Hematopoietic stem cells: from the bone to the bioreactor

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The *ex vivo* expansion of human hematopoietic stem cells is a rapidly developing area with a broad range of biomedical applications. The mechanisms of renewal, differentiation and plasticity of stem cells are currently under intense investigation. However, the complexity of hematopoiesis, the heterogeneity of the culture population and the complex interplay between the culture parameters that significantly influence the proliferation and differentiation of hematopoietic cells have impaired the translation of small scale results to the highly demanded large-scale applications. The better understanding of these mechanisms is providing the basis for more rational approaches to the *ex vivo* expansion of hematopoietic stem cells. Efforts are now being made to establish a rational design of bioreactor systems, allowing the modeling and control of large-scale production of stem cells and the study of their proliferation and differentiation, under conditions as similar as possible to those *in vivo*.

The possibility of repairing or replacing damaged tissues and organs has been a major concern for centuries, leading to the development of applications ranging from cosmetics to surgery. With the advent of the first successful organ transplantations, a new hope emerged, but it was later realized that it presented two main problems: the rejection caused by the immune system and the low availability of donors. In particular, one of the greatest demands in the health care system is the availability of blood and blood products with enhanced safety and stability, so needed for transfusion and treatment of leukemias. Other important applications encompass the use of stem cells used as gene therapy targets, to aid the immune system in its response to transformation (i.e. cancer) or infection with virus, therapy against autoimmune disorders, tissue repair therapies, or the use of stem cells for biosensor applications.

Stem cells

Stem cells can be thought of as 'backup' cells, because they lie in an undifferentiated state, waiting for a signal that will instruct them to proliferate and/or start following an increasingly restricted line of differentiation, in which their function is defined. They have multilineage differentiation capacity (i.e. they are multipotent), and possess self-renewal capability.

There are several problems concerning the isolation and identification of stem cells: because they are rare, they are difficult to track down; their morphology is not different from most cells within their tissue; and tests for the screening of stem cells (such as proliferation assays) are reasonably expensive and time-consuming. However, stem cells (as most cells, if not all) possess certain cell surface markers that enable the enrichment of a stem cell pool. These markers, also known in some instances as CD (cluster of differentiation), are surface proteins (or protein complexes) that help the cell to perform its specific role, and their functions range from adhesion molecules to ligand receptors. Hence, although a universal marker has not yet been found that is common to all stem cell types, stem cells in different tissues have their own unique set of stem-cell markers.

Here, we are interested in a particular set of stem cells, those responsible for the generation and maintenance of all blood cell types, including those of the immune system, which are known as hematopoietic stem cells (HSCs).

Hematopoietic stem cells

Hematopoietic stem cells are the cells responsible for blood cell renewal (hematopoiesis), including the generation of all myeloid and lymphoid cell lines (Fig. 1). Most of these cells are found within the bone marrow (BM) at a very low percentage (typically 0.01–0.05%), where they can also circulate into the peripheral blood (PB), at even lower percentages (~0.001%). Other sources of HSCs include umbilical cord blood (UCB) and fetal liver.

Types of HSCs

Not all HSCs are identical. Current knowledge reveals that they can be divided into three types: long-term self-renewing HSCs (LT-HSCs), short-term self-renewing HSCs (ST-HSCs) and multipotent progenitors (MPPs) which are apparently not self-renewing. Whereas LT-HSCs are thought to self-renew for the whole lifespan of an organism (in fact, the gold standard for defining a LT-HSC is its ability to engraft and repopulate a host hematopoietic system), ST-HSCs and MPPs have a shorter duration, being able to restore hematopoiesis in a lethally irradiated mouse only for up to four months [1]. The differentiation from LT-HSCs to ST-HSCs and from these to MPPs is

