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Searching for the Key to Expand Hematopoietic Stem Cells

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1. Introduction

Stem cells are characterized by their capacity to self renew and differentiate into progressively restricted cells that ultimately become limited to a specific cell fate. The two broad types of mammalian stem cells are: embryonic stem cells and adult stem cells.

Embryonic stem cells (ESC) are mostly derived from the undifferentiated inner mass cells of a blastocyst. These cells give rise during development of the embryo to all derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm. They do not contribute to the extra-embryonic membranes or the placenta. Ex-vivo, they can be cultured for extended periods of time and under the appropriate conditions, they can be also directed to differentiate into many specialized types of cells. These particular features are being exploited to use ESC as starting material for treatment of degenerative diseases and replacement of damaged organs. Although their potential is great, the promise of ESC-derived therapies will be unfulfilled unless several challenges are overcome. For example, the quite small production of ESC-derived cells obtained or the active immune rejection of the ESC-derived graft.

Unlike embryonic stem cells, the adult stem cells are already partially specialized. They have been found in most self-renewing tissues, including the skin, the brain, the intestinal epithelium and the hematopoietic system and have the primary role of maintaining and repairing the tissue in which they are found. They are located deep within organs in specialized areas known as the "stem cell niche" (Scadden, 2006). This microenvironment allows for their survival, self renewal, regulated proliferation and maintenance of their quiescence for long periods of time until the moment in which they are activated. *Ex vivo*, however, the capacity of stem cells to self-renew is limited, they exhibit poor survival and consequently their numbers sharply declines during experimental manipulation.

One of the more intriguing but highly debated areas of stem cell biology was the phenomenon described as plasticity or transdifferentiation. Numerous reports expressed opposing views

about this ability of stem cells to cross organ/tissue boundaries. These discrepancies have now been mostly passed over by current research showing that cell populations of one lineage might produce cells from other lineages by changing gene expression in response to micro-environmental cues (Jang and Sharkis, 2005; Theise, 2010).

Owing to their unique characteristic of plasticity, self-renewal capacity and potential to generate functional cell types, stem cells are particularly attractive for developing therapeutic settings that range from drug discovery protocols to cell transplantation and regenerative therapies. Nevertheless, several challenges including the need to identify the signals that influence the stem cell fate decisions and the application of this information towards the design of stem cell bioprocesses have to be overcome to accomplish the transition from fundamental science to functional technologies.

1.1 Hematopoietic stem cells

Hematopoietic stem cells (HSC) are probably the best characterized adult stem cell and often serve as a paradigm for other stem cells. Even though no morphological criteria to unequivocally identify such cells exist, HSC have been proven to be invaluable in the clinic. They are the only stem cells used routinely in cell based therapies, to treat numerous hematologic and non-hematologic malignancies as well as a range of both inherited and acquired diseases. This is typically due i) to the availability of a straight forward purification protocols using cell surface antigen selection and ii) to the possibility to perform reconstitution assays that rely on their clonal ability to reconstitute the entire hematopoietic system following transplantation into myeloablated recipients (Fig. 1). The same cell surface antigens, however, do not always conform to the same stem cell functional phenotype (Simonnet et al., 2009) and therefore the transplantation procedure constitutes undoubtedly the "gold standard" method for proving that a cell is indeed an HSC.

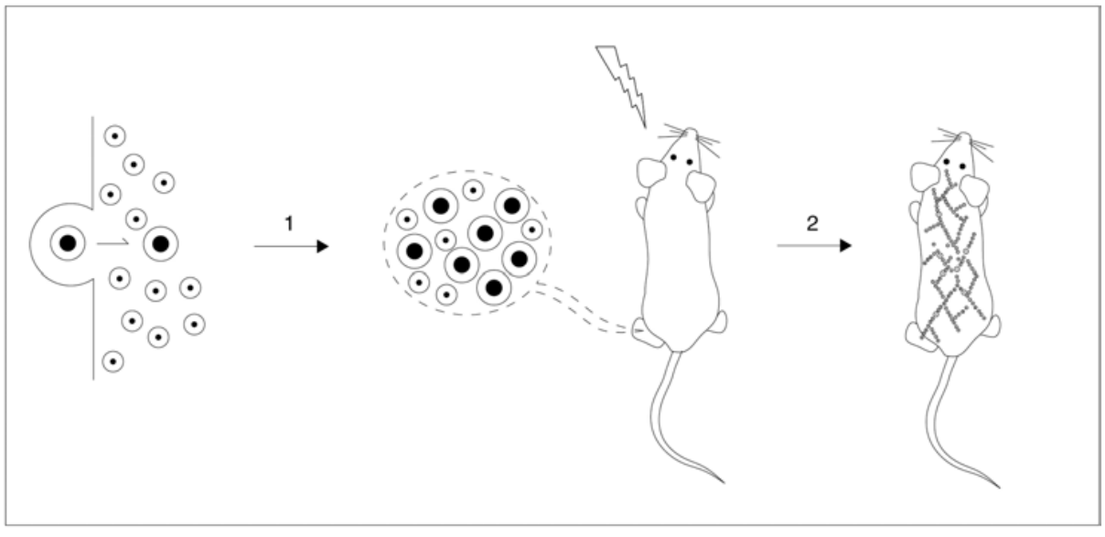

Fig. 1. A diagrammatic representation of a stem cell in its micro-environment and one stem cell induced to move out of the niche where it will undergo development. Following BM removal and cell surface antigen selection (1), cells are cultured *in vitro* and infused in a myeloablated mouse. Several weeks after (2), blood cells are regenerated in the transplanted mouse.

To maintain the steady-state of the stem cell compartment and to allow the regeneration of hematopoietic cells after transplantation or after hematopoietic injury, HSC divide asymmetrically or symmetrically. In an asymmetric self-renewing division, the two daughter cells adopt different fates, resulting in only one cell maintaining stem-cell properties. The symmetric self-renewing division refers to the process whereby both daughter cells retain stem cell properties. This type of cell division expands the stem-cell pool and is therefore critical for sustaining the HSC compartment and thus is a requirement for lifelong hematopoiesis.

The HSC fate decisions are dependent on concomitantly intrinsic HSC fate determinants and extrinsic signals delivered by the bone marrow (BM) niches were HSC resides. These niches are small cavities formed by heterogeneous types of cells, named stroma, that are positioned close to the BM longitudinal axis of the femur with more differentiated cells disposed in a graduated manner as the central longitudinal axis of the bone is approached. The attachment of HSC to the stroma via a network of adhesion molecules provide an environment that optimally balances signals that control self-renewal, proliferation and differentiation. Under normal physiological conditions, HSC are kept in a relatively low proliferative, quiescent state, protecting them from stress and preventing their depletion due to excessive proliferation (Jang and Sharkis, 2007). Recent data imply that these areas where HSC reside are hypoxic (Parmar et al., 2007).

To take advantage of the HSC plasticity capacities for therapeutic use, HSC may be withdrawn from their original niches, and placed on a novel non-hematopoietic environment. Once located in this novel medium, the reprogramming of the cell genome occurs and directs and/or contributes to their conversion into unrelated cell types (Fig. 2). The unexpected flexibility of HSC to produce non-hematopoietic cells was described for several cells/tissues (Quesenberry et al., 2010) including liver cells (Almeida-Porada et al., 2010; Jang et al., 2004), neurones (Mezey et al., 2000), lung epithelial (Abe et al., 2003) or connective tissues (Ogawa et al., 2010).

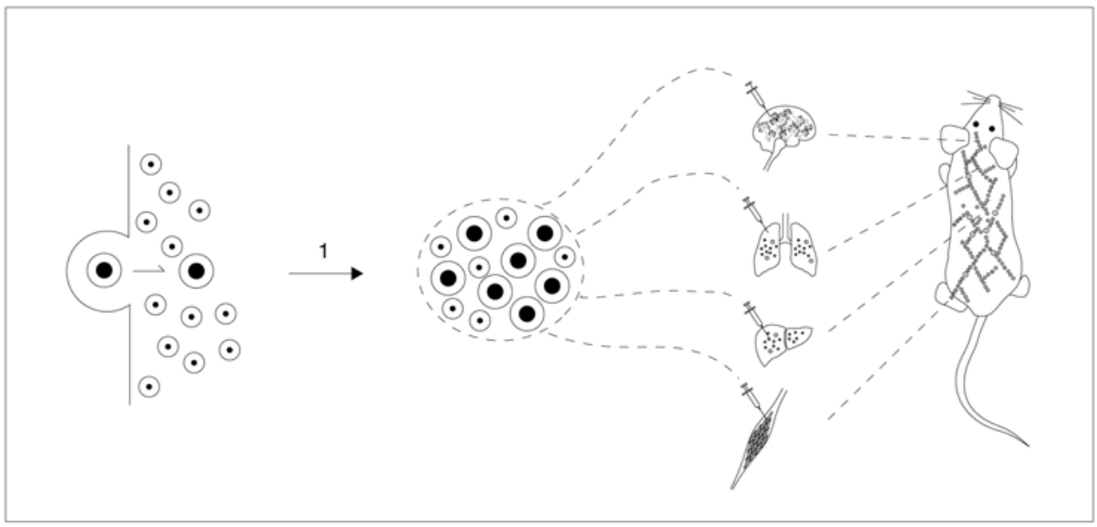

Fig. 2. A schematic representation of HSC plasticity. Hematopoietic cell are removed from the femoral bone (1) and the HSC-enriched population is cultured *in vitro*. Following an optional genetic modification, cells may be used to generate *in vivo* non-hematopoietic cell types.

The development of HSC-based therapies however, is to some extent prevented by the scarce representation of HSC in the BM and their finite lifespan *ex vivo*. Increasing their utilisation needs enhancement of hematopoietic stem cells availability or *de novo* generation of HSC. This presumes i) the development of robust methods to efficiently control HSC regulatory processes; ii) the therapeutic *in vivo* or *in vitro* expansion of HSC number and iii) the utilisation of optimized protocols to generate available HSC from ESC or IPSC.

2. Physiological pathways involved in the regulation of stem cells

HSC fate decisions are supported by the orchestration of several pathways such as Wnt, Notch and Hedgehog pathways that critically balance cell cycling and quiescence, leading to proliferation and apoptosis, self-renewal or differentiation (Fig. 3). The ultimate decision is dependent on hundreds of inputs including concentrations of different growth factors, cytokines, hormones, oxygen levels that must be integrated to subsequently activate these different signal transduction cascades. Understanding their regulation might led to the effective and more spread out utilization of HSC in clinical settings. The most relevant aspects of these pathways are briefly resumed below.

