

Identification of hematopoietic stem/progenitor cells: strength and drawbacks of functional assays

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A major challenge in hematopoiesis is to conceive assays that could bring useful insights into experimental and clinical hematology. This means identifying separately the various classes of hematopoietic progenitors that are produced sequentially during the progression from stem cells to differentiated functional cells. Standardized short-term colony assays easily quantify lineage-committed myeloid precursors, but identification of primitive cells, which have both the ability to repopulate durably myeloid and lymphoid lineages and perhaps to self-renew, still depends on *in vivo* assays. Whatever the assay, two important requisites have to be solved: one is the definition of appropriate read-outs that will depend solely on the function of these cells, and the second is to evaluate precisely their numbers and proliferative potential in quantitative assays. When evaluating hematopoiesis, three parameters have to be taken into account: (1) the lack of reliable correlation between the phenotype of a given cell and its function. This is especially problematic in post-transplantation situations where cells from transplanted animals are analysed; (2) functionally heterogeneous cells are identified in a single assay; and (3) ontogeny-related changes in hematopoietic cell proliferation and self-renewal that, in human beings, hampers the exploration of adult stem cells. Nevertheless, years of progress in the manipulation of hematopoietic stem cells have recently resulted in the purification of a cell subset that repopulates irradiated recipients with absolute efficiency.

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Introduction

In some tissues, the intestinal crypts, muscle fibers and skin, stem cells are located in a discrete unique anatomical location, and can be visualized directly on histological sections. This is not the case for hematopoietic stem cells in the bone marrow, even though their proximity to immature osteoblasts has recently been suggested (Calvi *et al.*, 2003; Zhang *et al.*, 2003). The

lack of specific markers and functional heterogeneity of even phenotypically pure populations explains why 30 years after their description by the Canadian and Australian hematological schools, indirect and retrospective functional assays remain essential to evaluate hematopoietic progenitor populations (Wu *et al.*, 1967; Iscove *et al.*, 1971; Metcalf and MacDonald, 1975; Abramson *et al.*, 1977). Despite their old-fashioned appearance in the era of genomics, practice of these multiple assays continues to yield a considerable amount of information that historically served to establish the concepts that regulate stem cell biology, and now extends to cancer stem cells and nonhematopoietic stem cells. If the fundamental rules of the assays have not changed, technological advances in cell purification, cell marking and production of purified regulatory factors through recombinant DNA technology have considerably improved their realization and reproducibility, recently leading to the purification to homogeneity of functional hematopoietic stem cells (Matsuzaki *et al.*, 2004). In this review, we deliberately chose to discuss the rationale and specificity of the various assays established to identify the different classes of progenitors. The phenotypical properties of these cells have been described elsewhere (Nakorn *et al.*, 2002, see also review on stem cells at: www.stemcell.com/technical/bulletins.asp) and will not be reviewed.

Principles underlying hematopoietic stem and progenitor cells assays

The hematopoietic system represents a continuum of cells with changing phenotype and properties as they progress from stem to differentiated cells. One could thus expect that in a steady-state situation, variations detected in the more mature compartments will faithfully reflect those occurring in stem cells. Unfortunately, both compartments are controlled by different factors and may vary independently from each other (Sauvaigeau *et al.*, 1995). This warrants the need for assays that evaluate each compartment independently.

All assays measure two cardinal parameters: cell proliferation (measured by the number of cells produced) and differentiative potential (estimated by the number of different lineages represented in its progeny). It was thus necessary to design experimental conditions

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that meet the specific requirements of each type of progenitor, while excluding the growth of others. However, since we cannot anticipate if conditions selected allow expression of the full differentiative potential of the cells under scrutiny, combining different assays will minimize the risk of underestimating their potential. Schematically, stem cells and their immediate progeny have to accomplish a high number of divisions (>15) in 'long-term assays' (>5 weeks) before they produce differentiated cells; in contrast, lineage-committed progenitor cells will differentiate in less than 3 weeks and five to 10 divisions in short-term assays. Besides this time boundary, our knowledge of molecules controlling the proliferation and differentiation of cells identified in short- and long-term assays is also quite different: growth factors required by committed progenitors are well characterized and most are available as recombinant proteins, whereas we know very little about the complex network of accessory cells and molecules regulating primitive/stem cells (see review no. 1 for details). Consequently, while short-term assays have been easily standardized, multiple conditions are still used in long-term cultures (LTC). Therefore, caution should be exercised when comparing data from different labs even though the assays used share the same name. This is particularly true for assays that detect human LTC-IC (long-term culture-initiating cells) and huSRC (human severe combined immunodeficient (SCID)-repopulating cells) (see later in text).

In 'clonal' assays, such as colony assays or single-cell cultures, the progeny of each progenitor remains well separated and its size is roughly proportional to the proliferative potential of the progenitor. In contrast, if cultures are seeded in liquid cultures with a functionally heterogeneous population of cells, it is impossible to discriminate the progeny from each individual progenitor. Consequently, the precise number of cells contributing to a given end point cannot be inferred only by measuring the total number of cells/lineages recovered. A precise evaluation of primitive hematopoietic cells is essential to understand the mechanisms leading to a gain (or loss) of response to a signal. Since these 'bulk' assays cannot discriminate between the recruitment of responding cells by a given signal, or its impact on their proliferative properties, quantitative assessment of primitive progenitors must be carried out differently. Phenotypic markers (CD34, CD38 and others) are not reliable indicators because they fluctuate independently of cell function (Sato *et al.*, 1999; Danet *et al.*, 2001). Furthermore, even if it were possible to work with highly purified stem cells, which are homogeneous for one property such as their longevity (Matsuzaki *et al.*, 2004), other properties such as self-renewal or multipotency may vary independently, identifying another level of heterogeneity. It is therefore not surprising that 'stemness' cannot be defined by one criterion or by a single gene (Ivanova *et al.*, 2002).

In theory, analysing single cells appears to be the best strategy to dissect this heterogeneity. Long-term lymphomyeloid reconstitution of myeloablated mice has

been achieved with single cells in about one-fifth of the recipients (CD34⁻ C-Kit⁺ Sca⁺; Osawa *et al.*, 1996) and lately with much greater efficiency through the use of dye exclusion (Benveniste *et al.*, 2003; Matsuzaki *et al.*, 2004). Single-cell transplantation was also used to explore the extrahematopoietic potential of cells with a given phenotype (Wagers *et al.*, 2002). *In vitro*, functional analysis of single cells is routinely used to analyse the direct impact of purified growth factors on survival and cycling (Terstappen *et al.*, 1991; Lansdorp *et al.*, 1993; Mayani *et al.*, 1993; Borge *et al.*, 1997; Bennaceur-Griscelli *et al.*, 2001) or the asymmetry of initial cell divisions (Brummendorf *et al.*, 1998; Takano *et al.*, 2004). However, recurrent work with single cells is difficult and time consuming. In addition, purified cells are isolated from the positive and negative influences imposed by their usual neighboring cells, and may thus exhibit quite a different 'behavior'.

