (SRC) that initiates the graft is very primitive and kinetic experiments suggest these cells are earlier in ontogeny than colony-forming progenitors (Lapidot et al., 1992; Cashman et al., 1994). Although the exact relationship of the SRC with the pluripotent human stem cell is unknown, this transplantation system provides a powerful tool to characterize primitive human cells and to create animal models of human hematopoietic diseases. One major application of this technology is the creation of animal models that reproduce human leukemic diseases (Lapidot et al., 1994; Kamel-Reid et al., 1989). A new class of primitive leukemic stem cells was identified and characterized based on their ability to initiate leukemia after transplantation (Lapidot et al., 1994).

We report here a further extension of this technology by creating an animal model for human genetic diseases. β-thalassemia and sickle cell anemia (SCA) are two common human genetic disorders of the β-globin gene that present special problems for gene therapy. The newly introduced globin genes must be introduced into stem cells, expressed only in erythroid cells and be precisely regulated in order to produce a functional hemoglobin molecule. An animal model was created by transplanting bone marrow cells from human β-thalassemia and SCA patients into immune-deficient mice. Primitive cells engrafted the murine bone marrow microenvironment and, in response to human cytokines, differentiated into multiple lineages including mature erythroid cells. This *in vivo* system provides a novel tool to evaluate gene therapy vectors and methods targeted to the correction of human stem cells from hemoglobinopathy patients.
3. Materials and methods

3.1 Bone marrow sample preparation

Bone marrow aspirates were obtained from 28 well-characterized patients diagnosed with β-thalassemia major (homozygous β0-thalassemia or compound heterozygous Hb β0/β+) or homozygous HbSS disease. Collection of samples was performed at the Hospital for Sick Children (Toronto, Canada). Written informed consent was obtained from each patient or a parent. Low-density leukocytes were collected after separation on Ficoll/Hypaque density gradients (Pharmacia, Piscataway, NJ). The ficoll-purified cells were washed twice in Iscove's modified Dulbecco's medium (IMDM; Gibco BRL, Burlington, Canada), containing 10% fetal calf serum (FCS; Sigma, St Louis, MO).

3.2 Transplantation of bone marrow cells into immune-deficient mice

The C.B17-scid/scid (SCID) mice and NOD/LtSz-scid/scid (NOD/SCID) mice were bred and maintained under sterile conditions at the Ontario Cancer Institute (Toronto, Canada). NOD/SCID mice were created by LDS by backcrossing the scid mutation ten generations onto the NOD/LtSz background. In accordance with our previously described transplantation protocol (Lapidot et al., 1992), the mice (6-8 weeks of age) were sublethally irradiated with 400 cGy from a 137Cs source immediately before intravenous infusion of 4-10 x 10^7 ficoll-purified bone marrow cells in 0.5-1.0ml IMDM medium with 10% FCS. Cytokines (PIXY321, 6.8μg/mouse [Immunex Corp., Seattle, WA] and huEPO, 20U/mouse [Ortho BioTech, Don Mills, Ontario]) were administered by intraperitoneal injection on alternate days. A few mice additionally received mast cell growth factor (huMGF, 10μg/mouse; Immunex Corp.).

3.3 DNA extraction and analysis

High molecular weight DNA was isolated from the bone marrow of transplanted mice and blotted using standard procedures. EcoRI digests of genomic DNA (1μg) were loaded into each lane and the blot was hybridized with a human chromosome 17-specific α-satellite probe (p17H8) (Waye and Willard, 1986). The proportion of human cell engraftment in the murine bone marrow was quantified by comparing the intensity of the characteristic 2.7kb band from the
samples to control human/mouse mixtures (0, 0.1, 1, 10 and 50% human DNA) (Kamel-Reid et al., 1989).

3.4 Hematopoietic progenitor cell assay

Human hematopoietic progenitors were assayed in culture conditions that were selective for human cells as previously described (Lapidot et al., 1992). Briefly, 2 x 10^5 bone marrow cells from transplanted mice were plated in 0.9% methylcellulose, 15% FCS, 15% human plasma, 5 x 10^{-5}M β-mercaptoethanol, 50ng/ml huMGF, 5ng/ml PIXY321, 10U/ml huIL3 (Immunex Corp.), 9U/ml huGM-CSF (Immunex Corp.) and 2U/ml huEPO. The cultures were incubated for 10-14 days at 37°C. Burst-forming-unit-erythroid (BFU-E), colony-forming-unit-granulocyte (CFU-G), macrophage (CFU-M), granulocyte/macrophage (CFU-GM) and CFU-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) were identified and scored on the basis of morphological criteria. Duplicate plates were scored for each mouse.

3.5 Flow cytometry

Three-color flow cytometry was performed on a FACScan (Becton Dickinson, Mountain View, CA) as previously described (Vormoor et al., 1994). Briefly, bone marrow cells from engrafted mice were subjected to erythrocyte lysis, washed in phosphate-buffered saline (PBS) and 1% BSA and incubated on ice for 30 minutes in presence of saturating amounts of human specific monoclonal antibodies. A PerCP-conjugated monoclonal antibody directed against the cell surface marker CD45 (HLe1, Becton Dickinson) was used for identifying the human cells. Cells of the erythroid lineage were detected by staining with anti-glycophorin A-FITC (glyco A; Amac Inc., Westbrook, ME) and human immature cells were detected by staining with anti-CD34-FITC (HPCA-1, Becton Dickinson) and anti-CD38-PE (Leu-17, Becton Dickinson). Bone marrow cells from the original patients and non-transplanted mice were stained with the same antibodies as positive and negative controls, respectively. IgG1 isotype controls conjugated to PE, FITC and PerCP were also included.

Data acquisition and analysis were performed using LYSIS II-software (Becton Dickinson). Dead cells were gated out by their intense staining with propidium iodide (PI).
3.6 RNA extraction and analysis

Following described protocols (Karlinsey et al., 1989), total RNA was prepared from engrafted mouse bone marrow and peripheral blood, as well as from erythroid colonies grown in vitro. Human globin chain expression was independently analyzed by RT-PCR (Furukawa et al., 1994) and RNase protection assay (Stamatoyannopoulos et al., 1993). Briefly, RT-PCR analyses were performed on total RNA that was reverse transcribed by M-MLV reverse transcriptase using random hexamers. An aliquot was submitted to 40-cycle amplification by PCR. γ- and β-globin cDNA were coamplified by a γβ primer set which is homologous to the γ- and β-globin cDNA, but also includes 1, 2 or 4-bp mismatches with the human δ-globin, mouse globin and human ε-globin cDNA, respectively. Human δ-globin and mouse globin cDNA should amplify, though less efficiently than β- and γ-globin. Distinction between the different globin cDNA can be achieved by restriction enzyme digestion of the 263 bp amplified product with BstEII, which is specific for human γ-globin cDNA, and DraIII, which specifically cuts the human β-globin cDNA, generating restriction fragments of 222/41 bp and 154/109 bp, respectively. For each RNase protection reaction, 50-2000ng of RNA was hybridized overnight at 50°C in presence of mouse α-, human β-/βS- and human γ-globin-specific probes, and then treated with RNase at room temperature for 40 minutes. The protected fragments were then separated on 6% polyacrylamide-8M urea gels and autoradiographed without an intensifying screen.

