Gene Therapy of Hematopoietic and Immune Systems: Current State and Perspectives

Maria Savvateeva[1], Fedor Rozov[1,2] and Alexander Belyavsky[1]
[1]*Engelhardt Institute of Molecular Biology, Russian Academy of Sciences*
[2]*University of Oslo, Centre for Medical Studies Russia, Moscow*
Russian Federation

1. Introduction

Hematopoietic stem cells (HSCs) present arguably the best entry point for gene therapy of hematopoietic and immune systems since genetically modified HSCs are long-lived and would eventually transfer the therapeutic constructs to all their descendants. However, gene therapy via HSCs, although conceptually simple, has proven to be a technically formidable problem that has yet to be solved successfully. Despite overtly positive results obtained in gene therapy experiments performed with mouse and larger animal models, these achievements did not translate into clinically acceptable outcomes for non-human primates and human patients, with exception of a few specific disease instances where a therapeutic gene brought about significant survival advantages to transduced cells (Cavazzana-Calvo et al., 2000, Schmidt et al, 2003). Major differences between outcomes of conceptually similar experiments in mice and primates underscore the notion that the fundamental principles governing functioning of hematopoietic system in small short-lived vs. larger long-lived animals differ significantly. Low degree of chimerism obtained in experiments with primates and humans is likely a result of intrinsically low efficiency of viral transduction of long-term repopulating (LTR) HSCs coupled with subsequent massive silencing of integrated constructs (Ellis, 2005; Horn et al, 2002). One may hypothesize that this situation reflects a better protection of hematopoietic system from external influences, in particular invasion of foreign genetic material, in longer-living animals.

However, our deepening knowledge of molecular mechanisms underlying functioning of HSCs within the organism provides hints as to what strategies may lead to the development of the efficient gene therapy via HSCs; some of these strategies are discussed below.

2. Improvements of vectors and ex vivo HSC transduction protocols

Numerous studies indicate that lentiviral vectors that are capable of transducing non-dividing cells may represent a more promising tool for introduction of genetic material into HSCs compared to retroviral vectors (Uchida et al, 1998, Case et al., 1999). This may be attributed to a largely quiescent nature of LTR HSCs, especially in larger animals (Cheshier et al., 1999, Shepherd et al., 2007). Since even lentiviral vectors transduce more efficiently dividing cells than quiescent ones (Trobridge et al., 2004), the current transduction protocols relied until recently on the use of culture conditions that induced entry of HSCs into cell

cycle but incidentally failed to maintain their stem cell status (Bunting et al., 1999). This situation seems to have been ameliorated after introduction of transduction protocols that rely on the use of serum-free media that lack factors inducing SC differentiation (Mostoslavsky et al., 2005) and novel growth factors that better preserve cell stemness (Zhang C et al., 2008). It remains yet to see whether these improvements are sufficient to significantly increase the efficiency of HSC gene therapy in clinical settings.

3. Selection of genetically modified HSCs in vivo: Negative selection

As current efficiency of transduction of human LTR HSCs with viral vectors appears to be quite low and there are no clinically proven protocols for expansion of these cells ex vivo, the most promising solution at hand to this problem is an in vivo selection of modified cells after their transduction and re-transplantation back to a patient. Conceptually, one might distinguish negative and positive in vivo selection strategies. The first one can be defined as a strategy that is aimed at elimination of stem and progenitor cells that do not bear integrated functional constructs. Positive selection implies a strategy that does not target the construct-negative stem cells but rather provides selective survival and growth advantage to the cells that bear the inserted construct. The negative selection gains presently much of attention and seems to be better poised for a clinical advancement in the near future. Arguably, the most promising and advanced variant of negative selection is based on the use of O6-MGMT as a selection marker and various alkylating compounds as selection agents (Davis et al., 2000, Ragg et al., 2000). Using this approach and multiple rounds of selection in vivo, overall peripheral blood chimerism has been driven in mice and larger animal models to levels higher than 75%. However, the clinical applicability of this technique is as yet unclear, as recent experiments performed by two research teams with non-human primates using MGMT-mediated selection produced rather conflicting results. One team demonstrated successful implementation of this strategy in monkeys, although with selection efficiencies and chimerism rates highly variable between individual animals (Beard et al., 2010), whereas another team reported a rather negligible increase in chimerism rates upon selection in vivo (Larochelle et al., 2009).

Various implementations of negative selection strategy are listed in the Table 1.