This underlines the complementary value of quantifying cells of interest in their cellular context (i.e. without purification), and this is usually performed using the method of the maximum likelihood. In this method, the number of progenitor cells with the desired function (e.g. the capacity to form B cells in LTC) is estimated by the best chosen end point, which reflects these 'unobservable' progenitors. Since the probability of these events is usually low, it follows the Poisson distribution. A linear correlation between the measured end point and the number of cells indicates that the only variable that influences the end point is the number of progenitors, and the Poisson statistical approach is applied to measure the frequency of progenitors that generate this end point (see below). The absence of linearity must be interpreted as a possible interaction between two populations and highlights the complementarity between single-cell versus population-based analyses.

Specific assays for distinct classes of hematopoietic progenitor cells

Establishment of assays to distinguish different stages along a developmental hematopoietic pathway has been accomplished in parallel with murine and human cells. Except for the much higher efficiency of murine as compared to human assays, particularly *in vivo*, the general principles governing these assays operate similarly in both species, and the progenitors that are identified 'fill' the same place in the puzzle. We did not separate murine from human assays, but we have indicated species-specific particularities when necessary.

In vitro assays (Figures 1–3)

Short-term *in vitro* assays The prototypes of the short-term assays are semi-solid colony assays, which identify and quantify lineage-restricted progenitors in well-standardized conditions (see www.stemcell.com/technical/manuals.asp for detailed description of colony assays). As their immobilized progeny accumulates in tight colonies with specific characteristics (composition, size,

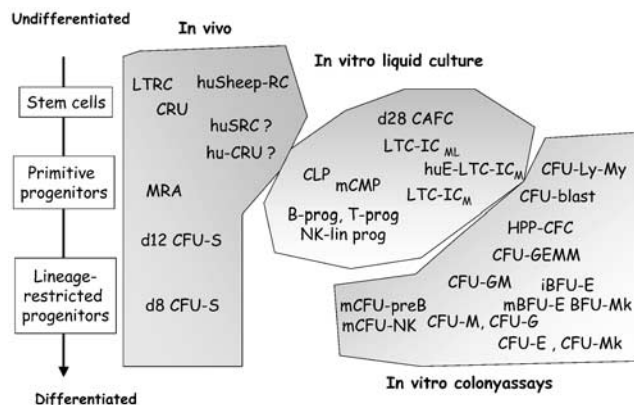


Figure 1 Various classes of hematopoietic stem/progenitor cells identified in *in vitro* and *in vivo* assays. The different cells identified have been clustered into three families according to the type of assay which served for their identification: *in vivo* transplantation assays on the left, *in vitro* long-term cultures in the middle panel and *in vitro* colony assays (right panel). Cells have been placed on the vertical axis according to their maturation stage, and fall within one of the three compartments' stem cells, primitive progenitors and lineage-restricted progenitors. However, this is sometimes rather speculative, since the relationships between cells identified in different assays are not directly demonstrated. 'hu' denote cells identified only in human tissues, 'm' those described only in murine assays. All others cells can be identified in assays initiated with human or murine cells

color, disposition), several types of progenitors (erythroid, granulocytic, macrophagic and megakaryocytic) can differentiate simultaneously in a given culture with no need to purify the input cells (Broxmeyer, 1984). Within a given lineage, progenitors with different levels of maturity can be recognized based on their sensitivity to cytokines, time required to generate differentiated cells and size of the colonies (these three parameters have been used to differentiate colony-forming unit erythroid (CFU-E) from mature and immature burst-forming unit erythroid (BFU-E) (Eaves and Eaves, 1978). Surprisingly, however, except for murine pre-B-cell precursors (Paige *et al.*, 1984), colony assays for lymphoid progenitors have not been well defined. Frequencies of colony-forming cells (CFC) among human CD34⁺ bone marrow cells average 15, 30% in CD34⁺CD38⁺ and 5% in the more immature CD34⁺CD38^{neg} population.

Short-term colony assays are not adequate for the detection of more immature progenitors: the lifespan of the viscous medium (methylcellulose, agar or plasma clot) does not extend beyond 3 weeks, which is too short for stem cells to produce a differentiated progeny, and it cannot be renewed. In the past, however, several classes of multipotent progenitors, colony-forming unit granulocyte-erythroid-macrophage-megakaryocyte (CFU-GEMM) (Ash *et al.*, 1981), blast progenitors (Leary and Ogawa, 1987) and lymphomyeloid progenitors (Lacaud *et al.*, 1998) have been identified based on the presence in a single colony of differentiated cells and/or progenitors belonging to more than one lineage. However, clonality of CFU-GEMM had to be proven to rule out that cells within a mixed colony were produced

by two overlapping restricted progenitors and not by a unique multipotent progenitor cell; replating of cells from the primary colony in secondary assays was required to unmask the full potential of blast progenitors. These limitations explain why these progenitors are not scored anymore now that alternative assays have been developed (Figure 1).

Cells that generate colonies will proliferate and differentiate in liquid cultures as well (i.e. without semi-solid support), in the presence of the same cytokines, but identification of their progeny will require flow cytometry analysis, and the benefit of an easy scoring of several progenitors in the same dish would be lost.

Long-term in vitro assays Any system designed to identify immature progenitors extends beyond 3–5 weeks. This time allows the immature progenitor to complete its differentiation and rules out any contribution by the surviving CFC. A common feature of these long-term systems is the presence of feeder cells that provide a substrate and a source of regulatory factors in an attempt to reconstitute the complexity of the *in vivo* marrow environment. Two different cell types, which probably overlap, have been identified in these assays, both in mouse and man: LTC-IC and cobblestone area-forming cells (CAFC). LTC-IC are defined by their ability, when cultured on supportive fibroblast monolayers, to give rise to daughter cell(s) detectable by standard *in vitro* colony assays (Dexter *et al.*, 1977; Coulombel *et al.*, 1983a). A CAFC integrates in the adherent layer, where it forms a 'cobblestone area', defined as a group of flattened, optically dense cells, tightly associated with adherent cells (Breems *et al.*, 1994; Ploemacher *et al.*, 1989).