3.7 Analysis of globin chain production

Single-cell suspensions of bone marrow, peripheral blood and erythroid colonies from transplanted mice were used to prepare cytological smears for immunofluorescent labeling with human anti-β- or βS- and anti-γ-globin chain antibodies (Stamatoyannopoulos et al., 1983). HbS production was also determined in engrafted mice using a functional sickling test. To hasten deoxygenation and polymerization of HbS, 0.5-1 x 10⁶ bone marrow cells or 20-30 pooled erythroid colonies were washed in PBS and treated at room temperature, in an oxygen-free environment, with an equal volume of 2% sodium metabisulfite (Sigma). Sickled cells could be detected after 30-60 minutes. Cells from untransplanted mice and non-SCA patients did not sickle under these conditions.
4. Results

4.1 Primitive cells from β-thalassemia and SCA patients engraft and proliferate in immune-deficient mice

Bone marrow cells from patients with β-thalassemia major or SCA were injected into the tail vein of sublethally irradiated SCID mice using our previously described protocol for the transplantation of normal human bone marrow cells (Lapidot et al., 1992). In three latter experiments, NOD/SCID mice were used as recipients. These mice have multiple defects in innate immunity (Shultz et al., 1995) and our preliminary experiments indicated that they were better recipients for human bone marrow transplants than SCID mice (unpublished data). The transplanted animals were treated every other day with human recombinant cytokines (PIXY 321 and huEPO, except as noted). One to four months post-transplantation, mice were euthanized and the extent of human cell engraftment was determined by morphologic and DNA analysis of the mouse marrow. Figure 1A shows the levels of human cell engraftment in a group of immune-deficient mice transplanted with bone marrow from two different β-thalassemia patients and two SCA donors. These results are representative of 24 patient samples obtained from well-characterized β-thalassemia major or SCA patients. The bone marrow from all of these mice was engrafted with human cells and there was a great degree of consistency amongst different mice transplanted with the same donor. Overall, seventy percent of the transplanted mice (n=66) contained high levels of human cells (5-60%; mean 18%) in the bone marrow (Figure 1B). Higher levels of human cells were seen in NOD/SCID mice compared to SCID mice.

Flow cytometry was carried out on the bone marrow from highly engrafted NOD/SCID mice to determine if immature cells had engrafted the mice. The cells were stained with anti-CD34 monoclonal antibodies. CD34 is a cell surface marker that is normally expressed on approximately 1% of human bone marrow cells; transplantation experiments have confirmed that human stem cells and progenitor cells express this marker. All four mice analyzed contained between 1.5-5% CD34+ cells (Figure 2, one mouse shown). CD38 is a cell surface marker whose expression increases with differentiation, therefore the CD34+CD38− fraction represents cells with a more immature phenotype (Terstappen et al., 1991). A small population of primitive
Fig.3-1. DNA analysis of the bone marrow of immune-deficient mice transplanted with hematopoietic cells from patients with hemoglobinopathies. A. A representative Southern analysis of mice transplanted with 4-10 x 10^7 bone marrow cells from patients diagnosed as β-thalassemia major (Thal-1 or Thal-6) or homozygous HbSS disease (SCA-19 or SCA-27). Each lane contains 1 µg of DNA from the bone marrow of individual SCID (Thal-1, Thal-6, SCA-19) or NOD/SCID (SCA-27) mice transplanted 4-8 weeks earlier. Mice were treated with human cytokines PIXY 321 and huEPO (mice in lanes 1, 3, 13 and 14 were additionally treated with huMGF). As outlined in the Methodology, the extent of human cell engraftment was determined by comparing the intensity of the 2.7kb α–satellite band in the sample lanes with the control human/mouse DNA mixtures. B. Summary of human cell engraftment. Data are presented from mice transplanted with cells from 24 donors including 16 β-thalassemia and 8 SCA patients, 4 other donors did not engraft the mice and were not included. The extent of human cell engraftment for each SCID recipient (empty symbol) and NOD/SCID recipient (filled symbol) was determined by DNA analysis (○) or by histology analysis of Wright stained slides (○). The horizontal lines specify the mean level of engraftment found in (n) mice examined 4-8 weeks after transplantation.
**Fig. 3-2.** Detection of immature human cells by flow cytometry of the bone marrow of a NOD/SCID mouse transplanted with human hematopoietic cells. Three-color flow cytometry analysis was performed on the bone marrow from a representative highly engrafted mouse (SCA 27.1) transplanted with SCA cells, 4 weeks post-transplantation (left panel) and an untransplanted mouse (right panel). Dead cells were excluded by their intense staining with PI. Human CD45$^+$ cells were gated as shown by the horizontal line on the histogram (top panel). This mouse contained 42% CD45$^+$ human cells. Human immature cells were identified by staining with anti-CD34-FITC and anti-CD38-PE monoclonal antibodies, immature cells are CD34$^+$CD38$^-$. The percentage given in each quadrant represents the percentage of cells in the total ungated population (10000 to 30000 events).
human CD34+CD38- cells were present in the murine bone marrow of one of these mice 4 weeks after transplantation of β-thalassemia cells (Figure 2).

4.2 Human erythroid cells develop in engrafted mice

To determine if human committed and multipotential progenitors were present in the bone marrow from the engrafted mice, mononuclear cells were plated in methylcellulose cultures that specifically supported the growth of human progenitors. Mice transplanted with cells from both β-thalassemia and SCA patients contained an average of 150 progenitors per $2 \times 10^5$ cells plated (Figure 3A). All of the myelo-erythroid committed progenitors were detected, including committed erythroid (BFU-E) (Figure 3B) and myeloid cells (CFU-G, CFU-M and CFU-GM), as well as multipotential CFU-GEMM (Figure 3C). BFU-E comprised approximately 10% of the total human progenitors present in the engrafted mice. Many of the BFU-E yielded large macroscopic colonies composed of several small clusters typical of immature erythroid progenitors (Figure 3B) (Eaves and Eaves, 1984). The continued presence of committed and multipotential progenitors for at least two to four months post-engraftment provides additional evidence that immature human cells are maintaining the progenitor pool in the engrafted mice.

