

# The Importance of Physiologically Inspired Physicochemical Parameters on Hematopoietic Stem-Cell Maintenance and Lineage-Specific Differentiation in *Ex Vivo* Cultures

## 6

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### 6.1. Introduction

#### 6.1.1. *The thesis for the chapter*

Stem cell localization reflects the needs of these cells for maintenance, renewal and need-driven differentiation. The stem cell niche is dictated not only by cells that co-occupy the body locale of the stem cells, but also by the physicochemical parameters or nutrient availability of that locale, including the pH, pO<sub>2</sub>, physical forces due to flow (or lack of thereof), and the amount (i.e. abundance, lack or limited supply) of nutrient and growth factors. These issues have largely remained out of the radar screen of modern stem cell biology and bioengineering, but they now emerge as potent, if not indispensable, modulators of stem cell fate. Here we focus on issues associated with *ex vivo* expansion of hematopoietic stem and progenitor cells (HSPCs).

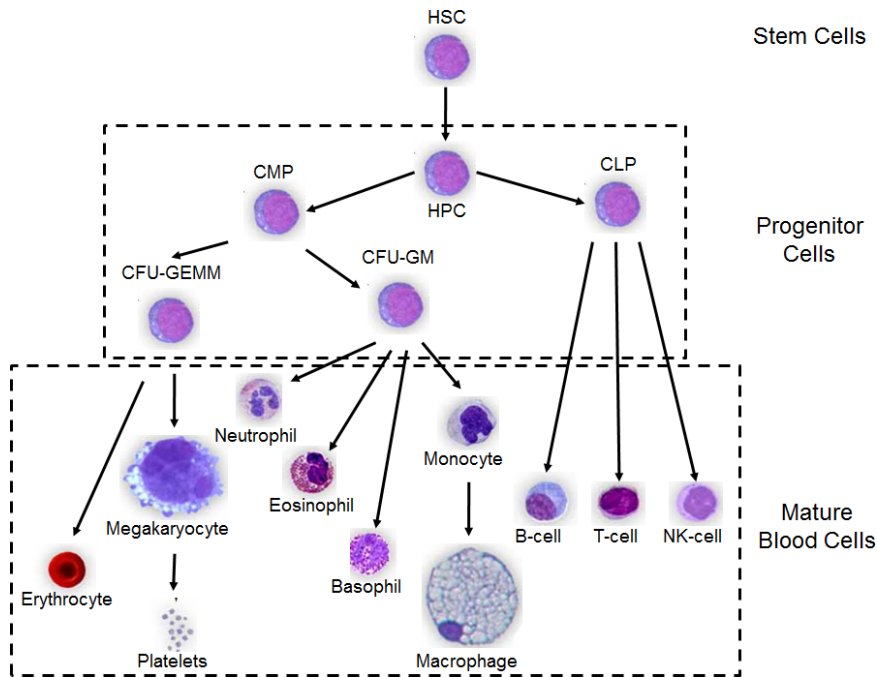
#### 6.1.2. *Hematopoietic culture applications and challenges, and the importance of the physicochemical parameters of the stem cell niche*

Stem and progenitor cells from the bone marrow (BM), mobilized peripheral blood (mPB), or cord blood (CB) can be used to restore hematopoietic activity in patients whose BM has been ablated by chemotherapy (Bertolini *et al.*, 1997; Brugger *et al.*,

1 1995; Decaudin *et al.*, 2004; Scheduling *et al.*, 2004; Shirvaikar *et al.*, 2008; Srouer  
2 *et al.*, 1999; Tura *et al.*, 2007; Verfaillie, 2002; Williams *et al.*, 1996). However,  
3 current protocols may be limited by donor cell availability and the long time (1.5–  
4 4+ weeks) needed to restore neutrophil and platelet counts to normal levels. By  
5 reducing the required harvest size, *ex vivo* expansion of hematopoietic progenitors  
6 has the potential to allow the following: (1) patients with low BM cellularity to serve  
7 as their own donors, (2) replacement of invasive BM harvests (1–2 liters) with an  
8 aliquot drawn in a doctor's office, (3) use of a single PB harvest, and (4) use of  
9 CB cells for adult transplants. Of equal or greater importance, *ex vivo* expansion  
10 has the potential to enhance the transplant outcome. For example, increased doses  
11 of granulocytic progenitors have been shown to decrease the variability in patient  
12 engraftment and increase the survival rate (Bacigalupo *et al.*, 1995; Perez-Simon  
13 *et al.*, 2003). In addition, by producing large numbers of post-progenitors with the  
14 potential to rapidly generate mature neutrophils and platelets, it may be possible to  
15 completely prevent cytopenias associated with high-dose chemotherapy (Timmins  
16 *et al.*, 2009). Finally, *ex vivo* expansion offers the potential for repeated cycles of  
17 chemotherapy and cell transplants from a single BM or PB harvest (that is aliquotted  
18 and frozen for subsequent culture). Other applications include production of cycling  
19 stem and progenitor cells for gene therapy, the expansion of dendritic cells for  
20 immunotherapy (Cui *et al.*, 2002; Di Nicola *et al.*, 1998; Saraya and Reid, 1996;  
21 Siena *et al.*, 1995), and production of red blood cells and platelets for transfusions  
22 (McAdams *et al.*, 1996b; Timmins and Nielsen, 2009). Clinical trials (Bertolini  
23 *et al.*, 1997; Brugger *et al.*, 1995; Williams *et al.*, 1996) have demonstrated that *ex*  
24 *vivo* expanded hematopoietic cells can be safely re-infused to immunocompromised  
25 patients who have undergone intensive chemotherapy for cancer.

26 Hematopoietic cultures remain among the most challenging culture systems  
27 because of the intrinsically heterogeneous and highly variable nature of the cultured  
28 cells. Heterogeneity and variability derive from both patient-to-patient variability  
29 and the complexity of hematopoietic differentiation (Figure 6.1). While some  
30 potential applications can be realized using standard hematopoietic culture tech-  
31 niques, effective control of the culture environment will be required to consistently  
32 produce the cell populations needed for the different applications noted above.  
33 There has been extensive research regarding the effects of hematopoietic growth  
34 factors (HGFs) on cell expansion and differentiation patterns (reviewed by Kelly,  
35 McAdams, Sauvageau, and Timmins and Nielsen (Kelly *et al.*, 2009; McAdams  
36 *et al.*, 1996a; Sauvageau *et al.*, 2004; Timmins and Nielsen, 2009)). Beyond  
37 cytokines, a fast-accumulating body of literature demonstrates that physiological  
38 parameters such as culture pH, pO<sub>2</sub> and flow-induced shear effects can also have  
39 dramatic effects, and this is the focus of the chapter. Bioengineering advances have

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**Figure 6.1.** The hematopoietic hierarchy showing a few of the differentiation steps for how hematopoietic stem cells in the bone marrow are committed and expand in numbers to generate all myeloid and lymphoid blood cells in the circulation.

1 not kept pace with the rapid developments in hematopoietic cell biology. The true  
 2 potential of *ex vivo* expansion of hematopoietic cells will only be realized when  
 3 culture systems or bioreactors of sufficient simplicity, flexibility, and economic  
 4 efficacy have been developed. Expansion protocols on the horizon are likely to be  
 5 used in a number of clinical trials in stem, progenitor and mature/differentiated  
 6 hematopoietic cell transplantation therapies. One challenge for the future will  
 7 be to further increase the productivity and reliability of the *ex vivo* expansion  
 8 protocols. In this context, the goal of stem cell bioengineering is to define culture  
 9 conditions (beyond media and cytokines) to improve the degree of control over the  
 10 proliferation and differentiation of specific progenitors and blood lineages. Such  
 11 goals will require increased understanding of the synergism between cytokines  
 12 and other culture parameters (pH, pO<sub>2</sub>, flow effects), and the engagement of  
 13 synthetic or semisynthetic matrices to present cytokines to cells, thus providing  
 14 a suitable “chemical” and “elastic” or protective microenvironment for the cells.  
 15 Such developments should be largely driven by understanding the natural milieu

1 or “niche” of the stem, progenitor and more differentiated cells, as well as the  
2 evolutionary forces that have shaped the “niche”. These aspects are especially  
3 emphasized in this chapter. One, of course, cannot exclude the engagement of novel,  
4 completely synthetic niches, but those will be hard to define rationally.