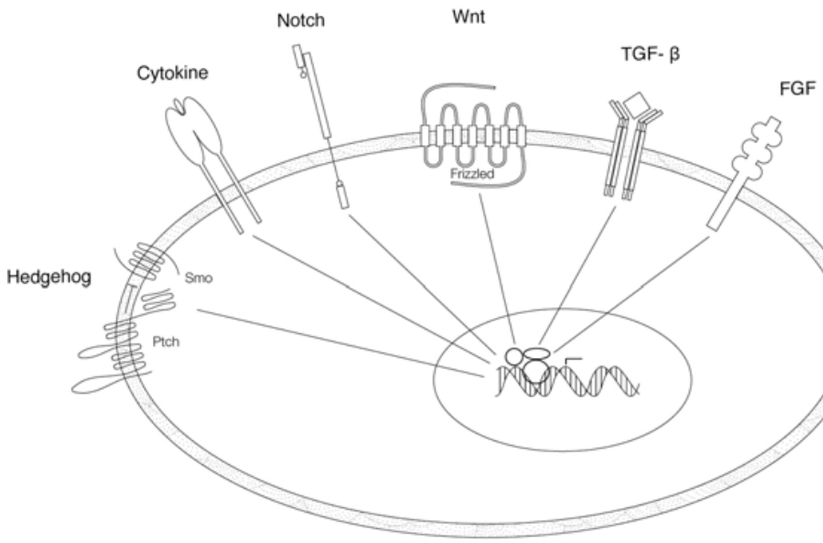

Fig. 3. A schematic representation of signaling pathways collectively influencing stem cell fate.

2.1 The hedgehog (Hh) signaling

In adult tissues, Hh signaling is involved in the maintenance of stem cells, regeneration and tissue repair where it governs processes like cell proliferation, cell renewal and differentiation. The three Hh ligand homologues: Sonic Hh, Indian Hh, and Desert Hh bind interchangeably the two related twelve-pass membrane Patched (Ptc) receptors. They relieve the inhibition of smoothened (SMO), a serpentine receptor resembling G protein–coupled receptors allowing activation of a family of zinc-finger transcription factors called GLI and the modification of the expression Hh target genes (Kasper et al., 2009).

The role of Hh signaling in HSC is controversial. Bhardwaj et al provided evidence for a role of Hh signaling in HSC (Bhardwaj et al., 2001). In this study, suppression of Hh signaling inhibited proliferation of HSC and addition of soluble SHh induced expansion of hematopoietic repopulating cells (Bhardwaj et al., 2001). More recent reports confirmed that suppression of the Hh pathway leads to a severe defect in HSC functions (Merchant et al., 2010; Trowbridge et al., 2006) whereas others reported that this pathway can be dispensable for HSC biology (Gao et al., 2009; Hofmann et al., 2009). In *Ptc1*+/−mice, which have increased Hh activity, activation of the Hh signaling pathway induces expansion of primitive blood cells under homeostatic conditions. However, when HSC are challenged to regenerate the blood system, persistent Hh activation leads to HSC exhaustion (Trowbridge et al., 2006). Furthermore, Indian Hh gene transfer can confer enhanced hematopoietic support ability to BM stromal cells, suggesting that it is involved in the interaction between HSC and the stromal cells. This leads to an increase in proliferation and repopulating capacity of primitive hematopoietic cells (Kobune et al., 2004). These results suggest a role for Hh signaling in balancing homeostasis and regeneration *in vivo*. In contrast, other reports show that Hh signaling is dispensable for adult HSC functions (Gao et al., 2009; Hofmann et al., 2009). In these studies conditional deletion of SMO, the only non redundant component of the Hh cascade, or pharmacologic inhibition of Hh signaling have no apparent effect on adult hematopoietic, including peripheral blood count, number or cell cycle status of stem or progenitor cells, hematopoietic colony-forming potential or long-term repopulating activity in *in vivo* assays. In agreement with this notion, genome-wide transcriptome analysis revealed that silencing the Hh signaling does not significantly alter the HSC-specific gene expression ''signature.'' Taken together, these conflicting data suggest that Hh signaling may influence HSC through more complex networks such as cell-niche interactions.

2.2 Fibroblast growth factor (FGF) signaling

FGF belongs to a family of heparin-binding polypeptides that shows multiple functions, including effects on cell proliferation, differentiation and survival (Baird, 1994). Twenty-four members of the FGF family have been identified in human and mice. FGFs bind and activate their cognate FGFRs that are encoded by four genes (FGFR1– 4). This results in receptor dimerization, tyrosine kinase autophosphorylation, and recruitment of signaling complexes. The FGF signal transduction proceeds by one, or a combination, of three main pathways: Ras/mitogen-activated protein kinase (MAPK) signaling; planar cell polarity/calcium; phosphoinotitide-3-kinase (PI3K)/Akt (extensively reviewed by Bottcher and Niehrs, 2005). Both FGF-1 and FGF-2 support HSC expansion when unfractionated mouse BM cells are cultured in serum-free medium (de Haan et al., 2003; Yeoh et al., 2006). Crcareva et al. confirmed that FGF-1 stimulates ex-vivo expansion of HSC (Crcareva et al., 2005). Conditional derivatives of FGFR-1 have also been used to support short-term HSC expansion and long-term HSC survival (Weinreich et al., 2006). This factor seems to also support *ex vivo* expansion of murine and human HSC in combination with other cytokines, i.e stem cell factor [SCF], thrombopoietin [TPO], insulin-like growth factor-2 [IGF-2], and fibroblast growth factor-1 [FGF-1] (Zhang and Lodish, 2005). Moreover, a recent study showed that addition of SCF, TPO, and FGF-1 to a mesenchymal stem cells (MSC) culture stimulates proliferation, maintenance of primitive immunophenotype, and expansion of CFU-initiating cells. This supports the notion that expansion of HSC requires complex stimulation of different signal cascades activated by soluble growth factors as well as adhesion proteins (Walenda et al., 2011).

2.3 Notch signaling

The Notch pathway is also an evolutionarily conserved mechanism that plays a fundamental role in regulating cell-fate decisions (Bolos et al., 2007). Four types of Notch receptors (Notch 1-4) and five Notch ligands (Jagged 1 and 2, Delta 1, 3 and 4) have been identified in vertebrates. Notch ligands are single-pass transmembrane proteins consisting of multiple EGF-like repeats and a characteristic DSL (Delta, Serrate, and LAG-2) domain (see for review Ohishi et al., 2003; Shimizu et al., 2000). One characteristic of this signaling pathway is the dual role of Notch as both a transmembrane receptor and a transcription factor in a system where no second messengers are used (Matsuno et al., 1995). Notch can have opposite functions in different self-renewing organs indicating that the outcome of Notch activation depends to a great extent on the cell context and the specific growth factors present in the microenvironment. For example, activation of Notch1 by Delta ligands 1 and 4 is required for inducing T-cell and inhibiting B-cell differentiation whereas Notch2 activation by Jagged1, and possibly Delta1, acts on HSC (Han et al., 2002; Radtke et al., 1999; Varnum-Finney et al., 2011).

A role for Notch in hematopoietic was initially suggested by detection of the human Notch1 gene in $CD34^+$ or lineage $(Lin)^-CD34^+$ hematopoietic cells (Milner et al., 1994). Transduction of murine HSC with a retrovirus expressing a constitutively active form of Notch1 induced the emergence of an immortalized pluripotent cytokine-dependent cell line capable of both myeloid and lymphoid repopulation *in vivo*, thereby demonstrating a role for Notch in HSC self-renewal (Varnum-Finney et al., 2000). Similar results were obtained using an immobilized form of the Notch ligand Delta-1 since incubation of murine HSC with immobilized Delta-1 and cytokines led to a several-log expansion of cells capable of short-term *in vivo* reconstitution (Varnum-Finney et al., 2003).

In contrast to the murine studies, only a modest or no increase in the progenitor numbers was achieved by expressing activated Notch-1 in human $CD34^+$ cord blood cells (Carlesso et al., 1999; Chadwick et al., 2007) or by incubation with Delta-1 (Jaleco et al., 2001), Delta-4 (Lauret et al., 2004) or Jagged-1 (Karanu et al., 2000; Karanu et al., 2001; Walker et al., 1999). This contrast with other reports showing that incubation of human cord blood cells with the immobilized Delta-1 combined with fibronectin fragments and cytokines induce a 100-fold increase in the number of $CD34^+$ cells compare to controls (Ohishi et al., 2002) and a 16-fold increase in SCID Repopulating Cells (SRC) number compared to uncultured cells. *In vivo* transplanted cells persisted 9 weeks post-transplantation and in secondary recipients, suggesting the presence of both long-term and short-term repopulating cells following culture of human cord blood cells on Delta-1 ligand (Delaney et al., 2010). The SRC enhancement by relatively low density of immobilized ligand and the preference to promote differentiation toward the T-cell lineage at higher ligand density revealed important ligand dose-dependent effects of Notch signaling (Delaney et al., 2005).

The engineered Notch ligand approach for *ex vivo* expansion of human cord blood cells is now under clinical investigation (http://clinicaltrials.gov/ct2/show/record/NCT00343798). In this phase 1 clinical trial, patients undergoing a myeloablative double cord blood transplantation are receiving one non-manipulated cord blood unit along with a second cord blood unit that has undergone Notch-mediated *ex vivo* expansion. These cells were safely infused and led to a significant reduction in the time needed for neutrophil

recovery (16 days in patients receiving the expanded unit, compared to 26 days in patients of the concurrent cohort). Similarly, preliminary evaluation of time needed for platelet recovery compared favourably in those patient receiving the expanded cell product compared with those receiving non-manipulated cells (Dahlberg et al., 2011). In addition, comparable overall survival and graft-versus-host disease risk of patient receiving non-manipulated cells was observed within the average follow-up of 354 days. The expanded cell population may also have retained long-term repopulating capacities as two patients display *in vivo* persistence of cultured donor cells. The lack of *in vivo* persistence in the remaining patients may either be due to loss of stem cell self-renewal capacity during *ex vivo* culture or to immune mediated rejection. Indeed, it has been well documented that in most of the patients who received two non-manipulated cord blood units for transplantation, only one contributes to persistent long-term engraftment. The mechanism responsible for this single donor dominance remains yet to be defined. Larger phase II/III studies are required to evaluate whether co-infusion of this expanded cell product decreases the occurrence of serious infection, improves survival, or affects duration of hospital stay (Delaney et al., 2010).

2.4 The transforming growth factor beta (TGFβ) superfamily

The TGFβ superfamily consist of a large collection of secreted proteins that regulate cell growth, differentiation, apoptosis, cellular homeostasis, and other functions in both the adult organism and the developing embryo. The more than 30 TGFβ family ligands are organized into three subgroups (reviewed in (Lyssiotis et al., 2011)). The TGFβ (which comprises SMAD and Activin/Nodal ligands), bone morphogenetic protein (BMP), and the growth differentiation factors (GDF). The TGFβ signaling leads to the phosphorylation of Smads by activated receptors resulting in their partnering with the common signaling transducer Smad4, and translocation to the nucleus. Once activated, Smads regulate diverse biological effects by partnering with transcription factors resulting in cell-state specific modulation of transcription (Kaivo-Oja et al., 2003).