4. Selection of genetically modified HSCs in vivo: Positive selection

Ongoing studies of the mechanisms controlling HSC self-maintenance and commitment continue to identify novel factors that bring about HSC expansion in vivo when over-expressed. A less than exhaustive set of these factors is listed in the Table 2. Arguably, the most extensively studied gene with such properties is the homeobox transcription factor HoxB4. Forced expression of HoxB4 in murine HSCs induces remarkable ex vivo and in vivo cell expansion without compromising their differentiation or inducing leukemic transformation (Sauvageau et al., 1995, Antonchuk et al., 2002). Similar effects were obtained using recombinant TAT-HOXB4 protein (Krosl et al., 2003). In some reports, HoxB4 and negative selection marker MGMT were used together to further increase percentage of modified HSCs (Chinnasamy et al., 2005). However, attempts to use HoxB4 for positive selection of HSCs in larger animals were much less successful, with a major expansion of short-term repopulating cells only (Zhang X et al., 2006). Besides, a significant number of leukemia occurrences apparently related to unregulated expression of HoxB4 were observed in these animals (Zhang X et al., 2008).

Slective marker	Selecting agent	Mode of action	References
O6-MGMT	BCNU, TMZ, other alkylating agents	MGMT protein functions to repair alkylated DNA caused by chemotherapeutic agents like BCNU or TMZ	Sawai et al, 2001; Zielske et al, 2003
Thymidylate synthase	5-fluorouracil (5-FU) 5-fluorodeoxy-uridine (5-FUdR)	Drug-resistant TS can protect bone marrow cells from 5-fluorouracil (5-FU) and related fluoropyrimidines that induce cessation of DNA and RNA synthesis, and subsequent cell death.	Bielas et al, 2009
Tyr22DHFR	Methotrexate	MTX acts on highly proliferative cells, blocking DNA synthesis through competitive inhibition of DHFR. Drug resistant dihydrofolate reductase such as Tyr22 (Tyr22DHFR) has the potential to selectively increase engraftment of gene-modified human hematopoietic cells	Gori et al, 2010
Multidrug resistance gene-1 (MDR)	Taxol, Paclitaxel	Overexpression of the multidrug resistance gene MDR1 in bone marrow cells results in protection from hematopoietic toxicity from chemotherapy drugs that are substrates for the MDR1 drug efflux pump	Cowan et al, 1999

Table 1. Strategies for negative selection of genetically modified HSC

Some other members of the HOX family, either alone or fused with specific cellular partners, are also able to induce expansion of hematopoietic progenitors in mice. Of particular importance is a fusion gene NUP98-HoxA10, which has a remarkable ability of multi-log expansion of murine repopulating cells ex vivo, exceeding that of HoxB4 (Ohta et al., 2007; Watts et al., 2011).

Recently, the powerful effect of overexpression of early acting transcription factor SALL4 on ex vivo expansion of human hematopoietic cells capable of long-term repopulation of NOD/SCID mice was demonstrated (Aguila et al., 2011). Significant ex vivo expansion could be also achieved using recombinant TAT-SALL4B protein.

There are at least a dozen of other genes that, when overexpressed, induce significant expansion of HSCs in mice in vivo. One of the most interesting groups of such factors are epigenetic regulators. Of particular interest is Bmi1, a member of Polycomb group, which is involved in regulation of mantenance of various adult stem cell types. Inactivation of Bmi1

leads to defect in HSC self-renewal (Park et al., 2003), whereas its enforced expression results a striking ex vivo expansion of multipotential progenitors and marked augmentation of HSC repopulating capacity in vivo (Iwama et al., 2004). In addition, enforced expression of Bmi1 in human CD34-positive cells leads to the ex vivo expansion of NOD/SCID repopulating cells (Rizo et al., 2008). Another Polycomb group gene that potentially could be used for positive selection is Ezh2; upon overexpression, it prevents HSC exhaustion (Kamminga et al., 2006). Forced expression of yet another epigenetic regulator, histone demethylase Fbxl10/Jhdm1b in HSCs abolishes exhaustion of the LTR HSCs following serial transplantation. This property of Ezh2 and Fbxl10/Jhdm1b makes them especially appropriate for schemes combining positive and negative selection since the latter one places very significant stress on hematopoietic system.