## 5 **6.2. Hematopoietic Background and Terminology**

### 6 **6.2.1. Hematopoiesis, stroma and in vitro** 7 **colony assays**

8 Hematopoiesis is the process of generating mature blood cells, which are produced  
9 in the average human at a rate of 400 billion per day (Koller and Palsson, 1993).  
10 Hematopoietic cells originate from a very small population of *totipotent stem cells*,  
11 which proliferate and differentiate to produce all blood–cell lineages in the body  
12 (Figure 6.1). Less primitive *pluripotent stem cells* may differentiate only into a subset  
13 of lineages. The intermediate stage cells that are committed to specific lineages,  
14 but still capable of significant proliferation are known as *progenitor cells*, while the  
15 final non-proliferative functional cells are termed *mature blood cells*. The *myeloid*  
16 *lineages* include the erythroid (E), granulocytic (G), monocytic (M), dendritic (DC),  
17 and megakaryocytic (Mk) lineages. The primary site of *in vivo* hematopoiesis is  
18 the red bone marrow (BM). The complex hematopoietic microenvironment in the  
19 BM cavities between blood vessels is potentiated by a variety of (mostly) non-  
20 hematopoietic supportive cells, collectively known as *stroma*. Stromal cells consist  
21 primarily of macrophages, endothelial and adventitial reticular cells, adipocytes,  
22 osteoblasts and osteoclasts, and differentiated non-hematopoietic stem cells known  
23 as mesenchymal stem cells (MSCs) (Caplan, 2009; Ho *et al.*, 2008; Morrison and  
24 Spradling, 2008) that provide both soluble and membrane-bound growth factors to  
25 hematopoietic cells. Stromal cells also produce an extracellular matrix that affects  
26 proliferation and differentiation. Colony-forming cell (CFC) or colony-forming  
27 unit (CFU) assays are *in vitro* assays that are widely used to assess the frequency  
28 and number of hematopoietic stem and progenitor cells (Coulombel, 2004). A small  
29 number of sample cells is placed in a semisolid medium containing a suitable mixture  
30 of growth factors, and incubated for 2 weeks. Cultures are then scored for the  
31 presence of colonies of 50 or more cells that originated from a single cell 2 weeks  
32 earlier. Colony types are assessed by color, with colonies containing: white cells  
33 termed colony-forming unit GM (CFU-GM), and red cells termed burst-forming-  
34 unit E (BFU-E). The long-term culture-initiating cell (LTC-IC) assay is the most  
35 common assay for more primitive cells. For this assay, sample cells are placed on a  
36 supportive stromal cell layer and maintained in culture for 5–8 weeks. After that, cells

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1 are placed into a colony assay to measure CFCs. During the 5–8 week culture period,  
2 CFCs differentiate into mature cells, and the more primitive LTC-ICs (believed to  
3 be a subset of the stem cells) differentiate into CFCs, which are then detected by  
4 the colony assay. A variant of this assay, known as long-term hematopoietic culture-  
5 initiating cell (LTHC-IC) (Traycoff *et al.*, 1995) eliminates the use of preformed  
6 stroma and offers substantial time savings (4 vs. 8 weeks total time) and improved  
7 reproducibility. *In vivo* assays (Gordon, 1993) and employing animal models provide  
8 more a sophisticated, clinically relevant assessment of HSC potency and potential.

9 **6.2.2. Sources of hematopoietic stem and progenitor**  
10 **cells (HSPCs)**

11 There are three main sources of hematopoietic stem and progenitor cells (Korbling  
12 and Anderlini, 2001). BM is collected under general anesthesia via multiple needle  
13 aspirations to the sternum and/or pelvis. Cord blood (CB) is obtained non-invasively  
14 from the umbilical cord of newborn infants. Mobilized peripheral blood (mPB)  
15 cells are generally obtained after a stem cell mobilization, which is achieved by  
16 the administration of one of several chemotherapeutic drugs and/or hematopoietic  
17 growth factors (HGFs) to the patient. Nucleated cells are then collected from the  
18 patients using a blood-processing machine (PB apheresis). The expansion potential  
19 of different sources of hematopoietic cells, and even of different samples of the  
20 same type of cells, is subject to large variations. CB has the greatest expansion  
21 potential (Cairo and Wagner, 1997; Lansdorp *et al.*, 1993), while PB has the greatest  
22 variation due to the wide variety of mobilization regimens and disease state of the  
23 patients (Janssen, 1995). CD34 is a surface glycoprotein of unknown function that  
24 is found on primitive cells from the quiescent stem cells to the highly proliferative  
25 progenitors (ca. 1% of collected mononuclear cells (MNCs) (Krause *et al.*, 1996)).  
26 Methods have been developed for the selection of CD34<sup>+</sup> cells (de Wynter *et al.*,  
27 1995). When compared to those for MNCs, cultures initiated with CD34<sup>+</sup> cells have  
28 much greater expansion potential. However, CD34<sup>+</sup> selection is expensive and often  
29 results in significant cell loss. A more recently explored source of hematopoietic cells  
30 is the placenta (Dzierzak and Robin, 2010), which is an available, otherwise wasted  
31 tissue like the umbilical cord. Finally, in the last few years, embryonic stem cells  
32 (ESCs) and induced pluripotent cells (iPCs) are explored as a longer-term, more  
33 accessible and stable source of HSPCs (Carpenter *et al.*, 2003; Choi *et al.*, 2009;  
34 Lengerke and Daley, 2010; Wang *et al.*, 2005). Like other applications of ESCs and  
35 iPCs, exploration of these cells as a practical source of HSPCs is still in its infancy,  
36 but is the focus of an ever-increasing research activity.

### 1 **6.2.3. Hematopoietic growth factors (HGFs) and** 2 **cytokines**

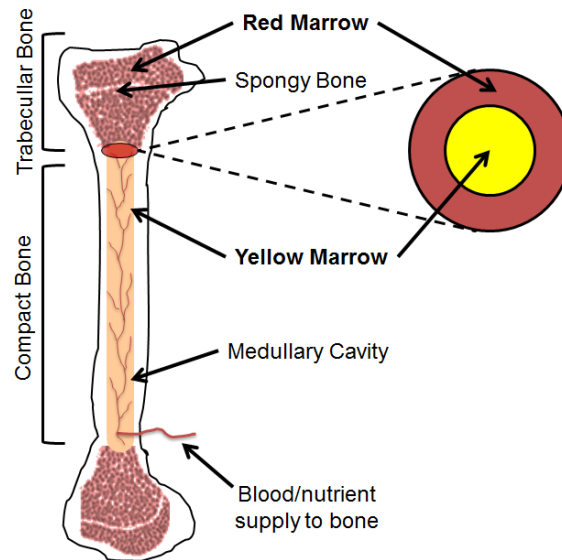
3 While stroma-containing cultures produce their own cytokines, stroma-free (com-  
4 monly called "suspension") cultures require the addition of exogenous HGFs (Audet  
5 *et al.*, 2002; Kelly *et al.*, 2009; Panuganti *et al.*, 2010; Sauvageau *et al.*, 2004).  
6 Cytokines essential for cell survival and proliferation are interleukin 3 (IL-3),  
7 stem cell factor (SCF), and/or flt3 ligand (FL). IL-3 prevents programmed cell  
8 death (apoptosis) of committed progenitor cells, and SCF (Brandt *et al.*, 1994)  
9 and FL (Takahira *et al.*, 1996) prevent apoptosis of early progenitor cells. FL is  
10 structurally and functionally similar to SCF except that it does not act on the E  
11 lineage (McKenna *et al.*, 1995). IL-6 acts in concert with other cytokines to induce  
12 cycling of additional stem and progenitor cells (Leary *et al.*, 1988). Thus, SCF/IL-  
13 3/IL-6 has become a common cytokine combination for broad expansion across  
14 multiple hematopoietic lineages. Granulocyte colony-stimulating factor (G-CSF)  
15 and IL-11 have also been shown to bring additional dormant primitive hematopoietic  
16 cells into the cell cycle (Leary *et al.*, 1992). GM-CSF is a broad-acting growth factor  
17 that induces proliferation of G, M, DC and E progenitors (Clark and Kamen, 1987).  
18 Erythropoietin (Epo) (Fisher, 2003; Kaushansky, 2006), thrombopoietin (Tpo) (de  
19 Sauvage *et al.*, 1994; Kaushansky, 2006) and G-CSF (Clark and Kamen, 1987;  
20 Kaushansky, 2006) are powerful proliferation and maturation factors for the E, Mk,  
21 and G lineages, respectively. Yet, in addition to the G-CSF mentioned above, Tpo  
22 and Epo have been found to promote the expansion of primitive hematopoietic stem  
23 cells, and thus, are frequently used in that context as well, not merely for promoting  
24 the expansion of their respective lineages. There are also cytokines which inhibit or  
25 enhance hematopoietic cell proliferation or differentiation (depending on the target  
26 cells and other cytokines used), including TGF- $\beta$  (Hatzfeld *et al.*, 1991) and TNF- $\alpha$   
27 (Rusten *et al.*, 1994).