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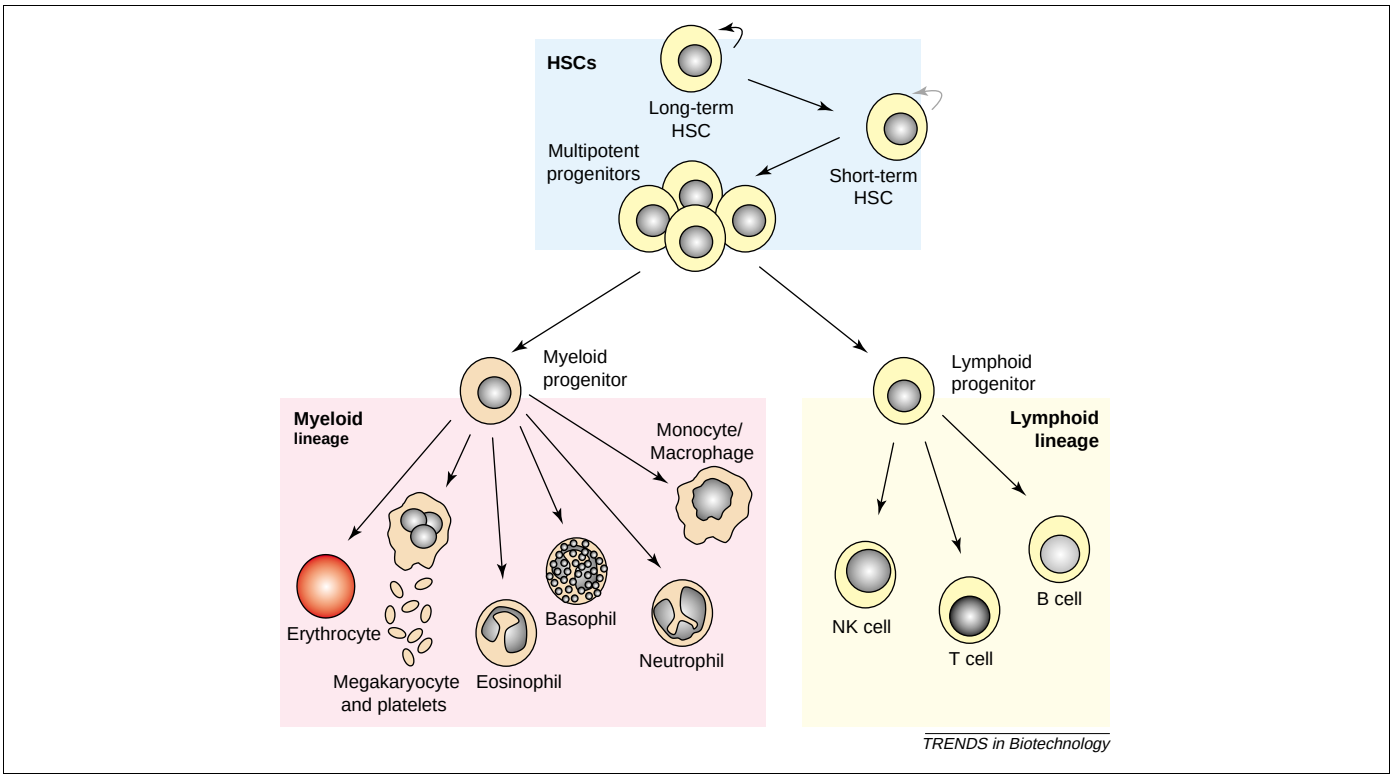

Fig. 1. The hematopoietic lineages of differentiation. The curved arrow indicates self-renewing ability. Two main lineages stem from hematopoietic stem cells (HSCs), originated from either myeloid or lymphoid progenitors, which in turn give rise to all blood (erythrocytes, megakaryocytes, eosinophils, basophils, neutrophils and monocytes/macrophages) and lymphatic [B cells, T cells and Natural Killer (NK) cells] cell types, respectively.

accompanied by a progressive loss of self-renewal ability and an increase of mitotic activity. After this proliferative activity, the final non-self-renewing MPPs then continue differentiation along either one of the two hematopoietic lineages (Fig. 1). It then becomes clear that LT-HSCs are the pivotal cells for use in HSC-based cell therapies. Regrettably, there is currently no way of isolating these within human BM or PB to relative homogeneity.