A significant number of studies have demonstrated that TGFβ inhibits proliferation of both murine and human HSC *in vitro*. It was suggest that TGFβ induces quiescence in HSC since its neutralization was showed to release early hematopoietic progenitors cells from quiescence (Hatzfeld et al., 1991; Yamazaki et al., 2009). In agreement with studies performed *in vitro*, injection of TGFβ1 into the femoral artery of mice effectively inhibits proliferation of multipotent hematopoietic progenitors in the BM, establishing an inhibitory role of TGFβ1 also *in vivo* (Goey et al., 1989). Despite a key role *in vitro*, TGFβ did not seem to provide the necessary signals that maintain quiescence and the stem cell pool *in vivo* (Larsson et al., 2005).

To block the entire Smad signaling pathway, the Smad7 was overexpressed in murine HSC using a retroviral gene transfer approach. Forced expression of Smad7 significantly increased the self-renewal capacity of HSC *in vivo* (Blank et al., 2006). In a similar approach using human hematopoietic cells, overexpression of Smad7 resulted in a shift from lymphoid-dominant engraftment toward the myeloid lineage, and an increase of the myeloid-committed clonogenic progenitor frequency in NOD-SCID mice (Chadwick et al., 2005). Instead, Smad4-deficient HSC displayed a significantly reduced repopulative capacity

of primary and secondary recipients (Karlsson et al., 2007). Because overexpression of Smad7 versus deletion of Smad4 would be anticipated to yield similar hematopoietic phenotypes, it is conceivable that Smad4 functions as a positive regulator of self-renewal independently of its role as a central mediator of the canonical Smad pathway. In the context of adult hematopoiesis, a high concentration of BMP-4 was shown to promote maintenance of human cord blood cells *in vitro*, while lower concentration of BMP4, BMP2 and BMP7 induced proliferation and differentiation of HSC (Bhatia et al., 1999).

2.5 Wingless-type (Wnt) pathway

Wnt proteins are secreted morphogens necessaries for basic developmental processes, such as cell-fate specification, progenitor-cell proliferation and the control of asymmetric cell division, in many different species and organs (Bejsovec, 2005; Moon et al., 2004). Wnt proteins bind to cell surface receptors of the Frizzled family which can translocate the signals to the nucleus and function as transcriptional activators through intracellular β-catenin. Different Wnt pathways are known but their clear separation and their independence remain controversial. There is one canonical pathway that acts on the stability of β-catenin and interacts with T cell transcription factors in the nucleus. There are many non-canonical pathways like the PCP and Wnt/Calcium pathways. The most distinctive differences between the canonical and non-canonical pathways include the specific ligands activating each pathway, ß-Catenin, LRP5/6 co-receptor, and Dsh-DEP domain independence, respectively, and the ability of the non-canonical pathways to inhibit the canonical pathway. Ligands that activate the non-canonical pathways are Wnt4, Wnt5a, and Wnt11.

Recent evidence based on genetic models suggests that canonical Wnt signaling, regulates HSC self-renewal. Active β-catenin promotes HSC proliferation and inhibits differentiation (Kirstetter et al., 2006; Scheller et al., 2006) whereas deficiency in β-catenin inhibits HSC self-renewal (Cobas et al., 2004; Luis et al., 2009; Zhao et al., 2007). Moreover, purified Wnt3a treatment of adult HSC increases self-renewal of murine HSC, as determined by *in vivo* reconstituting assays (Willert et al., 2003) and of human $Lin^{-}CD34^{+}$ cells as measured by immunophenotype and colony assays (Van Den Berg et al., 1998).

The role of the non-canonical pathways is not well defined, but surprisingly, their activation and consequently inhibition of the canonical pathway, appears also to be able to expand HSC. Murdoch et al. demonstrated that injecting mice with Wnt5a conditioned media prior to transplant of human umbilical cord blood cells increased engraftment more than 3-fold (Murdoch et al., 2003). Furthermore, culturing $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) cells with recombinant murine Wnt5a resulted in an enhancement of hematopoietic reconstitution in a BM transplant assay. Wnt5a seems to activate the non-canonical signaling pathways leading to a 3.5- fold more HSC in G0 phase (Nemeth et al., 2007).

Overexpression of Wnt4 led to a modest increase in HSC frequency as measured by phenotype and limiting dilution transplant assays and Wnt4-/- mice showed decreased frequencies of HSC in BM. Similar to the results obtained using Wnt5a, overexpression of Wnt4 led to an increase in the percentage of HSC in G0 (Louis et al., 2008). Whether Wnt4 and Wnt5a inhibit the canonical pathway in a similar fashion remains to be elucidated. These results show the importance of a balanced regulation of these two overlapping Wnt signaling pathways.

2.6 Cross-talk between these pathways

The individual contribution of these pathways to the hematopoietic development of HSC have been extensively addressed (Cerdan and Bhatia, 2010). However, there are many potential intersections along them and therefore the impact of their collective contribution towards influencing the fate of HSC should be carefully considered. Some of these intersection points are resumed below.

Ducan et al. provide a model for how HSC may integrate multiple signals to maintain the stem cell state. They showed that although the proliferation and survival of HSC exposed to Wnt proteins seem unaffected when Notch signaling is impaired, their ability to remain undifferentiated is substantially altered (Duncan et al., 2005). These results demonstrated that the Notch pathway is imperative in maintaining HSC in an undifferentiated state. These findings do not preclude the possibility that a stronger Wnt signal, such as activated β-catenin, may be able to overcome the consequences of loss of Notch signaling. Moreover, Wnt3a regulates the expression of established Notch target genes (Duncan et al., 2005) and the inhibition of GSK-3, a downstream target of Wnt signaling that affects HSC fate through mechanisms involving both Wnt and Notch target genes (Trowbridge et al., 2006). These findings suggest that these pathways could play a role in HSC self renewal using a common network of regulatory circuits with Wnt enhancing proliferation and survival, and Notch preventing differentiation (Blank et al., 2008).

Furthermore, there is substantial evidence for the cross-talk between the Wnt signaling pathway and FGFs and TGF-b by means of the association between Smad4 and Hox proteins. *Homeobox* (*hox*) genes encode transcription factors that function as regulators of hematopoiesis and are frequently dysregulated in human leukemia, particularly acute myeloid leukemia (Kroon et al., 1998). Recently, Wang et al described a mechanism whereby TGF-β/BMP inhibited the BM transformation capacity of HoxA9 and HoxA9-Nup98 fusion protein through a Smad4-dependent mechanism. Accordingly, Smad4 was shown to interact directly with HoxA9 and Nup98-HoxA9 fusion protein, thus precluding their DNA binding capacity and subsequent transcriptional activity (Wang et al., 2006). Smad4 also seems to participate in other signaling cascades such as Wnt or Notch (Itoh et al., 2004; Labbe et al., 2000).

These studies show the high interdependence between the different pathways, and the impact of their collective contribution on HSC self-renewal. This should be carefully considered when trying to expand HSC for clinical purposes.

2.7 Epigenetic control and HSC self-renewal

Epigenetic modifications, in addition to the intracellular pathways described in the previous section also play an essential role in regulating self-renewal, differentiation and tissue development. They induce gene expression regulation and can be grouped into three main categories: i) DNA methylation, ii) Histone modifications and iii) Nucleosome positioning. Recent studies suggest that epigenetic mechanisms contribute to establish the HSC unique characteristics. The following is a description of some of these examples.

2.7.1 Methylation of DNA

The most widely studied epigenetic modification in humans is cytosine methylation. DNA methylation occurs almost exclusively in the context of CpG dinucleotides that tend to cluster in regions called CpG islands. A group of enzymes, the DNA methyltransferases (DNMTs) tightly regulate both the initiation and maintenance of these methyl marks. DNA methylation can inhibit gene expression by various mechanisms. Methylated DNA can promote the recruitment of methyl-CpG-binding domain proteins which in turn recruit histone-modifying and chromatin-remodeling complexes to methylated sites. DNA methylation can also directly inhibit transcription by precluding the recruitment of DNA binding proteins from their target sites. In contrast, unmethylated CpG islands generate a chromatin structure favorable for gene expression (Portela and Esteller, 2010).

Methylation is controlled by at least 3 DNMTs: DNMT3a and DNMT3b for *de novo* methylation and DNMT1 for methylation maintenance. Conditionally disruption of *Dnmt3a*, *Dnmt3b*, or both *Dnmt3a* and *Dnmt3b* (*Dnmt3a/Dnmt3b*) showed that Dnmt3a and Dnmt3b function as *de novo* DNA methyltransferases during differentiation of hematopoietic cells. Unexpectedly, *in vitro* colony assays showed that both myeloid and lymphoid lineage differentiation potentials were maintained in Dnmt3a-, Dnmt3b-, and Dnmt3a/Dnmt3b-deficient HSC. However, Dnmt3a/Dnmt3b-deficient HSC, but not Dnmt3a- or Dnmt3b-deficient HSC, were incapable of long-term reconstitution in transplantation assays, suggesting a role for DNA methylation by Dnmt3a and Dnmt3b in HSC self-renewal (Tadokoro et al., 2007).

Conditional disruption of Dnmt1 in the mouse hematopoietic system revealed defects in self-renewal, niche retention, and in the ability of cells to give rise to multilineage hematopoiesis. Loss of Dnmt1 had specific impact on myeloid progenitor cells, causing enhanced cell cycling and inappropriate expression of mature lineage genes (Trowbridge et al., 2009). Consistent with these results, Broske et *al.* showed that Dnmt1 is essential for HSC self-renewal but dispensable for homing, cell cycle control and suppression of apoptosis but also implicated Dnmt1 in lymphoid differentiation (Broske et al., 2009).

2.7.2 Histone modifications and nucleosome positioning

A nucleosome is a histone octamer composed by a histone H3-H4 tetramer and two H2A-H2B dimers, around which DNA, 147 base pairs in length, is wrapped in 1.75 superhelical turns. Nucleosomes are connected by the so-called linker DNA and the histone H1. Histones post-transcriptional modifications, including acetylation, methylation, phosphorylation, ubiquitination, SUMOylation and ADP-ribosylation, occur predominantly in histone tails. They have important roles in transcriptional regulation as they can provide either an ON or OFF signature which result in the tight regulation of gene expression but display also important roles in DNA repair, DNA replication, alternative splicing and chromosome condensation. Nucleosomes act as barriers to transcription. They block access of activators and transcription factors to their sites on DNA and inhibit the elongation of the transcripts. The packaging of DNA into nucleosomes appears to affect all stages of transcription, thereby regulating gene expression. Nucleosome positioning plays also an important role in shaping the methylation landscape (Portela and Esteller, 2010).