## 28 **6.3. The Physiology Inspires: Bone Marrow** 29 **Organization, pO<sub>2</sub> and pH Levels, and the Impact** 30 **of Flow-Induced Shear Flow in Hematopoiesis**

### 31 **6.3.1. Physiological measurements and observations**

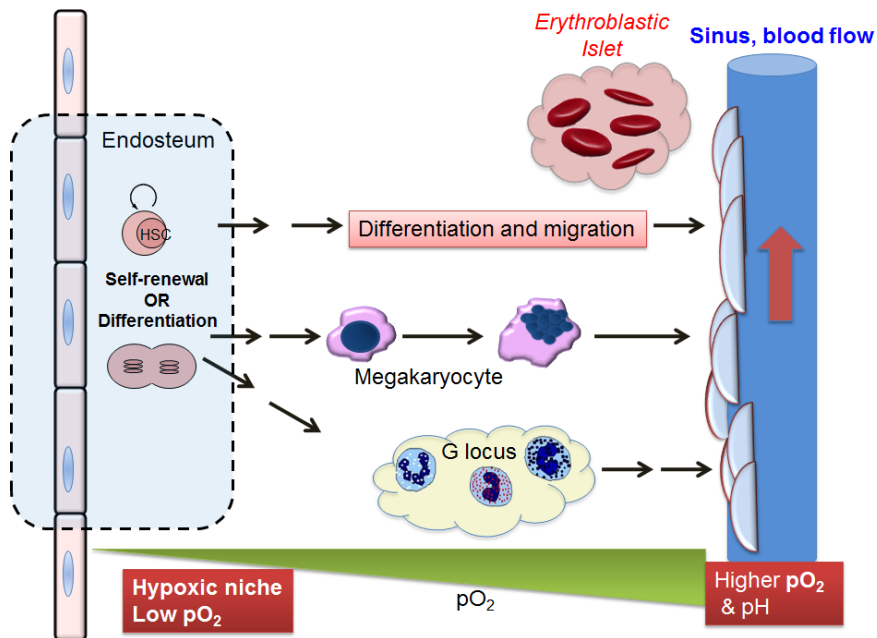
32 The organization of BM has been studied using primarily long bones (Figure 6.2)  
33 from rodents and rabbits (Chow *et al.*, 2001a; Chow *et al.*, 2001b; Kaplan *et al.*,  
34 2007; Lichtman, 1984; Mayani *et al.*, 1992; Tavassoli, 1974). The vasculature is  
35 dominated by the venous system, which is made up of vascular sinuses draining

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**Figure 6.2.** The long bone is the site of the red and yellow (fatty) bone marrow, the predominant site of adult hematopoiesis. The most primitive and thus, hematopoietic potent stem cells are assumed to be located mostly at the long bone endosteum, mostly in the red marrow. Copyright of the two parts of the figure is public: From Wikipedia (see separate attached PDF).

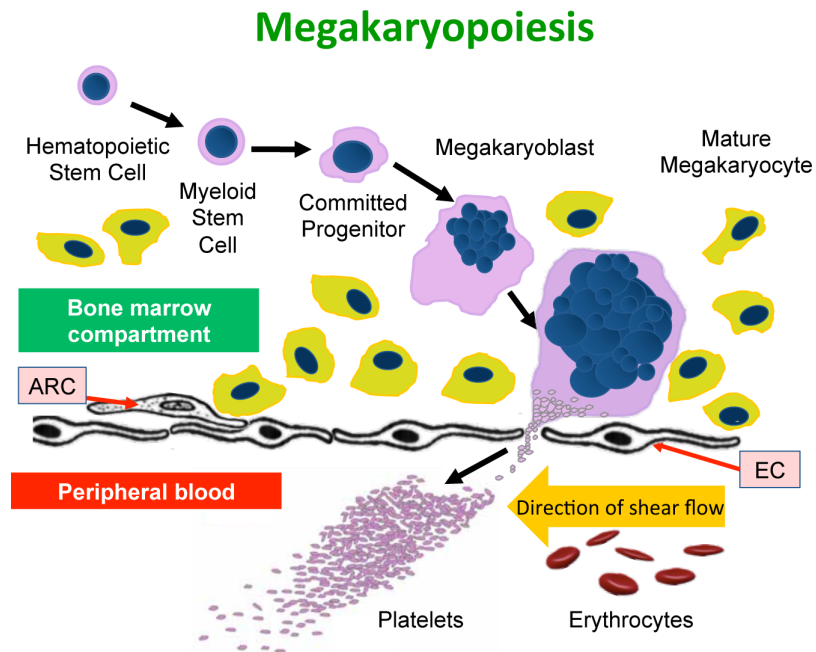
1 into the great central vein. In adults, the area around the central artery and central  
 2 veins is occupied by the fatty yellow marrow (found mostly in the compact part  
 3 area of the long bone), while the hematopoietically active red marrow resides  
 4 near the bone endosteum and (especially) in the trabecular part of the long bone  
 5 (Morrison and Spradling, 2008; ter Huurne *et al.*, 2010; Xie *et al.*, 2009) (Figure 6.2).  
 6 Hematopoiesis takes place in the extravascular spaces between the sinuses. The  
 7 venous system supports the passage of maturing hematopoietic cells, which are  
 8 being released from the BM hematopoietic compartment (BMHC). The thin sinus  
 9 wall is composed of endothelial cells, which line the sinus lumen, and adventitial  
 10 reticular cells, which form an incomplete abluminal outer coat. Hematopoietic cells  
 11 must cross the sinus wall to enter the circulatory blood. The most primitive stem and  
 12 progenitor cells reside in the extravascular (frequently referred to as perivascular)  
 13 areas, the furthest away from the sinuses and next to the endosteum. As cells  
 14 differentiate, they move closer to the sinus lining (Figure 6.3). Specifically, the  
 15 (motile) granulocytes (G cells) typically develop away from the sinus wall in G loci  
 16 and as they mature, they move near the wall where they develop microvilli that  
 17 facilitate their crossing into circulation. The (immotile) erythroid (E) cells develop



**Figure 6.3.** Schematic representation of the differentiation and migration of hematopoietic stem cells from the primitive endosteal niche (low, hypoxic pO<sub>2</sub>) to a bone marrow sinus (higher pO<sub>2</sub> and pH) for release of differentiated cells into the blood circulation.

1 in erythroblastic islets (one to two circular layers of E cells surrounding one or  
 2 two macrophages; diameter of ca. 50–80 μm) near the sinus wall, with mature E  
 3 cells bordering the vascular wall and immature cells (i.e., basophilic erythroblasts)  
 4 away from the wall. Megakaryocytes (Mks) differentiate in the BM vasculature:  
 5 they undergo a variation of the normal cell cycle, termed endomitosis, to form  
 6 polyploid cells (≥8N DNA content) (Vitrat *et al.*, 1998). Committed Mks migrate  
 7 from the hematopoietic BM compartment towards the endothelial lining of the BM  
 8 sinusoids, where they mature and extend long, branched cytoplasmic protrusions  
 9 (termed proplatelets) through gaps of the endothelium into the vasculature (Hattori  
 10 *et al.*, 2003; Schulze and Shivdasani, 2005) (Figures 6.3 and 6.4). Mk cells encounter  
 11 mechanical stresses as they deform to penetrate the gaps of the sinusoid walls, and  
 12 shear forces by the exposure to blood flow. The pulmonary circulation is another  
 13 important site of Mk maturation and platelet biogenesis (Dunois-Larde *et al.*, 2009):  
 14 Mks may enter the BM circulation and reach the lungs where they shed proplatelets.  
 15 As such, Mk cells encounter shear forces in circulation as well as mechanical strain  
 16 in the lung vasculature. Such stresses/forces were recently shown to be essential

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**Figure 6.4.** Bone marrow megakaryocytes migrate to and mature near the bone marrow sinuses, where they penetrate the endothelial sinus wall and release proplatelets into circulation under shear flow. A similar process takes place in the lung vasculature from less mature Mk that enter circulation and complete the Mk maturation process in the lungs.

1 for Mk maturation and proplatelet/platelet biogenesis (Dunois-Larde *et al.*, 2009;  
 2 Junt *et al.*, 2007). Additionally, Mk maturation is enhanced by oxidative stress (as  
 3 demonstrated by the positive impact of high  $O_2$  tensions on Mk maturation) as has  
 4 been shown by us (Mostafa *et al.*, 2000a) and, most recently, by others (Motohashi  
 5 *et al.*, 2010). Thus, Mk maturation and platelet release appear to be stress-induced  
 6 processes.

7 There is a wide variation in  $pO_2$  levels in different tissues of the human/animal  
 8 body and even within a single tissue due to frequent significant spatial heterogeneities  
 9 in tissue organization (Kietzmann *et al.*, 1999; Thomas *et al.*, 1994). In normal mouse  
 10 subcutaneous tissue, the mean measured  $pO_2$  was 30 mmHg and values as low as  
 11 12 mmHg were recorded (Helmlinger *et al.*, 1997). Similarly, low  $pO_2$  values have  
 12 been widely reported for other normal tissues (e.g. (Vaupe, 1996)). The lowest  $pO_2$   
 13 value in a tissue is found furthest from the vessel wall (Helmlinger *et al.*, 1997).  
 14 There are few direct  $pO_2$  and pH measurements in human BM. Ishikawa and Ito

1 (Ishikawa and Ito, 1988) reported a mean  $pO_2$  of  $52 \pm 15$  mmHg (4.85–8.8%) in  
2 healthy adult human BM. Pennathur-Das and Levitt reported the average  $pO_2$  in  
3 the BMHC as 5%  $O_2$  (Pennathur-Das and Levitt, 1987), while other measurements  
4 of bone marrow aspirates from normal human volunteer donors yielded mean  $pO_2$   
5 values of 6.6% (Harrison *et al.*, 2002). These  $pO_2$  values are approximately the same  
6 as mean values of other normal tissues (Helmlinger *et al.*, 1997)], and show that the  
7 overall level of oxygen tension in the BM is lower than that in circulation. Arterial  
8 blood has a  $pO_2$  of ca. 95–40 mmHg (12.5–5.25%) and a pH of 7.4, while venal  
9 blood has a  $pO_2$  of ca. 40 mmHg (5.25%) and a pH of 7.35 (McAdams *et al.*, 1997).  
10 The highest  $pO_2$  in the body is in the lungs (16%  $O_2$ ) (Kietzmann *et al.*, 1999;  
11 Mostafa *et al.*, 2000b). Thus, the sites of Mk maturation and platelet production  
12 have a substantially higher level of  $pO_2$  than the core of the BMHC, in which stem  
13 and early progenitors reside.