Another group of genes that might be used for positive selection are those that are frequently activated in predominant hematopoietic cell clones arising after retro- or lentiviral transduction, and are likely therefore to act as factors inducing in vivo expansion of these clones. The most prominent among such genes are MDS1/Evi-1 (Sellers et al., 2010; Métais & Dunbar, 2008), PRDM16 (Du et al., 2005; Ott et al., 2006) HMGA2 (Wang et al., 2010; Cavazzana-Calvo et al., 2010) and LMO2 (McCormack et al., 2003; McCormack et al., 2010). As a note of caution, forced expression of these genes may produce undesired effects; for example, expression of Evi-1 was reported to be associated with chromosomal instability (Stein et al., 2010).

In addition to protein factors, micro RNAs also have effect on HSC function and population size. In particular, miR-125a and miR-125b were shown to increase number of HSCs in vivo or enhance their repopulation capacity (Guo et al., 2010; Ooi et al., 2010).

Having focused on genes that expand stem cell population, one should not overlook another group of genes that exert an opposite effect, namely negative influence on HSC pool size. Thanks to RNA interference technology, suppression of gene expression in various cell types nowadays is nearly as simple as overexpression. If gene knockout or knockdown results in expansion of stem cell population, this property may potentially be used for positive selection. Among genes of interest in this respect are C/EBP alpha, Lnk and Nur77, to name a few. C/EBP alpha-deficient hematopoietic stem cells (HSCs) are hyperproliferative, have increased expression of Bmi-1 and enhanced competitive repopulating activity (Zhang et al. 2004; Heath et al., 2004). Inactivation of Lnk, inhibitory adaptor protein, leads to an expanded HSC pool with enhanced self-renewal (Bersenev et al., 2008). Mice with inactivation of both Nor-1 and Nur77 have abnormal expansion of HSCs and myeloid progenitors and develop lethal acute myeloid leukemia (AML).

Regardless of what gene is being used for positive selection, it is clear that its constitutive expression would eliminate one or more of the negative growth controls imposed on HSCs by organism, and thus increase risks of neoplastic transformation. Therefore, any clinically acceptable protocol for gene therapy using positive selection of transduced HSCs should be based on transient, tightly regulated gene expression. Given that positive selection, if correctly implemented, promises to provide significant advantages over negative selection schemes, further research into creation of robustly regulated expression systems for positive selection in HSCs seem to be fully warranted.

Gene	Observed effects	References
HOXB4	Overexpression of HoxB4 induces significant ex vivo and in vivo expansion of murine long-term repopulating HSCs.	Antonchuk et al., 2002; Sauvageau et al., 1995
NUP98-HOXA10	Enforced expression of NUP98-HOXA10 fusion protein results in significant expansion of murine repopulating cells ex vivo exceeding that of HoxB4.	Ohta et al., 2007; Watts et al., 2011
NF-Ya	Murine HSCs overexpressing NF-Ya demonstrate strongly increased in vivo repopulation.	Zhu et al., 2005
Bmi1	Enforced expression of Bmi1 leads to striking ex vivo expansion of multipotential progenitors and marked augmentation of HSC repopulating capacity in vivo.	Iwama et al., 2004; Rizo et al., 2008
Ezh2	Overexpression prevents exhaustion of long-term repopulating HSCs.	Kamminga et al., 2006
Fbxl10/ Jhdm1b	Same as above.	Konuma et al., 2011
Jab1	Mice with Jab1 overexpression have expanded HSC pool and develop a myeloproliferative disease.	Mori et al., 2008
HMGA2	Frequently found in the vicinity of integrated constructs in gene therapy trials; HMGA2-expressing cells have growth advantage in competitive repopulation and serial transplantation.	Cavazzana-Calvo et al., 2010; Ikeda et al., 2011; Wang et al., 2010
Evi-1	Frequently found in the vicinity of integrated constructs in gene therapy trials.	Métais & Dunbar, 2008; Sellers et al., 2010
PRDM16	Frequently found in the vicinity of integrated constructs in gene therapy trials.	Du et al., 2005; Ott et al., 2006
Sall4	Enforced expression results in ex vivo expansion of long-term NOD/SCID repopulating cells.	Aguila et al., 2011
MicroRNAs miR-125a, miR-125b	Forced expression of miR-125a was capable of increasing the number of HSCs cells several-fold. Overexpression of miR-125b enhances HSC function, as judged by serial transplantation.	Guo et al., 2010; Ooi et al., 2010
Lnk	Mice with Lnk inactivation have an expanded HSC pool with enhanced self-renewal.	Bersenev et al., 2008
Nur77/NR4A1 & Nor-1/NR4A3	Mice with inactivation of both Nor-1 and Nur77 have abnormal expansion of HSCs and myeloid progenitors and develop lethal acute myeloid leukemia.	Mullican et al., 2007
C/EBPα	C/EBP alpha-deficient HSCs are hyperproliferative and have enhanced competitive repopulating activity.	Heath et al., 2004; Zhang P et al. 2004;
Latexin	Mouse strains expressing lower latexin levels have increased numbers of HSCs.	Liang et al., 2007

