Humanizing Bone Marrow in Immune-Deficient Mice

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Abstract Humanized mice are useful for studying human hematopoietic stem cells (HSCs) and their niche. In particular, clonal study of human HSC enables precise comparison of in vivo behavior between murine and human HSCs. A single HSC is able to reconstitute hematopoiesis even after serial transplantations in mice. While the life span of somatic cells is over that of individual in mice, this is not the case in humans. Clonal studies of human HSCs clearly demonstrated their aging in hosts. Since murine studies have demonstrated that HSCs are protected from aging by their niche in bone marrow, the humanizing niche model will reveal the precise mechanism by which human HSCs are protected from exhaustion in vivo. Direct transplantation of human mesenchymal stem cells into mouse bone marrow results in reconstitution of the functional human hematopoietic microenvironment comprised of pericytes, myofibroblasts, reticular cells, osteocytes in bone, bone-lining osteoblasts, and endothelial cells. These humanized mouse models are essential for testing whether the insights on hematopoiesis from mouse studies are applicable to humans before clinical application.

Abbreviations ALP: alkaline phosphatase; BM: bone marrow; DP: double positive; FACS: fluorescence-activated cell sorter; GFP: green fluorescent protein; HME: Hematopoietic microenvironment; HMRC: hematopoietic microenvironment-reconstituting cells; HSC: hematopoietic stem cell; IBMT:

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intra-bone marrow transplantation; IL-2: interleukin 2; LAM-PCR: linear amplification-mediated-PCR; MSC: mesenchymal stem cell; PCR: polymerase chain reaction; PHA: phytohemagglutinin; SDF-1: stromal cell-derived factor 1; SRC: severe combined immunodeficiency (*scid*) mouse-repopulating cells; YFP: yellow fluorescent protein

1 Introduction

Production of blood cells over a lifetime is sustained by hematopoietic stem cells (HSCs) that are defined as cells having both the capacity for self-renewal and the ability to produce all mature hematopoietic lineages. The presence of HSCs was first demonstrated retrospectively by a gene-marking study [28]. Prospective identification was impossible until the successful purification of a single HSC with defined surface markers [30, 39, 50]. Then clonal studies of serial transplanted HSCs demonstrated that they intrinsically limit their potential for self-renewal, and the mean activity of individual stem cells was reduced with cell division [15]. Therefore, HSCs cannot maintain their immaturity by themselves but require a special environment, or niche, to keep themselves dormant in vivo for protection from exhaustion. Recent studies identify two types of stem cell niche in bone marrow (BM), the osteoblastic niche and the vascular niche, and several key molecules such as jagged-1, N-cadherin, CXCR-4, and angiopoietin-1 for niche function [3, 9, 25, 48, 55]. All of these essential findings on HSCs and the niche come from mouse studies. It is not clear whether these findings can be extrapolated to large animals including humans, since it is reported that HSCs behave differently between mice and felines [1]. Therefore, these findings must be tested in human HSCs before translation into clinical application.

To evaluate human HSC activity, xenotransplantation models are recognized as the most reliable surrogate assay. Immune-deficient mice have been widely used as recipients, since myeloid and lymphoid reconstitution can be easily attained in these mice by the transplantation of human HSCs [27, 45]. Severe combined immunodeficiency (*scid*) mouse-repopulating cell (SRC) assay has demonstrated characteristic features of human HSCs such as self-renewal and multilineage differentiation ability, frequency, surface markers, organization of hierarchy, and dynamic behaviors in vivo [5, 6, 7, 12, 16, 20, 31, 40, 49]. On the other hand, the human hematopoietic microenvironment has not been studied in vivo because of the lack of an appropriate model. Recently, Muguruma et al. established a human mesenchymal stem cell (MSC)-derived hematopoietic microenvironment-reconstituting cell (HMRC) model in immune-deficient mice [35].

In this review, we summarize recent results on clonal analysis of human HSCs and human microenvironment in the xenotransplantation model.