14 Marrow microphotographs (Chow *et al.*, 2001a; Chow *et al.*, 2001b; Lichtman,  
15 1981) show that there are as few as 4–8, to as many as 16–20 cells tightly packed  
16 between two sinuses or BM arterial vessels in any one direction. Thus, many  
17 hematopoietic cells (and especially the most primitive stem and progenitor cells)  
18 are 2–8 cells (or 20–80  $\mu\text{m}$ , assuming a conservative 10- $\mu\text{m}$  cell diameter) away  
19 from the closest sinus or BM arterial vessels. Another assessment of the distances of  
20 cells from sinuses or arteries is the size (80–1200  $\mu\text{m}$  in diameter) of the lymphocyte  
21 nodules (these nodules are between sinuses), in which lymphocytes are organized  
22 in the hematopoietic compartment of the human BM (Chow *et al.*, 2001a; Chow  
23 *et al.*, 2001b). The non-uniform vasculature of BM suggests spatial variations in  
24 pH and  $pO_2$  (Chow *et al.*, 2001a; Chow *et al.*, 2001b; McAdams *et al.*, 1997). It  
25 has been demonstrated experimentally that pH drops from 7.35 to ca. 7.1 within  
26 about 25  $\mu\text{m}$  from a blood vessel in normal subcutaneous tissue (rabbit) (Martin  
27 and Jain, 1994). Finally, it should be noted that the lowest pH and  $pO_2$  values  
28 are observed the furthest away from the vessel wall (Helmlinger *et al.*, 1997). In  
29 view of the fact that hematopoietic cells are metabolically very active (Chow *et al.*,  
30 2001a; Chow *et al.*, 2001b; Koller and Palsson, 1993), substantial pH and  $pO_2$   
31 (and possibly other nutrients such as glucose or cytokines) gradients exist in the  
32 BM hematopoietic compartment (Figure 6.3). This is further supported by several  
33 indirect studies, where it was noted that the most primitive stem cells that give rise to  
34 BM regeneration are relatively resistant to ionizing radiation (Allalunis *et al.*, 1983)  
35 (this is similar to the radioresistance of hypoxic cells in solid tumors (Helmlinger  
36 *et al.*, 1997)). It was thus suggested several years ago (Allalunis *et al.*, 1983) that “a  
37 hypoxic component of hematopoietic cells could be important in the regenerative  
38 process within the marrow after such myelotoxic trauma”. The most recent studies

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1 discussed in detail below have now established this hypothesis as a fact. In any case,  
2 it is important to note that the prevailing *mean BM* (i.e. blood in the arterial and  
3 venous system, and the hematopoietic tissue)  $pO_2$  is about one third of the normal  
4 atmospheric  $pO_2$  (i.e. 5–7% vs. 21%  $O_2$ ). Thus, the standard practice of culturing  
5 hematopoietic cells in 20%  $O_2$  incubators creates a hyperoxic environment, which  
6 may be detrimental to the cells. We note that most “low”  $pO_2$  studies (see below)  
7 on hematopoietic cells have been carried out under 5%  $O_2$ , a value which better  
8 approximates the likely  $pO_2$  value in the BM hematopoietic compartment near the  
9 sinus wall.

10 In summary, experimental evidence suggests that the pH,  $pO_2$ , and nutri-  
11 ent/cytokine concentration conditions in the BMHC are quite different from the  
12 conditions prevailing in the circulating blood and from those used in cell culture  
13 experiments and processes. We had proposed several years ago (Chow *et al.*, 2001a;  
14 Chow *et al.*, 2001b; Koller *et al.*, 1992c; LaIuppa *et al.*, 1998; McAdams *et al.*,  
15 1997; McAdams *et al.*, 1998; Yang *et al.*, 2002) that pH and  $pO_2$  gradients play  
16 an important role in cell differentiation and proliferation of hematopoietic cells in  
17 synergy with HGFs and cytokines, and had hypothesized that these effects can be  
18 exploited for practical *ex vivo* expansion applications. Both hypotheses have been  
19 now extensively supported by recent work in our laboratory and in many others, as  
20 detailed below.

### 21 **6.3.2. Modeling to estimate $pO_2$ and pH levels and** 22 **gradients in the BM hematopoietic** 23 **compartment (BMHC)**

#### 24 **6.3.2.1. Oxygen**

25 As discussed above, the BM is a tissue of complex architectural organization,  
26 which includes granulopoietic loci, erythroblastic islets, and lymphocytic nodules.  
27 Chow *et al.* (Chow *et al.*, 2001a; Chow *et al.*, 2001b) developed homogeneous and  
28 heterogeneous/composite Kroghian models to estimate  $pO_2$  distributions in the  
29 BM hematopoietic compartment (BMHC) and to conservatively simulate  $pO_2$ -  
30 limited cellular architectures. These models were built based on biophysical and  
31 metabolic data of hematopoietic cells and characteristics of the BM physiology.  
32 For the homogeneous Krogh models, they constructed a tissue cylinder solely  
33 occupied by various BM cell types. Krogh cylinders occupied by granulocytic  
34 progenitors (the most metabolically active stage of the most abundant cell type)  
35 provide a physiologically relevant limiting case of the smallest cylinder leading to

1 oxygen limitations, while adipocyte-occupied cylinders represent the other extreme  
2 of the thickest cylinders leading to oxygen limitation. Although the number of  
3 possible cellular architectures is large, all simulated  $pO_2$  profiles fall between  
4 those two extreme cases. For cellular architectures of physiological significance,  
5 more complex biophysical transport Kroghian models were considered, such as  
6 multilayer Kroghian models and two-dimensional Kroghian models. These models  
7 are built to simulate complex cellular architectures in which erythroid clusters  
8 or individual cells (megakaryocytes or adipocytes) are located in the BMHC,  
9 predominantly occupied by mature granulocytes.  $pO_2$  distributions in colony-type  
10 cellular arrangements (erythroblastic islets, granulopoietic loci, and lymphocytic  
11 nodules) were examined by multilayer Kroghian models. Simulation results based  
12 on these models show that most hematopoietic progenitors experience low  $pO_2$   
13 values, and that the most primitive stem cells, which are located even further away  
14 from BM sinuses, are likely located in a very low  $pO_2$  environment, commonly  
15 referred to as hypoxic.

#### 16 6.3.2.2. *pH*

17 Although pH gradients have been modeled using a similar effective diffusion  
18 and reaction model (Martin and Jain, 1994), this is not likely to prove effective  
19 in modeling pH gradients in tissues in view of the complexities associated with  
20 the bicarbonate buffer, and  $CO_2$  and lactate production and transport. Since the  
21 hematopoietic progenitors are very glycolytic and are metabolically almost as active  
22 as hybridoma and tumor cells (Chow *et al.*, 2001a), and since lactate formation is  
23 the main reason (Carlsson and Acker, 1988) for the generation of pH gradients in  
24 spheroids, it is possible to estimate upper and lower limits for pH gradients from  
25 analogous profiles in primary tissues (Helmlinger *et al.*, 1997) and tumors (Gorlach  
26 and Acker, 1994; Helmlinger *et al.*, 1997). There is a reasonable correlation between  
27  $pO_2$  and pH in primary subcutaneous tissue (Helmlinger *et al.*, 1997), and there is a  
28 good correlation between mean  $pO_2$  and pH profiles in tumors *in vivo* (Helmlinger  
29 *et al.*, 1997) or *ex vivo* (Gorlach and Acker, 1994). As would be expected, the slope  
30  $\Delta pH / \Delta pO_2$  appears to depend on cell metabolism. If this is accepted as generally  
31 valid, it is possible to use the metabolic characteristics of hematopoietic cells (Chow  
32 *et al.*, 2001a) and the estimated  $pO_2$  gradients (Chow *et al.*, 2001a; Chow *et al.*,  
33 2001b) to estimate pH gradients. One would surely conclude that areas of large  
34  $\Delta pO_2$  gradients are also areas of large  $\Delta pH$  gradients, and that pH levels and  $\Delta pH$   
35 gradients are likely to play a significant role in regulating stem- and progenitor-cell  
36 differentiation.