Table 2. Genes affecting in vivo expansion of HSCs

5. Expansion and selection of genetically modified HSCs ex vivo

Although much hope is currently invested into various schemes aimed at in vivo selection of gene-modified HSCs, a substantially simpler and arguably more elegant solution may be achieved if protocols for long-term culture and robust ex vivo expansion of HSCs could be developed. Very significant expansion of HSCs that occurs during embryonic development indicates that this might be eventually possible.

Over the last two decades, quite a few HSC culture protocols have been developed. The earlier established conditions involved cultivation in the presence of serum and cocktail of "classical" cytokines including SCF, IL3, IL6, FLT3L and TPO. Since bovine serum apparently contains factors that induce differentiation and/or apoptosis of HSCs, recent, more advanced protocols have been developed, which use defined, serum-free conditions that offer better reproducibility and minimize rapid loss of long-term repopulating HSCs during ex vivo culture and transduction with lenti- and retroviral vectors (Mostoslavsky et al., 2005).

In addition to classical cytokines, a number of new growth factors that have pronounced effect on HSC maintenance and expansion were identified in the last years. Among the most important are FGF1 (de Haan et al., 2003), IGFBP2 (Huynh et al., 2008), and several members of angiopoeitin-like family, in particular Angptl3 and 5 (Zhang et al., 2006).

Several major signaling pathways figuring prominently during embryonic development, in particular during specification of hematopoietic lineage, were shown to be important for adult HSC biology. Among those, Notch and Wnt pathways are currently considered as of the most immediate interest as far as HSC-niche interactions and ex vivo expansion are concerned. Stem and progenitor pool-enhancing properties of Notch signaling were demonstrated initially using constitutive Notch1 signaling in murine hematopoietic cells, which produced immortalized, cytokine-dependent stem cell-like cells (Varnum-Finney et al., 2000), and constitutive Notch4 signaling in human cord blood cells, which resulted in significant increase in cells repopulating immunodeficient mice (Vercauteren & Sutherland, 2004). Later on, culture of human CD34+ precursors with the immobilized Notch ligand Delta1 and cytokines was shown to result in a substantial increase in NOD/SCID-repopulating cells (Delaney et al., 2010); similar results were obtained for mouse cells with immobilized Jagged1 ligand (Toda et al., 2011).

As for Wnt signaling, initial studies indicated that overexpression of activated beta-catenin expanded the pool of HSCs in long-term cultures as judged by both phenotype and function. Wnt3a protein induced self-renewal of haematopoietic stem cells, whereas ectopic expression of inhibitors of the Wnt signalling pathway led to suppression of HSC growth in vitro and reduced reconstitution in vivo (Reya et al., 2003; Willert et al., 2003). Later publications demonstrated, though, that inactivation of the beta-catenin gene in bone marrow progenitors does not impair their ability to self-renew and reconstitute all hematopoietic lineages (Cobas et al., 2004), whereas activation of beta-catenin enforced cell cycle entry of hematopoietic stem cells, thus leading to exhaustion of the long-term stem cell pool (Sheller et al., 2006). Some recent studies demonstrate that it is the non-canonical Wnt signaling promoted by Wnt5a rather than the canonical one, that supports maintenance of competitive repopulating murine HSCs in culture (Buckley et al., 2011; Nemeth et al., 2007).

Yet another line of evidence indicates that activation of beta-catenin in the niche components rather than in HSCs may produce support of LTR cells ex vivo (Nemeth et al., 2009). Currently, there is little doubt that Wnt signaling plays important role in HSC biology, but the issue is apparently more complex than was implied by initial publications and remains highly controversial.

Other embryonic signaling pathways also might be exploited in HSC culture. Morphogens of the hedgehog family, namely Sonic and Indian hedgehogs, are able to support ex vivo expansion of human NOD/SCID repopulating cells (Bhardwaj et al., 2001; Kobune et al., 2004), despite the fact that in vivo Hedgehog signaling seems to not be necessary for adult murine hematopoietic stem cell function (Hofmann et al., 2009). BMP4, a member of BMP superfamily, is a critical component of the hematopoietic niche that regulates both HSC number and function (Goldman et al., 2009), and is able to expand NOD/SCID-repopulating cells in culture (Hutton et al., 2006).