Polycomb group (PcG) and Trithorax group (TrxG) proteins have emerged as key players in gene regulation and are thought to function coordinately to orchestrate DNA accessibility. These epigenetic regulators act antagonistically to either promote (TrxG) or repress (PcG) transcription through regulation of specific amino acid modifications in histones. It is not known how the PcG and TrxG proteins switch and balance between transcriptionally silenced heterochromatin (for example, enriched in histone H3 lysine 27 trimethylation, H3K27me3) and transcriptionally competent euchromatin (for example, enriched in histone H3 lysine 4 trimethylation, H3K4me3), respectively, during development.

In vertebrates, polycomb group proteins participate mainly in two complexes, Polycomb Repressive Complex (PRC) 1 and PRC2. Probably the best example of a chromatin-associated factor involved in self-renewal is BMI1, which is a component of PRC1. BMI1 is expressed in HSC and its expression decreases upon differentiation towards myeloid or erythroid cells, but is retained within the lymphoid compartments. Upon deletion of BMI1, no changes in the number of HSC in the fetal liver were observed, but in postnatal BMI1$^{-/-}$ mice, the number of HSC was markedly reduced. Targeted deletion of BMI1 in murine HSC impaired their competitive repopulation capacity (Park et al., 2003). *In vitro*, BMI1$^{-/-}$ HSC proliferated poorly and displayed an accelerated loss of multilineage differentiation potential and overexpression of BMI1 enhanced the self-renewal of HSC and enhanced their engraftment potential (Iwama et al., 2004).

Overexpression of BMI1 in cord blood CD34^{+} cells resulted in stem cell maintenance. After an *in vitro* culture period of 10 days, BMI1-overexpressing cells display a much better engraftment in NOD-SCID mice. Although the mechanisms involved remain to be elucidated, it was observed in single-cell assays that the percentage of CD34^{+}/CD38^{-} HSC undergoing apoptosis was reduced, whereas the percentage of quiescent HSC not undergoing cell cycle progression was increased upon BMI1 overexpression (Rizo et al., 2008). Lentiviral downmodulation of BMI1 in human cord blood CD34^{+} cells impaired long-term expansion, progenitor-forming capacity and stem cell frequencies, both in cytokine-driven liquid cultures and in BM stromal cocultures. This was associated with higher expression of p14ARF and p16INK4A and enhanced apoptosis, which coincided with increased levels of intracellular reactive oxygen species (ROS) and reduced FOXO3A expression (Rizo et al., 2008).

Another example of a chromatin-associated factor involved in self-renewal is the mixed lineage leukemia (MLL) protein, which encodes a trithorax-group chromatin regulator. Using *Mll*-deficient ESC to generate chimeras, Ernst et al. showed a cell-intrinsic requirement for MLL in the generation of lymphoid and myeloid populations in adult animals (Ernst et al., 2004). Moreover, MLL is often fused to the AF9 protein in leukemia and have been reported to impart leukaemia stem cell properties on committed hematopoietic progenitors. The leukemia stem cells generated can maintain the global identity of the progenitor from which they arose while activating a limited stem-cell- or self-renewal-associated programme (Krivtsov et al., 2006). Moreover, this MLL-AF9 fusion drives high-level expression of multiple *Hox* genes and can overcome Bmi1-deficiency to establish leukemic stem cells (Smith et al., 2011).

The studies described in this section establish that epigenetic alterations can modulate the self-renewal process. Epigenetic state in stem cells can be stably heritable or can be erased (partly or completely) by cell division. These changes might facilitate the transition of a progenitor cell to a self-renewing stem cell, or might prompt a stem cell to differentiate, divide or lose its ability to self-renew.

3. Compounds modifying HSC capacities

As described in the previous section, the strategy for stem-cell expansion involves activation of regulators that encourage HSC self-renewal and/or inhibition of pathways that mediate, differentiation or apoptosis by using primarily genetic modification approaches. An alternative strategy might imply pharmacological intervention by using a variety of small molecules. The term "small molecule" refers to a molecular entity that interacts with one or more molecular targets and effects a change in biological state while having minimal side effects. These small molecules, defined by a known structure, may be chemicals, proteins, small interfering RNAs or antibodies. Some of the most effective compounds for *ex vivo* maintaining or expanding HSC are reviewed below.

3.1 Regulation by cytokines

Cytokines are secreted proteins that regulate many aspects of hematopoiesis, such as, immune responses and inflammation. Numerous attempts have been made to use classic hematopoietic cytokines for the purpose of expanding HSC *in vitro*. Many interleukins, including interleukin (IL)-3, IL-6, and IL-11, Flt-3 ligand, TPO and SCF have extensively been investigated. In most cases, efforts to expand HSC have failed because of differentiation of HSC and subsequent loss of their reconstitution capacity. The combination of these molecules has however allowed maintaining HSC in culture for several days allowing their use in protocols for gene or cell therapies. Here we describe some examples of cytokines that were used to maintain HSC levels in culture.

3.1.1 Thrombopoietin (TPO)

TPO, acting through its receptor c-MPL, is the chief cytokine that regulates megakaryocyte production. However, several studies suggest that TPO can act to increase the *ex-vivo* expansion of HSC (Sitnicka et al., 1996). This effect was far more effective when used in combination with other cytokines including SCF, fms-like tyrosine kinase 3 ligand (FLT3-L), IL-3 or IL-6. Human cord blood cells expanded with this cytokine cocktail were shown to provide good short- and long-term platelet recovery and lymphomyeloid reconstitution in NOD-SCID mice (Ohmizono et al., 1997; Pineault et al., 2010). Further, a non peptidyl molecule agonist of c-MPL, NR-101, was found to be more efficient than TPO in expanding HSC. Indeed, 7 days culture of human cord blood $CD34^+$ or $CD34^+CD38^-$, treated with NR-101 induced a 2-fold increase in their number compare to TPO and a 2.9-fold or 2.3-fold increase in SRC numbers compared to freshly isolated $CD34^+$ cells or TPO-expanded cells respectively. As it was not more efficient than TPO in inducing megakaryocyte expansion, its effect seemed to be HSC specific. NR-101 treatment appeared to persistently activate STAT5 and to induce a long-term accumulation of HIF-1α (Nishino et al., 2009).

3.1.2 Angiopoietin-like 5 (ANGPLT5) and insulin-like growth factor binding protein 2 (IGFBP2)

Soluble growth factors, such as ANGPLT5 and IGFBP2, produced by the endothelium may enhance HSC expansion *ex vivo* when used with conventional cytokines. Although the addition of ANGPLT5 and/or IGFBP2 to a 10 days-human CD133+ cord blood cells culture has no effect upon the total nucleated cells number *in vitro*, it significantly enhances *in vivo* repopulation of NOD-SCID mice 2 months post-transplantation as well as secondary transplantation (Zhang et al., 2008a). These results were confirmed recently using human cord blood $CD34^+CD133^+$ cells cultured for 10 days in the presence of IGFBP2 and ANGPLT5. Expanded cells were shown to be capable of long-term multi-lineage and multi-site hematopoiesis in serial reconstitution in NSG mice (Drake et al., 2011).

3.1.3 Pleiotropin (Ptn)

Pleiotropin, which have mitogenic and angiogenic activities, has been found to be essential for maintenance of murine HSC. Mice transplanted with LSK $CD34^-$ cells treated with Ptn and a standard cocktail of cytokines showed 6-fold increase in HSC frequency compared to cells treated with cytokines alone. *In vivo,* systemic administration of Ptn was found to increase the number of BM LSK cells both in irradiated and nonirradiated mice, suggesting a role for this factor in the *in vivo* regeneration of HSC. Treatment of human cord blood $Lin^-CD34^+CD38^-$ cells with Ptn for 7 days induced a 4-fold increase in CFC content and a 3- or 7-fold improved engraftment at 4 or 7 weeks respectively in NOD-SCID mice compared with controls. This factor may activate the PI3-Kinase/AKT and Notch pathways by alleviating activation of its receptor, RPTP-β/ξ (Himburg et al., 2010).

3.2 Transcription factors: The HOX- family

3.2.1 HOXB4

The homeobox gene family member HoxB4 is the most investigated transcription factor for its potential to increase the self-renewal potential of HSC. HOXB4 belongs to a large family of transcription factors that share a highly conserved DNA-binding domain, the homeodomain. In mammals, there are 39 *Hox* genes grouped in four clusters referred to as A, B, C and D. In the hematopoietic system, 16 different *Hox* genes are transcribed during normal hematopoiesis. Primitive subpopulations express primarily genes of the A and B cluster (Giampaolo et al., 1995; Pineault et al., 2002; Sauvageau et al., 1994). Mice transplanted with marrow overexpressing HOXB4 resulted in a 47-fold increase of the competitive repopulating unit (CRU) numbers and did not develop leukemic transformation (Sauvageau et al., 1995). *HOXB4* overexpression in mouse HSC cultured for 14 days induced a primitive cell-specific growth advantage contrary to a progressive depletion of HSC usually observed under these conditions. Total cell growth (mostly mature cells) was enhanced by 2-fold, progenitors by 3-fold and HSC by 1000-fold in cells overexpressing HOXB4 (Antonchuk et al., 2002).

In humans, transient overexpression of HOXB4 in hematopoietic cord blood cells, did not increase proliferation of primitive progenitors, frequency of CFC, and LTC-ICs but induced an iincrease in myeloid differentiation (Brun et al., 2003). Other studies showed that

enforced high level of HOXB4 expression in human hematopoietic cord blood cells cultured for 24 hours induced a 5-10-fold increase in LTC-IC and a 4-fold increase in SRC (Buske et al., 2002). However, this HOXB4 overexpression markedly impaired the lymphoid and myeloerythroid differentiation (Schiedlmeier et al., 2003). Altogether these studies demonstrated that high levels of HOXB4 perturbed the myeloid differentiation program both *in vivo* and *in vitro* and are consistent with a dose dependant activity of HOXB4 to control the differentiation or self-renewal of HSC (Klump et al., 2005).