Flow cytometry was carried out on the bone marrow of highly engrafted mice to determine whether more mature human erythroid cells had developed from the committed erythroid progenitors (Figure 4). Cells were stained with the cell surface markers glycophorin A (glyco A) and CD45. Glyco A appears at the proerythroblast stage of differentiation and its expression remains at high and constant levels to the mature erythrocyte stage (Loken et al., 1987). In contrast, the CD45 antigen is gradually lost during red cell maturation (Loken et al., 1987). A large population of strongly glyco A+ cells, absent in control mice, was identified in the engrafted mice similar to the bone marrow of the original donor (Figure 4, top panel). Double labeling of the glyco A+ gated cells with CD45 showed the presence of both glyco A+CD45+ and more mature glyco A+CD45- cells, indicating that the mice exhibited a similar maturational process of erythroid development as the donor, although the mice had a higher proportion of immature erythroid cells (Figure 4, lower panel).
Fig. 3-3. Detection of human progenitors in the bone marrow of immune-deficient mice transplanted with human hematopoietic cells. A. Mean number of human progenitors (±SE) detected in the bone marrow of 44 mice transplanted with β-thalassemia (■) and SCA (▲) cells that were engrafted with ≥5% human cells. Cells (2 × 10^5) were plated in methylcellulose cultures that were selective for the growth of human colonies, as described in Methodology. B. A large macroscopic human erythroid colony (BFU-E) showing small clusters typical of primitive erythroid progenitors. C. A multilineage colony (CFU-GEMM) after 14 days of culture.
Fig. 3–4. Differentiation of human erythroid cells in the bone marrow of NOD/SCID mice transplanted with human SCA cells. Flow cytometry analysis was performed on the bone marrow of a highly engrafted mouse (SCA-27.1), 4 weeks after transplantation with SCA cells (left panel), on a non-transplanted mouse (middle panel) and on the donor SCA cells (right panel). This data is representative of four mice analyzed in a similar fashion. The cells were double stained with antibodies directed to CD45 and glycophorin A and dead cells were excluded by their intense staining with PI. The cell viability ranged from 87-93%. The histograms (top panel) show the presence of a population of strongly glycophorin A positive (glyco A⁺) cells in both the repopulated mouse bone marrow and the original sample, as compared to an untransplanted mouse. The glyco A⁺ cells were gated (horizontal line) and re-analyzed for CD45 expression (lower panel). Erythroid cells of more immature (glyco A⁺CD45⁺) and mature (glyco A⁺CD45⁻) maturational stages were found in the engrafted mouse bone marrow and in the original patient bone marrow. The percentages given in the different quadrants represent the percentage of cells in the total ungated populations (10000-30000 events).
4.3 Detection of human β- and γ-globin transcripts

Since the flow cytometry analysis demonstrated differentiation of human erythroid cells in the bone marrow of engrafted mice, human globin mRNA expression was analyzed in the peripheral blood, bone marrow and BFU-E. Total RNA was isolated from individual erythroid colonies and examined by RNase protection assay using probes specific for human β-, human γ-, and mouse α-globin mRNA. Both the β- and γ-globin mRNA were detected in 3/3 erythroid colonies (Figure 5A), correlating with the compound heterozygous Hb B0/B+ phenotype of the original patient. The absence of mouse α-globin mRNA also confirmed that the colonies were human. The polymerase chain reaction of reverse-transcribed RNA molecules (RT-PCR) revealed human globin gene transcripts in BFU-E (data not shown) and in the bone marrow of highly engrafted mice (Figure 5B, lanes 2 and 4). As expected in patients diagnosed with homozygous HbSS disease (SCA-27; Figure 5B, lane 1) or compound heterozygous Hb B0/B+, both β- and γ-globin mRNA were detected in transplanted SCID mice. In animals injected with bone marrow cells from homozygous B0-thalassemia, only γ-globin transcripts were found (data not shown). No human globin mRNA could be detected in the peripheral blood of transplanted mice (Figure 5B, lane 8). Total RNA extracted from non-transplanted SCID bone marrow or peripheral blood was used as a negative control (Figure 5B, lanes 3, 5 and 9).

4.4 Human β- and γ-globin protein production

In addition to the expression studies described above, human erythroid cells including mature erythrocytes were directly visualized in the bone marrow and erythroid colonies from transplanted mice by staining with FITC-labeled human specific anti-γ-, β- or β5-globin antibodies. Erythroid cells in the bone marrow from untransplanted mice and murine BFU-E did not stain with these antibodies (data not shown). High numbers of human erythroid cells staining positive for β-globin (Figure 6A) and γ-globin (Figure 6B) were found in the bone marrow of mice transplanted with cells from a compound heterozygous Hb B0/B+ patient. Human cells staining positive for β5-globin were also detected in the bone marrow of mice transplanted with cells from a homozygous HbSS disease patient (Figure 6C). In general, higher numbers of mature human erythroid cells were present in the NOD/SCID mice compared to SCID mice. Erythroid
**Fig. 3-5.** Detection of human erythroid cells, that express the human β- and γ-globin genes, in the bone marrow and erythroid colonies from immune-deficient mice transplanted with β-thalassemia major and homozygous SCA cells. A. RNase protection analysis of human globin gene expression in BFU-E colonies from β-thalassemia mice (Thal-11.1, 11.2, 11.3; the first two digits refer to the donor and the last digit represents the number assigned to each mouse) using probes specific for human β-, human γ- and mouse α-globin mRNAs. Controls were: lane 1, HuAγ transgenic mice; lane 2, Original bone marrow from patient Thal-11. This patient was a compound heterozygote (Hb β0/β+). B. RT-PCR analysis of human globin gene expression in the bone marrow and peripheral blood of mice transplanted with β-thalassemia (Thal-28) and SCA (SCA-27) cells. As outlined in Methodology, a primer set completely specific to human β- and γ-globin cDNA was used for the amplification, giving rise to an uncut (marked as U) fragment of 263 bp. BstEII restriction enzyme specifically cuts in the human γ-globin cDNA, producing fragments of 222 and 41 bp (not seen); DraIII cuts in the human β-globin cDNA and generates products of 154 and 109 bp. β- and γ-globin mRNA were both present in the donor cells (SCA-27, lane 1), in the bone marrow of a mouse transplanted with SCA cells (SCA-27.3, lane 2) and a mouse transplanted with β-thalassemia cells (Thal-28.3, lane 4). The patient Thal-28 was a compound heterozygote (Hb β0/β+, data not shown). No human globin mRNA was found in the peripheral blood of SCA-27.3 (lane 8). Negative controls include bone marrow (lanes 3 and 5) and peripheral blood (lane 9) from non-transplanted SCID mice as well as reagent controls (lanes 6 and 7).
Fig. 3-6. Detection of human erythroid cells stained with anti- β- or βS- and γ-globin antibodies in the bone marrow and erythroid colonies of immune-deficient mice transplanted with bone marrow cells from β-thalassemia major or SCA patients. Immunofluorescent staining of the bone marrow from mouse Thal-28.3 with FITC-conjugated antibodies directed against β-globin (A) and γ-globin (B). Bone marrow from mouse SCA-19.3 stained with antibodies directed against βS-globin (C). Human anti-γ-globin staining of erythroid colonies from mouse Thal-11.1 directly in the methylcellulose culture (D). Human anti-γ-globin staining (E) and anti-βS-globin staining (F) of cells from a BFU-E derived from mouse SCA-27.3. Functional sickling test of erythroid cells from an untransplanted mouse (G), bone marrow from mouse SCA-27.1 (H), and pooled BFU-E from mouse SCA-19.3 (I).
progenitor colonies from all mice transplanted with cells from β-thalassemia and SCA patients were positive for human globin proteins. Figure 6D shows a BFU-E from a representative mouse transplanted with β-thalassemia cells (Hb β0/β+) directly stained in the culture dish with anti-γ-globin antibodies. Cells from a BFU-E derived from a mouse transplanted with SCA cells stained positive with anti-γ-globin (Figure 6E) and anti-βS-globin antibodies (Figure 6F). Consistent with the RT-PCR, positively stained cells were below detectable limits in the peripheral circulation of all mice tested (data not shown).

A functional assay was also used to demonstrate the presence of mature erythroid cells expressing HbS in engrafted mice. Bone marrow cells (Figure 6H) or 20-30 pooled erythroid colonies (Figure 6I) were incubated in presence of sodium metabisulfite to hasten deoxygenation and polymerization of HbS; in both cases, sickled red cells were readily detected. No sickled cells were detected in the bone marrow of non-transplanted SCID mice (Figure 6G) or in the marrow of mice transplanted with β-thalassemia cells (data not shown).