Historically, in long-term bone marrow cultures, the adherent cell layer was generated from various bone marrow-derived mesenchymal cells, which proliferate *in vitro* (Dexter *et al.*, 1977). Most of the primitive progenitor cells (including d12 CFU-S and *in vivo* repopulating cells) were hidden in this layer where they proliferated cyclically and released their progeny (CFC and differentiated cells) in the nonadherent fraction. These could be maintained for several months in the mouse and 8–10 weeks in human (Figure 2) (Coulombel *et al.*, 1983a). Subsequently, variations have been introduced that simplified the procedure: test cells and stromal cells are collected separately; the first are phenotypically purified fractions from different fetal and adult tissues (most human LTC-IC are CD34⁺CD38^{low/neg}), the second either irradiated 4-week-old allogeneic long-term culture adherent layers (Sutherland *et al.*, 1989), or spontaneously immortalized murine bone marrow-derived stromal cell lines. Those murine feeders (MS-5, S17, AFT024, M210B4) are very popular because they support equally well the myeloid differentiation of LTC-IC from human hematopoietic sources, and lymphoid differentiation (see below), and are easier to handle than primary bone marrow cells because they are available in unlimited numbers

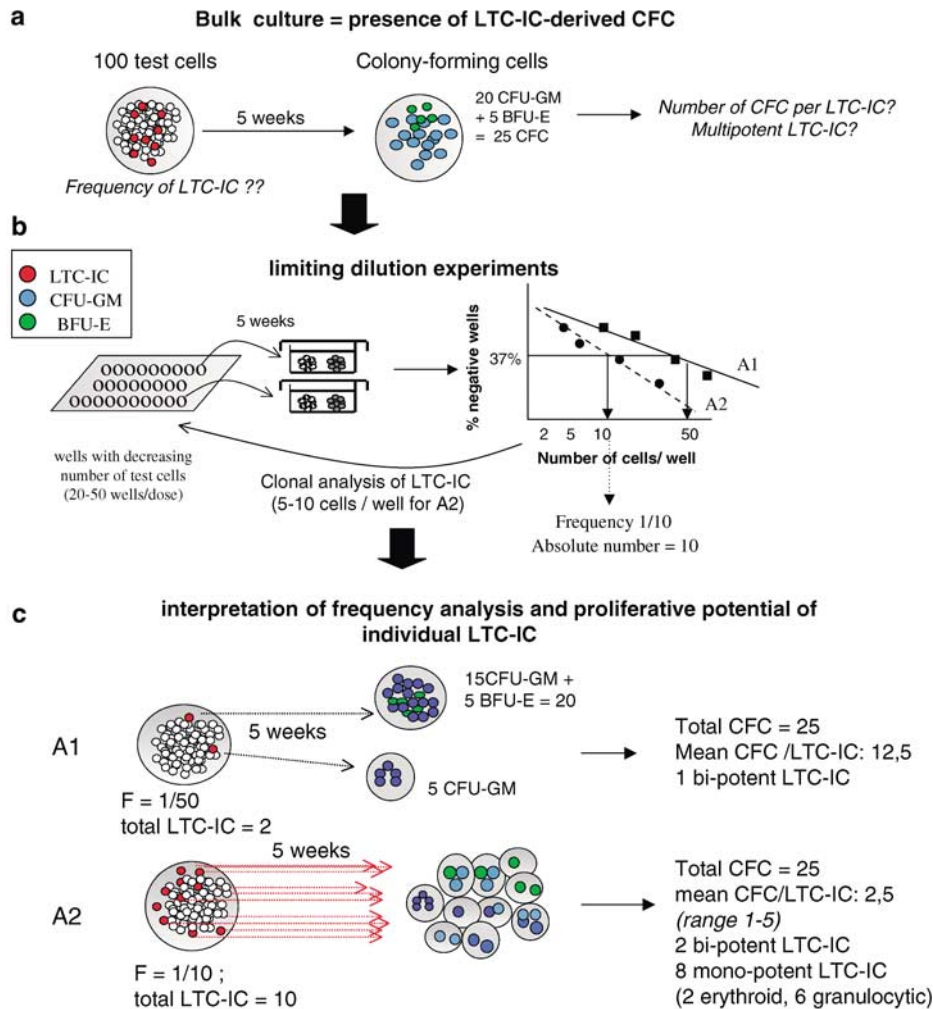


Figure 2 Rationale and procedure for quantitative assays. The example shown is that of LTC-IC, but this could apply to any other *in vivo* or *in vitro* assay. In (a) 100 cells are plated in long-term cultures and generate, after 5 weeks, a certain end point (here 25 CFC). This gives no information on the number of input LTC-IC (red) and on their individual potential. (b) To determine these two parameters, a limiting dilution experiment is performed, where decreasing numbers of cells are incubated as in (a), and individual wells are plated in colony assays after 5 weeks. The proportion of positive (negative) wells is defined (a positive well is defined as containing at least one CFC) and plotted against the number of input cells. Poisson statistics are used to calculate the frequency of LTC-IC in the input suspension. From this frequency, the absolute number of LTC-IC is calculated and the mean number of CFC per LTC-IC deduced. Precise assessment of the potential of individual LTC-IC can be assessed by plating wells with a number of cells, which gives less than one LTC-IC per well (deduced from the curve in (b))

(Sutherland *et al.*, 1991; Issaad *et al.*, 1993; Thiemann *et al.*, 1998; Punzel *et al.*, 1999a).

The presence of LTC-IC in the inoculum is identified retrospectively by the output of CFC in the culture after 5–8 weeks (Sutherland *et al.*, 1989) (Figure 2), but this gives no indication of the number of contributing input LTC-IC; the frequency of LTC-IC can be evaluated in limiting dilution experiments using Poisson's statistics (Sutherland *et al.*, 1990; Croisille *et al.*, 1994; Pettengell *et al.*, 1994) (Figure 2; Table 1). Comparison of a number of physical and phenotypic (e.g. CD38 expression) properties of CFC and LTC-IC and the 20–30-fold lower frequency of the latter have placed LTC-IC upstream of CFC. However, this LTC-IC compartment is highly heterogeneous: the number of clonogenic CFCs

produced at week 5 in the same conditions varies widely among LTC-ICs, from one to more than 50–100 CFCs per each LTC-IC (Petzer *et al.*, 1996; Zandstra *et al.*, 1997). In addition, if most LTC-IC produce only colony-forming unit granulocyte–macrophage (CFU-GM), CFU-G and/or CFU-M, 20–30% will also produce BFU-E, proving that some are multipotent (Issaad *et al.*, 1993) (Figure 2). In an attempt to identify a cell whose longevity will be closer to that expected from a stem cell, some have extended the LTC-IC assay up to 10–12 weeks and identified an 'extended LTC-IC or ELTC-IC', but its meaning is unclear and it most likely indicates the prolonged quiescence of the input cell (Hao *et al.*, 1996). Whether or not these differences identify a hierarchy of LTC-IC at different

