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UNIVERSITA' VITA-SALUTE SAN RAFFAELE

DOTTORATO DI RICERCA IN  
BIOLOGIA CELLULARE E MOLECOLARE

CRITICAL PARAMETERS AND MOLECULAR  
ANALYSIS OF LENTIVIRAL VECTOR-  
MEDIATED GENE TRANSFER INTO HUMAN  
HAEMATOPOIETIC STEM CELLS

Director of Studies: Prof. Luigi Naldini

Tesi di Dottorato di

Francesca Romana Santoni de Sio

Matricola 00008

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AUTHOR NO: W6102145  
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## DECLARATION

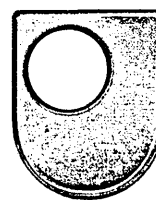
This thesis has been composed by myself and has not been used in any previous application for a degree. All the results presented here were obtained by myself, except for the statistical analysis, which was performed in collaboration with Prof. Mauro Gasparini (HSR-CUSSB, Milan and Politecnico di Torino, Italy) and the proteasome activity assay, which was performed in collaboration with Dr. Paolo Cascio (University of Turin, Italy).

All sources of information are acknowledged by means of reference.

Part of the work contained in this thesis has been published (see Appendix B):

Michele De Palma\*, Eugenio Montini\*, Francesca Romana Santoni de Sio\*, Fabrizio Benedicenti, Alessandra Gentile, Enzo Medico and Luigi Naldini. Promoter trapping reveals significant differences in integration site selection between MLV and HIV vectors in primary hematopoietic cells. *Blood*;105(6):2307-15, 2005 Mar 15. \*Equal contribution.

Francesca Romana Santoni de Sio, Paolo Cascio, Anna Zingale, Mauro Gasparini and Luigi Naldini. Proteasome Activity Restricts Lentiviral Gene Transfer into Hematopoietic Stem Cells and is Down-Regulated by Cytokines that Enhance Transduction. *Blood* 107(11):4257-65, 2006 Jun 1.



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Chapter 1.  
Introduction

## 1.1 Haematopoietic Stem Cells

Stem cells are immature, clonogenic cells with a high proliferative potential, which are able to self-renew and give rise to differentiated progeny.

In vertebrates they are classified in two groups: embryonic stem (ES) cells and adult stem cells. ES cells are found in the inner mass of the blastocyst and are able to generate all the differentiated cells of the organism (pluripotency). Adult stem cells derive from ES during foetal development and are tissue/organ specific stem cells, which are able to differentiate into a limited number of lineages (multipotency) and are responsible for homeostatic cell replacement and tissue regeneration, virtually for the entire life span of the organism (Harrison 1973). The frequency of adult stem cells in the total organ/tissue cell population is very low. Stem cells are slow-cycling under steady-state conditions and are able to balance self-renewal against differentiation when cycling.

Haematopoietic stem cells (HSCs) are adult stem cells that give rise to all blood cell lineages, including lymphoid (T and B) and myeloid cell lineages. Their existence was first postulated in the context of a clinical study, whose aim was the identification of a cell population able to protect patients that underwent lethal irradiation or chemotherapy. Although at that time the cause of death for irradiation was not known, it had been observed that death could be prevented if the haematopoietic tissues were protected (Jacobson, Marks et al. 1949). In the same period another group showed that intravenous injection of syngeneic bone marrow (BM) cells could prevent the death of lethally irradiated mice (Lorentz, Uphoff et al. 1951). Ten years later, Till and McCulloch identified a population of progenitor cells in the BM that were able to form myelo-erythroid colonies in the spleen of irradiated recipients (Till 1961; Wu, Till et al. 1967); they also showed that these colonies were clonal (Wu, Siminovitch et al. 1968; Wu, Till et al. 1968)

and that the colony-forming-cells, in many cases, were able to generate new colonies upon transplantation into secondary recipients (Siminovitch, McCulloch et al. 1963). Thus, the existence of a radioprotective cell population residing in the BM was postulated; these cells, which were able to self-renew and differentiate into different blood cell lineages, were termed haematopoietic stem cells (Siminovitch, McCulloch et al. 1963; Wu, Siminovitch et al. 1968). After 40 years of research HSC biology is still not completely understood, even though HSCs are still the best characterized stem cells.

### **1.1.1 Ontogeny**

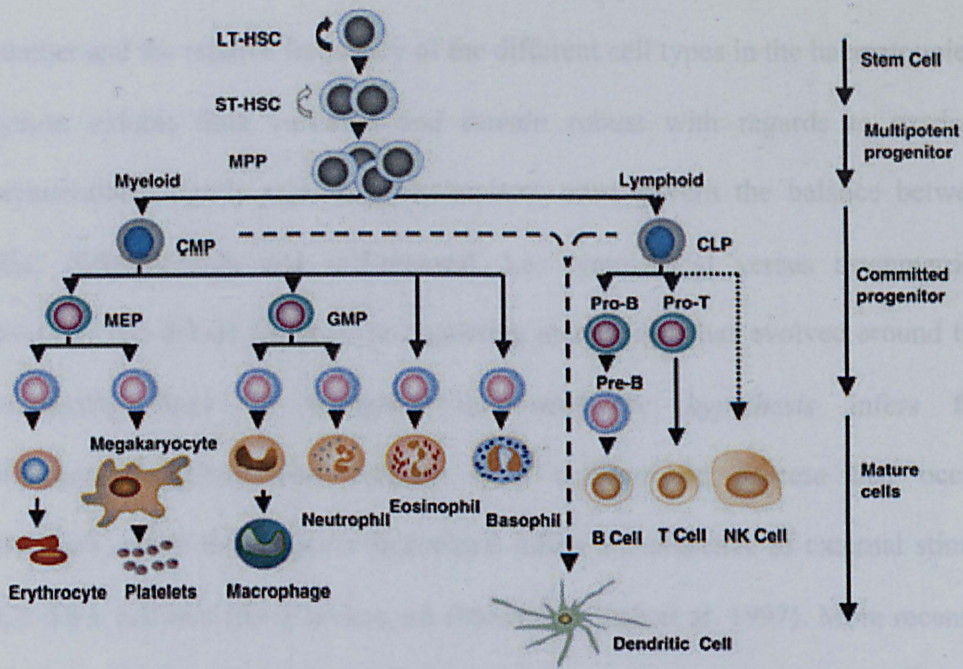
HSC development has been extensively studied in mammals, mainly in the mouse. The first site of haematopoiesis is the yolk sac, in which at embryonic day (E) 7.0 a population of progenitor cells emerges that originates primitive erythroblasts and macrophages. By rapid expansion the primitive erythroblasts and macrophages fill the nascent yolk sac capillary bed with flowing cells that begin to be propelled by the developing heart tube on E 8.25. At this time, multipotent progenitors giving rise to definitive lineages possibly also arise in the yolk sac (for a review of yolk sac haematopoiesis see (McGrath and Palis 2005). All these blood elements enter the bloodstream and through the umbilical cord vein migrate to the developing liver, where progenitor cell maturation takes place (Weissman, Papaioannou et al. 1978; Godin and Cumano 2005). In the mean time, at E 8.0, HSCs emerge in the mesoderm-derived paraaortic splachnopleure/aorta-gonad-mesonephros (P-Sp/AGM) region and migrate to the liver, where they mature (Muller, Medvinsky et al. 1994; Godin, Dieterlen-Lievre et al. 1995; Medvinsky and Dzierzak 1996). Starting from E 12.0 the foetal liver is the

primary site of haematopoiesis through birth (Morrison, Hemmati et al. 1995), although the spleen and BM become active sites of haematopoiesis prior to birth (E 18.0). However, the primary site of development of the HSCs that will support haematopoiesis in the adult is still debated. In fact, while there is some evidence that both the yolk sac and the P-Sp/AGM region play a role (Cumano, Ferraz et al. 2001; Matsuoka, Tsuji et al. 2001; de Bruijn, Ma et al. 2002), there are different interpretations about temporal and spatial emergence of these cells. Moreover, a number of studies have recently suggested that foetal placenta also functions as haematopoietic organ, which plays an important role in establishing the HSC pool during development (Alvarez-Silva, Belo-Diabangouaya et al. 2003; Gekas, Dieterlen-Lievre et al. 2005; Ottersbach and Dzierzak 2005). These new findings add a level of complexity to the established paradigms and prompt the need of revising the model for foetal haematopoiesis.

### **1.1.2 HSC fate decision**

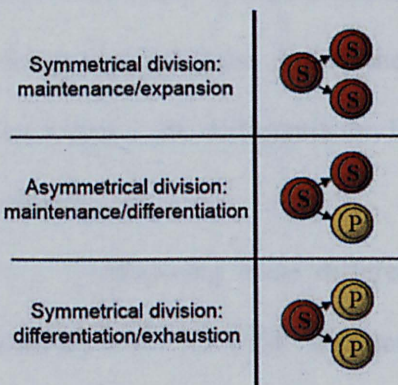
In the adult, HSCs reside mainly in the endosteal niche of BM, where they are associated to stromal cells, which support maintenance of stem cells and haematopoiesis by secretion of factors and cell-cell interactions. In order to generate mature blood cells, HSCs are engaged in a differentiation process towards multilineage progenitors; these cells can generate either common myeloid progenitors or take the lymphoid path to generate common lymphoid progenitors. These progenitors undergo further commitment steps that restrict their developmental potentials, in term of both proliferative and lineage potential, and end up with the generation of terminally differentiated progeny (Figure 1.1).





**Figure 1.1. Hematopoiesis.** LT-HSC: Long-term hematopoietic stem cell. ST-HSC: Short-term hematopoietic stem cell. MPP: multipotent progenitor. CMP: common myeloid progenitor. CLP: common lymphoid progenitor. MEP: megakaryocyte-erythrocyte progenitor. GMP: granulocyte-macrophage progenitor.

The ability of HSCs to differentiate without exhausting relies on the ability of stem cells to perform both symmetrical and asymmetrical divisions. Symmetrical divisions give rise to two daughter cells with the same potential of the dividing cell, whereas asymmetrical divisions give rise to a cell with the same potential of the dividing cell and a progenitor that has a higher degree of



**Figure 1.2. Symmetric and asymmetric divisions.** S: stem cell. P: progenitor cell.

commitment, lower self-renewing and proliferative potential and that will generate the differentiated progeny (Figure 1.2). Thus, symmetrical mitoses are responsible for the maintenance and expansion of the HSC compartment, while asymmetrical mitoses are responsible for both self-renewal and differentiation. Since both the absolute

number and the relative frequency of the different cell types in the haematopoietic system exhibit little variation and remain robust with regards to extrinsic perturbations, tightly regulated mechanisms must govern the balance between HSC differentiation and self-renewal, i.e. symmetrical versus asymmetrical division. The debate about these regulating mechanisms has evolved around two contrasting lines of thoughts: the *stochastic hypothesis* infers that differentiation/self-renewal decision is a cell-intrinsic process that occurs randomly, while the *inductive hypothesis* infers the existence of external stimuli that drive cell fate (for a review see (Morrison, Shah et al. 1997). More recently, on the basis of systems that model HSC development within a cell population, the *chaos hypothesis* has been proposed (Furusawa and Kaneko 2001). This theory is based on a chaotic behaviour at the level of a single HSC, where chaos does not represent disorder but a state of intracellular dynamic equilibrium sensitive to small variations and allowing HSCs to integrate diverse internal/external signals. According to this theory, lineage commitment and loss of multipotency are accompanied by a decrease in the complexity of the system. This hypothesis is in agreement with gene expression studies, which found low expression levels of many different lineage-commitment genes in HSCs; the expression of each lineage-related gene set is then enhanced or repressed during commitment, according to the differentiation lineage undertaken (see paragraph 1.1.4.1 Intrinsic signals).

Integrating these different hypotheses, it is now believed that the first driving force to HSC differentiation is an unknown random event at the intracellular level and that, in order to be effective, this choice must be consolidated by permissive external stimuli. In this view, HSC fate decision is the outcome of both stochastic events and external instructive signals and resembles

morphogenesis during embryonic development (for a comprehensive review on HSC cell fate determination see (Faubert, Lessard et al. 2004; Hoang 2004).

### 1.1.3 HSC maintenance

Since HSCs sustain haematopoiesis for the entire life span of the organism, they must use specific mechanisms to avoid the risk of damage from long replicative histories and to regulate the phenomenon of aging. It was initially proposed that a strategy exerted by HSCs to this aim is through *clonal succession*. In this model, the vast majority of the population is homeostatically maintained in a quiescent state, and one or at most some stem cells are actively supplying differentiated cells at any given time (Kay 1965; Lemischka, Raulet et al. 1986; Abkowitz, Linenberger et al. 1990). Accordingly, this model implies that cycling HSCs generate differentiated progeny until exhaustion and thus implies a reduction of HSC number with age. This hypothesis has been challenged by later studies that demonstrated that every 1-3 months all HSCs have replicated, supporting the evidence that they divide on a regular basis (Bradford, Williams et al. 1997; Cheshier, Morrison et al. 1999). Moreover, drawbacks and limitations of a work that supported the *clonal succession model* have been uncovered by successive studies. Indeed, Lemischka and coworkers (Lemischka, Raulet et al. 1986) used oncoretrovirus-mediated *ex-vivo* gene transfer to mark HSCs and track them *in vivo* after transplant. However, it has been later shown that retroviral vectors can integrate only in proliferating cells (Miller, Adam et al. 1990; Roe, Reynolds et al. 1993)(see paragraph 1.2 Gene transfer into haematopoietic stem cells). Thus, the authors marked and followed *in vivo* only HSCs that were already proliferating *ex vivo* and lost in their analysis cells that did not proliferate during the *ex vivo*

period. In addition, a gene therapy clinical trial has very recently shown that oncoretroviral integration is not neutral, as believed for a long time, and can activate growth-enhancing genes and induce *in vivo* expansion of selected clones (Ott, Schmidt et al. 2006). Thus, by using the oncoretroviral marking Lemischka *et al.* analysed HSC clones that were not only selected *in vitro* but also, possibly, selected *in vivo*.

Thus, the theory has been revised. In agreement with the *clonal succession model*, it is still believed that at any time the majority of HSCs is in a quiescent state and only a limited number enter cell cycle. However, this new view does not link cell cycle entry with differentiation and exhaustion and, thus, does not imply a reduction of HSC number. Indeed, when HSCs proliferate they can give rise to one or two HSCs with the same potential of the dividing cell (see paragraph 1.1.2 HSC fate decision); these cells go back to a quiescent state and maintain the HSC compartment.

A strategy exerted by HSCs to prevent exhaustion was proposed to be the maintenance of telomeres (Morrison, Prowse et al. 1996). However, contrasting results have been shown. Indeed, activity of telomerase (TERT), which compensates the telomeres shortening due to DNA replication and prevents induction of senescence, has been detected in different progenitor/stem cells, but has been found to be low or absent in HSCs (Samper, Fernandez et al. 2002; Allsopp, Morin et al. 2003; Allsopp, Morin et al. 2003). Moreover, it has been found that, whereas murine HSCs deficient in TERT show accelerated telomere erosion and an impaired long-term self-renew ability (Allsopp, Morin et al. 2003), over-expression of TERT does not induce an increase in self-renewal (Allsopp, Morin et al. 2003). Thus, even if in human HSCs the role of TERT still needs to

be clearly assessed, these findings support the idea that prevention of telomere length is a mechanism necessary, but not sufficient for HSCs maintenance.

#### 1.1.4 Molecular Mechanisms regulating HSCs

The homeostasis of the haematopoietic system mainly relies on the pathways undertaken by haematopoietic stem and progenitor cells (Figure 1.3). Whereas progenitors are mainly responsible for the short-term maintenance, HSCs are responsible for long-term maintenance. Thus, to sustain the system without undergoing exhaustion, HSCs must be tightly regulated. HSC regulation is a key issue not only for basic biology, but

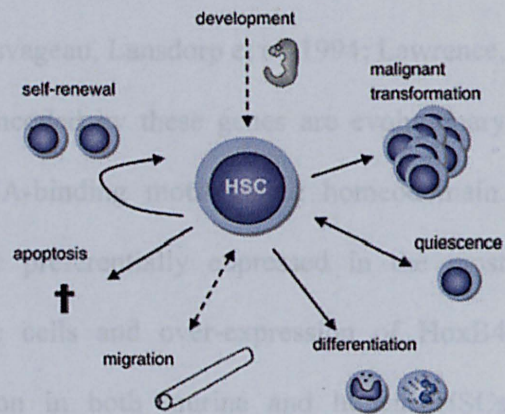


Figure 1.3. HSC fate

also for applied biology. Indeed, its understanding, and possibly its exploitation, is at the base of the development of efficacious gene and cell therapy protocols. Because of this, we will discuss in the following paragraphs the known HSC regulators. They are commonly divided in intracellular regulators (intrinsic signals) and external stimuli (extrinsic signals).

##### 1.1.4.1 Intrinsic signals

Intrinsic signals converge at the level of gene expression, and have been better characterized in the developmental pathways of HSCs than in the homeostatic maintenance of HSCs in the adult state.

Although the transcriptional machinery governing the HSC developmental pathways is very complex, some families of transcription factors involved in this process have been identified. Indeed, SCL/tal1, Rbnt2 (also known as LMO-2) and AML-1 genes have been shown to be essential for the generation of the haematopoietic system in the mouse (Warren, Colledge et al. 1994; Shivdasani, Mayer et al. 1995; Okuda, van Deursen et al. 1996; Lacaud, Gore et al. 2002). Another set of genes reported to be major players in regulating in haematopoiesis is the homeobox (Hox) genes family (Sauvageau, Lansdorp et al. 1994; Lawrence, Sauvageau et al. 1995). The proteins encoded by these genes are evolutionary preserved and characterized by a DNA-binding motif called homeodomain. Among them, HoxB3 and HoxB4 are preferentially expressed in the most primitive population of haematopoietic cells and over-expression of HoxB4 favours self-renewal over differentiation in both murine and human HSCs (Sauvageau, Lansdorp et al. 1994; Sauvageau, Thorsteinsdottir et al. 1995; Thorsteinsdottir, Sauvageau et al. 1999; Antonchuk, Sauvageau et al. 2001; Buske, Feuring-Buske et al. 2002). Moreover, HoxB4, directly delivered or fused to HIV-TAT protein, has been shown to sustain haematopoietic stem/progenitor cell (HSPC) expansion *in vitro* (Amsellem, Pflumio et al. 2003; Kros, Austin et al. 2003). Other Hox proteins have been identified as regulators of early haematopoiesis, such as Hox9, Hox8 and Hox6 (Lawrence, Helgason et al. 1997; Izon, Rozenfeld et al. 1998; Shimamoto, Tang et al. 1999; Kappen 2000; So, Karsunky et al. 2004). However, a relatively mild effect on haematopoiesis was observed as consequence of the deficiency of each single Hox gene, whereas a more profound defect has been shown in mice lacking Hox cofactors, such as Pbx1 and Meis1 (DiMartino, Selleri et al. 2001; Hisa, Spence et al. 2004), or upstream regulators, such as Mll (Ernst, Fisher et al. 2004), suggesting redundant

and compensating functions of Hox gene(s). The role of these genes in haematopoiesis is still under investigation, but it is now known that they target cell cycle genes, and that Hox proteins interact with both nuclear factor and signal transducers (for a comprehensive review on Hox regulation of HSCs see (Abramovich and Humphries 2005).

A factor believed to play a critical role in maintaining HSCs, both during embryogenesis and in the adult, is the zinc-finger transcription factor Gata-2 (Tsai, Keller et al. 1994; Tsai and Orkin 1997). Indeed, the disruption of the gene encoding for it leads to a reduction of all haematopoietic precursors and the enforced expression blocks normal haematopoiesis (Persons, Allay et al. 1999). Similarly, the TEL locus, encoding an Ets family transcription factor, seems to have a role in BM haematopoiesis (Wang, Swat et al. 1998).

More recently, the polcomb group gene Bmi-1 has been shown to be absolutely required for HSC maintenance. Its expression is enriched in stem and progenitor cells in the adult (Lessard, Baban et al. 1998; Lessard and Sauvageau 2003) and it has been shown that its absence leads in mice to a progressive impairment of BM HSC and committed progenitors function (van der Lugt, Domen et al. 1994; Lessard, Schumacher et al. 1999). Moreover, it has been demonstrated that knock-down of Bmi-1 strongly reduces HSC self-renewing ability (Park, Qian et al. 2003). In this study Park *et al.* also studied HSC expression pattern and found a significant up-regulation of the cyclin-dependent kinase inhibitors (CKIs) p16<sup>INK4a</sup> and p19<sup>ARF</sup> when Bmi-1 was knocked-down. Thus, they proposed a model in which Bmi-1, by down-regulating the expression of these two CKIs, maintains self-renewing proliferation and prevents senescence and apoptosis (Park, Qian et al. 2003).

Indeed, some CKIs have been proposed as players in the control of HSPCs. Cheng *et al.* found that p21<sup>cip1/waf1</sup> is highly expressed in the quiescent fraction of BM cells and that its absence leads to cell-cycle entry and premature exhaustion of murine HSCs (Cheng, Rodrigues *et al.* 2000). The same group has also demonstrated that mice lacking p27<sup>kip1</sup>, a CKI belonging to the same family of p21, have normal HSC number, cell-cycling and self-renewal, but they show an increase in progenitors (Cheng, Rodrigues *et al.* 2000). Among the family of CKIs encoded by the INK4 locus, p16 expression is highly enriched in HSPCs and down regulated during differentiation (Furukawa, Kikuchi *et al.* 2000). In mice its absence seems not to lead to defects in haematopoiesis (Serrano, Lee *et al.* 1996), possibly due to activation of compensating mechanisms, while *in vitro* p16<sup>INK4a</sup> seems to negatively regulate HSPC proliferation (Park, Qian *et al.* 2003). Moreover, recently Ito *et al.* (Ito, Hirao *et al.* 2004) have showed a reduced HSC self-renewal in mice knocked-down for ataxia telangiectasia mutated (ATM) gene, which is responsible for genomic stability maintenance in stress conditions (Shiloh 2003). They associated this defect with an intracellular increase of reactive oxygen species that upregulated p16<sup>INK4a</sup> and p19<sup>ARF</sup> by activating p38 MAPK (Ito, Hirao *et al.* 2004; Ito, Hirao *et al.* 2006) and thus suggested a role of the INK4 CKIs in HSC regulation. p18<sup>INK4</sup> has also been proposed as HSC regulator, since its absence in mice induces an increase in self-renewal (Yuan, Shen *et al.* 2004).

All these findings suggest that the balance between self-renewal and differentiation in HSCs may be obtained through the balance of different CKIs classes and support the idea that HSC maintenance is tightly linked to the maintenance of quiescence. The role of CKIs has been confirmed by a recent gene expression study, in which Weissman's group analysed different subsets of



murine HSPCs and found that expression of several CKIs is up-regulated in the quiescent fraction (Passegue, Wagers et al. 2005). In the same work, the authors showed a precise pattern of expression of the cyclin genes for each haematopoietic population analysed and proposed that the activation of different cyclins may evolve in specific cellular outcomes.

Understanding the molecular mechanisms governing HSC behaviour is important not only for basic biology studies, but also for health issues. Indeed, the extensive clonal proliferation and the long-term self-renew ability make HSCs more susceptible to genetic alteration; this, together with the capacity to counteract the phenomenon of senescence, raises the issue of the development of cancer. In fact, it has been shown that in cancers of the haematopoietic system the initially transformed cell is a primitive stem cell that took an aberrant developmental pathway, which can be initially driven by a deregulation of critical intrinsic factors (Bonnet and Dick 1997; Reya, Morrison et al. 2001; Jordan 2002).

In the recent years, with the advent of new high-throughput gene expression analyses, several groups have attempted to define the complete molecular phenotype of HSCs (Phillips, Ernst et al. 2000; Zhou, Chen et al. 2001; Ivanova, Dimos et al. 2002; Ramalho-Santos, Yoon et al. 2002; Akashi, He et al. 2003; Georgantas, Tanadve et al. 2004; Venezia, Merchant et al. 2004). Surprisingly, in most of these studies many lineage-restricted gene sets have been found to be expressed in HSCs. These findings have supported the development of the *ground state model* (Enver and Greaves 1998). This model, which is now widely accepted by the scientific community, proposes that HSCs display a molecular “ground state” composed of low levels of different commitment-related transcripts and that the commitment/differentiation process takes place by

selecting and amplifying the appropriate set of available transcripts and possibly repressing the remaining. This “lineage priming” is believed to be maintained by a promiscuous state of wide-open chromatin, which allows the simultaneous binding of multiple competing/alternative lineage specific transcription factors (for a review see (Akashi 2005)).

#### 1.1.4.2 Extrinsic signals

In the adult, HSCs reside mainly in the BM, where they are anchored to stromal cell and extracellular matrix through adhesion molecules. As aforementioned, the BM microenvironment (niche) provides factors that profoundly influence HSC fate. Some of the pathways involved in HSC regulation in the adult are also active during embryogenesis.

*Wnt and Notch pathways.* The Wnt/ $\beta$ -catenin pathway has been recently implicated in the regulation of HSC self-renewal. Indeed, over-expression of constitutively activated  $\beta$ -catenin in murine HSCs prevents differentiation while promoting self-renewal (Reya, Duncan et al. 2003). Moreover, Wnt3a purified protein was shown to induce HSC proliferation without apparent differentiation *in vitro* (Willert, Brown et al. 2003). Interestingly, the activation of this pathway increased the expression of HoxB4 (see paragraph 1.1.4.1 Intrinsic signals) and Notch1 (Reya, Duncan et al. 2003). Indeed, activation of Notch1 transmembrane receptor has been shown to promote HSC self-renewal (Varnum-Finney, Purton et al. 1998; Karanu, Murdoch et al. 2000; Varnum-Finney, Xu et al. 2000; Karanu, Murdoch et al. 2001; Ohishi, Varnum-Finney et al. 2002), although critical targets for the Notch signalling pathway in HSCs still need to be elucidated. A very

recent study proposed glycogen synthase kinase-3 (GSK-3) as modulator of gene targets of Wnt and Notch-1 (Trowbridge, Xenocostas et al. 2006). In this work, an enhancement of human and murine HSC self-renewing proliferation was shown upon administration of GSK-3 inhibitors *in vivo* and this enhancement was correlated to an increased expression of Notch-1 target genes (Trowbridge, Xenocostas et al. 2006).

*TGF- $\beta$  superfamily of ligands.* Notch1 ligand, Jagged-1 has been shown to be highly expressed on activated osteoblasts and to mediate the maintenance of Notch1 activated HSCs (Calvi, Adams et al. 2003). Recently, osteoblasts have been proposed by several groups as potent regulators of HSCs in the BM niche. Beyond the Jagged-1/Notch1 interaction, two other mechanisms have been suggested. The first is the homotypic N-caderin interaction between osteoblasts and HSCs and the second is the bone morphogenetic protein (BMP) signalling, which has been shown to control the number of primitive HSCs (Zhang, Niu et al. 2003). However, a different group demonstrated that different BMPs induce opposite effects on human HSPCs *in vitro*, some inducing block of proliferation and maintenance of primitive phenotype and others inducing proliferation and differentiation (Bhatia, Bonnet et al. 1999). BMPs belong to the TGF- $\beta$  superfamily of ligands; another component of this family, TGF- $\beta$ 1, has been shown to be a negative regulator of HSC proliferation and appears to be a regulator of quiescence *in vitro* (for a review see (Fortunel, Hatzfeld et al. 2000). In contrast, recent studies demonstrated that mice, in which TGF- $\beta$  receptors were knocked-down, showed normal HSC self-renewal and proliferation (Larsson, Blank et al. 2003; Larsson, Blank et al. 2005). Moreover, *in vitro* experiments have shown opposite effects, depending on the concentration, of TGF- $\beta$ 2 on

HSCs, while TGF- $\beta$ 2 knock-down reduced HSC self-renewal *in vivo* (Langer, Henckaerts et al. 2004). The apparent contrasting results obtained *in vitro* and *in vivo* can be explained by compensatory mechanisms operated by other factors present in the BM niche but absent in the *in vitro* culture. All together these findings suggest an important role of TGF- $\beta$  family in regulating HSC compartment and strongly support the concept that the outcome of HSC fate decision is context dependent and regulated by a complex cross talk of many different pathways. Indeed, a possible mechanism exerted by TGF- $\beta$ 1, behind the Smad signalling activation, is the down-regulation of the expression of some cytokine-receptor, such as c-kit (stem cell factor – SCF – receptor), Flt3 (fms-like tyrosine kinase 3) and IL6-receptor (Sansilvestri, Cardoso et al. 1995).

*Cytokines.* Several cytokines, such as SCF, Flt3-Ligand, IL6 and thrombopoietin (TPO), have been shown to promote growth of murine and human HSPCs *in vitro* (Petzer, Zandstra et al. 1996; Sitnicka, Lin et al. 1996; Ueda, Tsuji et al. 2000; Nakauchi, Sudo et al. 2001). However, some receptors, such as FLT3, were not found to be expressed in HSCs (Adolfsson, Borge et al. 2001), whereas others, such as c-kit and TPO receptor (c-mpl), were found; knock-out of these receptors induce in mice severe HSC deficiencies (Miller, Rebel et al. 1996; Kimura, Roberts et al. 1998), suggesting a role of these cytokines in promoting HSC *in vivo* maintenance.

Flt3-Ligand, SCF, IL6 and TPO, upon binding to their receptor, activate the JAK2/STAT5 pathway (Bacon, Tortolani et al. 1995; Sattler, Durstin et al. 1995; Linnekin, Mou et al. 1997; Piekorz, Nemetz et al. 1997; Hayakawa, Towatari et al. 2000). In agreement with a positive role of these cytokines in HSC maintenance, recent studies have shown that knock-down of STAT5 in mice

reduce HSC self-renewal and multipotency, while its constitutive activation induce expansion of multipotent progenitors and promote HSC self-renewal (Bunting, Bradley et al. 2002; Snow, Abraham et al. 2002; Kato, Iwama et al. 2005). Another interesting study has shown that SHIP (SH2-containing inositol 5-phosphatase), a negative regulator of numerous signal transduction pathways, is probably involved in regulation of the cytokine-receptors pathways involved in HSC maintenance, because its knock-down in mice induce defects in HSC homeostasis and this defect is linked to an enhanced cytokine responsiveness (Liu, Shalaby et al. 1998; Helgason, Antonchuk et al. 2003).

*Tie2/Angiopoietin1 pathway.* HSC cell cycle regulation has been linked to the Tie2/Angiopoietin1 signalling pathway. The activation of this pathway by the ligand, which is produced by both endothelial cells and HSCs, through the tyrosine kinase receptor Tie2 maintained HSC self-renewal and is thought to play a critical role in the maintenance of quiescence (Takakura, Huang et al. 1998; Arai, Hirao et al. 2004). Moreover, Angiopoietin1 increases the interaction of HSC integrins and fibronectin present in the extracellular matrix and this interaction was shown to enhance in turn growth and survival of HSCs (Yokota, Oritani et al. 1998).

*Inorganic compounds.* Recent reports have proposed a role of oxygen in the regulation of HSCs in BM niche. Most primitive cells would reside in hypoxic areas, where low oxygen levels would enhance survival and expansion of self-renewing HSCs (Danet, Pan et al. 2003; Ceradini, Kulkarni et al. 2004). Moreover, very recently Scadden's group has proposed the high concentration of calcium ions at the BM endosteal surface as signal for HSC localization. The

calcium-sensing receptor expressed by HSCs would help in retaining these cells in close physical proximity to the endosteal surface and, thus, the regulatory niche (Adams, Chabner et al. 2006).

*Negative regulators.* HSC fate is determined by the balance of positive and negative stimuli in the BM niche but, beyond TGF- $\beta$ 1, negative regulators are less known. Some studies proposed tumor necrosis factor- $\alpha$  and interferon- $\gamma$  as players in the negative regulation of haematopoietic progenitors (Jacobsen, Ruscetti et al. 1992; Maciejewski, Selleri et al. 1995; Zhang, Harada et al. 1995; Yang, Dybedal et al. 2005).

Overall, a big effort has been made by several groups in the last fifteen years to understand the molecular mechanisms involved in HSC regulation. Nonetheless, the high level of complexity, the difficulty of studying HSCs in physiological conditions and possibly the difference between mouse and human haematopoiesis have not yet allowed to fully address it.

#### *1.1.4.2.1 Homing*

HSCs are mostly BM-resident cells. However, under stress conditions and, to a lesser extent; in physiologic conditions HSCs can migrate within the BM or to extra-medullary organs. In a BM transplant setting, transplanted HSCs must migrate through the blood, cross the vasculature and seed in the BM niche. This migration requires an active process termed homing. Like other HSC features, homing is tightly regulated by factors expressed in the BM microenvironment, such as adhesion molecules and haematopoietic cytokines. Although the

molecular mechanisms are not fully understood, major advances have been made in the recent years in the elucidation of the homing process and the molecular and cellular pathways involved.

The Stromal cell Derived Factor 1 (SDF-1)/CXCR4 chemokine receptor 4 (CXCR4) interaction has been proposed as a fundamental axis regulating retention, migration and mobilization of HSCs during homeostasis and stress (for a review see (Cottler-Fox, Lapidot et al. 2003). Lapidot's group has shown that HSC homing to the BM requires functional interaction between CXCR4 expressed on the surface of HSCs and SDF-1 expressed by BM endothelium and stromal cells (Peled, Petit et al. 1999; Kollet, Spiegel et al. 2001). Moreover, it has been shown that over-expression of CXCR4 leads to increased cell motility and increased HSC engraftment (Brenner, Whiting-Theobald et al. 2004; Kahn, Byk et al. 2004). Very recently, a role for protein kinase C  $\zeta$  (PKC  $\zeta$ ) as intracellular signal transducer downstream to CXCR4 activation has been shown. Indeed, PKC  $\zeta$  translocates to the membrane upon SDF1 stimulation and increases CXCR4 expression and matrix metalloproteinase 9 (MMP-9) secretion (Petit, Goichberg et al. 2005; Goichberg, Kalinkovich et al. 2006). Proteolytic enzymes, such as MMP-9 and MMP-2, then facilitate HSC homing by degrading extracellular matrix and increasing HSC motility. Nevertheless, it has been shown that some cell surface peptidases, such as CD26, have a negative role in the HSC homing process. Indeed, inhibition of CD26 has been shown to enhance HSC homing in response to SDF-1, possibly because CD26 is responsible for cleavage and inactivation of SDF-1 itself (Christopherson, Hangoc et al. 2002; Christopherson, Hangoc et al. 2004).

Adhesion molecules, such as integrins and selectins, mediate rolling and adhesion of homing cells before extravasation. Very late antigen 4 (VLA-4) and

VLA-5 integrins have been found to participate in HSC homing to the BM (van der Loo, Xiao et al. 1998; Kollet, Spiegel et al. 2001). Moreover, SDF-1 has been shown to increase adhesiveness of the integrins VLA-4 and LFA-1 to their respective endothelial ligands (Peled, Petit et al. 1999).

Together, these results propose SDF-1 as key player in regulating HSC homing and suggest that several pathways are involved in its action.

