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Post-Transduction Events in Retrovirus-Mediated Gene Therapy Involving Hematopoietic Stem Cells: Beyond Efficiency Issues

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Abstract Numerous incremental technological improvements have occurred recently in the application of therapeutic retrovirus-mediated gene transfer into hematopoietic stem cells (HSCs). Improved transduction efficiencies are now reaching levels that may correct some inherited or acquired disorders. Novel retroviral vector systems likewise offer the possibility for an expanded portfolio of treatment approaches. Most importantly, however, investigators are now also focusing efforts on post-transduction events to fully impact correction. Here we describe recent advances in the field, with a special emphasis on the role of post-transduction processes, for correction of disorders or treatments that involve HSCs or their progeny. J. Cell. Biochem. Suppl. 38: 46–54, 2002. © 2002 Wiley-Liss, Inc.

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Gene augmentation and gene replacement hold promise for the therapy of numerous inherited and acquired disorders. The hematopoietic system, which is derived from pluripotent hematopoietic stem cells (HSCs), represents a fundamental bellwether for the development and implementation of this type of therapy. Importantly, HSCs and their progeny are associated with the pathogenesis of many diseases. Equally important from a gene therapy perspective, HSCs are a target cell population that is relatively accessible from bone marrow or mobilized peripheral blood. Proven methods also exist for the preparation of hosts for the successful transplantation of gene-modified HSCs. Indeed, gene transfer and therapy studies using HSCs have been the focus of several laboratories [see for example, Karlsson, 1991; Rosenberg et al., 2000; Williams and Smith, 2000]. In addition to retrovirus-mediated transfer strategies, numerous other viral and nonviral gene transfer technologies have targeted HSCs [Medin and Karlsson, 1997a].

Recombinant retroviruses were among the first gene delivery agents utilized for marking [Joyner et al., 1983] and therapeutic [Willis et al., 1984] gene transfer studies involving the hematopoietic system. In fact, the first overall clinical application of this general therapeutic approach in any system occurred in 1989 when Rosenberg et al. [1990] utilized a recombinant retrovirus to transfer the gene for neomycin phosphotransferase into tumor infiltrating lymphocytes. Four other landmark studies also occurred in the early 1990s. In the first study, gene marking was used to trace the origin of malignant disease relapse in children receiving high-dose chemotherapy and autologous bone marrow transplantation [Brenner et al., 1993]. In that study, persistence of vector-marked hematopoietic cells, at relatively high levels, was observed post-transplantation. The next three studies utilized recombinant retrovirus-mediated gene transfer in the first attempt to use gene therapy to correct an inherited disease characterized by a single gene defect, that being adenosine deaminase deficiency. Interestingly, each study utilized a different hematopoietic

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cell gene transfer target. One study focused on peripheral blood T lymphocytes [Blaese et al., 1995], another focused on peripheral blood lymphocytes and bone marrow HSCs [Bordignon et al., 1995], and a third focused on cord blood HSCs [Kohn et al., 1995]. In these collective studies, the level of gene transfer was relatively low and patients were maintained on enzyme replacement during and after gene-modified cell therapy. Nonetheless, it appears that some level of immune system reconstitution, that persisted for a number of years, may have occurred as a result of the gene therapy.

One fundamental advantage of recombinant retroviruses as therapeutic gene transfer agents is that retroviral genomic integration into the host cell chromosome offers the possibility of stable, long-term expression of the therapeutic or marking transgene. Furthermore, integration allows the progeny of the retrovirally-infected parent cell to also express the transgene. Therapeutic recombinant retroviruses are constructed using "gutted" wild-type virus backbones and are packaged by passage through engineered cells designed to provide necessary viral assembly products in *trans*. The origin and nature of the packaging cell line determines the tropism of the recombinant virus. The resulting virions bind to receptors on target cells, are internalized, integrate into host cell chromosomes after reverse transcription of their single-stranded RNA molecules into double-stranded DNA, and migrate through the DNA pore complex. Retroviruses, especially derivatives of murine oncoretroviruses which have been employed in the clinic, are non-pathogenic and can transfer up to about 10 kb of foreign DNA. One theoretical concern regarding retrovirus-mediated gene transfer is that integration utilizes viral integrases that function in a fairly random manner, which may induce insertional mutagenesis or dormant oncogene activation. However, no adverse events of this type have been reported in any pre-clinical or clinical study involving recombinant retroviral gene transfer to date.