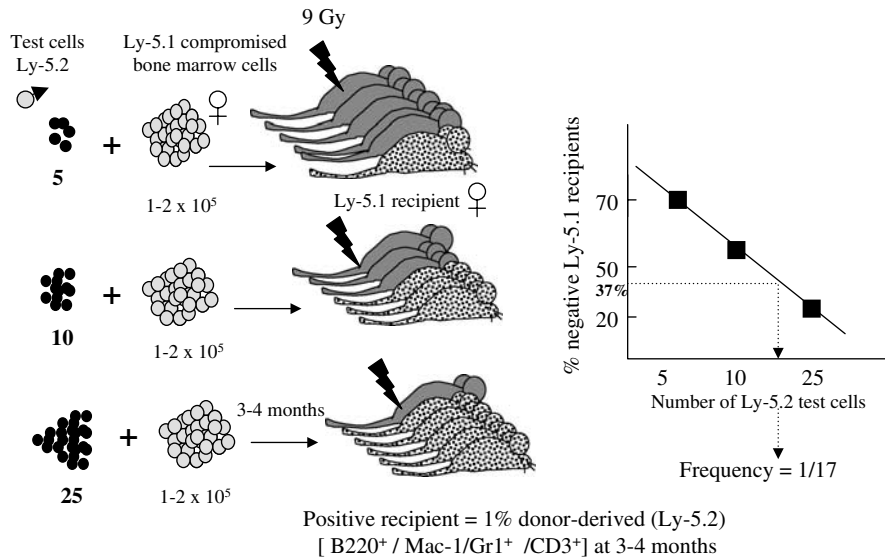


Figure 3 Procedure for the CRU assay to quantify murine stem cells *in vivo*. Decreasing numbers of test cells, which differ from the recipient cells by a sex marker (here male) or a congenic marker (here Ly-5 polymorphism), are injected to groups of female recipients that were lethally irradiated. A fixed number of compromised cells of the same strain and gender as those of the recipient are injected as radioprotective. They contribute minimally to the long-term repopulation. After several weeks, the recipients are killed and the proportion of positive recipients is determined (here, a positive recipient, dotted mice, has been defined as containing at least 1% donor-derived multilineage cells in the peripheral blood). Since there is a linear relationship between the number of injected cells and the proportion of negative recipients, Poisson's statistics can be applied to derive the frequency of CRU

Table 1 Frequencies of human immature progenitors in different hematopoietic tissues

Source/phenotype	LTC-IC ^a	SRC	CAFC
<i>Fetal liver cells</i>			
CD45 ⁺	0.7/10 ³	0.2/10 ³	
CD34 ⁺		0.09–0.2/10 ³	
CD34 ⁺ 38 ⁻	40/10 ³	25/10 ³	
<i>Cord blood cells</i>			
MNC	0.07–0.4/10 ³	0.002–0.03/10 ^{3b}	0.3–1/10 ³
CD34 ⁺	4–100/10 ³	0.06–0.2/10 ³	40–100/10 ³
CD34 ⁺ 38 ^{low/neg}	100–500/10 ³	1–2/10 ³	
<i>Adult bone marrow</i>			
MNC	0.03–0.1/10 ³	0.003/10 ³	0.3/10 ^{3c}
CD34 ⁺	1–80/10 ³	0.008–0.02/10 ³	0.36/10 ³
CD34 ⁺ CD38 ⁻	20–100/10 ³	0.1–1/10 ^{3b}	
<i>Mobilized peripheral blood</i>			
MNC	0.004→0.1/10 ^{3d}	0.0002/10 ³	
CD34 ⁺	2→20/10 ³	0.01/10 ³	15/10 ³
CD34 ⁺ CD38 ⁻	4–6/10 ³		

MNC: light-density mononuclear cells. ^aVariations in the values are explained by the different feeders used in the LTC-IC assays. ^bVariation is partly explained by the use of NOD-SCID or NOD-SCIDβ2M recipients. ^cSome 'false' cobblestone areas may be detected when the assay is performed with MNC. ^dArrow indicates the shift in the values when unmanipulated and mobilized peripheral blood (post-G-CSF) are used

maturation stages or reflect the stochastic induction of differentiation in an homogeneous population is unknown.

Differentiated lymphoid cells are typically not detected in LTC maintained in standard myeloid conditions, although their progenitors are present (Phillips, 1980); however, in these cultures, the differentiation of B-cell progenitors can be easily induced by switching the medium from 'myeloid' to 'lymphoid' by withdrawing horse serum, hydrocortisone and lowering the proportion of fetal calf serum (Whitlock *et al.*, 1984). These 'switch' conditions have led to the identification of various lymphomyeloid progenitors (Berardi *et al.*, 1997) such as LTC-IC_{ML} (Lemieux *et al.*, 1995) or ML-IC (Punzel *et al.*, 1999b). Interestingly, the same feeder cells are equally competent for the support of myeloid and B lymphoid differentiation of LTC-IC. T cells are never produced, although their progenitors are maintained, and some LTC-IC may have this potential.

Murine CAFC counted on days 28–35 copurify with primitive stem cells giving long-term repopulation in irradiated recipients (Ploemacher *et al.*, 1991; Szilvassy and Cory, 1993) (Table 2), and in human beings, week-6 CAFC are thought to be equivalent to the week 5 LTC-IC (Pettengell *et al.*, 1994), and week 12 CAFC to ELTC-IC, although this has been questioned (Denning-Kendall *et al.*, 2003) (Table 1). This one-step CAFC assay is often preferred to LTC-IC because of its easy visual screening, but the assay must be performed at limiting dilutions to avoid overlap of cobblestone areas; it gives no information on the heterogeneity or multipotency of individual CAFC, and stromal cells must be prescreened since only some will integrate CAFC.

Thus, if LTC-IC and CAFC are the best approximation of an *in vitro* stem cell assay in human beings, the

Table 2 Frequencies and absolute numbers of selected populations of mouse hematopoietic cells in different tissues

Source/phenotype	LTC-IC	CAFC d35	CFU-S d12 ^a	CRU/LTRC ^a
<i>Fetal liver cells</i>				
Unfractionated Sca1 ⁺ Lin ⁻		2.7–5.0/10 ⁵ —	1/10 ⁴ (2300 tot) (1/10 ⁴ or 950 tot) —	1/17 000 or 1100 (1/12 000 or 700) 1/39 (1/88)
<i>Adult bone marrow</i>				
5FU – unfractionated	1/27–50 000	0.7–1.8/10 ⁵	1/5700 (3500 tot) (1700 tot)	1/13 000 (1/220 000)
5FU – in prestimulation media for 2 days ^b				1/2500
Sca ⁺ Lin ⁻	1/42 (1/84)	1/15	2.4/10 ⁵	1/40–1/76
Endoglin ⁺ Sca ⁺ Rh ^{low} ^c				1/3
Lin ⁻ SP Rho ^{-d}				1/2.5
SP ^e tip ^e CD34 ⁻ C-Kit ⁺ Sca1 ⁺ Lin ⁻				1/1

LTC-IC: long-term culture-initiating cell; CAFC: cobblestone area-forming cell; CFU-S d12: colony-forming unit spleen day 12; LTRC: long-term repopulating cells (HSCs); CRU: competitive repopulation unit. ^aNumbers and proportions within parentheses indicate data obtained in *W^{kit}* mutant recipients. ^bPrestimulation media includes serum and IL-3 + IL-6 + SF as described (Sauvageau *et al.*, 1995). Determination of CRU frequency performed by Jana Krosil and Guy Sauvageau. ^cChang-Zheng Chen, *et al.* (2003). *Immunity*, **19**, 525–533. ^dUchida N *et al.* (2003). *Exp Hematol*, **31**, 1338–1347. ^eMatsuzaki Y *et al.* (2004). *Immunity*, **20**, 87–93

rapid loss of erythroid potential, the lack of lymphoid differentiation and the heterogeneity of these progenitors should be kept in mind.

