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Hematopoietic Stem Cells Are Pluripotent and Not Just “Hematopoietic”

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Abstract

Over a decade ago, several preclinical transplantation studies suggested the striking concept of the tissue-reconstituting ability (often referred to as HSC plasticity) of hematopoietic stem cells (HSCs). While this heralded an exciting time of radically new therapies for disorders of many organs and tissues, the concept was soon mired in controversy and remained dormant for almost a decade. This commentary provides a concise review of evidence for HSC plasticity, including more recent findings based on single HSC transplantation in mouse and clinical transplantation studies. There is strong evidence for the concept that HSCs are pluripotent and are the source for the majority, if not all, of the cell types in our body. Also discussed are some biological and experimental issues that need to be considered in the future investigation of HSC plasticity.

Introduction

Many tissues and organs in our body possess variable but significant regenerative capacity. Some organs such as bone marrow (BM), skin, and the mucosa of the gastrointestinal tract are characterized by life-long cell turnover. In these organs the mature cells have finite life spans and are continually replaced by more primitive progenitor cells. Others such as skeletal muscle and liver are characterized by limited cell turnover in the steady-state, but are capable of significant regeneration following tissue injury and loss of constituent cells. To account for the cell turnover in these organs, the concept of stem cells, cells that can self-renew and generate progeny committed to differentiation in specific pathways, emerged decades ago. Further, it was generally held that stem cells possess organ/tissue specificity; for example, hematopoietic stem cells (HSCs) generate only blood cells.

Against this long-held belief, striking tissue-reconstituting capability of HSCs (often referred to as HSC plasticity) was reported about a decade ago and suggested exciting new avenues of therapy for disorders of many organs and tissues. However, these reports were soon followed by others with negative results and papers offering different interpretations, as exemplified in the disputes among a number of major laboratories [1–3]. Our laboratory has also been engaged in the studies of HSC plasticity using single-cell HSC transplantation and has obtained unequivocal evidence for the HSC-origin of fibroblasts/myofibroblasts,

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adipocytes, and osteo-chondrocytes, major cell types comprising connective tissue. This commentary consists of a brief summary of our studies of connective tissue and of studies reported from other laboratories indicating an HSC-origin of other major cell types. We believe these findings strongly support the concept that HSCs are the source for the majority, if not all, of the cell types in our body. Also discussed in this commentary are the clinical implications of these observations and a number of biological and experimental issues that need to be considered in the future investigation of HSC plasticity.

Single Hematopoietic Stem Cell Transplantation

Our studies were based on the belief that only single HSC transplantation provides definitive information about HSC plasticity. For this purpose, an efficient method for generating mice exhibiting high-level, multi-lineage hematopoietic engraftment was necessary. While a number of methods for single cell transplantation had been proposed [4–6], we did not find these to be sufficiently efficient for our purpose. We, therefore, devised a method combining single cell deposition of BM cells that are highly enriched for HSCs with short-term cell culture [7, 8]. As donors, we chose the mice that had been genetically engineered to ubiquitously express enhanced green fluorescent protein (EGFP) [9]. Here, Lin[−], Sca-1⁺, c-kit⁺, CD34[−] BM cells [5] or Lin[−], Sca-1⁺, CD34[−] side population (SP) [6] BM cells from transgenic EGFP mice were individually deposited into each of 96-well culture plates and cultured for one week in the presence of Steel factor (SF; c-kit ligand) and interleukin-11 or a combination of SF and granulocyte colony-stimulating factor (G-CSF). Previously, we had observed that both interleukin-11 and G-CSF, in synergy with SF [10] support proliferation of cell cycle dormant primitive multipotential progenitors. Because the majority of HSCs are dormant in cell cycle and do not begin cell division until a few days after initiation of the cell culture [8], transplantation of clones consisting of 20 or fewer cells after one week of incubation significantly raised the efficiency of generating mice with high level multilineage engraftment [7, 8]. Two months to one year after transplantation, only mice revealing high-level multi-lineage engraftment by donor EGFP⁺ cells were selected for studies of tissue reconstitution. In order to exclude the possibility that the observed results were artifacts of short-term cell culture, we also carried out transplantation of 100 un-cultured Lin[−], Sca-1⁺, c-kit⁺, CD34[−] BM cells in each study and made similar observations to those seen in the clonally engrafted mice. In most of the studies, we excluded the possibility of cell fusions by carrying out male-to-male or female-to-male transplantation and analyzing the number of Y-chromosomes in the EGFP⁺ cells.