Historically, the relatively low efficiency of retrovirus-mediated gene transfer into HSCs has limited progress in the clinic; indeed, inefficiencies of transfer into primate HSCs still remain a significant obstacle. Note that it is beyond the scope of this prospect to address the multitude of recent advances in HSC biology. For our discussion, HSCs will be defined as a population of cells contained within the CD34 + subset of mononuclear cells that is capable of repopulating ablated individuals with full reconstitution of all blood cell lineages. In addition, while other cells may be more primitive, they are often in lower abundance than the relatively rare CD34 + cell, so that without some type of amplification schema (see below), they are not likely to be present in high enough numbers to effect therapy for many disorders. Importantly, it has also recently been shown that CD34 + cells can also differentiate into non-hematopoietic cells and tissues, which suggests that HSC-targeted gene therapy may eventually be harnessed for an even broader array of therapeutic applications (see below).

The observed inefficiencies of recombinant oncoretrovirus-mediated gene transfer into HSCs likely relates to both a low expression of retroviral receptors on HSCs and the largely quiescent nature of this pluripotent population. The second consideration affects efficiency due to the fact that oncoretrovirus-mediated gene transfer requires cell replication for viral integration [Miller et al., 1990]. Nonetheless, significant incremental improvements in the efficiency of retrovirus-mediated gene transfer into HSCs have been recently observed [see for example, Williams and Smith, 2000]. Such improvements (Table I) include the use of different classes of recombinant retroviruses, adaptation of alternative viral pseudotypes in packaging recombinant virions, addition of attachment factors that co-localize retrovirus and target cells during the infection course, and improvement in cytokine combinations that better support the transduction process targeting HSCs.

The adaptation of recombinant lentiviruses, based on the human immunodeficiency virus [Poznansky et al., 1991; Shimada et al., 1991] and other lentiviruses such as the feline

TABLE I. Recent Advances in Increasing the Efficiency of Retrovirus-Mediated Gene Transfers to HSCs

Strategies to enhance co-localization of virus and cells (fibronectin fragment, "spinoculation")
Employment of alternative viral psuedotypes
Refined cytokine usage to support transduction
New viral systems including recombinant lentiviruses
Serum-free cell isolation and transduction conditions to minimize recipient immune response
Use of high titer produce clones isolated as single cells by selection (drug selection, flow cytometric methods)

immunodeficiency virus [Poeschla et al., 1998], represents a major conceptual, but pragmatically yet unproven, advance for the gene transfer field. Lentiviruses are able to integrate their DNA into non-dividing, although not fully quiescent cells [Korin and Zack, 1998]. As such, this approach may be particularly relevant to gene transfer to HSCs. Further studies will be required to determine, whether the recombinant lentivirus-mediated infection approach can truly improve gene transfer efficiency into long-term repopulating HSCs, however. Successful clinical application of the lentivirus-based gene therapy approach will also require satisfactory responses to safety and regulatory issues, along with successful packaging of recombinant lentiviruses in clinical grade and scale.

