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Self-renewal and lineage restriction of hematopoietic stem cells

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Over the past decade, the purification and characterization of hematopoietic stem cells have ascertained their presence at the clonal level although they had hitherto existed conceptually. Now we have begun to understand their functions in molecular terms. Several important works indicative of such a new era in stem cell biology have been published recently. In particular, Bmi1, which belongs to the Polycomb group of genes, has been implicated as one of the basic molecules to maintain the proliferation capacity in hematopoietic stem cells. We need to seek other similarly important molecules for their functions. Perhaps studying interactions among genes is one of the most exciting subjects in stem cell research.

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Abbreviations

CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CRU	competitive repopulating unit
E	erythroblast
ES	embryonic stem
HSCs	hematopoietic stem cells
KO	knockout
KSL	Kit ⁺ , Sca-1 ⁺ , Lin ⁻
m	macrophage
M	megakaryocyte
MAS	mean activity in stem cell
n	neutrophil
PcG	Polycomb group

Introduction

Hematopoietic stem cells (HSCs) are defined by their two functions: self-renewal and multilineage differentiation capabilities. The two major goals of HSC study are to understand these capacities at the molecular level and to clarify the mechanisms regulating self-renewal and lineage commitment. It is well known that serial transplantation eventually results in poor reconstitution. Our recent quantitative assessment of *in vivo* self-renewal at the

clonal level has suggested that the self-renewal capacity of HSCs is extensive but also limited. Early work by Hayflick demonstrated that cultured normal human cells have a population doubling limit. Telomerase is a ribonucleoprotein enzyme consisting of a catalytic reverse transcriptase component (TERT) and an RNA template component. Cell senescence is associated with telomere shortening in human somatic cells where telomerase is not expressed. A low level of telomerase activity has been detected in an HSC population, but it is not sufficient for preventing progressive shortening of their telomeres after transplantation. It was expected that overexpression of TERT may lead to enhancement of their self-renewal ability. However, it has recently been shown that this is not the case [1•].

From our point of view, there are two research directions to address the limit of self-renewal capacity. One is a search for molecules responsible for this limitation. Enforced gene expression and gene knockout (KO) studies have provided great information on positive and negative regulators for proliferation ability at the level of HSCs as briefly summarized herein. The other is to study developmental hematopoiesis. The HSC pool is enormously expanded during development, but not much in adult bone marrow and spleen. Self-renewal and lineage restriction of HSCs should be spatially and temporally regulated.

A simple explanation for *in vivo* HSC expansion would be that certain embryonic cells can do what adult HSCs cannot. Adult HSCs have been detected as long-term multilineage repopulating cells by transplantation assay using lethally irradiated mice as recipients. Novel assays would be needed to know the existence of embryonic HSCs with limitless self-renewal capacity, if they are in hematopoietic organs from embryonic though adult life. The recent discovery of multipotent adult progenitor cells [2] and observation of HSC expansion in long-term bone marrow culture with FGF-1 [3] have suggested that certain types of culture system can be applied to their detection. Alternatively, adult HSCs may acquire properties of embryonic ones as a result of epigenetic changes induced by *in vitro* culture.

Another possibility is that HSCs must stay in proper niches to self-renew: once they are removed from such sites, they may be destined to differentiate. To verify this attractive idea, the niche localization has to be specified in hematopoietic tissues and molecules interplaying between HSCs and their microenvironment should be identified.