Evidence for the Hematopoietic Stem Cell Origin of Connective Tissue Cells

Hematopoietic Stem Cell Origin of Fibroblasts/myofibroblasts

The studies based on single HSC transplantation yielded unequivocal evidence that the major cellular components of connective tissue, i.e., fibroblasts/myofibroblasts, adipocytes and osteo-chondrocytes are derived from HSCs. As summarized in a brief review [11], we discovered that HSCs generate via non-fusion mechanisms, many types of fibroblasts/myofibroblasts, including glomerular mesangial cells of the kidney [7], brain microglial cells and perivascular cells [12], tumor-associated fibroblasts/myofibroblasts [13], inner ear fibrocytes [14] and the fibroblasts/myofibroblasts in the adult heart valves [15]. Other laboratories, also using the single HSC transplantation approach, reported that fibroblasts/myofibroblasts seen at the site of myocardial infarction [16] and liver stellate cells [17] are also derived from HSCs. We have also succeeded in the culture of fibroblasts from EGFP⁺ BM cells from mice engrafted by a single HSC [18]. Time-course flow cytometric analyses of the cultured EGFP⁺ BM cells revealed gradual increase in expression of collagen I and

discoidin domain receptor 2 (DDR2), a collagen receptor, and concomitant gradual loss of CD45 during 3 weeks of incubation. As for the precursors of the fibroblasts, both fibroblast colony-forming units (CFU-F) [19, 20] and peripheral blood fibrocytes [21] were found to be derived from the bone marrow of the recipients of single HSC transplantation [18]. This cell culture study was consistent with the results of the transplantation studies and supported the concept of an HSC origin of fibroblasts/myofibroblasts and their progenitors [11].

Hematopoietic Stem Cell Origin of Adipocytes

We next confirmed the HSC-origin of adipocytes using both single HSC transplantation and primary culture [22]. When adipose tissues from mice reconstituted by a single EGFP⁺ HSC were examined, immunohistochemical analysis revealed the presence of EGFP⁺ adipocytes that stained positive for leptin, perilipin and fatty acid binding protein 4. Diet containing rosiglitazone, a peroxisome proliferator-activated receptor-gamma (PPAR γ) agonist, significantly enhanced the number of the EGFP⁺ adipocytes in the tissues of these mice. When EGFP⁺ bone marrow cells from the engrafted mice were cultured under adipogenic conditions, all the cultured cells stained positive with oil red O and sudan black B and exhibited the presence of abundant mRNA for many adipocyte markers. Finally, clonal adipocyte culture of hematopoietic progenitors that were sorted based on Mac-1 expression suggested that adipocytes are derived from HSCs via progenitors for monocytes/macrophages. We have observed EGFP⁺ adipocytes in both white and brown (unpublished observation) adipose tissues. While there had been a controversy in the literature regarding the ability of BM cells to give rise to adipocytes [23–26], our *in vivo* and *in vitro* studies unequivocally demonstrated the HSC origin of adipocytes.

Hematopoietic Stem Cell Origin of Osteo-chondrocytes

Bone and cartilage, together with tendon/ ligament and adipose tissues constitute mesenchymal connective tissues. Following documentation of the HSC origin of fibroblasts/myofibroblasts and adipocytes, we discovered that bone cells (osteoblasts and osteocytes) and chondrocytes are also derived from HSCs [27]. The paraffin sections of the bones and cartilages from the mice reconstituted with a single HSC revealed infrequent presence of EGFP⁺ osteocytes and chondrocytes. We interpreted this to reflect the known slow cellular turnover in these tissues [28]. In order to facilitate generation of new EGFP⁺ osteocytes and hypertrophic chondrocytes from HSCs, we induced non-stabilized tibial fracture in the engrafted mice. When mice were sacrificed at 7 days, 2 weeks and 2 months after fracture, the paraffin sections of the callus sites showed numerous EGFP⁺ hypertrophic chondrocytes, osteoblasts and osteocytes. These cells stained positive for Runx-2 and osteocalcin, supporting the concept that HSCs generate bone cells and chondrocytes. Our observations are consistent with the observation by Olmsted-Davis, et al. [29] that a single SP cell and 3000 SP cells can generate osteoblasts in culture and *in vivo*, respectively.