2 Clonal Analysis of Human Hematopoietic Stem Cells in NOG Mice

Unlike murine HSCs that have been purified and analyzed at the single-cell level, retrovirus-mediated gene marking is the only strategy for in vivo clonal analysis of human HSCs. Since retrovirus vectors essentially integrate at random, each genomic integration site serves as a distinct clonal marker that can be used to trace the progeny of individual stem cells after transplantation. Gene-marking studies in SRC assays have successfully elucidated the in vivo kinetics of human HSCs such as proliferation, self-renewal, and multilineage differentiation [2, 18, 32, 33, 37].

A major shortcoming of using the NOD/SCID mouse model is a lack of reproducible human T-lymphocyte repopulation. Consequently, the multilineage differentiation capacity of SRCs in NOD/SCID recipients has been assessed by reconstitution of only B-lymphoid and myeloid lineages. Since a close relationship between B-lymphocyte and macrophage differentiation has been indicated [8, 14, 21, 34], current analyses cannot clearly distinguish true HSCs from lineage-restricted progenitors such as B-lymphocyte/macrophage progenitors. Thus, the multilineage differentiation and self-renewal of HSCs represented by a single SRC are yet to be proven.

NOD/SCID/γc^{null} (NOG) mice were recently generated by crossing NOD/Shi-*scid* mice with mice expressing a form of the IL-2R γ chain lacking the cytoplasmic region that were reported to have defective NK cells [22]. They have defective T, B, and NK cell activities so that they demonstrate human T cell development in their thymus, in addition to myeloid and B-lymphoid reconstitution, when transplanted with $CD34^+$ cells. Furthermore, these T cells bear polyclonal $\alpha\beta$ TCR and respond not only to mitogenic stimuli, such as PHA and IL-2, but also to allogenic human cells. These results indicate that functional human T lymphocytes can be reconstituted from $CD34^+$ cells in NOG mice [19, 46, 52].

HSCs can be identified as thymus-repopulating cells and distinguished from short-lived oligo- or monopotent progenitors in NOG mice. Thymopoiesis requires constant recruitment of progenitors into the thymus, which eventually produces mature T-lymphocytes in a relatively short period of time [17]. Therefore, to maintain thymopoiesis in recipient mice, transplanted HSCs must divide without loss of thymus-repopulating activity. While several classes of SRCs that differ in their proliferative and self-renewal potential have been reported [16, 20, 31], analyzing the thymus-repopulating activity of these cells provides a unique way to distinguish and identify long-term self-renewing stem cells within the SRCs.

Yahata et al. established a novel strategy to analyze both self-renewal and multilineage differentiation of a single human thymus-repopulating SRC clone in NOG recipient mice [54] using linear amplification-mediated-PCR (LAM-PCR) that verifies individual genomic virus integration sites by direct sequencing [44] (Fig. 1). The identification of specific clones in fluorescence-activated cell sorter (FACS)-sorted lymphomyeloid lineage populations by their unique molecular markers allowed us to assess how individual clones contribute to the specific lineages during long-term