Molecular basis for self-renewal and lineage commitment

To better understand the molecular basis of HSC self-renewal and lineage restriction, several candidate genes have recently been the focus of interest. Humphries and co-workers have studied members of the clustered homeobox gene family extensively. Some of these genes have already been shown to play roles in definitive hematopoiesis. In particular, Overexpression of *HoxB4* in HSCs resulted in a ~50-fold expansion of competitive repopulating units (CRUs) *in vivo* [4]. Furthermore, it has been described that there is a remarkable growth advantage in *HoxB4*-transduced HSCs over untransduced ones when cultured *in vitro* [5]. *Pbx1* encoding a TALE homeodomain transcription factor has been considered to interact with *HoxB4*. It has been reported that knock down of endogenous *Pbx1* expression enhances *in vivo* expansion of *HoxB4*-transduced HSCs, suggesting that *Pbx1* suppresses the effects of *HoxB4* [6]. Despite a large increase in CRU number *in vivo* as well as *in vitro*, the quality of each CRU has never been evaluated. It is important to compare the amount of repopulating activity in each CRU regenerated with that in normal CRU before expansion. We have suggested the mean activity in stem cell (MAS, RU/CRU) be used for this purpose [7]. It is known that embryonic stem (ES) cells do not contribute to hematopoietic reconstitution when transplanted into myeloablated adult mice. Surprisingly, the ectopic expression of *HoxB4* has been shown to support the development of HSCs from ES cells *in vitro* [8]. *HoxB4* can be one of the important molecules regulating repopulating activity, but it is unclear how this gene works in HSC development from ES cells and their expansion in culture.

The Polycomb group (PcG) genes and the counteracting trithorax group genes have been implicated as upstream regulators of Hox genes. Among PcG proteins, *Bmi1*, *Rae28*, *M33*, *Mph1*, *Ring1a* and *Ring1b* together constitute a multimeric complex. Null mutation mice for these genes similarly display skeletal transformations and hematopoietic defects. Regarding the function of HSCs, *Bmi1*- or *Rae28*-deficient mice have exhibited the progressive hematopoietic defect with increase of age. This defect has appeared more severe in *Bmi1* than in *Rae28* [9,10,11]. Long-term repopulating activity in fetal liver and adult bone marrow cells of *Bmi1*-deficient mice was too low to be detected, indicating a crucial role of *Bmi1* in maintenance of proliferation capacities of HSCs and progenitors [10,12]. In addition, the work has provided an excellent insight into how *Bmi1* functions in leukemic stem cell emergence [10].

β -catenin is a component of the Wnt signaling pathway. Its activating mutations have been implicated in tumorigenesis. Very recently, it has been demonstrated that overexpression of such a stable form of β -catenin expands

the pool of HSCs *in vitro* [13]. As β -catenin-expressing HSCs could grow consistently in culture with SCF alone for two months, special attention should be paid to their leukemic transformation. Purification of Wnt proteins has been difficult because of their inefficient secretion from transfectants and insolubility. At last, however, several Wnt proteins have been successfully purified and Wnt3a protein has thus been shown to act on HSCs as a growth factor [14]. The data suggest that it can induce the self-renewal of HSCs *in vitro*. Frizzled proteins are receptors for Wnt proteins. Certain members of the Frizzled family are expressed in HSCs. Their binding specificities to members of the Wnt family remain obscure. It is of interest to examine whether or not any particular members of Wnt proteins have different effects on HSCs through their specific receptor signaling.

HSCs reside in a small subpopulation of bone marrow cells termed the 'side population' when stained with the fluorescent dye Hoechst 33342. The side-population phenotype of HSCs is due to their expression of *Bcrp1*, an ATP binding cassette transporter that is responsible for dye efflux. The dye efflux property of HSCs does not seem directly linked with their self-renewal and differentiation capacities [15].