Molecular and cellular mechanisms of hematopoiesis

The dynamics of hematopoiesis is relatively complex, involving a delicate interplay of molecular and cellular factors. An intimate understanding of these mechanisms is necessary for the development of a rational approach to the establishment of an *ex vivo* culture of HSCs, namely regarding the need for signaling molecules to be present in the culture medium and the environment around the cells (usually known as niche) which provides the essential physical and physiological support.

Signaling between cells is achieved by means of secreted glycoproteins, also known as cytokines, which, depending on their presence and concentration, can induce stem cells to differentiate along a certain lineage, stop differentiation or possibly even undergo apoptosis (cellular programmed suicide). Several cytokines are used in combination for the *ex vivo* culture of HSC (listed in Table 1). For instance, although the mechanism of induction of stem-cell self-renewal remains largely unknown, it was recently shown that in mice it seems to depend on the presence of stem cell factor (SCF) and thrombopoietin (Tpo) [2,3], and on the activation of the gp130 receptor molecule [4]. It is also noteworthy that leukemia inhibitory factor (LIF) was found to maintain murine embryonic stem cells undifferentiated [5].

The extracelular environment is also of the utmost importance. It is now well established that within the BM there are niches in which HSCs grow and mature, consisting of a support of stromal cells (adipocytes, endothelial cells, macrophages and fibroblasts, collectively known as stroma) immersed in an extracelular matrix including collagen-like molecules and proteoglycans. This environment is responsible for the fixation of HSCs to adhesion molecules found at the surface of the extracelular matrix and for the stromal cell secretion of growth factors that promote HSC differentiation. The recent discovery that CD164, a sialomucin expressed in HSC, is involved in adhesion to stroma and might be a potent negative regulator of hematopoietic progenitor cell proliferation [6] is an example of the key role of niches in HSC culture.

The above example illustrates that there are some cell-surface markers specific to HSCs. In fact, the most common way of identifying and separating HSCs relies on specific markers that are expressed preferentially at their surface (Table 2). However, these markers are not completely exclusive to HSCs and therefore any selection based on them still yields a heterogeneous population. The best selection strategies are based on the combination of several markers, either by positive (presence of the marker) or negative (absence) selection. The most widely used combination of markers for HSCs is $CD34^{+}/CD38^{-}$, although recently there seems to be evidence that a more primitive HSC population might be $CD34^{+}$ (reviewed in [7]), and therefore current selection strategies are usually

Table 1. Cytokines used in human hematopoietic stem cell (HSC) culture *ex vivo*

Cytokine (abbreviation)	Function	Refs
Fms-like tyrosine kinase-3 ligand (Flt-3L, FL)	Potentiates the effects of other cytokines; promotes survival of HSCs	[55–59]
Granulocyte CSF (G-CSF)	Mobilization of HSCs to peripheral blood	[60–62]
Interleukin 3 (IL-3)	Together with IL-6, promotes proliferation of HSCs	[55,58,63]
Interleukin 6 (IL-6)	Together with IL-3, promotes proliferation of HSCs	[55,57,59,64,65]
Interleukin 10 (IL-10)	Helps proliferation of HSCs	[63,66]
Interleukin 11 (IL-11)	Shortens the G_0 period of the cell cycle of HSCs	[57]
Jagged-1	Regulates HSC self-renewal	[67,68]
Platelet-derived growth factor (PDGF)	Mitogen for connective tissue cells	[59]
Stem cell factor (SCF)	Growth factor for HSC progenitor cells	[55,58,63]
Thrombopoietin (TPO)	Stimulator of megakaryocytopoiesis	[56–59]

CSF, colony-stimulating factor; *Fms*, fibromyalgia syndrome gene.

based on the removal of cells bearing specific markers that indicate unwanted differentiated cells.