To increase the effect of HOXB4, a *NUP98-HOXB4* fusion gene was engeeniered since the fusion of *Hox* genes with the nucleoporine gene *NUP98* is often reported in leukemia. Ohta et *al.* observed, in a murine transplantation model, a 300-fold increase in CRUs among NUP98-HOXB4-overexpressing cells compared to only 80-fold increase with HOXB4 alone. An even higher increase (2000-fold) was observed using the *NUP98-HOXA10* fusion gene that, in contrast to HOXB4, blocks terminal differentiation and leads to a sustained output of cells with a "primitive" phenotype (Pineault et al., 2005; Pineault et al., 2004). The authors did not observe any long-term hematological defect in recipients repopulated with NUP98-HOXA10 expanded HSC (Ohta et al., 2007). However, these results contrast with those obtained by Watts et al. in a nonhuman primate stem cell transplantation model. Transplantation of comparable doses of HOXB4- and NUP98-NUP98-HOXA10-overexpressing cells revealed that HOXB4 contributed more to early hematopoiesis whereas NUP98-HOXA10 contributed more to later hematopoeisis. The emergence of a deleterious effect, such as leukaemia, could not be monitored due to the short survey period of the study (Watts et al., 2011).

In 2006, Zhang et al. investigate the ability of HOXB4 to expand HSC in a clinically relevant nonhuman primate competitive repopulation model. They found an initial 56-fold advantage for the *HOXB4*-transduced cells which decline significantly over time (Zhang et al., 2006). In addition, the first appearence of myeloid leukemia linked to HOXB4 expression were observed two years later, both in the original group of monkeys (1 out of 2) and in dogs (2 out of 2) that received cells transduced with a HOXB4 expressing vector (Zhang et al., 2008b). None of the 40 dogs and monkeys that received cells transduced with control vectors developed leukemia. Besides, a profound growth inhibition and a rapid cell differentiation was induced by siRNA knocking down HOXB4 using a cell line derived from the leukemic cells of one animal. The direct implication of HOXB4 in the development of leukemia can not be certify since analysis of the vector insertion sites in the genome of all tumors revealed insertion of the transgene near or within protooncogenes, such as *c-myb* and *PRDM16* (Zhang et al., 2008b).

To avoid the use of retroviral vectors, Amsellem et al. generate an MS-5 stromal cell line secreting HOXB4 to expand human cord blood hematopoietic cells. Using a 5-week long term culture system, they show a 4-fold increase in LTC-IC and 2.5-fold increase in SRC in NOD-SCID mice. This expansion did not appear to interfere with myeloid or lymphoid differentiation. However, the coculture system might not be suitable for clinical applications (Amsellem et al., 2003). To avoid this issue, Krosl et al. used a soluble recombinant HOXB4 protein fused to a small peptide derived from the HIV TAT protein. TAT-HOXB4 treatment of murine HSC for 4 days expanded approximately 4- to 6-fold and were 8-20 times more numerous than non treated HSC. This TAT-HOXB4 expanded population retained its normal *in vivo* potential for differentiation and long-term repopulation (Krosl et al., 2003).

The capacity of soluble HOXB4 to expand human HSC was verified using several recombinant human HOXB4 proteins. The N-terminal-tat and C-terminal histidine-tagged version of HOXB4 (T-HOXB4-H) had the highest activity in expanding CFC (10-fold) and LTC-IC (15-fold), and a 1.5- to 2.7-fold increase in SRC (Tang et al., 2009).

3.2.2 Other HOX family proteins

Surveys of *Hox* gene expression in HSC enriched populations showed dominancy of the *Hox*A cluster. In d14.5 fetal liver populations enriched for HSC, the expression of HOXA4 is a log higher than that of HOXB4. The fact that during this phase of development HSC undergo their major expansion, combined with the high homology and functional redundancy found within *Hox* paralog groups, suggests a putative role of HOXA4 to expand HSC with negligible or null oncogenic potential. HOXA4 overexpressing HSC expanded 6.6-fold after a week of culture. Although HOXA4 expressing HSC produced mature myeloid and lymphoid progeny in irradiated recipient mice, B-cell progenitors were preferentially expanded compared to myeloid progenitors (Fournier et al., 2011).

HOXC4, another member of the *Hox* family, is also expressed in proliferating hematopoietic cells suggesting a role in the control of normal proliferation. Using retroviral gene transfer in human $CD34^+$ cells, Daga et *al.* showed that HOXC4 induced an *in vitro* expansion of committed cells and early hematopoietic progenitors, with the most striking effect on LTC-IC (13-fold expansion) (Daga et al., 2000). These results are consistent with those of Amsellem and Fichelson who showed a more efficient expansion of human $CD34^+/CD38^{low}$ cells on MS-5 cell line secreting HOXC4 compared to those secreting HOXB4. The simultaneous presence of HOXB4 and HOXC4 seems synergize to improve expansion (Amsellem and Fichelson, 2006). However, the *in vivo* effect of HOXC4 still remains to be established.

All these observations clearly implicated Hox family proteins in HSC self renewal but further studies are required to determine if the use of these compounds could be suitable for clinical applications.

3.3 Chemical compounds

The low efficiency obtained with purified proteins and the safety concerns when attempting to expand HSC with viral vector-mediated gene transfer (Baum et al., 2003) lead to searching for alternative and safer approaches. One of these promising strategies involved the use of chemical compounds.

Chemical molecules constitute a particularly useful tool for modifying biological signaling pathways since they can be arrayed in chemical libraries for high-throughput analysis, and they can be withdrawn from the biological system once the desired effect is obtained. The use of a small molecule allows the study of the kinetics of a response in a more subtle and graduated way that is not possible with gene disruption techniques. These molecules may be further transposed into drugs for therapeutic use. Their use is rapid and cost-effective.

What are the sources of molecules available?

Historically, the pharmaceutical companies gathered the collections of molecules accumulated during the year in-house companies. These molecules can come from two different sources, one from natural origin and the other from chemically-synthesized compounds. Several companies have pooled their collections through partnerships to increase the size and diversity. At present, a large collection of oriented chemical libraries is available. In the milieu of academia, access to these collections is almost impossible unless a very restrictive partnership is framed. The number of screenable drug candidates have dramatically increased in the last years, and might account for 10 000 to 1 000 000 compounds. The difficulty to use these large collections resides in the ability to order millions of natural products, many of which are available in only limited amounts and are not yet completely characterized or even purified. Further, to identify a molecule producing the desired biological effect, different concentrations covering several orders of magnitude should be initially screened. This is why their widespread use has not yet been generalized and most discoveries to date are mainly available through the pharmaceutical industry. During the past ten years, various companies have specialized in the provision of all-purpose or targeted libraries. ChemBridge, ChemDiv, Asinex, Prestwick, Maybridge, enamine, Interbioscreen, TimTec can be mentioned as examples of commercially available collections. These libraries are relatively diverse and oriented "drug-like" (Kugawa et al., 2007). Small-molecule compounds approved for use as drugs may also be "repurposed" for new indications and studied to determine the mechanisms of their beneficial and adverse effects. A comprehensive collection of all small-molecule drugs approved for human use would be invaluable for systematic repurposing across human diseases, particularly for rare and neglected diseases, for which the cost and time required for development of a new chemical entity are often prohibitive. Major efforts are now underway to produce comprehensive collections of these small molecules amenable to high-throughput screening (Huang et al., 2011).

During the last ten years, cell-based phenotypic and pathway-specific screens using synthetic small molecules have provided new insights into stem cell biology and help to identify a number of small molecules that can be used to selectively (a) control self-renewal of embryonic and adult stem cells; (b) expand therapeutically desirable mature cell types; (c) control lineage commitment; and (d) enhance the reversion of lineage-restricted cells back to the multipotent or pluripotent state. All four practices are beginning to find application in therapeutic settings.

In this section we will focus on chemical compounds that were used to expand HSC. However, the most important question to keep in mind is whether the *in vitro* expanded cells preserve their capacities to regenerate hematopoiesis *in vivo* (Fig. 4).

3.3.1 Chromatin-modifying agents

Valproic acid (VPA) and chlamydocin are histone deacetylase (HDAC) inhibitors that exert their activity by interacting with the catalytic site of HDACs.

VPA was first studied by De Felice et al. on human $CD34^+$ cells isolated from cord blood, mobilized peripheral blood and BM. They showed that VPA preserves the CD34+ population after 1 week (40-89%) or 3 weeks (21-52%) of culture with cytokines and VPA increases H4 acetylation levels at specific sites on *HOXB4* and AC133 (De Felice et al., 2005).

In 2008, using a two step culture system, Seet et al. showed that VPA induced a 2-fold expansion of human cord blood $CD34^+CD45^+$ cells. Higher numbers of treated cells resided in the S phase compare to controls. VPA-treated cells reconstituted hematopoiesis in NOD-SCID mouse with a 6-fold higher efficiency compare to control cells. The advantage of using VPA resides on the fact that this molecule is clinically well-known since it has been used for more than 25 years to treat neurologic disorders (Seet et al., 2009). Chlamydocin, was showed to enhance Thy-1 expression on human $CD34^+$ cells and to display a 4-fold increase in SRC in NOD-SCID (Young et al., 2004).

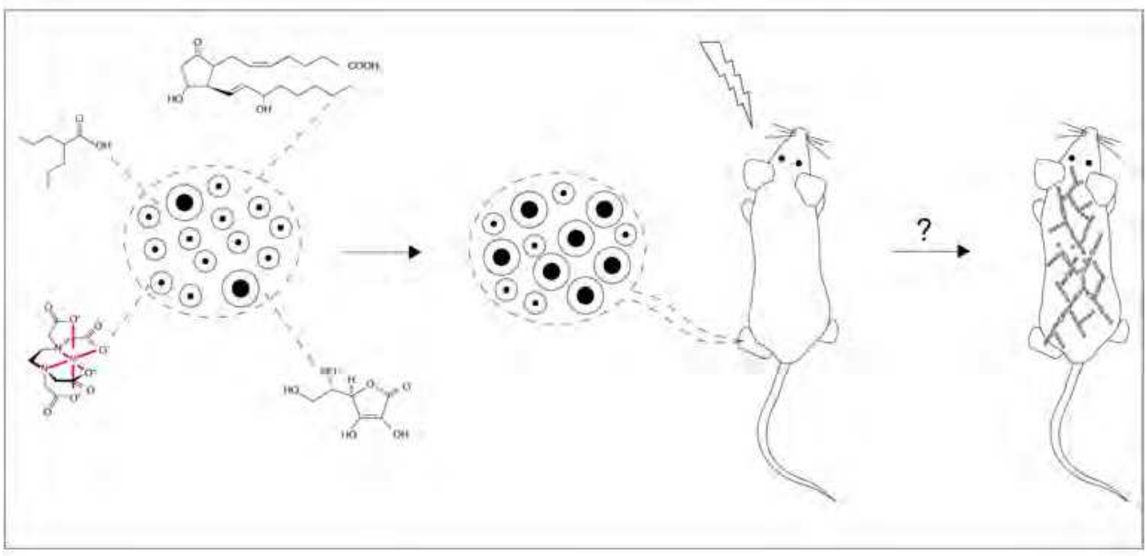

Fig. 4. A diagrammatic representation of an experimental design typology to test the effect of molecules on HSC expansion. Each molecule is added individually to the *in vitro* culture of HSC and the expansion capacities are then measured. However, infusion of the treated cells in myeloablated mice is essential to answer the question (**?**) on whether the HSC treated with the selected molecule have still the capacity to regenerate blood cells in transplanted animals.