Overall, the field of gene therapy has had an undulating history. Early euphoria over the seemingly limitless possibilities that this therapeutic approach offered was soon tempered by an inability to show clear and reproducible clinical responses. Publication of the Orkin-Motulsky report in 1995 verbalized shortcomings and instituted guidelines for self-correction; however, it is clear that scientific, clinical, and ethical concerns still pose obstacles to further progress. Another setback occurred with a fatal immune response observed in a patient in a clinical trial in 1999 mediated by a recombinant adenovirus [Lehrman, 1999]. Yet with this historical background and notwithstanding some of the difficulties mentioned above, it is now intriguing to witness a renewed sense of optimism for clinical gene therapy, especially for retrovirus-mediated gene therapy. This time the optimism appears to be well-founded. Many more practical basic science studies on the technology of gene transfer itself have now been completed. Indeed, the sum of the aforementioned incremental advances in efficiency of retrovirus-mediated gene transfer now meets a minimal threshold for effectiveness for some disorders (see below). Certainly, the possibility that lentivirus-based vectors may further enhance gene transfer efficiencies into pluripotent HSCs has created a great deal of excitement. Stem cell research itself, with its demonstration of alternative HSC sources and broad HSC plasticity, has allowed investigators to envision new indications and therapy using gene-modified HSCs. A final and extremely important advance, which we will emphasize in this Prospect, has been the observation that post-gene transduction events (Fig. 1) can play critical roles in the efficacy of gene therapy. Advances in post-transduction strategies will synergize with other advances targeting HSCs and play a pivotal role in future investigations.

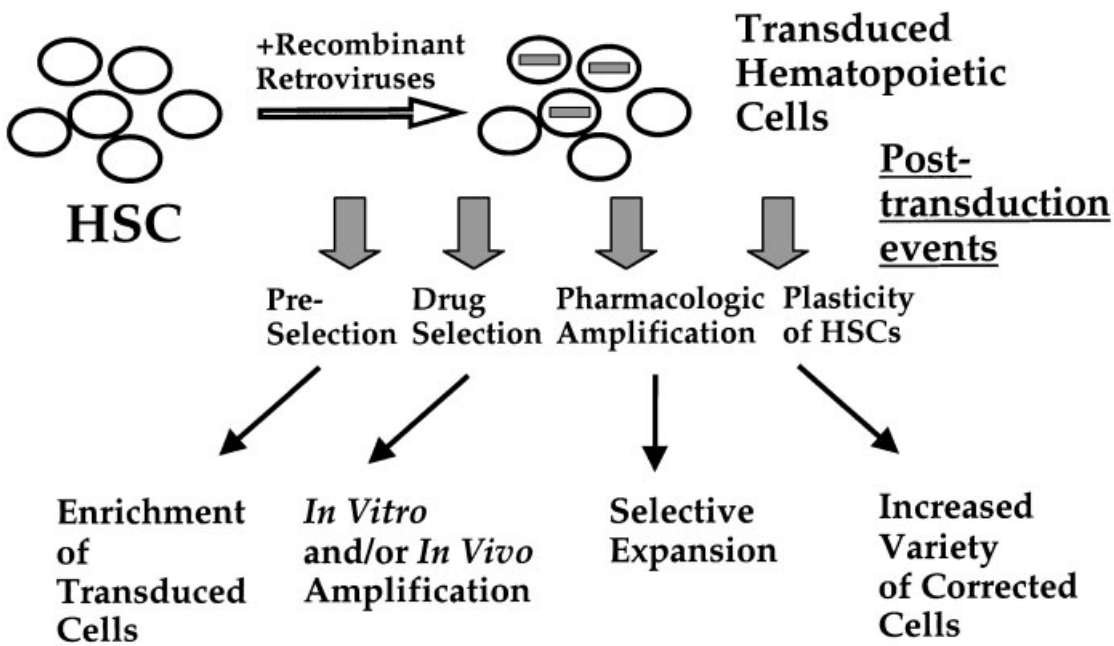

Fig. 1. Post-transduction events to increase the percentages and utility of gene-corrected cells.