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## 6.4. The Stem-Cell Niche as Related to Low Oxygen Tension

### 6.4.1. *The importance of the hypoxic endosteum as a site for stem cell renewal and maintenance*

It is now well accepted that HSCs reside in discrete microenvironmental domains within the bone marrow that are collectively termed the “stem cell niche” (Schofield, 1978). The quiescent state is now accepted as a characteristic and indispensable property for the maintenance of HSCs and likely all primitive stem cells (Arai *et al.*, 2004). Several recent studies suggest that the true and most primitive, and thus potent, HSCs reside next to the endosteal surface of the bone in proximity to stromal cells and osteoblasts, and that hematopoietic differentiation proceeds radially towards the longitudinal axis of the marrow (Nilsson *et al.*, 2001; Schofield, 1978; Winkler *et al.*, 2010; Xie *et al.*, 2009). Levesque *et al.* (Levesque *et al.*, 2007) used confocal laser scanning microscopy to show that, in the murine system, the endosteum at the bone BM interface is hypoxic, with constitutive expression of hypoxia-inducible transcription factor-1 alpha (HIF-1 $\alpha$ ), and that HSPC mobilization induces expansion of the hypoxic areas in the BM due to the proliferation of myeloid progenitors that occurs during HSPC mobilization. Genetic studies have confirmed that the endosteal region of the bone marrow is the principal site of HSC niche and hematopoiesis (Calvi *et al.*, 2003); (Zhang *et al.*, 2003). The unique microenvironment of the endosteum not only represents the anatomical location where the HSCs reside, but also potentiates signaling cascades that maintain definitive stem cell properties and regulates the stem cell pool, presumably by interacting with stroma cells that regulate stem cell function (Dao *et al.*, 2007; Jones and Wagers, 2008; Morrison and Spradling, 2008; ter Huurne *et al.*, 2010; Zhang *et al.*, 2003). In addition, residence of adult HSCs in this BM niches is essential for their quiescence and long-term functions (Schofield, 1978; Wilson and Trumpp, 2006; Morrison and Spradling, 2008; Nilsson *et al.*, 2001; ter Huurne *et al.*, 2010). Arai *et al.* (Arai *et al.*, 2004) have shown that that HSCs expressing receptor tyrosine kinase Tie2 are quiescent and anti-apoptotic, and adhere to osteoblasts in the endosteum BM niche. *In vitro*, the interaction of Tie2 with its ligand angiopoietin-1 (Ang-1) induced cobblestone formation, while *in vivo*, it maintained the long-term repopulating activity of HSCs. Ang-1 enhanced the ability of HSCs to become quiescent and induced HSC adhesion to the bone.

As recently discussed (Winkler *et al.*, 2010), it is hypothesized that vascular niches, which are abundantly supplied with nutrients and oxygen by sinusoidal blood, may represent “proliferative niches,” whereas endosteal niches, which are

1 generally inaccessible to blood nutrients and maintain a low oxygen concentration,  
2 could represent more “quiescent niches” for HSCs (Figure 6.3). Building on the  
3 seminal work of Parmar *et al.* (Parmar *et al.*, 2007), and in order to investigate  
4 the functional attributes of these niches, Winkler *et al.* (Winkler *et al.*, 2010)  
5 perfused murine BM with the vital DNA dye Hoechst 33342 *in vivo* before BM  
6 cell collection. These experiments aimed to assess the position of HSCs and multi-  
7 potent myeloid progenitors relative to blood flow. Independently, Xie *et al.* (Xie  
8 *et al.*, 2009) used real-time imaging to identify the location and frequency of  
9 HSCs in murine long bones. These findings suggest that the most potent HSC  
10 niches are low in oxygen concentration and supported by locally produced growth  
11 factors due to poor access by blood flow. In summary, the endosteal niches are now  
12 firmly established as the hypoxic environment for HSC maintenance and possibly  
13 renewal.

#### 14 **6.4.2. Low oxygen tension or mild hypoxia is an** 15 **essential element of the stem-cell niche and** 16 **not only for hematopoietic stem cells**

17 As discussed above, HSCs and the supporting cells of the stem cell niche found  
18 within the endosteum are predominantly located at the lowest end of an oxygen  
19 gradient in the BM with the implication that regionally defined hypoxia plays a  
20 fundamental role in regulating stem cell function (Mazumdar *et al.*, 2010; Parmar  
21 *et al.*, 2007; Winkler *et al.*, 2010). Although hypoxia is not generally encountered  
22 in most healthy tissues, as discussed above, BM represents a unique tissue type  
23 given its complicated hierarchical organization emanating from a small population  
24 of resting stem cells, all in the endosteum area and mostly in the trabecular part  
25 of the long bones (Xie *et al.*, 2009). The data discussed above seem to strongly  
26 support the hypothesis that stem cell metabolism is regulated by very low oxygen  
27 levels and oxygen gradients within the stem-cell niche (Mohyeldin *et al.*, 2010).  
28 Supporting this hypothesis, the very primitive Lin<sup>-</sup>Sca<sup>+</sup>Kit<sup>+</sup> (LSK) cells express  
29 high levels of glycolytic and oxidative repair proteins, indicating that primitive  
30 HSCs cells are adapted to anaerobic metabolism (Unwin *et al.*, 2006). Exposure  
31 to high levels of oxygen damages the functions of HSCs (Cipolleschi *et al.*, 1993;  
32 Ivanovic *et al.*, 2000a; Parmar *et al.*, 2007), explaining further why HSCs prefer  
33 to reside in areas of low pO<sub>2</sub>. Yet, the significance of hypoxia signaling in HSCs  
34 has been only recently explored. Takubo *et al.* (Takubo *et al.*, 2010) and Simsek  
35 *et al.* (Simsek *et al.*, 2010) have shown that hypoxia regulates the metabolic state  
36 of HSCs and protects their integrity by controlling HIF-1 $\alpha$ . Mazumdar *et al.*  
37 (Mazumdar *et al.*, 2010) have shown that oxygen regulates stem cell maintenance

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1 through HIF-1 $\alpha$ -controlled Wnt/ $\beta$ -catenin signaling. Tothova *et al.* (Tothova *et al.*,  
2 2007) have shown that the FoxO family of transcriptional regulators (a subgroup of  
3 Forkhead transcription factors which modulate responses to oxidative stress, DNA  
4 damage, cell cycle arrest and general homeostasis) are necessary for protecting the  
5 HSC compartment from oxidative stress, and that *in vivo* treatment of mice, with  
6 defective FoxO gene expression, with the antioxidant N-acetyl-S-cysteine reversed  
7 the FoxO gene defects. Taken together, this body of recent work provides molecular  
8 understanding for studies published several years ago whereby *ex vivo* maintenance  
9 and expansion of primitive HSCs was shown to be greatly improved by maintaining  
10 the cell cultures at very low oxygen levels (1–5% O<sub>2</sub>) (Cipolleschi *et al.*, 1993; Danet  
11 *et al.*, 2003; Ivanovic *et al.*, 2000a; Ivanovic *et al.*, 2000b; Koller *et al.*, 1992a; Koller  
12 *et al.*, 1992b; Koller *et al.*, 1992c).

13 The impact and (likely) necessity for low oxygen tension and/or hypoxia for  
14 stem cell maintenance and expansion is now becoming more broadly accepted  
15 as being applicable to other stem cells such as the BM mesenchymal stem cells  
16 (MSCs)(Grayson *et al.*, 2007; Grayson *et al.*, 2006a; Hirao *et al.*, 2006; Rosova *et al.*,  
17 2008), neuronal stem cells (Mazumdar *et al.*, 2010; Shingo *et al.*, 2001), as well as  
18 embryonic stem cells (ESCs) and induced pluripotent cells (iPCs) (recently reviewed  
19 (Mohyeldin *et al.*, 2010)). These findings suggest that the stem cell niche of these  
20 various tissues is likely anoxic due to mass transfer resistances (diffusion and lack  
21 of perfusion) similar to that established in the BM tissue (Chow *et al.*, 2001a; Chow  
22 *et al.*, 2001b), as already discussed.

## 23 **6.5. Beyond the Stem Cell Compartment: Low Oxygen** 24 **Tension Impacts Proliferation and Differentiation** 25 **Potential of Various Hematological Lineages, and** 26 **More Broadly Stem Cell Differentiation**

### 27 **6.5.1. Phenomenological studies**

28 Beyond stem cells, oxygen tension can also greatly influence both cell proliferation  
29 and differentiation of hematopoietic lineages. The role of pO<sub>2</sub> with respect to  
30 desirable outcomes from hematopoietic cultures has been long recognized, starting  
31 with hematopoietic colony assays on semisolid media. The size and number of  
32 hematopoietic colonies in semisolid medium is significantly enhanced under low  
33 pO<sub>2</sub> (Bradley *et al.*, 1978; Broxmeyer *et al.*, 1989; Broxmeyer *et al.*, 1990; Katahira  
34 and Mizoguchi, 1987; Maeda *et al.*, 1986; Rich, 1986; Rich and Kubanek, 1982). In  
35 early studies in liquid cultures, pO<sub>2</sub> effects have been quite variable, with optimal  
36 production reported at 20% O<sub>2</sub> for murine BM cultures (Cipolleschi *et al.*, 1993)