Besides being useful as a means of identifying and separating cells, some surface cell markers are receptors for cytokines which, when bound to the receptor, initiate a cascade of intracellular reactions known as signal transduction. As would be expected, this is the mechanism by which cells are told to 'do something', such as proliferating, differentiating or even undergoing apoptosis. The elucidation of the molecular mechanisms involved in signal transduction (reviewed in [8]) is already providing clues as to how HSC self-renewal and differentiation is accomplished.

Stem cell plasticity

The possibility that stem cells of a given tissue might differentiate into cells of a different tissue is known as plasticity, or transdifferentiation (reviewed in [9]). However, recent findings question some of the results achieved, interpreting them as either cell fusion between embryonic stem (ES) cells and adult stem cells [10] or heterogeneity in stem-cell populations [11]. Yet, if this phenomenon proves to be true, it will solve the problem of having to use ES cells (more versatile than adult stem cells) and the possibility of re-creating *ex vivo* the niches in which to drive differentiation will prove to be an inestimable tool for research and therapy [12].

Ex vivo expansion of HSCs

The development of a stable *in vitro* system for the growth of HSC will be a valuable tool for the study of the mechanisms controlling hematopoiesis. Successful *ex vivo* models enable the study of the dynamics and mechanisms of cell differentiation and organ development. Further, meaningful pharmacological studies can be performed in such systems. The need for improved cultivation methods is driven by the fact that many potential therapeutic applications are limited by the availability of stem cells or their derivatives, because donor-derived cell samples consist of only small numbers of cells despite the fact that large amounts of cells are needed for research and especially for cell therapies. The *in vitro* propagation of undifferentiated HSC populations remains largely undeveloped and is considered a major technical challenge because of the complex kinetics of the heterogeneous starting culture population, the transient nature of the subpopulations of interest, the lack of invariant measures and the complex interactions between the culture parameters. The pursued aim is the *in vitro* maintenance and expansion without differentiation of transplantable HSCs while maintaining their ability to home, engraft and differentiate into appropriate receptive tissues.

Culture parameters

The first HSC cultures were performed using serum-containing media. In addition to its nutritional role, serum protects the cells from shear stresses within the reactor and influences the transduction of mechanical forces that affect (either negatively or positively) surface receptors [13]. However, the addition of serum to a culture medium makes its composition undefined, adds uncontrolled variability, and complicates the clinical use of cells thus cultured because of increased regulatory hurdles. Interactions between known and unknown serum components complicate *in vitro* cell culture studies on the effects of specific medium components. Serum-free media have supported excellent expansion of highly purified HSCs, stimulated by multiple cytokines [14], and recently serum-free culture conditions were developed that support the *ex*

Table 2. Markers of human hematopoietic stem cells (HSCs)

Name	Function	SWISS-PROT entry[a]
$CD34^{-}$ or $^{-}$	Sialomucin, possibly involved in cell–cell adhesion	P28906
Thy 1^{-} (CD90)	Possibly involved in cell–cell interaction	P04216
$CD38^{-}$	ADP-ribosyl cyclase 1, synthesizes cyclic ADP-ribose; indicates differentiation to both erythroid and myeloid progenitors	P28907
c-Kit^{-} (CD117)	Tyrosine kinase receptor for stem cell factor (SCF)	P10721
$AC133^{-}$ (CD133)	Prominin-like protein, function unknown	O43490
lin^{-} (lineage)	Generic designation for several markers of blood cell lineages	–

A plus (+) sign indicates presence of the marker in HSCs, whereas a minus (−) indicates its absence.
[a]http://www.expasy.ch/sprot/

vivo expansion and maintenance of HSCs while maintaining their engraftment capability [15].