Particularities in the assessment of lymphoid development

Experimental conditions that allow lymphoid differentiation of primitive progenitors have been established in 1990 in human beings, but 15 years earlier in the mouse. This illustrates the divergence in the control of human and murine lymphoid differentiation, which contrasts with the similarity of controls operating in the myeloid lineages of both species. A second difficulty in human beings is that lymphopoiesis is evaluated in a xenogenic environment, which explains why mature functional B- and T-lymphocytes are not produced restricting the experimental exploration to the early compartment of lymphoid progenitors. This has now been successfully carried out as described in a very recent publication (see below and Traggiai *et al.*, 2004).

However, the lack of biological assays has not hampered our understanding of the progression from stem cells to mature immune effectors; rather than on the identification of individual progenitors in functional assays as in myeloid lineages, establishment of the lymphoid hierarchy relies on the molecular dissection of V(D)J gene rearrangements associated to the ordered sequence of T- or B-cell receptors (TCR or BCR), TCR- β -selection and positive or negative selection of thymocytes. The major input of *in vitro* or *in vivo* assays in the mouse has been to help understand at what early stage of the hematopoietic hierarchy lymphoid and myeloerythroid potentials begin to segregate. This has been carried out essentially by analysing *in vitro* the potential of single cells purified from fetal tissues (fetal liver, thymus) or adult bone marrow based on the combination of cell surface antigens. These studies have led to the identification of a common lymphoid progenitor (Kondo *et al.*, 1997) devoid of myeloid potential, and also to a large number of marrow precursors with multiple lymphocyte differentiation possibilities and

reduced myeloid differentiation potential both in mice (Yokota *et al.*, 2003) and human beings (Hao *et al.*, 2001; Reynaud *et al.*, 2003), leading to disparate models of B lymphopoiesis in which filiation with one another remains unclear (Kincade *et al.*, 2000). Cell surface molecules such as (B220, AA4.1, CD43, C-Kit, CD24) have contributed to this progress since they allow the purification of functionally distinct populations. Unfortunately, the mouse B-cell populations defined by these surface antigens are not shared with human beings (Reynaud *et al.*, 2003).

In mice, the Rag-deficient mutants, which lack conventional populations of B and T cells, provide an excellent background for studying the molecular and cellular mechanisms of murine lymphoid differentiation *in vivo* (Chen *et al.*, 1994; Spanopoulou, 1996). In contrast, designing appropriate lymphoid assays *in vitro* face specific difficulties, which are heightened in humans: (1) T-cell lymphoid differentiation (and perhaps commitment) occurs outside the bone marrow in the thymic cortex and medulla, but thymic-derived stromal cells do not support T lymphopoiesis from primitive progenitors. (2) Our knowledge of growth factors and other molecules controlling early lymphoid differentiation is poor and those operational in mice (IL-7, FLT-3L) are not so critical in humans. (3) Age-related decrease in the lymphoid potential of primitive progenitors explains why most lymphoid assays in human beings are performed with fetal and not adult bone marrow- or peripheral-mobilized cells, which compromises the study of immune reconstitution posttransplantation or viral diseases such as HIV.

(a) *B-cell assays*: Pre-B (CFU pre-B) (Paige *et al.*, 1984; Lemieux and Eaves, 1996) and NK precursor cells have been identified in semi-solid colony assays in mouse but not human beings. Discrimination of B from non-B colonies on the visual aspect of the colony is not accurate enough to bypass the analysis of B220 or CD19 expression on plucked colonies. B-cell potential is therefore frequently assessed in liquid culture on stromal feeders. Murine Dexter-type long-term bone marrow

cultures have been adapted very rapidly to the identification of B-lineage progenitors (Whitlock *et al.*, 1984), a strategy that regularly failed in standard human beings LTC (LeBien, 1989). This was solved when murine stromal feeders were substituted for human marrow-derived adherent cells, which has become the standard procedure to identify the B-potential of test cells (Sys-1, S17, MS-5, AFT024) as those used for LTC-IC (Cumano *et al.*, 1990; Baum *et al.*, 1992; Rawlings *et al.*, 1997; Robin *et al.*, 1999b; Barker and Verfaillie, 2000); the detection of B220⁺ and/or CD19⁺ cells after 5–6 weeks in low serum conditions identifies the presence of B-cell progenitors in the inoculum. Although maturation of human B-cells stops at the pre-B/immature B-cells, with very few IgM⁺ cells, it can be promoted by the addition of CD40 ligand and IL-4 (Fluckiger *et al.*, 1998). Human cytokines are usually unnecessary unless very early progenitors are assessed, and the real benefit of IL-7 or FLT-3L on human B-cell precursors is unclear, in contrast to the well-recognized role of IL-7 in murine B-cell differentiation. Cytokines may even be deleterious in human B-cell assays, by promoting the development of granulocytes and macrophages that inhibit B-cell development through the release of cytokines such as interferon α (Lin *et al.*, 1998), IL-6 (Nakamura *et al.*, 2004) or others.

This observation points to a serious problem, which is the antagonism between the conditions required for B- and granulomacrophagic or B- and T-cell differentiation; this complicates the simultaneous detection of both potentials from single multipotent cells. Since the same stromal feeder (MS-5, S17) usually supports both myeloid and B-cell pathways, this can be solved by either switching the medium after 2 weeks or by finding a compromise in culture ingredients, which allows the development of both lineages, although neither will be optimal (Hao *et al.*, 1998; Robin *et al.*, 1999b).

(b) *T-cell assays*: The progression from triple negative thymocytes to mature T cells *in vitro* depends on the reconstitution of a thymic three-dimensional architecture such as that realized in fetal thymus organ culture (FTOC) or reaggregates cultures (Anderson *et al.*, 1996), and monolayers of thymic stromal cells almost never substitute for the intact thymic environment (Rosenzweig *et al.*, 1996). However, this has been recently questioned by the induction of extrathymic differentiation in murine primitive progenitors (CD117⁺ Sca1^{hi} CD24^{lo} Lin⁻) grown on a stromal cell line (OP-9) expressing the Delta-1 Notch ligand (Schmitt and Zuniga-Pflucker, 2002). The Notch-signaling pathway has emerged as a crucial determinant of T-cell commitment (Radtko *et al.*, 2004) and its ligands as potent modulators of cell fate.