### **1.1.5 HSC transdifferentiation**

As discussed above, the adult stem cell has been believed for long time to be able to differentiate only in the lineages belonging to the resident organ/tissue. However, several recent studies have claimed that adult stem cells may transdifferentiate in response to particular external stimuli. Transdifferentiation is the conversion of a cell of one tissue lineage into a cell of an entirely distinct lineage, with concomitant loss of the tissue-specific markers and function of the original cell type, and acquisition of markers and function of the transdifferentiated cell type. Indeed, some groups have reported that BM cells contribute to the formation of non-blood cells within different tissues (Asahara, Murohara et al. 1997; Ferrari, Cusella-De Angelis et al. 1998; Gussoni, Soneoka et al. 1999; Petersen, Bowen et al. 1999; Lagasse, Connors et al. 2000; Theise, Badve et al. 2000; Jackson, Majka et al. 2001; Krause, Theise et al. 2001; Lyden, Hattori et al. 2001; Orlic, Kajstura et al. 2001; Grant, May et al. 2002; Theise, Henegariu et al. 2002). Surprisingly, a significant number of following studies failed to detect these contributions in similar experimental system (Castro, Jackson et al. 2002; Wagers, Sherwood et al. 2002; Choi, Uchino et al. 2003; De Palma, Venneri et al. 2003; Ono, Yoshihara et al. 2003; Vallieres and Sawchenko



2003). The reason for this apparent irreproducibility of the first set of experiments is not entirely clear, but could be partially linked to different models and detection strategies. There are also two alternative explanations. The first is related to the purity of the test population; in fact, it is possible that the supposed transdifferentiation was in reality due to the contribution of distinct contaminant cells, since most of the studies inferred such activity following transplant of large numbers of heterogeneous populations. The second is a mechanism known as cell-cell fusion; by this mechanism a haematopoietic cell could contribute to the formation of a non-haematopoietic tissue fusing to a resident cell, rather than transdifferentiating. Thus, after a first wave of excitement, the scientific community is now cautious about adult stem cell transdifferentiation, which is now believed to be, if real, extremely rare.

### **1.1.6 Markers and purification**

HSCs are rare cells among the haematopoietic cell population residing in the BM and their isolation from the BM is further complicated because these cells lack a distinctive phenotype. The use of flow cytometry techniques allowed the isolation of HSPCs, based on the expression of surface markers (Visser, Bauman et al. 1984; Muller-Sieburg, Whitlock et al. 1986). Nevertheless, to date, a single HSC-specific marker has not been identified.

The most studied animal model for haematopoiesis is the mouse, where several HSC markers have been discovered. Among these markers, the most used are c-kit (the tyrosine kinase receptor of stem cell factor – SCF), Thy-1 (a transmembrane protein present on HSCs and lymphocytes) and Sca-1 (Stem Cell associated Antigen); moreover, since HSPCs do not express lineage markers, it is

possible to use a combination of lineage specific antigens to negatively select differentiated/committed cells (Baum, Weissman et al. 1992; Morrison, Wandycz et al. 1997). Using this combination of cell markers it is possible to isolate a population of cells, called KTLS ( $c\text{-kit}^{\text{positive}}/\text{Thy-1}^{\text{low}}/\text{Lineage}^{\text{negative/low}}/\text{Sca-1}^{\text{positive}}$ ) highly enriched in HSCs (Uchida and Weissman 1992; Uchida, Tsukamoto et al. 1998). Moreover, very recently, it has been shown that SLAM receptors are differentially expressed in haematopoietic stem versus progenitor cells, and that they can be used to isolate a highly pure population of HSCs (Kiel, Yilmaz et al. 2005; Yilmaz, Kiel et al. 2006).

Less predictive surface markers have been established for the isolation of human HSCs. Among these, the more widely used is CD34, a heavily glycosylated type I transmembrane protein belonging to the sialomucin-like adhesion molecule family. This antigen was initially discovered in an attempt to develop antibodies specifically directed against human marrow cells, but not mature blood cells (Civin, Strauss et al. 1984). CD34 is expressed by stem/progenitor cells and is down-regulated during differentiation (Civin, Strauss et al. 1984; Katz, Tindle et al. 1985; Andrews, Singer et al. 1986; Strauss, Rowley et al. 1986; Andrews, Singer et al. 1989; Krause, Fackler et al. 1996); it is also expressed by small vessel endothelial cells and by some fibroblasts (Fina, Molgaard et al. 1990; Brown, Greaves et al. 1991). It has also been found that the more primitive haematopoietic progenitors are  $\text{CD34}^{\text{positive/bright}}$ , while the more committed ones are  $\text{CD34}^{\text{positive/dim}}$  (Andrews, Singer et al. 1989; Krause, Ito et al. 1994); this different level of expression allows for further enriching the population of primitive HSPCs. The physiological function of this molecule is still not yet clear, although its involvement in adhesion and localization of HSPCs in the BM niche has been postulated (Fina, Molgaard et al. 1990; Healy, May et al.

1995). In the BM niche, the CD34<sup>positive</sup> cells represent 1.5% of the mononucleated cells, while in the peripheral blood they are more rare (0.1-0.5%) (Civin, Strauss et al. 1984; Civin, Banquerigo et al. 1987; Loken, Shah et al. 1987). Although CD34 is commonly used to purify HSPCs, there is some evidence that its expression may vary with the activation stage of the cells (Osawa, Hanada et al. 1996; Bhatia, Bonnet et al. 1998; Sato, Laver et al. 1999; Zanjani, Almeida-Porada et al. 1999; Dao, Arevalo et al. 2003; Hess, Karanu et al. 2003; Zanjani, Almeida-Porada et al. 2003). A confounding issue is that whereas some studies suggested that the more primitive HSCs are CD34<sup>negative</sup> and that they are the precursors of the CD34<sup>positive</sup> population (Osawa, Hanada et al. 1996; Bhatia, Bonnet et al. 1998; Sato, Laver et al. 1999), other studies proposed that CD34<sup>positive</sup> cells transplanted in mice can generate *in vivo* CD34<sup>negative</sup> cells (Dao, Arevalo et al. 2003).

In association with CD34, other antigens can be used to further enrich HSCs. The most frequently used marker is CD38 (Terstappen, Huang et al. 1991; Cardoso, Li et al. 1993; Randall, Lund et al. 1996; Novelli, Ramirez et al. 1998). This transmembrane protein has a higher expression in committed progenitors (both lymphoid and myeloid) than primitive HSCs, so that its use in combination with CD34 (CD34<sup>positive</sup>CD38<sup>negative</sup> selection) allows for the enrichment of more pure stem cells (Xiao and Dooley 2000). Moreover, human HSCs, similar to murine HSCs, do not express surface markers that are associated with the terminal maturation of blood cells, thus enabling the selection of HSPCs based on the exclusion of cells expressing lineage-specific markers (lineage negative selection).

Other selection procedures have been developed based on markers expressed by CD34<sup>+</sup> cells. One such marker is CD133 (also known as AC133), which is expressed only by the CD34<sup>positive/bright</sup> fraction (Miraglia, Godfrey et al.

1997; Yin, Miraglia et al. 1997). Others are the vascular endothelium grow factor receptors 1 and 2 (VEGFR-1 and VEGFR-2 or KDR), c-kit (CD117; stem cell factor receptor), CD90 (Thy-1) and CD109 (Craig, Kay et al. 1993; Murray, Bruno et al. 1999; Ziegler, Valtieri et al. 1999; Hattori, Heissig et al. 2002).

Beyond the use of surface markers, it has been shown the possibility to use metabolic markers for HSPCs, such as rhodamine and Hoechst 33342 dye loading. The dye efflux depends on the expression of membrane pumps. High expression of such membrane pumps is associated with primitive HSPCs (Goodell, Brose et al. 1996; Goodell, Rosenzweig et al. 1997). The Hoechst 33342-low cells, termed side population (SP), were found to overlap with HSPCs in the mouse (Goodell, Brose et al. 1996). SP cells have been identified also in the human BM (Goodell, Rosenzweig et al. 1997) and in foetal liver (Uchida, Fujisaki et al. 2001). However, Hoechst 33342 intercalates into DNA and is slightly toxic, thus its use for the selection of HSCs is not suitable for transplantation. Moreover, the dye efflux properties could be related to the cell cycle status of the cells (Cai, Weiss et al. 2004). Another metabolic marker associated with immature cells is the aldehyde dehydrogenase (ALDH), whose expression correlates with high stem cell activity *in vivo* (Storms, Trujillo et al. 1999; Fallon, Gentry et al. 2003; Hess, Meyerrose et al. 2004).

It should be noted that all the strategies described above allow for enrichment of HSCs together with more committed progenitors. This heterogeneous population is useful for transplant purposes (because committed progenitors radioprotect the host), but it hampers the study of stem cell biology. To this aim, in the future it would be desirable to develop sorting procedures that enable the isolation of pure primitive HSCs.

### 1.1.7 Functional Assays

Because of the lack of specific markers and the functional heterogeneity of any population enriched in HSPCs, indirect and retrospective functional assays are required to characterize HSCs.

Two sets of functional assays have been developed in the last 30 years: the *in vitro* assays and *in vivo* assays. The first set typically evaluates two parameters: cell proliferation and differentiative potential. Indeed, primitive progenitor must accomplish a high number of divisions before they produce differentiated cells, whereas lineage-committed progenitors differentiate in a shorter time. However, the *in vitro* assays fail to assess some features that are critical for the characterization of HSCs: long-term self-renewal and multilineage differentiation. The *in vivo* assays are used to evaluate these latter parameters.

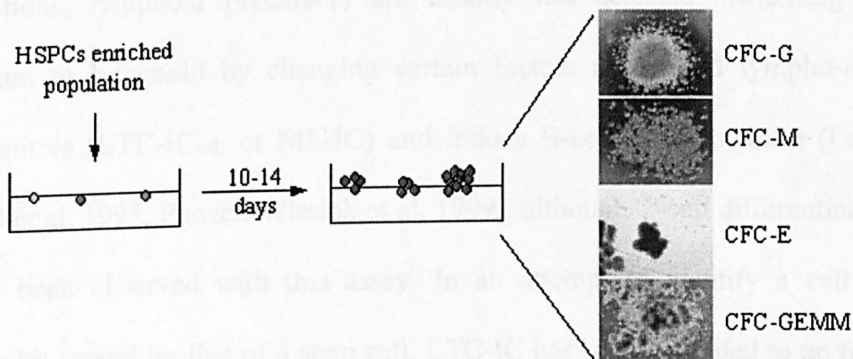
#### 1.1.7.1 *In vitro* assays

*In vitro* assays can be divided in *short-term* and *long-term* assays. *Short-term* assays identify committed progenitors, while *long-term* assays are used to characterize primitive progenitors.

##### 1.1.7.1.1 *Short-term in vitro* assays

The prototype of this kind of assay is the Colony-Forming Cell (CFC) assay. This is a clonogenic assay, which is based on the ability of haematopoietic progenitors to give rise to different cell lineages. Haematopoietic cells are seeded at low density in a semi-solid medium containing grow-factors that induce differentiation. As they are immobilized, the progenies of each single cell accumulate in tight colonies, which are scored, 2 weeks after seeding, for morphology. Several types of myeloid progenitors can differentiate

simultaneously in the same culture medium (Broxmeyer 1984), whereas CFC assay culture medium for lymphoid progenitors have not been well established. In this assay the number and type of haematopoietic progenitors seeded is evaluated, distinguishing granulocyte colonies (CFC-G), macrophage colonies (CFC-M) and erythroid colonies (CFC-E or BFU-E) (Figure 1.4). Moreover, progenitors at different maturation stages can be recognized based on time required to generate differentiated cells and the size of colony. Large colonies composed of different types of progenitors and differentiated cells are identified as colony-forming cell granulocyte-erythroid-macrophage-megacaryocyte (CFC-GEMM). It has been postulated that these CFC-GEMM colonies indicate the presence of a high proliferative multipotent progenitor in the culture (Ash, Detrick et al. 1981).



**Figure 1.4. CFC assay.** HSPCs are seeded at low-density in semi-solid medium. After 10-14 days colony number and morphology are scored by microscopy. For a detailed description of the assay see relative paragraph in the Methods section.

However, clonality of these colonies had to be proven to rule out that cells within a mixed colony were produced by two overlapping restricted progenitors and not by one multipotent progenitor cell. Only replating of cells from the primary colony in secondary assays can unmask the full potential of the multipotent progenitors. Still, more primitive immature progenitors cannot be detected by this assay, because the time of culture is too short for these progenitors to produce differentiated cells.

### 1.1.7.1.2 *Long-term in vitro assays*

Every system designed to detect immature progenitors extends beyond 5 weeks. This long-term culture eliminates mature progenitors that exhaust by the time of analysis. A common feature of the long-term systems is the use of feeder cells that provide growth factors, which surrogates the complexity of the BM niche.

The most frequently used *long-term in vitro* assay is the Long-Term Cell-Initiating Culture (LTC-IC). In this assay haematopoietic cells are seeded on a fibroblast monolayer and their ability to give rise to daughter cells able to form colonies is evaluated after 5-8 weeks by performing standard CFC assays (Sutherland, Eaves et al. 1989). Since cells are usually maintained in myeloid conditions, lymphoid precursors are usually not detected; switching of the medium to lymphoid by changing certain factors can reveal lympho-myeloid progenitors (LTC-IC<sub>ML</sub> or ML-IC) and induce B-cell differentiation (Lemieux, Rebel et al. 1995; Punzel, Wissink et al. 1999), although T-cell differentiation has never been observed with this assay. In an attempt to identify a cell whose longevity resembles that of a stem cell, LTC-IC has been extended to up to 10-12 weeks (ELTC-IC), but it is still unclear if this extension of the culture period can help identifying more primitive progenitors (Hao, Thiemann et al. 1996).

An alternative *long-term in vitro* assay is represented by the cobblestone area-forming cell (CAFC) assay. The difference between CAFC and LTC-IC is the use of a different feeder layer that allows the growth of dense hematopoietic colonies tightly associated with adherent cells (Ploemacher, van der Sluijs et al. 1989; Breems, Blokland et al. 1994). Thus, re-seeding of cells in semi-solid medium is not required in this assay. LTC-IC and CAFC assays have been though for long time to identify the same progenitor population, indeed it has been shown that human week-6 CAFC are equivalent to week-5 LTC-IC (Pettengell, Luft et

al. 1994). However, this view has been recently challenged (Denning-Kendall, Singha et al. 2003).

The discussed *long-term* assay are, to date, the best performing *in vitro* assays available to characterize haematopoietic progenitors, however it should be highlighted that they fail to detect lymphoid differentiation. Thus, other assays have been developed in order to determine the lymphoid potential of haematopoietic progenitors. B-cell potential is usually assessed in liquid culture on stromal feeders after 5-6 weeks in low serum conditions (Cumano, Dorshkind et al. 1990; Baum, Weissman et al. 1992; Barker and Verfaillie 2000; Ficara, Superchi et al. 2004). T-cell potential is more difficult to assess *in vitro*, mainly because of the need of the thymus three-dimensional structure for T-cell maturation. As surrogate of thymic architecture, murine foetal thymus organ cultures (FTOC) have been used to identify human T-cell progenitors, even though this chimera (human/mouse) does not allow for sustained proliferation and full differentiation of human progenitors (Barcena, Galy et al. 1994; Plum, De Smedt et al. 1994; Robin, Bennaceur-Griscelli et al. 1999; Weekx, Snoeck et al. 2000). A promising strategy to assess T-cell differentiation is the use of genetically enginred OP9 murine stromal cells. These cells, which have been shown to support *in vitro* the differentiation of hematopoietic progenitors into multiple lineages, including B cells, have been shown to support T-cell differentiation of early murine progenitor cells when enginred to costitutively express the Notch ligand Delta-1 (Schmitt and Zuniga-Pflucker 2002).



### 1.1.7.2 *In vivo* Assays

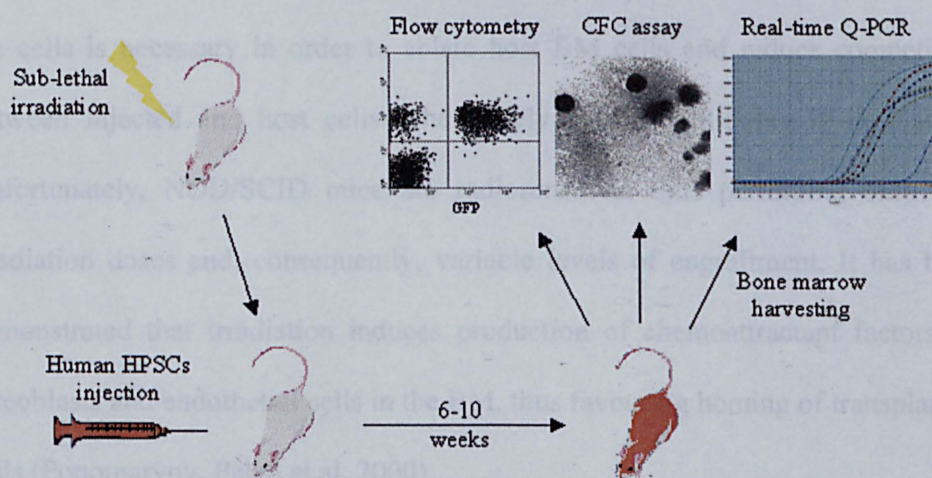
*In vitro* assays can identify haematopoietic progenitors, but not primitive stem cells, because they cannot assess self-renewal and full multilineage (lymphomyeloid) differentiation. In order to assess these features it is necessary to use indirect and retrospective assays in which human cells are transplanted into an animal model (xenotransplant model). This setting allows for tracking the injected cells for a long period (monitoring self-renewal) and to analyse their ability to produce different mature cell lineages (monitoring multilineage differentiation). It should be noted that the outcome of these assays is affected also by the ability of HSCs to home to the BM of the recipient.

The readouts of the xenotransplant models are usually flow cytometry analysis with specific antibodies of BM, peripheral blood and/or other haematopoietic organs (i.e. spleen or thymus) in order to evaluate the extent of the human graft and evaluate differentiation of the human cells towards the different lineages. Some *in vitro* assays, such as CFC assay, are also usually performed from cells derived from BM of transplanted mice, in order to verify the ability of engrafting cells to give rise to clonogenic progenitors following the *in vivo* time window. Recently, a novel set of tracking technologies based on magnetic nanoparticles and quantum dots have been proposed (Gao, Cui et al. 2004; Perez, Josephson et al. 2004) to detect by *in vivo* imaging techniques (MRI or laser-based macro illumination systems) limiting numbers of cells in a non-invasive experimental setting. However, because these molecules are lost during cell proliferation, their application in HSC xenotransplant settings is limited to the tracking of HSC homing and do not allow the long-term evaluation of HSC engraftment. Moreover, issues regarding toxicity and catabolism of these particles

and the need of refining the resolution of analysis have limited the applications of these *in vivo* imaging techniques to date.

In 1961 Till and McCulloch developed the first *in vivo* repopulation assay, transplanting murine BM, and identified a population of cells that were able to give rise to mixed colonies in the spleen of mice recipient 12 days after the transplant (Till 1961). These Colony-forming Units-Spleen (CFU-Ss) were initially regarded as HSCs. However, later studies demonstrated that the short time in which colonies were generated was not enough for stem cells to differentiate and that these CFU-Ss were progenitors rather than true primitive stem cells (Morrison and Weissman 1994; van der Loo, van den Bos et al. 1994). In the following years, transplant models that characterize murine HSCs have been established, although transplant models are still missing that satisfactorily characterise human HSCs.

The most used xenotransplant model for human HSC characterization is the NOD/Lt-Sz-Scid/Scid (NOD/SCID – nonobese diabetic / severe combined immunodeficient) mouse (Shultz, Schweitzer et al. 1995) (Figure 1.5).



**Figure 1.5. SRC assay.** NOD/SCID mice are sublethally irradiated 24 hours before intravenous injection of human HSPCs. 6-10 weeks after transplant mice bone marrow is harvested and engraftment and transduction levels are determined by flow cytometry, CFC assay or Real-time quantitative PCR. For a detailed description of the assay see relative paragraph in the Methods section.

In pioneering studies Dick's group showed that intravenous injection of human BM or cord blood (see paragraph 1.1.8 Sources) cells into sub-lethally irradiated SCID mice resulted in engraftment of primitive cells that were able to produce LTC-ICs, CFCs as well as differentiated myeloid, lymphoid and erythroid cells (Lapidot, Pflumio et al. 1992; Vormoor, Lapidot et al. 1994; Dick 1996). Because of these properties, the primitive cells were considered to be closely related to HSCs and were defined as SRCs (SCID Repopulating Cells). Later studies showed that SCID mice were not ideal recipient of human transplants, because they possessed antigen non-specific immunity, and proposed NOD/SCID mice as a better model to support human engraftment (Larochelle, Vormoor et al. 1995; Lowry, Shultz et al. 1996; Pflumio, Izac et al. 1996; Cashman, Lapidot et al. 1997). Indeed, in NOD/SCID mice the DNA repair gene defect of SCID mice, which severely impairs B- and T-cell development, is combined with impaired natural killer cell activity, absence of complement activity and the defect in macrophage function of the NOD mice (Shultz, Schweitzer et al. 1995). As in BM transplantation clinical settings, conditioning of the recipient before injection of the cells is necessary in order to ablate host BM cells and reduce competition between injected and host cells. The widely used conditioning is irradiation. Unfortunately, NOD/SCID mice are radiosensitive, thus permitting only low irradiation doses and, consequently, variable levels of engraftment. It has been demonstrated that irradiation induces production of chemoattractant factors by osteoblasts and endothelial cells in the BM, thus favouring homing of transplanted cells (Ponomaryov, Peled et al. 2000).

Kinetics studies showed that only 0.1% of the injected CFCs and LTC-ICs are detectable in the BM of transplanted immunodeficient mice 2 days after cell injection and that there is a large expansion of these cells over the next 4 weeks,

implying their origin from primitive cells (Vormoor, Lapidot et al. 1994; Cashman, Lapidot et al. 1997). Nevertheless, it should be highlighted that many cells were transplanted in the abovementioned studies, so that it was not possible to determine if the differentiated progeny arose from one primitive multipotent HSC or from more than one albeit committed progenitors. In order to address this issue, limiting dilution experiments were performed that showed that the model was reliable in assessing engraftment of a single human multipotent HSC (Bhatia, Wang et al. 1997; Conneally, Cashman et al. 1997; Wang, Doedens et al. 1997). The NOD/SCID model, however, shows two major limitations that do not allow for the assessment of all HSC features. The first is the short life span of these mice, which die because of thymomas at about six months of age and thus do not allow long-term monitoring of transplanted SRCs. The second is that they do not support human T-cell development and thus do not allow assessing full multilineage differentiation of SRCs.

To overcome these limitations, in the recent years many different mouse models able to support human haematopoiesis were developed.  $\beta 2$ -microglobulin knock-out / NOD/SCID mice do not develop thymomas and have a longer life-span, however they still do not support T-cell differentiation (Kollet, Peled et al. 2000), whereas beige-nude-SCID (bnx) mice support T- but not B-cell development (Dao and Nolta 1998). Recently, more promising models, such as RAG-2<sup>-/-</sup> $\gamma$ -chain<sup>-/-</sup> and NOD/SCID $\gamma$ -chain<sup>-/-</sup> mice, were proposed (Weijer, Uittenbogaart et al. 2002; Traggi, Chicha et al. 2004; Shultz, Lyons et al. 2005), but the usefulness of these new strains needs to be further explored.

Because by definition stem cells are able to self-renew for the entire life-span of the organism they belong to, human stem cell self-renewal should be evaluated in a model that enables the follow-up of transplanted cells for a time

window as long as human life-span. In this view, non-human primates would appear as more adapted than mice to characterize human HSCs. Unfortunately, the existing immunological barriers do not allow transplant of human cells in primates, thus requiring transplantation in utero when foetuses are immunologically tolerant. This strategy has been successfully applied by Zanjani's group, which used the sheep as large animal model. They showed persistence of human cells several years after transplant, with myeloid and lymphoid (T and B) differentiation (Porada, Porada et al. 2004). However, the complexity of these techniques may limit the use of these models.

An alternative approach to test HSC self-renewal in a long-term setting is serial transplantation in immunodeficient mice, in which BM of engrafted animals is transplanted into secondary recipients and, possibly, from secondary to tertiary mice. In this setting, only long-term self-renewing HSCs will maintain the ability to engraft and repopulate, whereas less primitive HSC will exhaust. The feasibility of this approach has been shown by different groups (Peled, Petit et al. 1999; Guenechea, Gan et al. 2001; Ailles, Schmidt et al. 2002; Dao, Arevalo et al. 2003). Moreover, taking advantage of clonal tracking and gene marking by lentiviral vectors, we have identified a single multipotent and long-term self-renewing HSC able to repopulate primary and secondary recipient and differentiate into both lymphoid and myeloid lineages (Ailles, Schmidt et al. 2002).

### **1.1.8 Sources**

For both research and clinical applications three tissue sources are widely used to purify HSCs: BM, mobilised peripheral blood (MPB) and umbilical cord blood (CB).

Although the presence of SRCs in all of these sources has been demonstrated (Lapidot, Pflumio et al. 1992; Vormoor, Lapidot et al. 1994; Hogan, Shpall et al. 1997), SRCs seem to be more abundant and/or endowed with higher proliferative potential, engraftment and self-renewal ability when isolated from CB (Wang, Doedens et al. 1997; Rosler, Brandt et al. 2000). CB is more easily available than the other tissues and for this reason it is the most exploited source of HSCs for research purposes. However, in a clinical setting, CB may not contain sufficient numbers of HSCs for transplantation in adult individuals (Abboud, Xu et al. 1992; Rubinstein, Carrier et al. 1998). To solve this issue many efforts have been made to define a protocol for CB-derived HSC expansion (see paragraph 1.2.1.2 HSC culture and gene transfer).

For a long time, BM has been used as a source of HSCs for clinical applications. Later on, MPB has been exploited as an alternative source. MPB is enriched in HSCs thanks to the injection of growth factors, such as granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF) and/or SCF into the donor. These factors induce proliferation and/or mobilization of BM HSCs. Some clinical transplantation trials that used MPB as source of HSPCs have suggested that MPB-derived HSPCs induce a more rapid myeloid recovery than the BM-derived counterparts (Bensinger, Weaver et al. 1995; Korbling, Przepiorka et al. 1995).

As discussed above (see paragraph 1.1.4 Molecular mechanisms regulating HSCs), external stimuli have an important role in regulating HSC features. For this reason, it is commonly believed that HSCs derived from different sources possess different characteristics, even though a comprehensive analysis is still missing. Indeed, some studies demonstrated different frequency of progenitors, cell cycle status, gene expression profiles and engraftment ability of HSCs derived

from CB, BM or MPB. In particular, it has been found that MPB-derived HSPCs are less cycling and more susceptible to apoptosis than BM-derived HSPCs, and this has been linked to a different gene expression profile in the two cell population (Uchida, He et al. 1997; Steidl, Kronenwett et al. 2002). Moreover, a lower engraftment ability and proliferative potential of BM- and MPB-derived HSPCs have been shown when compared to CB-derived HSPCs (Ueda, Yoshida et al. 2001; Ng, van Kessel et al. 2004; Theunissen and Verfaillie 2005).

Because of the differences in the biological properties of HSCs derived from different sources, the most appropriate source has to be chosen according to each application.

## 1.2 Gene transfer into haematopoietic stem cells

HSCs are attractive targets for the gene therapy of a variety of inherited and acquired genetic diseases, including immunodeficiencies, metabolic disorders and haemoglobinopathies. These disorders have been treated for a long time by allogeneic bone marrow transplantation. However, the development of graft versus host diseases in the transplanted patients is not infrequent in this setting. To overcome this problem, transplant of gene corrected autologous HSCs has been proposed as alternative therapy. HSCs can be transduced *ex vivo* and be re-infused in the donor where they self-renew and generate large numbers of gene corrected, differentiated progeny, theoretically for the lifespan of the host. To be efficacious, the genetic material must be inserted into the target cell chromatin in order to be maintained during HSC proliferation and differentiation processes. Moreover, efficient gene transfer should occur in primitive HSCs, without affecting their features.

Several gene transfer methods have been developed. Non-viral approaches take advantage of physical delivery methods (i.e. direct DNA injection, electroporation, nucleofection, gene guns) and chemical approaches (i.e. liposomes). The low frequency of integration into the target cell chromatin has limited the application of gene transfer in HSC by non-viral approaches. A new non-viral approach based on a transposon system (i.e. sleeping beauty transposon) has been recently developed; although it should ensure integration of the genetic material into the target cell chromatin, efficiency and safety of this system still need to be evaluated in HSCs (for a review see (Hackett, Ekker et al. 2005)). The more widely used viral approaches are based on Retrovirus, Adenovirus, Adeno-Associated virus, Herpes Simplex virus and Flavivirus (Tomanin and Scarpa 2004) and take advantage of the viral life cycle to transfer genetic material into target cells. Since viral methods are usually more efficient than non-viral methods, they have been largely studied as tool for HSC gene transfer. Moreover, some viral vectors allow for stable integration into the cell chromatin. Nevertheless, concerns about the safety of the viral sequences contained within the gene transfer vector, the risk of insertional mutagenesis and the potential immunogenicity of the vector particles have been raised.

### **1.2.1 HSC gene transfer by viral vectors**

During the last twenty years the viral vectors most exploited for HSC transduction (abortive infection) have been those derived from Retroviruses, mainly because of their ability to integrate into the target cell chromatin.

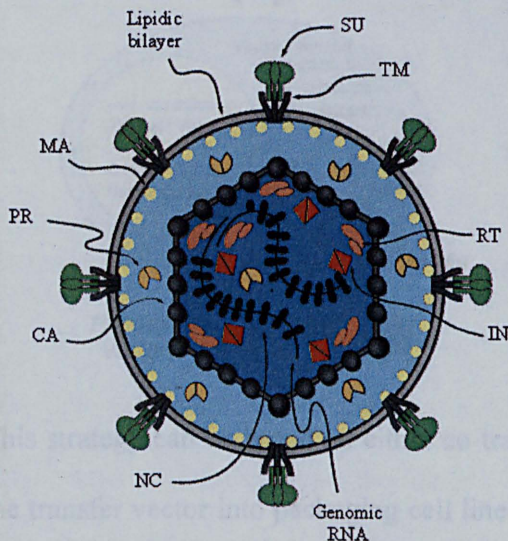
In addition to retroviral vectors, other viral vectors have been tested. Vectors derived from Adenovirus have high titers, wide tropism and the ability to



transfer relatively large genes. However, their high immunogenicity, their inability to integrate in the host chromatin and the absence of viral receptors on HSCs have limited their application. Recently, new strategies to generate hybrid adenoviral vectors that overcome some of these limitations have been developed, but definitive evidence of their efficiency is still missing (for a review see (Marini, Shayakhmetov et al. 2002)). Contrasting results have been shown about HSC transduction by Adeno-Associated virus-derived vectors (Alexander, Russell et al. 1997; Hargrove, Vanin et al. 1997; Nathwani, Hanawa et al. 2000). However, their tendency to remain in episomal forms limits their application in the haematopoietic field.

### 1.2.1.1 Retroviruses-derived vectors

Retroviruses are RNA viruses, whose replication strategy is based on an intermediate DNA form (provirus), which stably integrates into the infected cell

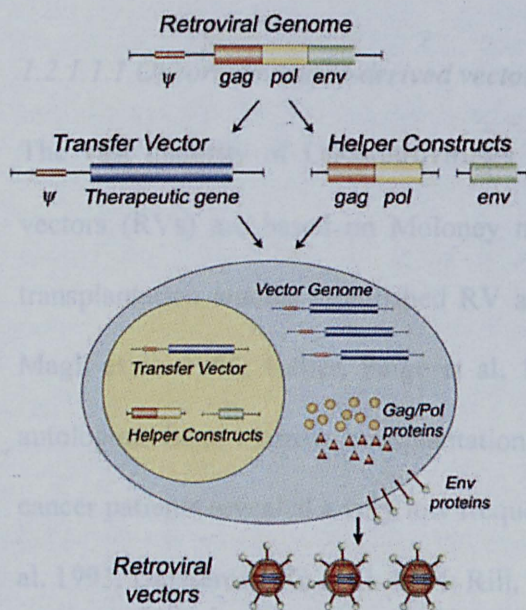


**Figure 1.6. Retroviral virion.** The following viral proteins are depicted: SU: surface; TM: transmembrane; RT: reverse transcriptase; IN: integrase; NC: nucleocapsid; CA: capsid; PR: protease; MA: matrix.

chromatin. Retroviruses possess a RNA genome, a proteic core (capsid) and a lipidic envelope, in which viral glycoproteins are inserted (Figure 1.6). This family of viruses has been divided into three subfamilies: Oncoviruses, Lentiviruses and Spumaviruses (Coffin, Hughes et al. 2000). To date, vectors for HSC gene transfer have been developed from viruses

belonging to each of these subfamilies. However, while oncoretroviral and lentiviral vectors have been extensively studied, Spumavirus-derived vectors have been applied only recently to the HSC field.

Vector design is based on the spatial separation between *cis*-acting and *trans*-acting sequences present in the retrovirus genome. Indeed, retroviral vectors are constructed starting from a wild-type virus, from which portions of the genome (*trans*-acting sequences) that encode for protein requested for the viral life cycle have been removed. Since these types of transfer vector contain only *cis*-acting sequences, production of retroviral vectors relies on the supply of the products of the deleted sequences by separated plasmid(s) (Figure 1.7) (Kay, Glorioso et al. 2001).



**Figure 1.7. Retroviruses-derived vectors.** Retroviral vectors are constructed by splitting *cis*- and *trans*-acting sequences of the retrovirus genome in different constructs. *Cis*-acting (regulatory) sequences, including the encapsidation signal ( $\psi$ ), are present only in the transfer vector containing the gene of interest, whereas *trans*-acting (encoding for viral proteins) sequences are provided by helper plasmids for the production of the vectors.

This strategy can be based on either co-transfection procedures or transfection of the transfer vector into packaging cell lines stably producing the viral proteins. In both cases, all the structural proteins needed for vector production are present only in the producer cells. Indeed, in the target cells the sequences encoding for

viral protein will be absent, because they lack the signals for encapsidation, and thus replication of the vector will be inhibited, unless recombination events occur.

Retroviral tropism is dictated by the envelope glycoproteins. Thanks to the viral assembling process and to the strategy used to generate these vectors, it is possible to exchange the wild-type envelope proteins with other envelope proteins (a process termed pseudotyping) and, thus, modify the tropism of the vector. Different envelopes have been shown to efficiently pseudotype retroviral vectors and target haematopoietic cells (Movassagh, Desmyter et al. 1998; Kelly, Vandergriff et al. 2000; Gatlin, Melkus et al. 2001; Ailles, Schmidt et al. 2002; Sandrin, Boson et al. 2002).

#### *1.2.1.1.1 Oncoretroviruses-derived vectors*

The vast majority of Oncoretroviruses (also known as  $\gamma$ -Retroviruses)-derived vectors (RVs) are based on Moloney murine leukaemia virus (MLV). *In vivo* transplantation studies established RV ability to transduce murine HSCs (Dick, Magli et al. 1985; Keller, Paige et al. 1985). However, initial clinical trials of autologous bone marrow transplantation with retrovirally gene marked cells on cancer patients revealed a very low frequency of transduced cells (Brenner, Rill et al. 1993; Deisseroth, Zu et al. 1994; Rill, Santana et al. 1994; Dunbar, Cottler-Fox et al. 1995), even though transduced cells were present up to 18 months after transplantation, suggesting transduction of primitive HSCs (Dunbar, Cottler-Fox et al. 1995). These results uncovered both the potential and the limitations of RVs in HSC gene transfer; these limitations are mainly related to the inability of RVs to transduce quiescent cells, because they need nuclear membrane disruption, and

thus mitosis, to have access to chromatin (Miller, Adam et al. 1990; Roe, Reynolds et al. 1993).