Accessory cells, such as stroma, have an important role in conditioning the environment in which expansion takes place, by producing some of the cytokines that regulate hematopoiesis. It has been reported that serum-free cultures with pre-established stroma were the most effective in maintaining the cells in their primitive phenotype, whereas in cultures supplemented with various combinations of recombinant human growth factors, HSC differentiation prevailed [16]. In addition to cytokine supplementation, the endogenous production of cytokine and growth factors, both known and yet to be identified, are likely to affect the long-term outcome of HSC cultures [17]. It has been suggested that direct stroma contact is beneficial for expansion and is required for conservation of total graft quality, provided that exogenous cytokines are supplemented to override some existing stroma contact-mediated proliferation-blocking effects [18]. Still, the role of stroma-contact is not fully understood as experiments in which UCB cultures were separated from the feeder layer by microporous membranes showed that no-contact cultures, which allow only small molecules to traffic between stroma cells and UCB, improved the expansion results [19].

Because the cytokine microenvironment is dynamic, the composition of the culture system can change dramatically as culture time progresses and the endogenous production of factors that have a suppressing effect on HSC self-renewal might significantly influence culture output. Designing successful HSC expansion cultures thus requires strategies to maintain an appropriate balance between stimulatory and inhibitory modulators of HSC function [20]. Physicochemical parameters such as pH, oxygen and glucose have significant effects on endogenous cytokine production and hence on stem cell responses.

Cells experience growth inhibition and even toxicity at high oxygen concentrations and anoxia at low oxygen concentrations. Oxygen tension also modulates the expression of cytokine receptors, transcription factors and lineage-specific markers [21]. Oxygen tensions ranging from 1% to 10% (*in vivo* oxygen tensions in BM are in the range of 2–7%) were shown to enhance both the size and number of hematopoietic colonies obtained in semi-solid media [22]. Nitric oxide, hydrogen peroxide and oxygen radicals, all inhibitors of cell proliferation, are produced in lower amounts in a controlled environment of 5% oxygen, compared with atmospheric 21% oxygen. Low oxygen concentrations promote immature BFU-E maintenance *ex vivo* [23], whereas 20% oxygen was observed to significantly enhance total megakaryocyte (Mk) number, polyploidy and pro-platelet formation, when compared with 5% oxygen in culture [21]. The observation that more mature cells might be less sensitive to changes in their oxygen environment than their immediate precursors suggests that the definition of an 'optimal' oxygen tension might depend not only on the cytokines used to stimulate the cells, but also on the developmental potential of the cells being stimulated.

Many studies have shown that medium acidification causes growth inhibition [22,24,25]. Some indication of differences in the survival and differentiation of HSCs into granulocyte-macrophage (optimal pH 7.2–7.4) versus erythroid lineages (optimal pH ~7.6) have been documented [22]. Also, the pH range 7.35–7.40 promotes differentiation, maturation and apoptosis of Mk cells, whereas lower pH (= 7.1) extends the expansion of primitive Mk progenitor cells [24]. Unless controlled, HSC cultures might experience pH variations up to 0.5 pH units [25], largely because of the formation of lactate. However, most studies have addressed the effect of pH on the differentiation of stem cells rather than setting optimum pH ranges for their expansion. Furthermore, pH was often an observed variable rather than a controlled parameter, highlighting the need to perform further studies in controlled bioreactors, in which the effects of pH and lactate can be studied separately. Attention should also be drawn to the way pH is controlled because the addition of sodium hydroxide or ammonium nitrite seems to influence culture behavior differently (P.W. Zandstra, pers. commun.).

Bioreactor design and operation

Static culture systems such as T-flasks have been the most widely used culture devices for expanding HSC since the pioneering work of Dexter and colleagues [26]. However, these systems have several limitations, including: lack of mixing, resulting in critical concentration gradients for pH, dissolved oxygen, cytokines and metabolites; difficult or even impossible online monitoring and control; low process reproducibility; repeated handling required to feed cultures or obtain data on culture performance; and productivity limited by the number of cells that can be supported by a given surface area [27,28].

The use of bioreactor systems is an alternative approach to standard flask cultures of cells *in vitro*. Advanced bioreactors are required when a large number of cells is needed, accessory cells are used and high cell densities are desired. A deeper and broader insight into the influence of exogenous input on HSC expansion and commitment is only achievable in closely monitored and tightly controlled bioreactors. A few studies have been performed with different types of bioreactors for the *ex vivo* expansion of cells, involving perfusion chambers, stirred reactors, hollow fiber, rotating and packed bed reactors (Table 3). Figure 2 depicts the main type of reactors used so far for the expansion of HSCs.

