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Stabilizing hematopoietic stem cells in vitro

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Abstract

Hematopoietic stem cells (HSCs) can stably regenerate all lineages of the adult blood and immune systems following transplantation via a combination of self-renewal and multipotent differentiation. HSCs are therefore an important cell type in both basic research and in the clinic, where HSC transplantation is a curative therapy for a range of diseases. However, as a rare bone marrow cell population, the characterization and collection of HSCs can often be challenging. This has led to a large search for in vitro culture conditions that support the growth of functional HSCs and the in vitro stabilization of the HSC state represents a major challenge in the field. Here, we review recent progress towards stabilizing HSCs in vitro.

Keywords

hematopoietic stem cell; hematopoiesis; ex vivo; in vitro; stem cell culture

Introduction

The hematopoietic system is made up of a number of specialized cell types including oxygen-carrying red blood cells, megakaryocyte-producing platelets, and pathogen-fighting innate and adaptive immune cells (including monocytes, neutrophils, T lymphocytes, and B lymphocytes). Hematopoiesis (blood formation) from hematopoietic stem and progenitor cells (HSPCs) within the bone marrow (and other hematopoietic organs e.g. spleen) is essential for sustaining blood and immune functions[1-3]. The most primitive hematopoietic cell type, the hematopoietic stem cell (HSC), retains throughout life the unique capacity for both self-renewal and multipotent differentiation[4]. Self-renewal allows HSCs to repopulate the HSC pool while multipotent differentiation allows HSCs to differentiate into any mature

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adult hematopoietic cell type. HSCs initially differentiate into hematopoietic progenitor cells (HPCs), which lack self-renewal capacity but can retain multipotent differentiation potential[2,4]. As HPCs differentiate further, they become progressively more lineage restricted before forming a mature hematopoietic cell type. Loss of homeostasis within the hematopoietic system by either over- or under-production of certain lineages, blocked differentiation, or loss of hematopoietic cell function, results in morbidity and mortality. Understanding the mechanism of hematopoiesis therefore represents a major goal in biomedical research.

Besides their biological importance, HSCs are also significant from a therapeutic standpoint. HSC transplantation (HSCT) represents a curative therapy for a number of malignant and non-malignant diseases[5,6]. The aim of HSCT is the reconstitution of a healthy hematopoietic system within the patient. This is usually achieved through collecting healthy allogeneic HSCs from a suitable immune-matched donor and transplanting them into the recipient who has received bone marrow conditioning (usually radiation and/or chemotherapy), which destroys their hematopoietic system. Successful engraftment of donor HSCs leads to long-term hematopoietic system reconstitution, and is key to the curative ability of HSCT. Autologous HSCT involves using the patient's own HSCs and is also used clinically, for example in high-dose radiation/chemotherapy solid cancer treatments, where such a therapy would otherwise cause bone marrow failure. Autologous HSCT has been used in the context of gene therapy to treat certain congenital blood diseases such as primary immunodeficiencies, where patient HSCs are collected, "corrected" by transferring missing genes by retrovirus or lentivirus, and then returned to the patient[7].

Given the large interest in HSC biology and clinical use of HSCT therapies for over half a century, it is perhaps surprising that HSCs are still poorly maintained outside the body[8]. For example, donor HSCs for HSCT are transplanted fresh or after only minimal in vitro culture to avoid loss of activity. Improvements in expansion of HSCs could dramatically improve engraftment success rates and also open up the wider use of umbilical cord blood HSCs in HSCT; cord blood is an accessible source of HSCs but often do not contain sufficient numbers of HSCs for transplantation[9]. Even improvements in the maintenance of HSCs in vitro during gene correction (e.g. lentiviral or retroviral transduction, or more recent CRISPR/Cas9 gene editing[10,11]) could have a major impact on access to therapy. The in vitro stabilization of HSCs therefore represents a major challenge in the stem cell field. Efforts to overcome this challenge have stimulated a range of studies into the mechanism of HSC self-renewal[1,12,13] and the in vivo HSC microenvironment (niche)[3,14]. Here, we review the recent progress towards stabilizing adult HSC in vitro. To provide context for non-HSC experts, we start with a summary of HSC assays and nomenclature before focusing our discussion on recent progress (in the last 1-2 years) and landmark papers in this field.