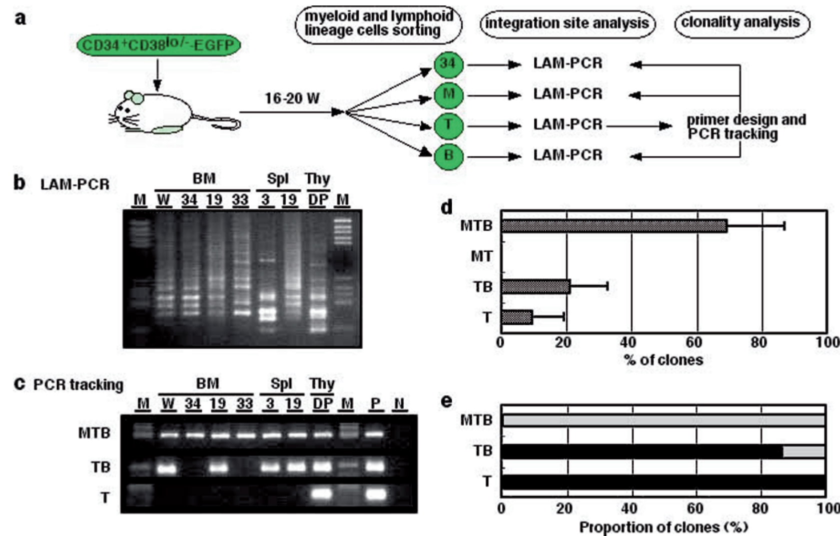

Fig. 1 Clonal analysis of primary transplanted SRCs [54]. **a** Study design. **b** Representative LAM-PCR profiles of SRCs. Each band represents a different insertion locus in the assayed material. **c** DP-derived T-lymphoid insertion sites were traced by PCR. The clones detected in all lymphomyeloid lineage cells were designated as multipotent type (MTB). Clones restricted in T-lymphoid and B-lymphoid cells were p-TB. Clones detected in T-lymphoid cells were p-T. **d** Relative frequencies of each clone type detected in primary SRCs. **e** The proportion of clones detected in the $CD34^+$ cell population. *Gray bars* represent the clones detected in $CD34^+$ cells. *Black bars* represent the clones not detected in $CD34^+$ cells

hematopoiesis in vivo. They focused on the CD4/CD8 double-positive (DP) immature thymocyte population as a starting point for analysis of the clonal capacity of human HSCs. The study presents direct clonal evidence that a single human HSC had the capacity to produce lymphoid and myeloid lineage cells and that self-renewal division of multilineage clones resulted in expansion of SRC populations in primary and secondary NOG recipients during long-term hematopoiesis.

The finding that the same multipotent HSC clone was detected in paired secondary recipients (MTB-MTB type) indicates that a single SRC clone can self-replicate to produce two daughter cells with multilineage differentiation and self-renewal potential, leading to the in vivo expansion of SRCs. On the other hand, one of the daughter clones of the pair was no longer found in the $CD34^+$ cell population in approximately half of MTB-MTB clone pairs. Furthermore, the phenotype was not retained in 11.1% of MTB-MTB clones. Considering that 100% of MTB clones in primary recipients possess the stem cell phenotype, these results indicate that SRCs with the stem cell phenotype progressively decrease during serial transplantation, leading to exhaustion of SRCs. This is consistent with the finding that the proportion of clones with the stem cell phenotype decreased as the clone committed to specific lineages. By assessing the phenotype of self-replicated multilineage clone

pairs, determined by the presence of common clones in the CD34$^+$ cell population, they were able to reveal the status of HSCs during aging. It is possible that the extensive replication required for hematopoietic reconstitution in the recipient may result in loss of the stem cell phenotype. The data indicate that although the total SRC population appears to expand, the ability of individual SRCs may become restricted during long-term hematopoiesis in vivo.

3 Humanizing Hematopoietic Microenvironment in Mice

The stem cell niche is a key determinant of stem cell development. We are beginning to understand the murine HSC niche and the molecular mechanisms that govern the fate of murine HSCs [3, 9, 25, 48, 55], but there exists a paucity of data on the cellular and molecular microenvironmental regulation of human hematopoiesis in vivo , due largely to a lack of good experimental tools. Although the identification of SRCs has facilitated detailed characterization of human HSCs in vivo, the key niches that function in human cell repopulation have not been identified.

Mesenchymal stem cells (MSCs) present in BM are thought to give rise to cells that constitute the hematopoietic microenvironment (HME) [43]. MSCs have been isolated from BM and various tissues from humans and many other species, expanded in culture, and shown to differentiate into osteocytes, chondrocytes, adipocytes, and myoblasts under defined conditions in vitro [41]. In culture, MSCs produce a number of cytokines and extracellular matrix proteins and express cell adhesion molecules, all of which are involved in the regulation of hematopoiesis [13, 29]. They also support the development of hematopoietic colonies in vitro. However, in contrast to HSCs, MSCs have only been defined and isolated by physical and functional properties in vitro. Consequently, little is known about their phenotypic and functional characteristics in vivo.