Overall, there was a correlation between human cell engraftment and erythroid differentiation. Erythroid cells expressing globin were detected in 10/11 (91%) mice that contained ≥10% total human cells in their bone marrow while only 4/9 (44%) mice with 1-10% total human cells had erythroid cells. Erythroid cells were not detected in mice (0/8) that were engrafted with <1% human cells.
5. Discussion

5.1 Primitive human cells engraft immune-deficient mice

This report provides the first evidence that genetically deficient bone marrow cells from patients with human β-thalassemia and SCA can engraft immune-deficient mice and reproduce the cellular and molecular defect observed in the donors. The level of human cell engraftment and the number and differentiation profile of the progenitors that developed in the mice was similar to our previous experience with normal bone marrow or cord blood. Several lines of evidence suggest that primitive cells are responsible for maintaining the graft of SCA or β-thalassemia bone marrow in the transplanted mice. The bone marrow of engrafted mice contained high levels of multiple lineages of human hematopoietic cells in response to cytokine treatment. The continued presence of high numbers of multipotential and committed progenitor cells in cytokine treated mice for at least two to four months indicates that a more immature cell is maintaining the progenitor population. A significant population of CD34+ and more primitive CD34+CD38− cells were found in the murine bone marrow. These data, together with our previous experiments on the engraftment of normal human bone marrow (Lapidot et al., 1992) or cord blood (Vormoor et al., 1994) into SCID mice, provide strong evidence that primitive human cells, earlier in ontogeny than colony-forming progenitors, are responsible for engrafting the murine bone marrow microenvironment and maintaining the graft for at least 4 months. Conclusive evidence that the SRC is a pluripotent stem cell will come from tagging the engrafting cells with a retrovirus and using the novel integration site as a lineage marker (Dick et al., 1985).

5.2 Differentiation of erythroid cells

The higher levels of erythroid cells that developed in the bone marrow of NOD/SCID recipients, compared to SCID mice (Lapidot et al., 1992), enabled us to carry out a more detailed flow cytometry analysis of human erythroid differentiation in transplanted mice. We found that the entire human erythroid lineage from BFU-E to mature erythrocytes developed in the murine bone marrow. As expected, these cells expressed human γ-, β- or βS-globin typical of the genetic deficiency of the donor. In the case of SCA transplanted mice, the mature erythrocytes could also be induced to sickle when incubated in vitro under reduced oxygen tension. Despite the active human erythropoiesis seen in the bone marrow of erythropoietin treated mice, mature
erythrocytes were rarely seen in the peripheral blood. The absence of mature erythrocytes in the blood could be due to reduced erythroid maturation. Consistent with this idea, the bone marrow from transplanted mice contained a higher proportion of immature glyco A+CD45+ compared to more mature glyco A+CD45- cells normally seen in human bone marrow. An alternate possibility is an increased turn-over of mature human erythrocytes in transplanted mice. We and others have found that mature human red blood cells have a very short half-life after IV transplantation into SCID mice (unpublished data).

5.3 Implications for gene therapy

The development of an in vivo system that assays both primitive human hematopoietic cells and genetically deficient erythroid cells provides a powerful pre-clinical tool to evaluate gene therapy methods directed to the genetic manipulation of human stem cells with human globin expression vectors. The hemoglobinopathies present special problems for gene therapy because the globin gene needs to be efficiently introduced into human stem cells and then expressed permanently at high but precise levels specifically in the erythroid cells in order to produce a functional hemoglobin molecule. One major problem is the difficulty in designing globin vectors because globin gene regulation is very complex and the regulatory elements that function in retrovirus vectors have not been precisely defined. Transfer of globin vectors without the 5' regulatory sequences into murine stem cells resulted in long-term expression of the human globin gene specifically in erythrocytes at very low levels (<1%) (Dzierzak et al., 1988). Inclusion of the four DNAase hypersensitive sites from the locus control region into the retrovirus vector increased the level of expression by ~10 fold (Plavec et al., 1993). However, vectors containing the locus control region are prone to rearrangements and lower virus titer. Another major problem for gene therapy is the development of conditions for efficiently infecting human pluripotent stem cells with retrovirus vectors. Past studies with human cells have focused on introducing globin vectors into BFU-E (Bender et al., 1988). The SCID transplant system provides the means of assaying primitive human cells based on their ability to repopulate the entire hematopoietic lineage in vivo. We (Dick et al., 1991) and Nolta et al. (Nolta et al., 1994) have previously reported that low numbers of genetically manipulated cells could be detected in transplanted immune-deficient mice. However, these mice were engrafted with low levels of human cells and no mature erythroid cells developed in the mice. As described in chapter 2, we
have used the improved transplant system described here with NOD/SCID mice and cytokine treatment to assay the efficiency of gene transfer into SRC from human bone marrow or cord blood. The differential susceptibility to retroviral infection of SRC as compared to CFC and LTC-IC highlights the need to use appropriate assays for pre-clinical studies and the need to develop new vectors and approaches for efficiently transducing human stem cells (Larochelle et al., 1996).

The models for β-thalassemia and sickle cell anemia we describe here provide unique experimental systems to assess new globin vector constructs and conditions specific for gene transfer into primitive human hematopoietic cells and for their ability to maintain long-term effective levels of globin expression in the erythroid lineage. Effectiveness can be determined by measuring both levels of globin expression and by monitoring correction of the cellular defect found in hemoglobinopathies such as red blood cell sickling. These models may also provide a suitable system to evaluate inducers of fetal hemoglobin production (Perrine et al., 1993). Although this report has focused on hemoglobinopathies, the approach we describe here should be applicable to create animal models for any genetic defect involving the human hematopoietic system.
6. References


Cashman et al., 1994 Proliferation and expansion of CD34+ human progenitor cells in the marrow of NOD/SCID mice. *Blood*. 84: 368a-


FOURTH CHAPTER

CONCLUSIONS
More than three decades ago, the identification of CFU-S by Till and McCulloch (Till and McCulloch, 1961) and the development of simple *in vitro* assays for CFC simultaneously by Sachs and Metcalf (Pluznik and Sachs, 1966; Bradley and Metcalf, 1966) established an experimental paradigm that has influenced hematopoietic research until the present time. However, the inability of CFC to proliferate in secondary cultures and the heterogeneity within the CFU-S population suggested their production from an earlier, more immature compartment of hematopoietic stem cells capable of both self-renewal and multilineage differentiation. Ultimately, long-term reconstitution of the hematopoietic system of a recipient animal after transplantation was shown as the only definitive means of identifying and characterizing the hematopoietic stem cell.

Although murine transplantation studies were instrumental in the identification and characterization of hematopoietic cells with long-term repopulating potential, parallel investigations on human hematopoietic stem cells have been impeded by the absence of *in vivo* assays equivalent to those developed in the mouse. Over the last few years, significant progress has occurred in the development of new approaches to the assessment and characterization of primitive human hematopoietic cells likely to include those with long-term *in vivo* repopulating potential. One such approach makes use of immune-deficient mice to create *in vivo* models of human hematopoietic reconstitution (Dick, 1991). Alternatively, long-term bone marrow cultures also appear to be maintained by cellular entities with characteristics of very primitive cells (Dexter et al., 1977; Gartner and Kaplan, 1980). Moreover, clonogenic assays have recently been modified to support the maintenance of immature CFU-blasts endowed with replating capacity (Nakahata and Ogawa, 1982). Which of these assays truly measure human stem cell function? What is the relationship between the cells assayed in each system? The retroviral gene marking approach used to address these questions also points to a corollary problem: are human stem cells infectable with retroviral vectors? The data presented in this thesis address these fundamental problems of human stem cell biology and human stem cell gene therapy, and thereby provide new insights into the identification and characterization of this cell type.