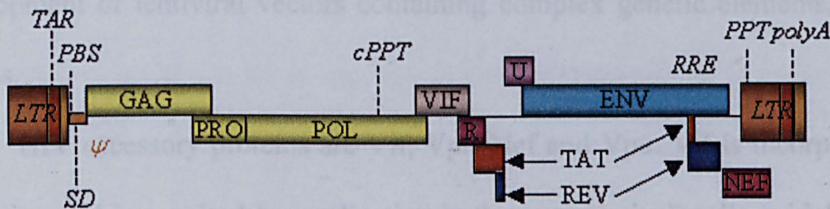
Since HSCs are mostly non-proliferating cells, they require a cytokine stimulation that trigger them to the cell cycle in order to be transduced by RVs. Thus, all the protocols developed for retroviral vector-mediated HSC gene transfer involve a prolonged *ex vivo* manipulation and/or induction of cell cycle entry and proliferation (Larochelle, Vormoor et al. 1996; Conneally, Eaves et al. 1998; Dao, Taylor et al. 1998; Barquinero, Segovia et al. 2000; Guenechea, Gan et al. 2001), conditions that were shown to compromise HSC potential, in particularly by decreasing HSC frequency and long term repopulating ability (Glimm, Oh et al. 2000; Guenechea, Gan et al. 2001). Nevertheless, three successful clinical trials for SCID disease have demonstrated that RVs can be useful tools for HSC-based gene therapy (Cavazzana-Calvo, Hacein-Bey et al. 2000; Aiuti, Slavin et al. 2002; Gaspar, Parsley et al. 2004). Two issues, however, should be highlighted: 1) there is still not evidence that haematopoietic reconstitution was due to stem rather than progenitor cell engraftment; 2) a major factor in the successful haematopoietic reconstitution by gene-corrected cells was the selective growth advantage conferred *in vivo* to transduced cells, that enable the amplification of a small input of transduced HSPCs.

Finally, other concerns have been raised about HSC transduction by RVs. The first is linked to the discovering of promoter silencing mechanisms that shut down transgene expression in these vectors (Challita and Kohn 1994; Gram, Nielsen et al. 1998; Klug, Cheshier et al. 2000; Zentilin, Qin et al. 2000; Pannell and Ellis 2001). Another issue is related to vector safety, since insertional mutagenesis leading to leukaemia associated to retroviral vector insertion has been demonstrated in a recent clinical trial (Hacein-Bey-Abina, von Kalle et al.

2003; Hacein-Bey-Abina, Von Kalle et al. 2003) (see paragraph 1.2.1.4 Safety of retroviral vectors).

### 1.2.1.1.2 Lentiviruses and derived vectors

Lentiviruses are complex retroviruses. The most studied lentivirus is the human immunodeficiency virus 1 (HIV-1). Lentiviruses differ from oncoretroviruses for their genome, which encodes for the structural proteins common to all retroviruses and for a set of regulatory and accessory proteins, which are mainly responsible for the pathogenicity of the virus (Anderson and Hope 2004) (Figure 1.8).



**Figure 1.8. HIV genome.** The principal HIV *cis-acting* (italic font) and *trans-acting* (regular font) sequences are indicated. LTR: long terminal repeat; it contains the viral promoter/enhancer region. PBS: primer binding site of the retrotranscriptase. SD: splice donor.  $\psi$ : encapsidation signal. (c)PPT: (central) polypurine tract. RRE: Rev-responsive element. PolyA: polyadenylation signal. GAG, PRO, POL and ENV genes are present in all retroviruses. GAG encodes for the structural proteins Capsid, Matrix and Nucleocapsid. PRO encodes for the Protease enzyme. POL encodes for the enzymes Retrotranscriptase and Integrase. ENV encodes for the envelope glycoproteins Surface and Transmembrane. VIF, VPR (R), VPU (U), TAT, REV and NEF genes encode for regulatory and accessory proteins and are characteristic of the HIV genome.

HIV regulatory proteins are TAT and REV. TAT is a potent *trans*-activator of transcription that, binding to the TAR sequence in the U3 LTR region, activates the transcription of HIV genome. Alternative splicing of the full-length transcript generates the array of mRNAs required for expression of all viral genes. The full-length transcript itself also serves as a translational template, as well as the source of new viral genomes. Thus, the unspliced genome must be transported from the nucleus to the cytoplasm, an event that ordinarily does not occur in the cell. This problem is overcome by REV, which binds the RRE sequence of the

transcribed genome and allows nuclear export of the unspliced RNA. Since TAT induces a number of potentially detrimental cellular responses (for a review see (Peruzzi 2006), it has been deleted in the design of late generation lentiviral vectors. This is possible if the U3 region of the 5' LTR in the transfer vector construct is replaced by constitutively active promoter sequences, thus rendering TAT dispensable (see below Figure 1.10). Instead, REV is the only regulatory/accessory protein that has been maintained in the design of late generation lentiviral vectors. Indeed, its activity is required to express the transfer vector RNA. The presence of REV in the vector system has also allowed the development of lentiviral vectors containing complex genetic elements, such as introns.

HIV accessory proteins are Vif, Vpr, Nef and Vpu. Vif is incorporated in the virion and is required for replication in “nonpermissive” cells, which include the natural targets of HIV-1 (lymphocytes, monocytes, dendritic cells, and brain microglia). In “permissive” cells (most HIV-1-infectable cell lines), Vif is completely dispensable. In “nonpermissive” cells delta-Vif HIV are unable to complete reverse transcription (von Schwedler, Song et al. 1993). This phenotype can be explained by recent studies that demonstrated that Vif counteracts the activity of a potent restrictive factor of HIV-1 replication incorporated in the virions produced by “nonpermissive” cells (see paragraph 1.2.1.3.2 APOBEC proteins). It has also been suggested that Vif stabilizes the viral core and, associating with the HIV genomic RNA, directs efficient reverse transcription by affecting the nucleic acid components of the RT complex (Ohagen and Gabuzda 2000) (Dettenhofer, Cen et al. 2000).

Vpr is present in the viral particle and it has been shown to induce G<sub>2</sub> cell cycle arrest (He, Choe et al. 1995). As expression of the viral genome is optimal

in the G<sub>2</sub> phase of the cycle, Vpr increases virus production by delaying infected cells at this point of the cell cycle. Vpr has also proposed to play a role in the nuclear import of the preintegration complex (see below).

Nef has several roles in the HIV-1 life cycle. One of these is the down-regulation of the trans-membrane molecules CD4 and MHC-I; down-regulation of CD4, which is the receptor for HIV envelope glycoproteins, helps budding of the virus from infected cells by preventing binding of the envelope proteins to this molecule at the cell membrane level, whereas MHC-I down-regulation protects infected cells from recognition and killing by cytotoxic T lymphocytes (Mangasarian, Foti et al. 1997). In addition, Nef enhances virion infectivity, likely because it is involved in the uncoating and reverse transcription processes (Aiken and Trono 1995; Aiken 1997). The exact mechanism of these effects remains to be elucidated.

Vpu mainly acts facilitating virus release. It down-regulates CD4 expression in the ER to prevent interaction with HIV-1 envelope proteins during virus budding and stimulates the release of virions possibly by acting as ion channel. Together with Nef, Vpu also down-regulates expression of MHC-I molecules in infected cells (for a review on Vpu see (Bour and Strebel 2003)).

All the HIV accessory proteins have been deleted in the design of late generation lentiviral vectors. This means that these proteins are dispensable in the vector system. Indeed, Vif is only required during HIV-1 assembly in cells which have a “nonpermissive” phenotype. 293T cells, in which vector particles are produced, are “permissive” cells, and thus Vif is not required for infectious particle assembly. Vpu down-regulates CD4 expression to prevent interaction with HIV-1 envelope proteins. However, the vectors have a heterologous envelope and are produced in cells that do not express CD4. Vpu is also believed

to stimulate the release of virions from the cell, but this effect was not observed in 293T cells. As with Vpu, the CD4-downregulating activity of Nef would obviously not be required in this system. However, Nef also functions to promote the infectivity HIV-1 virions. The lack of a requirement for Nef may be explained by the use of the VSV-G envelope. Aiken (Aiken 1997) showed that pseudotyping of HIV-1 with VSV-G, which changes the mechanism of target cell entry to an endocytic pathway rather than direct fusion with the plasma membrane, markedly suppressed the requirement for Nef. Finally, the lack of a requirement for Vpr in most cell types can be explained by the redundancy of nuclear import signals present in the PIC, as described below. It should be highlighted, however, that the dispensability of the accessory proteins in the lentiviral vector system has been demonstrated in permissive cell types. It cannot be excluded that in some less permissive primary cells, which could also express restrictive factors (see paragraph 1.2.1.3 Retroviral restriction in mammalian cells), some accessory proteins can be needed for efficient transduction.

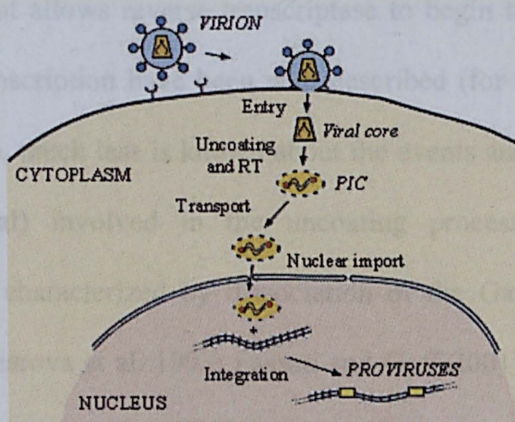
Beside the regulatory/accessory proteins encoded by lentiviruses and not by oncoretroviruses, a major difference between these two families or retroviruses resides in the early steps of their life cycle. Indeed, lentiviruses, but not oncoretroviruses, are able to cross the intact nuclear membrane and thus can integrate into the chromatin of non-proliferating cells (Weinberg, Matthews et al. 1991; Lewis, Hensel et al. 1992; Lewis and Emerman 1994). Because of the relevance of the issue for gene transfer applications, the early stages of lentiviral life cycle will be discussed.



### 1.2.1.1.2.1 Early stages of lentivirus life cycle

The early part of lentivirus life cycle is usually divided in different phases, although some of them are strictly linked and occur at the same time. The early

**Figure 1.9. Early stages of lentivirus life cycle.** The incoming virion particle contains two single stranded RNA molecules within a protein core, surrounded by a lipid bilayer. Upon receptor binding and fusion of the viral and cellular membrane the core is released into the cytoplasm (Entry). The core is then disassembled (uncoating) and the RNA genome is retrotranscribed in linear double stranded DNA (RT: reverse transcription), giving rise to the viral preintegration complex (PIC). The PIC is transported through the cytoplasm and through the nuclear membrane. The viral DNA integrates into the host chromatin to form the provirus.



stages of lentivirus life cycle are: entry, uncoating, reverse transcription, nuclear import and integration into the host cell chromatin (Figure 1.9).

Entry is mediated by binding of the viral envelope glycoproteins to specific cell membrane receptors. To enter the target cells only one membrane molecule is needed for most retroviruses, whereas for HIV-1 a receptor for initial binding (CD4) (Maddon, Dalglish et al. 1986) and a co-receptor (the chemokine receptor CCR5 or CXCR4) (for a review see (Berger, Murphy et al. 1999) for the fusion between virus and cell membrane are needed. Following fusion, the viral core is released into the target cell cytoplasm. Indeed, HIV entering is pH-independent as indicated by HIV resistance to drugs blocking endosome acidification (McClure, Marsh et al. 1988).

Immediately after release into the cytoplasm the viral core undergoes a progressive disassembly, known as uncoating, and the viral RNA genome is retrotranscribed into double stranded DNA by the viral reverse transcriptase. The trigger for the beginning of DNA synthesis is not known. However, it appears that

initiation of reverse transcription is strictly linked to the uncoating process (Zhang, Dornadula et al. 2000) and it is possible that the exposure of the retrotranscription complex to the significant concentration of deoxiribonucleotides in the cytoplasmic environment is what allows reverse transcriptase to begin to act. While the steps of the reverse transcription have been well described (for a review see (Telesnitski and Goff 1997), much less is known about the events and the proteins (both cellular and viral) involved in the uncoating process. Disassembly of the lentiviral core is characterized by dissociation of the Gag encoded Capsid protein (Bukrinsky, Sharova et al. 1993; Fassati and Goff 2001). Still, the composition of the preintegration complex (PIC; also known as reverse-transcription complex), which is the result of the viral core uncoating, is still not fully understood. This is mainly because of technical limits in the isolation of the particles and the fact that the vast majority of the particles present in the cytoplasm are not on a productive pathway for infection. However, the complexes isolated by most protocols retained the Pol encoded proteins (reverse transcriptase, protease and integrase), whereas the majority of the Gag encoded matrix and nucleocapsid proteins, initially believed to be associated with the PIC (Bukrinsky, Sharova et al. 1993; Miller, Farnet et al. 1997), are lost during uncoating (Fassati and Goff 2001). Some cellular proteins have also been found in the HIV PIC. Cyclophilin A, a peptidyl-prolyl isomerase, is brought into the target cell inside the viral particle and is apparently important to allow uncoating and reverse transcription (Franke, Yuan et al. 1994; Braaten, Franke et al. 1996). Moreover, the high mobility group protein HMG I(Y) seems to be recruited by the PIC and has been proposed to be important for integration (Farnet and Bushman 1997).

Interestingly, it seems that oncoretroviral core persists longer than that of HIV, since MLV nucleocapsid, matrix and even capsid proteins are found to be associated to the PIC in the vicinity of the nuclear membrane (Bowerman, Brown et al. 1989; Risco, Menendez-Arias et al. 1995). This difference in uncoating may be relevant to the pathways lentiviruses and oncoretroviruses use to access the nucleus, as uncoating is likely to be necessary for the ability of HIV to migrate through the nuclear pore (see below).

To reach the nuclear membrane, the particles must travel through the cytoplasm. HIV transport has been shown to exploit the cellular cytoskeleton. In particular, initial movements at the cell periphery occur in association with actin (Bukrinskaya, Brichacek et al. 1998), while subsequent translocation toward the nucleus takes place along the microtubule network, likely by interaction of HIV with the dynein-dependent motor complex (McDonald, Vodicka et al. 2002). After reaching the nuclear envelope HIV PIC is translocated through the nuclear pore, most likely by relying on the cellular nuclear import machinery. For a long time the most favoured model for HIV nuclear import has been that the PIC itself is karyophilic. Indeed, some viral proteins such as matrix, integrase and Vpr have been shown to contain nuclear localization signals (NLSs) in addition to the *cis*-acting element central polypurine tract (cPPT) (Bukrinsky, Haggerty et al. 1993; Heinzinger, Bukrinsky et al. 1994; Gallay, Swingler et al. 1995; Gallay, Hope et al. 1997; Vodicka, Koepp et al. 1998; Zennou, Petit et al. 2000; Bouyac-Bertoia, Dvorin et al. 2001). However, these results are controversial, since HIV with mutations in each of the identified NLSs still infects non-dividing cells (Fouchier, Meyer et al. 1997; Reil, Bukovsky et al. 1998; Dvorin, Bell et al. 2002). Moreover, very recently Emerman's group has shown that deletions in all four of the described NLSs do not abrogate the ability of the virus to infect non-dividing

cells (Yamashita and Emerman 2005). The same group previously showed that the capsid protein is involved in the HIV infectious phenotype in non-dividing cells (Yamashita and Emerman 2004), although capsid is not nucleophilic and is not associated with HIV PIC. Thus, they have proposed a new model in which the determinant step for the ability of HIV to cross the nuclear membrane is the uncoating process. Although there is not agreement about the viral components needed for nuclear translocation, this translocation is likely to be mediated by the importin/karyopherin cellular machinery.

Like all retroviruses, HIV then integrates into the host chromatin. The process of integration of the linear viral DNA is carried out by the viral integrase protein (for a review see (Brown 1997). Several cellular DNA binding proteins have been described to interact with integrase. A major regulator of integration is the barrier to auto-integration factor (BAF), which indirectly promotes integration in the target DNA by preventing integrase-mediated autointegration of the viral genome (Lee and Craigie 1998). Other three proteins have been proposed as positive regulators of HIV integration. HMG I (Y) dramatically stimulates integration reactions *in vitro*, probably by inducing changes in DNA structure (Farnet and Bushman 1997). Similarly, the integrase interactor 1 (Ini1), a subunit of the SWI/SNF chromatin remodelling complex, has been proposed to stimulate the *in vitro* DNA-joining ability of integrase (Kalpana, Marmon et al. 1994). Finally, the lens epithelium-derived growth factor (LEDGF/p75), a protein implicated in the regulation of gene expression and in cellular stress responses, has been found to interact with integrase and seems to be absolutely required to dock the PIC to the host chromatin (for a review see (Ciuffi and Bushman 2006). For a long time integration of lentiviruses and more in general of all retroviruses was believed to occur randomly into the host chromatin. Nevertheless, recent

reports have challenged this notion and indicated specific biases for integration into transcriptionally active genes (see paragraph 1.2.1.4 Safety of retroviral vectors).

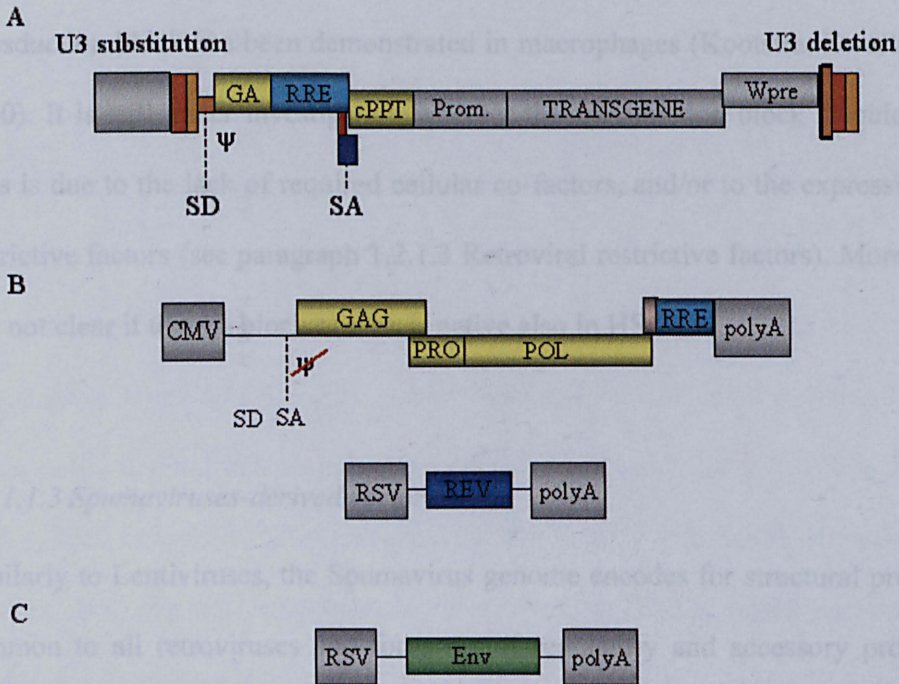
Overall, despite the intensive study of HIV in the last twenty years, many steps in the lentiviral life cycle are still not fully understood. Elucidating the mechanisms and identifying the cellular factors involved in these processes will lead on one hand to discover new ways to inhibit HIV replication and on the other to improve lentiviral vectors for gene transfer.

#### *1.2.1.1.2.2 Lentiviruses-derived vectors*

To broaden significantly the scope of HSC-based gene therapy, transduction of the majority of HSC without compromising their biological properties would be required. Lentiviral Vectors (LVs) are good candidates to this aim.

The most studied lentivirus is HIV-1. Because of its tropism for human cells and the acquired knowledge of its structure, many efforts have focused on the design of HIV-1 derived vectors. Starting from initial constructs (Naldini, Blomer et al. 1996), which contained all HIV-1 sequences, several studies have been performed to limit viral sequences/proteins without losing transduction efficiency. The state of the art of HIV-1 derived vectors is a packaging system constituted by four plasmids, in which all the accessory proteins have been deleted, and a Self-Inactivating (SIN) transfer vector (Figure 1.10) (Dull, Zufferey et al. 1998; Zufferey, Dull et al. 1998; Follenzi, Ailles et al. 2000; Delenda 2004). In this design the sequence homologies between the different plasmids have been greatly reduced, making recombination and production of replication competent lentiviruses very unlikely to occur. Moreover, the SIN design of the transfer vector, in which the enhancer/promoter sequence (U3) of HIV-1 has been deleted,

strongly improved safety. In fact, the SIN-Long Terminal Repeat (SIN-LTR) cannot initiate transcription of the vector genome and, thus, should not allow mobilization of the vector, for instance following superinfection by HIV; the SIN-LTR should also reduce the potential impact of the vector on flanking gene expression. Finally, the SIN design improved vector performance, allowing for the use of internal promoters, which can be selected from a large variety of cellular transcription regulatory elements or viral sequences, according to the application.



**Figure 1.10. Four plasmids system for the production of late generation LVs.** A) Transfer vector: the HIV 5'LTR U3 has been substituted with the Raus Sarcoma Virus or Citomegalovirus promoter-enhancer sequence; the 3'LTR U3 sequence containing the HIV promoter-enhancer region has been deleted. Upon retrotranscription 3'LTR is copied at the 5' obtaining a Self-Inactivating (SIN) vector. Wpre: Woodchuck post-regulatory elements. B) Packaging constructs encoding HIV structural proteins. CMV: citomegalovirus enhancer-promoter sequence. C) Envelope construct encoding for the envelope glycoproteins. RSV: Raus sarcoma virus enhancer-promoter. For other abbreviations see Figure 1.8 legend.

The main advantage of using LVs is their ability to transduce non-proliferating cells (Naldini, Blomer et al. 1996). In the HSC field, this would imply no needing for prolonged stimulation and, thus, a reduce risk of affecting

HSC features. Others and we have shown efficient transduction of CB SRCs after a short incubation with LVs (Uchida, Sutton et al. 1998; Miyoshi, Smith et al. 1999; Guenechea, Gan et al. 2000; Ailles, Schmidt et al. 2002). Although LVs transduce non-proliferating cells, they seem to be unable to transduce quiescent T lymphocytes, which must exit from G<sub>0</sub>, and presumably progress to the G<sub>1</sub>b phase of the cell cycle, in order to be infected by HIV-1, or transduced by LVs (Korin and Zack 1999; Unutmaz, KewalRamani et al. 1999; Wu and Marsh 2001; Cavalieri, Cazzaniga et al. 2003; Verhoeven, Dardalhon et al. 2003). A similar transduction block has been demonstrated in macrophages (Kootstra, Zwart et al. 2000). It is still under investigation whether the transduction block in quiescent cells is due to the lack of required cellular co-factors, and/or to the expression of restrictive factors (see paragraph 1.2.1.3 Retroviral restrictive factors). Moreover, it is not clear if this G<sub>0</sub> block of LVs is active also in HSCs.

#### *1.2.1.1.3 Spumaviruses-derived vectors*

Similarly to Lentiviruses, the Spumavirus genome encodes for structural proteins common to all retroviruses and for a set of regulatory and accessory proteins. Despite the name of the prototype Spumavirus, Human Foamy Virus (HFV), which is now known to be a chimpanzee virus (Nemo, Brown et al. 1978; Herchenroder, Renne et al. 1994; Schweizer and Neumann-Haefelin 1995), FVs are not endemic in human populations (Schweizer, Turek et al. 1995). This, coupled to their non-pathogenicity in their natural hosts (non-human primates), has supported the idea that HFV-derived vectors (FVV) could be safer than vectors derived from other retroviruses. In addition to safety considerations, the broad host and tissue tropism of FV (Russell and Miller 1996; Hill, Bieniasz et al.

1999), and the ability of FVV to package up to 9.2 kb of foreign genetic material (Trobridge, Josephson et al. 2002; Vassilopoulos, Josephson et al. 2003), made FVV promising gene transfer tools.

FVs have a characteristic cell cycle, in which reverse transcription occurs in cells that produce virions rather than in infected target cells, so that the functional genome is double-stranded DNA (Yu, Baldwin et al. 1996; Moebes, Enssle et al. 1997; Yu, Sullivan et al. 1999). Beside this, they differ from RVs and LVs also for their cell cycle requirements for transduction. Indeed, it has been recently shown that FV vectors for transgene expression ultimately require mitosis, but a transduction intermediate is stable and persists in non-dividing cells for some days (Trobridge and Russell 2004). This means that cells that are non-proliferating during exposure to the vector can be transduced by FV vectors only if they divide in the following short period.

Thus, even though some studies have reported efficient transduction of SRCs by FV-derived vectors (Josephson, Vassilopoulos et al. 2002; Leurs, Jansen et al. 2003; Josephson, Trobridge et al. 2004), it is still under investigation whether these vectors can transduce more primitive HSCs, which possibly are quiescent during transduction and will not divide soon after.

#### 1.2.1.2 HSC *ex vivo* culture and gene transfer

The development of protocols that maintain HSCs in culture without affecting their biological properties is strictly linked to the knowledge of extrinsic signals regulating HSC *in vivo*. Since the majority of these stimuli still need to be elucidated, the specific combination of most suitable factors for *ex vivo* culture and gene transfer remains to be determined.



Several studies have addressed this issue. IL-6, IL-3, SCF, Flt3L and TPO have been the most studied factors; they have been shown to preserve function, allow a moderate expansion and aid in transduction of HSPCs (Bodine, Karlsson et al. 1989; Bodine, Orlic et al. 1992; Petzer, Zandstra et al. 1996; Bhatia, Bonnet et al. 1997; Dao, Hannum et al. 1997; Glimm and Eaves 1999; Piacibello, Sanavio et al. 1999; Shpall, Quinones et al. 2002; McGuckin, Forraz et al. 2004). As discussed above, more recently, some of the newly discovered players in HSC regulation have been exploited *in vitro* (see paragraph 1.1.4 Molecular mechanisms regulating HSCs). BM stromal cells or extra-cellular matrix fragments have been used to ameliorate HSPC culture and gene transfer (Moritz, Patel et al. 1994; Nolte, Smogorzewska et al. 1995; Hanenberg, Xiao et al. 1996; Dao, Hashino et al. 1998; Yokota, Oritani et al. 1998). Also, it has been shown that *in vitro* exposure to SDF-1 increased haematopoietic progenitors proliferation *in vitro* and HSC engraftment *in vivo* (Lataillade, Clay et al. 2000; Glimm, Tang et al. 2002). Two different groups have also recently proposed the histone deacetylase inhibitor valproic acid as stimulator of HSPC proliferation and self-renewal (Bug, Gul et al. 2005; De Felice, Tatarelli et al. 2005). Bug *et al.* have shown down-regulation of p21 and up-regulation of HoxB4 induced by valproic acid that could explain this effect.

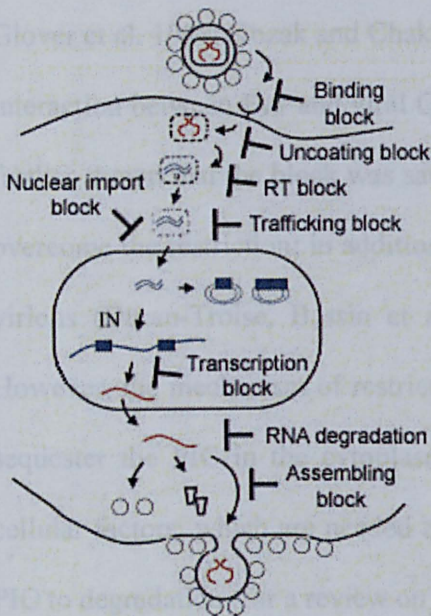
Beyond the choice of the best factors to maintain HSPCs, an issue that has been investigated in the gene transfer field is the period of cell culture. Since RVs have been the most exploited vectors for HSC gene transfer, most of the designed protocols included a several days of cell stimulation, in order to induce cell proliferation. Some of these protocols have been shown to have a therapeutic potential in clinical trials (Cavazzana-Calvo, Hacein-Bey et al. 2000; Aiuti, Slavin et al. 2002; Gaspar, Parsley et al. 2004). However, the benefits observed are

partially due to a selective advantage of the transduced cells. Only long-term analysis of the patients' graft will determine if transduced cells were progenitor, short-term or long-term stem cells. On the other hand, in NOD/SCID xenotransplant models, Dick's group showed that 4 days in culture strongly reduced HSC engraftment ability in a competitive setting (Mazurier, Gan et al. 2004), supporting the idea that minimal HSC manipulation is warranted. LVs are good candidates to efficiently transduce HSCs without extensive manipulation. Nevertheless, in apparent contrast with the lack of LV requirement for target cell proliferation, we have found a significant enhancement in gene transfer in the presence of a combination of early-acting cytokines (IL6, SCF, TPO and Flt3L) (Ailles, Schmidt et al. 2002). Although cytokine enhancement of SRC transduction by LVs has now been reproduced in other works (Zielske and Gerson 2003), it remains to be established whether the gain in gene transfer could be offset, even for a short cytokine exposure, by a decreased engraftment or long-term repopulation capacity of the transduced cells. Indeed, a recent study has shown that even a short period of exposure to SCF, Flt3L, IL6 and IL3 is detrimental for MPB-derived haematopoietic progenitors homing (Ahmed, Ings et al. 2004).

Finally, it should be highlighted that the differences in the biological properties imply different cytokine requirements for the *in vitro* maintenance and gene transfer of HSCs derived from different sources (see paragraph 1.1.8 Sources). Indeed, the different proliferative potential, susceptibility to apoptosis, and cell cycle status can strongly influence the ability of the cells to grow *in vitro* and the susceptibility to transduction. Thus, specific protocols for HSCs derived from CB, BM and MPB are under investigation.

### 1.2.1.3 Retroviral restriction in mammalian cells

Retroviruses, like all viruses, exploit cellular factors in order to establish productive infection. More recently, it has been demonstrated that host factors can



**Figure 1.11. Retrovirus life cycle and restrictions.** RT: retrotranscription. IN: integration.

also inhibit retroviral infection. Such factors may affect several infection steps, both in the early phases (i.e. viral entry, uncoating, reverse transcription, nuclear import and integration) and in the late stages (i.e. transcription, translation, assembling and budding) (Figure 1.11). Because of the recent advances in the field and the relevance of the issue also for gene transfer applications, in this paragraph only the restrictive factors

acting in the early phases of retroviral life cycle will be discussed.

#### 1.2.1.3.1 Restrictive factors targeting the capsid

The first antiretroviral gene to be identified is the Fv1 locus in the mouse (Pincus, Hartley et al. 1971; Lilly and Pincus 1973; Rowe, Humphrey et al. 1973; Pincus, Hartley et al. 1975). Two major alleles were identified: Fv1<sup>n</sup>, found in NIH Swiss mice, blocked the replication of the B-tropic MLV oncoretrovirus, but allowed replication of N-tropic strain of the same virus; and Fv1<sup>b</sup>, found in Balb/c mice, allowed replication of B-tropic viruses and blocked N-tropic strains. The block was found to be dominant and to act at early stages of infection, after reverse transcription and before integration into the host chromatin (Jolicoeur 1979;

Yang, Kiggans et al. 1980). Later on, the determinant of the Fv1 tropism was identified as a specific amino acid residue in the capsid (CA) domain of the gag protein (Boone, Myer et al. 1983; DesGroseillers and Jolicoeur 1983; Boone, Glover et al. 1988; Kozak and Chakraborti 1996). These studies demonstrated that interaction between Fv1 and viral CA is needed for the restriction activity. It was further shown that the block was saturable, indeed increasing doses of virus could overcome the restriction; in addition, the block was abrogated also by inactivated virions (Duran-Troise, Bassin et al. 1977; Bassin, Duran-Troise et al. 1978). However, the mechanism of restriction of Fv1 still needs to be identified. It may sequester the PIC in the cytoplasm, thus inhibiting the binding to the PIC of cellular factors, which are needed by the virus. Alternatively, Fv1 may target the PIC to degradation (for a review on Fv1 see (Goff 1996).

More recently, interest in retroviral restrictive factors increased when human cells were found to have specific resistance to N-tropic MLV (Towers, Bock et al. 2000; Besnier, Ylinen et al. 2003). The restriction of this MLV strain, pseudotyped with Vesicular Stomatitis Virus (VSV) envelope in order to be infective for non-murine cells, was ascribed to the Ref1 gene in different mammalian cells, including pig, cow and non-human primate cells (Towers, Bock et al. 2000). The Ref1 block is similar to that of Fv1. Indeed, it is dominant, it is directed against the same CA amino acid residue as Fv1 and is abrogated by pre-exposure to restricted virus (Cowan, Hatzioannou et al. 2002; Munk, Brandt et al. 2002; Towers, Collins et al. 2002). However, Ref1 seems to act earlier than Fv1 in the viral life cycle, by blocking reverse transcription. Interestingly, Ref1 restriction in human cells has later been shown to act also against a distantly related lentivirus, the equine infectious anaemia virus (EIAV) (Hatzioannou, Cowan et al. 2003). The non-similarity in CA sequences between MLV and

EIAV, thus suggested the presence in these two viruses of similar conformational structures that are recognized by Ref1.

At the same time of Ref1 identification, several studies showed that lentiviruses, including HIV-1, were restricted in non-human primates by a factor, which was called Lv1 (Besnier, Takeuchi et al. 2002; Cowan, Hatzioannou et al. 2002; Munk, Brandt et al. 2002; Hatzioannou, Cowan et al. 2003). The identification of Lv1 was a very relevant finding for the virology field, because it provided a molecular mechanism for the well-known species-specificity of Lentiviruses. Like Fv1 and Ref1, Lv1 was found to target the CA protein and to be saturable (Kootstra, Munk et al. 2003; Owens, Yang et al. 2003).

Very recently, a major breakthrough in the field was achieved with the cloning of the gene responsible for Lv1 activity in rhesus macaque (Stremlau, Owens et al. 2004). The gene was found to encode TRIM5 $\alpha$ , a member of the Tripartite Motif (TRIM) family. In the same year, 4 different groups demonstrated that African green monkey TRIM5 $\alpha$  gene encoded for the activities, which were previously identified as Ref1 and Lv1 (Hatzioannou, Perez-Caballero et al. 2004; Keckesova, Ylinen et al. 2004; Perron, Stremlau et al. 2004; Yap, Nisole et al. 2004). Although the mechanism of action was similar between TRIM5 $\alpha$  and Fv1, an obvious sequence homology between TRIM5 $\alpha$  and Fv1 was not found. Later, it was shown that the restriction activity of Fv1 ectopically expressed in human cells was dependent on TRIM5 $\alpha$  expression (Keckesova, Ylinen et al. 2004), suggesting the involvement of a murine TRIM5 $\alpha$ -closely-related protein cooperating with Fv1.