Defining HSC fate decisions

In adults, HSCs are a rare cell population and largely remain in a quiescent G0 (dormant) state throughout their life in the bone marrow, entering cell cycle infrequently unless hematopoietic stress occurs[1]. The current dogma is that once in culture, an HSC may retain quiescence, undergo self-renewal via symmetric or asymmetric cell division, differentiate

(thereby losing self-renewal), undergo senescence, or die (through apoptosis or other cell death mechanisms) (Figure 1). While cell death and senescence are relatively easy to assess within in vitro cultures, tracking the retention and expansion of HSCs (vs. HPCs) is more challenging because the only functional assay for an HSC is transplantation. It is worth noting that phenotypic identity and functional identity of HSCs are not necessarily the same[4,15]. Even the best phenotypic markers fail to identify functional HSCs at 100% purity and the HSC population displays significant heterogeneity[16,17]. This also means that any starting bulk cell population in an HSC culture is unlikely to contain functional pure HSCs and instead represents a mixture of HSCs with self-renewal potential and HPCs without.

In mice, functional HSCs reconstitute multilineage hematopoiesis long-term (usually defined as 16-24 weeks) in an irradiated recipient, while HPCs only reconstitute hematopoiesis partially or transiently[15]. Similar reconstitution potential can be assessed for human HSCs by transplantation into sub-lethally irradiated immunodeficient mice, although the xenogeneic nature of this limit the full assessment of HSC function[18]. HSCs can be subdivided into short-term (ST) and long-term (LT)-HSCs, with LT-HSCs being able to reconstitute hematopoiesis serially in secondary recipient mice, a demonstration of potent self-renewal potential[15]. By contrast, ST-HSCs can only reconstitute primary recipients and therefore lack potent self-renewal potential (and likely are more similar to a progenitor than a stem cell). An ultimate goal of the field is the stable maintenance and expansion of functional LT-HSCs.

Maintaining HSC function in vitro through quiescence

To expand the absolute number of HSCs in a culture, symmetric self-renewal cell division is needed. Unfortunately, most traditional in vitro HSC cultures appear to induce HSC differentiation during cell proliferation, or at best asymmetric self-renewal (generating one HSC and one non-HSC) to maintain HSCs. Instead, stably maintaining HSCs in vitro was first achieved through sustaining the HSC quiescent state in vitro. One of the first demonstrations of in vitro maintenance of quiescent HSCs was through the use of transforming growth factor-beta (TGFb)[19], with recombinant TGFb able to maintain HSCs in a dormant state in vitro for several weeks without cell division. Following transplantation into irradiated recipients, the cultured HSCs functioned normally, reconstituting multi-lineage hematopoiesis long-term. More recently, a combination of low cytokine concentrations, lipids, and hypoxia have been identified to support in vitro HSC quiescence[20]. Through screening a number of media conditions, Takubo and colleagues generated in vitro culture conditions that maintained functional HSCs with minimal cell division for over a month[20]. Interestingly, they found that HSC quiescence required high fatty acids and lipid carrying bovine serum albumin (BSA).

Identification of self-renewal agonists

While maintenance of quiescent HSCs may be useful, to achieve in vitro expansion of HSCs, self-renewal through cell division is required. To survive and grow in vitro, HSCs require exogenous stimulation and in vitro HSC cultures are commonly supplemented with a cocktail of hematopoietic cytokines that are aimed at promoting the survival, proliferation,

and self-renewal of HSCs. A number of different cytokines have been reported, but HSC cultures typically include stem cell factor (SCF), thrombopoietin (TPO), interleukin-11 (IL-11), Flt3 ligand (Flt3l), and/or IL-6 (and are reviewed elsewhere[8]). Alongside these cytokines, fetal bovine serum or some form of serum albumin supplement (such as BSA or human serum albumin) plus insulin are also usually added. Besides providing the buffering protein serum albumin, these supplements also contain other bioactive molecules, including fatty acids and proteins, which can support HSC self-renewal. However, depending on the batch of serum albumin, differentiation-inducing factors can also be found in these supplements[21], and in general these types of cultures only support modest expansion of HSCs.