**1. Genetic manipulation of human hematopoietic cells**

Permanent correction of human disorders affecting the hematopoietic system requires that genes must be introduced into pluripotent stem cells because mature cells and multipotent or
lineage-restricted progenitors cannot permanently reconstitute the entire hematopoietic system (Anderson, 1992). Genetic manipulation of human stem cells poses several challenges. While retroviruses can efficiently transduce murine pluripotent stem cells (Dick et al., 1985; Lemischka et al., 1986; Jordan et al., 1990; Keller et al., 1985), extrapolation of these methods to other large mammals (Bodine et al., 1993; Schuening et al., 1991) resulted in very low numbers of gene marked engrafted cells, suggesting that there may be species differences in the characteristics of stem cells. In addition, current gene transfer procedures employed in human clinical trials (Dunbar et al., 1995; Kohn et al., 1995; Cornetta et al., 1996; Deisseroth et al., 1994; Brenner et al., 1993) were optimized using in vitro CFC and LTC-IC assays. However, these progenitors may not share the same biological properties as stem cells, resulting in methods that have little relevance for stem cell gene transfer. The relevance of the currently employed methods of gene transfer into repopulating cells has been evaluated in this thesis (chapter 2) by systematically comparing the efficiency of transduction into human CFC, LTC-IC and SRC (Larochelle et al., 1996). These studies have provided fundamental new insights into the organization of the human hematopoietic hierarchy and present important implications for stem cell gene therapy.

1.1 Identification of a novel human hematopoietic cell

1.1.1 Differential susceptibility of SRC to retroviral infection

Human CB or BM cells were infected using procedures previously developed for transduction of progenitors by co-culture on high titer virus producing cell lines (Dick et al., 1991). While the majority of clonogenic progenitors (up to 80%) and LTC-IC (up to 70%) were readily transduced after two days of coculture, the levels of genetically manipulated human cells found in highly engrafted mice were uniformly low (0-0.1%). Since mice contained high numbers of unmarked human progenitors and mature cells, unmarked SRC must have initiated the graft. This result was further supported by examination of the kinetics of engraftment which indicated that only 0.1-1% of the original number of CFC and LTC-IC were present two days after transplantation (Cashman et al., 1994). Over the next four weeks, their number increased logarithmically to almost restore the numbers originally injected. Also, ³H-TdR suicide studies indicated that a high proportion of the CFC were in cycle over the first month after transplantation indicating that the increase in human progenitors was due to proliferation and differentiation from primitive engrafting cells that are even earlier than LTC-IC (Cashman et al.,
Hence, we conclude that most CFC and LTC-IC are incapable of long-term engraftment of NOD/SCID mice and are therefore distinct from SRC.

1.1.2 Cell surface characterization of SRC

The cell surface characterization of SRC provides additional independent support that SRC are distinct from most CFC and LTC-IC. The SRC were found exclusively (100%) in the CD34^+CD38^- fraction after cell purification. Since >70% of LTC-IC and >97% CFC are found in the CD34^+CD38^- fraction (Sauvageau et al., 1994), these data confirm that SRC are distinct from CFC and at least the majority of LTC-IC that read out in the standard 5 week LTC-IC assay.

Since LTC-IC are detected in both the CD38^+ and CD38^- subfractions of the CD34^+ cells, it has become apparent that these long-term culture-initiating cells represent a heterogeneous population of cells. Recently, long-term BM cultures maintained for extended periods of time (12 weeks) have also been described (E-LTBMC) and have served to provide further evidence of the heterogeneity within the LTC-IC compartment (Hao et al., 1995). These studies have suggested that LTC-IC with the highest proliferative capacity persist after longer times in culture (12 weeks) and are from the CD34^+CD38^- fraction. Therefore, since only a proportion of LTC-IC have been infected in the gene marking studies described before, it is possible that the transduced LTC-IC may derive primarily from the CD34^+CD38^- fraction, while the more primitive LTC-IC in the CD34^+CD38^- fraction may be resistant to retroviral infection. Hence, LTC-IC defined as CD34^+CD38^- may have SRC activity. This issue can be clarified by comparison of the efficiency of infection of CD34^+CD38^- and CD34^+CD38^+ cells prior to engraftment.

1.1.3 Working model of human hematopoiesis

Figure 4-1 incorporates the recent data discussed above in a working model of normal human hematopoiesis consistent with information gained over the last decades from investigations that made use of in vivo SCID transplantation assays, long-term bone marrow cultures, in vitro colony assays, and flow cytometric analysis of cell surface markers. In combination with earlier data, the gene marking studies and cell purification analyses presented here provide the strongest evidence that SRC defines a novel human hematopoietic cell that is more immature than any other cell type detected to date.
Fig. 4-1. Schematic representation of the human hematopoietic system incorporating the recent data from gene marking and cell purification studies. Clonogenic assays detect progenitor cells committed to a specific lineage (e.g. BFU-E), as well as multipotential progenitors that produce all myeloid/erythroid lineages (CFU-GEMM). LTC-IC appear to represent the most primitive cells assayed in vitro. Ultimately, SRC specify novel human hematopoietic cells defined by their long-term and multilineage repopulating ability in SCID mice. SRC appear to detect the most primitive cells. The horizontal lines depict a potential overlap between the various populations of cells (e.g. some LTC-IC may have SRC activity).
1.2 Implications for gene therapy

The inefficient infection of SRC observed in the marking studies described before is concordant with the low level of gene transduction reported in large mammals (Bodine et al., 1993; Schuening et al., 1991) and human clinical trials (Dunbar et al., 1995; Kohn et al., 1995; Cornetta et al., 1996; Deisseroth et al., 1994; Brenner et al., 1993). This inability to infect human SRC, in contrast to CFC and LTC-IC, highlights the need for appropriate assays that predict the outcomes in human clinical trials.

The development and optimization of gene therapy protocols would be aided by animal models that reproduce human genetic diseases. Chapter 3 of this thesis presents the development of such models for β-thalassemia and sickle cell anemia (Larochelle et al., 1995). These two diseases present special problems for gene therapy because the globin gene must be expressed in a strictly regulated fashion in the erythroid lineage. Bone marrow cells from pediatric patients with β-thalassemia and sickle cell anemia were transplanted into sub-lethally irradiated SCID or NOD/SCID mice. The highly engrafted erythropoietin-treated mice contained multiple lineages of human progenitor cells as well as mature human erythrocytes. These observations indicate that immature human hematopoietic cells engrafted the murine BM and differentiated along the erythroid lineage in response to erythropoietin. The human erythroid cells and human BFU-E recovered from the marrow of engrafted mice expressed the original disease phenotype of the donor as demonstrated by β-, γ- or β⁺-globin expression and, for sickle cell anemia mice, by the characteristic sickling observed under reducing conditions in an in vitro assay. This system can thus be used to evaluate gene transfer into primitive human cells, longevity of expression in the appropriate lineage, and correction of the diseased cellular phenotype. The same approach should also be applicable to other single defects such as Gaucher’s disease and severe combined immunodeficiency disease.