Earlier, Horwitz, et al. [30, 31] had performed clinical BM transplantation in children with osteogenesis imperfect (OI). OI is a genetic disorder resulting from an abnormal amount and/or structure of Type I collagen and is characterized by osteopenia, fragile bones and skeletal deformities. While statistical significance of their observations could not be established because of the small number of the cases, their clinical observations and laboratory parameters suggested the benefits of BM transplantation. Parallel with the single HSC transplantation, we tested the efficacy of HSC transplantation in a mouse model of OI [32]. Homozygous OI mice are excellent recipients for transplantation of normal HSCs because the fast turnover of osteoprogenitors has been observed [33, 34]. We transplanted 50 Lin[−] Sca-1⁺ c-kit⁺ CD34[−] SP BM cells from the EGFP mice into irradiated OI mice and analyzed changes in bone parameters using longitudinal micro-computed tomography (micro-CT) 3 to 6 months post-transplantation. When the mice showed high levels of

hematopoietic engraftment, dramatic improvements in 3D micro-CT images of long bones from these mice were observed. Morphometric assessment of the bone parameters showed an increase in bone volume, trabecular number and trabecular thickness with a concomitant decrease in trabecular spacing. Analysis of a non-engrafted mouse and another mouse that was transplanted with BM cells from OI mice showed continued deterioration in the bone parameters. These findings supported the clinical observations by Horwitz, et al. [30, 31] and further suggested the contribution of HSCs to the osteo-chondrogenic regeneration in OI.

Evidence for Hematopoietic Stem Cell Origin of Hepatocytes, Skeletal Muscle Cells, Lung Epithelial Cells and Cardiomyocytes

Earlier reports from other laboratories consistently supported the abilities of HSCs to generate hepatocytes and skeletal muscle cells. Regarding HSC origin of hepatocytes, Petersen, et al. [35, 36] first described that transplanted rat BM cells can migrate to the liver and differentiate to hepatic oval cells, progenitors for hepatocytes. It was then reported that syngeneic transplantation of BM cells that are highly enriched for HSCs into lethally irradiated female mice causes hepatocyte reconstitution by donor cells [37]. Finally, Lagasse, et al. [38] described that transplantation of as few as 50 Lin⁻ c-kit^{high} Sca-1⁺ Thy-1^{low} BM cells restores liver function of fumarylacetoacetate hydrolase-negative mice, a model for human hereditary tyrosinemia. Subsequently, it was reported that fusion between donor hematopoietic cells and recipient liver cells is the primary cause for the apparent BM-derived hepatocytes [39], although fusion was not the mechanism for generation of human hepatocytes from cord blood cells in immune incompetent mice [40]. These findings reflect the fact that anisokaryosis is a common physiological feature of hepatocytes. Regeneration of skeletal muscle cells by BM cells was demonstrated using transplantation studies of skeletal muscle cells in two different models, with the first by Farrari et al. [41] and then by Bittner et al. [42]. Gussoni, et al. [43] then reported that transplantation of highly purified hematopoietic stem cells to mdx mice, an animal model of Duchenne's muscular dystrophy, resulted in significant reconstitution of muscle cells. These studies were followed by a detailed demonstration of the transition from BM cells to multinucleate muscle fiber via satellite cells [44] and the demonstration that a single HSC can regenerate skeletal muscle cells via myeloid intermediates [45].

Regarding lung epithelial cells, Krause and colleagues [46] presented evidence for the ability of HSCs to generate lung epithelial cells by using single HSC transplantation while others reported negative evidence for this concept. Readers are referred to a review [47] for a detailed summary of this controversy. Similarly, the premise of HSC-derived cardiomyocytes was mired in controversy. The initial exciting report of regeneration of cardiomyocytes from HSCs following ischemic injury [48, 49] was questioned in subsequent negative reports [50, 51] and was later attributed to the low incidence cell fusion between donor hematopoietic stem cells and recipient heart cells [52]. The turnover of human cardiomyocytes was reported to be extremely slow [53]. Therefore, the low-level cell fusion should not be dismissed as an anecdotal observation. Rather, it may represent the physiological speed of regeneration of cardiomyocytes, reflecting their unique syncytial structure.