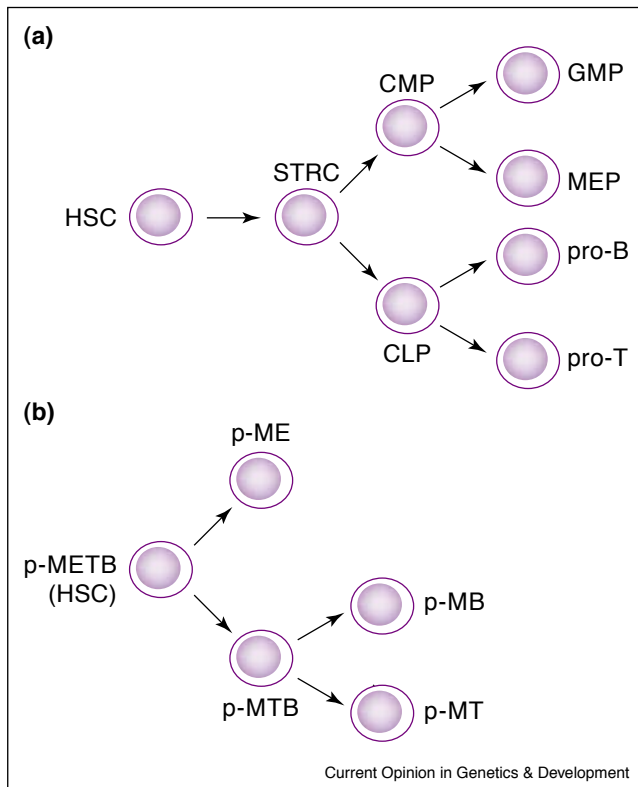
KO mouse studies have suggested that some transcriptional factors like SCL, GATA-2 and AML1 play essential roles in early development or specification of HSCs. As these mutant mice are embryonic lethal, the roles of these genes in the function of adult HSCs have not been elucidated. Conditional gene targeting is useful for addressing this problem. Studies of SCL conditional KO mice have revealed that continued expression of SCL is not required for HSC function but their differentiation along erythroid and megakaryocytic lineages is perturbed at the level of progenitors [16,17].

It is too complex to study the molecular basis of *in vivo* lineage commitment of HSCs. If we have an *in vitro* model system to work on, analysis of transgenic or KO mice would provide us more valuable information on molecules of interest.

The mode for hematopoietic stem cell differentiation

Two interesting models have recently been proposed to explain how lineage-restricted progenitors are generated from HSCs (Figure 1). Akashi, Weissman *et al.* have proposed that lineage commitment of HSCs first takes place when their progeny with multilineage differentiation potential give rise to common myeloid progenitors (CMPs) [18] and common lymphoid progenitors (CLPs) [19]. This model is based on phenotypic identification of CMPs and CLPs in adult mouse bone marrow. They have similarly shown the presence of CMPs and CLPs in the fetal liver [19,20]. This model seemed to have been

Figure 1



HSC differentiation models (a,b). Common myeloid progenitor (CMP), common lymphoid progenitor (CLP), megakaryocyte-erythrocyte-restricted progenitor (MEP), granulocyte-monocyte-restricted progenitor (GMP), short-term repopulating cell (STRC), myeloid, erythroid, T and B cell progenitor (p-METB), myeloerythroid progenitor (p-ME), myeloid, T and B cell progenitor (p-MTB), myeloid and B cell progenitor (p-MB), myeloid and T cell progenitor (p-MT).

accepted by many researchers. However, in their proposal, a progenitor that gives rise to both CMP and CLP is still obscure. The 'short-term repopulating cell' is a candidate for such an intermediate cell, but the differentiation potential of this cell has not been clarified at the clonal level. Short-term repopulating cells likely represent a heterogeneous population in differentiation potential. Jacobsen and co-workers have found an interesting subpopulation in Kit^+ , Sca-1^+ , Lin^- (KSL) bone marrow cells [21]. KSL cells were subdivided into Flt-3-positive (FL^+KSL) and -negative (FL^-KSL) fractions. The majority of $\text{CD34}^-\text{KSL}$ cells highly enriched for HSCs were included in FL^-KSL fraction. FL^+KSL were able to give rise to CLP and neutrophil and macrophage (nm) progenitors, but not to erythroblasts (E) and megakaryocytes (M). These data are consistent with our own unpublished results. Moreover, T cell development occurring independently of CLP has been suggested recently [21]. We have obtained data showing that $\text{CD34}^-\text{KSL}$ cells asymmetrically give rise to nmEM and uni-, bi-, or tri-potent progenitors (H Ema, unpublished data). Thus, a