Retroviral Gene Therapy of Acquired or Inherited Disorders: Role of In Vivo Post-Transduction Events in Overcoming Low Infection Efficiency

The critical contribution of post-transduction events to the success of oncoretrovirus-mediated gene therapy was clearly illustrated in a truly landmark report published on the genetic correction of human severe combined immunodeficiency-X1 disease (SCID) [Cavazzana-Calvo et al., 2000]. In that study, autologous bone marrow-derived CD34 + cells were transduced ex vivo with a recombinant oncoretroviral vector encoding the gamma chain (γc) of the IL-2 receptor complex. Transplantation of the transduced cells, which were administered without myeloablative or immunosuppressive conditioning, normalized immune function in two pediatric SCID patients. Due to aforementioned technical advances, from an historical perspective the efficiency of gene transfer into CD34 + cells in this study was fairly high (20%–40% and 36% per patient, respectively) and likely contributed to the clinical success. However, more importantly, it is likely that the transduced cells had a distinct in vivo growth advantage relative to nontransduced cells. As such, a critical post-transduction event occurred; because the transduced cells were rendered capable of responding to cytokines that require γc cytokine receptor chain signaling, marked amplification of the corrected T and NK lymphocyte progenitor cells likely occurred in the patients. It is important to note that this potential post-transduction selective pressure may have been reduced in the previously described gene therapy trials involving adenosine deaminase deficiency since patients received enzyme replacement therapy concurrent with gene therapy.

An in vivo selective advantage may have also improved the results of a recombinant retroviral clinical trial involving HSCs transduced with the human multidrug resistance 1 gene (mdr-1) [Abonour et al., 2000]. In that study, peripheral blood CD34 + cells from 11 patients were mobilized and collected, transduced with a retroviral vector encoding the human mdr-1 gene, and transplanted after a cycle of dose-intensive chemotherapy. Patients received the chemotherapy agents carboplatin and VP-16 (etoposide; which is transported by the mdr-1 protein). For the clinical gene transfer field, this study was a significant success, as gene-marked colony-forming units (5%–15% positive) were observed from bone marrow biopsies one year post-transplant in approximately 60% of recipients. Although further studies will be required to fully assess the role of etoposide administration in the success of this strategy, this result further suggests that post-transduction events such as in vivo selective pressure may be critical to gene therapy efficacy in some cases.

In one of our laboratories (Medin), we have demonstrated that another distinct post-transduction mechanism, termed "metabolic cooperativity", contributes significantly to gene therapy success in murine models of an inherited disorder called Fabry disease [Siatskas and Medin, 2001]. In this case the observed effect is not through selective amplification of transduced cells (as above). Instead, overexpression of the therapeutic factor (α-galactosidase A; α-gal A), originating in transduced murine and human hematopoietic cells, leads to intracellular correction along with secretion of α-gal A into the culture medium in vitro and into plasma in vivo. We have found that this secreted α-gal A can then traffic to uncorrected bystander cells and tissues and be taken up and used to reduce the substrate accumulation that characterizes this disease. Thus a relatively low efficiency of gene transfer into HSCs typically can result in measurable systemic therapeutic efficacy. In our murine model of Fabry disease, we have demonstrated that this approach corrects the defect in clinically relevant organs, that these effects are stable over time, and that the therapeutic effect can be transferred by secondary bone marrow transplantation to other Fabry mice recipients [Takenaka et al., 2000]. HSCs are important in Fabry disease since a major source of lipid accumulation is from the breakdown of erythrocytes by macrophages. Thus gene-corrected hematopoietic cells and progeny can target the deficiency at the origin. The Fabry disease model is an important one for lysosomal storage disorders and HSC gene therapy in general, as it demonstrates that correction of the enzyme deficiency at the stem cell level can abrogate disease processes both in HSC progeny and in tissues. In addition, because lipid accumulation in blood vessels accounts for the primary clinical manifestations of Fabry disease, the observation that CD34 + cells can differentiate into vascular endothelial cells [Asahara et al., 1997] provides an opportunity to study the ability of HSC gene

transfer to modulate endothelial cell-mediated processes.