Systemic administration of MSCs for facilitation of BM transplantation has been proposed based on the in vitro characteristics of MSCs [23]. In recent studies, cotransplantation of human MSCs and HSCs resulted in increased chimerism or accelerated hematopoietic recovery (or both) in animal models and in humans [26, 38], suggesting a role for MSCs in the engraftment and repopulation of HSCs. Although the existence of donor MSCs has been documented in the BM of recipient animals after MSC infusion [4], the methods used to detect engraftment, such as polymerase chain reaction (PCR) or staining of cytospin samples, could not unambiguously distinguish engraftment from cell survival or nonspecific lodgment on the vascular bed. There is no physical evidence that transplanted human MSCs have indeed engrafted in the BM of adult animals and directly participated in the enhanced engraftment of HSCs.

To assess the engraftment, spatial distribution, and lineage commitment of MSCs as well as their roles in hematopoiesis in vivo, Muguruma et al. transplanted green fluorescent protein (GFP)-marked human MSCs into the tibiae of NOD/SCID mice by intra-bone marrow transplantation (IBMT) [35], a method previously

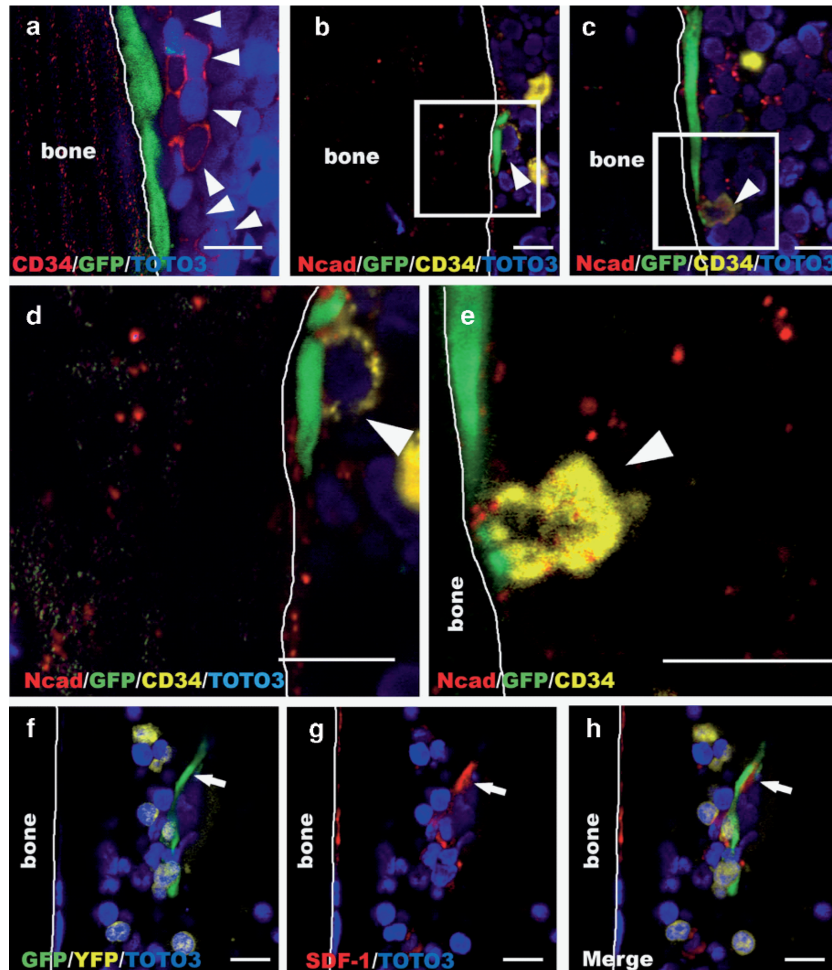

Fig. 2 Expression of N-cadherin and SDF-1 by HMRCs that interact with human hematopoietic cells [35]. **a** CD34+ cells (*arrowheads*) appear to colonize near the bone-lining HMRCs. **b–c** Bone-lining HMRCs colocalize with CD34+ cells (*arrowheads*) through the asymmetrical expression of N-cadherin. **d–e** Higher magnifications of **b** and **c**. **f–h** An HMRC in the endosteal hematopoietic parenchyma (*arrow*) expresses SDF-1 and interacts with a few YFP+ hematopoietic cells. All *bars* represent 10 μm

shown to improve the engraftment of both hematopoietic and nonhematopoietic cells in mice [24, 53].