For years, T-cell differentiation in mice has been assessed *in vitro* in FTOC, a procedure recently applied to identify human T-cell progenitors as well (Plum *et al.*, 1994; Robin *et al.*, 1999a). Thymic lobes from most mouse strains can be used, provided that they are collected at E14, and depleted of their endogenous T-cell populations, which is unnecessary if thymuses from immune-deficient SCID or nonobese diabetic (NOD)-

SCID strains are used (Plum *et al.*, 2000). A small fraction of cells first enter the thymus, a specific recognition/migration process which is poorly understood, and differentiates through the ordered sequence described *in vivo* through the double-positive (DP) CD4⁺CD8⁺ stage to the single positively selected CD4⁺ or CD8⁺ T cells if test cells are of murine origin (Anderson *et al.*, 1996). This process is very successful in mice and can even be applied to analyse single cells (Godin *et al.*, 1995; Kawamoto *et al.*, 1997), but is fraught with several limitations in chimeric human/mouse FTOC: proliferation is low, and human T-cell differentiation stops at the DP cells with very limited further T-lymphocyte selection (Barcena *et al.*, 1994; Plum *et al.*, 1994; Robin *et al.*, 1999a; Weekx *et al.*, 2000). Quantification of T-cell progenitors by limiting dilutions (and even single cells can be assessed in reaggregate cultures) can be attained in FTOC and as low as 100 CD34⁺ cord blood cells can generate CD4^{high} T cells in this system (Robin *et al.*, 1999a). Although the rationale to use human embryonic thymus is obvious, this is not feasible: these tissues are rare, and HLA typing would be necessary to discriminate recipient from allogeneic donor cells (Galy *et al.*, 1995).

However, it is likely that the assessment of human T-cell differentiation will dramatically improve in the near future, since three novel strategies have been recently successfully introduced: (1) as mentioned above, the OP9 murine stromal cells expressing the Notch ligand Delta-1 allows the T-cell differentiation of early murine progenitor cells (Schmitt and Zuniga-Pflucker, 2002) and embryonic stem cells (Schmitt *et al.*, 2004) in the absence of a thymic environment. Although not yet adapted to human cells, these conditions should facilitate analysis of the T-cell potential of individual cells. However, Delta-1 inhibits B-cell differentiation (Jaleco *et al.*, 2001), an antagonism that may hamper the simultaneous detection of B and T-cells from single progenitors. (2) Second, robust T-cell proliferation and differentiation have been recently obtained in the thymus of NOD-SCID mice treated by anti-CD122 antibody (Kerre *et al.*, 2002), and in NOD-SCID γ c^{-/-} mice (Hiramatsu *et al.*, 2003), after the infusion of human fetal CD34⁺ cells. (3) Finally, injection of CD34⁺ cord blood cells directly into the liver of newborn RAG- γ c-null mice has led to the development of a functional adaptive immune function (Traggiai *et al.*, 2004).

In vivo assays

A hierarchy of transplantable hematopoietic cells has been defined in the murine system (and to a lesser extent human) using *in vivo* assays, which also assess different types of progenitor/stem cells. Two end points are measured, longevity and multipotentiality. Whereas longevity of *in vitro* assays is expressed in weeks, it is measured in months for *in vivo* assays, allowing to identify 'true' murine stem cells with certainty by their ability to function (i.e. produce a lymphomyeloid progeny). Finally, the ability to home to the bone

marrow of recipients, previously considered as a poorly controlled variable leading to underestimation of the number of stem cells, may now be viewed as a cardinal criteria defining an LTRC (Benveniste *et al.*, 2003; Matsuzaki *et al.*, 2004).

Identification and quantification of long-term reconstituting cells in mice Schematically, a continuum of transplantable cells exists, which are classified in three groups: short-lived cells such as day 12 CFU-S (colony-forming unit spleen) that form mixed colonies in the spleen of the recipient in 12 days (McCulloch and Till, 1964; Magli *et al.*, 1982); second, pre-CFU-S (Hodgson and Bradley, 1979) and marrow-repopulating activity (MRA) (Lord and Woolford, 1993), identified in the bone marrow of recipients 2 weeks after transplantation, and defined by their ability to produce CFU-S in secondary recipients; third, long-term reconstituting cells (LTRC) that produce differentiated cells of multiple lymphoid and myeloid lineages for months in bone marrow and peripheral lymphoid organs. These long-lived clones are best identified by analysing donor granulocytes, T- and B-lymphocytes in the peripheral blood of recipients 3–4 months after transplantation. Thus, *in vivo*, longevity, rather than multipotentiality, which also characterizes CFU-S and MRA, is the best criteria of stem cell ‘activity’. Importantly, the majority of long-lived clones are multipotent (Jordan *et al.*, 1990; Keller and Snodgrass, 1990).

Even if the presence of donor-derived peripheral blood cells in recipients acknowledges the activity of LTRC, it provides no information on the number of functional clones. This information is crucial to explore if external signals (Reya *et al.*, 2003) or selected transduced genes (Sauvageau *et al.*, 1995), which lead to an increase in engraftment, act through cell recruitment or increased proliferation of existing clones. As described for LTC-IC, this can be carried out by analysing *in vivo* the proportion of single cells, which will read out as LTRC after exposure to these signals (Reya *et al.*, 2003; Takano *et al.*, 2004); however, quantification is more readily attained based on the competitive repopulating unit (CRU) assay: Decreasing number of donor test cells (differentiated from recipient and radioprotective cells by a specific sex or congenic marker) are injected and the proportion of positive recipients for a donor-derived repopulation (see later) is measured (Figure 3). However, since recipients are lethally irradiated, short-lived radioprotective progenitors (containing CFU-S and MRA) must be coinjected with test cells to prevent cell death caused by myeloablation, since purified test cells may require several weeks to produce effector cells (Jones *et al.*, 1990). In the original description, the protective ‘helper’ cells were derived from normal bone marrow cells of the same gender as that of the recipient and had been serially transplanted to compromise their long-term potential and minimize competition with donor test cells (Szilvassy *et al.*, 1990). Thus, test cells will ‘compete’ with the few endogenous stem cells spared by the conditioning

regimen and, to a lesser degree, with the helper cells. Practically, decreasing numbers of test cells are injected with a fixed number ($1-2 \times 10^5$) of compromised radioprotective cells (Figure 3). In these CRU assays, the criteria used for effective repopulation changed over the years depending on the method used (e.g. Southern blot versus flow cytometry). Current criteria, mostly dependent on flow cytometry analysis, require that $\geq 1\%$ of donor-derived cells in both myeloid (Mac-1⁺) and lymphoid (B220⁺) lineages are identified 3–4 months following transplantation. A more rigorous assessment would include T cells and erythroid repopulation as well (Trevisan and Iscove, 1995). CRU frequencies are then calculated using Poisson’s statistics, which predict that mice are reconstituted by a single LTRC when 63% of hosts are reconstituted (Table 2).

The CRU assay was initially performed using myeloablated recipient. CRU detection in C-Kit mutant (e.g. W41/W41) mice is easier to assess because reconstitution by single HSCs is very dominant in this setting (quantum effect) (Miller and Eaves, 1997; Antonchuk *et al.*, 2001).

As mentioned earlier, it was previously believed that the number of CRUs in the input suspension was underestimated because HSCs were believed to seed with an efficiency of 5–20%. However, this is a matter of debate, because single cells from a highly purified subset of murine bone marrow have shown a 100% efficient homing ability (Benveniste *et al.*, 2003; Matsuzaki *et al.*, 2004).