A final illustration of the important role of post-transduction effects in retrovirus-mediated gene therapy of HSCs is found in immune therapy studies. Dendritic cells (DCs) are potent antigen presenting cells derived from CD34+ cells [Liu, 2001] that can function as cellular immunologic adjuvants for vaccine therapies. Investigators have suggested that transduction of human DCs with recombinant retroviruses may allow presentation of immunogenic antigens in the proper HLA context to induce potent and specific immune responses, including both CD4+ and CD8+ T cell immunity. Such an approach may be particularly relevant to cancer vaccines, as was demonstrated in principle for melanoma antigens [Reeves et al., 1996]. Together, our laboratories have recently undertaken studies to adapt this approach to antigens implicated in prostate cancer including prostate specific antigen (PSA) and prostate specific membrane antigen (PSMA) [Medin et al., 2001]. In initial experiments, we have found that murine DCs can be effectively transduced with genes encoding these cancer antigens. Our data indicates that the transduced DCs can present tumor antigen to enhance host B and T cell immune responses and that vaccination of hosts with the transduced DCs can enhance host rejection of specific antigen-expressing tumor cells. Although further advances in this area of immuno-gene therapy may be realized by additional gains in the efficiency of retrovirus-mediated gene transfer, enhancement of post-transduction events will likely be even more critical. For example, in immuno-gene therapy the transferred product is processed by the DC target population into immunogenic peptides that can be presented into MHC class I or class II presentation pathways to CD8+ or CD4+ T cells, respectively, with both cell responses likely to facilitate immune-mediated anti-tumor responses. As such, one focus of our work will be to develop gene transfer methods that facilitate and optimize both class I and II presentation of tumor antigens.

An additional focus of our work, which represents another example of a post-transduction event, will be to optimize the host CD4+ and CD8+ T cell response to a given transferred gene. CD4+ and CD8+ T cells can both exist in functionally-defined subsets, based primarily on their pattern of cytokine secretion [Mosmann et al., 1986; Croft et al., 1994]. CD4+, Th1-type cells and CD8+, Tc1-type cells preferentially secrete the type I cytokines IL-2, and IFN-γ, whereas CD4+, Th2-type cells and CD8+, Tc2-type cells primarily secrete the type II cytokines IL-4, IL-5, and IL-10. Such Th1/Th2 and Tc1/Tc2 T cell subsets are cross-regulatory, for example, with Th2 cells down-regulating Th1-driven immune responses. This cytokine-based immune regulation has been shown to have great in vivo significance in both murine models [Sher et al., 1992] and in human disease states [Romagnani, 1994]. In general, Th1- and Tc1-type immune responses favor the generation of cell-mediated immunity, including anti-tumor immunity. In our own studies, we have shown that allospecific Th1 and Tc1 cells can mediate potent anti-leukemia responses, with Th2 cells actually abrogating such type I immunity against cancer [Fowler et al., 1996]. In light of these findings, we hypothesize that in the setting of retrovirus-mediated immuno-gene therapy using tumor antigen-tranduced DC, shifting of the host CD4+ and CD8+ T cell immunity towards a Th1 and Tc1 cytokine profile will enhance the gene therapeutic effect. To this extent, ongoing studies in our labs are now evaluating the role of host Th1/Th2 and Tc1/Tc2 subsets in the post-transduction modulation of retrovirus-based immuno-gene therapy.

Retrovirus-Mediated Gene Therapy of Acquired or Inherited Disorders: Role of Positive or Negative Selection in Post-Transduction Events Overcoming Low Infection Efficiency

We, and others have undertaken studies to increase the percentage of retrovirally-corrected cells that are returned to, or are resident in, recipient animals [reviewed in Medin and Karlsson, 1997b]. One method utilized has been drug selection, including negative selection. In this approach, investigators insert a drug-selectable gene (such as mdr-1, as mentioned above) into the recombinant retroviral vectors. In the presence of drugs transported by the mdr-1 protein product, such as etoposide, the transduced cells have a selective growth advantage with resultant negative selection of the non-transduced and endogenous host hematopoietic cells. Although this specific procedure has been shown to be effective in vivo [Sorrentino et al., 1992], in one case animals developed leukemia [Bunting et al., 1998]. Furthermore, for many

disorders including Fabry disease, associated local and peripheral toxicities of the selective agents are not likely to be well tolerated.