In addition to the use of secreted proteins to for ex vivo HSC culture, one apparent trend of the last years is the application of low-molecular weight chemicals, in particular agonists or inhibitors of particular intracellular signaling pathways, for ex vivo culture. Thus, specific inhibitor of p38 kinase induces self-renewal and ex vivo expansion of HSCs as shown by the in vitro cobblestone area forming cell assay and serial transplantation (Wang et al., 2011). GSK-3β inhibitors, which stimulate Wnt signaling, were shown to promote engraftment of cultured HSCs (Ko et al., 2011; Trowbridge et al., 2006). Of significant clinical interest is the finding that ex vivo treatment with stabilized prostaglandin E2 enhances frequency of both hematopoietic progenitors and long-term repopulating HSCs present as analyzed by competitive transplantation (North et al., 2007). According to other data, only the short-term repopulating HSCs are expanded by this treatment, though (Frisch et al., 2009).

The initial studies demonstrating substantial degree of expansion of HSCs ex vivo relied the use of stromal cells as feeder layers (Moore et al., 1997). Based on the substantial progress in identification of HSC niches in bone marrow, there is currently a revival of interest in development of protocols for co-culture of HSC with stromal cell layers (Chou & Lodish, 2010; De Toni et al., 2011). These stromal cells produce a range of factors that significantly improve the maintenance and expansion of HSCs in culture, most likely by mimicking more or less successfully niche conditions. Very prominent components of the HSC niche are cell surface proteins, in particular cell adhesion molecules. The importance of cell-cell interactions was highlighted by the study by Wagner et al., 2007, indicating that maintenance of primitive hematopoietic progenitors by stromal lines is associated with expression of cell adhesion proteins rather than with secretory profiles of these lines. In particular, N-cadherin was shown to be an important component of the osteoblastic HSC niche (Zhang et al., 2003). However, importance of N-cadherin for HSC-niche interactions was later questioned (Kiel et al., 2007), thus rising substantial controversy. In an elegant in vitro study Lutolf et al. (2009) have shown that N-cadherin, as well as Wnt3a, are the only proteins among those tested that were capable of supporting self-renewal divisions of HSCs in vitro. N-cadherin expression was also shown to be important for maintenance of long-term repopulating cells in culture (Hosokawa et al., 2010). Ability of stromal cell line FMS/PA6-P to support primitive murine hematopoietic cells was found to depend critically on N-CAM expression (Wang et al., 2005). Yet another cell adhesion protein, namely mKirre, plays a prominent role in hematopoietic supportive capacity of OP9 stromal cells (Ueno et al., 2003).

Quite promising developments occur currently in the field of 3-D culture (Yuan et al., 2011; Tan et al., 2010; Miyoshi at el., 2011). Despite a relative paucity of data related to the 3-D culture of HSCs, available publications demonstrate significant advantages of this technique and indicate that in combination with correctly chosen or gene-modified stromal cell layers, 3-D culturing may eventually lead to creation of artificial niche that will be able to support substantial expansion of human HSCs ex vivo.

A question of paramount importance for the field is whether specific combinations of soluble factors will be able to attain a bone fide ex vivo expansion of HSCs, or this goal can only be achieved if specific cell surface proteins produced by the niche cells are also employed in the process, or perhaps the only way to the eventual success is the use of supporting stromal cell layers for ex vivo culture? As a number of molecules that contribute to the maintenance of HSCs in vitro and in vivo continues to rise, and there is a steady improvement in techniques for culturing HSCs, chances are that within a matter of a few years, key combination(s) of specific factors and modes of their application that can produce robust self-renewal and expansion of human HSC ex vivo will be identified. Table 3 provides a list, albeit incomplete, of factors and chemicals that, in addition to "classical" cytokines, are being used for maintenance and expansion of HSCs ex vivo.