Several groups have confirmed and extended the importance of TRIM5 $\alpha$  as a retroviral restrictive factor. An interesting finding has been that the various

primate TRIM5 $\alpha$  proteins could restrict a variety of retroviruses, but, generally, not the viruses normally found in that species. These findings supported the idea that TRIM5 $\alpha$  was selected during evolution as cellular defence against cross-species transmission.

Despite the efforts made, the mechanism of action of TRIM5 $\alpha$  still needs to be defined. TRIM proteins are characterized by a RING domain, which is typically involved in protein-protein interaction and found in many E3 ubiquitin-ligase, one or two B boxes and a coiled-coil region. TRIM5 $\alpha$  and other family components contain the SPRY domain, in which the restriction specificity has been mapped (Stremlau, Perron et al. 2005; Yap, Nisole et al. 2005). Three main hypotheses have been formulated about the TRIM5 $\alpha$  mechanism of restriction, based on the structure of the protein: 1) TRIM5 $\alpha$  traps the virus in the cytoplasm and avoids nuclear translocation; this speculation is based on the ability of TRIM5 $\alpha$  to aggregate in large structures that bind different proteins. 2) TRIM5 $\alpha$  binding to CA interferes with CA modification needed by the vector to uncoat. 3) TRIM5 $\alpha$  targets the entering virus to degradation. This last hypothesis was based on the finding that other TRIM5 isoforms manifested ubiquitin ligase activity. Although induction of viral degradation has been the most accredited hypothesis since TRIM5 $\alpha$  has been discovered, very recently it has been shown that the TRIM5 $\alpha$  owl monkey-specific variant restricts HIV-1 independently of the proteasome system (Perez-Caballero, Hatzioannou et al. 2005), thus suggesting either that other proteases are involved in the restriction or that the virus is trapped (and/or uncoating is inhibited) rather than be degraded following TRIM5 $\alpha$  binding.

Other TRIM proteins have been assayed for restriction activity. TRIM19 has been proposed as viral restrictive factor in human cells. TRIM19, also known as PML, was originally identified as fusion partner in the oncogenic activation of the retinoic acid receptor (de The, Lavau et al. 1991). However, whereas restriction by TRIM19 has been demonstrated for some viruses (Doucas, Ishov et al. 1996; Chelbi-Alix, Quignon et al. 1998), contrasting results have been shown about the role of TRIM19 in HIV-1 infection (Bell, Montaner et al. 2001; Turelli, Doucas et al. 2001; Yap, Nisole et al. 2004). TRIM1 has been found to act against MLV, but not HIV (Yap, Nisole et al. 2004) (for a review on TRIM family and retroviral restriction see (Nisole, Stoye et al. 2005).

Very recently a novel HIV restrictive factor has been discovered. It has been termed Lv2 and, although it remains to be characterized, it has been shown to exert a more complex mechanism of restriction. Indeed, Lv2 is specific for both the entry pathway (dictated by the viral envelope) and the capsid proteins of the virus (Schmitz, Marchant et al. 2004).

#### *1.2.1.3.2 APOBEC proteins*

In the last years another factor has been implicated in retroviral restriction in human cells and has been shown to exert an intriguing mechanism for viral resistance. This restriction factor has been originally identified as dominant restrictive player in a T cell line non-permissive for HIV-1 infection, and called CEM15 (from the name of the non-permissive cell line) (Sheehy, Gaddis et al. 2002). Sheehy *et al.* have also found two interesting features of CEM15: it has to be packaged into the nascent virion to restrict it in the successive infection and its activity is overcome by the HIV-1 accessory protein Vif. By sequencing they have

found close homology of CEM15 to the apolipoprotein B mRNA editing enzyme catalytic polypeptide 1 (APOBEC1) (Teng, Burant et al. 1993; Sheehy, Gaddis et al. 2002). This finding suggested a RNA editing ability of CEM15, but its main mechanism of action has been identified only when several groups sequenced retroviral DNA from cells that have been infected with Vif-deficient HIV-1 that were produced in the presence of CEM15 (Harris, Bishop et al. 2003; Lecossier, Bouchonnet et al. 2003; Mangeat, Turelli et al. 2003; Zhang, Yang et al. 2003). These authors have shown that CEM 15, now designated APOBEC3G, induced hypermutation in the retroviral plus strand DNA, in which up to 25% of all deoxyguanosine (G) sequenced were mutated to deoxyadenosine (A). This hypermutation then induced the degradation of the viral genome or, in the case of successful reverse transcription and insertion into the cell chromatin, the production of non-functional viral proteins.

As aforementioned, APOBEC3G restriction is overcome by HIV-1 Vif protein. A number of studies have addressed this issue and, through the analysis of virions produced in presence of APOBEC3G with or without Vif, have demonstrated that APOBEC3G is not packaged into the budding virus when Vif is present (Conticello, Harris et al. 2003; Kao, Khan et al. 2003; Marin, Rose et al. 2003; Sheehy, Gaddis et al. 2003; Stopak, de Noronha et al. 2003). This exclusion has been shown to be mediated by the ability of Vif to target APOBEC3G for ubiquitination and thus proteasomal degradation (Marin, Rose et al. 2003; Sheehy, Gaddis et al. 2003; Stopak, de Noronha et al. 2003).

Later, it has been shown that APOBEC3G can act against other viruses, such HBV (Turelli, Mangeat et al. 2004) and that other members of the APOBEC family, such as APOBEC3F and APOBEC3B, have anti-retroviral activity (Bishop, Holmes et al. 2004; Liddament, Brown et al. 2004; Wiegand, Doehle et



al. 2004; Zheng, Irwin et al. 2004). All these findings support a dominant role of these deaminating enzymes in the cellular defence against viral infection.

Finally, several publications have identified G-to-A hypermutations in subjects with HIV-1 infection (Borman, Quillent et al. 1995; Deacon, Tsykin et al. 1995; Huang, Zhang et al. 1998; Janini, Rogers et al. 2001). These results would suggest some activity of APOBEC3G even in presence of Vif. Indeed, a very recent work has supported this hypothesis, suggesting that APOBEC3G can be induced in macrophages by interferon- $\alpha$  and overcomes the effect of Vif (Peng, Lei et al. 2006)(Peng G 2006 j exp med 203). Other studies have added further complexity to the APOBEC3G mechanism of action model, proposing that its activity can be dissociated from cytidine deaminase activity and that it can act in the target cell without the need to be incorporated in the virion (Chiu, Soros et al. 2005; Newman, Holmes et al. 2005; Strebel 2005). Chiu *et al.* have also shown the existence of two different forms of APOBEC3G in quiescent and activated CD4 T cells, one enzymatically active and the other inactive, respectively (Chiu, Soros et al. 2005). The expression of the active form in quiescent T cells could account for the HIV block observed in these cells.

In conclusion, although it is now clear that mammalian cells exploit several defence mechanisms against early steps of retroviral infection, our knowledge about retroviral restrictive factors and their mechanisms of action is still partial. A deeper understanding of these mechanisms will help to find strategies on one hand to counteract viral infection and on the other hand to improve gene transfer efficiency.

#### 1.2.1.4 Safety of retroviral vectors

Integration of a transgene into the cell chromatin may ensure stable expression of the gene product in the target cell and its progeny. As discussed above, because of this feature, integrative vectors, such as retrovirus-based vectors, have been the preferred choice for gene delivery into HSCs. On the other hand, vector integration represents an insertional mutagen that may significantly affect the expression of cellular genes found at, or near to, the insertion site, and may consequently alter the cell growth control and eventually trigger cell transformation. Indeed, integrated LTR sequences may act as promoter/enhancer recruiting ubiquitous and cell type-specific transcription factors to upregulate expression of flanking genes. Although the mechanisms of proto-oncogene activation by retroviral insertional mutagenesis has been well described (for a review see (Mikkers and Berns 2003), their occurrence at a relatively high frequency using replication-defective gene transfer vectors had not been anticipated until recently.

Recent clinical trials of severe combined immunodeficiency gene therapy have highlighted the therapeutic potential RV-mediated gene transfer into HSCs (Cavazzana-Calvo, Hacein-Bey et al. 2000; Aiuti, Slavin et al. 2002; Gaspar, Parsley et al. 2004). Unfortunately, in one X-linked Severe Combined Immunodeficiency (X-SCID) trial, three out of eleven successfully treated subjects developed leukaemia caused by the clonal outgrowth of transduced cells (Hacein-Bey-Abina, von Kalle et al. 2003). Transformed cell growth was triggered by vector integration close to the LMO2 proto-oncogene, resulting in its over-expression, and a putative synergistic interaction between LMO2 and the IL-2R common  $\gamma$ -chain therapeutic transgene (Hacein-Bey-Abina, Von Kalle et al. 2003; Fischer, Abina et al. 2004). Synergistic interaction is controversial, still the

occurrence of this adverse event has prompted a reassessment of the insertional mutagenesis risk by retroviral vectors. Recent reports have also challenged the notion that retroviral integration occurs randomly in the target cell chromatin, and indicated specific biases for integration into transcriptionally active genes for both MLV and HIV (Schroder, Shinn et al. 2002; Wu, Li et al. 2003).

All these recent findings have triggered the scientific community to deeply study and compare integration site selection among different integrating vectors to evaluate the risk-benefit ratio in gene therapy applications and to develop safer vectors. Towards these aims, several groups have engaged in high-throughput retrieval of vector integration sites. Instead, we have used a novel and complementary approach based on genetic trapping, which allows quantitative and comparative assessment of some vector-specific integration biases by probing hundred thousands integrations at once in different types of target cells, including primary cells tested in different conditions (De Palma, Montini et al. 2005) (see appendix B).

Some studies have shown that MLV and MLV-derived vectors have a strong bias for integration next to the promoter region (Laufs, Gentner et al. 2003; Wu, Li et al. 2003), a behaviour which may increase the risk of transcriptional deregulation of the targeted endogenous gene. On the other hand, the superior proficiency of LVs at integrating into primitive HSCs, a feature that may provide a critical advantage over RVs for the success of gene therapy, may also confer an increased risk of insertional mutagenesis. Even though the advanced SIN-LTR design of LVs may reduce the risk of insertional mutagenesis, a complete evaluation of the oncogenicity of RV and LV is still missing.

To this aim, a number of groups are currently developing mouse models assessing the oncogenic potential of vector-mediated gene transfer in HSCs. Upon

transplanting wild-type mouse HSCs treated with increasing doses of RV expressing the multidrug resistance 1 (MDR1) gene, Modlich *et al.* reported the occurrence of tumours harbouring multiple integrations, several of which mapped close to known proto-oncogenes (Modlich, Kustikova *et al.* 2005). Moreover, using a tumour-prone mouse model, very recently it has been shown a dose-dependent acceleration of tumour onset triggered by RV, whereas tumourigenesis was unaffected by LV (Montini, Cesana *et al.* 2006). These results suggest a low oncogenic potential of LVs. Since the RV tested in this work had full LTR, whereas the LV had a SIN-LTR, it is likely that, beside the different integration site selection patterns of the two vectors, the lack of transcriptionally active LTRs was a major determinant of the low genotoxicity of lentiviral vectors observed in this model.

Overall, obtaining a reliable assessment of the genotoxic risk of retroviral vectors integration is needed to validate vector safety and to advance the prospective applications of HSC-based gene therapy.

### 1.3 Scope of the work

HSCs are attractive targets for the gene therapy of a variety of inherited diseases. Because of their self-renewing ability, multipotency and high proliferative potential, HSCs may reconstitute all haematopoietic lineages in a transplanted host. For gene therapy to be efficacious, effective gene transfer must be reached into HSCs without inducing detrimental effects on their biological properties. Because of their ability to transduce non-proliferating cells, LVs have been proposed as efficient tools for HSC gene transfer. Although HSCs can be transduced by LV in very short *ex vivo* culture, they display low permissivity to the vector, requiring high vector doses and exposure to cytokines to reach high-frequency gene transfer. Indeed, we have found that a significant enhancement in gene transfer was obtained by transducing the cells in the presence of a combination of early-acting cytokines (IL6, SCF, TPO and Flt3L) (Ailles, Schmidt et al. 2002).

First aim of this project was to establish in stringent xenotransplant models whether cytokine-stimulation affected HSC properties and whether cytokine enhancement of LV transduction was linked to the induction of cell cycle progression in HSCs. To determine whether cytokine exposure decreased engraftment or repopulation capacity of the transduced cells, we have performed competitive repopulation assays, by comparing engraftment ability of stimulated versus unstimulated SRCs. To determine whether cytokine enhancement of transduction was due to an early induction of cell cycle progression in HSCs, we performed an S-phase suicide assay. This assay was also used to determine whether triggering HSCs, which are mostly quiescent, to the G<sub>1</sub> phase of the cell cycle was required in order to transduce them. In this regard, it has been shown

that LVs are restricted in quiescent T lymphocytes, which must exit from G<sub>0</sub> of the cell cycle in order to be infected by HIV-1, or transduced by LVs (Unutmaz, KewalRamani et al. 1999; Cavalieri, Cazzaniga et al. 2003; Verhoeven, Dardalhon et al. 2003). Progression into late G<sub>1</sub>, however, commits the cells to DNA replication and mitosis, and may thus impair the long-term repopulating ability of HSCs, a feature that is difficult to assess in the NOD/SCID model.

Second aim of this work was to determine the rate-limiting factor(s) that make HSCs poorly permissive to LV transduction, and to eventually develop strategies to overcome these restrictions. Indeed, the impaired transduction may be due to the lack of required cellular co-factors, and/or the expression of restrictive factors (Chiu, Soros et al. 2005). Some of these factors may affect viral uncoating, reverse transcription, and/or target the internalised viral particles to degradation by the ubiquitin-proteasome pathway. Thus, we evaluated the role of the ubiquitin-proteasome pathway in LV-mediated transduction of hematopoietic stem and progenitor cells.

Third aim was to investigate integration site selection of LV in HSPCs. Indeed retroviral vector integration may have detrimental effects on HSPCs, for instance by altering gene expression and inducing aberrant developmental pathways. Analysis of integration site selection can help to predict the consequence of the insertional mutagenesis induced by these vectors. We used a promoter trapping approach built into LV and RV (MLV) vectors to evaluate integration site selection in the target cell genome.

Overall, the results of this project will help to develop better protocols for HSC gene therapy and improve understanding of the interactions between target cells and LVs.

# Chapter 2.

## Methods

## 2.1 Reagents and suppliers

### 2.1.1 Chemicals

All chemicals were obtained from FLUKA or Sigma-Aldrich.

Other chemicals used during the study were obtained from the following sources:

Lymphoprep	Axis-Shield PoC AS
MG132	Calbiochem, EMD Biosciences
PS341	Millennium Pharmaceuticals
CH296 (retronectin)	Takara BIO

### 2.1.2 Radiochemicals

<sup>3</sup>H–Thymidine (25 Ci/mol): Amersham Biosciences, GE Healthcare.

### 2.1.3 Enzymes and buffers

All DNA modification enzymes and buffers were obtained from Roche.

All restriction enzymes and buffers were obtained from either Roche or New England Biolabs.

AmpliTaq Platinum polimerase enzyme was supplied by Invitrogen.

### 2.1.4 Bacterial strains

The Top10 bacterial strain was supplied by Invitrogen (code number 44-0301).

### 2.1.5 Cell culture

Cells were incubated at 37°C in a 5% CO<sub>2</sub> incubator.



All tissue culture plastic ware (tissue culture plates and tubes) was obtained from BD Falcon.

Trypsin-EDTA solution: Phosphate Buffered Saline 0,05% trypsin, 4mM EDTA pH 7,3.

Media, sera and supplements:

IMDM	Sigma-Aldrich
RPMI 1640	Euroclone
StemSpan	Stem Cell Technologies
MethoCult GF4434	Stem Cell Technologies
Foetal Bovine Serum (FBS)	BioSera
Trypsin	Invitrogen
L-Glutamine	Invitrogen
Penicillin/Streptomycin	Invitrogen
HEPES	Invitrogen
Polybrene	Sigma-Aldrich

All cytokines were supplied by Peprotech.

Phosphate buffered saline (PBS) was obtained from Sigma-Aldrich.

### 2.1.6 Antibodies

All antibodies for flow cytometry were supplied by DAKO A/S.

## 2.2 Plasmids

LVs were produced by co-transfection of 4 plasmids: transfer construct, packaging construct, Rev-encoding construct and envelope construct. The retroviral trap vector was produced by co-transfection of 3 plasmids: transfer construct, packaging construct and envelope construct. All the plasmids contain the gene encoding for Ampicillin resistance.

### *Transfer constructs:*

pRRL.sin.PPT.hPGK.eGFP.pre (Follenzi, Ailles et al. 2000) and pRRL.sin.PPT.hPGK.YFP.pre, obtained by replacing the fragment *Sall-SpeI* of pRRL.sin.PPT.hPGK.YFP.pre with the fragment *Sall-SpeI* containing YFP from pRRLsin.hPGK.YFP (kindly provided by Tom Dull, Cell Genesis) were used in competitive repopulation experiments.

pCCLsin.PPT.hPGK.eGFP.pre (Dull, Zufferey et al. 1998; Zufferey, Dull et al. 1998; Follenzi, Ailles et al. 2000) was used in all the other experiments, excluding promoter trapping experiments.

Lentiviral and Retroviral Trap (LT and RT, respectively) were used for promoter trapping experiments. A Puro<sup>R</sup>-GFP fusion gene was cloned into the promoter trap pROSA-GFNR (Medico, Gambarotta et al. 2001) in place of GFNR.PGKneo, and the resulting expression cassette (Splice Acceptor site.Puro<sup>R</sup>-GFP.polyA) was excised by *AflII/NheI* digestion, and cloned in reverse orientation into the HIV-based self-inactivating (SIN) lentiviral vector pRRL.SIN.cPPT.PGK.eGFP in place of PGK.eGFP or into the MLV-based SIN retroviral vector pRkat43.3.PGK.YFP (Roberts MR j immunol 1998) in place of PGK.YFP, to generate LT and RT vectors, respectively.

### *Packaging constructs:*

pCMV $\Delta$ R8.74 (Dull, Zufferey et al. 1998) was used in the production of vectors used in competitive repopulation assay and in the production of the LT vector.

pMDLg/pRRE (Dull, Zufferey et al. 1998) was used to package vectors used in all the other experiments.

pCMV.GAG.POL (Naldini, Blomer et al. 1996) was used in the production of the RT vector.

*Rev-encoding construct:*

pRSV-REV (Dull, Zufferey et al. 1998).

*Envelope constructs:*

pMD2.VSV-G (Follenzi, Ailles et al. 2000) was used for VSV-G-pseudotyped vectors.

phCMV-10A1 (Sandrin, Boson et al. 2002) was used for A-MLV amphotropic-pseudotyped vectors.

phCMV-RD114/TR was used for RD114/TR-pseudotyped vectors.

### 2.3 Transformation of competent bacteria and plasmid preparation

Plasmid DNA (0,5-5ng) to be transformed was mixed with 50 $\mu$ l of chemically competent Top10 cells (Invitrogen) prepared specifically for the heat shock mediated transformation method. The mix was incubated on ice for 30 minutes, then 1 minute in a water bath at 42°C, and then returned to ice for a further 5 minutes. The mix was then plated onto Luria Bertani (LB) agar plates, containing 100 $\mu$ g/ml ampicillin and placed overnight in a 37°C bacterial incubator. The following day, colonies were picked and placed into 1 ml of LB media containing carbenicillin (ampicillin homologue), and grown over day with agitation at 37°C.

Ten  $\mu$ l of the over day culture were placed in 200ml of LB media containing carbenicillin, and grown overnight shaking at 37°C. The following day, cell suspension was measured by spectrophotometer analysis ( $\lambda=600\text{nm}$ ) to check for optimal cell density (about 1-1,5 O.D.) and subjected to large-scale plasmid DNA preparation.

Plasmid DNA preparation was performed using High Purity Plasmid Purification Systems (Marligen Biosciences). This system is based on alkaline lysis, followed by DNA purification on an anion exchange resin. After elution, plasmid DNA was then desalted and concentrated by alcohol precipitation, washed in 70% ethanol and resuspended in TE buffer (10mM Tris-HCl pH 8,0, 0.1 mM EDTA).

## 2.4 Vector production

### 2.4.1 Transfection

Reagents:

2X HBS: 281mM NaCl, 100mM HEPES, 1,5mM  $\text{Na}_2\text{HPO}_4$ , pH 7,12, 0,22 $\mu$ M filtered, stored at  $-20^\circ\text{C}$  or  $-80^\circ\text{C}$ .

2.5M  $\text{CaCl}_2$ : tissue culture grade (Sigma-Aldrich), 0,22 $\mu$ M filtered, stored at  $-20^\circ\text{C}$ .

0.1X TE buffer: 10mM Tris (pH 8,0), 1mM EDTA (pH 8,0) diluted 1:10 with  $\text{dH}_2\text{O}$ , 0,22 $\mu$ M filtered, stored at  $4^\circ\text{C}$ .

$\text{dH}_2\text{O}$ : endotoxin-free, tissue culture grade (Sigma-Aldrich).

Vector stocks were prepared by calcium phosphate transfection and concentrated by ultracentrifugation. Vectors were produced by transfection of human embryonic kidney 293T cell line (containing the mutant gene of SV40 Large T Antigen, see paragraph 2.5 Cell lines), because these cells are optimal DNA recipients in transfection procedures and the backbones of the vector construct contain SV40 origin of replication.

$9 \times 10^6$  293T cells *per* dish were seeded and incubated in 15 cm dishes, 24 hours before transfection in IMDM, 10%FBS, Penicillin (25U/ml), Streptomycin (25U/ml). Two hours before transfection medium was replaced.

To produce LVs, plasmid DNA mix was prepared by mixing 9 $\mu$ g pMD2-VSV-G or 22,5 $\mu$ g phCMV-10A1 or 12,5 $\mu$ g phCMV-RD114/TR, 16,25 $\mu$ g pCMV $\Delta$ R8.74 or 12,5 $\mu$ g pMDLg/pRRE, 6,25 $\mu$ g pRSV-REV and 25 $\mu$ g transfer construct together *per* dish. To produce RT vector, plasmid DNA mix was prepared by mixing 9 $\mu$ g pMD2-VSV-G, 15 $\mu$ g pCMV.GAG.POL and 20 $\mu$ g of transfer construct together *per* dish. The plasmid solution was made up to a final volume of 1125 $\mu$ l with 0.1XTE/dH<sub>2</sub>O (2:1) in a 15 ml polypropylene tube. Finally 125 $\mu$ l of 2.5M CaCl<sub>2</sub> were added. The precipitate was formed by drop wise addition of 1250 $\mu$ l 2X HBS solution to the 1250 $\mu$ l DNA-TE-CaCl<sub>2</sub> mixture while vortexing at full speed. The precipitate was added to 293T cells immediately following addition of the 2X HBS and cells were incubated at 37°C. 14-16 hours after transfection, medium was replaced. 24 hours after medium changing, supernatant was collected, filtered through 0.22 $\mu$ m pore nitrocellulose filter and ultracentrifugated at 19.500 rpm in SW32Ti rotor (Optima L-60 preparative Ultracentrifuge; Beckman) 2 hours at room temperature for VSV-pseudotyped vectors or 7000rpm in SW32Ti rotor (Optima L-60 preparative Ultracentrifuge;

Beckman) 12 hours at 4°C for A-MLV- and RD114/TR-pseudotyped vectors. Pellets containing the vector were resuspended in a volume of sterile PBS (Sigma-Aldrich) representing 1/500 of the starting medium volume, pooled and rotate on a wheel at room temperature for 1 hour. The concentrated vector preparation was then divided into small aliquots (25-50µl) and stored at -80°C. Once thawed, vector aliquots were not frozen again (each freeze and thaw approximately halves vector titer).

#### **2.4.2 Vector particle amount determination**

Vector particle amount was determined by immunoenzymatic assay, using the Alliance HIV-1 p24 antigen ELISA kit (PerkinElmer), following manufacturer's instructions.

Serial dilutions of the vector stock (see below vector titer determination) were plated in triplicate in microplate wells, which are coated with a highly specific mouse monoclonal antibody to the HIV-1 capsid protein p24, and vector particle envelope was lysed by 5% Triton X-100. The captured antigen was then complexed with biotinylated polyclonal antibody to HIV-1 p24, followed by a streptavidin-HRP (horseradish peroxidase) conjugate. The resulting complex was detected by incubation with ortho-phenylenediamine-HCl (OPD), which produces a yellow colour that is directly proportional to the amount of HIV-1 p24 captured. The absorbance of each microplate well was determined using a microplate reader (Versa Max, Molecular Devices) ( $\lambda$  490nm) and quantity of p24 protein was calculated using an HIV-1 p24 antigen standard curve.

### 2.4.3 End-point titration

End-point titration is performed by transducing a permissive target cell line with serial dilutions of the vector preparation.

The protocol used was as follows:

- Plate  $5 \times 10^4$  HeLa or 293T cells per well in 3,5cm wells (in order to have the following day  $10^5$  cells/well approximately)
- The following day, thaw a vector aliquot and prepare serial ten-fold dilutions of 2X viral stock (from  $10^{-3}$  to  $10^{-8}$ ) in IMDM 10% FBS
- Aspirate medium from cell wells and add 500 $\mu$ l of medium 16 $\mu$ g/ml polybrene (2X final polybrene concentration).
- Add 500 $\mu$ l of serial dilutions (one dilution *per* well)
- Incubate at 37°C in a 5% CO<sub>2</sub> incubator for 72 hours (for expression titer – FACS analysis) or for 10-15 days, dividing them (for integration titer – Real-time Quantitative-Polimerase Chain Reaction (Q-PCR) analysis)
- Detach cells and, for expression titer, fix them in 1ml/sample PBS, 1% paraformaldehyde, 2% FBS; for integration titer see paragraph 2.12
- Perform FACS analysis or Q-PCR analysis

The titer is defined as number of transducing units per milliliter (TU/ml) of vector preparation. Expression titer is based on the assumption that a single vector copy integrated in the host genome will give a positive cell. Assuming that all the cells are equally susceptible to transduction, following Poisson distribution for random independent events, a single transduction event, and not more, has occurred in most positive cells, when the percentage of positive cells in the total population is below 25%. It follows that expression titer must be calculated from a sample corresponding to a vector dilution where positivity of cells ranges between 1% (to ensure an acceptable signal over the instrument noise) to 25%, in order not

to underestimate the titer when multiple transduction events per cell have occurred. Integration titer must be calculated from a sample corresponding to a vector dilution where vector copies *per* cells ranges between 0,01 and 0,25. Proof of linearity must be obtained showing that different dilutions in the optimal testing range yield linear increase in transduction frequency.

The equation to calculate expression titer is:

$$\text{Titer (TU/ml)} = (\text{number of cells at the time of vector addition}) \times \\ \times (\% \text{ transgene+ cells}/100) \times (\text{dilution factor})$$

The equation to calculate integration titer is:

$$\text{Titer (TU/ml)} = (\text{number of cells at the time of vector addition}) \times \\ \times (\text{vector copies per cell}) \times (\text{dilution factor})$$

For FACS and Q-PCR analyses see relative paragraphs below.

Titers of vectors used in this project ranged between  $10^9$  and  $10^{10}$  TU/ml (vector stock concentrated 500x).

#### **2.4.4 Infectivity of the vector stocks**

Infectivity is a reliable parameter to evaluate the quality of the vector stock and can be defined as the transducing activity *per* unit of physical particle, where the first parameter is expressed as TU/ml, as obtained by end-point titration and the second as ng p24/ml, as determined by immunoenzymatic assay.

Since one ng of p24 could theoretically contain  $1.2 \times 10^7$  particles, if all the particles in the vector stock were infectious, a titer of  $10^8$  TU/ml would correspond to a p24 concentration of about 10 ng/ml. Indeed, a concentration in the range of 500-1000 ng p24/ml is more reasonably expected. This is due to two major reasons. The first reason is that end-point titer fails to estimate the real



content of infectious particles in a vector preparation, because vector particles move by Brownian motion in the medium and only a fraction of them has the chance to get in contact with a cell in the monolayer and transduce it in the time window of the assay. The second reason instead is crucially linked to the quality of the vector batch tested. In fact, the efficiency of packaging of infectious particles is lower than what is predicted by theoretical calculations, and a good fraction of the total p24 protein is not assembled into infectious virions.

Vector infectivity can be calculated by the following equation:

$$\text{Infectivity} = (\text{TU/ml})/(\text{ng p24/ml}) = \text{TU/ng p24}$$

Infectivities were  $10^4$ - $10^5$  TU/ng p24 for all vector types.

#### Notes:

When the vector titer is calculated on a permissive cell line, refractory targets may need to be transduced employing a high number of TU *per* ml. It should be taken into account the fact that the expression *TU/cell*, which is equivalent to MOI (multiplicity of infection) is an arbitrary definition, because it does not consider the volume in which the transduction is performed, and therefore the particle concentration. Within this context, it is well acknowledged that vector concentration in the transduction medium is more important than the absolute number of particles available for each cell, since only a fraction of them comes into contact with the target, especially when cells are cultured in monolayers in a large volume of medium.

## 2.5 Cell lines

HeLa (ATCC Number CCL-2), 293T (originally called 293tsA1609ne, and derived from 293 cells, a continuous human embryonic kidney cell line transformed by sheared Type 5 Adenovirus DNA, by transfection with the tsA 1609 mutant gene of SV40 Large T Antigen and the Neo<sup>r</sup> gene of E. Coli - ATCC Number CRL-1573; (Graham, Smiley et al. 1977) and K-562 (ATCC Number CCL-243) cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM; Sigma-Aldrich) and U-937 (ATCC Number CRL-1593.2) in RPMI-1640 (Euroclone), both supplemented with 10% foetal bovine serum (FBS; BioSera), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2mM L-glutamine. To detach adherent cells PBS 0,05% trypsin, 4mM EDTA (pH 7,3) is used.

## 2.6 Haematopoietic stem/progenitor cell (HSPC) enrichment

### 2.6.1 Human HSPCs

Cells from human subjects were obtained with informed consent according to the Declaration of Helsinki, and to a protocol approved by the San Raffaele Scientific Institute Bioethical Committee.

Mononuclear cells were obtained from human cord blood scheduled for discard by density separation, and CD34<sup>+</sup> cells were isolated by positive selection.

Mononuclear cells separation protocol used was as follows:

- dilute cord blood 1:2 in PBS
- stratify 30ml of diluted cord blood on 15ml Lymphoprep (Axis-Shield PoC AS)

- spin at 1500 rpm in Heraeus Megafuge 1.0 (Kendro Laboratory products) 30 minutes at room temperature, without brake
- remove plasma upper layer containing platelets
- transfer the interphase between plasma and lymphoprep in a new tube; this fraction contains mononuclear cells, whereas Lymphoprep lower layer contains granulocytes and erythrocytes
- wash cells in 25ml PBS, spinning at 1000 rpm in Heraeus Megafuge 1.0 (Kendro Laboratory products) 10 minutes at room temperature
- pour off supernatant and resuspend all cells in 20ml MACS buffer (PBS pH 7.2, 0.5 % Bovine Serum Albumin – BSA, Sigma-Aldrich - and 2mM EDTA)
- count cells an aliquot of cells in a Burker chamber after a 1:50 dilution (1:10 in acetic acid to lyse erythrocytes possibly remained, followed by 1:5 dilution in trypan blue (Invitrogen) to exclude dead cells)

Mononuclear cells were then subjected to CD34 positive selection, by using the MACS Indirect CD34 Progenitor Cell Isolation Kit. The magnetically labelled cells were enriched on LS separation columns in the magnetic field of the MidiMACS magnet (all Miltenyi Biotech), following manufacturer's instructions. Cells were centrifuged at 1000 rpm in Heraeus Megafuge 1.0 R (Kendro Laboratory products) 10 minutes at 4°C, resuspended in 300µl MACS buffer *per* 10<sup>8</sup> cells and incubated with 100µl Fc receptors blocking reagent (to block aspecific binding of the antibody) together with 100µl apten-conjugated anti-CD34 antibody *per* 10<sup>8</sup> cells 20 min at 4°C. Cells were then washed with 20ml MACS buffer as above, resuspended in 400µl MACS buffer *per* 10<sup>8</sup> cells and incubated with a secondary magnetic beads-conjugated anti-aptent antibody 20 min at 4°C. Cells were washed again, resuspended in 1ml MACS buffer *per* 10<sup>8</sup> counted cells and subjected to two rounds of separation on LS columns.

Flow through was discarded. CD34+ cells, recovered by removing the column from the magnetic field and by using the plunger, were then counted and either frozen in IMDM 40% FBS, 10% Dimethyl Sulfoxide (DMSO) or used fresh.

### **2.6.2 Murine HSPCs**

Bone marrow cells were harvested from six-week-old C57Bl/6 or FVB mice (both from Charles River Laboratories) and subjected to negative selection by using the StemSep Mouse Hematopoietic Progenitor Cell Enrichment kit and the Green StemSep Magnet with StemSep 0.6" Negative Selection Gravity Columns (all Stem Cell Technologies), following manufacturer's instruction.

Bone marrow cells were harvested by flushing from femurs and tibias from mice with IMDM 5% FBS and counted in a Burker chamber after a 1:50 dilution (1:10 dilution in acetic acid to lyse erythrocytes, followed by a 1:5 dilution in trypan blue (Invitrogen) to exclude dead cells). IMDM was poured off after spinning at 1000 rpm in Heraeus Megafuge 1.0 (Kendro Laboratory products) 10 min at room temperature, and cells were resuspended in 25ml PBS 2% FBS and centrifuged again (washing step). Cells were resuspended at a concentration of  $5 \times 10^7$  cells/ml in PBS 2% FBS, 5% rat serum and incubated 15 min at 4°C to block aspecific binding of antibodies. Cells were then incubated with 35µl/ml StemSep Enrichment Cocktail, which contains a combination of rat biotinilated monoclonal antibodies anti-mouse lineage specific markers (CD5, CD11b, CD45R/B220, Gr-1, Neutrophils (7-4) and TER119) 15 min at 4°C. After washing with PBS 2% FBS, cells were resuspended at  $5 \times 10^7$  cells/ml in PBS 2% FBS, incubated with 100µl/ml Anti-Biotin tetrameric antibody complexes 15 min

at 4°C and then with 60µl/ml magnetic colloid 15 min at 4°C. Cells were then loaded on a primed and equilibrated column in the magnetic field. Flow through, which contains Lin<sup>-</sup> cells, was harvested and cells were washed, resuspended in StemSpan medium (Stem Cell Technologies) and counted. Murine HSPCs were used fresh.

## 2.7 Transduction

### 2.7.1 Human HSPCs

CD34<sup>+</sup> cells were incubated at a concentration of  $5 \times 10^5$ - $1 \times 10^6$  cells/ml in Stem Span serum-free medium (Stem Cell Technologies) with or without the following cytokines, tested individually or in combination: 20 ng/ml recombinant human-Interleukin 6 (rh-IL6), 100 ng/ml rh-Stem Cell Factor (rh-SCF), 100 ng/ml rh-fms-like tyrosine kinase 3 Ligand (rh-FLT3L), and 20 ng/ml rh-thrombopoietin (rh-TPO) (all from Peprotech). Transduction was carried out for 20-24 hours with the indicated concentration of VSV-pseudotyped LV, or  $10^8$  TU/ml where not indicated.