Perfusion chambers

Palsson and collaborators developed a perfusion culture system based on flat small-scale cell culture chambers with an attached stromal layer for the expansion of HSCs [29–31]. The design of such a reactor was driven by the observation that increase of medium exchange rates and the provision of soluble growth factors leads to an extended *ex vivo* proliferation of human bone marrow cells [29,32,33]. This system was the basis of a patented cell production system [34,35] owned by Aastrom Biosciences and tested in clinical trials [36]. The geometry of the perfusion chambers affected cell growth and differentiation [37]. Radial flow-type chambers provided the most uniform environment because of the absence of walls parallel to the

Table 3. Bioreactors most frequently used for the culture of hematopoietic stem cells (HSCs)

Bioreactor Type	Culture mode	Progenitor cell expansion[a]	Refs
Perfusion chamber	Small scale perfusion chamber (3 ml)	3-fold LTC-IC	[29,30]
	Large scale perfusion chamber (30 ml)	7.5-fold LTC-IC	[31]
	Grooves perpendicular to the direction of flow	LTC-IC maintenance	[38]
Stirred tank	Batch suspension culture in controlled bioreactor (working volume 120 ml)	6-fold hematopoietic progenitors	[69]
	Batch suspension culture in spinner flask (total volume 250 ml) with periodical cytokine addition	2.6-fold BFU-E	[39]
	Batch suspension culture in controlled bioreactor (working volume 550 ml)	8-fold CFU	[48]
	Batch culture with microcarriers in spinner flask (total volume 250 ml) with periodical cytokine addition	1.8-fold BFU-E	[39]
	Batch suspension culture in spinner flask (working volume 20 ml) with periodical medium replacement	7-fold LTC-IC	[40]
	Batch culture of microencapsulated cells in spinner flask (working volume 50 ml with 12–13 ml of capsules) with daily exchange of 10 ml of medium	11-fold BFU-E	[41]
Fluidized bed	173 working volume. Bed of gelatine-modified open porous glass carriers (32 ml)	No expansion	[47]
Fixed (packed) bed	173 ml working volume. Bed of gelatine-modified open porous glass carriers (20 ml)	7-fold CFU-GM	[47]
	28 ml working volume. Bed of macroporous collagen microcarriers (10 ml)	114-fold CFU-GM	[48]
	1 ml working volume. Bed of macroporous collagen microcarriers	N/A	[48]
Airlift	Batch suspension culture in spinner flask (total volume 250 ml) with periodical cytokine addition	0.8-fold BFU-E	[39]
Hollow Fiber	Medium from a 250 ml reservoir was passed into the hollow fiber cartridge (1.0 sq ft fiber surface area, 10 000 MW cutoff)	No expansion	[39]

[a]Indicative values of expansion of HSCs achieved in experiments with each bioreactor type are provided. Expansion comparison should, however, take into account other factors such as culture medium, physicochemical conditions, medium exchange rates, etc. BFU-E, Burst-forming units erythroid; CFU, Colony-forming unit; GM, Granulocyte monocyte; LTC-IC, Long-term culture-initiating cell; N/A, Not available.

flow path that creates slow-flowing regions. Later, the flatbed perfusion chamber was modified to retain cells through the addition of grooves perpendicular to the flow direction, at the chamber bottom [38]. This type of reactor allowed the *ex vivo* expansion of HSCs with cytokine supplementation but in the absence of stroma.