The phenotypes of transplanted MSCs and their progeny at 10 weeks after transplantation were investigated in detail. MSCs were preferentially localized to the endosteal region (73.8 ± 18.4%), frequently within five cells from the surface of the bone. When human MSC-derived GFP-expressing cells (GFP+ cells) were found away from the endosteum, they were often associated with the vasculature.

Those vasculature-associated GFP+ cells expressed α-SM actin, the expression of which has been documented in pericytes, SM cells of the vascular wall, as well as myofibroblasts in BM [11]. A total of 59.9 ± 21.6% of GFP+ cells found in BM were positive for α-SM actin.

Two other types of GFP+ cells that were negative for α-SM actin were also present in BM: flattened cells located in the hematopoietic cords but not specifically associated with the vasculature and cells characterized by long cytoplasmic extensions, so-called reticular cells that are considered to be the predominant cells of the HME [51]. BM was often interspersed with a fine network of cell processes that expressed alkaline phosphatase (ALP), an enzyme that distinguishes reticular cells from the stromal component of acid phosphatase- or nonspecific esterase-expressing macrophages. A total of 28.2 ± 11.2% of GFP+ cells in BM were ALP positive.

In addition, GFP+ cells were found within or on the surface of the bone. Cells in the bone stained positive for osteocalcin, a specific marker of mature osteoblasts and osteocytes, indicating an active participation in skeletal remodeling. Furthermore, GFP+ cells on the bone surface resembled spindle-shaped osteoblasts, a key component of the stem cell niche in murine hematopoiesis [3, 9, 55]. These cells expressed osteopontin and N-cadherin, both of which are involved in regulating murine HSCs [36, 47]. These results indicate that within 10 weeks after transplantation, human MSCs differentiated into pericytes, myofibroblasts, reticular cells, osteocytes in bone, bone-lining osteoblasts, and endothelial cells, which constitute the three-dimensional structure of hematopoietic parenchyma and provide the milieu of hematopoiesis. These cells were designated as human MSC-derived hematopoietic microenvironment-reconstituting cells (HMRCs).

CD34+ cells adhered to HMRCs on the bone surface and appeared to proliferate along the endosteal surface, suggesting the existence of specific local signals between CD34+ cells and bone-lining HMRCs (Fig. 2a). Bone-lining HMRCs associated with CD34+ cells through the colocalization of N-cadherin (Fig. 2b-e). In addition, an HMRC in the endosteal hematopoietic parenchyma expressed stromal cell-derived factor 1 (SDF-1) and interacted with a few YFP+ human hematopoietic cells (Fig. 2f-h), although SDF-1 was not detected in ex vivo expanded MSCs by immunofluorescence analysis. In BM, SDF-1 is constitutively expressed by osteoblasts, endothelial cells, and BM stromal cells [42]. In addition to its well-established role in homing and retention of HSCs in BM, SDF-1 has been implicated in regulating the status of primitive HSCs both in vitro and in vivo [10]. Therefore, HMRCs may contribute to the maintenance of primitive human HSCs through N-cadherin-mediated interactions and the production of SDF-1.

4 Conclusion

Clonal study of human HSCs enables precise comparison of in vivo behavior between murine and human HSCs. A single HSC is able to reconstitute hematopoiesis even after serial transplantations in mice. While the life span of somatic cells is over that of individual in mice, this is not the case in humans. Clonal studies of

human HSCs clearly demonstrated their aging in hosts. Since mouse studies have been demonstrated that HSCs are protected from aging by their niche in BM, the humanizing niche model will reveal the precise mechanism by which human HSCs are protected from exhaustion in vivo.

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