2. Future directions of the SRC assay

The experiments described in this thesis, together with the data accumulated over the past decade, provide a starting point for developing a quantitative assay for SRC. Such an assay will set the stage for a more complete analysis of optimal conditions and cytokines to promote survival, differentiation or self-renewal of SRC; for identifying receptors expressed at the surface
of SRC; for providing a direct means for purification and quantitation of these cells; and for evaluating the role of novel genes in the regulation of primitive human hematopoietic cells. These prospects will provide novel insights into the development of genetic therapy, BM purging, cytokine treatment, BM transplantation, and herald the reality of developing new therapeutic vistas for the treatment of hematopoietic diseases.

2.1 Optimization of gene transfer into SRC

The SCID transplantation assay provides the basis to enable a mechanistic understanding of the barrier to transduction of primitive human SRC. This may point the way to the development of improved methods and vectors of gene transfer specific for human pluripotent stem cells.

2.1.1 Determination of mechanisms of SRC resistance to retroviral infection

Although retroviruses are currently the most efficient gene transfer vectors, they can only infect and integrate into cells that express a specific receptor and that are undergoing active cell division. Thus, the difficulty encountered in transducing human hematopoietic stem cells may be related to the lack of appropriate viral receptors at the surface of these cells or, alternatively, to culture conditions inadequate to stimulate their proliferation.

Viral receptors

Indirect evidence has already been provided that retroviral receptors may be down-regulated in primitive hematopoietic cells (Crooks and Kohn, 1993). The envelope gene of the retrovirus determines the host range of the virus. Two classes of viruses have been shown to infect human cells, those expressing the amphotropic envelope protein (Figure 1-4B) and those expressing the gibbon-ape leukemia virus (GALV) (Figure 1-4C). The receptors for both viral envelope proteins have been cloned (Miller and Miller, 1994; van, M et al., 1994) and antibodies directed against the GALV receptor are now available. The hypothesis of a lack or reduced expression of viral receptors on stem cells may therefore be tested using multiparameter flow cytometry to separate human cells into receptor expressing (Rec⁺) and non-expressing (Rec⁻) fractions and by comparing the ability of each population to reconstitute the hematopoietic system of immune-deficient mice. Engraftment of rec⁺ cells would provide evidence of retroviral expression on human repopulating cells; alternatively, engraftment of rec⁻ cells would indicate a lack of appropriate receptors on these cells.
Culture conditions for infection

Problems encountered in transducing human repopulating cells may also be due, in part, to the inability of retroviruses to integrate into non-replicating cells as a result of culture conditions inadequate to stimulate the proliferation of these cells. Most of the infection experiments performed to date and described in chapter 2 have used a 48-hour period of co-culture on retroviral packaging cell lines preceded by one day of prestimulation in the presence of IL-3, IL-6 and SCF. As discussed before (see §1.3.3, chapter 1), a number of additional cytokines, including G-CSF, IL-11, IL-12, LIF and FL, have also been classified as early-acting synergistic factors and may be required for the entry of dormant cells into the cell cycle. An extensive menu of negative regulators have also been described (see §1.3.3, chapter 1) and inclusion of selected members to the pre-stimulation cocktail may also enhance the susceptibility of SRC to retroviral infection. Preliminary investigations (unpublished) testing various combinations of positive and negative regulators indicate that it may be possible to expand primitive human repopulating cells ex vivo without the loss of their stem cell properties, and to delineate the nature and timing of their initial proliferative responses to specific factors.

Although it is becoming apparent that intimate contact of hematopoietic stem cells with stromal elements is essential for these cells to express their properties (see §2.2.1, chapter 1), efficiency of gene transfer performed on stromal support has not yet proven to be superior to efficiencies obtained with other methods of transduction. Moreover, as described in chapter 2, the 30/35 FN and CH-296 fragments of fibronectin have already been tested for their ability to increase retroviral-mediated gene transfer into SRC (Larochelle et al., 1996). Although FN allows survival of SRC maintained in culture for at least 3 days, it was unable to contribute to higher levels of gene transfer into these cells.

2.1.2 Evaluation of novel vectors for gene transfer

Novel vehicles for gene transfer are being developed to overcome the mechanistic resistance of human repopulating cells to retroviral infection. The SRC assay offers the unique opportunity to evaluate these novel transduction methods without the need to proceed to clinical trials.
Vectors with viral receptor-independent infection capacity

A pseudotyped murine leukemia virus-derived vector has recently been produced by replacing the retroviral envelope glycoprotein by the G glycoprotein of vesicular stomatitis virus (VSV-G) (Yee et al., 1994; Emi et al., 1991; Burns et al., 1993). VSV-G interacts with a phospholipid component (phosphatidylserine) of the host membrane to mediate viral entry by membrane fusion (Mastromarino et al., 1987; Schlegel et al., 1983). Since viral entry is not dependent on specific protein receptors, VSV has a very broad host range, and may be useful for stem cell infection. In addition, VSV can withstand the shearing forces of ultracentrifugation and, therefore, may be concentrated to very high titers ($\geq 10^9$ cfu/ml) without loss of infectivity. A limitation to the application of this recombinant vector is the toxicity of the VSV-G protein when stably expressed in cultured cells. However, large-scale production of VSV-G pseudotyped MoMuLV vector has recently been generated by transfection of a 3T3 cell line with a plasmid containing the VSV-G cDNA driven by a tetracyclin-inducible promoter (Yang et al., 1994).

The putative lack of retroviral receptors on human stem cells may also be overcome by altering the tropism of current retroviral vectors. For instance, a chimeric protein containing the erythropoietin polypeptide and part of the envelope protein of ecotropic MoMuLV has recently been used to engineer viral surface epitopes (Kasahara et al., 1994). The murine virus became several times more infectious for murine cells bearing the EPO receptor, and it also became infectious for human cells expressing EPO receptor (Kasahara et al., 1994). This type of tissue-specific targeting by means of ligand-receptor interactions could be achieved by targeting the c-kit or CD34 surface antigen and may conceivably be useful for the infection of human SRC.

Dividing and quiescent cells have been shown to be equally susceptible to adenoviral infection, but the inability of adenoviruses to integrate into host genome has limited their potential for gene transfer (see §3.1.1, chapter 1). Adenoviral vectors expressing GALV or amphotropic receptors may be used here to infect SRC, rendering these cells transiently susceptible to retrovirus infection.

Vectors for infection of quiescent cells

Advances in the area of human stem cell biology may eventually permit the efficient purification of HSC and their reliable ex vivo expansion without lineage commitment and differentiation. This would allow for their more efficient transduction using MoMuLV-based
vectors. Alternatively, a lentiviral vector based on human immuno-deficiency virus has recently been developed that may be of value for the efficient transduction of quiescent stem cells (Naldini et al., 1996). In contrast to MoMuLV-based vectors, this HIV-based construct has been shown to transduce heterologous sequences into nondividing cells, including HeLa cells and rat fibroblasts arrested in their cell cycle, as well as human primary fibroblasts and terminally differentiated neurons. This property is due, at least in part, to the nuclear localization signal (NLS) on the HIV capsid which allows transport of the reverse transcribed viral genome across the intact nuclear membrane even in the absence of mitosis (Bukrinsky et al., 1993). A vector based on HFV of the Spumavirinae subfamily has also been constructed and shown to transduce stationary-phase cultures more efficiently than murine leukemia virus vectors (Russell and Miller, 1996).