1 and 5% O<sub>2</sub> for CB MNC cultures (Koller *et al.*, 1992a; Koller *et al.*, 1992c).  
2 Subsequent, more detailed and better controlled studies have established that the  
3 effects of thrombopoietin (Tpo), Epo and G-CSF on megakaryocytes, erythrocytes  
4 and granulocytes, respectively, are dependent on pO<sub>2</sub> (Hevehan *et al.*, 2000; LaIuppa  
5 *et al.*, 1998; Mostafa *et al.*, 2000b; Mostafa *et al.*, 2001). As discussed, in the  
6 case of megakaryocytes (Mks), relatively undifferentiated progenitor cells mature  
7 adjacent to BM sinus walls, extend protrusions (proplatelets) into blood vessels,  
8 and subsequently release platelets within the sinusoidal space or in lung capillaries  
9 (Junt *et al.*, 2007). Platelet release can be viewed as the culmination of terminal Mk  
10 differentiation, and because the sites for platelet release have higher levels of pO<sub>2</sub>  
11 than the core of the BM, where stem and progenitor cells reside, this example  
12 highlights the "oxygen paradigm" as it relates to hematopoietic differentiation.  
13 HSCs reside in a low oxygen state and move to a more oxygenated region to  
14 differentiate. In support of this hypothesis, Mks generated from primary human  
15 CD34<sup>+</sup> cells collected from G-CSF mobilized peripheral blood show increased  
16 markers of differentiation, such as greater expansion, increased polyploidization,  
17 and increased proplatelet formation (Mostafa *et al.*, 2000a). Moreover, pO<sub>2</sub>  
18 modulates the expression of cytokine receptors, transcription factors, and lineage-  
19 specific markers in cultured human megakaryocytes (Mostafa *et al.*, 2001). During  
20 cultures of cord blood and bone marrow-derived mononuclear cells, reduced pO<sub>2</sub>  
21 resulted in a substantial increase in both the number and frequency of granulocyte-  
22 macrophage colony-forming units (CFU-GM), erythroid burst-forming units  
23 (BFU-E), and granulocyte erythrocyte macrophage megakaryocyte colony-forming  
24 units (CFU-GEMM) (Koller *et al.*, 1992a). Low pO<sub>2</sub> also stimulates the activation  
25 or differentiation of cells generated from bone marrow precursors, including cells of  
26 the monocyte-macrophage lineage (Bradley *et al.*, 1978); (Broxmeyer *et al.*, 1990);  
27 (Koller *et al.*, 1992a).

28 Furthermore, low pO<sub>2</sub> is an integral component of the native BM microen-  
29 vironment of the human MSCs. Decreased (3%) pO<sub>2</sub> is also a key parameter that  
30 influences the *in vitro* proliferative lifespan and differentiation potential of BM-  
31 derived MSCs (Grayson *et al.*, 2006b). In these experiments, culturing cells at  
32 reduced pO<sub>2</sub> resulted in increased proliferation, but reduced the differentiation  
33 capacity of MSCs into adipogenic progeny. Similarly, hypoxia blocks the growth and  
34 differentiation of osteoblasts (and thus bone formation), while strongly stimulating  
35 osteoclast formation (and thus bone resorption). pO<sub>2</sub> also affects the differentiation  
36 of many other stem and progenitor cells, including neuronal (Studer *et al.*, 2000)  
37 and cytotrophoblast stem cells, (Genbacev *et al.*, 1996), and ESCs (Ezashi *et al.*,  
38 2005) among others.

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**6.5.2. Reactive-oxygen species (ROS) and antioxidants**

Several mechanisms may be responsible for the effects of pO<sub>2</sub> on hematopoietic cultures. The recent work on the importance of mild hypoxia on the maintenance and, possibly, renewal of the most primitive stem cell compartment was discussed above (Cipolleschi *et al.*, 1993); (Ivanovic *et al.*, 2000a); (Parmar *et al.*, 2007; Takubo *et al.*, 2010) (Mazumdar *et al.*, 2010; Simsek *et al.*, 2010). It is clear that ROS play a significant role in terms of both damage and signaling in the stem cell compartment, and apparently also at the more differentiated states of progenitors and beyond (Ito *et al.*, 2006; Nordberg and Arner, 2001; Sauer *et al.*, 2001; Tothova *et al.*, 2007). The literature on molecular signaling of the impact of O<sub>2</sub> or ROS on cells (stem cells and other) has grown enormously and is beyond the scope of this chapter, but a recent review (Naka *et al.*, 2008) provides an up-to-date summary on the impact of ROS on HSCs. In this section, our aim is to review the somewhat older literature on practical issues in the context of HSPC cultures related to O<sub>2</sub> effects and ROS toxicity, as it remains largely unknown and is not covered in the aforementioned 2008 review.

Decreased toxicity at low pO<sub>2</sub> was suggested several years ago by studies that have shown increased numbers of colonies in assays supplemented with the reducing agents 2-mercaptoethanol and  $\alpha$ -thioglycerol (Bradley *et al.*, 1978; Ishikawa and Ito, 1988; Katahira and Mizoguchi, 1987; Rich and Kubanek, 1982). Increased colony formation due to  $\alpha$ -thioglycerol is obtained both at low and high pO<sub>2</sub>, but enhancement is generally greater at 20% O<sub>2</sub>. Meagher *et al.* (Meagher *et al.*, 1988) measured increasing levels of superoxide and H<sub>2</sub>O<sub>2</sub> with time in BM cultures. Addition of catalase (which scavenges H<sub>2</sub>O<sub>2</sub>) or mannitol (which scavenges hydroxyl radicals) increased BFU-E production by 100% and 50%, and CFU-GM production by 50% and 25%, respectively. Superoxide dismutase had only a small effect, even though it decreased superoxide to undetectable levels. These results and the effects of the three additives on H<sub>2</sub>O<sub>2</sub> levels suggest that H<sub>2</sub>O<sub>2</sub> inhibits maintenance of hematopoietic stem and progenitor cells. The effects of pO<sub>2</sub> may also be related to specific effects on individual cell types. Macrophages produce cytokines in an O<sub>2</sub>-dependent manner (Rich, 1986), while T cells and macrophages cocultured under 5% O<sub>2</sub> combine to produce a factor that stimulates BFU-E proliferation (Pennathur-Das and Levitt, 1987). In our own work, we have observed increased IL-6 production under 5% O<sub>2</sub> in cultures of CB MNC, both in suspension and on irradiated stroma (Koller *et al.*, 1992c).

1 Oxygen and ROS levels are widely known (Fiegl *et al.*, 2009; Ito *et al.*, 2006;  
2 Nakata *et al.*, 2004; Nordberg and Arner, 2001; Schwarz *et al.*, 2009) to modulate  
3 gene expression of transcription factors, cytokines and growth factors, and, as  
4 such one would anticipate, a large interaction network whereby changes in O<sub>2</sub>  
5 or ROS concentrations will alter the locally produced growth factors, cytokines,  
6 and chemokines in the complex BM environment. Indeed, there are substantial  
7 literature as to how O<sub>2</sub> levels impact both cytokine production and regulation of  
8 cytokine receptor levels in hematopoietic and related cells (Fiegl *et al.*, 2009; Rogers  
9 *et al.*, 2008; Schwarz *et al.*, 2009; Tenen *et al.*, 1997). For example, transcription of  
10 the IL-6 and TNF- $\alpha$  genes is activated under low O<sub>2</sub> conditions due to upregulation  
11 in DNA binding of certain members of the C/EBP family of proteins (Yan  
12 *et al.*, 1995). The presence of C/EBP binding sites, which could potentially act  
13 as O<sub>2</sub>-responsive elements in the M-CSFR, GM-CSFR, and G-CSFR promoters,  
14 suggests that receptor expression may change with pO<sub>2</sub>. The importance of C/EBP $\alpha$   
15 was demonstrated in C/EBP $\alpha^{-/-}$  animals in which G-CSFR mRNA levels were  
16 reduced (Tenen *et al.*, 1997). The evident analogy between hypoxia-inducible factor-  
17 1 (HIF-1) binding to the Epo gene, which results in increased Epo production  
18 during O<sub>2</sub> deprivation (Fandrey, 1995; Wang *et al.*, 1995; Wang and Semenza,  
19 1993a; Wang and Semenza, 1993b) and C/EBP binding to the genes mentioned  
20 above, implicates changes in DNA-binding activity as the link between pO<sub>2</sub> and  
21 the perturbation of cytokine networks. This is an area of great practical interest  
22 in the context of stem cell bioengineering, aiming to achieve precise phenotypic  
23 outcomes.

## 24 **6.6. The Largely Underappreciated Impact** 25 **of Culture pH on Hematopoietic Stem and** 26 **Progenitor Expansion**

27 Based on the pH value of arterial and venal blood, most hematopoietic cultures  
28 have been performed at a pH of 7.4. However, as noted above, it is likely that pH  
29 varies within the BM. pH significantly affects the properties and differentiation  
30 of hematopoietic cells (McAdams *et al.*, 1997; McAdams *et al.*, 1998; Yang *et al.*,  
31 2002). A rise in intracellular pH has been associated with the activation of platelets,  
32 lymphocytes and neutrophils (Ozaki, 1992). pH can also be a powerful regulator  
33 of cell proliferation and cytokine secretion. Rich (Rich, 1988) has shown that the  
34 optimal proliferation of murine macrophages occurs between pH 7.6 and 8.0, and  
35 that Epo secretion by macrophages varies with pH<sub>e</sub>. It has also been reported that  
36 pH affects colony formation from murine BM cells (Zipori and Sasson, 1981).  
37 The myeloid lineage has demonstrated (Fischkoff and Rossi, 1990) that HL-60

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1 (promyelocytic leukemia) cells, which normally differentiate into monocytes when  
2 cultured at pH 7.2, will instead differentiate into eosinophils when cultured at  
3 pH 7.6. In another study employing the HL-60 cell line, decreasing culture pH  
4 (7.4, 7.2, and 7.0) increased the CD13 surface protein (a human aminopeptidase  
5 N/metalloprotease which is one of the earliest markers of commitment to the  
6 myeloid lineage, and involved in several key function of myeloid cells including viral  
7 infections) surface concentration without affecting CD13 mRNA levels. Decreasing  
8 culture pH also decreased the average HL.60 cell size, as well as glucose consumption  
9 and lactate production rates. Endo *et al.* have investigated the effects of pH on the  
10 human erythroid cell lines KU-812 and K562 (Endo *et al.*, 1994) and found that a  
11 significantly greater proportion of cells acquire a mature erythroid phenotype when  
12 cultured at pH 7.6 than when cultured at pH 7.4. The pH in static hematopoietic  
13 cultures has been reported to decline over time. The higher the cell density, the  
14 greater the pH decline, which results from metabolic acid production (Schultz  
15 *et al.*, 1992; Zipori and Sasson, 1981).