In contrast to this strategy of in vivo selection, we have been pursuing strategies to enrich for transduced cells in vitro prior to transplantation. Fluorescence enrichment based on co-expression of therapeutic genes and the green fluorescence protein in bicistronic retroviral vector constructs has been previously employed for the positive selection. In an important study describing the utility of this approach, flow cytometry-enriched transduced cells have been shown to repair a genetic deficiency in a murine model of erythropoietic protoporphyria [Pawliuk et al., 1999]. A problem here, however, is that fluorescent protein-based enrichment requires use of high-speed flow cytometric sorting; a technology that is not yet practical enough to isolate large number of transduced cells for clinical utility. Immune responses have also been observed to overexpressed fluorescent proteins as they are of heterologous origin. We have been developing methods to enrich transduced hematopoietic cells with an aim towards direct clinical applicability. To this extent, we have engineered bicistronic retroviral vectors that co-express both the gene therapeutic product (α-gal A) and a cell surface antigen (human CD25) that can be utilized for positive cell enrichment. This method has allowed us to enrich transduced cells from a variety of sources to $>90\%$ purity by positive selection using clinically-relevant bulk immuno-affinity isolation methods [Qin et al., 2001]. In our Fabry disease murine model, we have shown that this positive selection method leads to increased therapeutic enzyme activity in clinically relevant organs after transplantation compared with nonselected cells [Qin et al., 2001]. Recently we have also adapted this technique to transduced murine HSCs (derived from 5-FU treated donor mice) and observed an even more potent enhancement of corrective effects after transplantation (Siatskas et al., submitted). Based on these promising results, we will pursue further studies using positively-selected transduced HSC populations, both in lysosomal storage disease models and in the DC-based immunogene therapy studies.

CONCLUDING REMARKS

In addition to advances mentioned above, a very exciting era is beginning with the recent spate of discoveries concerning the plasticity of HSCs. Pluripotent HSCs are being found in unexpected sites of origin and are also found to be able to program into a variety of cells and tissues not normally thought to be hematopoietic in nature [for example see review by Mertelsmann, 2000, and other authors in this issue]. Retrovirus-mediated correction of these cells opens the door to treatment for a variety of disorders once thought to be inaccessible. It remains to be seen, however, if this observed plasticity is more than just phenomenology and whether these cells can be effectively harnessed to mediate correction of acquired or inherited diseases in the clinic. It also remains to be seen what the effects will be of retrovirus-mediated gene transfer on function and differentiation starting with these cell populations and whether enough cells to effect therapy can be collected and manipulated. Sub-fractionation of starting cell populations will also be important. For example, in some lysosomal storage disorders (excluding Fabry disease), it would be interesting to know which cells of hematopoietic origin have increased ability to home to the brain and nervous tissue and to provide remodeling (and thus enhancing localized metabolic cooperativity). Selection of target cells is a component of this that is also important. As an example, we are undertaking studies in the Fabry mouse model to determine exactly which sub-population of hematopoietic cells that are contributing most to the observed metabolic cooperativity effects in vivo. It may be thus possible to focus gene transfer and amplification strategies to these cells specifically.