2.2 Characterization of SRC

2.2.1 Identification of human stem cells by gene transfer

Gene marking studies based on optimized gene transfer procedures will provide the most conclusive way to demonstrate the pluripotentiality of SRC. Because of the ability of retroviral vectors to randomly integrate into a large number of chromosomal locations, the site of integration serves as a unique clonal marker that allows the examination of individual stem cell clones as they repopulate the hematopoietic system of the transplanted immune-deficient mice; detection of the same integration in both myeloid and lymphoid lineages provides definitive demonstration of pluripotent stem cell engraftment.

This approach, originally exploited for the characterization of murine stem cells (see §1.1.5, chapter 1), has recently been applied to the bnx mouse transplantation model to provide exciting evidence documenting the pluripotent character of primitive human hematopoietic cells capable of repopulating these immune-deficient recipients (Nolta et al., 1996). Human CD34+ bone marrow cells were transduced with NeoR-containing retroviral vectors in a stromal culture system and used for long-term engraftment into immune-deficient bnx mice. Although engraftment in this model is relatively low (< 10%), gene-marked human lymphoid and myeloid populations were recovered from the marrow of the mice 7-11 months after transplantation. Gene-marked myeloid and T cells clones were expanded ex vivo and, using inverse PCR, common proviral integration sites were seen in myeloid and lymphoid populations, as
corroborated by sequencing of the PCR fragments. This analysis thus confirmed engraftment of \textit{bnu} mice with a cell capable of multilineage differentiation.

Consistent with the observations described in chapter 2 of this thesis using the NOD/SCID transplantation system, the frequency of gene transfer into the pluripotent \textit{bnu}-repopulating cell was low. These low levels of transduction necessitate the use of large numbers of animals and require the development of very sensitive methods of detection. Optimization of gene transfer procedures into human stem cells should facilitate the application of this approach to the NOD/SCID transplantation model. The high levels of engraftment obtained in this system together with high efficiency gene transfer will also make it possible to evaluate the self-renewal capacity of SRC following transplantation into secondary recipients; detection of the same integration sites in multiple lineages of all recipients will assess the self-renewal ability of the marked pluripotent SRC. Maintenance in secondary animals of a separate clonal marker in the myeloid and lymphoid lineages would rather suggest self-renewal of a lineage-restricted SRC. Ultimately, transplantation of the same transduced specimen to both human and mouse, and detection of the same integration sites in multiple lineages of both recipients for extended periods of time will allow the definitive assimilation of SRC to the human pluripotent stem cells.

\subsection*{2.2.2 Characterization of SRC by limiting dilution assays}

Limiting dilution assays (LDA) have been utilized to determine the frequency of murine pluripotent stem cells within marrow populations (see §1.2.1, chapter 1). This pointed the way to the elaboration of a quantitative assay for murine HSC that contributed to delineate the exact hierarchical structure of the murine hematopoietic system. In a similar fashion, limiting dilution analysis may also be applied to the quantitation of human SRC for the development of a quantitative assay for these cells (Wang and Dick, in preparation). The numerous applications of such an assay have been outlined above.

When applied to the quantitation of SRC, LDA are performed by injecting various doses of cells ($10^4$-$10^8$) per group of replicate mice. Individual mouse within each group is analyzed for an all-or-nothing response, as measured by the presence or absence of human cells in the BM of each animal. The Poisson probability theory is then applied to describe the relation between the average number of cells tested per mouse and the number of negatively responding mice per group (Porter and Berry, 1964; Taswell, 1981). The dose corresponding to 37\% negatively
responding mice indicates the frequency of SRC within the test cell population (e.g. normal BM or CB). Preliminary studies suggest a frequency of SRC in adult BM of $\sim 1/3 \times 10^6$ and $\sim 1/8 \times 10^5$ in umbilical CB (Wang and Dick, in preparation). Although not corrected for seeding efficiency, SRC appear rarer than CFC and LTC-IC (Figure 4-1), thus providing an additional evidence that SRC are distinct from most progenitors.

As discussed above, retroviral marking provides the most direct approach to demonstrate the pluripotentiality of SRC. Limiting dilution analysis combined with clonality assays presents an alternative, albeit more indirect, system to evaluate the multipotential character of SRC. Clonality assays based on X-chromosome inactivation have already shown their utility by providing evidence for the existence of human pluripotent HSC (see §2, chapter 1). Clonality can be assessed in any cell lineage in the majority of female subjects by molecular analysis of patterns of X-chromosome inactivation. Following the principle of lyonization, each somatic cell in female subjects randomly inactivates one X-chromosome early in embryogenesis and transmits its pattern of inactivation to all progeny. This pattern of X-inactivation serves as a clonal marker and can be followed using a polymorphic gene, such as G6PD, PGK, HPRT, M-27β or HUMARA, expressed on the X-chromosome. Hence, in a mouse injected with a limiting dose (i.e. one SRC) of human cells from a female donor, detection of the same pattern of inactivation in both lymphoid and myeloid lineages would suggest a proliferation of this SRC in both lineages. Although binomial statistics may be applied to confirm repopulation from a single clone (i.e. only one SRC), this approach provides only an indirect confirmation of SRC pluripotentiality and its limited resolution (only two possible markers: active or inactive X-chromosome) does not permit the elucidation of the possible dynamics within the entire stem cell population.

2.2.3 Purification of SRC

The initial purification experiments presented in chapter 2 indicated a 1600-fold enrichment of SRC in the CD34+CD38- cell fraction. Further purification of SRC will provide a tool to define a more complete surface phenotype for these cells; to potentially separate SRC into pluripotent and lineage-restricted subfractions; to distinguish normal SRC from neoplastic cells for purging strategies; to determine their direct response to cytokines; and to identify novel genes specifically expressed in this purified population (see §2.3).
Multiparameter flow sorting has evolved as a clear favorite to achieve high levels of purity. In this procedure, SRC can be selected on the basis of expression or lack of expression of multiple cell surface receptors (CD34, CD38, Thy-1, CD71, CD45RO, CD45RA, HLA-DR, c-kit and lineage associated antigens; see §2.1.3, chapter 1) as well as on the basis of light scattering properties and uptake of rhodamine-123. Counterflow centrifugal elutriation (CCE) has also been reported to isolate marrow populations on the basis of their size and density. CCE may be used to separate SRC from more mature progenitors in a way reminiscent of the isolation of CFU-S from the more primitive murine hematopoietic stem cells (Jones et al., 1990). Finally, it is also possible to take advantage of the non-cycling state of SRC for their purification. This was well exemplified by the recent study of Beraldi et al. who combined the stimulating activities of hematopoietic growth factors and the cytotoxic effect of 5-FU to isolate a population of primitive human hematopoietic cells (Berardi et al., 1995) (see §2.1.2, chapter 1).