Identification of primitive human progenitors using immune-deficient mice If longevity *in vivo* is the most reliable parameter to identify murine LTRC function, then how can we measure this activity with human cells? At first, non-human primates could appear as more adapted than mice for identification of human stem cells given their size (allowing iterative samplings) and lifespan; however, the immunological barrier requires transplanting human cells *in utero* at a precise period when fetuses are immunologically tolerant. This strategy has been successfully pioneered by the group of E Zanjani (Srouf *et al.*, 1992; Zanjani *et al.*, 1994), which convincingly showed the persistence of human cells several years after their transplant in sheep; and they have also highlighted important species-specific differences between mice versus large animal models for assessing stem cells *in vivo*. An alternative which has also been explored in monkeys is to work in an autologous transplant setting (Norol *et al.*, 2002; Horn *et al.*, 2003) and extrapolate the results to the human situation. In both models, costs and space limit the use of such hosts.

These limitations prompted the development of a mouse model. Lethally irradiated wild-type recipients are not suitable and recipients with a genetic defect leading to a permanent and profound immune deficiency tolerating xenografts have been used. Since the first description by J Dick in 1988, many strains of engraftment of human cells in bg/nu/xid mice (Kamel-Reid and Dick, 1988) have been tested. Interestingly,

immune deficiency *per se* does not guarantee successful engraftment, and the genetic background of the recipient has to be taken into account: thus human cells do not develop when injected intravenously in 6- to 8-week-old *RAG*^{-/-} mice or *RAG*^{-/-} hosts crossed to γ c-null mice, but they do extensively proliferate and differentiate if injected in the liver of these newborn mice (Traggiai *et al.*, 2004). SCID (Kamel-Reid and Dick, 1988; Lapidot *et al.*, 1992; Vormoor *et al.*, 1994) and NOD-SCID (Larochelle *et al.*, 1996; Pflumio *et al.*, 1996; Cashman *et al.*, 1997) strains accept human transplants. However, the striking preferential development of B-cells (often >80% human cells), and the lack of T- and NK-cell differentiations in these strains has raised some concerns about the normal development of human stem cells in the murine environment. Reducing or abolishing NK activity by genetic manipulation of the hosts (NOD-SCID β 2-microglobulin-null (Kollet *et al.*, 2000) and NOD-SCID γ c^{-/-} (Hiramatsu *et al.*, 2003) or by infusing antibodies against the asialoprotein or the IL-2R β (Kerre *et al.*, 2002; Shultz *et al.*, 2003) have strikingly improved permissiveness towards human T and even NK lymphoid differentiation. This underlines the important role of NK cells and additional host innate immune factors, and suggests that adapted models will be designed to allow the full differentiative potential of human primitive progenitors to be assessed.

Despite their immune deficiency and hypersensitivity to ionizing radiation, immune-deficient hosts require mild irradiation (3.5–4 Gy) to allow the implantation of human cells in mouse tissues, and the dose of irradiation has been positively correlated with the proportion of engrafted human cells. Surprisingly, there is no need for supplementing mice with human cytokines if more than 500 cells are injected (Bonnet *et al.*, 1999); this is in keeping with the ability of murine stromal cells to support LTC-IC in the absence of any additional human cytokines, which indicates that signals regulating early progenitors either cross species barriers or if human specific, are produced in sufficient amounts by the transplanted human cells.

Evaluation of human hematopoiesis in the bone marrow, spleen and thymus of the recipients at various time points has revealed two compartments of transplantable hematopoietic cells with distinct biological properties. Short-term repopulating cells are detected within 2 weeks after the graft by their production of myeloid cells (particularly erythroid) and disappear 4–6 weeks later (Glimm *et al.*, 2001). They are identified only in NOD-SCID γ c^{-/-} or NOD-SCID β 2M, or after NOD-SCID treatment by anti-asialoprotein (Ishihara *et al.*, 1992) or anti-CD122, perhaps because they are very sensitive to NK cell rejection (Hogan *et al.*, 2002; Shultz *et al.*, 2003). Whether or not they have any physiological relevance in clinical transplantation is unknown.

The progeny of long-term repopulating cells (called here human SCID-repopulating cell or huSRC) is evaluated around 8–10 weeks. Typically, the proportion of human CD45⁺ cells in the bone marrow of NOD-SCID recipients injected with 10–50 × 10³ CD34⁺ cord blood cells, which is highly variable at weeks 4–6

(1–20% CD45⁺ cells) stabilizes by around 30–60% at weeks 10–12 (Pflumio *et al.*, 1996; Cashman *et al.*, 1997). At any time point, CD19⁺ B cells predominate from CD34⁺CD19⁺ pro-B in the bone marrow to CD19⁺IgM⁺ immature B-cells located in the spleen, indicating that human pre-B cells have migrated to this peripheral lymphoid organ (Hogan *et al.*, 1997). In contrast, and as expected, differentiated cells from the myeloid lineages are poorly represented, but there is a relative inflation of CD34⁺ cells. In contrast to murine transplant assays, where engraftment is evaluated by analysing peripheral blood cells, here, because of their reduced terminal differentiation and poor marrow egress, the characterization of these human CD34⁺ precursors requires to be analysed using *in vitro* assays seeded with the recipient's bone marrow. These assays show normal relative proportions of clonogenic cells and LTC-IC (Pflumio *et al.*, 1996; Cashman *et al.*, 1997).

Defining the place of huSRC in the hematopoietic hierarchy is difficult and relies on its phenotype and frequency, calculated in limiting dilution experiments where test cells are injected with carrier cells as described to quantify murine CRU (Wang *et al.*, 1997; Bhatia *et al.*, 1997; Conneally *et al.*, 1997) (Table 1). However, since analysing donor differentiated cells in the host peripheral blood is not possible here, multiple alternative criteria have been proposed to define a 'positive' recipient, thus introducing some confusion (see also Figure 4). Positivity can be defined by the presence of 0.1–1% human DNA in the bone marrow of recipients, or of at least 1% CD45⁺ human cells in the host marrow. However, these criteria will identify cells that probably do not repopulate both lymphoid and myeloid lineages. More discriminating read-outs include the simultaneous detection of CD34⁻CD19/20⁺ B-lineage cells and CD45/71⁺CD15/66b⁺ mature myeloid cells, or B cells and CFU-GM. In this case only, the assay detects the human counterpart of the murine CRU (Holyoake *et al.*, 1999) (Figure 4). The output of LTC-IC and CFC per CRU can also be calculated, which allows to compare the intrinsic potential of CRU from ontogenically different sources (Holyoake *et al.*, 1999). Use of these more or less specific end points explains the variation in the measures of engraftment, and in the extent of huSRC expansion in response to cytokines (Conneally *et al.*, 1997; Gammaitoni *et al.*, 2003; Rollini *et al.*, 2004). However, if mice are analysed long enough after the graft, the total level of engraftment may be considered as a measure of the population of lymphomyeloid repopulating cells as was demonstrated in mice (Jordan and Lemischka, 1990).