Even with a renewed sense of optimism in general in the utility of retrovirus-mediated gene transfer to effect therapy and to target new cell populations and applications, there is still not the blind euphoria that once existed in this field. This time there is more caution. Important and difficult issues remain to be solved. Chief among these are what are appropriate models for studying outcomes employing transduced HSCs? Do small animal results transfer into clinical efficacy? Historically this has not always been the case. So what are appropriate models for studying outcomes? A number of investigators have switched to surrogate assays for human HSC repopulation that involve transplantation of isolated HSCs into immune-deficient mice. Yet these models are not perfect, either. Here, even with supplemented

cytokines, there is variety in cells studied and results obtained. Arguments have also been made that this is effecting selection for human cells capable of growing in a mouse. It remains to be seen if these models and future derivatives fully recapitulate human hematopoiesis in a standard, reproducible fashion. Large animal models, on the other hand, remain limited and take a longer time for the results of transplantation of gene-modified cells to come to fruition. Important results have indeed been seen in sheep models and in primates but few labs have the practical experience or resources to pursue these studies. Another issue is standardization of results. Not only does recombinant retroviral vector titer mean different things to different labs, but evaluations of hematopoietic assays for gene transfer outcomes are quite varied as well.

Other post-transduction events are also important and are the focus of study in a number of laboratories. For example, what happens to the recipient cell after the transduction course? Clearly engraftment of modified HSCs is altered. Can engraftment be renewed or maintained? Should other cells be transplanted simultaneously to best effect therapy? In our studies, would we benefit from co-transplanting specifically reactive T cells generated in vitro to optimize immuno-gene therapy outcomes? Furthermore, what about homing to important tissues for a particular disorder? In our work, what is the appropriate location of injection of transduced cells to best facilitate trafficking for optimal antigen presentation in immuno-gene therapy? Is overexpression of appropriate homing signals or co-stimulatory factors in tandem with cancer antigens necessary to get maximal self-immune responses? Plenty of work needs to be done on host conditioning as well as maintaining the fidelity of transduced cells during and after engraftment. This is especially important since, for a number of disorders (possibly the majority), aggressive conditioning of the host in order to get graft acceptance will not likely be part of the clinical therapeutic regimen.

Long-term gene expression mediated by recombinant retroviral vectors is also not a given. Some investigators maintain that retroviruses are subject to "promoter shut-down" due to host responses-most often thought to be related to promoter methylation in vivo. Does functional pre-enrichment of positively transduced cells possibly abrogate this long-term as has been shown in one study [Kalberer et al., 2000]? Will these results transfer into other systems where the transferred proviral promoter system has not evolved for expression in an ecotropic host?

Another very promising area of research in retrovirus-mediated gene therapy has emerged in the last few years. That of selective expansion of transduced cells. In this approach a given gene transfer efficiency is accepted but post-transduction events such as specific and reversible pharmacological dimerization of cytokine receptors allows selective expansion of transduced cells [see for example, Neff and Blau, 2001]. Other investigators have added homeobox genes to recombinant retroviral vectors [Thorsteinsdottir et al., 1999] to enhance expansion and repopulation while others have constructed responsive fusion polypeptides between hormone responsive proteins and cytokine receptors [Xu et al., 1999] to modulate selective amplification as well. It remains to be seen if these modified cells are fully functional and can repopulate large animal models without leading to unrestricted growth. Nonetheless this approach remains very interesting and exciting and will be the focus of a number of laboratories in the future.

In conclusion, it appears that the field of retrovirus-mediated gene therapy is at a crossroads. Great conceptual and technical advances provide a strong foundation for future developments in therapeutic gene transfer. In this prospect we have detailed numerous examples, whereby progress in gene therapy has been realized through attention to cellular and molecular mechanisms that arise after gene transduction. On an optimistic note, we believe that these examples signal that the field of gene therapy has likely progressed from a largely technical field to one that has truly embraced emerging areas of stem cell biology, immunology, and molecular medicine. Successful implementation of new gene transfer therapies will, therefore, require a multi-disciplinary approach that encompasses new technical advances in vector design, identification of new cellular effectors, and augmentation of post-transduction mechanisms that enhance gene-mediated regulation of disease.

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