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EDITORIAL

Cell division tracking and expansion of hematopoietic long-term repopulating cells

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The combined use of rigorous assays for quantitating transplantable stem cell numbers and precise cell labeling and tracking procedures have provided definitive evidence that stem cell self-renewal divisions can occur *in vitro* in the absence of stromal feeder layers. These findings set the stage for defining conditions that may alter the ability of these cells to maintain their primitive status when mitogenically activated.
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Clinical treatments involving the use of myeloablative regimens are dependent on the availability of a graft containing a sufficient number of hematopoietic stem cells to ensure permanent reconstitution of the entire hematopoietic system. In recent years, much interest has arisen in the potential of previously unexplored sources of hematopoietic stem cells which could benefit from, or require, being exposed to conditions that induce their proliferation and expansion *ex vivo* prior to being transplanted. Over the last few years, many studies have described cytokine combinations that support the production *in vitro* of large numbers of murine and human myeloid progenitors detectable as colony-forming cells (CFC) in semi-solid cultures. However, it is also now widely appreciated that very few, if any, of these cells have long-term *in vivo* engrafting potential. Moreover, a unique phenotype that can be functionally associated with this latter activity has not yet been identified. Thus, progress in addressing whether and how the expansion of hematopoietic stem cells can be achieved *in vitro* has been limited to studies that have used quantitative methods to analyze changes in the number of cells with long-term multi-lineage repopulating activity before and after culture (eg using the competitive repopulating unit, or CRU, assay[1]).

Because primary marrow stromal cell layers have been known for a long time to be capable of stimulating the proliferation and differentiation of very primitive hematopoietic cells,[2–4] a number of investigators have sought to determine whether stem cell self-renewal divisions can also occur in stromal cell-containing cultures. As summarized in Table 1, simple measurements of the total numbers of *in vivo* repopulating cells (CRU) present after various periods of time have shown that there is a rapid decline in this population within a few weeks in cultures containing feeders of primary marrow cells.[5–8] Nevertheless, clonal analysis[9] and retroviral marking[5] strategies have both demonstrated that this net decline occurs in the face of a significant expansion of a small proportion of the input CRU. Somewhat better overall results have since been obtained by groups using various cell lines, such as the S17 line, CFC034, 2012, AFT024,[10,11] AGMS3[12] and DAS104–4[13] cells as feeders. Recent studies have suggested that the improved stem cell maintenance obtained with some of these cells may be associated with their expression of notch ligands[14,15] or cytokines that signal through gp130, like LIF.[16]

Table 1 Stem cell numbers in cultures containing stroma

Stroma	*Days of culture*	*Effect on stem cell numbers (relative to input)*	*Ref.*
S17	7	Decrease	7
DAS104-4	7	Maintenance	13
S17	10	Decrease	21
S17	14	Decrease	16
S17 (+LIF)	14	Maintenance	16
S17	21	Maintenance	10
2012	21	Maintenance	10
CFC0342	21	Maintenance	10
NMF[a]	28	Decrease	5
NMF[a]	28	Decrease	6
NMF[a]	28	Decrease	7
NMF[a]	28	Decrease	8
2012	28	Maintenance	10
AFT024	28	Maintenance	10
AFT024	42	Maintenance	11

[a]Normal marrow-derived feeder layer.

With the cloning of the first cytokines known to be expressed by stromal cells, their potential to stimulate CRU amplification *in vitro* in the absence of stroma was explored. As summarized in Table 2, incubation of murine stem cell-containing suspensions in media supplemented with serum and Steel factor (SF, also known as stem cell factor or c-kit-ligand) plus one or more of IL-6, IL-11, Flt3-ligand (FL), or thrombopoietin (TPO)[17–19] has usually been found to result in a slight reduction of the CRU population within the first 4 to 7 days, although in one report evidence of murine stem cell expansion after 6 days in culture with SF plus IL-11 was inferred from serial transplant experiments.[20] In contrast, the use of serum-free media plus a combination of SF and IL-11 with[21] or without FL[18] has, for the first time, allowed modest net increases in murine CRU to be obtained *in vitro*. Similarly, when $CD34^+CD38^-$ cells isolated from human cord blood have been cultured in serum-free media supplemented with SF, FL, IL-3, IL-6, G-CSF, small but significant increases in the number of human cells able to regenerate lymphoid and myeloid cells in irradiated immunodeficient mice were demonstrable.[22,23] In 4 day cultures with murine cells, inclusion of anti-TGF-β was also found to improve CRU maintenance and allow a ~4-fold increase in repopulating cells to be achieved.[24]

Interestingly, inclusion of even low concentrations of either IL-1 or IL-3 was found to abrogate the repopulating cell expansions that would otherwise have occurred in the cultures of murine cells.[25] Negative effects of IL-3 on the *in vitro* expansion of human long-term culture-initiating cells (LTC-

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Table 2 Stem cell numbers in cultures without stroma