Stirred reactors

Stirred bioreactors provide a homogeneous environment and are easy to operate, allowing sampling, monitoring and control of culture conditions (Fig. 2). Typical operating modes include batch, fed-batch and perfusion mode (medium exchange with retention of cells by means of an external filtration module or of internal devices such as spin filters). HSCs do not require surface attachment to grow and have been successfully cultured in stirred bioreactors with improved performance, as mixing overcomes diffusion limitations of static culture systems. Stirred suspension culture systems are relatively simple and readily scalable. In addition, their relatively homogeneous nature makes them suited for the investigation of different culture parameters. However, it is not clear to what extent stirred cultures can support accessory cells, unless microcarriers are added [39,40] or cells are microencapsulated [41]. Furthermore, HSCs are relatively sensitive to shear, and agitation is thought to affect surface marker expression, including cytokine receptors [42], which profoundly affect which cells expand and to what extent expansion occurs. Although many studies have shown that lineage commitment and mature cell function are stabilized by cell–cell and cell–extracellular matrix interactions [18,43], stem cell self-renewal might be more efficient in the absence of these signals [19,22].

Packed and fluidized bed reactors

Because no cell–cell or cell–matrix interactions are possible in suspension systems, and the fluid dynamics are not characteristic of the *in vivo* environment, alternative designs to mimic the *in vivo* intricate structure of the bone marrow environment were attempted. For that purpose, packed bed reactors were designed to provide a 3D scaffolding for cell attachment and culture [44–48]. In these systems, an initial attachment-dependent stromal cell culture is started on the bed particles whereupon HSCs can be co-cultivated. Despite the relatively small scale of these bioreactors (up to 500 ml working volume), screening and optimization of culture conditions is advantageously performed in a miniaturized loop reactor containing a fixed bed of collagen microcarriers with a working volume of 1 ml that can be placed in a conventional 12-well culture plate [49]. A fluidized bed bioreactor system was also used [47]. However, in the fluidized bed the carrier movement inhibited adhesion of HSCs to stromal cells.

Other reactor types

Hollow fiber and air-lift reactors were used [39], but poor expansion was obtained. Recently, a new low-shear, suspension culture method in a rotating bioreactor was developed that enabled the culture of mixed cell populations coalescing into 3D structures (spheroids) that reproduce some of the *in vivo* characteristics of the organs