A-MLV or RD114/TR pseudotyped LVs were used at a concentration of  $3 \times 10^7$  TU/ml, preloaded for 2 hrs on non-tissue culture treated dishes coated with recombinant human Fibronectin Fragment (CH296 – Takara BIO) following manufacturer instructions.

For the proteasome inhibitor experiments, CD34<sup>+</sup> were transduced as described above with VSV-pseudotyped LV and MG132 (Calbiochem, EMD Biosciences) or PS-341 (Millennium Pharmaceuticals) was added to the transduction medium at the indicated concentration and washed out with the vector after the 20-24 hours transduction period.

For promoter trapping experiments, CD34<sup>+</sup> were stimulated 36 hours with the abovementioned combination of cytokines before transduction with 10<sup>7</sup> TU/ml (integration titer) of LT and RT in the first experiment or with 10<sup>7</sup> TU/ml of RT and 5x10<sup>7</sup> TU/ml LT in the second experiment. Prestimulation was needed in order to allow comparable level of transduction by RT and LT.

In all the experiments, after the transduction period CD34<sup>+</sup> cells were washed and maintained in IMDM medium 10% FBS containing 20 ng/ml rh-IL6, 100 ng/ml rh-SCF, and 20 ng/ml rh-Interleukin 3 (rh-IL3) (all from Peprotech) for suspension culture and/or plated for Colony Forming Cell Assay (see below) and/or transplanted into NOD/SCID mice (see below). CD34<sup>+</sup> cells subjected to S-phase suicide assay were treated with the S-phase specific killing agent before seeding and/or transplant (see relative paragraph below). HSPCs grown in suspension were analysed by FACS 2 weeks after transduction.

In promoter trapping experiments puromycin selection was performed adding 1µg/ml puromycin (Sigma-Aldrich) to the suspension culture medium 3 days after transduction.

### **2.7.2 Murine HSPCs**

For the proteasome inhibitor experiments FVB Lin<sup>-</sup> cells were incubated at a concentration of 1x10<sup>6</sup> cells/ml in Stem Span serum-free medium (Stem Cell Technologies) in presence of 20 ng/ml recombinant mouse - rm - IL3, 100 ng/ml rm-SCF, rm-FLT3L, and rm-TPO (all from Peprotech) and transduced for 24 hours with 10<sup>8</sup> TU/ml of VSV-pseudotyped LV. MG132 (Calbiochem, EMD Biosciences) was added to the transduction medium at the indicated concentration and washed out with the vector after the transduction period.

For promoter trapping experiments  $10^6$  cells/ml C57Bl/6 Lin<sup>-</sup> were stimulated for 24 hours in StemSpan serum-free medium in presence of the abovementioned cytokines, before transduction with  $5 \times 10^7$  TU/ml of LT and RT. Prestimulation of the cells was necessary to reach efficient transduction by both vector types.

In all the experiments, after the transduction period Lin<sup>-</sup> cells were washed and maintained in IMDM medium 10% FBS containing the combination of cytokines abovementioned for suspension culture and analysed by FACS 1 or 2 weeks later (for promoter trapping experiments or proteasome inhibitor experiments, respectively).

### 2.7.3 Cell lines

U937 cells were seeded at  $10^6$  cells/ml in 1,25 cm wells and transduced with  $2 \times 10^5$  LV TU/ml. K562 were seeded at  $2 \times 10^5$  cells/ml in 1,25 cm wells and transduced with  $5 \times 10^5$  LV TU/ml. HeLa and 293T cells were seeded at  $5 \times 10^4$  cells/ml in 3,5cm wells and transduced the following day with  $5 \times 10^4$  LV TU/ml. MG132 (Calbiochem, EMD Biosciences) was added to the transduction medium at the indicated concentration and washed out with the vector after the transduction period. All cell lines were transduced for 12 hours, then washed and kept in culture 2 weeks before FACS analysis.

### 2.8 S-phase suicide assay

CD34<sup>+</sup> cells transduction was performed using  $10^8$  LV TU/ml as described above, in presence or absence of cytokines. Cells were then washed with serum-free

medium, plated at a concentration of  $2 \times 10^6$  cells/ml and exposed to 20  $\mu\text{Ci/ml}$  of high specific activity  $^3\text{H}$ -Thymidine (25 Ci/mmol - Amersham Biosciences, GE Healthcare) or at a concentration of  $5 \times 10^5$  cells/ml and exposed to  $2 \times 10^{-6}\text{M}$  cytosine 1- $\beta$ -D-arabinofuranoside (Ara-C - Sigma-Aldrich). Cells were incubated for 16 hours with  $^3\text{H}$ -t or 12 hours with Ara-C and then washed twice with serum-free medium and maintained, as aforementioned, in IMDM medium 10% FBS containing 20 ng/ml rh-IL6, 100 ng/ml rh-SCF, and 20 ng/ml rh-IL3 (all from Peprotech) for suspension culture and plated for Colony Forming Cell Assay and/or transplanted into NOD/SCID mice (see relative paragraphs below).

To assess killing efficiency of the S-phase specific killing agents, the number of cells plated in Colony Forming Cell Assay for each sample was calculated on the basis of the number of cells counted before the exposure to  $^3\text{H}$ -t or Ara-C. Thus, killing efficiency was calculated by scoring colonies derived from S-phase specific killing agent-treated cells, using colonies derived from untreated cells as control.

## 2.9 Human HSPC Colony Forming Cell (CFC) Assay

CFC assay was used to assess transduction, and possible toxicity of the treatments, in haematopoietic committed progenitors. When performed on NOD/SCID derived bone marrow cells, it was used also as indirect demonstration of the presence of SCID Repopulating Cells. Indeed, the presence of CFCs in the bone marrow of transplanted mice 6-10 weeks after transplant indicates the presence of a more primitive stem cell that generated them, since originally transplanted CFCs exhaust before analysis.



After transduction, CD34+ cells were washed, counted (excepted for S-phase suicide assay; see relative paragraph above) and seeded at a density of 1000 cells/ml in semisolid medium (MethoCult GF4434 - Stem Cell Technologies). Cells were mixed by vortexing and 1,1ml of cell suspension were plated in a 3,5cm dish. Semisolid medium composition is as follows: IMDM 1% methylcellulose, 30% FBS, 1% BSA,  $10^{-4}$ M 2-mercaptoethanol, 2mM L-glutamine, 50ng/ml rh-SCF, 10ng/ml rh-granulocyte macrophage-colony stimulating factor (rh-GM-CSF), 10ng/ml rhIL3, 3U/ml erythropoietin (EPO). When CFC assay was performed on NOD/SCID bone marrow derived cells, cells were plated at a density of  $2,5 \times 10^5$  cells/ml.

For puromycin selection in promoter trapping experiments, CD34+ cells were plated 24 hours after transduction in semisolid medium containing 1µg/ml puromycin (Sigma-Aldrich).

Each sample was plated in duplicate and dishes were put in a humid chamber in order to avoid drying of the semisolid medium. Cells were incubated for 2 weeks and then scored by light and fluorescence microscopy for number and transgene expression.

### **2.9.1 CFC analysis by Polimerase Chain Reaction (PCR)**

Individual, well-isolated colonies were plucked 10-14 days after plating into 0,5 ml of PBS, vortexed, left at room temperature for 30 minutes, then vortexed again and centrifuged at 2000 rpm in Heraeus Biofuge pico (Kendro Laboratory products) for 10 minutes. Pellets were resuspended in 25 µl of lysis buffer (20 mM TrisHCl pH 8,4, 50 mM KCl, 1,5 mM MgCl<sub>2</sub>, 0,45% Tween-20, 0,45%

Nonidet P-40, and 1,5 mg/ml proteinase K) and incubated at 56°C for 1 hour and 95°C for 15 min. PCR was carried out on 5 µl of the preparation.

Amplification was performed in a total volume of 50 µl, with AmpliTaq Platinum enzyme (Invitrogen) in 1.5 mM MgCl<sub>2</sub> with 0.5µM forward and reverse primers. PCR conditions were: one cycle of 94°C for 2 minutes; 30 cycles of 94°C for 30 seconds, annealing (for temperatures see below primer sets) for 30 seconds, and 72°C for 30 seconds; one cycle of 72°C for 7 minutes.

20µl of the PCR products were analysed by agarose gel electrophoresis.

For promoter trapping experiments a primer set specific for GFP was used (5' GCAGAAGAACGGCATCAAGGT 3' and 5' GAACTCCAGCAGGACCATGTGA 3'). Temperature of annealing was 60°C for this primer set and it yielded a 202 bp band.

For the analysis of colonies in the competitive repopulation assay two primer sets were used, one specific for GFP (not complementary to YFP) (5' CCCTCGTGACCACCCTGAC 3' and 5' TGCTCAGGGCGGACTGGGT 3') and one specific for YFP (not complementary to GFP) (5' ACCCTCGTGACCACCTTCGG 3' and 5' CTTTGCTCAGGGCGGACTGGTA 3'). Temperature of annealing were 60°C for YFP and 66°C for GFP primer set. Both primer sets yielded a 454 bp band.

## 2.10 SCID Repopulating Cell (SRC) Assay

In order to assess performance and transduction of human HSCs, we performed the SRC assay. The xenotransplant model used is the NOD/LtSz scid/scid (NOD/SCID) (nonobese diabetic/severe combined immunodeficient) mouse

(Shultz, Schweitzer et al. 1995) transplanted with cord blood derived CD34+ cells.

NOD/SCID mice were obtained from the British Columbia Cancer Research Center (Vancouver, Canada) or from Charles River (Charles River Laboratories Inc.) and maintained in ventilated cages in a Specific Pathogen Free (SPF) facility, according to approved protocols. All procedures were performed according to protocols approved by the Animal Care and Use Committee of the Fondazione San Raffaele del Monte Tabor (IACUC 244) and communicated to the Ministry of Health and local authorities according to Italian law.

Six- to eight-week-old mice were sub-lethally irradiated (350 cGy; Rad Gil EN 60601-1, Gilardoni) 24 hours before transplant. The following day, transduced CD34+ cells were washed and resuspended in PBS at a density of  $1.5 \times 10^6$  cells/ml approximately. Mice were then heated under an infrared heat lamp and transplanted by intravenous injection in the tail vein of 250  $\mu$ l (about  $4 \times 10^5$  cells) of cell suspension *per* mouse using an U-100 insulin syringe (1ml, gauge 26) (Terumo). Mice were maintained with sterile acidified water (8 mM HCl), containing 16  $\mu$ g/ml neomycin sulfate (Sigma-Aldrich).

Competitive repopulation assay was performed using cells transduced in different conditions and marked with distinguishable vectors. Each non competitively repopulated (reference) mouse received  $4 \times 10^5$  CD34+ cells transduced in presence or absence of cytokines with either LV-GFP or LV-YFP; each competitively repopulated mouse received  $2 \times 10^5$  CD34+ cells transduced in presence of cytokines with LV-GFP or LV-YFP and  $2 \times 10^5$  CD34+ transduced in absence of cytokines with LV-YFP or LV-GFP, respectively.

In S-phase suicide assay transduced CD34+ cells, both Ara-C-treated and untreated, were not counted before transplant and the number of cells to be

injected was calculated on the basis of the number of cells plated before treatment. CD34+ cells were injected together with  $5 \times 10^5$  CD34-negative accessory cells. Accessory cells were used to substitute the killed progenitors cells in supporting the first stages on engraftment. The same number of accessory cells was transplanted also with untreated cells in order to avoid any variability in the read out due to a possible contribution of these cells to repopulation. CD34-negative cells were harvested from the flow-through of CD34 positive selection, counted and frozen in FBS 10% DMSO; they were thawed the day before transplantation, counted and seeded in IMDM 10% FBS at a density of  $10^6$  cells/ml. Before transplants viable CD34-negative cells were counted by using trypan blue (1:5 dilution) (Invitrogen) and pooled with CD34+ cells.

Six to ten weeks after transplant, mice were euthanised by CO<sub>2</sub> inhalation and bone marrow cells were harvested by flushing from femurs and tibias in 5ml IMDM 5% FBS *per* mouse. The volume of each sample was then made up to 15 ml with IMDM 5% FBS and cells were counted after a 1:10 dilution in 3% acetic acid to exclude red cells from the count.  $6 \times 10^5$  cells/sample were used for FACS analysis,  $5 \times 10^5$  cells/sample for CFC assay and the remaining cells ( $10^7$ - $2 \times 10^7$  cells/sample) for Q-PCR analysis (see relative paragraphs)(Ailles, Schmidt et al. 2002).

## 2.11 Flow cytometry

Since all the vectors used in this work encode for a fluorescent protein, transduction was assessed mainly by flow cytometry. The instrument used was FACSCalibur (Becton Dickinson Immunocytometry Systems).

### 2.11.1 Assessment of transduction efficiency in *in vitro* cultures

To determine expression titer, HeLa or 293T cells were detached by trypsin-EDTA solution, washed with PBS, fixed with PBS, 1% paraformaldehyde, 2% FBS (1ml/sample) and analysed by FACS for transgene expression (GFP or YFP).

To assess transduction efficiency cells were grown for at least 5 days after transduction to reach steady state GFP expression and to rule out pseudotransduction (detection of GFP protein associated with the virus particles during vector production and transferred to the target cells during infection) and then is diluted out of the cells as they divide). Adherent cells were detached by trypsin-EDTA solution, washed with PBS 2% FBS and resuspended in PBS 2% FBS containing 10µg/ml 7-amino-actinomycin D (7AAD) (Sigma-Aldrich). Cells grown in suspension were washed with PBS 2% FBS and resuspended in PBS 2% FBS containing 10µg/ml 7AAD. Only viable, 7AAD-negative cells were analysed for transgene expression.

Untransduced cells were used as negative control to set parameters and gates. 10,000-20,000 events were scored.

### 2.11.2 Cell cycle analysis

CD34+ cells, exposed or not to Ara-C, were washed with PBS and resuspended in 0,5mL DNA staining buffer *per* 10<sup>6</sup> cells (PBS 1% saponin containing 300KU RNase and 50 µg/ml Propidium Iodide – PI (Sigma-Aldrich). Cells were incubated in the dark for 10 minutes on ice and then analysed by FACS for DNA staining. Cell aggregates and dead cells were eliminated from further analysis by gating individual cells in the FL3-area versus FL3-width plot. 100,000 events were scored.

### 2.11.3 NOD/SCID bone marrow analysis

Cells from bone marrow were retrieved by flushing from femurs and tibias of NOD/SCID mice in 5ml/sample IMDM 5% FBS. The volume of each sample was then brought to 15 ml with IMDM 5% FBS and cells were counted after a 1:10 dilution in 3% acetic acid to exclude red cells from the count.

$6 \times 10^5$  cells/sample were transferred into a 5ml polystyrene round-bottom tube and centrifuged at 1000 rpm in Heraeus Megafuge 1.0 (Kendro Laboratory products) 10 min at room temperature. Cells were resuspended in 5ml/sample 7% ammonium chloride solution (Stem Cell Technologies) and incubated 20 min on ice to lyse red cells. Cells were then centrifuged at 1200 rpm in Heraeus Megafuge 1.0 (Kendro Laboratory products) 7 min at 4°C, resuspended in 0,5ml PBS 2% FBS, 5% human serum and 5% mouse serum and incubated on ice for 10 minutes to block aspecific binding sites of the antibodies. Cells were then divided into 100  $\mu$ l aliquots and 10  $\mu$ l of each of the following human-specific Cy5-phycoerythrin- (for the competitive repopulation assays) or phycoerythrin- (for other assays) conjugated antibodies were used:

anti-IgG1 (10 $\mu$ g/ml): isotype control

anti-hCD45 (20,0 $\mu$ g/ml): pan-leukocyte marker (used to determine the extent of the human graft)

anti-hCD34 (10,0 $\mu$ g/ml): haematopoietic progenitor marker

anti-hCD13 (12,5 $\mu$ g/ml): myeloid marker

anti-hCD19 (17,5 $\mu$ g/ml): B-lymphoid marker

(all from DAKO A/S). Cells were incubated in the dark 20 min on ice and then washed by adding 1ml PBS 2% FBS and spinning at 1200 rpm in Heraeus Megafuge 1.0 (Kendro Laboratory products) 7 min at 4°C. Cells were then resuspended in 0,3 ml PBS 2% FBS containing 2  $\mu$ g/ml PI for viable staining and

analysed by three-colour flow cytometry. The percentage of antibody-positive/transgene-positive cells was determined after excluding nonviable cells. PI was not used in combination with Cy5 phycoerythrin-conjugated antibodies and YFP, because of emission wavelength overlapping.

A gate was set to exclude at least 99.9% of cells labelled with the isotype control. Mice were considered engrafted if hCD45+ cells exceeded 1% of total mononuclear bone marrow cells.

In the competitive repopulated mice, since both GFP and YFP signals emit in the FL-1 channel whereas only YFP emits in the FL-2 channel, we calculated the frequency of GFP-expressing cells by subtracting the fraction of YFP-expressing cells, scored in FL-2, from the percentage of cells scored positive in FL-1.

Untransplanted mice were used as negative control. We scored 10,000-100,000 events.

## 2.12 Genomic DNA extraction and Real-time Quantitative-PCR (Q-PCR) analysis

In order to evaluate vector copy number *per* cell and, in some cases, percentage of human cells in NOD/SCID bone marrow, Q-PCR was performed on genomic DNA extracted from transduced cells either grown in culture or derived from mouse bone marrow. Cells in culture were grown for at least 10 days before analysis to exclude non-integrated vector forms from the analysis.

### 2.12.1 Genomic DNA extraction

Genomic DNA extraction was performed by using the Blood and Cell Culture DNA kit (Midi kit; Qiagen). This system is based on cell lysis, followed by DNA purification on an anion exchange resin.

Cultured cells were harvested (for cells grown in suspension) or detached by trypsin-EDTA solution (for adherent cells) and washed with ice-cold PBS by spinning at 1000 rpm in Heraeus Megafuge 1.0 R (Kendro Laboratory products) 10 min at 4°C.

Nuclei precipitation protocol was as follows:

- resuspend cells with ice-cold PBS to a concentration of  $5 \times 10^6$ - $1 \times 10^7$  cells/ml
- add 1 volume of ice-cold Buffer C1 and 3 volumes of ice-cold distilled water, mix by inverting tubes and incubate 10 min on ice to lyse cells preserving the nuclei
- spin 2000 rpm in Heraeus Megafuge 1.0 R (Kendro Laboratory products) 15 min at 4°C and discard supernatant
- resuspend cells in 1ml ice-cold Buffer C1 and 3ml ice-cold distilled water
- spin 2000 rpm in Heraeus Megafuge 1.0 R (Kendro Laboratory products) 15 min at 4°C and discard supernatant to remove residual cell debris
- resuspend cells in 5ml Buffer G2 (lysis buffer) containing 0,4mg/ml RNase A

For bone marrow derived cells nuclei precipitation was not performed and pellets of cells were directly resuspended in 5ml Buffer G2 containing 0,4mg/ml RNase A.

Samples were then incubated 1 hour at 37°C in a water bath to degrade RNA. Lysis was then performed by adding proteinase K to the suspension to a final concentration of 0,4mg/ml and incubating samples over night at 50°C in a water bath.



The following day samples were vortexed at maximum speed 10 seconds and applied to equilibrated anion exchange resin columns, washed and eluted. After elution, genomic DNA was precipitated by adding 0,7 volumes of room temperature isopropanol and inverting tubes several times. DNA was then transferred by winding the floccule onto a loop into a 1,5ml tube containing 1ml of 70% ethanol. Samples were then centrifuged to remove precipitated salts at 14,000 rpm in Heraeus Biofuge fresco (Kendro Laboratory products) 15 min at 4°C. After removal of supernatant, DNA was air-dried for 15 min, resuspended in 100-200µl TE (10mM Tris, 1mM EDTA; pH 8,0) and dissolved on a shaker at 55°C for 2 hours.

Genomic DNA was finally quantified by spectrophotometer analysis and run on a 0,8% agarose gel to check for possible RNA contaminations and degradation of the sample. DNA was then stored at -20°C.

### **2.12.2 Real-time Q-PCR**

Q-PCR was performed in Optical 96-well Fast Thermal Cycling Plates (Applied Biosystem) on ABI PRISM 7900 Sequence Detector System (Applied Biosystem), using the following thermal cycling conditions: one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, 40 cycles at 95°C for 15 seconds and 60°C for 1 min. For each sample, the Sequence Detector System 2.1 software provided an amplification curve constructed by relating the fluorescence signal intensity ( $\Delta R_n$ ) to the cycle number. Cycle threshold (Ct) was defined as the cycle number at which the fluorescence signal was more than 10 SD of the mean background noise collected from the 3rd to the 15th cycle. Vector copy number was determined by using as standard curve serial dilutions (200ng, 100ng, 50ng, 25ng and 12,5 ng) of

genomic DNA extracted from a human cell line clone stably containing a known number of LV-GFP integrations (copy number of the standard curve was previously defined by Southern Blot analysis). To determine vector copy number *per cell*, LV copy numbers were normalized to human DNA content, which was assessed either by spectrophotometry or by Q-PCR. When assessed by Q-PCR, human DNA content was quantified by using the same standard curve used for vector copy number assessment. When engraftment in NOD/SCID mice was assessed by Q-PCR, murine DNA content was determined by using as standard curve serial dilutions (200ng, 100ng, 50ng, 25ng and 12,5 ng) of genomic DNA extracted from mouse bone marrow derived cells.

Each sample was run in triplicate in a total volume of 25µl/reaction, containing 12,5µl TaqMan Universal Master Mix (4304437; Applied Biosystem), 100ng of sample DNA and one of the following amplification systems at the following concentrations.

For integration titer on promoter trapping vectors oligonucleotides and probe complementary to the GFP sequence (common to both LT and RT) were used:

forward primer: 5' – CAGCTCGCCGACCACTA – 3' (900nM)

reverse primer: 5' – GGGCCGTCGCGAT – 3' (900nM)

probe: 5' – 6-FAM – CCAGCAGAACACCCCC – MGB – 3' (200nM)

For vector copy number analysis of transduced HSPCs grown in suspension two systems were used, one is the system complementary to the GFP sequence described above and the other is complementary to the LV encapsidation signal psi ( $\psi$ ) sequence:

forward primer: 5' – TGAAAGCGAAAGGGAA – 3' (200nM)

reverse primer: 5' – GACTTCGCGCGTGCC – 3' (200nM)

probe: 5' – VIC – AGCTCTCTCGACGCAGGACTCGGC – TAMRA – 3'  
(200nM)

In these samples human DNA content was assessed by Q-PCR by using oligonucleotides and probe complementary to the human Telomerase Reverse Transcriptase (hTERT) sequence (Sozzi, Conte et al. 2003):

forward primer: 5' – GGCACACGTGGCTTTTCG – 3' (200nM)

reverse primer: 5' – GGTGAACCTCGTAAGTTTATGCAA – 3' (600nM)

probe: 5' – 6-FAM – TCAGGACGTTCGAGTGGACACGGTG – TAMRA – 3'  
(200nM)

For the analysis of the genomic DNA extracted from NOD/SCID bone marrow derived cells three different systems were used. To detect vector sequence oligonucleotides and probe complementary to the LV Rev Responsive Element (RRE) sequence were used:

forward: 5' – TGAGGGCTATTGAGGCGC – 3' (600nM)

reverse: 5' – TGCCTGGAGCTGCTTGATG – 3' (600nM)

probe: 5' – 6-FAM – TTGCAACTCACAGTCTG – MGB – 3' (200nM)

To quantify human DNA content the hTERT system described above was used.

To quantify murine DNA amount oligonucleotides and probe complementary to the murine  $\beta$ -actin gene were used:

forward: 5' – AGAGGGAAATCGTGCGTGAC – 3' (300nM)

reverse: 5' – CAATAGTGATGACCTGGCCGT – 3' (750nM)

probe: 5' – VIC – CACTGCCGCATCCTCTTCCTCCC – MGB – 3' (200nM).

The level of engraftment in these mice was determined as the ratio between human DNA and total DNA content (human and murine).

## 2.13 Proteasome activity assays

Cellular extracts from cord blood CD34+ cell, stimulated or not with cytokines for the indicated time, and from cell lines were prepared as previously described (Mo, Cascio et al. 1999) with minor modifications.

$10^6$  cells *per* sample were harvested or detached by trypsin-EDTA solution, if adherent, and washed three times with ice-cold PBS in 50ml tubes. Pellets were resuspended in 200 $\mu$ l of ice-cold lysis buffer (50mM Tris/HCl pH 7,5, 1mM dithiothreitol, 0,25M sucrose, 5mM MgCl<sub>2</sub>, 0,5mM EDTA, 2mM ATP), transferred in 1,5ml tubes and sonicated. Samples were then centrifuged at 13000 rpm in Heraeus Biofuge fresco (Kendro Laboratory products) 30 min at 4°C. Supernatants were then transferred into clean 1,5ml tubes and frozen at -80°C.

Peptidase activities of proteasome were assayed by monitoring the production of 7-amino-4-methylcoumarin (amc) from fluorogenic peptides as previously described (Cascio, Call et al. 2002). Briefly, Suc-LLVY-amc (for the chymotrypsin-like activity), Boc-LRR-amc (for the trypsin-like activity) and Ac-YVAD-amc (for the caspase-like activity) were used at a final concentration of 100 $\mu$ M in 20mM Tris-HCl pH 7,5, 1mM ATP, 2mM MgCl<sub>2</sub>, 0,1% BSA. Reactions were started by adding an aliquot of cellular extract and the fluorescence of released amc (excitation, 380 nm; emission, 460 nm) was monitored continuously at 37°C with a Carry Eclipse spectrofluorimeter. Background activity was determined by addition of the proteasome inhibitors MG132 (for the chymotrypsin and the caspase-like activities) and  $\beta$ -lactone (for the trypsin-like activity) at a final concentration of 10 $\mu$ M and 20 $\mu$ M respectively. Assays were calibrated using standard solutions of free fluorophores.

Consumption of the substrates at the end of incubation never exceeded 1%. Concentration of DMSO (in which stock solution of peptides and inhibitors were prepared) in the assays never exceeded 0.7%, which does not affect proteasome activity (Akopian, Kisselev et al. 1997).

## 2.14 Statistical analysis

For the Competitive Repopulation Assay we established a statistical model for %FP, the percentage of SRC expressing the transgene. Such a percentage is the response variable of interest and its distribution may be affected by several variables, including the cytokine treatment (variable “cyt”, + or -), the marker (variable “dye”, YFP or GFP), the cell preparation (variable “batch”) and the xenotransplant model, (variable “tx-mod”, reference or competitively repopulated). Taking into consideration that each reference mouse generates only one measurement (either %YFP or %GFP), while each competitively repopulated mouse generates two non-independent measurements (%YFP and %GFP), we modelled the presence of within-mouse correlated pairs of observations treating the variable “mouse” as a random effect. According to the statistical literature (see (Searle, Casella et al. 1992) for a comprehensive treatment), random effects represent specific sources of randomness in addition to natural variability underlying all biological experiments. Since %FP are percentages, which are by definition constrained between 0 and 100, we transformed them into their logodds

$$\text{lofp} = \log (\%FP/(100-\%FP)).$$

A linear mixed effect model for response variable “lofp” was constructed with fixed effect variables “cyt”, “dye”, “batch” and “tx-mod” and random effect

variable “mouse”. The model was fitted using routine `lme` from the contributed package `nlme` (Pinheiro and Bates 2000) to the free software R (RdevelopmentCoreTeam 2004).

To compare results obtained in other experiments, we performed the following tests. In most cases, t-tests were not appropriate either because percentages are naturally bounded between 0 and 100, and cannot be modeled by normal distributions, or because the normal distribution assumption for the relevant statistics is not appropriate when there are too few observations. We used nonparametric tests based on ranks: Wilcoxon signed rank test for paired samples and Wilcoxon-Mann-Whitney rank sum test for independent samples. Calculations were done using the function `wilcox.test`, native to the free software R (RdevelopmentCoreTeam 2004).

A standard sign test was used for the data in Figure 4.6.

Chapters 3-5.

Results

### 3. Effect of early-acting cytokines on HSC features and transduction by LVs

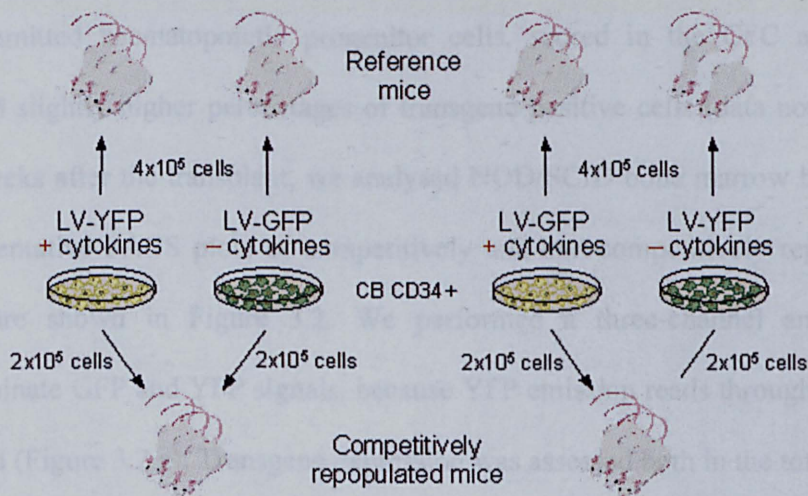
#### 3.1 Effect of cytokine stimulation on repopulating ability of human HSCs

We have previously demonstrated that Cord Blood (CB)-derived HSCs can be efficiently transduced after a short incubation with a lentiviral vector (LV) (20-24 hours) in serum free conditions, in the absence of cytokine stimulation. Nevertheless, we have also found that the presence of a cocktail of early-acting cytokines (SCF, TPO, Flt3L and IL6) during the transduction period further enhanced transduction efficiency, reaching on average more than 70% of SCID Repopulating Cells (SRCs) transduced (Ailles, Schmidt et al. 2002). When we transplanted CD34+ cells transduced in the presence or absence of cytokines in a NOD/SCID model, we observed similar levels of engraftment between stimulated and unstimulated SRCs, suggesting that exposure to cytokines did not compromise SRC engraftment ability. However, the limited follow-up of the NOD/SCID mice and the heterogeneity of the engrafting SRCs did not allow analysing HSC long-term repopulating ability, so that we could not evaluate a possible detrimental effect of cytokines on long-term HSCs and on their clonogenic and proliferation ability. Thus, in this work we aimed to establish in a more stringent model whether the gain in gene transfer could be offset, even for a short cytokine exposure, by a decreased engraftment or long-term repopulation capacity of the transduced cells.

In order to stringently assess the engraftment and repopulation capacity of human cord blood SRCs after the short *ex vivo* culture with and without the combination of cytokines, we performed a competitive NOD/SCID mouse repopulation assay. We transduced CB CD34+ cells in both culture conditions



with either of two late generation self-inactivating LVs (Follenzi, Ailles et al. 2000) differing only for the transgene (Green Fluorescent Protein - GFP - or Yellow Fluorescent Protein - YFP). We injected half of the cells transduced with cytokines by LV-YFP and half of the cells transduced without cytokines by LV-GFP into the same NOD/SCID mouse (YFP+*cyt*/GFP-*cyt* mice, n=12). In order to avoid any transgene-specific effect we also swapped transgene and culture condition in a parallel group of mice (YFP-*cyt*/GFP+*cyt* mice, n=8). In parallel, we transplanted mice with the same total number of cells transduced in only one culture condition, and with either of the two vectors, and obtained four experimental groups of reference, non-competitively repopulated mice (GFP+*cyt*, GFP-*cyt*, YFP+*cyt*, YFP-*cyt* mice, n=4,6,5,5 per group, respectively). A scheme of the experimental plan is shown in Figure 3.1.



**Figure 3.1. Competitive repopulation assay: experimental strategy.**

Competitive repopulation assay was performed using cells transduced in different conditions and marked with distinguishable vectors. Each non competitively repopulated (reference) mouse received  $4 \times 10^5$  CD34+ cells transduced in presence or absence of cytokines with either LV-GFP or LV-YFP; each competitively repopulated mouse received  $2 \times 10^5$  CD34+ cells transduced in presence of cytokines with LV-GFP or LV-YFP and  $2 \times 10^5$  CD34+ transduced in absence of cytokines with LV-YFP or LV-GFP, respectively.

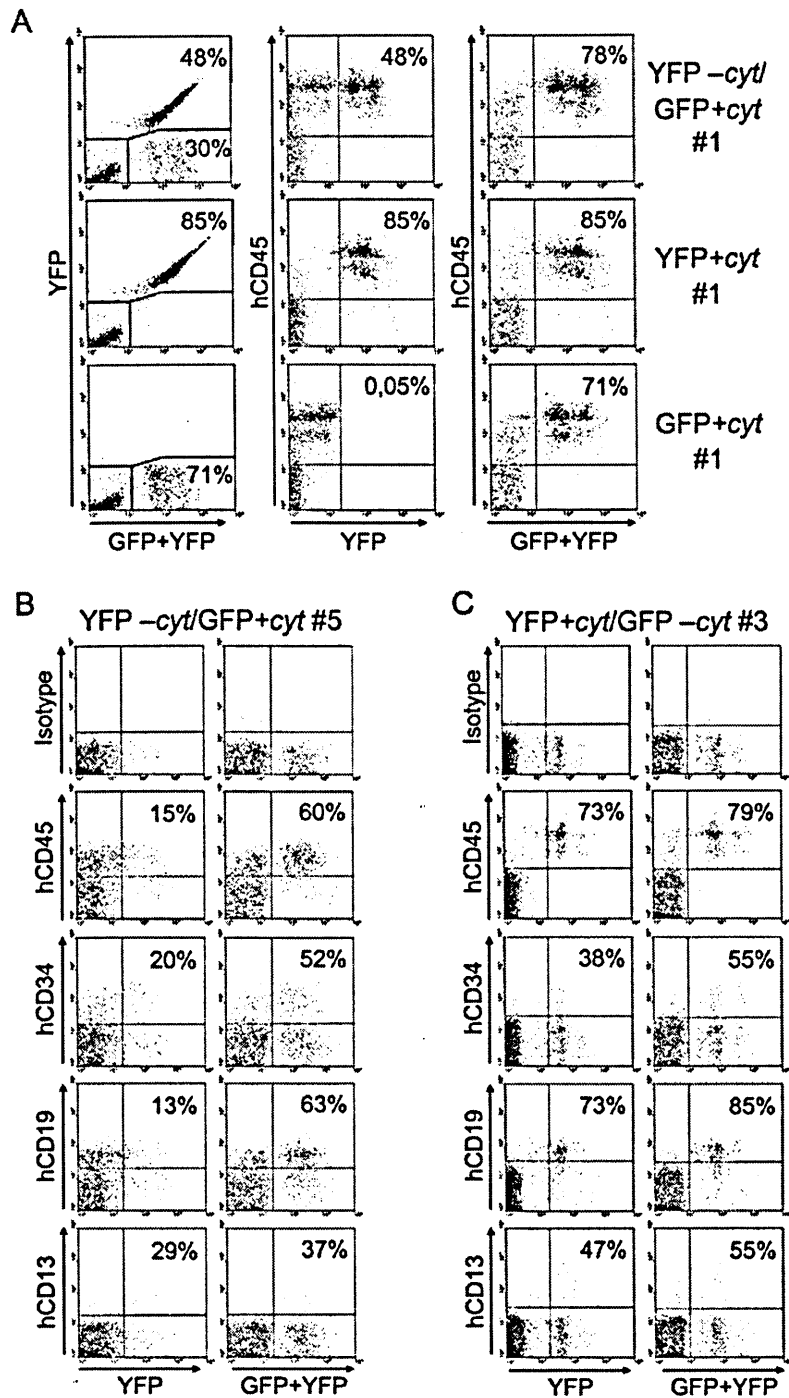
Aliquots of the transduced cells were kept in culture and plated in CFC assays for assessment of transduction. FACS analysis of suspension cultures showed on average 41% and 20% frequency of transgene-expressing cells, one

week after transduction with and without cytokines, respectively, with the LV-GFP vector and 49% and 27%, with and without cytokines, respectively, with the LV-YFP vector (average of three experiments performed; Table 3.1).