Another HDAC inhibitor, trichostatin A (TSA), and 5-aza-2'-deoxycytidine (5azaD), a DNA methyl transferase inhibitor where shown to act in synergy to yield a 12.5-fold increase of human $CD34^+CD90^+$ cells after 9 days of culture in comparison to the input cell numbers, a 9.8-fold increase in the numbers of CFU and a 9.6-fold increase in SRC. Several genes implicated in HSC self-renewal including *HOXB4*, *BMI1*, *GATA2*, *P21*, and *P27* were up-regulated in the 5azaD/TSA-treated cells (Araki et al., 2006; Araki et al., 2007).

3.3.2 Copper chelator tetraethylenepentamine (TEPA)

Several clinical observations have suggested that copper plays a role in regulating HSC development. Peled et al. reported that modulation of cellular copper content might shift the balance between self-renewal and differentiation (Peled et al., 2005; Peled et al., 2002). This group cultured human $CD34^+$ cord blood cells with the copper chelator TEPA during extended periods of time and showed a higher percentage of early progenitors ($CD34^+CD38^-$, $CD34^+CD38^-Lin^-$) in the TEPA-treated cultures compared with controls and a 1- to 3-log-fold

expansion of $CD34^+$ cells compare with that of controls. They cultured human $CD133^+$ cord blood cells during 3 weeks, in order to use a clinically suitable protocol, and found that the median output value of $CD34^+$ cells increased by 89-fold, $CD34^+CD38^-$ by 30-fold and CFU by 172-fold over the input values. Moreover, the $CD34^+$ cells expanded with TEPA appeared to show improved NOD-SCID engraftment compare to control cells (Peled et al., 2004a; Peled et al., 2004b). Based on these data, a phase 1 trial was initiated. In this study, a portion of a single cord blood unit was cultured with TEPA and cytokines for 21 days and co-infused with the remainder of the untreated cell fraction. Although this methodology showed a 219-fold expansion of total nucleated cells *in vitro*, it did not improve the time to neutrophil or platelet recovery (de Lima et al., 2008). A phase 2/3 study is under way in more than 28 centers in the United States, Europe, and Israel, to evaluate the safety and efficacy of this approach ("StemEx") in 100 patients with advanced hematologic malignancies (http://clinicaltrials.gov/ct2/show/NCT00469729).

3.3.3 Oxygen, reactive oxygen species and antioxidants

Low oxygen levels were also described to play a beneficial role on HSC expansion *in vitro*. This is consistent with the observation that protection of HSC *in vivo* is achieved by a predominantly low-oxygen environment of the stem-cell niche (Cipolleschi et al., 1993; Eliasson and Jonsson, 2010).

The positive effect of hypoxia on the survival and/or self-renewal of the HSC population *in vitro* was demonstrated quantitatively on human marrow cells with $Lin^-CD34^+CD38^-$ phenotype which are enriched in SRC. A significant increase in SRC after 4 days was found in cultures under 1.5% O_2 compared to normoxic conditions. The positive effect of hypoxia on SRCs is short-lived but their engraftment into immmunocompromised mice was to some extent improved (Danet et al., 2003).

Similar studies have been performed with cord blood cells (Hermitte et al., 2006). The authors reported preferential survival of primitive HSC among cord blood $CD34^+$ cells in cultures under 0.1% O_2. After 72 hours, cells were 1.5 and 2.5 times more in quiescence (G0) at 3% and 0.1% O2. At 0.1% O2, 46.5%+/-19.1% of divided cells returned to G0 compared with 7.9%+/-0.3% at 20%. This shows a return of the cycling $CD34^+$ cells into G0, a quiescent state that characterizes steady-state HSC.

During the process of HSC purification or mobilization from the BM to the peripheral blood, the cells go across different levels of oxygenation until reach maxima in culture assays. Furthermore, cell factors added to these cultures can lead to an abnormal increase in reactive oxygen species (ROS) in the HSC and to a ROS stress that might change their properties and functions (Hao et al., 2011; Ito et al., 2006; Pervaiz et al., 2009). These ROS are unstable reactive molecular species possessing an unpaired electron that are produced continuously in cells as a byproduct of metabolism. They participate in vital signal transduction pathways but they can also oxidize DNA, proteins, and lipids leading to cell differentiation, senescence, and apoptosis. Notably, the mouse long-term repopulating HSC capacities were found in a Ros^{low} population (Jang and Sharkis, 2007). This cell population has a higher self-renewal activity than a Ros^{high} population both *in vitro* and *in vivo*. Moreover, distinct metabolic profiles of HSC reflect their location in the hypoxic niche (Simsek et al., 2010; Takubo et al., 2010).

The continual production of ROS in the *in vitro* culture (Iiyama et al., 2006) might be overcome by the addition of antioxidants. These molecules will maintain the ROS at a low level, thereby regulating the proliferation, growth, signal transduction, and gene expression of the cells (Chen et al., 2008).

Antioxidants are classified into enzyme and non-enzyme antioxidants. Enzyme antioxidants include superoxide dismutase, catalase, and glutathione peroxidase. Non-enzyme antioxidant includes vitamin C.

The application of enzyme antioxidants is limited because of the poor stability and ease of inactivation (Wojcik et al., 2010). However, when culturing mouse HSC in the presence of catalase, the number of short-term or long-term HSC with LSK immune markers was significantly increased and the stem cells begin to degenerate as the catalase is removed (Gupta et al., 2006).

Ascorbic acid (vitamin C) is a natural water-soluble antioxidant but under some conditions such as the air, heat, light, alkaline substances, enzymes and trace amount of copper oxide and iron, oxidation of vitamin C could be accelerated and the oxidative products lead to the damage of cellular DNA. The ascorbic acid 2-phosphate (AA2P), one derivative of vitamin C, is stable at 37°C in cell culture media and has no cytotoxic effect; therefore it might constitute an advantageous antioxidant (Duarte et al., 2009). Reducing oxidative stress by N-acetyl-L-cysteine (NAC) may enhance the viability and engraftment of HSC as treatment of gene corrected BM mononuclear cells or purified CD34(+) cells from FANCA patients with the reducing agent NAC showed increased CFC (Becker et al., 2010).

Although the current amplification under normal oxygen can expand a certain number of HSC, the application of glutathione for stem cell mobilization and re-infusion as well as the application of AA2P in the *in vitro* amplification culture of cells may become effective methods for protecting the hematopoietic reconstitution capacity of HSC (Hao et al., 2011). Moreover, *in vitro* culturing HSC-enriched samples under O2 concentrations that more closely resemble the BM environment (low O2 concentrations, 1–3%) might also improve their expansion and preserve proper stem cell functions for engraftment.

3.3.4 PGE2

Prostaglandin E2 (PGE2) was first identified as capable of enhancing HSC formation in zebrafish, following a high-toughput chemical screen. This effect was also tested using murine transplantation assays. When murine BM cells where briefly treated *ex vivo* by PGE2, a 3-fold increase in the CFU number and a 3.3-fold increase of SRC 6 weeks post transplantation were observed (North et al., 2007). Hoggatt et al. confirmed enhanced murine HSC engraftment following PGE2 exposure as they observed a 4-fold increase in HSC 20 weeks after transplantation. The increase in chimerism was still present in primary recipient 32 weeks post-transplant and in secondary recipients without additional PGE2 treatment. Several studies were performed to determine whether the action of PGE2 on HSC could be the result of an increase in HSC numbers, homing capability, proliferation, survival, or a combination thereof. Hoggatt et al. observed a significant increase in homing of PGE2-treated LSK cells. This was partially attributed to an increase in CXCR4 expression, a SDF1α specific receptor. This effect also occurs in

human HSC, since PGE2-treated cord blood cells transplanted into NOD-SCID mice displayed an enhanced homing to marrow. In addition, PGE2 treatment increased survivin expression, reduced intracellular active caspase-3 that lead to enhanced HSC survival and increased the percentage of cycling cells (Hoggatt et al., 2009). Frish et al. treated mice *in vivo* with PGE2 by intraperitoneal injection twice a day for 16 days. They observed a significant increase of the LSK population without inhibiting their differentiation. The treatment expands preferentially the short-term-HSC/MPP subpopulation since this advantage was lost 6 weeks post-transplant in primary recipients and in secondary transplants. The disparities between these studies may be the result of the extended exposure of mice to PGE2 compared with a short term pulse used hitherto (Frisch et al., 2009).

Goessling et al. briefly treated human cord blood $CD34^+$ cells *in vitro* with dimethyl-PGE2 (dmPGE2). They showed that dmPGE2 treatment decreased apoptosis, increased 1.4-fold the CFU number and enhanced engraftment of unfractionated and $CD34^+$ cord blood cells after xenotransplantation in NOD-SCID mice. Using a non-human primate transplantation model, they found no significant enhancement of CD34+-treated cells engraftment but showed that dmPGE2 treatment had no negative impact on HSC function, including multilineage repopulation, even 1 year post-transplantation. They suggested that these results reflect suboptimal compound dosing and anticipate the use of 50μM rather than 10μM of dmPGE2 in future transplantation assays (Goessling et al., 2011). Based on these data, this brief *ex vivo* incubation with dmPGE2 is currently being tested in a phase 1 clinical trial in which adults with hematologic malignancies receive a non-myeloablative conditioning treatment followed by double-unit cord blood transplantation in which 1 of the 2 cord blood units has been incubated with dmPGE2 before infusion (http://clinicaltrials.gov/ct2/show/ NCT00890500).

3.3.5 Aryl Hydrocarbon receptor (AhR) antagonists

Using a high-throughput screen based on CD34/CD133 expression, Boitano et al identified a purine derivative (StemRegenin1 or SR1) capable of *in vitro* enhancing the levels of a $CD34^+$ cell population derived from blood of mobilized donors. SR1 added to human $CD34^+$ cells cultured for 5 weeks led to a 10-fold increase in total nucleated cells, a 47-fold increase in $CD34^+$ cells and a 65-fold increase in CFU. $CD34^+$ cord blood cells cultured in the presence of SR1 for 3 weeks revealed a 17-fold increase in SRC content in NOD-SCID Gamma (NSG) primary recipient and a 12-fold increase in the number of secondary SRC compared to input (Boitano et al., 2010). Additional screens followed by a quantitative structure-activity relationship identified three novel compounds (i.e SR2, SR3 and SR4), structurally distinct from SR1, that expand the number of human $CD34^+$ cells. Experiments that aimed to determine the ability of cord blood derived human HSC expanded with these molecules to engraft NSG mice are still undergoing (Bouchez et al., 2011). SR1, SR2, SR3 and SR4 were showed to act as antagonists of AhR signaling. Indeed, this receptor has been implicated in HSC biology and hematopoietic disease through numerous factors including c-MYC, HES-1, PU.1, C/EBP, β-catenin, CXCR4, and STAT-5 (Singh et al., 2009). However, the precise mechanism whereby an AhR inhibitor might induce HSC self-renewal remains unknown.