Over the years, a number of attempts have been made to identify molecules and culture conditions that support symmetric self-renewal of HSCs. Probably the most successful small molecule self-renewal agonists to date are UM171 and StemRegin-1 (SR1), which together in combination with cytokines can support ~30-fold expansion of human LT-HSCs from umbilical cord blood during a two-week culture[22]. Recent mechanistic studies of UM171 suggest that it drives HSC self-renewal through modulating inflammatory signaling to limit the accumulation of cell-damaging reactive oxygen species (ROS)[23]. Both UM171 and SR1 have now been evaluated in clinical trials, both demonstrating feasibility and safety for cord blood transplantation[24,25]. More recently, other small molecules, including histone de-acetylase inhibitors have also shown efficacy in expanding human HSCs in vitro[26]. Transgene overexpression has also recently been used to induce HSC self-renewal in vitro, with Mikkola and colleagues using expression of a key self-renewal factor, MLLT3, to induce human HSC expansion in vitro[27].

Exciting improvements in human HSC expansion have recently been demonstrated through the use of zwitterionic hydrogels in 3-dimensional (3D) HSC cultures[28]. These 3D cultures afforded an impressive 73-fold expansion of LT-HSCs during a 24-day culture. Interestingly, the expansion efficient in 3D cultures was limited to use of zwitterionic hydrogels, with polyethylene glycol-based 3D cultures offering only modest support for HSCs. Mechanistic studies suggested that these 3D hydrogel cultures at least in part supported HSC self-renewal through inhibiting aerobic metabolism and suppressing of DNA-damaging ROS.

In vitro HSC stabilization has also been recently been attempted through mimicking the in vivo HSC microenvironment. Frenette and colleagues achieved this by developing a novel stromal co-culture system using genetically revitalized primary bone marrow mesenchymal stromal cells (MSCs)[29]. In this system, overexpression of five transcription factors; *Klf4, Ostf1, Xbp1, Irf3,* and *Irf7,* in MSCs was used to preserve their HSC-supportive activities in vitro and could maintain and modestly expand HSCs (~7-fold). Interestingly, the revitalized MSCs also appeared to support HSCs at least in part through reducing accumulation of DNA damage[29].

Removal of self-renewal antagonists

An alternative approach to those described above has been the identification and removal of self-renewal antagonists from HSC cultures. For example, HSCs are often cultured in

commercially available liquid media, which were not specifically developed for HSC growth and can include inhibitory factors. Even simple changes to these media such as removing the amino acid tryptophan can improve HSC expansion in vitro[30]. More recently, the high concentration of calcium in standard medias were found to reduce HSC maintenance in vitro[31] through regulating mitochondrial metabolism[31,32]. However, it was suggested that low concentrations of calcium inhibit calpain proteases, which play a role in reducing the known HSC self-renewal antagonists, Ten-eleven translocated (Tet) enzymes such as Tet2[33]. Notably, a Tet2 co-factor, ascorbic acid (vitamin C), was also recently identified as an HSC self-renewal antagonist[34,35].

As mentioned above, serum albumin supplements such as FBS are one of the most variable components of HSC media. Even purified bovine serum albumin-fraction V display large batch-to-batch variability in HSC maintenance and expansion[21]. It is therefore perhaps unsurprising that most success to date in expanding functional HSCs has been achieved through the use of albumin-free conditions[36,37]. We recently found that serum albumin can be replaced with polyvinyl alcohol (PVA) in HSC media. In combination with optimized concentrations of SCF and TPO, and the use a fibronectin matrix, this albumin-free approach afforded long-term expansion of mouse HSCs, for over 1-2 months. Limiting dilution assays estimated ex vivo expansion at between 236-899-fold, and symmetric self-renewal was also demonstrated by clone-splitting transplantation experiments. While human HSCs can also be maintained in PVA-based media, further work is needed to demonstrate long-term expansion of human HSCs in these conditions.