6. Pre-conditioning and transplantation regimens

A common practice in the field of HSC gene therapy is a transduction of HSCs using viral vectors in the ex vivo setting. The advantages of this strategy include elimination of non-target transduction events, higher transduction efficiency and better control over the overall process. However, the opposite side of the coin in this case is the necessity for transduced cells to compete with the bone marrow-resident ones, which is likely to lower significantly the degree of chimerism after gene therapy. For efficient repopulation of hematopoietic system with gene-modified HSCs, extensive myeloablative treatments eliminating resident HSCs are usually performed. However, since these treatments are of generalized character and connected with substantial risks of morbidity and mortality, especially for elderly patients, they should preferably be avoided whenever possible. A combination of nonmyeloablative pre-conditioning of the recipient animals with in vivo selection strategy can be used to achieve substantial degrees of chimerism (Davis et al., 2000, Zielske et al., 2003). Additional ways to develop more appropriate pretreatment conditions involve the use of molecules that disrupt key signaling pathways within HSCs or niche components thus inducing HSC loss, as was shown for the case of inactivation of c-kit or mpl signaling by neutralizing antibodies (Czechowicz et al., 2007; Yoshihara et al., 2007), and for combined poly(I:C)/5-fluorouracil (5-FU) treatment (Sato et al., 2009). The other approach for nonmyeloablative HSC transplantation is based on disruption of HSC-niche interactions thus aiding in the stem cell mobilization (Chen et al., 2006). This alternative might grow into clinically relevant technique if the efficiency of current protocols for mobilization of HSCs is further improved. The more HSCs are mobilized into circulation and used for viral transduction, the higher is ratio of transduced vs. resident stem cells and better chances to achieve significant engraftment and chimerism of gene-modified cells without resorting to drastic myeloablative regimens. Although current combinations of mobilizing agents (Ramirez et al, 2009) demonstrate much higher mobilization rates than the initially used G-CSF, there is still a long way to go before this strategy may equal or surpass myeloablative pre-conditioning in its efficiency.

Factor	Observed effects	References
FGF1	FGF1 under serum-free conditions stimulates expansion of serially transplantable, long-term repopulating HSCs.	de Haan et al., 2003
Angptl2, 3 and 5	Proteins of angiopoeitin-like family provide 20- to 30-fold net expansion of long-term HSCs according to reconstitution analysis.	Zhang C et al., 2006
IGFBP2	IGFBP2 enhances ex vivo expansion of mouse HSCs.	Huynh et al., 2008
IL32	IL-32 significantly induces the proliferation of HSCs in culture.	Moldenhauer et al., 2011
Delta 1, Jagged1 (Notch ligands)	Culturing murine or human cells with surface-immobilized Notch ligands resulted in expansion of primitive hematopoietic population.	Delaney et al., 2010; Toda et al., 2011;
Wnt3a, Wnt10b (Wnt canonical pathway)	Wnt3a protein induces self-renewal of haematopoietic stem cells. Wnt10b enhances growth of hematopoietic precursors.	Willert et al., 2003; Congdon et al., 2010
Wnt5a (Wnt non-canonical pathway)	Wnt5a inhibits canonical Wnt signaling and supports maintenance of competitive repopulating murine HSCs in culture.	Nemeth et. al, 2007; Buckley et al., 2011
Shh, Ihh	Sonic hedgehog and Indian hedgehog support ex vivo expansion of human NOD/SCID repopulating cells.	Bhardwaj et al., 2001; Kobune et al., 2004
Bmp4	BMP4 expands NOD/SCID-repopulating cells in culture.	Hutton et al., 2006
TAT-HOXB4 fusion protein	TAT-HOXB4 protein produces significant ex vivo expansion of murine HSCs.	Krosl et al., 2003
TAT-NF-Ya fusion protein	TAT-NF-Ya protein treatment produces several-fold increase in the percentage of human cells repopulating immunodeficient mice.	Domashenko et al., 2010
TAT-SALL4B fusion protein	TAT-SALL4B fusion protein rapidly expands long-term NOD/SCID repopulating cells.	Aguila et al, 2011
Prostaglandin E2	Ex vivo incubation with PGE2 increases the frequency of long-term repopulating HSCs as measured by competitive transplantation.	North et al., 2007
SB203580	SB203580, specific p38 inhibitor, leads to increase in HSC self-renewal and ex vivo expansion.	Wang et al., 2011
StemRegenin 1	SR1, aryl hydrocarbon receptor antagonist, provides substantial increase in cells engrafting into immunodeficient mice.	Boitano et al., 2010
zVADfmk, zLLYfmk	Cord blood CD34+ cells cultured in presence of zVADfmk or zLLYfmk (inhibitors of caspases and calpains, respectively) have a higher ability for engraftment in NOD/SCID mice.	Imai et al., 2010; Sangeetha et al, 2010;
GSK-3 inhibitors	Pretreatment with GSK-3 inhibitors (BIO or CHIR-911) promotes engraftment and repopulation of ex vivo-expanded HSCs.	Ko et al., 2011; Trowbridge et al., 2006
Rapamycin	HSCs cultured in vitro in the presence of mTOR inhibitor rapamycin demonstrate enhanced engraftment.	Rohrabaugh et al., 2011
Copper helators	Copper chelator tetraethylenepentamine increases long-term ex vivo expansion and engraftment capabilities of blood progenitors.	Peled et al., 2004
N-cadherin	N-cadherin expression on stromal cells is important for maintenance of long-term repopulating cells in culture.	Hosokawa et al., 2010
N-CAM	N-CAM expression on stromal cells supports primitive murine hematopoietic cells.	Wang et al., 2005
mKirre	mKirre is responsible for hematopoietic supportive capacity of OP9 stromal cells.	Ueno et al., 2003