Formulation of a New Paradigm

For decades, BM was thought to contain two types of stem cells, HSCs and MSCs. In this model, HSCs produce blood cells while MSCs are responsible for maintenance of a number of mesenchymal cells such as adipocytes, chondrocytes and bone cells. Our series of single HSC transplantation studies documenting the HSC origin of fibroblasts/myofibroblasts,

adipocytes, osteo-chondrocytes and CFU-F, the precursors of MSCs [20, 54], contradict this popular belief. Despite the long history of MSC research, the definition of MSCs has remained unclear and their surface phenotype unknown as pointed out in critical reviews [55, 56]. MSCs, therefore, usually denote plastic-adherent multipotential cells grown in culture and the evidence for multipotentiality of MSCs has been obtained mostly *in vitro*. Recognizing the persistence of this ambiguity in the “stemness” of MSCs, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed that the plastic-adherent multipotential cells grown from a variety of tissues and organs be termed “multipotent mesenchymal stromal cells” thus, saving the abbreviation MSCs [57]. Our documentation of the HSC origin of fibroblasts, CFU-F, fibrocytes [18], adipocytes [22] and osteo-chondrogenic cells [27, 32] is consistent with the concept that MSCs are derived from HSCs.

The documentation presented above of the HSC origin of the cells constituting connective tissue, hepatocytes, skeletal and cardiac muscle cells and lung epithelial cells, logically leads to a radically new paradigm that HSCs are not just “hematopoietic,” but are pluripotent. HSCs may support regeneration of the majority of, if not all, cells in our body. This new paradigm promises exciting new avenues in the treatment of numerous medical conditions and in future biomedical research.

Clinical Implications

Hematopoietic Stem Cell Transplantation for Hereditary Collagen Disorders

The new paradigm predicts that many more types of genetic disorders would be treatable by HSC transplantation. Genetic collagen disorders appear to be the first important candidates for HSC transplantation since fibroblasts/myofibroblasts are the major source of collagen. A total of 28 types of collagen are variably expressed in different organs and connective tissues and hereditary deficiencies of these collagens cause various types of human genetic disorders. Collagen is long, fibrous structural protein, which major function is to provide tensile strength to different organs and connective tissues. Our observation of the HSC origin of bone cells [27] provides scientific rationale for the results of clinical BM transplantation in OI, a Type I collagen disorder, carried out by Horwitz and his colleagues [30, 31, 58] and for our preclinical observations in a mouse model of OI [32]. Similarly, the HSC origin of fibroblasts/myofibroblasts [11] provides scientific rationale for the remarkable success of umbilical cord blood transplantation in the treatment of patients with Recessive Dystrophic Epidermolysis Bullosa (RDEB), disorder of collagen VII [59]. The same investigator's group also reported that the pathology of a mouse model of RDEB was ameliorated by transplantation of unmanipulated BM cells from wild-type mice, but not by non-hematopoietic BM cells [60]. While the details of the “non-hematopoietic BM cells” were not elaborated in the paper, it appears that the cells tested included MSCs.

We predict that Alport syndrome, caused by mutations of genes encoding for type IV collagen, will be the next indication of clinical HSC transplantation. Alport syndrome is characterized by progressive renal failure, deafness and lens dislocation of the anterior lens capsule. Deterioration of the basement membranes in renal glomeruli and the inner ear labyrinth is the typical pathologic finding. In about 85% of patients, there is X-linked inheritance of mutations in the gene for $\alpha 5(\text{IV})$ collagen chain. While progression of the disease varies depending on the types of mutations, renal failure and deafness develop by age 30 in the juvenile form. The outcome of kidney transplantation is known to be worse than patients with other forms of renal failure [61]. Mouse models for both X-linked [62] and autosomal [63] forms of Alport syndrome exhibit typical functional and morphologic deterioration of the kidney and cochlea. BM transplantation was shown to be effective in reversing the renal pathologies in animals with autosomal Alport syndrome [64, 65]. These