3.3.6 SALL4

The transcription factor SALL4 was reported to play a role in maintaining ES cell pluripotency through interaction with Oct4 and Nanog (Wu et al., 2006; Yang et al., 2010). It was recently showed that overexpression of SALL4 can expand *ex vivo* human mobilized HSC from peripheral blood (Aguila et al., 2011). SALL4-transduced cells seemed capable of *ex vivo* expansion of both, $CD34^+CD38^-$ and $CD34^+CD38^+$ cells and showed enhanced stem cell engraftment and long term repopulation capacity in NOD-SCID mice. Moreover, human $CD34^+$ cells cultured 3 to 4 days with a soluble SALL4 fusion protein (TAT-SALL4B) showed a 10-fold increase in total mononuclear cells, a 8-fold increase in $CD34^+$ cells and a 10-fold increase in the CFU number compare to controls (Aguila et al., 2011). However, *in vivo* studies with this fusion protein still have to be conducted to validate that these expanded cells are still able to reconstitute hematopoiesis in transplanted recipients.

4. *De novo* generation of HSC

Considering the interest in HSC expansion for treatment of both malignant and non-malignant diseases as well as their use in gene therapy and the difficulty to obtain *ex vivo* expansion of HSC without loss of their regeneration capacities, relevant methods to produce *de novo* HSC have emerged.

4.1 Obtaining HSC from ESC

One of these methods was initiated 20 years ago when ESC could be cultivated *in vitro* and directed to generate hematopoietic cells (Wiles and Keller, 1991). Since then, culture conditions were constantly optimized and allowed the differentiation into specific hematopoietic lineages such as erythroid and myeloid lineages, T and B lymphocytes and megakaryocytes (for review see Sakamoto et al., 2010). These protocols were then adapted to human (h) ESC. These cells like their murine counterparts, are karyotypically stable, capable of prolonged self-renewal, and might differentiate into most cell types. These properties might be exploited for therapeutic benefits to cure many human degenerative diseases and resulted in intense biomedical studies.

Different methods were established to generate hematopoietic progenitors and specific lineages from mouse ESC including embryoid bodies formation, coculture with stromal cells, and direct differentiation in coated plates using a mixture of cytokines and growth factors without stromal cells (Tian and Kaufman, 2008). These protocols were then optimized for efficient differentiation of hESC into early mesodermal cells (Bernardo et al., 2011) and for obtaining defined hematopoietic precursors from ES cells (Chiang and Wong, 2011; Salvagiotto et al., 2011).

The ultimate goal of these strategies is to produce HSC capable of robust, long-term, multilineage engraftment to alleviate blood cells diseases; however the numbers and the capacities of the *de novo* cells generated are not quite sufficient to fulfill the clinical challenge. At present, multipotent hematopoietic progenitors (short-term HSC) with limited engrafting ability in transplanted mice were obtained (Woods et al., 2011). Other groups reported efficient generation of cells that mostly produce the myeloid lineage following long term engraftment or produce CD34+ hematopoietic precursors that have phenotype similar

to adult HSC but might best correspond to the embryonic stage of yolk-sac, aortogonadal-mesonephros (AGM), and/or fetal liver stage of hematopoiesis (Melichar et al., 2011; Narayan et al., 2006 and for review : Tian and Kaufman, 2008). More recently, the polycomb group protein Bmi1 was shown to promote more than 100-fold increase of hematopoietic cell development from ESC (Ding et al., 2011).

Since short-term HSC could be generated from ESC, an attractive option to increase the number of clinically competent HSC would be to find a molecule that dedifferentiate from short-term or mature hematopoietic cells to the long-term HSC population. Such a strategy might be valuable, since de-differentiation of somatic cells mediated by a chemical has been achieved in other systems. This is the case for reversine or 2-(4-morpholinoanilino)-6-cyclo-hexylaminopurine. This chemical compound was reported to induce reversal of mouse myoblast cell line, C2C12, to become multipotent progenitor cells, which can re-differentiate into osteoblasts and adipocytes (Chen et al., 2004). The de-differentiation activity of reversine however is not conserved across all cell lineages, since in certain cell types, it acts as a potent differentiation-inducing molecule (D'Alise et al., 2008).

To support the generation of long-term repopulating HSC from mouse ESC, other groups tested intrinsic regulators of adult HSC (Schuringa et al., 2004; Wang et al., 2005b). However, the use of many of these compounds, such as HoxB4, did not improve the expected engraftment efficiency *in vivo* (Wang et al., 2005a).

An unfavorable complication for the use of ESC in producing HSC is that lifelong use of drugs is required to prevent rejection of the transplanted cells. In order to make ESC practical for therapeutic use, it would be necessary to create a new stem cell line for each patient that needs treatment. Serious technical and ethical problems are associated with this issue.

4.2 Obtaining HSC from induced pluripotent stem cells

An alternative to the utilization of ES cells to produce *de novo* HSC arise from one of the most transformative accomplishments performed in the last years: the discovery that transient overexpression of a small number of defined transcription factors can reprogram the differentiated cells and become pluripotent populations. These cells are commonly referred to as Induced Pluripotent Stem Cells (iPSC) and have definitively broken the dogma commonly accepted that differentiated cell types generally lack the ability to revert back to a less specialized state.

4.2.1 Reprogramming somatic cells to pluripotency

The direct reprogramming of somatic cells to pluripotency was demonstrated in 2006, when Takahashi and Yamanaka converted adult mouse fibroblasts to iPSC by overexpressing four transcription factors: octamer-binding transcription factor 4 (OCT4), sex determining region Y-box 2 (SOX2), Kruppel-like factor 4 (KLF4), and cytoplasmic Myc (c-MYC) in mouse embryonic fibroblasts using retroviruses (Takahashi and Yamanaka, 2006). The transcription factors originally used for reprogramming differentiated cells are not stringently necessary to achieve this process as some of them can be replaced by other factors. Yu et al. were able to reprogram human fibroblasts with a distinct set of transcription factors comprising OCT4,

SOX2, NANOG, and LIN28 (Yu et al., 2007). Krüppel-like transcription factors (Klf2 and Klf5) and the orphan nuclear receptor, Esrrb, can replace Klf4 (Nakagawa et al., 2008 and for review see Feng et al., 2009).

The derived iPSC exhibited typical ESC morphology and were similar to ESC in their regenerative potential (Takahashi and Yamanaka, 2006) and their capacity to differentiate into cells of all three germ layers, the ectoderm, mesoderm, and endoderm. Because iPSC are generated without the need to destroy an embryo, their discovery has further energized the field of regenerative medicine and stem cell biology. Patient-specific therapeutic cells derived from induced pluripotent stem iPSC may bypass the ethical issues associated with ESC and avoid potential immunological reactions associated with allogenic transplantation. These human disease–specific iPSC provide a unique and previously unavailable resource for studying the pathophysiology of various important human diseases.

The therapeutical hope of iPSC is based on three issues: 1) The ability to generate iPSC from any tissue of the organism, and further differentiate them according to the patient needs, particularly into a wide range of primary human cell types, many of which are unavailable for routine use; 2) The ability to generate iPSC from patients with any disease; 3) The possibility of using patient-derived iPSC for drug development.

The therapeutic potential of such iPSC (schematized in Fig. 5) was demonstrated in a proof-of-principle study using a humanized sickle cell anemia mouse model (Hanna et al., 2007). In this study, mice could be rescued after transplantation with hematopoietic progenitors obtained *in vitro* from autologous iPSC. This was achieved after correction of the human sickle hemoglobin allele by gene-specific targeting.

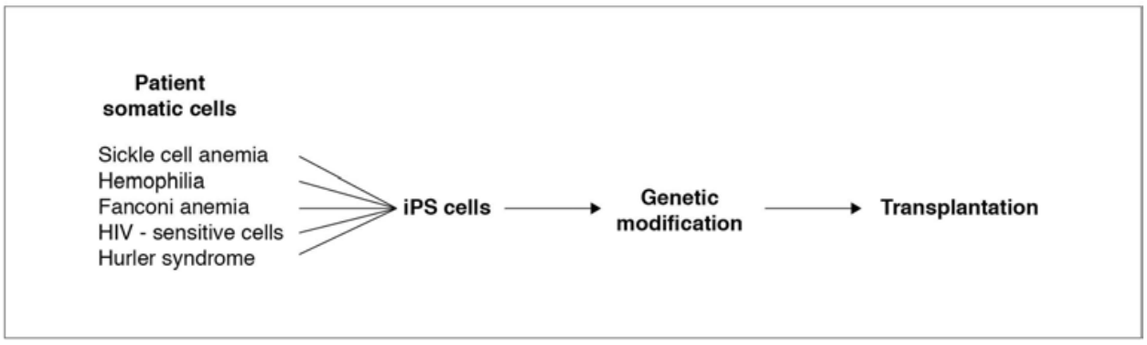

Fig. 5. Studies performed to validate the therapeutic potential of iPSC.

Also in mice, Xu et al. cured hemophilia by transplantation of cells that were generated from murine wild-type iPSC. These murine experiments suggest that human iPSC can be utilized for regenerative and therapeutic applications (Xu et al., 2009). Most recently, patient-specific iPSC have been established. Raya et al. reprogrammed dermal fibroblasts and/or epidermal keratinocytes of Fanconi anemia patients to generate iPSC, which were genetically corrected with lentiviral vectors encoding FANCA or FANCD2, to obtain hematopoietic progenitors of the myeloid and erythroid lineages that are phenotypically normal, that is, disease-free (Raya et al., 2009). Similar strategies were performed to correct the Hurler syndrome (Tolar et al., 2011) and for the production of macrophages from iPSCs which were resistant to HIV infection (Kambal et al., 2011).

The enthusiasm surrounding the clinical potential of iPSC is tempered by key issues regarding their safety, efficacy, and long-term benefits. Fully realizing the biomedical potential of iPSC in a clinical setting will require addressing certain limitations inherent to the process. First, need to find alternative strategies to remove non-viral or non-integrative vectors to overcome their potential deleterious effects. Although expression of the exogenous reprogramming factors is eventually silenced during iPSC cell generation, there is a significant risk of tumorigenesis if these exogenous genes are inadvertently reactivated. Second, it will be essential to increase the number of cells with specific phenotype. Third, it will be necessary to improve the efficiency of reprogramming (0.001–3% of cells are reprogrammed) since this is a slow and inefficient process (Jaenisch and Young, 2008; Nakagawa et al., 2008; Wernig et al., 2008).