**Table 3.1. Competitive Repopulation Assay. FACS analysis of HSPC suspension culture**

	% transgene positive cells	
	- <i>cyt</i>	+ <i>cyt</i>
LV-GFP	14	31
	26	43
	20	49
Average	20	41
LV-YFP	29	54
	40	58
	13	34
Average	27	49

In committed haematopoietic progenitor cells, scored in the CFC assay, we reached slightly higher percentages of transgene positive cells (data not shown). Ten weeks after the transplant, we analysed NOD/SCID bone marrow by FACS. Representative FACS plots of competitively and non-competitively repopulated mice are shown in Figure 3.2. We performed a three-channel analysis to discriminate GFP and YFP signals, because YFP emission reads through the GFP channel (Figure 3.2 a). Transgene expression was assessed both in the total human CD45+ cell population, and, in order to verify transduction of multi-potent HSCs, in the progenitor, lymphoid and myeloid fractions (Figure 3.2 b,c). All repopulated mice (>1% hCD45+ cells, 40 mice out of 44 assayed; the numbers of mice studied *per* group given above refer to the successfully repopulated mice) showed multi-lineage engraftment by transduced SRCs. Human engraftment level and frequency of transgene expression for all the positive mice are shown in Table 3.2.



**Figure 3.2. Competitive repopulation assay: representative FACS Analysis.**

Three-channels FACS analysis of mouse BM ten weeks after transplant of human CD34+ cells transduced with either LV-GFP or LV-YFP. A) Frequency of transgene expression in the human cell (hCD45+) graft. FACS plots from a competitively repopulated mouse, showing both GFP and YFP-positive cells (upper panels), and two reference mice, showing either YFP or GFP-positive cells (middle and lower panels), as indicated. Percentages of transgene-positive cells detected in each channel (FL-2: YFP; FL-1: GFP + YFP, FL-3: human CD45) are given in the respective quadrant. B) and C) Multilineage engraftment by transduced SRCs. Percentages shown represent the fraction of transgene-expressing cells among the indicated lineage marker-positive cells (scored in the FL-3 channel). Because both GFP and YFP signals emit in the FL-1 channel, we calculated the frequency of GFP-expressing cells by subtracting the fraction of YFP-expressing cells, scored in FL-2, from the percentage of cells scored positive in FL-1. Mouse numbers as in Table 3.2.

**Table 3.2. Competitive Repopulation Assay. SRC analysis**

Reference Mice				Competitively Repopulated Mice			
<b>GFP +<i>cyt</i></b>				<b>YFP+<i>cyt</i> / GFP-<i>cyt</i></b>			
Mouse	%hCD45 <sup>A</sup>	%GFP <sup>B</sup>	%YFP <sup>B</sup>	Mouse	%hCD45 <sup>A</sup>	%GFP <sup>B</sup>	%YFP <sup>B</sup>
1	45	71	-	1	18	32	27
2	41	80	-	2	10	40	17
3	64	83	-	3	19	6	73
4	16	69	-	4	15	7	65
Average	41	76	-	5	6	9	82
<b>GFP -<i>cyt</i></b>				<b>YFP-<i>cyt</i> / GFP+<i>cyt</i></b>			
Mouse	%hCD45	%GFP	%YFP	Mouse	%hCD45	%GFP	%YFP
1	66	60	-	6	40	1	85
2	53	58	-	7	3	26	31
3	17	23	-	8	4	47	17
4	18	33	-	9	25	24	20
5	20	35	-	10	15	20	16
6	59	37	-	11	45	68	22
Average	39	41	-	12	9	18	11
<b>YFP +<i>cyt</i></b>				<b>YFP-<i>cyt</i> / GFP+<i>cyt</i></b>			
Mouse	%hCD45	%GFP	%YFP	Mouse	%hCD45	%GFP	%YFP
1	40	-	85	1	58	30	48
2	24	-	71	2	15	33	33
3	50	-	78	3	5	44	22
4	2	-	35	4	7	6	85
5	50	-	44	5	40	45	15
Average	33	-	63	6	10	50	8
<b>YFP -<i>cyt</i></b>				<b>YFP-<i>cyt</i> / GFP+<i>cyt</i></b>			
Mouse	%hCD45	%GFP	%YFP	Mouse	%hCD45	%GFP	%YFP
1	45	-	82	7	30	67	20
2	15	-	53	8	43	51	2
3	60	-	63	Average	26	41	29
4	19	-	18				
5	11	-	18				
Average	30	-	47				

<sup>A</sup> percentage of human cells in the mouse BM

<sup>B</sup> percentage of human cells expressing the indicated transgene

As expected for the NOD/SCID model, mice were engrafted by human cells to variable levels, ranging from 2 to 66% of their bone marrow cells. The engraftment level was slightly lower in competitively repopulated than in non-

competitively repopulated mice (21% versus 35% of CD45+ cells on average, respectively, n=20,20).

The frequency of transgene-expressing cells was on average consistently higher for SRCs transduced with cytokines, in all permutations of vector type and experimental condition tested. As previously observed (Mikkola, Woods et al. 2000; Ailles, Schmidt et al. 2002), the frequency of transgene-expressing cells was higher, in each condition tested, for the SRC progeny than for the *in vitro* suspension cultures.

To assess whether the cytokine stimulation had an impact on the repopulating capacity of the transduced SRCs we developed a statistical model, in which all the experimental parameters (see paragraph 2.14 Statistical analysis) were taken in consideration and the relevant hypotheses tested. The response variable of interest was the percentage of transduced SRCs, which in the statistical model was termed %FP. Its distribution might be affected by several variables, including cytokines stimulation (“cyt”), the transgene (“dye”), the cell preparation (“batch”), and the xenotransplant model (“tx-mod” - non-competitively or competitively repopulated).

Results of the statistical analysis are shown in Table 3.3. We confirmed that cytokines had a significant effect on the frequency of transgene-positive cells, with an estimated positive effect in the logodds scale equal to +1.044. This means that if the mean frequency of transduction is for example 30% for unstimulated cells (-cyt), the estimated mean frequency increases to 55% when stimulated (+cyt).

Because the interaction between the parameters “cyt” and “tx-mod” (competitive or not) was not significant, we concluded that cytokine stimulation had a positive significant impact on SRC transduction without changing

significantly its extent and direction between reference and competitively repopulated mice, thus showing that cytokine-stimulated SRCs had an engraftment capacity similar to that of unstimulated cells, even in a stringent repopulation model.

**Table 3.3. Competitive Repopulation Assay. P-values for testing the significance of each variable**

	p-value
(Intercept)	0.0009
dye <sup>A</sup>	0.5670
cyt <sup>B</sup>	0.0115
tx-mod <sup>C</sup>	0.0007
batch <sup>D</sup>	0.0975
cyt/tx-mod interaction	0.7737

<sup>A</sup> marker: GFP or YFP

<sup>B</sup> cytokine treatment: plus or minus

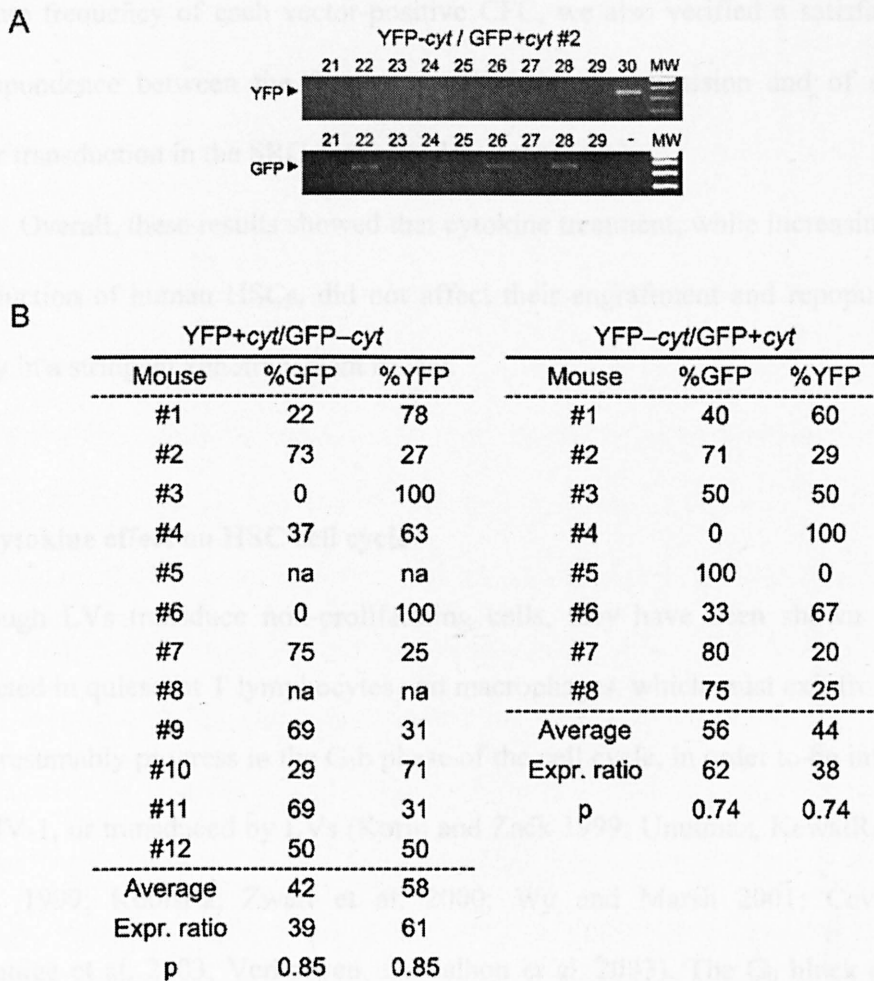
<sup>C</sup> xenotransplant model: competitive or reference. This variable is significant, with the competitively repopulated mice having a significantly lower %FP than the reference, since the competitively repopulated mice received half dose of cells for each condition with respect to the reference, and both types of injected cells contributed similarly to the graft.

<sup>D</sup> batches of CD34+ cells

Variable "tx-mod" was also significant, with the competitively repopulated mice having a significantly lower %FP than the reference; this was expected, since the competitively repopulated mice received half dose of cells for each condition with respect to the reference, and both types of injected cells contributed similarly to the graft. Variables "dye" and "batch" were not significant, showing no evidence of either a difference between YFP and GFP or differences across cell preparations.

In order to confirm our findings at the vector DNA level, we performed PCR analysis, with specific primers for each transgene, on DNA extracted from human colonies derived from the bone marrow of engrafted mice (Figure 3.3). Colonies were plucked either randomly, to assess the absolute frequency of vector-positive CFCs for each vector type, or for positive fluorescence by

microscopy, to more quickly assess the relative proportion of YFP and GFP vector among transgene-expressing CFCs.



**Figure 3.3. Competitive repopulation assay: vector DNA analysis of bone marrow-derived CFCs.**

Single CFCs were plucked randomly, lysed and analyzed by PCR, using two sets of primers specific for either GFP or YFP sequences. A) Agarose gel showing a representative analysis of ten colonies from the indicated mouse, scored for YFP (upper panel) and GFP (lower panel). B) Relative proportion of GFP and YFP-positive colonies. Values were calculated from the total number of colonies scored positive for transgene DNA for each mouse, and averaged. For reference, the average relative proportion of GFP and YFP-expressing cells measured in the mouse bone marrow by FACS, calculated from the data in Table 3.2 (Expr. ratio: expression ratio) is shown. Comparison between the two proportions did not show a statistically significant difference. P-values were calculated by signed rank two-sided tests.

The relative proportions of YFP and GFP expressing cells in competitively repopulated mice can be calculated from the data in Table 3.2. When we compared it to the relative proportions of YFP and GFP vector-positive colonies in the human graft, as assessed by PCR analysis and shown in Figure 3.3 b, we

found that there was no evidence of a statistically significant difference between the distributions of the two groups. In a small panel of mice scored for the absolute frequency of each vector-positive CFC, we also verified a satisfactory correspondence between the frequency of transgene expression and of actual vector transduction in the SRC progeny (data not shown).

Overall, these results showed that cytokine treatment, while increasing LV transduction of human HSCs, did not affect their engraftment and repopulating ability in a stringent xenotransplant model.

### **3.2 Cytokine effect on HSC cell cycle**

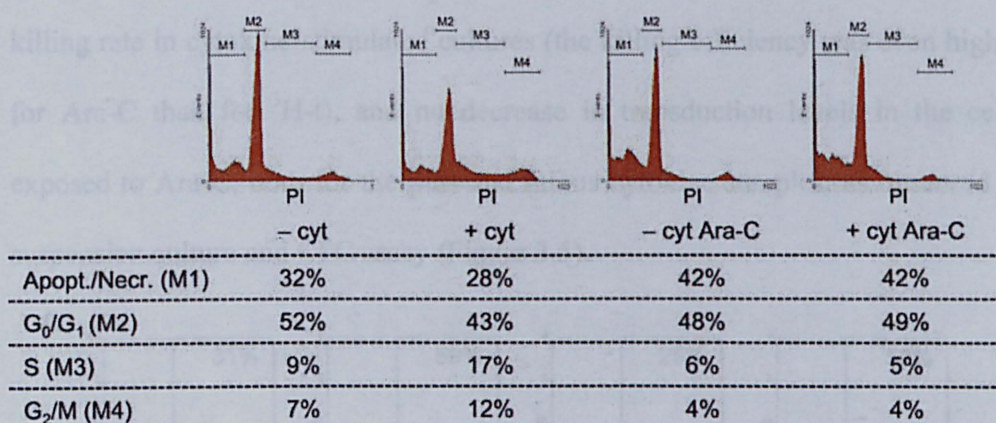
Although LVs transduce non-proliferating cells, they have been shown to be restricted in quiescent T lymphocytes and macrophages, which must exit from  $G_0$ , and presumably progress to the  $G_1$  phase of the cell cycle, in order to be infected by HIV-1, or transduced by LVs (Korin and Zack 1999; Unutmaz, KewalRamani et al. 1999; Kootstra, Zwart et al. 2000; Wu and Marsh 2001; Cavalieri, Cazzaniga et al. 2003; Verhoeyen, Dardalhon et al. 2003). The  $G_0$  block of LV transduction could be active in HSCs as well, implying that quiescent HSCs are not transducible. Thus, a possible explanation for the cytokine enhancement of LV transduction could be that only the SRCs in the  $G_1$  phase of the cell cycle are susceptible to transduction, and that cytokines enhance transduction triggering the exit of SRCs from  $G_0$ . Progression into late  $G_1$ , however, commits the cells to DNA replication and mitosis, and may thus impair the long-term repopulating ability of HSCs (Glimm, Oh et al. 2000; Guenechea, Gan et al. 2001), a feature that is difficult to assess in the NOD/SCID model.



Since we could not measure the cell cycle status of the few SRCs contained in the CD34+ population, we performed an S-phase suicide assay on the transduced cells. In a first set of *in vitro* experiments, we transduced with LV-GFP CB CD34+ cells with or without cytokines, and then exposed the cells or not to <sup>3</sup>H-thymidine (<sup>3</sup>H-t) for 16 hrs, as described (Ponchio, Conneally et al. 1995). This approach relies on the ability of high-specific activity <sup>3</sup>H-t to deliver a lethal dose of radiation exclusively to cells passing through S-phase (Becker, McCulloch et al. 1965), and on the use of a sufficiently prolonged exposure time to allow all the cells in late G<sub>1</sub>, G<sub>2</sub> or M phase, to enter S-phase (Ponchio, Conneally et al. 1995). After <sup>3</sup>H-t treatment, we performed CFC assay. The results showed that <sup>3</sup>H-t killed the majority of cytokine-stimulated cells (>65% of plated CFC) and a much smaller fraction of non-stimulated cells (19%), indicating that cytokines triggered the committed progenitors into S-phase, as expected. If the enhancement of transduction observed in cytokine-stimulated cells were due to their cell cycle progression, the transduced cells should be preferentially killed in cytokine-stimulated cultures, abrogating the observed difference in transduction between stimulated and unstimulated cells. When scoring transduction frequency in the surviving culture, we found 56% and 33% GFP+ cells in cultures not exposed to <sup>3</sup>H-t, and 56% and 31% GFP+ in cultures exposed to <sup>3</sup>H-t, and either stimulated or not with cytokines, respectively (average of 2 experiments performed with similar results). These results strongly suggested that the cytokine enhancement of haematopoietic stem/progenitor cell (HSPC) transduction did not require progression into the S-phase of the cell cycle.

In order to safely perform a similar analysis in the more relevant SRC fraction of the CD34+ cells, we used the cytosine 1-β-D-arabinofuranoside (Ara-C) as killing agent. Ara-C was previously shown to be as effective as <sup>3</sup>H-t in

killing cycling human HSPCs and it was used to evaluate the cell cycle status of these cells (Dresch, El Kebir et al. 1983; Lemoli, Tafuri et al. 1997; Ivanovic, Bartolozzi et al. 1999; Lucotti, Malabarba et al. 2000). Similarly to the experiments described above, we treated CD34+ cells with or without cytokines, and exposed them or not to Ara-C. To verify the cell cycle status of the cells after treatment, we stained them with propidium iodide and analysed by FACS (Figure 3.4). We observed that cytokines increased the fraction of cells in S-phase, 17% for the +cyt condition versus 9% for the -cyt condition, and that this difference was completely abrogated when the cells were exposed to Ara-C.



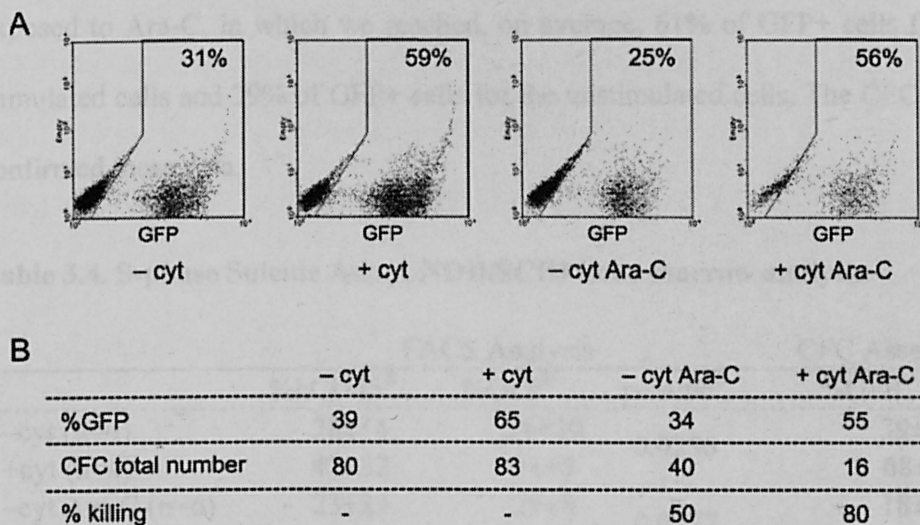
**Figure 3.4. S-phase suicide assay: HSPC cell cycle analysis.**

Human CD34+ cells were stimulated (+cyt) or not (-cyt) with cytokines for 18 hrs, exposed or not to Ara-C for further 12 hrs, as indicated, and immediately analyzed. Upper panels, FACS histograms of Propidium Iodide (PI) staining. Lower panel, distribution of the analyzed cell populations in the indicated cell cycle phases, marked as M1 to 4 in the histogram. Apopt./Necr.: apoptotic/necrotic cells.

It should be noted, however, that propidium iodide staining does not allow a precise quantification of the fraction of cells in the S-phase of the cell cycle. This is because this analysis is based only on the evaluation of the amount of DNA present in the cells (cells in the G<sub>2</sub>/M phases have a 2 fold higher amount of DNA when compared to cells in the G<sub>0</sub>/G<sub>1</sub> phases, whereas cells in the S-phase have a quantity of DNA that is intermediate between G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M cells); in this setting it is also possible that we scored in the S-phase fraction cells in the G<sub>2</sub>/M

phase that underwent apoptosis. In order to have a more precise quantification of cells in the S-phase in the different conditions tested, bromodeoxyuridine incorporation experiment would be required. However, we decided not to perform further cell cycle analyses on the bulk CD34+ population and chose to prove the killing efficacy of Ara-C treatment in the more relevant fraction of hematopoietic progenitors (see below).

We then transduced CD34+ cells with LV-GFP, exposed them or not to Ara-C, performed *in vitro* assays, and injected the cells into NOD/SCID mice. The *in vitro* results reproduced the findings obtained with <sup>3</sup>H-t, showing a higher killing rate in cytokine-stimulated cultures (the killing efficiency was even higher for Ara-C than for <sup>3</sup>H-t), and no decrease in transduction levels in the cells exposed to Ara-C, both for the plus and minus cytokine samples, as observed in suspension culture and CFC assay (Figure 3.5).



**Figure 3.5. S-phase suicide assay: HSPCs assayed *in vitro* by suspension culture and CFC assay.**

Human CD34+ cells were transduced with LV-GFP in presence (+cyt) or absence (-cyt) of cytokines for 18 hrs, exposed or not to Ara-C for further 12 hrs, as indicated, and analyzed after 1 (A) or 2 (B) weeks of culture. A) Representative FACS analysis of GFP expression in suspension culture. Percentages of GFP-positive cells are given. B) CFC assay. GFP+ colonies were scored by fluorescence microscopy; CFC total number is expressed as number of colonies per 10<sup>3</sup> CD34+ cells plated; percentage of killing is calculated as the ratio between the number of colonies arising from the Ara-C treated and untreated populations. Average values of two experiments.

Interestingly, despite the seemingly equal distribution along the cell cycle of cultures exposed to Ara-C and the higher Ara-C killing rate in the stimulated cells fraction, the transduction efficiency remained much higher for the cytokine-stimulated cells. For transplantation, the transduced cells were matched for the number of viable cells prior to the Ara-C treatment, and injected together with CD34<sup>neg</sup> accessory cells. Accessory cells were used in order to substitute the killed progenitors cells in supporting the first stages on engraftment. NOD/SCID mice were killed 6-8 weeks after transplant and bone marrow was harvested and analysed by FACS; bone marrow cells were also plated in methylcellulose for CFC assay (Table 3.4). FACS analysis showed, for the cells not exposed to Ara-C, a higher SRC transduction efficiency when cytokines were used, reaching on average 91% of GFP+ cells for the stimulated cells and 54% for the unstimulated cells. Interestingly, we observed a similarly significant difference in the cells exposed to Ara-C, in which we reached, on average, 61% of GFP+ cells for the stimulated cells and 29% of GFP+ cells for the unstimulated cells. The CFC assay confirmed these data.

**Table 3.4. S-phase Suicide Assay. NOD/SCID bone marrow analysis**

	FACS Analysis			CFC Assay
	%hCD45 <sup>A</sup>	%GFP <sup>B</sup>	p-value <sup>C</sup>	%GFP <sup>D</sup>
-cyt (n=4)	28±14	54±20	0.0286	39±14
+cyt (n=4)	42±32	91±5		68±15
-cyt Ara-C (n=6)	23±23	29±8	0.0277	18±13
+cyt Ara-C (n=5)	7±6	61±22		45±22

All values are expressed as mean±SD of two experiments performed

<sup>A</sup> percentage of human cells in the mouse BM

<sup>B</sup> percentage of human GFP expressing cells

<sup>C</sup> p-value calculated by rank sum two-sided test

<sup>D</sup> percentage of human GFP expressing colonies, scored by fluorescence microscopy

n: number of mice *per* group

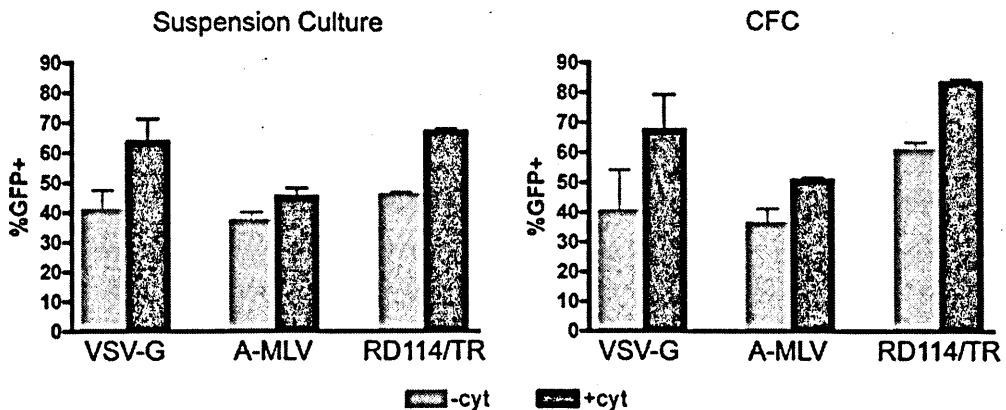
Because the cytokine enhancement of SRC transduction was not abrogated by Ara-C treatment, we conclude that it was not due to cell progression into S-phase.

Ara-C treatment decreased both the engraftment and the transduction levels in both stimulated and non-stimulated SRCs, indicating that a fraction of SRCs were sensitive to Ara-C, most likely because they were induced to proliferate early after transplant, and that the quiescent SRC fraction was mainly responsible for repopulating the mice transplanted with Ara-C exposed cells. Because the SRCs successfully engrafting after Ara-C treatment were transduced to substantial levels, these data indicated that LVs transduced cells that were not committed to early cell cycle progression and thus strongly suggest that LVs are able to transduce quiescent SRCs *ex vivo*.

### **3.3 Cytokines effect on LV entry pathway**

We then aimed to better understand how cytokines affect LV transduction in HSPCs. In order to verify whether the difference observed in the transduction efficiency between cells stimulated and not stimulated reflected a difference in the entry efficiency of VSV-G-pseudotyped vector, we tested vectors pseudotyped with the MLV amphotropic (A-MLV) and the modified form of the feline endogenous retrovirus RD114 (RD114/TR) envelopes. Indeed, these vectors exploit different routes to enter the target cell: A-MLV- and VSV-G-pseudotyped LV entry is pH dependent (is mediated by endosome), whereas RD114/TR pseudotyped LV entry is pH independent (envelope fuses to the plasma membrane). If cytokines affected the specific pathway of VSV-G-pseudotyped vector entry, we postulated that the enhancement of transduction would be envelope-dependent and possibly abolished by changing it. We performed some

experiments transducing CB CD34+ cells with the three vectors above mentioned, all carrying the same PGK-GFP expression cassette, and for each vector we used the two protocols, with and without cytokine stimulation. We then plated the cells for suspension culture and for CFC assay. As shown in Figure 3.6, both in liquid culture and in CFC analysis, we observed a higher transduction level for the protocol with cytokine stimulation for all vectors tested. Importantly, the difference between stimulated and unstimulated cells transduced with the RD114/TR-pseudotyped vector is comparable to that one of cells transduced with the VSV-G-pseudotyped vector. Since these two vectors exploit different entry pathways, these results indicate that the cytokines did not modulate a specific entry pathway.



**Figure 3.6. Cytokine effect on HSPC transduction by LV pseudotyped with different envelopes.**

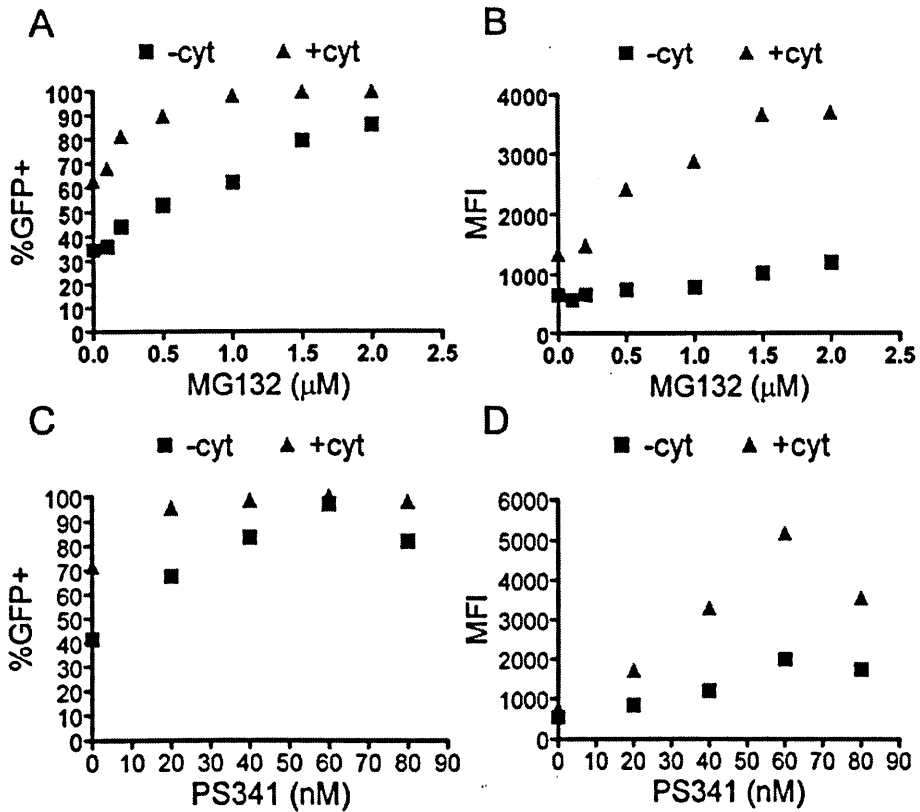
Human CD34+ cells were transduced with LV-GFP pseudotyped by the indicated envelope, with (+cyt) and without (-cyt) cytokines, and analyzed after 2 weeks of culture. Each bar represents the average frequency and standard error (two to six experiments performed, depending on the envelope) of GFP expressing-cells in suspension culture and CFC assay, as indicated. For suspension culture analysis  $p=0.022$  for the VSV comparison,  $0.097$  for the A-MLV comparison and  $0.058$  for the RD114/TR, calculated by signed rank two-sided tests.

## 4. Restriction of LV transduction in HSPCs

### 4.1 Effect of proteasome inhibition on HSPC lentiviral gene transfer

We then evaluated other factors influencing the post-entry steps of viral infection. Because of its emerging effector role in many cellular response pathways and its postulated antiviral activity observed in earlier studies of HIV-infected lymphocytes (Schwartz, Marechal et al. 1998), we assessed the influence of the proteasome on LV transduction of HSPCs.

In a first set of experiments we transduced CD34+ cells with the standard dose of LV-GFP, with or without cytokine stimulation, and added different doses of the reversible peptide aldehyde proteasome inhibitor MG132 (Kisselev and Goldberg 2001) during the transduction period. After transduction cells were washed in order to remove the inhibitor, and kept in suspension culture. After two weeks, FACS analysis revealed a substantial MG132 dose-dependent increase in the frequency of GFP-expressing cells and in their Mean Fluorescence Intensity (MFI), suggesting increasing vector copy number *per* cell, both for the stimulated and not stimulated cells (Figure 4.1 a,b). Remarkably, at concentrations ranging from 1 to 1,5 $\mu$ M MG132, we were able to transduce virtually all the stimulated cells, and to very high MFI, and up to 90% of the non-stimulated cells. We were not able to further increase the transduction level in non-stimulated cells without reaching toxic doses of MG132. To verify that the observed enhancement of transduction was specifically dependent on proteasome inhibition, we repeated these experiments using the more powerful peptide boronate inhibitor PS-341 (bortezomib) and obtained very similar results to those observed with MG132, although the higher toxicity of PS-341 prevented testing doses higher than 80 nM (Figure 4.1 c,d).

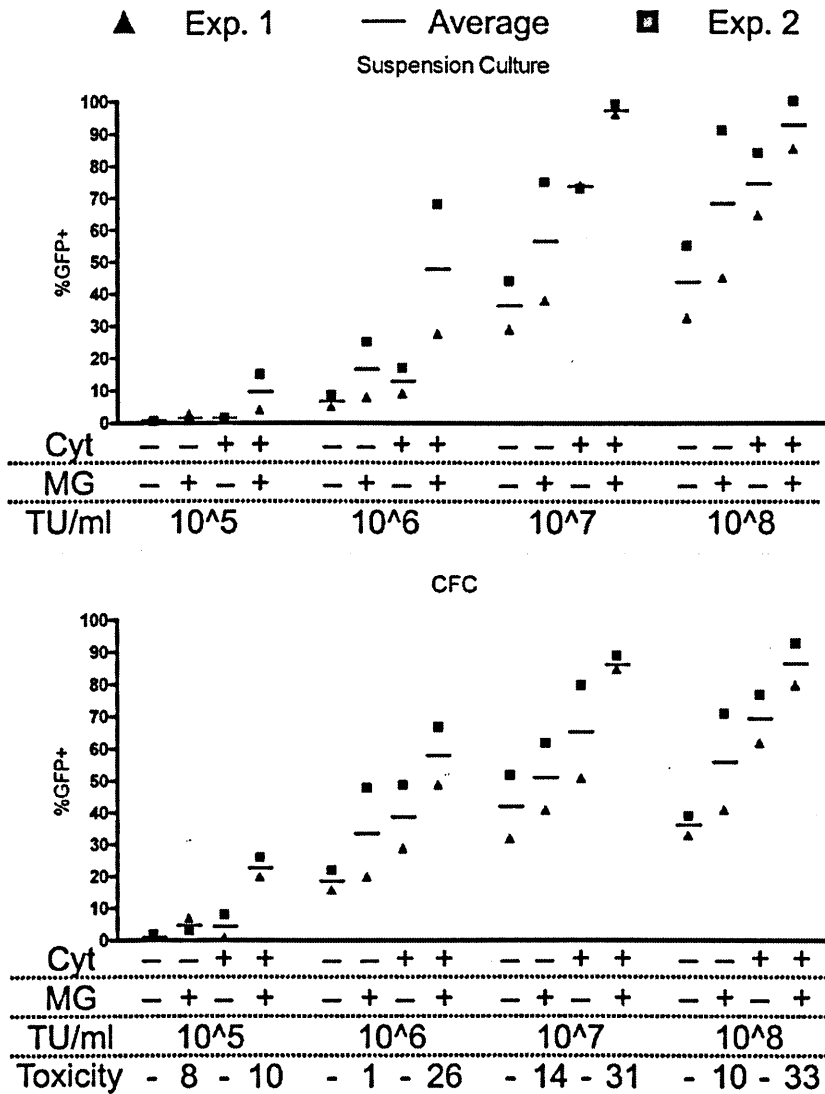


**Figure 4.1. HSPC transduction by LV in presence of increasing doses of proteasome inhibitors.**

CD34<sup>+</sup> cells were transduced with  $10^8$  TU/ml of LV-GFP with (+cyt) or without (-cyt) cytokines stimulation in the presence of increasing doses of the proteasome inhibitors MG132 (A and B) and PS-341 (C and D), and analyzed after two weeks of suspension culture (A and C) Percentage of GFP<sup>+</sup> cells, and (B) and (D) Mean Fluorescence Intensity (MFI). Average of two experiments per panel.