reports are consistent with our independent findings that HSCs give rise to glomerular mesangial cells [7] and inner ear fibrocytes [14]. We believe that, in the engrafted Alport mice [64, 65], normal HSCs generated fibroblasts/myofibroblasts in the glomeruli and inner ear and synthesized normal type IV collagen. HSC origin and fast turnover (estimated to be 2% in rats) of glomerular mesangial would also explain the disappointing outcome from clinical kidney transplantation. While there are no preclinical transplantation data, many other genetic collagen disorders, e.g. Ehlers-Danlos syndrome, congenital defects in type I, III or V collagen, will become indications for HSC transplantation. Replacement of affected connective tissue cells with normal cells by HSC transplantation should ameliorate and/or prevent the occurrence of the pathologies in these disorders.

Hematopoietic Stem Cell Transplantation for Other Hereditary Disorders

As introduced earlier in this perspective, the preclinical observations made by Lagasse, et al. [38] in fumarylacetoacetate hydrolase-deficient mice strongly indicate that HSC transplantation would likely restore liver functions of patients with hereditary tyrosinemia. Similarly, preclinical transplantation studies indicate the use of HSCs in the treatment of Duchenne's muscular dystrophy [43]. Of particular interest to us is the potential use of HSC transplantation in the treatment of genetic obesity, such as leptin deficiency. The scientific rationale for supplying leptin from normal adipose tissue has been provided by the preclinical transplantation of white adipose tissues to the affected mice [66] and HSC origin of adipocytes documented in our laboratory [22]. Here, the challenge will be induction of neo-adipogenesis in the recipients of HSC transplantation since adipocyte turnover is slow [67]. If successful with the use of agents such as PPAR γ agonist, the patients should benefit from a life-time supply of leptin from newly-generated normal adipocytes to curb appetite. These are some examples of new indications for HSC transplantation and we believe that many more genetic disorders will prove treatable with HSC transplantation. What is needed is unequivocal documentation of HSC origin of more cell types and relevant preclinical demonstrations.

Use of Hematopoietic Stem Cells for Non-Genetic Medical Disorders

The new paradigm promises to also bring radically new approaches to the treatment of non-genetic medical problems. Many preclinical animal studies have already shown that use of granulocyte-colony-stimulating factor (G-CSF) in the treatment of brain stroke reduces the size of stroke and yields various functional improvements [68–71]. While the precise mechanism of the effectiveness of the G-CSF treatment is unknown, enhanced mobilization of fibroblasts/myofibroblasts from their progenitors and HSCs resulting in smaller scars is a likely mechanism. Non-union and mal-union after fracture continue to be important orthopedic issues. Although bone repair is generally a rapid and efficient process, 5–20% of fractures fail to heal, resulting in delayed unions or persistent non-unions. It is possible that properly timed use of G-CSF administration may enhance fracture healing and prevent non-unions. These represent only a couple of examples of tissue regeneration based on HSCs. Appreciation of the fundamental concept that HSCs are the source of many tissues and cells in our body is likely to result in the development of radically new approaches in the general bio-medical fields.

Suggestions for Future Studies of Hematopoietic Stem Cell Plasticity

Single HSC transplantation will remain obligatory for unequivocal documentation of the extent of HSC plasticity. Our decade-long investigations of HSC plasticity using this technique made us aware of a number of biological and technical issues that are pertinent to the subject.

Levels of Hematopoietic Engraftment

To prevent false negative conclusions, studies must be performed on mice whose hematopoiesis is highly reconstituted by the donor HSC. As pointed out clearly by Theise, et al. [2], there is ambiguity about the levels of hematopoietic reconstitution in mice examined by Wagers, et al. [1, 3] who opined against HSC plasticity. In our laboratory, we used only the recipient mice exhibiting higher than 50% hematopoietic engraftment.