2.3 Identifying genes and factors involved in stem cell regulation

A priori, self-renewal and commitment of stem cells to terminal differentiation represent opposite outcomes, and normal hematopoiesis requires a regulated balance between these two outcomes. However, when the genetic make-up of critical genes is altered, the balance can be shifted so that self-renewal exceeds differentiation, resulting in the eventual over-abundance of undifferentiated cells that possess significant proliferative capacity. Hence, characterization of genes and their product involved in normal and leukemic stem cells is fundamental to understand how decision-making normally occurs and how leukemic transformation impairs the normal developmental program.

The experiments presented in this thesis describe the functional properties of primitive human long-term repopulating cells and suggest features of regulatory mechanisms. As illustrated in this section, the development of a quantitative assay for SRC together with novel molecular tools now provide optimism that many descriptive aspects of stem cell biology will soon be supported by elucidation of molecular and genetic mechanisms regulating normal and leukemic stem cell behavior.

2.3.1 Genes and factors involved in normal hematopoiesis

Many years of in vitro studies have defined a range of cytokines that promote growth and differentiation of clonogenic progenitors (Ogawa, 1994; Ogawa, 1993). In particular, the use of
the CFU-blast assay has demonstrated the ability of certain cytokines to shorten the G₀ period (see §1.3.3, chapter 1). Some combinations of soluble factors have also been successfully used to maintain stem cell competitive repopulation ability in short-term suspension cultures (see §1.3.3, chapter 1). However, in vitro expansion of pluripotent stem cells in these experiments was not documented. In fact, the single most convincing suggestion that stem cells may be stimulated into cell cycle by defined factors has been obtained in the mouse from the enhanced infectibility of such cells by retroviruses (Dick et al., 1985). More recently, the c-kit [reviewed in (McNiece and Briddell, 1995)] andflt3/flk2 [reviewed in (Lyman et al., 1994)] ligands have been presented as the most likely candidates for factors central to normal stem cell behavior. However, no finding has yet clearly demonstrated their critical role in stem cell self-renewal.

In combination with the recently developed methods to create global cDNA libraries from small cell numbers (Brady et al., 1995), the population of SRC exclusively found in the CD34⁻CD38⁻ fraction after cell purification can now be used to identify the specific cytokines, cytokine receptors or other genes that may regulate their developmental program. For instance, specific primers for c-kit and flt3/flk2 ligands and their receptors could be used to screen such libraries and determine which factors act on the most immature subpopulation. An extensive array of known genes susceptible to play a role in stem cell regulation may also be analyzed, including HOX gene family members, genes for signalling molecules, genes identified in lower organisms such as xenopus and zebrafish involved in hematopoietic induction of mesoderm in the developing embryo, and genes that play a critical role in cell fate determination during embryonic development (e.g. Notch) (Artavanis-Tsakonas et al., 1995). The panel of cDNA isolated from the different purified fractions may thus provide a comprehensive catalogue of gene expression that can be probed indefinitely as new genes become available.

Recently, subtractive hybridization (Robertson et al., 1992) and differential display (Liang and Pardee, 1992) have also been used successfully to identify and isolate differentially expressed genes between two related populations of cells. In the first method, cDNA can be cloned by multiple rounds of subtractive hybridation and amplification by PCR (Robertson et al., 1992); the second approach involves a systematic amplification of messenger RNA using specific primers followed by a display of their products on a denaturing polyacrylamide gel (Liang and Pardee, 1995). Comparison between the purified CD34⁻CD38⁻ SRC population and the CD34⁺CD38⁺
SRC-depleted fraction could permit isolation of genes specifically expressed in SRC. The same approach may be used to identify genes differentially expressed in normal stem cells compared to leukemic cells. As described below, characterization of such genes may be achieved using the CML model of leukemic progression developed in NOD/SCID mice.

2.3.2 Genes involved in leukemic progression

Progression of chronic phase CML into aggressive blast phase cells is an interesting model to study genetic events involved in leukemic progression. The SRC assay in combination with retrovirus insertional mutagenesis may provide a powerful approach to identify oncogenes important in leukemic progression. Replication competent retroviruses are potent mutagens of somatic and germ cells of the mouse, causing both gene activation and inactivation (van Lohuizen M. et al., 1991). Cloning of these insertion sites has provided a rich source of new genes important for murine leukemic progression (Ben-David and Bernstein, 1991). A similar approach may be adopted to identify novel human oncogenes. Blast phase CML cells grow more aggressively in NOD/SCID mice than chronic phase CML, thereby providing a useful endpoint to attest for leukemic progression (Sirard et al., 1996). Hence, mice engrafted with chronic phase CML cells can be infected with a replication competent retrovirus (e.g. GALV) to permit in vivo spread of the virus; in animals where aggressive human leukemia arises, the genes surrounding the retrovirus insertion site may be cloned and analyzed for their involvement in leukemic progression.

The SCID transplantation system may also be used for the development of functional assays to determine the role of oncogenes in the progression of human leukemia. The feasibility of this approach may be established by introducing various oncogenes (e.g. ras, myc, bcr/abl, and genes isolated by subtractive hybridization or differential display) into chronic phase CML using engineered replication-deficient retroviruses. Outgrowth of acute leukemia in mice transplanted with these transduced cells would support the involvement of these specific oncogenes in the progression of leukemia. These experiments could serve as a paradigm to functionally examine the biological consequence of expressing any gene that could perturb the growth and/or differentiation of hematopoietic cells. For instance, a number of class I HOX genes have been shown to be expressed during hematopoietic development (Lawrence and Largman, 1992; Sauvageau et al., 1995; Sauvageau et al., 1994), suggesting a putative regulatory role for these
genes. At least two HOX members are involved in chromosomal translocations associated with pre-B ALL (E2A-PBX) (Kamps et al., 1990) or T ALL (HOX 11) (Dube et al., 1991). Interestingly, when introduced into murine stem cells with retroviruses (Hawley et al., 1994; Kamps and Baltimore, 1993), both translocations induce myeloid leukemia. Insights into the nature of this species-specific difference may be gained, as described above, using the leukemic progression model developed in NOD/SCID mice. These studies could also be extended by infecting normal human BM cells with a variety of engineered retroviral vectors expressing candidate oncogenes, singly or in combination, thereby creating experimental models that reproduce the entire leukemogenic process. Together, these studies may lead to a better understanding of leukemogenesis and of the molecular control of normal stem cell development.

3. Summary

The specific resistance of SCID-repopulating cells (SRC) to retroviral infection and the preliminary results of their purification have served to identify and characterize a new cellular element of the human hematopoietic hierarchy. Further characterization of this cell using improved gene transfer strategies, limiting dilution assays and various purification procedures will culminate in the development of a quantitative assay for SRC and may validate its use as an assay for pluripotent hematopoietic stem cells of human origin. Such a system will be invaluable to decipher the mechanisms of poor retroviral infection of stem cells and evaluate novel gene therapy protocols for numerous hematopoietic diseases, such as hemoglobinopathies. Together with recently developed molecular tools, the SCID transplantation assay also heralds the reality of identifying new genes involved in normal and leukemic stem cell regulation that could shed global insights into the process of stem cell differentiation and self-renewal in other tissues.

Despite the dramatic advances in the characterization and clinical application involving the hematopoietic stem cells, the final quantum leap for their ultimate characterization, isolation and manipulation awaits further investigation. Nevertheless, in the next years, the reality may well go beyond the current aspirations underlying the old myth of the hematopoietic stem cell...
3. References


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