Serum	*Source of stem cells*	*Cytokines present*	*Days of culture*	*Effect on stem cell numbers (relative to input)*	*Ref.*
15%	Mouse	SF/IL-3/IL-6/TGF-β	4	Decrease	24
15%	Mouse	SF/IL-3/IL-6/anti-TGF-β	4	Increase	24
None	Human	SF/FL/IL-3/IL-6/G-CSF	4	Increase	23
None	Mouse	SF/FL/IL-11	5	Increase	21
None	Human	SF/FL/IL-3/IL-6/G-CSF	5–8	Increase	22
30%	Mouse	SF/IL-6	7	Decrease	35
20%	Mouse	TPO	7	Decrease	19
20%	Mouse	FL/TPO or FL/IL-6	7	Decrease	19
None	Mouse	SF/FL/IL-11/TPO	10	Maintenance	21
None	Mouse	SF/FL/IL-11	10	Increase	21
20%	Mouse	FL/IL-11	14	Increase	18
20%	Mouse	SF/IL-11	14	Increase	18

IC), a population that is very closely related to CRU,[22] has also been demonstrated.[26] However, the relative concentrations of IL-3 that elicit this type of negative effect on human stem cell self-renewal appear to be higher than the concentrations of IL-3 that have a similar effect on murine stem cells.

Given the minimal expansions of stem cell numbers seen to date, the question as to whether the repopulating cells detected in cultured populations are the result of cell division (self-renewal events) has remained controversial. To investigate this question, we and others have begun to look specifically into the cell division kinetics of repopulating cells stimulated *in vitro* with various cytokines. Cell divisions can be tracked by time course studies of cells labeled with fluorescent dyes such as PKH-2,[27] PKH-26,[27–29] PKH-67[13] and 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE).[30,31] All of these markers confer a measurable and relatively stable level of fluorescence on the cells initially labeled. Their subsequent proliferation activity can then be inferred from the detection of a decreased level of fluorescence. Conversely, their failure to divide (without loss of viability) is indicated by the maintenance of a highly fluorescent phenotype.

In one such study, mouse bone marrow cells were labeled with PKH-2 and then cultured for 7 days in the presence of SF, IL-3 and IL-6, at the end of which the majority of the repopulating cells still detectable were found to be contained within a population of cells whose fluorescence could not be distinguished from the starting population. This was interpreted as evidence that the initial stem cells had remained quiescent over this entire period.[27] Similar results were obtained when more stringently sorted lin$^-$Sca-1$^+$c-kit$^+$ cells were cultured with either TPO or FL/Il-6.[19] On the other hand, the vast majority of human lin$^-$CD34$^+$Thy-1$^+$ bone marrow cells stained with PKH-26 and cultured in TPO plus SF and FL[29] could be shown to have divided within 6 days. Although this was not accompanied by a net expansion of cells with *in vivo* repopulating activity, all such cells were in the fraction of cells that had divided (PKH-26^{lo}). The latter result suggests that some stem cell self-renewal divisions can take place in cultures lacking stromal cells, even though these do not occur at a frequency sufficient to counteract a concomitant loss of stem cell activity (due to their differentiation and/or death).

Both PKH-2 and PKH-26 have thus been powerful tools to document kinetic changes *in vitro* in populations of primitive hematopoietic cells. Nevertheless, the heterogeneity in staining intensity obtained with either of these dyes does not allow small differences in the numbers of divisions executed to be resolved. For example, in some of the studies just described, the possibility that one or two stem cell self-renewal divisions had occurred could not be excluded because of the breadth of the gate used to sort the PKH-2^{bright},[27] or PKH-26^{high}[19,29] or PKH-67^{high}[13] cells which spanned several (sometimes up to nine) division equivalents. The high resolution tracking possible with CFSE-labeling[31] obviates these problems and may thus be better suited to such studies.

CFSE was first used to track the homing of lymphocytes[32] and hepatocytes.[33] After cells are exposed to CFSE, the intensity of cellular fluorescence decreases rapidly in the first 15 h,[31,32] but then stabilizes. This allows cells to be reliably followed for at least 8 weeks[32] unless they proliferate, which causes their fluorescence to be reduced with each cell division. In contrast to the PKH dyes mentioned, for CFSE it has been formally demonstrated by cell cycle experiments that the fluorescence of labeled cells is exactly halved at each successive mitosis.[34] CFSE labeling could thus be used to establish the B cell differentiation events that occur in sequential cell generations.[30] The improvement of the division tracking method implemented by Nordon *et al*[31] underscores the prediction of Hodgkin *et al*[30] that CFSE 'potentially allows the generation of comprehensive differentiation maps for stem cells undergoing development *in vitro* and *in vivo*.'

We have recently used CFSE labeling to show that the majority of lineage marker-negative (lin$^-$) mouse bone marrow cells cultured in serum-free medium supplemented with FL, SF and IL-11 will undergo up to five divisions within 3 days and up to seven divisions within 4 days. In addition we found that the rate at which these cells proliferate *in vivo* (post-transplant) is similar (manuscript in preparation). Interestingly, when the proliferative history of the repopulating cells (CRU) in the cultures was examined, the majority of these still detectable after 3 days were in the fraction that had not yet divided whereas, 1 day later, CRU were detected exclusively in the fraction of cells that had divided. These studies provide independent confirmation by cell division tracking that totipotent hematopoietic stem cells defined by the most rigorous quantitative assay available will proliferate *in vitro* in defined media in response to FL, SF and IL-11 stimulation. Since this cytokine combination can also produce a net increase in CRU numbers,[21] it would appear that this increase must be largely due to CRU cell division (self-renewal) events. This system now offers interesting opportunities to explore the intracellular signaling steps that are necessary for this response to be achieved.

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