16 In cultures of primary HSPCs, which routinely experience pH variations  
17 of as much as 0.5 units, pH was found to have substantial effects both on  
18 progenitor cloning efficiency (as measured in liquid cultures) and on progenitor cell  
19 differentiation (as measured in methylcellulose cultures) (McAdams *et al.*, 1997;  
20 McAdams *et al.*, 1998). Cloning efficiencies of PB BPU-E in erythroid (E) cultures  
21 were ninefold higher at low pH (7.1) compared to pH (7.6). A small pH increase  
22 of 0.2 units over physiological values consistently produced significant reductions  
23 (42–85%) in cloning efficiencies for all cell types and cytokine combinations tested.  
24 Methylcellulose cultures of CB mononuclear (MNC) and PB MNC cells determined  
25 that differentiation of CFU-GM into progeny was optimal between pH 7.2 and 7.4.  
26 The differentiation of erythroid progenitors (BFU-E) progressively increased as pH  
27 was increased from 6.95 to 7.6. Flow cytometric analyses of key E surface markers  
28 CD71 and CD45RA demonstrated that E differentiation proceeds faster at high  
29 pH and is blocked at an intermediate stage by low pH. Methylcellulose cultures  
30 of PB CD34<sup>+</sup> cells exhibited similar patterns to the MNC cultures. In human  
31 primary Mk cultures from peripheral blood CD34<sup>+</sup> cells, higher pH promotes Mk  
32 cell differentiation, maturation, and apoptosis. Specifically, higher pH resulted in  
33 an earlier and higher polyploidization of CD41<sup>+</sup> Mk cells and an earlier onset  
34 of Mk cell apoptosis. Faster depletion of CD34<sup>+</sup> cells and an earlier peak in the  
35 fraction of larger colony-forming Mk cells (BFU-Mks) were also observed at higher  
36 pH. To sum, even small variations in pH substantially affected the performance  
37 of human hematopoietic cultures, with the E and Mk lineages being particularly  
38 sensitive, while PB progenitors are more sensitive to pH variations than CB  
39 progenitors.

## 6.7. Apoptosis, its Impact on Hematopoiesis and its Relation to $pO_2$ and pH

Apoptosis or programmed cell death is an essential process involved in removing superfluous or damaged cells (Kroemer *et al.*, 1995). With blood tissue being the most proliferative organ *in vivo*, apoptosis plays a major role in regulating hematopoiesis (Koury, 1992). The role of ROS in the apoptotic death of HSCs has been recently reviewed (Naka *et al.*, 2008). Beyond HSCs, it has been suggested that suppressing apoptosis (rather than inducing proliferation) may be an important regulatory role of lineage-specific cytokines such as Epo, Tpo and G-CSF (Lacronique *et al.*, 1997; Philpott *et al.*, 1997; Zauli *et al.*, 1997). In addition, other cytokines (TNF- $\alpha$  and TGF- $\beta$ 1) have been shown to potently induce apoptosis (Koury, 1992; Kroemer *et al.*, 1995; Sachs, 1996). Apoptosis is an important regulator of the function of mature granulocytic cells at sites of inflammation. Changes in surface marker expression allow apoptotic neutrophils to be phagocytosed intact, preventing the release of proinflammatory mediators (Dransfield *et al.*, 1995). Apoptosis may also play a significant role in regulating the proliferation and maturation of granulocytic progenitors, as indicated by G-CSF-induced suppression of apoptosis in PB CD34<sup>+</sup> progenitors (Philpott *et al.*, 1997) and the increased susceptibility to apoptosis of granulocytes in G-CSFR-deficient mice (Liu *et al.*, 1996). Similarly, studies of the Mk lineage have provided evidence that the natural fate of Mk cells is apoptotic death, which may be a trigger for platelet release (Zauli *et al.*, 1997). The role of Tpo appears to be the suppression of apoptosis in the Mk lineage long enough to allow significant differentiation and maturation to the platelet-forming stage. The features of cells stressed by oxidative damage and those undergoing apoptosis are remarkably similar (Payne *et al.*, 1995). For example, when a cell contains unrepaired DNA damage, p53 protein expression increases and induces the apoptotic pathways (Polyak *et al.*, 1997). The importance of  $pO_2$  and ROS in apoptosis is also indicated by the ability of antioxidants and free radical scavengers to inhibit apoptosis *in vitro* (Hockenbery *et al.*, 1993; Ito *et al.*, 2006; Kroemer *et al.*, 1995; Sakagami *et al.*, 1996). Beyond apoptosis, however, the production of ROS is an important part of signaling during hematopoietic differentiation. Notably, it has been shown that ROS produced in response to stimulation of hematopoietic cells by lineage-specific HGFs (GM-CSF, IL-3, SCF, and Tpo) are necessary for lineage-specific activation of hematopoietic cells predominantly through the tyrosine phosphorylation (Sattler *et al.*, 1999).

Culture or tissue pH affects the intracellular pH by a variety of mechanisms. Decreased intracellular pH ( $pH_i$ ) and/or alteration in the set point of  $Na^+/H^+$  antiporters as an initial step in apoptosis has been established in many cell types

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1 (Morana *et al.*, 1996; Reynolds *et al.*, 1996). Intracellular pH is an important  
2 parameter in the activation of apoptotic cascades (Hansen *et al.*, 2006) and notably  
3 of caspase-independent cascades (Kitanaka and Kuchino, 1999). Intracellular pH  
4 is also an important parameter affecting the cellular redox state, which determines  
5 how cells respond to apoptotic signals due to oxidative stress (Schafer and Buettner,  
6 2001). It was reported that the occurrence of DNA fragmentation correlated with  
7 intracellular acidification of up to 1 pH unit in the model human hematopoietic  
8 (myeloid cell) line HL-60 (Barry and Eastman, 1992; Barry *et al.*, 1993). A pH-  
9 sensitive DNase II (active below pH 6.8) was identified as the mediator of DNA  
10 digestion in this system, in the myeloid cell line ML-1 (human myeloid leukemia)  
11 and in an IL-2-dependent T cell line (Li and Eastman, 1995; Morana *et al.*,  
12 1996). In neutrophils, this endonuclease is the only detectable DNase (Gottlieb  
13 *et al.*, 1995). In some studies, artificial reduction of  $\text{pH}_i$  has resulted in the  
14 induction of apoptosis (Perez-Sala *et al.*, 1995). It has been also shown that  
15 intracellular alkalinization can block apoptosis (Rajotte *et al.*, 1992). The ability  
16 of G-CSF to protect neutrophils from apoptosis depends on activation of a vacuolar  
17  $\text{H}^+$ ATPase that increases  $\text{pH}_i$  (Gottlieb *et al.*, 1995). Other studies have shown  
18 the anti-apoptotic properties of SCF and GM-CSF are mediated through a similar  
19 mechanism (Caceres-Cortes *et al.*, 1994). Given that extracellular pH often affects  
20  $\text{pH}_i$  (Novikova *et al.*, 1993), these findings suggest that apoptotic regulation may be  
21 one of the mechanisms behind the effects of pH on hematopoietic cells, as discussed  
22 above.

## 23 **6.8. Shear Forces in Stem Cell and Hematopoietic** 24 **Differentiation**

### 25 **6.8.1. Biomechanical forces in stem-cell biology**

26 The original perception that the stem cell niche (see above) is physically a nearly  
27 static one (i.e. it lacks any appreciable fluid motion, whereby only low Reynolds  
28 number interstitial flow may be present), would lead to the conclusion that  
29 shear and other mechanical forces do not affect stem cell biology and progenitor  
30 differentiation. This perception is changing fast over the last few years for all  
31 stem cell types, including MSCs (Park *et al.*, 2010; Yamamoto *et al.*, 2005), ESCs  
32 (Adamo *et al.*, 2009; Ahsan and Nerem, 2010; Masumura *et al.*, 2009; Saha *et al.*,  
33 2006), and HSCs (Collins *et al.*, 1998a; Dunois-Larde *et al.*, 2009). However,  
34 when it comes to the differentiation of hematopoietic stem and progenitor cells,  
35 shear forces are of physiological significance for most lineages, and notably for the  
36 Mk lineage, which we discuss in some detail below. Both mature and immature

1 hematopoietic cells are released into circulation and subjected to a spectrum  
2 of shear and biomechanical forces; in this context, the expectation that such  
3 forces would affect the differentiation and maturation of hematopoietic cells is a  
4 logical one.