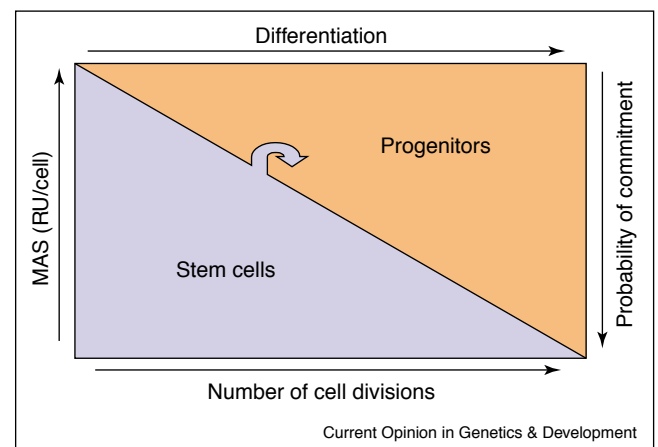
progenitor for CMP and CLP may not lie at the main branching point for lineage commitment of HSCs.

Kawamoto, Katsura *et al.* [22[•]] have proposed another interesting model for HSC differentiation, as shown in Figure 1b. They have analyzed the differentiation potential of fractionated fetal liver cells. A unique culture called the multilineage progenitor assay has been used to detect myeloid, B- and T-cell lineage differentiation potentials. The multilineage progenitor assay has been invented by modifying fetal thymic organ culture. In addition to T cell progenitor (T), B cell (B), myeloid (M, namely nm) and E progenitors can be detected by this assay. They demonstrated the presence of clonogenic progenitor cells for METB, MTB, ME, MT and MB lineages in KSL fetal liver cells [22[•]]. They have claimed that CLPs have never been detected in the fetal liver, contradicting the observation by Akashi, Weissman *et al.* [19]. As it was reported that some fetal liver CLPs had macrophage differentiation potential, p-MTB can be equivalent to CLP. There are two unique points in their model. First, the lineage commitment of HSCs is defined by the appearance of progenitor cells for either the ME or MTB lineage (Figure 1b). Second, B- or T-cell differentiation potential is accompanied by myeloid differentiation potential in the early stages of lineage restriction. This model is only based on detection of a variety of distinct progenitor cells. To verify the model, each subset of progenitors should be prospectively separated and characterized. Of greater interest is a relationship between HSCs and their committed progeny.

The concept for self-renewal and lineage commitment

In general, HSCs are considered to make a choice of either self-renewing or committing to differentiation. Their fate decision may not be as simple as this. First,

Figure 2



Generation-age hypothesis proposed by Rosendaal, Hodgson and Bradley [23]. The authors have added the MAS to their model.

apoptosis should also be tightly regulated in HSCs. Second, HSCs show a variety of repopulating activity levels, suggesting their heterogeneity in nature. The generation-age hypothesis [23] is our preferred concept [24]. We have incorporated the MAS into this idea (Figure 2). As stem cells divide, the amount of repopulating activity per cell is progressively reduced. Simultaneously, the likelihood of lineage commitment increases. MAS level can be interpreted as an indicator of degree of self-renewal capacity [7]. In the strict sense, a stem cell may not regenerate daughter cells with the exact same activity. Therefore, HSCs should be measured quantitatively to evaluate self-renewal capacity. Lineage commitment in HSCs can be considered to occur when at least one of their differentiation potentials is lost. Dedifferentiation of progenitors has never been formally described despite its possibility [25].

Conclusions

On the basis of gene-expression studies of an HSC population, genes of interest have been selected and characterized mostly by using gene overexpression and targeting strategies. Because a number of genes expressed in HSCs have been known by now [26–28], more genes are likely to be found to play crucial roles in HSC functions. For a large-scale screening of functional genes, application of RNA interference may serve as a powerful tool. Sooner or later, the complexity of multiple gene expression in HSCs should be better understood. So far, signal transduction in HSCs is poorly understood despite the growing knowledge of various signaling molecules in many other cell types. This lack of understanding stems from the difficulty in obtaining the sufficient number of HSCs for such experiments, but this may be resolved through the use, for instance, of KO mice in which HSCs proliferate or *in vitro* culture. Epigenetic regulation of gene expression in HSCs may also be of great interest.

Acknowledgements

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