In a second set of experiments we evaluated the effect of a constant concentration of MG132 - 1  $\mu$ M, chosen because it showed the highest difference in response between stimulated and non-stimulated cells - on CD34<sup>+</sup> cells transduced with or without cytokines, and different LV amounts. The same number of cells were treated or not with MG132, transduced and cultured as above, and plated in the CFC assay to test the toxicity of MG132. As shown in Figure 4.2, the proteasome inhibitor substantially enhanced transduction at each vector dose, both for the stimulated and not stimulated cells, whether assayed in suspension cultures or by CFC ( $p=0.008$  and  $p=0.016$  for MG132 treated vs. untreated cells, in +cyt and -cyt conditions, respectively, both for suspension culture cells and CFC; signed rank test).

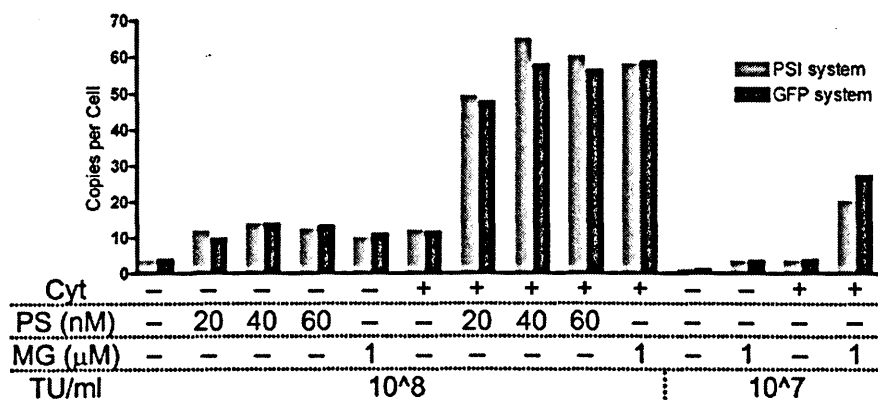




**Figure 4.2. Effect of proteasome inhibition on HSPC transduction by LV.** CD34+ cells transduced with or without cytokines (Cyt) stimulation with increasing doses of LV-GFP (TU/ml), and in the absence or presence of 1 $\mu$ M MG132 (MG), as indicated, and analyzed two weeks after. Upper panel, percentage of GFP-positive cells in suspension culture; lower panel, GFP-positive colonies from CFC assays. Toxicity was calculated from the ratio between the number of colonies arising from MG132-exposed and not exposed cells and shown as percentage of CFC killed. Average (—) and individual values (triangles and squares) from two experiments. Statistical analysis reported in the text.

Concerning the drug toxicity, the stimulated cells showed higher toxicity in response to the inhibitor than the non-stimulated cells. A possible contributing factor to the higher toxicity, however, was the extremely high GFP expression level, and vector integration load, reached in the stimulated cells, as indicated by an increasing percentage of CFC death with increasing LV dose (Figure 4.2 CFC and data not shown).

In order to confirm at the molecular level that the enhancement of transgene expression, observed when proteasome inhibitors were used, was due to an increase in integrated vector copies, we performed real-time Quantitative PCR (Q-PCR) analysis on the DNA of transduced progenitors after 3 weeks of suspension culture. We used two primers-probe systems to amplify different vector sequences and found that both proteasome inhibitors (PS341 and MG132) increased from three- to seven-fold the average vector copy number *per cell*, both in non-stimulated and cytokine stimulated cells. The enhancement was dependent on the inhibitor dose, remarkably reaching an average of 14 and 61 copies *per cell* in non-stimulated and stimulated cells, respectively, at the highest doses of vector and inhibitor (Figure 4.3).



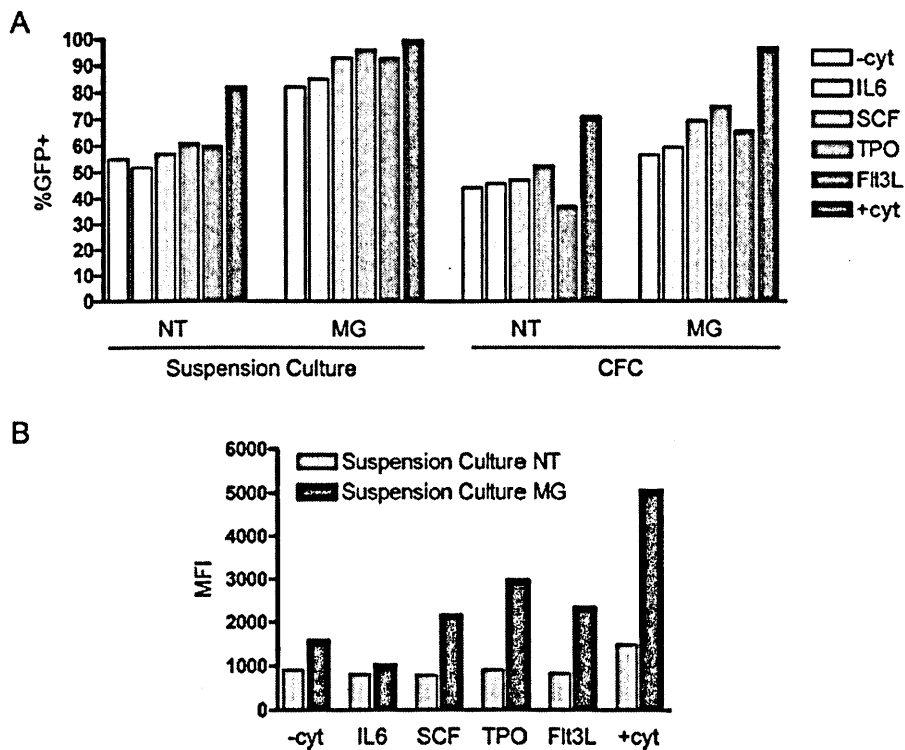
**Figure 4.3. Effect of proteasome inhibition on HSPC transduction by LV: molecular analysis.**

Real-time quantitative PCR analysis of DNA extracted after 3 weeks of suspension culture from HSPCs stimulated or not with cytokines (Cyt), transduced with different doses of LV-GFP (TU/ml) and treated or not with different doses of PS341 (PS) or MG132 (MG), as indicated. Vector sequences were amplified with a system annealing to the  $\psi$  of the vector (PSI system) and a system annealing to the GFP sequence (GFP system). For each condition average of three detections is shown.

Overall, these data indicated that the proteasome effectively restricted LV gene transfer in HSPCs.

## 4.2 Cytokine effect on proteasome activity of HSPCs

We assessed the contribution of individual cytokines to the observed enhancement of LV transduction by taking advantage of the response amplification mediated by MG132. We transduced CD34+ cells with or without 1  $\mu$ M MG132 and in the absence or presence of IL6, SCF, TPO and Flt3L, tested separately or in combination (Figure 4.4). Each cytokine tested induced only a modest increase in gene transfer as compared to the unstimulated cells, both when the transduced cells were scored in liquid culture and in CFC assay.

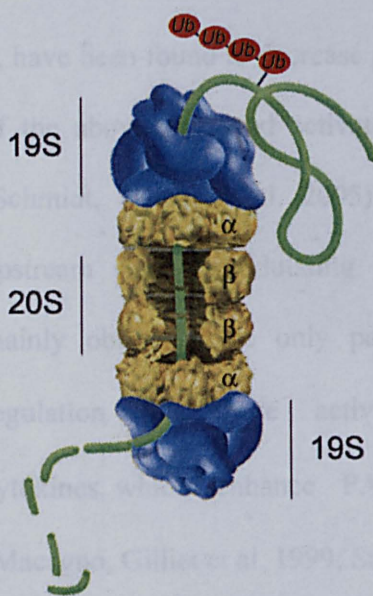


**Figure 4.4. Contribution of individual cytokines to enhanced HSPC transduction by LV.** A) Percentage of GFP+ cells in suspension culture and colonies (CFC) derived from CD34+ cells transduced with  $10^8$  TU/ml of LV-GFP without (-cyt) or with the indicated cytokine, added separately (IL6, SCF, TPO, Flt3L) or all together (+cyt), and with (MG) or without (NT, not treated) 1 $\mu$ M MG132. B) Mean Fluorescence Intensity (MFI) of suspension culture cells shown in A. One experiment of two performed with similar results is shown.

Although the effect of the separately tested cytokines was barely detectable in the absence of MG132, addition of the proteasome inhibitor during transduction revealed a higher response than that seen in non-stimulated cells. When comparing cells stimulated with only one of the cytokines with cells stimulated by

their combination, the effect of cytokines on gene transfer appeared to be synergistic. This was indicated by comparing both the average fluorescence of the transduced cells, which reflected the average number of transduced vector copies *per cell*, and the frequency of transduction, which reached saturation at 100%.

The 26S proteasome is an ATP-dependent, multifunctional proteolytic complex found both in the nucleus and in the cytoplasm of all eukaryotic cells. It is responsible for the degradation of a large variety of cell proteins and is essential for many cellular regulatory mechanisms. It consists of 50 subunit organized in the 20S proteolytic core particle sandwiched between two 19S regulatory complexes (Figure 4.5) (for a review on proteasome structure and function see



**Figure 4.5. A schematic of the 26S proteasome. Ub: ubiquitin.**

(Kisselev and Goldberg 2001). The 20S proteasome is a hollow cylindrical particle consisting of four stacked rings; the 2 outer rings are called alpha and the two inner beta. Each beta ring contains three different catalytic activities. All these active sites face the inner chamber of the cylinder, and the only way for substrates to reach this chamber

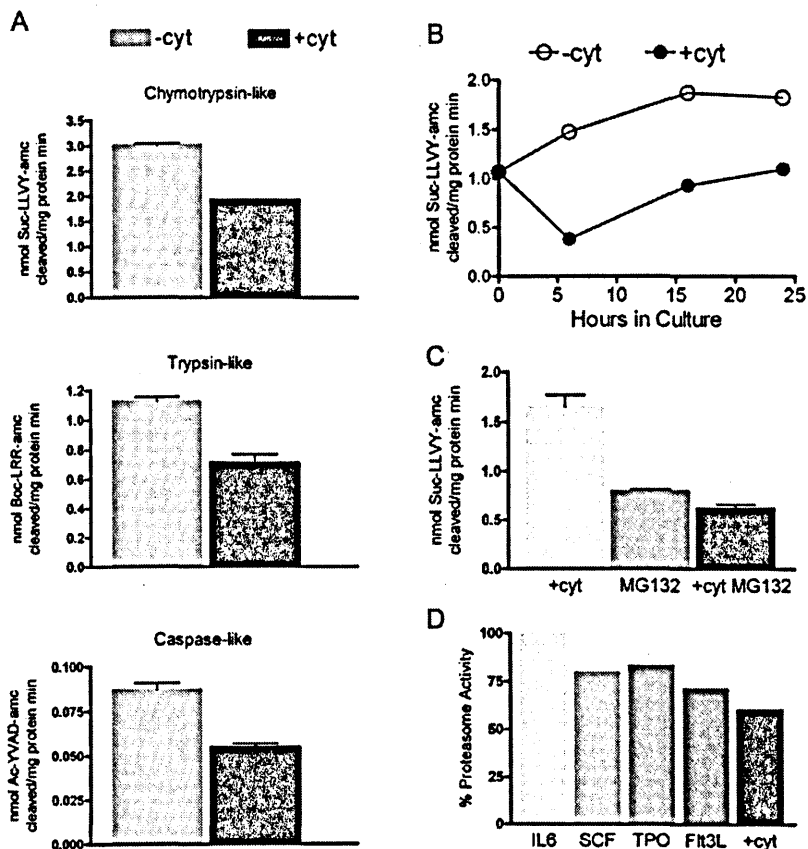
is through the gated channels in the alpha ring. The 19S regulatory particles are associated to the alpha rings and are responsible for recognition, unfolding and linearization of polyubiquitinated substrates and inject them into the 20S particle by using complex ATP-dependent mechanisms. This complex structure, which isolates the catalytic sites from the cytoplasm, ensures that proteins digestion is isolated from the surrounding cytoplasm and prevents uncontrolled destruction of the bulk of cellular proteins. As aforementioned, the 20S core particle contains 6

active sites (3 on each beta ring), which differ in their specificities. Two proteolytic sites termed “chymotrypsin-like” cut preferentially after hydrophobic residues, two termed “trypsin-like” cut preferentially after basic residues and two termed “caspase-like” cut preferentially after acidic residues.

Although in the recent years several molecules have been discovered to be associated with the described basic components of the 26S proteasome, the pattern of regulation of the activity of this complex is very poorly understood. Some cellular factors, such as PA28 (also called 11S) and PA200, have been found to enhance proteasome activity by directly associating with 20S and some cellular, such as PI31 and PR39, and viral molecules, such as HIV TAT and HBV protein x, have been found to decrease proteasomal activity by competing for the binding of the abovementioned activators (for a review on proteasome regulation see (Schmidt, Hanna et al. 2005). However, the mechanisms of action and the upstream signals modulating the expression of activators/inhibitors are still mainly obscure. The only pathways known to be involved in proteasome regulation are those activated by interferon  $\gamma$  and proinflammatory cytokines, which enhance PA28 in professional antigen presenting cells (Macagno, Gilliet et al. 1999; Strehl, Seifert et al. 2005).

Having found that proteasome restriction is active in HSPCs against LV and that cytokine stimulation increases LV gene transfer in these cells, we then asked if there could be a link between the cytokines we used and the proteasome activity. Thus, we tested proteasome activity in CD34+ cells stimulated or not with cytokines. We assayed the proteasomal chymotryptic-, tryptic- and caspase-like activity by using specific fluorogenic peptide substrates and subtracting background activity (caused by non-proteasomal degradation) by addition of the proteasome inhibitors MG132 (for the chymotrypsin and the caspase-like

activities) and  $\beta$ -lactone (for the trypsin-like activity). We calculated reaction velocities from the slopes of the initial linear portions of the reaction progress curves and found that, for all activities tested, cytokine-stimulated CD34+ cells displayed a significantly lower specific activity, compared to not stimulated cells (global p-value=  $2 \times 10^{-18}$ ; calculated by sign test on two experiments with 18 observations) (Figure 4.6 a).



**Figure 4.6. Proteasome activities in HSPCs.**

CD34+ cells were treated as indicated, lysed and tested for proteasome peptidase activities. Peptidase activities were assayed by monitoring the production of 7-amino-4-methylcoumarin (amc) from fluorogenic peptides: Suc-LLVY-amc for the chymotrypsin-like activity, Boc-LRR-amc for the trypsin-like activity and Ac-YVAD-amc for the caspase-like activity. A) Peptidase activities of cells exposed to cytokines (+cyt) or not (-cyt) for 24 hours. Statistical analysis reported in the text. B-D) Chymotrypsin-like activity of cells kept in culture for the indicated times with (+cyt) or without (-cyt) cytokines (B); exposed to cytokines (+cyt), to MG132 (MG132), or to both treatments (+cyt MG132) for 24 hours (C); exposed to the indicated cytokine for 24 hours (D). Values in A-C are mean $\pm$ SD of 3 measurements from one of two experiments with similar results, performed with different cell preparations. Values in D are the average of two experiments, and are expressed as percentage of the proteasome activity of unstimulated cells.

Of note, because apoptosis has been recently shown to decrease the proteasome activity (Sun, Butterworth et al. 2004), and the unstimulated cultures may contain a higher fraction of apoptotic cells, the difference between unstimulated and stimulated cells may even be higher than measured.

It should be noted that we measured proteasome activity at saturating doses of substrate ( $V_{max}$ ) and thus we could not distinguish if the difference observed was due to a difference in enzymatic activity or in amount of enzyme in the two samples. Unfortunately, the measurement of the affinity constant ( $K_M$ ), which could be easily done by performing the assay at non-saturating doses of substrate, is not predictable of the actual activity/kinetic of the proteasome (two enzyme may have same  $K_M$  but different kinetics). The best assay to determine the activity of the proteasome would be to measure the number of molecules of substrate degraded by a single molecule of enzyme *per second* ( $k_{cat}$ ), by performing the measurement on purified proteasomes. On the other hand, Western Blot analysis performed by using antibodies against different subunits of the proteasome would determine the amount of enzyme present in the different samples. However, the unavailability of high numbers of primary HSPCs precluded the use of the latter two assays and thus we relied on the  $V_{max}$  measurement, which is widely used and accepted for testing *in vitro* proteasome activity.

In order to better understand the kinetic of the effect of cytokines on proteasome activity, we then performed a time-course analysis. We seeded CD34+ cells in serum-free medium with or without cytokines, harvested them at different time points and assayed the chymotryptic-like proteasome activity, which accounts for 90% of the total activity of this complex (Figure 4.6 b). Interestingly, we observed that the proteasome activity of fresh (non-cultured)

cells was similar to that of cells stimulated for 24 hours, whereas unstimulated cells progressively up-regulated proteasome activity in culture. Cytokines not only prevented proteasome activity up-regulation, but they also rapidly and strongly down-regulated it. Indeed, at the earliest time point analysed (6 hours) proteasome activity of stimulated cells accounts for less than 50% of the initial activity. Moreover, if we compare unstimulated with stimulated cells at this time point, the difference in specific activity is much more profound than that observed at 24 hours. Indeed, at 24 hours stimulated cells activity was 60% of unstimulated cells, whereas at 6 hours it was 30%.

In the *in vitro* experiments in which we used proteasome inhibitors on stimulated and unstimulated CD34+ cells, we observed an additive effect of cytokines and proteasome inhibitor in enhancing transduction (see Figures 4.1-4.4). In order to verify if this additive effect was present also at the proteasome activity level, we measured the proteasome activity 24 hours after treatment with cytokines, MG132 or their combination (Figure 4.6 c). MG132 down-regulated the proteasome activity more effectively than the cytokines, and the combination was slightly but not significantly more effective than the drug alone.

Finally, we assessed the contribution of each individual cytokine to the observed proteasome activity down-regulation (Figure 4.6 d). Interestingly, we found that 3 out of 4 cytokines present in the complete cocktail affected proteasome activity. Indeed, we observed a moderate down-regulation of proteasome activity when CD34+ cells were exposed to SCF, TPO or Flt3L, while no effect was seen with IL6. However, no cytokine tested alone reached the effect of the cocktail, indicating an additive effect of the three cytokines on proteasome activity down-regulation.

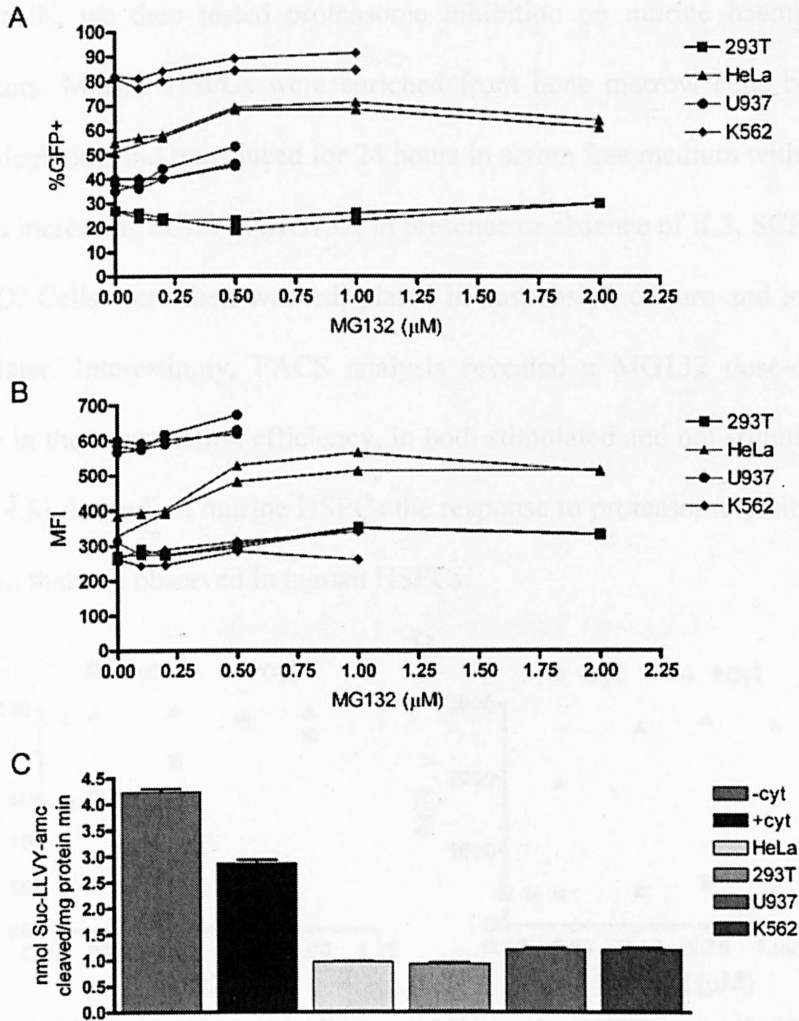


Overall, the rapid onset and the substantial extent of proteasome activity down-regulation induced by cytokines in HSPCs indicate a likely role of this response in mediating the enhancement of LV transduction. However, because the addition of cytokines to cells exposed to proteasome inhibitor further enhanced transduction without likely inducing a comparatively stronger proteasome activity down-regulation, it is conceivable that other pathways activated by cytokines beside their effect on the proteasome contribute to the increased permissiveness to LV transduction.

#### **4.3 Proteasome restriction in different cell types**

In order to verify if proteasome restriction of LVs was common to different cell types, we evaluated the effect of MG132 on the transduction of different cell lines, either of haematopoietic origin (K562 and U937) or not (293T and HeLa). Cells were transduced with a constant LV-GFP dose and with increasing MG132 doses, washed and analysed two weeks later by FACS. Surprisingly, FACS analysis showed that MG132 effect on the level of transduction was much lower for all the cell lines tested than that observed in HSPCs (Figure 4.7 a,b; compare with Figure 4.1). To exclude the possibility that proteasome inhibitor was not effective because the level of transduction for some cell lines had already reached saturation without the drug, we repeated the experiments using lower doses of vector and obtained similar results (data not shown). Then, we asked whether the responsiveness to proteasome inhibition could be linked to the proteasome activity of the cells. Thus, we assayed the proteasome chymotryptic-like activity in all cell types tested. Interestingly, we found a profound difference between haematopoietic progenitors and the cell lines

tested. Indeed, the activity in all cell line extracts was about one third to one fourth of that found in HSPCs, stimulated or not with cytokines, respectively (Figure 4.7 c).

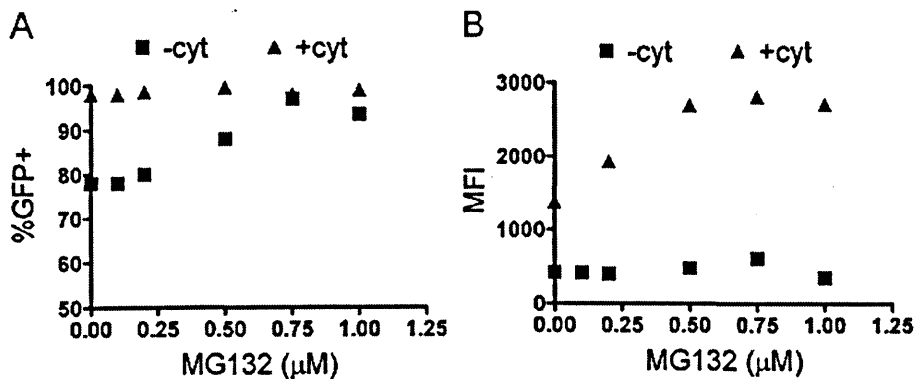


**Figure 4.7. Effect of proteasome inhibition on LV transduction of different cell lines.** A) Frequency of GFP+ cells and B) MFI of 293T, HeLa, U937 and K562 cells transduced with LV-GFP in presence of increasing concentrations (from 0.1 to 2 μM) of MG132 for 12 hours. Missing points at the highest doses in K562 and U937 curves are due to cell death because of MG132 toxicity. Two to three experiments are shown for each cell line. C) CD34+ cells exposed (+cyt) or not (-cyt) to cytokines for 20-24 hours, HeLa, 293T, U937 and K562 cells were lysed and tested for proteasome chymotrypsin-like activity. Chymotrypsin-like activity was assayed by monitoring the production of 7-amino-4-methylcoumarin (amc) from fluorogenic peptide Suc-LLVY-amc. Values are mean±SD of 3 measurements from one of two experiments with similar results.

We also tested the proteasome inhibitor on several primary differentiated human hematopoietic cells, such as T and B lymphocytes and monocytes, and in none of these cells we observed an increase in the level of LV transduction (data not

shown), suggesting that proteasome restriction is related to progenitor cells and that it is not active in differentiated cells.

In order to determine if proteasome restriction was a specific feature of human cells, we then tested proteasome inhibition on murine haematopoietic progenitors. Murine HSPCs were enriched from bone marrow cells by lineage marker depletion and transduced for 24 hours in serum free medium with LV-GFP and with increasing doses of MG132, in presence or absence of IL3, SCF, FLT3L, and TPO. Cells were then washed, plated in suspension culture and analysed 2 weeks later. Interestingly, FACS analysis revealed a MG132 dose-dependent increase in the transduction efficiency, in both stimulated and not stimulated cells (Figure 4.8). Indeed, in murine HSPCs the response to proteasome inhibition was similar to that one observed in human HSPCs.



**Figure 4.8. Effect of proteasome inhibition on LV transduction of murine HSPCs.** FVB mice bone marrow-derived lineage negative cells were transduced with  $10^8$  TU/ml of LV-GFP, with (+cyt) or without (-cyt) cytokines stimulation and in the presence of increasing doses of the proteasome inhibitor MG132. Cells were analyzed two weeks after transduction. A) Percentage of GFP+ cells. B) Mean Fluorescence Intensity (MFI). Average of two experiments is shown.

These results suggest that proteasome restriction of LV is a specific feature of HSPCs, both human and murine, and that it could be linked to the high proteasome activity observed in these cells.

#### 4.4 Effect of proteasome inhibition on SRC lentiviral gene transfer

We then verified the effect of proteasome inhibition on SRC transduction. We transduced CD34+ cells stimulated or not with cytokines with  $2 \times 10^7$  TU/ml of LV-GFP and with or without  $0.7 \mu\text{M}$  MG132, performed *in vitro* assays, and transplanted them into NOD/SCID mice. We chose lower vector and MG132 doses than those used in previous experiments to better detect drug-dependent enhancement of transduction and reduce a possible toxicity. As shown in Table 4.1, suspension culture analysis confirmed our previous results, showing a strong enhancement in gene transfer when proteasome inhibitor was present during transduction in both cytokine-stimulated and unstimulated cells, whether assayed in suspension cultures or by CFC assay.

**Table 4.1. Proteasome inhibition. Analysis of HSPCs assayed *in vitro***

	% GFP <sup>A</sup>	
	Suspension culture <sup>B</sup>	CFC assay <sup>C</sup>
- cyt	42±21	40±8
- cyt MG	63±13	59±4
+ cyt	66±17	63±6
+ cyt MG	90±9	86±14

Average±Standard deviation are shown (n=4 *per* group)

<sup>A</sup>Percentage of GFP expressing cells or colonies, assessed by :

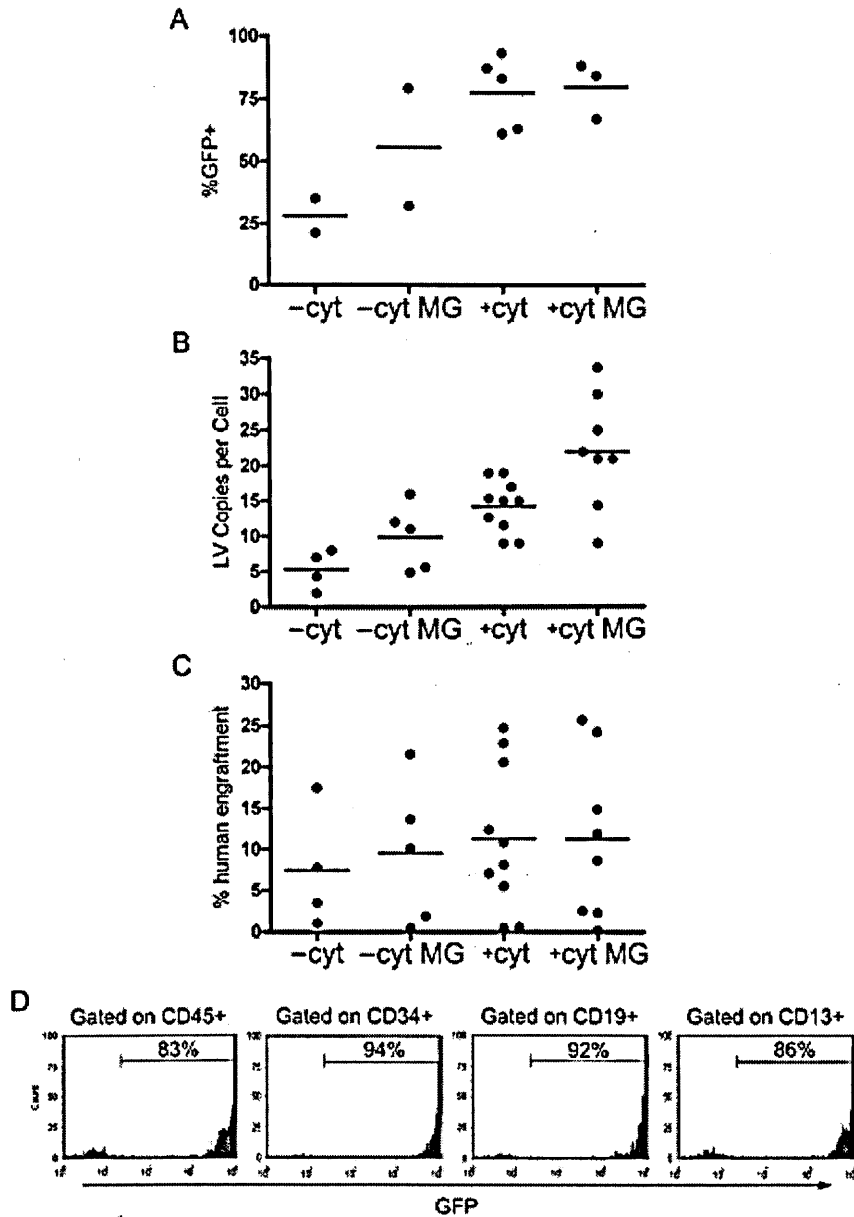
<sup>B</sup>FACS analysis

<sup>C</sup>Fluorescence microscopy analysis

MG:  $0,7 \mu\text{M}$  MG132

Six weeks after transplant we harvested NOD/SCID bone marrow and analysed it by FACS and Q-PCR. FACS analysis showed again a higher transduction frequency in stimulated cells compared to unstimulated. Moreover, we observed higher transduction levels in cells transduced without stimulation in presence of proteasome inhibitor compared to cells transduced without stimulation in absence of proteasome inhibitor (-cyt MG versus -cyt). However, since we reached such a

high frequency of transduction in the stimulated cells, any possible further enhancement by MG132 addition could not be detected (Figure 4.9 a).



**Figure 4.9. Effect of proteasome inhibition on SRC transduction.**

CD34+ cells were transduced with LV-GFP without any stimulation (-cyt), in the presence of 0.7 $\mu$ M MG132 (-cyt MG), in the presence of cytokines (+cyt) or in the presence of both cytokines and 0.7 $\mu$ M MG132 (+cyt MG), and transplanted in NOD/SCID mice. Mice bone marrow was analyzed 6 weeks after transplant by FACS (A) or by quantitative real time PCR (B and C). A) Percentage of GFP-positive cells in the human graft. B) Number of LV integrated copies *per* human cell. C) Percentage of human engraftment, calculated as ratio between human and total (human+murine) DNA. Each dot represents a mouse, for each group the average (—) is shown. D) FACS analysis of a mouse transplanted with cells transduced in presence of cytokines and MG132 and carrying 34 LV copies *per* cell; multilineage repopulation is shown by a similar percentage of GFP+ cells in all the indicated lineages. Percentages were calculated within the gated populations. Human engraftment level was 15%.

Thus, we performed Q-PCR on genomic DNA extracted from bone marrow-derived cells. We developed specific primers and probe systems to evaluate the number of integrated vector copies *per* human cell and determine the level of human engraftment *per* mouse. Indeed, we used a system of primers and probe complementary to the LV Rev Responsive Element (RRE) sequence, a system complementary to the human Telomerase Reverse Transcriptase (TERT) gene and a system complementary to the mouse  $\beta$ -actin gene (Figure 4.9 b,c). Proteasome inhibitor increased the average vector copy number by 2-fold in unstimulated cells and 1.5-fold in stimulated cells. When proteasome inhibitor was used on cells transduced in presence of cytokines we could reach the remarkable number of 34 copies *per* human cell in the graft (highest score) and an average of 23 copies *per* cell (Figure 4.9 b; +cyt MG). Moreover, it should be highlighted that, consistently with the results shown before, cytokine treatment alone increased 3-fold the vector copy number *per* cell. The differences between the four groups were statistically significant (p-value=0.002; n=27).

We also tested the percentage of human cells in the graft of all the mice to verify if such a high vector load and/or the potential MG132 toxicity could have affected SRC engraftment ability. Importantly, we obtained similar engraftment levels in all groups tested (p-value= 0.46; n=27). In addition, when we analysed BM-derived cells for the presence of the different lineages by FACS, we observed multilineage engraftment by transduced cells in all groups. A representative analysis of multilineage engraftment is shown in Figure 4.9 d. The mouse analysed in this panel carries the highest vector copy number scored. These findings suggest that, at least in this model, proteasome inhibition does not have a detrimental effect on SRC repopulating ability.

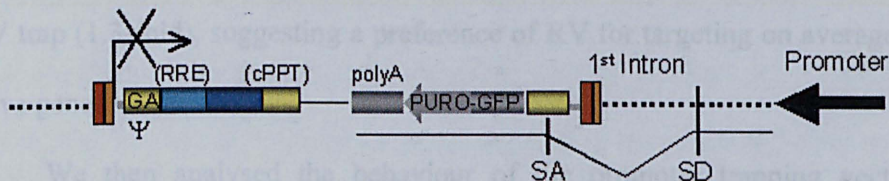
In conclusion, these results prove that proteasome effectively restricts lentiviral transduction in HSCs and highlight a new strategy to overcome the low permissiveness of human HSCs to LV gene transfer.

## 5. LV integration site selection in HSPCs

As aforementioned, the aim of gene therapy is to reach efficient and safe gene transfer. Thus, having evaluated LV transduction efficiency in HSPCs we aimed to evaluate some safety features of LVs. We investigated integration site selection of LV in HSPCs and compared it with integration site selection of MLV-derived Retroviral Vectors (RV). Indeed, vector integration can have detrimental effects on HSPCs, mainly by altering gene expression and inducing aberrant developmental pathways and analysis of integration site selection can help to predict the consequence of the insertional mutagenesis induced by these vectors.