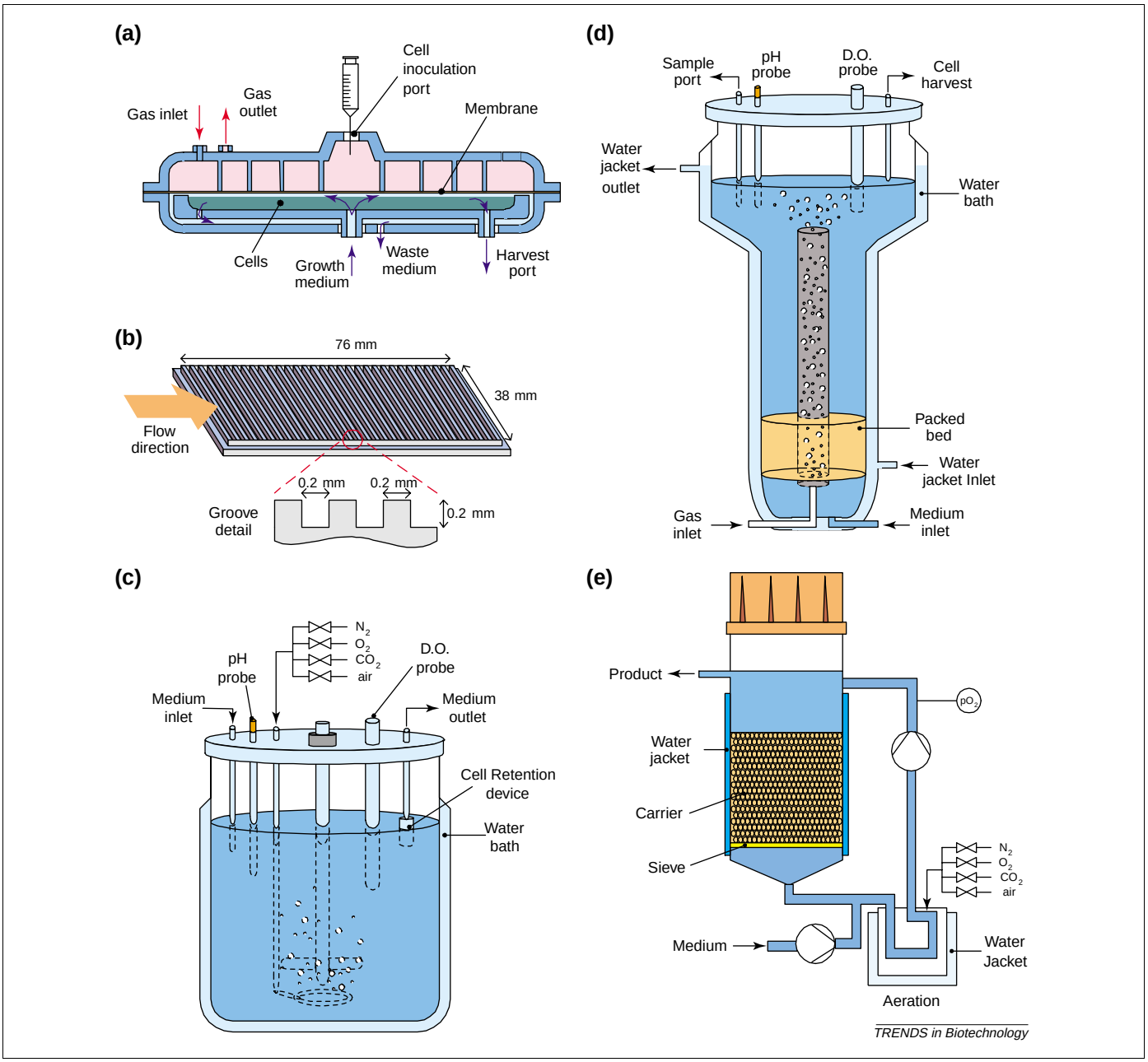

Fig. 2. Bioreactors more frequently used for the culture of hematopoietic stem cells: (a) perfusion chamber; (b) grooved surface of perfused chamber; (c) stirred tank; (d) air-lift fixed-bed; and (e) fixed bed or fluidized bed bioreactor.

from which they were derived [50]. Attempts of culturing HSCs in such systems are currently underway.

Culture monitoring and modelling

Evaluating kinetics in hematopoietic cultures is complicated by the distribution of cells over various stages of differentiation and by the presence of cells from different lineages. Thus, an observed response is an integral response from several cell populations. Models predicting isolated or distributed populations by incorporating data such as rates of growth, death, differentiation, transition between quiescence and active cycling, concentrations of one or two cytokines, glucose uptake and lactate production rates, dissolved oxygen and pH have been established [51–54]. Nevertheless, most models only deal with a very limited set of inputs and were derived from a few off-line experimental measurements and from cultures performed in poorly controlled settings (mostly T-flasks and spinner flasks). The implementation of online monitoring capabilities and the use of automated control systems such as the triggering of appropriate changes in the supply rates of medium and gas components (oxygen, carbon dioxide) are essential not only to the expansion of HSCs, but will also provide a means to study, under controlled and reproducible conditions, the effects of multiple parameters on the cultivation and the kinetics of the production of specific cells, oxygen consumption and carbon dioxide production, nutrient depletion, and cytokine production and consumption, which are not yet well known. Only then can the kinetic analysis, together with the hydrodynamic and mass transfer characterization of the bioreactors, be integrated in a model framework that

might be used for the establishment and the control of optimal operational conditions and for the design of new, up-scalable bioreactors for the effective *ex vivo* production of HSCs.

Conclusion

The true potential of *ex vivo* expansion of HSCs will only be realized when culture systems of sufficient simplicity, flexibility and economic efficacy have been developed. This review indicates that the development of bioreactors is very important for the suitable cultivation of HSCs. The aimed mimicry of *in vivo* conditions constitutes a major technical challenge that still remains largely undeveloped.

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