Marrow homing activity, which is bypassed *in vitro*, is a major protagonist *in vivo*; on a quantitative basis, it has been admitted that only a minor fraction of injected human cells returns to the bone marrow of recipients (around 5% for human CD34⁺ or CAFc), and that this property fluctuates with the position of the cells in the cell cycle (Cashman and Eaves, 2000; Srour *et al.*, 2001; Jetmore *et al.*, 2002; Szilvassy *et al.*, 2003; Uchida *et al.*, 2003) and is maximal in quiescent cells. These changes may interfere with the assay particularly after cytokine

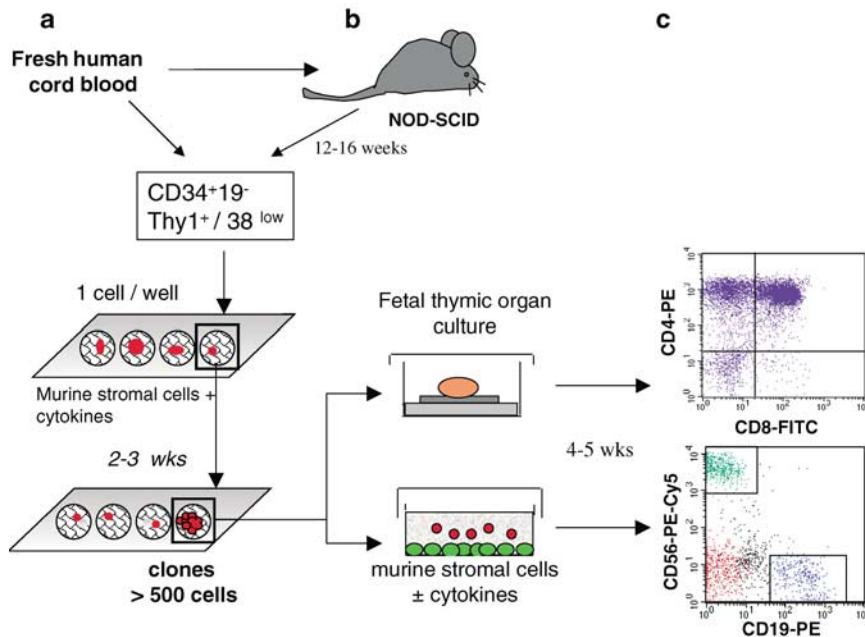


Figure 4 Identification at the clonal level of multipotent transplantable cells in human cord blood. Identification of these cells is done in three steps: in (a), cells are collected either from fresh cord blood or from the bone marrow of NOD-SCID mice transplanted 16 weeks before with CD34⁺ cord blood cells. Single CD34⁺CD19⁻CD38⁻ (or Thy-1⁺) are sorted and plated onto MS-5 murine stromal cells with cytokines, and clones that contain more than 500 cells are collected 2–3 weeks later. In (b), cells from each clone are divided in two aliquots: one is grown in conditions appropriate for the differentiation of B, NK and myeloid cells, and the other in an FTOC culture. In (c), the production of differentiated cells belonging to these lineages is evaluated 5 weeks later by flow cytometry: CD19⁺ B cells, CD56⁺ NK cells and C15⁺ myeloid cells are identified in stroma-based long-term cultures, and CD4⁺CD8⁺ thymocytes in FTOC. Since all these differentiated cells arose from a single cell, this proves the multipotency of the initial cells. Their frequency in cord blood can be calculated from the number of positive wells (from Robin *et al.*, 1999b)

exposure, which activates test cells. However, this should be re-examined in view of the very high homing efficiency of a subset of purified murine LTRC (Benveniste *et al.*, 2003; Matsuzaki *et al.*, 2004).

Clinical relevance of assays

A major application of CFC, LTC (myeloid and/or lymphoid) or SRC assays both in mouse and man, has been to sort out a hierarchy of relationships between the various classes of progenitors identified. But then, what is the value of such assays for the clinics? Based on analysis of frequency, position in the cell cycle, phenotypic markers, multiple studies in the mouse have shown that CRU assays, some LTC-IC, days 28–35 CAFC identify closely related populations although the degree of overlap is unknown (Szilvassy and Cory, 1993). In human beings, such precise comparisons have yet to be made. Results obtained in nonhuman primates which compare autologous reconstitution of genetically marked cells infused in parallel in irradiated primates and NOD-SCID mice have suggested that huSRC do not measure LTRC (Horn *et al.*, 2003) although this issue remains unresolved (Morris *et al.*, 2004). However, in human beings, three parameters should be taken into consideration: (1) NOD-SCID mice rapidly develop thymic lymphomas and mortality is important beyond

the third month, a time which is much too short to pretend measuring human stem cells. Nevertheless, one can argue that this is valid, since in the mouse or sheep, measuring the end points at 10 weeks or 4 months yield equivalent conclusions. (2) More problematic is the ontogeny-related decrease in the frequency and potential of human primitive progenitors, which precludes any long-term analysis of the function of huSRC/CRU from adult stem cells as opposed to fetal cells, and most successful studies demonstrating regeneration or amplification of huSRC/CRU have been carried with fetal human sources (Conneally *et al.*, 1997; Gammaitoni *et al.*, 2003; Rollini *et al.*, 2004). (3) There is fluctuation in the homing properties, as mentioned above.

The clinical usefulness of human hematopoietic assays remains relatively unexplored: few studies have tried to correlate the numbers of LTC-IC or huSRC with the outcome of transplantation (Breems *et al.*, 1996) or appraisal of the quality of the graft, which is achieved by measuring the amount of CD34⁺ cells. The length and constraints inherent to the realization of LTC-IC and huSRC assays are an obvious explanation. A huge amount of work has been dedicated to find conditions that will lead to a net increase in LTC-IC and huSRC, with no loss of their potential. Although this has been achieved by different combinations of cytokines and feeder cells, the magnitude of this increase does not

exceed a few-fold, which is disappointing for a therapeutic goal (McNiece and Briddell, 2001), but the recent description of novel critical signals (bioactive Wnt3A, Notch ligands, BIO, recombinant Hoxb4) may change this view (Ohishi *et al.*, 2002; Amsellem *et al.*, 2003; Krosl *et al.*, 2003; Murdoch *et al.*, 2003; Sato *et al.*, 2004).

A second clinical impact of these assays is about their role in the characterization of leukemic stem cells in disorders such as CML (Lapidot *et al.*, 1994; Sutherland *et al.*, 1996; Lewis *et al.*, 1998; Wang *et al.*, 1998) and AML (Bonnet and Dick, 1997), and the discrimination between residual normal versus leukemic progenitors based on their differential behavior in biological assays (Coulombel *et al.*, 1983b), which has formed the basis of therapeutic strategies (Chang *et al.*, 1986; Eaves *et al.*, 1998). This has led to the experimental demonstration that the leukemic population can also be organized in a

hierarchy with only a small subset of leukemic cells proliferating extensively and generating a large population of leukemic cells which even though cancerous have lost some of their proliferative potential (Sutherland *et al.*, 1996; Bonnet and Dick, 1997, see also Warner *et al.*, 2004, this issue). This concept of tumoral stem cells has now been extended to solid tumors (Pardal *et al.*, 2003) (see articles by Al-Haji and Sing and Dirks, 2004, this issue; Kondo *et al.*, 2004).

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