In order to evaluate LV versus RV integrations in HSPCs, we developed genetic traps based on both vectors. We introduced a promoter trapping construct (Medico, Gambarotta et al. 2001) into LV and RV (MLV-based) and compared their trapping efficiency using a puromycin resistance-GFP (Puro<sup>R</sup>-GFP) reporter. We cloned the Puro<sup>R</sup>-GFP gene between a strong cellular splice acceptor (SA) and a polyadenylation (polyA) site (Medico, Gambarotta et al. 2001), and inserted the resulting expression cassette in reverse orientation into SIN RV (Roberts, Cooke et al. 1998) and SIN LV (Follenzi, Ailles et al. 2000), immediately upstream to the modified 3' LTR. The reverse orientation prevents reporter expression from the 5' LTR of the transduced vector when the LTR is not fully inactivated, as in the case of the SIN RV (De Palma, Montini et al. 2005)(see Figure 2 enclosed article). With this cassette design reporter expression is dependent on vector

integration downstream to a transcriptionally active cellular promoter (Figure 5.1). Indeed, only if the vector integrates with the appropriate orientation within a promoter-proximal intron or exon of a transcribed gene, a fusion messenger transcript will be produced and likely processed to express the reporter. The placement of the expression cassette close to the 3' end of the vector leaves most viral sequences outside of the fusion transcript, reducing their potential influence on reporter expression. The frequency and average level of reporter expression may thus readout the frequency of vector integration into active genes, their average expression level, and may uncover preferential integration in the proximity of cellular promoters. If target site selection differs between RVs and LVs according to these parameters, it will also result in quantifiable differences in their promoter trapping efficiency.



**Figure 5.1. A schematic of the vector trap integrated within the first intron of a cellular gene.**

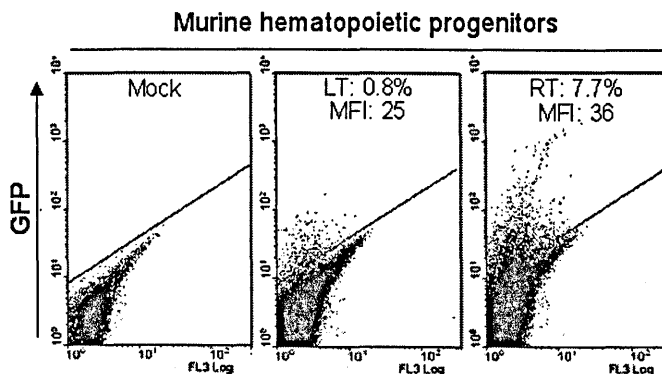
The expression cassette is placed in reverse transcriptional orientation with respect to the vector framework.  $\Psi$ , viral encapsidation signal, including the 5' portion of the gag gene (GA); RRE, rev responsive element, and cPPT, central polypurine tract (LT only); polyA, polyadenylation signal; SD and SA, splice donor and acceptor sites.

We transduced human 293T cells with VSV-pseudotyped stocks of each vector trap. By analysing cell populations containing similar amounts of integrated vector, we found that promoter trapping efficiency, calculated as the ratio between expression and integration, was significantly higher (5 to 6 fold) for the RV than the LV trap (see Figure 1b enclosed article). We reproduced these findings in murine cell lines, such as liver progenitor MLP29 cells (see Figure 1c enclosed article) and endothelial H5V cells. Trapping efficiency was calculated



from cultures expressing the reporter gene in less than 20% of the cells to avoid the possibility that multiple trapping events *per* cell led to non-linear dose-response between integration and expression. These data, together with the mapping of LV- (LT) and RV-based (RT) promoter traps integration sites (see Table 1 and Figure 3 enclosed article), indicated that RV has a higher propensity than LV to integrate into promoter-proximal region. These results are consistent with data recently obtained by high-throughput mapping of RV and LV integrations (Hematti, Hong et al. 2004; Mitchell, Beitzel et al. 2004). Interestingly, also in primary cells, such as activated human peripheral blood mononucleated cells, RT trapping efficiency was higher than that of LT (on average 4 times) (see Table 2 enclosed article) and, as seen with continuous cell lines, the GFP MFI after puromycin selection was higher for the MLV than the HIV trap (1.3-fold), suggesting a preference of RV for targeting on average more active genes.

We then analysed the behaviour of the promoter trapping vectors in HSPCs.

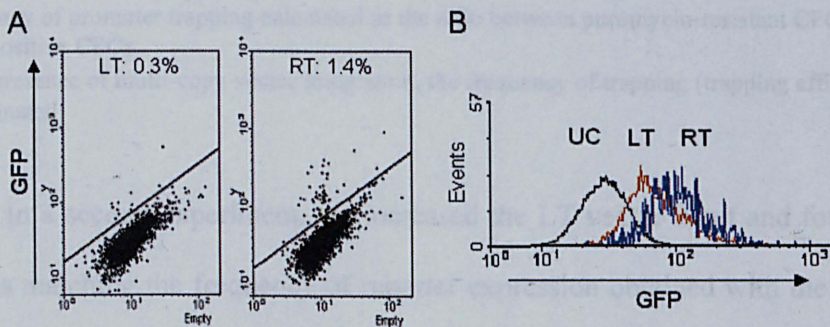


**Figure 5.2. Transduction of murine HSPCs by LT and RT.**

FACS analysis of GFP expression in murine hematopoietic progenitors transduced with matched amounts of the indicated vector traps and analyzed 5 days post-transduction. The frequency and MFI of GFP-positive cells are indicated. One experiment of two performed with similar results is shown.

Murine HSPCs were enriched from bone marrow cells by lineage marker depletion, stimulated with IL3, SCF, FLT3L, and TPO for 24 hrs to reach efficient transduction by both vector types, and transduced with doses of LT and RT matched to yield comparable vector integration amounts. FACS analysis performed after 5 days of culture indicated a sharp difference in reporter gene expression, with the cultures transduced by RT containing a higher frequency of GFP-expressing cells and reaching a higher GFP MFI (Figure 5.2).

CB-derived human CD34<sup>+</sup> cells were stimulated for 36 hrs in medium containing cytokines and then transduced with RT and LT. This protocol was chosen in order to allow comparable transduction levels by RT and LT. We then treated or not the transduced cells with puromycin, and analysed them by FACS for GFP expression, and by clonogenic assays to determine the frequency of puromycin-resistant haematopoietic colonies. In a first experiment, cells were transduced with matched amounts of either vector to yield an average 20% transduction frequency, in order to allow calculating trapping efficiency from the assumption that vector-positive CFCs obtained in these conditions contain a single vector copy.



**Figure 5.3. Transduction of human HSPCs by LT and RT.**

A) FACS analysis of GFP expression in CD34<sup>+</sup> cells stimulated for 36 hrs with a cytokine cocktail, transduced with matched vector amounts yielding low-copy (<1) integration *per* cell, and analyzed after 1 week in suspension culture. The percentage of GFP-positive cells is indicated. Representative results of two experiments are shown. B) FACS analysis of GFP expression (histogram) of puromycin-selected cells from the populations shown in A). UC, unselected cells.

Consistently with the results obtained in other cell types, the frequency of GFP-positive cells in suspension culture (Figure 5.3 a), and the frequency of puromycin-resistant CFCs (Table 5.1, exp1) were significantly higher for cells transduced by RT than LT. PCR analysis for vector-specific sequences in haematopoietic colonies confirmed similar frequency of integration by both vectors at the predicted level (Table 5.1, exp1), showing that also in human HSPCs RT trapped cellular promoters more efficiently than LT. Furthermore, the RT was expressed to an higher extent both in the absence of puromycin selection (not shown), or following selection (Figure 5.3 b).

**Table 5.1. Promoter trapping. CFC analysis of CD34+ cells transduced with LT and RT**

	% vector positive CFCs <sup>A</sup>	% puro-resistant CFCs <sup>B</sup>	Trapping efficiency (%) <sup>C</sup>
LT exp1	23	0.5	2
RT exp1	18	3.3	18
LT exp2	96	7.1	<<7*
RT exp2	23	4.9	21

CFC assays of the same cells shown in Figure 5.3 (exp1) and from a different experiment that used a higher input of LT vector (exp2), plated in semi-solid medium containing or not puromycin.

<sup>A</sup>Frequency of vector-positive CFCs determined by PCR performed on unselected CFCs

<sup>B</sup>Frequency of puromycin-resistant CFCs calculated from the ratio between CFCs grown in medium with and without puromycin

<sup>C</sup>Frequency of promoter trapping calculated as the ratio between puromycin-resistant CFCs and vector-positive CFCs

\*In the presence of multi-copy vector integration, the frequency of trapping (trapping efficiency) is overestimated

In a second experiment, we increased the LT vector input and found that cultures matching the frequency of reporter expression obtained with the RT had multi-copy HIV integration in the CFCs (Table 5.1, exp2). Interestingly, also in these latter transduction conditions (low-copy RT versus multi-copy LT integration), puromycin-selected RT-transduced cells expressed the reporter gene to a higher extent than LT-transduced cells (not shown).

These results provide direct, functional evidence that RVs preferentially integrate close to highly active promoters in HSPCs, and can efficiently exploit them for proviral expression, whereas LVs do not have the same integration bias.

Chapter 6.  
Discussion

## 6.1 Early-acting cytokines and HSC engraftment

Using a stringent assay of competitive xenograft repopulation, we show that human CB HSCs, transduced to high efficiency by a short exposure to LV in presence of SCF, TPO, IL6 and Flt3L, maintain comparable repopulation capacity as cells transduced in the absence of stimulation. Both types of cells contributed similarly to the multi-lineage human graft, and yielded similar frequencies of haematopoietic progenitors, when the mouse BM was analysed 10 weeks after transplant. We also show that the enhancement of LV gene transfer is dependent on the synergy of all four cytokines.

Our marking studies provides rigorous, and until now lacking, proof that early-acting cytokines, which are currently investigated for *ex vivo* HSC expansion, do not impair stem cell properties in the conditions tested. This is in apparent contrast with the results obtained in the NOD/SCID xenotransplant model by Dick's group (Mazurier, Gan et al. 2004). They showed that cytokine-stimulated (with SCF, TPO, IL6, Flt3L and G-CSF) HSCs had an impaired engraftment ability with respect to non-stimulated HSCs in a competitive setting. Although the cocktail of cytokines they used is very similar to ours, they stimulated HSCs for 4 days, whereas we stimulated the cells only for 24 hours. Since we did not observe the detrimental effect on HSCs they showed, this indicates that, beside the choice of the cytokines, the period of *ex vivo* culture is critical to maintain HSC properties.

Also, previous studies reported a decreased homing capacity of CB CFCs after exposure to some of the cytokines used in this study (Ahmed, Ings et al. 2004). Although our results do not suggest such impairment, we cannot exclude that cytokine-stimulated cells may compensate for a decreased homing capacity with a higher *in vivo* self-renewal and/or clonogenicity. A detailed clonal analysis

of the human graft in the repopulated mice may help clarifying these issues, although the biological heterogeneity and the small number of SRCs engrafting each mouse (Ailles, Schmidt et al. 2002; Mazurier, Gan et al. 2004) make very difficult to reliably correlate differences in graft clonal composition to the experimental conditions used for the transplant.

Cytokines may exert anti-apoptotic activity and/or trigger cell growth and replication, according to the target cell type and condition. Although the cell viability tended to decrease more rapidly in unstimulated CD34+ cells, the total number of cells in culture did not change significantly between the two conditions in the short time used in this study, and the SRCs contained within unstimulated cells remained fully competent to engraft and repopulate the mice. On the other hand, because the SRC assay does not provide for extended long-term follow-up and does not support T-lymphopoiesis, we must caution that our analysis cannot exclude detrimental effects of the cytokines on these more-difficult-to-assess stem cell features. Further studies in newly described xenograft models (Weijer, Uittenbogaart et al. 2002; Traggiai, Chicha et al. 2004; Shultz, Lyons et al. 2005) or, more likely, the follow-up of patients treated with LV-mediated HSC gene therapy in upcoming trials will address these issues.

As previously observed (Mikkola, Woods et al. 2000; Ailles, Schmidt et al. 2002), the average gene expression level was higher *in vivo*, in the SRC progeny, than in the *in vitro* outgrowth of the same transduced cell culture, both for cells stimulated and not stimulated with cytokines. This difference indicates a higher transduction rate of SRCs than the more committed progenitors assayed *in vitro*, and may be due to a higher permissiveness of SRCs to LV transduction, or, more likely, to the fact that actively cycling cells, such as committed progenitors, may transit through mitosis during the initial steps of transduction, after viral

entry and before integration, and thus dilute the vector genomes between the daughter cells, as compared to quiescent cells.

## 6.2 HSC cell cycle and LV transduction

The enhancement of SRC transduction by cytokines was not abrogated by treating the cells with the S-phase poison Ara-C after transduction and before transplantation. Thus, we conclude that this enhancement was not due to induction of cell progression into S-phase. Ara-C treated cells yielded a lower, although not statistically significant, level of human cell engraftment in transplanted mice (see Table 3.4), suggesting that a fraction of SRCs, which are induced to proliferate shortly after transplantation, may become sensitive to the delayed effect of Ara-C *in vivo*. We also observed that Ara-C decreased the transduction level of both cytokine-stimulated and non-stimulated SRCs. This suggests that a fraction of SRCs may become sensitive to Ara-C *in vivo* and that cells in this fraction are more permissive to LV. These more permissive cells may be those exiting from quiescence; because of an earlier S-phase induction upon transplantation, they may become sensitive to the delayed effect of Ara-C *in vivo*. The cells less susceptible to LV may be the quiescent cells; because of a delayed S-phase induction upon transplantation, they may be resistant to Ara-C. Because the SRCs successfully engrafting after Ara-C treatment were transduced to substantial levels, these findings strongly suggest that LVs are able to transduce quiescent SRCs. Because previous studies could not rule out that SRCs transduced by LV in the absence of cytokine stimulation represented the fraction of cells already committed to the cell cycle, our data now provide evidence that primitive human HSCs can be transduced as quiescent cells. In this respect, HSCs may behave



differently than lymphocytes that restrict LV transduction, and HIV-1 infection, in the G<sub>0</sub> phase of the cell cycle.

Indeed, even though HIV and HIV-derived vectors can infect/transduce non-proliferating, including terminally differentiated and post-mitotic, cells (Weinberg, Matthews et al. 1991; Lewis, Hensel et al. 1992; Lewis and Emerman 1994; Naldini, Blomer et al. 1996), they are restricted in naïve T cells that are in the G<sub>0</sub> phase of the cell cycle (Korin and Zack 1999; Unutmaz, KewalRamani et al. 1999; Wu and Marsh 2001; Cavalieri, Cazzaniga et al. 2003; Verhoeven, Dardalhon et al. 2003). It is still not clear at what stage of the viral cycle this block is active; what is known is that exiting from quiescence and entering the G<sub>1b</sub> phase, without the need for full activation and proliferation, is sufficient for rendering the cells permissive. Several hypotheses have been proposed to explain this phenomenon, including lack of sufficient levels of dNTPs or lack of necessary transcription factors, such as NFAT, in G<sub>0</sub> cells, but all of them have been disproved in certain experimental settings (Kinoshita, Chen et al. 1998; Korin and Zack 1999; Ducrey-Rundquist, Guyader et al. 2002; Oswald-Richter, Grill et al. 2004). Very recently APOBEC3G has been proposed as mediator of the G<sub>0</sub> block in T cells (Chiu, Soros et al. 2005). Although previous studies demonstrated that this cytidine deaminase restricts HIV only when incorporated into viral particles and only if the viral accessory protein Vif is absent, Chiu *et al.* have proposed a new model for APOBEC3G restriction. According to this model, APOBEC3G is not affected by Vif and can block HIV infection by impairing reverse transcription in quiescent target T cells as part of a low molecular mass ribonucleoprotein complex (for a comprehensive review on APOBEC family and retroviral restriction see (Harris and Liddament 2004; Cullen 2006). However, despite the big effort made to identify defects in HIV infection of resting T cells,

it should be outlined that *in vivo* situation could differ from what has been observed *in vitro*. Indeed, it has been shown that HIV can productively infect naïve quiescent T cells in human lymphoid tissue *ex vivo* and *in vivo* (Zhang, Schuler et al. 1999; Eckstein, Penn et al. 2001; Kinter, Moorthy et al. 2003). Thus, it is possible that the G<sub>0</sub> block of HIV and LV infection in T lymphocytes is related to the *in vitro* setting rather than reflecting the *in vivo* scenario, which could resembles what observed with HSCs: an inefficient, but still effective, transduction of G<sub>0</sub> cells.

### 6.3 Proteasome restriction of LV

Whereas HSCs can be transduced by LV in very short *ex vivo* culture, they require high vector concentration and stimulation with cytokines to reach high gene transfer frequency. Indeed, to obtain similar level of gene transfer in cell lines considered to be permissive to LV, such as HeLa or 293T, a vector dose that is between 100 and 1000 fold lower than what is needed for HSPCs is sufficient. This indicates that HSCs display low permissivity and/or some level of restriction to LV.

In the last years several groups have reported that mammalian cells express factors able to counteract retrovirus and lentivirus infection. Beside the aforementioned deaminating enzymes, different families of cytosolic proteins that inhibit replication by targeting viral uncoating have been identified (for a review on retroviral restrictive factors see (Goff 2004). Although the mechanism of action of these factors is mostly unknown, it was proposed that they could act by targeting the entering virus for degradation by the ubiquitin-proteasome pathway. Starting from these considerations, we tested if inhibition of proteasome could

rescue the restrictive phenotype of HSPCs. Treating the cells with either of two reversible proteasome inhibitors at the time of transduction increased significantly the frequency of transduced cells, indicating that this proteolytic particle represents a major rate-limiting factor in the LV transduction pathway, as it was previously observed for lymphocytes infected by HIV-1 (Schwartz, Marechal et al. 1998). These findings are relevant for both gene therapy application and basic biology studies.

### **6.3.1 Relevance for gene therapy applications**

For gene therapy applications, proteasome inhibitors can be useful tools to enhance LV gene transfer efficiency in HSPCs. Indeed, direct measurement of integrated vector copy numbers in HSPCs transduced in the presence and absence of proteasome inhibitors showed a dramatic increase in transduction of cells exposed to the drugs. When we determined the ratio of vector particles administered that were able to integrate into the target cell chromatin using the best protocol (cytokine stimulation), we discovered that, without proteasome inhibition, only 10% of the transducing units used gave rise to effective transduction (see Figure 4.3; 10 copies *per* cell, using  $10^8$  TU/ml and  $10^6$  cells/ml, which means 100 transducing units *per* cell); when the proteasome inhibitor was present during the transduction, more than 50% of the total administered transducing units integrated into the target cells (50-60 copies *per* cell at highest dose of both PS341 and MG132). This indicates that a large excess of vector particles is uptaken but does not become integrated, and confirm at a molecular level that proteasome effectively restricts LV transduction in HSPCs. The possibility to enhance transduction efficiency may be particularly useful when

using low infectivity LVs, such as LV encoding multiple transgenes or complex expression cassettes, for which reaching high particle concentration may be a challenge. Moreover, our findings may be of particular interest in the context of HSCs derived from BM and MPB. Indeed, these cells are known to be less susceptible to transduction than CB-derived HSCs, and, thus, the proteasome inhibition approach may be even more useful in this setting to enhance transduction efficiency. Although in this work we have shown the effect of proteasome inhibition on HSCs derived only from CB, we have tested MG132 on MPB-derived HSPCs and observed a significant increase in gene transfer efficiency (data not shown).

We have investigated if the proteasome inhibitor can be used to enhance gene transfer in different cell types. Interestingly, we have found a much lower, if any, enhancement of transduction in all the cell lines tested (both haematopoietic and not) as compared to the enhancement obtained in HSPCs. We correlated the response to proteasome inhibition to the proteasome activity, with an enhancement of transduction due to proteasome inhibition related to high basal proteasome activity of the target cells. We have also tested proteasome inhibition on several primary differentiated hematopoietic cells, such as T and B lymphocytes and monocytes, and we have not observed an enhancement in LV gene transfer (data not shown). These data suggest that proteasome restriction may be a specific feature of HSPCs.

Two recent works (Groschel and Bushman 2005; Wei, Denton et al. 2005) reported increased HIV infection of some cell lines by proteasome inhibition. However, the cells were scored 2 days after infection, thus without ruling out transient effects of the treatment on the reporter gene expression. In addition, Groschel *et al.* (Groschel and Bushman 2005) used high doses of inhibitor and

correlated the observed effect with the induction of target cells arrest in the G<sub>2</sub>/M phase of the cell cycle. It is likely that because of the specific experimental conditions these observations involved a mechanism different than that observed in our work. Indeed, an effect due to an induction of the arrest in the G<sub>2</sub>/M phase in HSPCs is not likely since, at least in absence of cytokine stimulation, HSPCs are mostly not proliferating (see Figure 3.4). Moreover, we performed cell cycle analyses on HSPCs and did not observe significant changes in the cell cycle distribution between cells treated and not with the proteasome inhibitor (data not shown).

### 6.3.2 Biological significance

As aforementioned, the discovery of proteasome restriction of LV is relevant not only for gene therapy applications, but also for basic biology studies. Our findings can help to better understand the innate cellular defence against HIV. Indeed, although in this work we have documented evidence only of a restriction against an HIV-derived vector, we have indications that proteasome restriction is active also against a VSV-G-pseudotyped HIV (data not shown). The most intriguing issue is the mechanism by which the proteasome controls lentiviral infection. There are many open questions that need to be addressed and one of them regards the specificity of the restriction. Enhancement of gene transfer by proteasome inhibition has been shown for Adeno-Associated Vectors (AAV) (Duan, Yue et al. 2000; Douar, Poulard et al. 2001; Yan, Zak et al. 2002; Denby, Nicklin et al. 2005; Hacker, Wingenfeld et al. 2005). Although the mechanism is not elucidated, Yan *et al.* showed an increase in capsid ubiquitination when proteasome was inhibited and proposed that this enhanced ubiquitination promoted the completion

of the AAV latent life cycle. This mechanism is probably specific for the life cycle of AAV and it is in contrast to what has been suggested for HIV. Indeed, Swartz *et al.* (Schwartz, Marechal et al. 1998) suggested that the incoming HIV is targeted by ubiquitin to proteasomal degradation. Thus, it is possible that different mechanisms are involved in HIV and AAV proteasome restriction. We cannot exclude that the restriction we reported is active against other viruses. However, our preliminary results would indicate some specificity for LV. In fact, proteasome inhibition did not enhance RV transduction either in human or in murine HSPCs (data not shown).

The specificity of proteasome restriction is strictly linked to the molecular mechanism of the restriction itself and to the step in the infection process that is affected. Since we used VSV-G-pseudotyped vectors, we cannot formally exclude that the antiviral response mediated by the proteasome can be induced by or is specific to the VSV pathway of entry, rather than to HIV. However, both published results (Schwartz, Marechal et al. 1998) and our results using alternate-pseudotyped LV and VSV-G-pseudotyped RV (data not shown) show that proteasome restriction is not VSV-G specific and that it is active also against non-pseudotyped HIV. Thus, it is reasonable to suggest that the infection/transduction early block maps to a step following entry. Despite the intensive study of HIV biology in the last 20 years, the processes and the cellular proteins involved in the viral journey from the cell surface to the cell genome are not fully understood (for a review on HIV post-entry steps see (Dvorin and Malim 2003; Lehmann-Che and Saib 2004; Yamashita and Emerman 2006). In particular, uncoating and nuclear import still need to be clearly elucidated and the studies about proteasome restriction may help to identify novel players in these processes.

We have formulated different hypotheses to explain the mechanism of this restriction. The different mechanisms we propose are not mutually exclusive and could all be involved in controlling transduction. The most obvious hypothesis is that the proteasome acts directly by degrading the uncoating vector core in the cytosol, most likely after it has been targeted for degradation by a specific ubiquitin conjugation system. Ubiquitination and targeting to proteasome of entering virus by a restrictive factor was postulated in the first studies about TRIM5 $\alpha$  (Stremlau, Owens et al. 2004), however a recent work has described the activity of this factor as independent from proteasome activity (Perez-Caballero, Hatziiannou et al. 2005). An alternative hypothesis considers an indirect action of the proteasome: it could either degrade or modulate the activation of cytosolic factors affecting the early steps of transduction. Several cellular response and signalling pathways, such as the heat-shock response, NF- $\kappa$ B activation, apoptosis and cell cycle progression, among many others, are rapidly regulated by the proteasome activity (for a review see (Glickman and Ciechanover 2002; Goldberg 2005) and this modulation may in turn affect the outcome of lentiviral infection. Of particular interest is the activation of NF- $\kappa$ B, because this transcription factor is involved in the inflammatory signalling pathways and it could possibly link what we described as proteasome restriction to the innate immune response to viruses. A third hypothesis implies a role of proteasome as chromatin interacting factor. Indeed, a growing body of evidence demonstrates that components of the proteasome are involved in the regulation of gene transcription. Although for a long time it has been postulated that only the 19S, and thus only the non-proteolytic subunit of the complex, was involved in gene regulation, recently a role of the proteolytic 20S particle has been also demonstrated (Morris, Kaiser et al. 2003; Gillette, Gonzalez et al. 2004). 20S proteasome has been shown to

regulate different steps in transcription by regulating turnover, exchange and recruitment of activators/repressors, by recruiting RNA polymerase and by degrading RNA polymerase in response to DNA damage (for a review see (Collins and Tansey 2006). Thus, the nuclear proteasome could influence LV transduction by affecting integration rather than uncoating or nuclear import. Indeed, proteasome could regulate chromatin accessibility, act during DNA damage response, which is activated by viral integration, and/or change the availability of some transcription factors interacting with the vector (see paragraph 6.5.1 Integration site selection).

#### 6.4 Early-acting cytokines and proteasome activity

In this project we have confirmed our previous results (Ailles, Schmidt et al. 2002), which showed that SCF, TPO, FLT3L and IL6 improved LV transduction of HSCs. Then, we have investigated how the cytokines can enhance permissivity of these cells to gene transfer. Having found that they do not act by inducing early progression to the S-phase of the cell cycle or affecting LV pathway of entry, we asked if they could act at a post-entry level. Post-entry restrictive factors targeting uncoating, such as TRIM5 $\alpha$ , are typically identified by saturating their activity with increasing viral input (Towers, Collins et al. 2002). When we performed dose-response curves in unstimulated and stimulated cells, we did not observe different slopes in the two conditions (data not shown). Thus, we could not overcome the relative resistance of unstimulated, as compared to cytokine-stimulated, cells by increasing the vector input. This suggests that a saturable restrictive factor is not present in unstimulated cells and that cytokines do not enhance transduction by eliminating a cytosolic restriction factor. Instead, we



found that the enhancement of LV transduction by cytokines in HSPCs may involve, at least in part, down-regulation of the proteasome activity in target cells.

By directly testing the proteasome activities in stimulated and unstimulated HSPCs, we showed that they were significantly down-regulated by exposure to the cytokines. Importantly, this cytokines response was rapid, with a maximal effect at the earliest time point analysed (6 hours), in which the proteasome activity of stimulated cells was 30% of the proteasome activity of unstimulated cells. Interestingly, these early time points (6-12 hours) correspond to the period in which the first steps of LV transduction take place. Although it is difficult to estimate the actual level of inhibition reached in cells treated with MG132, the activity recovered after 24 hour exposure was similar to that recovered from cells exposed for 6 hours to cytokines, suggesting that the extent of down-regulation induced by the latter was substantial. However, when we tested the proteasome activity of cells treated with cytokines and MG132 together we did not observe a significant additive effect of the two on the down-regulation of proteasome activity as compared to cells treated with MG132 alone, whereas we observed an additive effect of cytokines and MG132 in enhancing the transduction (see Figures 4.1-4.3). This suggests that other mechanisms, beside proteasome down-regulation, are probably involved in the enhancement of LV permissiveness induced by cytokines.

The down-regulation of proteasome activity in HSPCs as an early response to cytokine stimulation is a novel and intriguing finding, as it likely affects several other cellular functions than permissiveness to gene transfer, and may represent a crucial effector in the signal transduction pathways triggered by the cytokine receptors. Until now and to our knowledge, rapid modulation of proteasome activity by extracellular signals has only been reported for the immunoproteasome

in dendritic cell (Macagno, Gilliet et al. 1999; Strehl, Seifert et al. 2005). Interestingly, SCF, TPO, and Flt3L all had an additive effect on the proteasome activity. Thus, the proteasome may represent a regulatory hotspot at the crossroad of multiple intracellular signalling pathways. Further studies will identify the signalling pathways from the cytokine receptors to the proteasome.

### 6.5 Safety of LV gene transfer in HSPCs

Our studies indicate that, according to the absence or presence of cytokines, HSC gene transfer can be tuned to limit the average level of vector integration, and reduce the risk of insertional mutagenesis, or instead to maximize the frequency of transduction and extent of transgene expression. Because LV integration preferentially targets active genes, the choice between these two conditions may also affect the potential spectrum of integration sites, making less or more likely that cytokine-responsive, growth-related genes are targeted. In preliminary studies performed using a promoter-trapping approach, however, we did not observe differences in the average expression level of vector integration sites between HSPCs transduced in the two conditions.

The use of proteasome inhibitors during transduction further increased HSC transduction by LV. Since we obtained a very high vector copy number in SRCs transduced in presence of cytokines and proteasome inhibitor, further studies are needed to assess the impact on the target cell genome and evaluate the possible consequences of the increased insertional mutagenesis. However, as shown in Figures 4.1-4.3, using proteasome inhibitors in absence of cytokine stimulation allows high-frequency transduction without necessarily increasing the average vector copy number to extremely high levels, thus providing a possible

new strategy to optimise gene transfer. It is conceivable that both minimal and maximal thresholds of vector copy numbers providing the best risk-benefit ratio will have to be set according to each proposed gene therapy application. Moreover, integration sites analysis comparing cells transduced in presence or absence of proteasome inhibitors would be interesting in light of new findings that suggest a role for proteasome in gene transcription (see above and for a review (Collins and Tansey 2006). If these results, mainly obtained in yeast, were confirmed, inhibition of proteasome could also affect the integration site selection of LVs.

#### **6.5.1 Integration site selection**

We used a promoter trap built into LV and RV (MLV) vectors to evaluate integration site selection in the target cell genome. We found a highly significant difference in trapping efficiency between the two vectors and showed that this effect was dependent on a different integration pattern of the two viruses within transcribed genes.

Our study provides direct, functional evidence that RVs preferentially integrate close to highly active promoters. Such results are consistent with recent studies that analysed integration sites and reported preferential occurrence within  $\pm 5\text{kb}$  from transcription start sites of cellular genes (Wu, Li et al. 2003; Hematti, Hong et al. 2004; Mitchell, Beitzel et al. 2004). Notably, with the promoter trapping approach used here, we were able to screen several hundred thousand integrations in primary HSPCs, for both RV and LV. By using a functional readout, our analysis did not rely on the sometimes uncertain “in silico” identification of genes and transcription regulatory elements and on our current

knowledge of the overall extent of genome transcription, and avoided possible biases due to the vector-genome junction retrieval procedures.

Remarkably, one out of five RV integrations in human HSPCs resulted in reporter gene expression. Because only integrations occurring downstream of a promoter and with the proper orientation will express the reporter, the RV bias for integration proximal to active promoters may even be higher than previously estimated. This surprisingly high trapping efficiency may be dependent on specific experimental conditions, such as the inclusion of the wPRE element in the trap that enhances reporter gene expression by stabilizing the transcript (Zufferey, Donello et al. 1999). In addition, by comparing RV and LV integration in similarly infected cells, we consistently found that the RV trap was expressed to a slightly but significantly higher level than the LV trap, suggesting preferential RV integration into a subset of more actively transcribed genes. However, we cannot exclude that the different integration pattern of RV and LV within transcription units may play a role in this finding, given the complex interplay of factors affecting reporter protein expression, including mRNA and protein stability. If verified, the finding of RV integration into a subset of more actively transcribed genes will highlight the specificity of the rules governing integration site selection by different integrating viruses, making a strong case for virus-specific interactions with cellular proteins tethering the integration complex to selected gene sets. Data validating this hypothesis are currently emerging by high-throughput analysis of RV and LV integrations. Targeting of a highly transcribed gene subset by RV may reflect tethering of its integration complex by cellular components recruited at highly active or induced (growth-responsive) promoters. Promoter targeting may have evolved in the parental retroviruses to provide a fraction of their progeny with a supplementary cellular promoter, less susceptible

to silencing by genome surveillance mechanisms than the LTR promoter (Bestor 2000). The proximity and functional relationship frequently established between RV proviruses and flanking cellular promoters in our study indicate that integration of enhancer-rich wild-type MLV LTR is likely to up-regulate transcription from cellular promoters and, depending on proviral position and orientation, overexpress the cognate genes, including proto-oncogenes. These results correlate with the well-known oncogenicity of wild-type  $\gamma$ -retroviruses (Mikkers and Berns 2003), and underline the risk of insertional mutagenesis by RVs.

HIV and HIV-derived vectors have been reported to integrate more frequently within transcribed genes than outside of them (Schroder, Shinn et al. 2002; Mitchell, Beitzel et al. 2004). However, since they insert throughout the transcriptional unit without showing a bias for promoter-proximal integration (Wu, Li et al. 2003) and our data), the promoter trapping efficiency of LV traps was much lower than that observed for RV traps. Preferential intragenic integration of LVs was recently reported in primary haematopoietic cells (Imren, Fabry et al. 2004; Mitchell, Beitzel et al. 2004) and integration intensity was shown to positively correlate with transcriptional intensity in one study (Mitchell, Beitzel et al. 2004). Preferential insertion throughout transcriptionally permissive chromatin may benefit the lentivirus lifestyle, which spread horizontally in the cognate host species, target interphase chromatin crossing the nucleopore, and express their own transcription regulators.

These results, together with the advanced LV engineering that fully inactivates the HIV LTR, suggest that LVs provide a safer integrating tool for gene therapy. Although further studies are required in order to fully confirm it, the first evidence of a low genotoxicity of LV integrations has been recently reported

(Montini, Cesana et al. 2006). Still, it remains to be determined the role of the LTR in the genotoxic risk of RVs and LVs. Indeed, Montini *et al.* tested a full LTR RV and a SIN-LTR LV. Thus, it is likely that, beside the different integration site selection patterns of the two vectors, the presence of transcriptionally active LTRs was a major determinant of the genotoxicity of RVs observed in this model. Indeed, very recently Baum's group has tested an improved SIN-LTR RV design and observed a reduced transforming capacity of the SIN constructs with respect to the corresponding LTR vectors in an *in vitro* culture system (Modlich, Bohne et al. 2006; Schambach, Bohne et al. 2006). In conclusion, a full assessment of the actual genotoxic risk of retroviral vectors integration is still missing and further studies are needed to validate vector safety and to advance the prospective applications of HSC-based gene therapy.

## 6.6 Concluding remarks

In this project we have evaluated several aspects of LV transduction of HSPCs and assessed efficient protocols for safe HSC gene transfer. Moreover, we have brought to light novel cellular players in the cell host-virus interaction. Further studies will determine, on one hand, the safety of the proteasome inhibitor approach for HSC gene therapy and, on the other, the molecular mechanisms of proteasome restriction of LVs.

Chapter 7.  
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