Use of PVA appears to minimize the introduction of differentiation-inducing factors associated with serum albumin supplements[36], while similarly stabilizing the activity of recombinant cytokines in liquid media[38]. However, the exact function of PVA in HSC cultures is still incompletely understood. Interestingly, the functions of PVA in supporting HSC self-renewal and proliferation could be dissociated through modulating the chemical structure of PVA[36]. PVA is formed from hydrolysis of polyvinyl acetate, which can be incompletely hydrolyzed (generating a polymer containing a mixture of alcohol and acetate domains) or completely hydrolyzed (generating a polymer containing only alcohol domains). Incompletely (~87%) hydrolyzed PVA supported rapid proliferation and expansion of HSCs while completely (>99%) hydrolyzed PVA could support expansion of HSCs but with significantly reduced cell proliferation. These results that further optimization of PVA structure may be possible to further improve HSC expansion.

Besides the use of PVA to reduce the introduction of exogenous self-renewal antagonists, limiting the buildup of differentiation-inducing factors secreted by HSCs and HPCs within the culture was also important to expand HSCs more than a week in vitro. Long-term in vitro HSC cultures required regular complete media changes, which were essential for the maintenance and expansion of mouse HSCs in long-term in vitro cultures[36,37]. A similar build of secreted differentiation-inducing factors in human HSC cultures was previously been observed by Zandstra and colleagues[39], who suggested that this could be overcome using an automated batch-fed method, where HSC cultures are constantly provided with fresh media to limit a concentration buildup of negative factors. Taken together, observations highlight that self-renewal antagonists can be both found with common HSC medias and

build up within the HSC cultures, for example by cell secretion of differentiation-inducing factors or metabolic activity (e.g. in the case of ROS).

Conclusions

In conclusion, exciting progress has been made over the last couple of years in stabilizing HSCs in vitro through adding self-renewal agonists and removing self-renewal antagonists. In particular, the application of new reagents has also offered improvement, such as the use of zwitterionic hydrogels for 3D culture[28] and use of PVA[36]. It will be in interesting to understand whether these different approaches are synergistic and can further improve HSC stability and expansion in vitro in combination. These new systems provide important tools for experimental hematology, to study the mechanism of hematopoiesis and to develop new therapeutic strategies for clinical application.

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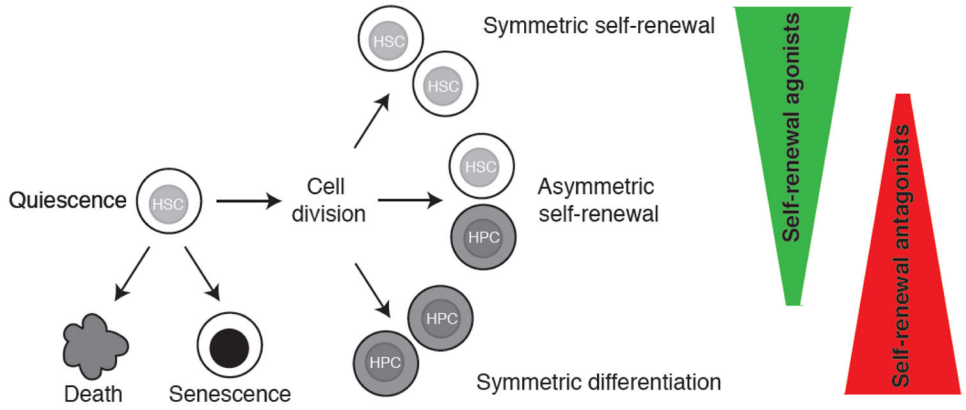

Figure 1: In vitro cell fates for hematopoietic stem cells

Hematopoietic stem cells (HSCs) can be either maintained in quiescence, or stimulated into cell cycle where symmetric self-renewal, asymmetric self-renewal, or symmetric differentiation is possible. HSCs differentiate into hematopoietic progenitor cells (HPCs). Alternatively, HSCs may undergo cell death or senescence. Recent improvements in the in vitro expansion of HSCs have been made through identifying and modulating both agonists and antagonists of HSC self-renewal.