### 5 **6.8.2. The importance of biomechanical forces** 6 **in megakaryocytic differentiation**

7 Mk cells are subjected to mechanical forces (strain) as Mk cells squeeze through  
8 gaps in the endothelial lining of the BM sinusoids (Figures 6.3 and 4), as well  
9 as shear forces due to blood flow in the BM microvasculature or when Mk cells  
10 enter circulation and mature in the pulmonary vasculature (Dunois-Larde *et al.*,  
11 2009). Such forces result in enhanced Mk maturation, (pro)platelet shedding and  
12 enhanced platelet biogenesis both *in vivo* (Junt *et al.*, 2007) and *in vitro* (Dunois-  
13 Larde *et al.*, 2009). The data in these recent studies suggest that shear/mechanical  
14 forces engage specific signaling (which is so far unexplored) that enhances platelet  
15 biogenesis. Although shear effects on endothelial cell biology is a well-established  
16 field, they remain mechanistically unexplored in Mk differentiation. Activation  
17 of p53 signaling in endothelial cells in response to flow-induced shear stress has  
18 been established (Zeng *et al.*, 2003). Our recent work (Fuhrken *et al.*, 2008;  
19 Fuhrken *et al.*, 2007) supports the hypothesis that p53 is a regulator of Mk  
20 maturation, modulating Mk-cell polyploidization in a specific manner, whereby  
21 p53 senses the cellular stress associated with polyploidization and regulates the  
22 process by controlling endomitosis vs. apoptosis. Furthermore, preliminary data  
23 from our laboratory suggest that shear flow alters p53 acetylation and thus, p53  
24 activation. It appears that p53, as a known regulator of stress response, is also  
25 involved in integrating mechanical stresses during Mk maturation and proplatelet  
26 release.

### 27 **6.9. Learning from Nature: Case Studies of How** 28 **to Integrate the Effects of pO<sub>2</sub>, pH, Fluid-forces,** 29 **and Medium Perfusion in Processes to Generate** 30 **Desirable Progenitor and Mature** 31 **Hematopoietic Cells**

32 In this section, the goal is to show how these biophysical parameters (pO<sub>2</sub>, pH,  
33 perfusion, biomechanical forces) have been, or can be, explored to bring about  
34 desirable culture outcomes in the context of stem- and progenitor-cell cultures and

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1 bioengineering. We discuss three cases where one or more of these parameters were  
2 integrated in achieving a desirable outcome.

3 **6.9.1. Perfusion and low  $pO_2$  levels for expanding**  
4 ***the primitive HSPC compartment***

5 Since the most primitive compartment of hematopoiesis in the BM takes place under  
6 homeostatic perfusion (albeit at low rates), and low  $pO_2$  conditions, such conditions  
7 would be expected to provide superior performance of the expansion of HSPCs. The  
8 development of perfusion bioreactors for stroma cocultures with control of  $pO_2$  at  
9 about 5% demonstrated superior performance compared to any other published  
10 culture system, and notably so for the expansion of the most primitive LTC-IC cells  
11 (Koller *et al.*, 1993). Similarly, the beneficial effect of perfusion on LTC-IC output  
12 in the absence of a stromal layer was also demonstrated (Sandstrom *et al.*, 1996;  
13 Sandstrom *et al.*, 1995).

14 **6.9.2. Stirred hematopoietic bioreactors with pH and**  
15  **$pO_2$  control and perfusion operation for**  
16 ***expanding myeloid cells***

17 A stirred bioreactor would have several advantages over static-culture systems and  
18 flat-bed bioreactors: inherent homogeneity, ability to sample more easily for culture  
19 characterization, ability to implement process-parameter control, and ease of scale-  
20 up to clinical size. On the other hand, successful growth of hematopoietic cells in  
21 stirred reactors may be problematic due to the shear sensitivity of the cells and  
22 the lack of characteristics present in static systems (e.g. cell-to-cell contact and  
23 a local microenvironment around the cells). Such bioreactors would be suitable  
24 for the expansion of mononuclear cells into granulocytes/ neutrophils, as these  
25 cells apparently grow as suspension cultures in defined media and with defined  
26 cytokine combinations (Collins *et al.*, 1996; Hevehan *et al.*, 2000; Timmins and  
27 Nielsen, 2009). PB MNCs, CB MNCs, and PB CD34<sub>+</sub> cells grew well in spinner  
28 flasks when compared to control T-flask (static) cultures (Collins *et al.*, 1998a).  
29 Culture proliferation in spinner flasks was dependent on both agitator design and  
30 RPM, and when optimized, provided better culture outcomes compared to static  
31 controls. When combined with a cell dilution feeding protocol, cultures of PB  
32 and CB MNCs in stirred bioreactor with pH and  $pO_2$  control provided better  
33 outcomes in the expansion of total cells and CFU-GM progenitor cells (Collins  
34 *et al.*, 1998b).

1 **6.9.3. A three-stage process for the production of**  
2 **megakaryocytic cells and platelets from**  
3 **human CD34<sup>+</sup> cells, and the importance of**  
4 **biomechanical forces in a scaled-up process**

5 As discussed, megakaryopoiesis requires HSPC commitment (Figure 6.1) to the  
6 megakaryocytic cell (Mk) lineage. Because the degree of Mk ploidy directly  
7 correlates with the number of platelets produced (Mattia *et al.*, 2002), it is  
8 necessary to increase the ploidy of cultured Mks similar to what is observed  
9 *in vivo*, thus making it possible to obtain several thousand platelets per Mk  
10 (Mattia *et al.*, 2002). After commitment of HSPCs to Mk differentiation, the  
11 road to platelet production requires expansion of Mk progenitors and committed  
12 Mks, endomitosis accompanied polyploidization, maturation, and Mk apoptosis  
13 accompanied proplatelet release (Figure 6.4). This is a set of sequential steps, each  
14 dependent on the previous one, while amplifying the capacity of the system in  
15 terms of cell numbers. HSPCs move from the endosteum to the BM sinuses as  
16 they commit to become Mks (Figure 6.3) and penetrate the endothelial walls of the  
17 BM sinuses to release proplatelets (Figure 6.4). As discussed, a set of committed  
18 Mks can directly enter circulation through the BM sinuses to reach maturation and  
19 release proplatelets in the lung vasculature. pH and pO<sub>2</sub> levels increase from the  
20 endosteum to sinuses (Figure 6.3), and different sets of cytokines are important  
21 for various stages of differentiation (Panuganti *et al.*, 2010). Moreover, pO<sub>2</sub> and  
22 pH levels in blood circulation and the lung vasculature are considerably higher  
23 than in the endosteum region of the BM and, for that matter, than in most of the  
24 BM hematopoietic compartments between sinuses. Thus, Mk maturation requires  
25 higher pO<sub>2</sub> and pH settings, while HSPC and Mk progenitor settings require lower  
26 pO<sub>2</sub> and pH settings and different cytokine combinations. We thus argued that *ex*  
27 *vivo* Mk or platelet production would be better achieved by dividing the process into  
28 three separate phases (Panuganti *et al.*, 2010). Environmental conditions and growth  
29 factors must be optimized separately for Mk commitment and progenitor expansion,  
30 mature Mk production and Mk polyploidization; we set out to optimize such process  
31 in terms of cytokine combinations, and the pO<sub>2</sub> and pH settings (Panuganti *et al.*,  
32 2010).

33 In the actual scaled-up process, while the early phase of HSPC expansion  
34 and early Mk commitment can be carried in a suspension reactor as discussed  
35 above, the process of Mk maturation, involving cell size enlargement due to  
36 polyploidization and controlled cell death for proplatelet release, will require a  
37 “protective” environment such as a packed-bed bioreactor (Sullenbarger *et al.*,  
38 2009). As discussed, shear/mechanical stresses play a crucial role, both *in vivo* (Junt

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1 *et al.*, 2007) and *in vitro* (Dunois-Larde *et al.*, 2009), in the biogenesis of functional  
2 platelets. Thus, the ability to control Mk maturation under flow conditions is critical  
3 for providing tissue engineering solutions for designing processes to achieve large-  
4 scale production of platelets (Sullenbarger *et al.*, 2009). Incorporating shear flow in  
5 the design of processes aiming to optimize platelet release from *in vitro* generated  
6 human Mks is a necessary step for achieving *ex vivo* platelet production, which is a  
7 goal of great practical significance in transfusion medicine (Reems *et al.*, 2010).

8 **6.10. Summary**

9 The stem cell niche and the microenvironment, in which progenitor cells  
10 differentiate to produce mature hematopoietic cells to be release into circulation, is  
11 characterized by low O<sub>2</sub> tension and O<sub>2</sub> gradients, pH gradients, cytokine gradients,  
12 variable BM perfusion rates (due to variable vascularization of the BM; low near  
13 the endosteum, high away from the endosteum; Figure 6.3), and in some cases,  
14 exposure to flow-induced shear forces or biomechanical forces due to cell penetration  
15 through endothelial gaps. We have reviewed the physiological, anatomical and,  
16 where available, molecular evidence for how such parameters and gradients affect  
17 the maintenance and differentiation of stem cells, significantly the expansion,  
18 differentiation and phenotypic characteristics of progenitor and differentiated cells.  
19 We have also discussed applications and integration of these parameters in process  
20 to generate stem, progenitor and differentiated hematopoietic cells for applications  
21 in regenerative and transfusion medicine.

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