Tissue Turnover Time and Stimulation of Tissue Regeneration

Consideration of the physiological turnover time of the target issue/cell type is important. For studies of the issue/cell types with slow turnover, it will be necessary to employ methods to stimulate their regeneration. For example, the turnover of osteo-chondrocytes is extremely slow [28]. We therefore induced non-stabilized fractures in the engrafted recipient mice for robust generation of hypertrophic chondrocytes and subsequent neo-osteogenesis. We also tested transplantation in the mouse model of OI whose osteoprogenitor turnover is known to be fast [33, 34]. The turnover of adipocytes is also slow as reported for human adipocytes [67] and as observed by us in a mouse model [22]. Therefore, administration of a PPAR γ agonist to the engrafted mice was required for stimulation of adipogenesis and clear-cut documentation of the HSC-origin of the newly generated adipocytes. Further, we examined the tissues of the transplanted mice for studies of HSC plasticity. This approach proved to result in enhanced stem cell engraftment as compared to the parabiotic animal model employed by Wagers, et al. [1, 3] because generalized radiation toxicity is likely to have caused opening of the tissue niches for regeneration [2].

Gradual Process of Tissue Specification and Its Implications

Daily turnover of glomerular mesangial cells in rats was estimated to be 1% [72]. Reflecting this rapid turnover, many EGFP⁺ glomerular mesangial cells were observed in the kidneys of the recipient mice as early as 2 months after single HSC transplantation [7]. Wagers, et al. [1] failed to recognize the presence of HSC-derived mesangial cells because they deemed CD45⁺ cells as hematopoietic and excluded them from tissue cells of HSC origin. Expression of hematopoietic markers such as CD45 [73] and CD34 [74] by the glomerular mesangial cells has been observed, particularly in the kidneys of animals with pathologies. In our cell culture study of surface phenotypes of BM cells, we observed gradual loss of CD45 expression and concomitant gradual acquisition of fibroblast markers by the newly generated fibroblasts [18]. The observed gradual transition from CD45⁺ hematopoietic cells to mesangial cells suggest that the tissue specification process in general involves multiple stages of differentiation and sets of progenitors possessing different capabilities of niche recognition. This process further implies that the current and popular site-directed transplantation of stem cells [75] may be futile attempts to induce tissue regeneration. Open and intact tissue niches and hematopoietic progenitors that recognize the niches are the likely prerequisites for tissue specification. Presentation of abundant HSCs and their direct descendants to the injury sites with broken niches will not hasten tissue regeneration. Further elucidation of the basic mechanism of tissue specification and identification of the progenitors suited for individual transplant aims appears to be essential.

Remaining Puzzles

While we have uncovered unexpected tissue regeneration by HSCs, we could not obtain evidence for HSC contribution to two types of tissues with active cell renewal, including vascular endothelial cells and skin. Following the reports of *in vitro* and *in vivo* evidence for BM-derived circulating endothelial progenitors by the Asahara-Isner group [76–79], investigators in a number of laboratories reported *in vitro* evidence for circulating precursors of endothelial cells, suggesting a BM origin of endothelial cells. There also has been *in vivo*

evidence for adult vasculogenesis supported by BM stem cells [80], including a study based on single HSC transplantation [81] and a report that myeloid lineage progenitors generate vascular endothelial cells [82]. Against these reports, however, at least two independent transplantation studies [83, 84] refuted the premise that BM cells give rise to adult vascular endothelium. We also did not observe EGFP-labeled endothelial cells in our model, even though we examined numerous mice transplanted with EGFP⁺ BM cells that were variously enriched for HSCs. Newly formed capillaries in the sites of middle cerebral artery occlusion [12] or transplanted tumors [13] did not contain BM-derived endothelial cells. The only donor-derived cell type we observed repeatedly around the capillary vessels were pericyte-like perivascular cells [13], which is in agreement with the results from Ziegelhoeffer, et al. [83] and Rajantie, et al. [84]. Similarly, we did not observe HSC-derived keratinocytes in the skin of the recipient mice although we did not pursue this subject vigorously. It is possible that EGFP expression by these tissues is weak because we have also noted variable tissue EGFP expression by the EGFP⁺ donor cells, e.g. peripheral blood T-cells [7, 13] and cells in the urogenital tract (personal observation) in the engrafted mice. Studies based on different models and careful re-examination of the experimental conditions may yet uncover more cases of HSC plasticity.

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