4.2.2 Improving reprogramming somatic cells to pluripotency

To overcome the potential deleterious effects of viral vectors or oncogenes, and to improve the efficiency of the process toward a potential clinical application, a powerful alternative is offered by using small molecules. Several small molecules were reported to improve the reprogramming process by lowering the epigenetic barrier to initiate pluripotency (for reviews see Feng et al., 2009; Lyssiotis et al., 2011). Consistent with this notion, small molecules that affect reorganization of chromatin architecture, a rate limiting step during the reprogramming of a somatic genome, have been identified (Blelloch et al., 2006; Hochedlinger and Jaenisch, 2006; Huangfu et al., 2008a). In particular, the HDAC inhibitor VPA was shown to strongly increase reprogramming efficiency in the absence of c-Myc in both mouse and human cells and to allow 2-factor reprogramming (Oct4 and Sox2) of human fibroblasts in the absence of Klf4 and c-Myc (Huangfu et al., 2008b). Other epigenetic regulators such as BIX01294, a G9a histone methyltransferase inhibitor; BayK8644, an L-type calcium channel agonist and the two DNA methyltransferase inhibitors, AzaC and RG108 (summarized in Fig. 6), substantially increased reprogramming efficiency (Lukaszewicz et al., 2010; Shi et al., 2008).

The low efficiency of reprogramming (Hong et al., 2009) might also result from the accumulation of ROS (Parrinello et al., 2003). Consistent with this, Esteban et al. found that vitamin C strongly increases the reprogramming efficiency (Esteban et al., 2010). This is in line with the study reporting that hypoxic conditions improve the efficiency of iPSC production generated from mouse or human somatic cells (Yoshida et al., 2009). Co-treatment with VPA synergizes this effect. Other molecules including the MEK inhibitor PD0325901, the GSK3 inhibitor CHIR99021 combined with tranylcypromine, kenpaullone, SB-431542, and the TGF-β signaling inhibitor called RepSox (Ichida et al., 2009) were reported to enhance reprogramming or to replace viral vectors or oncogenes (Li and Ding, 2009; Pan and Thomson, 2007).

With the continued use of high-throughput screening to identify more chemicals that could assist in reprogramming, we may be closer to the goal of using a chemical-only cocktail to reprogram somatic cells to iPSC. These pluripotency gene activators may be then used in combination with specific differentiation modulators to achieve the production of the desired cell type.

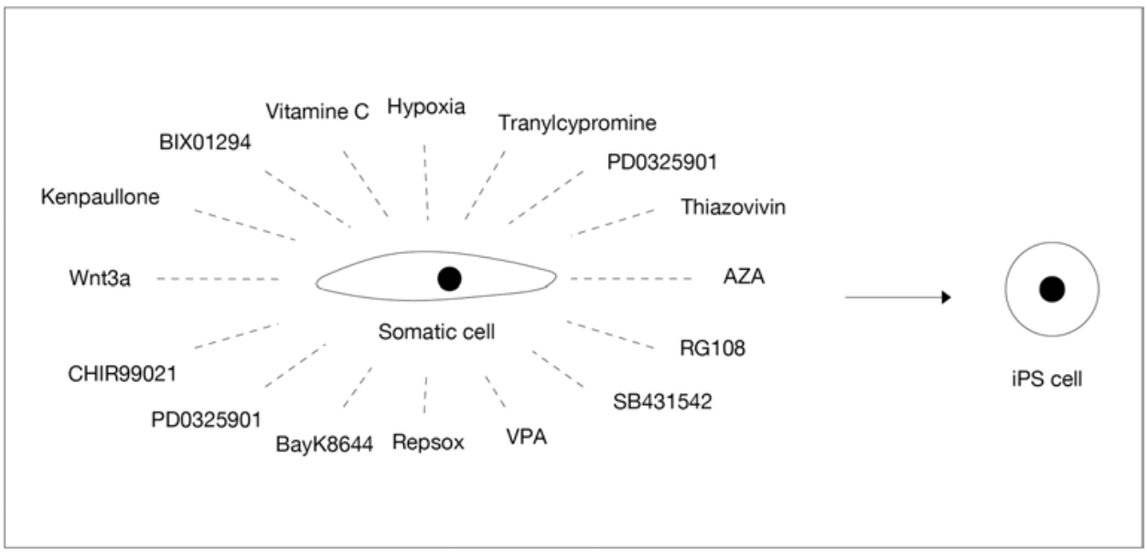

Fig. 6. Chemicals used to enhance reprogramming or to replace core reprogramming factors.

4.2.3 Obtaining HSC from induced pluripotent stem cells

Differentiation of iPSC into hematopoietic lineage have been achieved using a combination of specific cytokines and growth factors (Sakamoto et al., 2010) and have already demonstrated from both mouse and human iPSC (Lengerke et al., 2009; Niwa et al., 2009; Woods et al., 2011). However, the number of cells obtained in view of therapeutic use is still insufficient and small molecules that might expand the production of hematopoietic cells have yet to be found. Studies in this direction are beginning to emerge. For example, Wnt signaling, in particular WNT3a, mediates the stimulation of hemoangiogenic cell development and increase hematopoietic differentiation from ESC and iPSC (Wang and Nakayama, 2009; Wang et al., 2010). However, the conditions to generate human HSC capable of robust, long-term, multilineage engraftment from iPSC are still hoped for.

5. Conclusion

The ex-vivo expansion of HSC represents a promising approach to obtain large enough quantities for therapeutic intervention in cell and gene therapy protocols. Derivatives of hESCs and iPS cells are also expected to be employed as *de novo* HSC source for therapeutic settings. However, as described in the previous section, practical and ethical issues must be settled before clinical practice can begin. In both cases, the chemical biology approach using small molecules as tools or drugs holds unquestionably greater promise in the outcome of the final goal.

Even though a few molecules are being tested in clinical assays, the ideal soluble factor that enables to increase the number of rare HSC during the *ex vivo* growth culture without limiting their regeneration capacities has yet to be found. Most attempts have been unsuccessful because i) suitable expansion *in vitro* has been mostly correlated with loss of

the regenerative capacities of HSC *in vivo;* ii) no straight forward method allows the association of *in vitro* observations with the *in vivo* outcome; iii) testing the *in vivo* effect of each molecule independently would be costly, time-consuming and would need an imposing number of mice which is ethically inconceivable.

In an attempt to develop new tools that might overcome some of these limitations, we have developed an innovative screening strategy to identify molecules for their potential to improve the *in vitro* HSC self-renewal and proliferation while preserving the HSC regenerative capacities *in vivo* (Sii Felice K, Grosselin J, Leboulch P, Tronik-Le Roux D, manuscript in preparation). Our approach is based on stem cells labeling with specific barcodes before exposure to the molecules (Fig. 7). Then, prior to their infusion in myeloablated mice, all the treated cells are pooled. Several weeks after transplantation, the identification of barcodes present in the blood and the BM of transplanted mice will enable the precise retrospective quantification of the initial effect of the molecule.

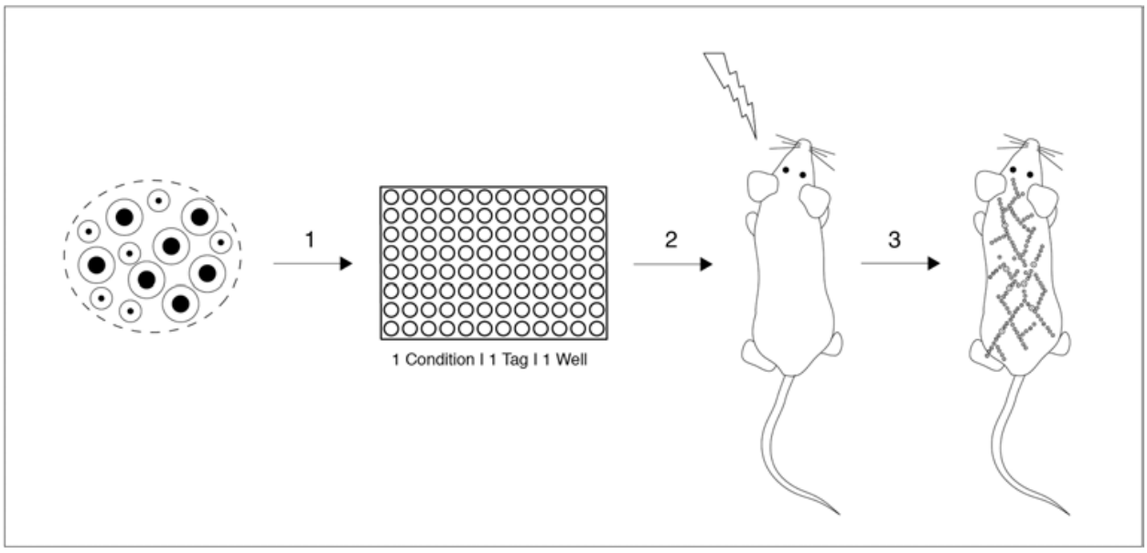

Fig. 7. Schematic representation of the strategy developed to simultaneously test dozens of molecules. Each well contains barcoded-HSC (1) treated by a particular molecule. After several days of *in vitro* culture (2), all the cells are pooled, infused in myeloablated mice. The identification of barcodes in blood and BM of transplanted mice will enable the precise retrospective quantification of the initial effect of the molecule.

This strategy might facilitate the development of high-throughput screening for fast and effective identification of small molecules that can be used to burst the production of HSC. This will undoubtedly accelerate the promise of regenerative medicine as a routine therapeutic modality for many blood diseases as well as for gene and cell therapy.

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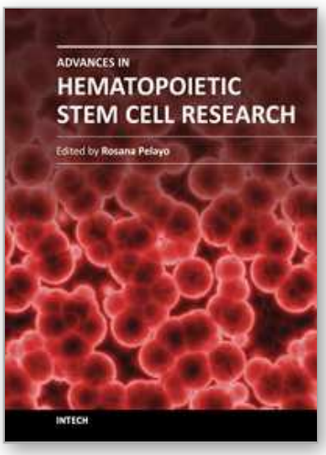

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This book provides a comprehensive overview in our understanding of the biology and therapeutic potential of hematopoietic stem cells, and is aimed at those engaged in stem cell research: undergraduate and postgraduate science students, investigators and clinicians. Starting from fundamental principles in hematopoiesis, Advances in Hematopoietic Stem Cell Research assemble a wealth of information relevant to central mechanisms that may regulate differentiation, and expansion of hematopoietic stem cells in normal conditions and during disease.

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