Table 3. Proteins and compounds affecting ex vivo maintenance and expansion of HSCs (“classical” cytokines not listed)

There are reports indicating that the engraftment of gene-modified stem cells might be significantly improved by their direct intra-bone transplantation (Mazurier et al., 2003). As irradiation commonly used for preconditioning also damages hematopoietic niche, in particular mesenchymal stem cells, HSC co-transplantation with MSCs was tested and showed promising results (Masuda et al., 2009).

Even a more radical departure from the accepted strategies for HSCs would be in situ transduction of HSCs using systemic or intra-bone delivery of viral vectors (McCauslin et al., 2003, Pan, 2009). Currently, this is a rather hypothetical approach due to serious safety concerns connected with potential off-target modifications of non-hematopoetic cells. However, this strategy alleviates the need for hazardous pre-conditioning treatments and will become a viable alternative with further development of modified viral envelops (Zhang X & Roth, 2010) that target vectors specifically to hematopoietic stem and progenitor cells while minimizing off-target events.

7. Safety: Vector genotoxicity, transposon vectors and other issues

The genotoxicity issue is currently the most immediate and direct safety concern related to the gene therapy using HSCs. Several otherwise successful gene therapy trials of severe combined immunodeficiency using retroviral vectors have resulted in occurrence of leukemia in a significant percentage of patients. Substantial efforts were thus devoted to elucidation of integration patterns and clonal population structure in the hematopoietic compartment after viral transduction, both in experimental models and in clinical trials. The obtained results, although not unanimous, demonstrate nevertheless a frequent occurrence of oligoclonal hematopoiesis after gene therapy, with viral integration sites tending to concentrate in the vicinity of a limited number of genes preferentially involved in growth and proliferation control such as above mentioned Evi-1, PRDM16 or HMGA2. Although upregulation of these genes rarely led to overt neoplastic transformation, it is nevertheless clear that the patients with oligoclonal hematopoiesis are at substantial risk of acquiring leukemias at some future time point.

Various strategies are being currently developed to minimize the risk of neoplastic transformations of HSCs after viral transduction. The most promising approaches include using lentiviral instead of retroviral vectors, and insulators to shield cellular oncogenes from activation by strong viral promoters (Puthenveetil et al., 2004). Insulators, however, tends to significantly reduce viral titers (Nielsen et al., 2009), relatively inefficient (Uchida et al., 2011) and do not provide guarantee against insertional activation of potential oncogenes such as HMGA2 (Cavazzana-Calvo et al., 2010). Another approach is to use promoters specific for differentiated cells that are expected to produce negligible activation of oncogenes in stem cells. However, such promoters tend to provide comparably lower expression levels, and although this might be improved by addition of strong enhancers (Gruh et al., 2008), it is far from certain that such combinations would not activate nearby cellular promoters.

Transposon vectors offer an exciting alternative to retro- and lentiviral vectors. The transposon-based gene delivery combines advantages of integrating viral vectors with those of plasmid vectors. Permanent genomic integration of transposon vectors provides long-term expression, whereas there are significantly fewer constraints on vector design and use

of various function elements like insulators. Transposon systems are inherently less immunogenic than viral delivery systems, whereas their cargo capacity generally exceeds that of retro- and lentiviral vectors (Zayed *et al*., 2004). Initial experiments with transposons were plagued by low efficiency of integration, but continuous improvements in molecular design of transposases have significantly increased the efficiency of integration process (Mátés et al., 2009). Currently, transposons based on Sleeping Beauty (SB) system represent the most advanced version of this technology (reviewed by Ivics & Izsvák, 2011), although other system such as piggyBac are also being perfected (Yusa et al., 2011) and may offer some advantages, such as larger cargo capacity, over the SB system (Lacoste et al., 2009).

Although stable SB transposon-mediated gene transfer into hematopoietic cells was reported (Xue et al., 2009), efficient vector delivery to HSCs remains poorly resolved issue, which is currently being addressed by using electroporation or hybrid lentiviral-transposon vectors (Staunstrup et al., 2009). Although certain undesired effects such as SB transposase cytotoxicity were observed, it seems that they might be minimized by controllable mRNA delivery (Galla et al., 2011). Compared to lenti- and retroviral vectors that show preferential integration near active genes, SB transposon vectors demonstrate nearly random integration profiles (Moldt et al., 2011), although this property might not be shared by other transposon systems (Huang et al., 2010).

Another serious safety concern is a direct consequence of a current low efficiency of transduction of LTR HSCs, which necessitates the use of myeloablative pre-conditioning and negative selection strategies to eliminate competing endogenous HSCs and increase chimerism levels. Negative selection strategies using in particular alkylating drugs place a significant stress upon hematopoietic system. However, as demonstrated by Xie et al., 2010, repetitive hematopoietic stress by busulfan administration in a nonhuman primate may rapidly lead to reduction of polyclonality and eventually to cytopenia. In addition, potential long term mutagenic effects of alkylating agents are largely unknown, thus adding more uncertainty as to correct assessment of risks and benefits of this strategy. Apparently, in order to tackle efficiently the problem of low transduction efficiency, it is not sufficient to rely on the use of negative selection only, but is also important to achieve substantial improvements in ex vivo stem cell culturing, expansion and transduction efficiency. Promising approaches also involve use of positive ex vivo and in vivo selection and in situ transduction strategies.

8. Novel technologies

In the recent few years, a group of new exciting and very powerful technologies, namely cell reprogramming using specific combinations of transcription factors and/or micro RNAs appeared (Takahashi & Yamanaka, 2006; Miyoshi et al., 2011). Much hope is invested into development of strategies aiming at derivation of patient-specific induced pluripotent (iPS) cells similar to embryonic stem (ES) cells, with their subsequent differentiation into hematopoetic cells capable of long-term hematopoiesis. In addition to this indirect reprogramming strategy, methods for direct reprogramming that bypass derivation of iPS cells are also being elaborated. There is one report stating that ectopic expression of Oct4 transcription factor in human fibroblasts is sufficient to convert them into hematopoietic cells with in vivo engraftment capacity (Szabo et al., 2010). However, whether the published

technique may result in production of bona fide hematopietic stem cells capable of long-term reconstitution, remains to be seen. It should be noted that such a goal has not yet been achieved for ES or iPS cells. If efficient reprogramming into HSCs were possible, the perspectives would look staggering. First of all, since starting primary cell populations such as mesenchymal stem/progenitor cells can be propagated for many generations and are amenable for selection of efficient vector integration events, it will be possible to obtain cell populations in which the majority of reprogrammed HCS-like cells bear functioning transgenes, thus increasing efficiency of gene therapy many-fold. Besides, if this technology were able to generate ex vivo significantly more reprogrammed cells with HSC properties than is possible to obtain from a patient, this would establish basis for a radically increase in a level of chimerism after transplantation, thus further improving the efficiency of gene therapy. Of course, the safety issues, in particular potential epigenetic and genome instability of reprogrammed cells that might result in neoplastic transformations, must be addressed especially carefully in this case.

9. Conclusion

Current protocols of gene therapy of hematopoietic and immune system, despite significant efforts by numerous teams worldwide, demonstrate as yet a relatively modest clinical efficiency. However, there are sufficient reasons to assume that many rather inconspicuous yet significant recent technical developments are preparing the field for a decisive breakthrough in the near future. In addition, new cutting- edge technologies such as direct cell reprogramming are entering the scene and may eventually present a radically different and a more efficient solution of the problem. Given all these considerations, the future of gene therapy of blood and immune system diseases looks definitely bright.

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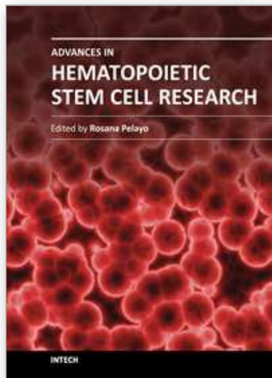

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This book provides a comprehensive overview in our understanding of the biology and therapeutic potential of hematopoietic stem cells, and is aimed at those engaged in stem cell research: undergraduate and postgraduate science students, investigators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

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