HUMAN GENE THERAPY 22:659–668 (June 2011)

DOI: 10.1089/hum.2010.237

Contributions of Gene Marking to Cell and Gene Therapies

Cecilia N. Barese and Cynthia E. Dunbar

Abstract

The first human genetic modification studies used replication-incompetent integrating vector vectors to introduce marker genes into T lymphocytes and subsequently into hematopoietic stem cells. Such studies have provided numerous insights into the biology of hematopoiesis and immune reconstitution and contributed to clinical development of gene and cell therapies. Tracking of hematopoietic reconstitution and analysis of the origin of residual malignant disease after hematopoietic transplantation has been possible via gene marking. Introduction of selectable marker genes has enabled preselection of specific T-cell populations for tumor and viral immunotherapy and reduced the threat of graft-versus-host disease, improving the survival of patients after allogeneic marrow transplantation. Marking studies in humans, murine xenografts, and large animals have helped optimize conditions for gene transfer into $CD34^+$ hematopoietic progenitors, contributing to the achievement of gene transfer efficiencies sufficient for clinical benefit in several serious genetic diseases such as X-linked severe combined immunodeficiency and adrenoleukodystropy. When adverse events linked to insertional mutagenesis arose in clinical gene therapy trials for inherited immunodeficiencies, additional animal studies using gene-marking vectors have greatly increased our understanding of genotoxicity. The knowledge gained from these studies is being translated into new vector designs and clinical protocols, which we hope will continue to improve the efficiency, effectiveness and safety of these promising therapeutic approaches.

Introduction

One of the greatest success stories of gene transfer is the inclusion of genes or truncated gene sequences in hematopoietic stem cells or mature blood lineages to "mark" a target cell population and track their contributions to hematopoiesis or immune function over time. Such genes or gene sequences, most frequently delivered via retroviral vectors, are not inherently therapeutic by themselves, but rather integrate into the cells' genome without disturbing normal cellular functions. The persistence of these gene sequences in the progeny of transduced cells has wide-ranging applications. Since the 1990s, gene marking has been used in both experimental and clinical situations. Marking of T cells or hematopoietic progenitor cells has provided invaluable information regarding cell fate and therapeutic potential. Analyses of marker genes in tumor cells revealed new information regarding the origin of residual disease after autologous transplantation. Preclinical studies using gene marking enabled researchers to optimize *ex vivo* manipulation and transduction of hematopoietic stem cells (HSCs), increasing the efficiency of gene transfer and eventually resulting in clinically efficacious gene therapy in patients with genetic immunodeficiencies and storage disorders. This review will focus on how these gene-marking approaches have contributed to the fields of hematopoietic stem cell transplantation (HSCT), T-cell immunotherapy, and gene therapy. Finally, we will summarize recent insights into the risks of vector integration genotoxicity, using animal models and marking vectors. These results have informed the search for safer methods of gene delivery to HSCs and have important implications for future gene therapy applications.

Available Marker Genes

Because marker genes are designed to facilitate the identification and tracking of gene-marked cells *in vitro* and *in vivo*, ideally they should be safe, nontoxic, nonimmunogenic, and easily monitored and they should facilitate positive immunoselection if purification of marked cells is desirable. Genetically marked cells can be followed either by analysis for expression of a transgene product or via marker detection of vector DNA sequences. Depending on the experimental or clinical situation, there are a number of factors to consider in selecting the optimal gene-marking approach. Table 1 summarizes the major approaches studied to date.

Antibiotic resistance genes such as *neo* were used extensively for the first decade of gene marking and gene therapy

Hematology Branch, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20852.

studies. The *neo* marker gene had practical advantages for vector production, served as an unequivocal DNA tag for transduced cells, and appeared minimally toxic and immunogenic. However, fluorescent proteins such as green fluorescent protein (GFP) rapidly supplanted *neo* for most marking applications following their discovery, given the ease of analysis of GFP-marked cell populations via flow cytometry, microscopy, or even whole-animal imaging. Cell surface molecules, such as truncated forms of the nerve growth factor receptor (ΔNGFR) or CD34, have also been developed, and allow efficient and straightforward immunoselection of transduced cells prior to administration. Recently, a series of marking lentiviral vectors containing drug-selectable fluorescent proteins (LeGo) became available and have great potential for multicolor marking and tracking studies (Weber *et al.*, 2010) Transduction of cells with marking vectors containing independent DNA "bar codes" is another intriguing approach for sophisticated clonal tracking of transduced cells (Gerrits *et al.*, 2010).

Marking T Cells

The high incidence of resistance to conventional cancer chemotherapeutics stimulated the development of T-cell immunotherapies. Interest in knowing whether tumor-reactive T cells homed in on and persisted in tumors stimulated the early application of gene-marking techniques in immunotherapy protocols. A summary of the pioneering studies using gene-marked T cells and the lessons learned from these clinical trials follows.

In 1989, Rosenberg *et al.* conducted the first study using genetically modified cells in a human clinical trial, using a replication-incompetent integrating retrovirus carrying the *neo* marker gene to transduce autologous tumor-infiltrating lymphocytes (TIL) purified from melanoma patients (Rosenberg *et al.*, 1990). The marker gene *neo* was introduced into TIL from 10 patients with metastatic melanoma, and selection of transduced cells was performed by continuous culture in G418 following transduction, prior to re-infusion of the TIL into the patients. Peripheral blood and tumor biopsy specimens analyzed for *neo* gene sequences by PCR revealed the presence of transduced TIL in tumor tissues for up to 64 days post-infusion, with less consistent detection in the circulation, indicating that at least some of the gene-modified TIL homed to the tumor and persisted long term. These early studies demonstrated the potential for adoptive cell therapies and suggested that infusion of genetically

Table 1. Summary of Genetic Marking Approaches

Gene marker	*Advantages*	*Disadvantages*
NEO	No need of high technology methods for detection (Dick *et al.*, 1985) Most data indicates no impact on cell function (Wu *et al.*, 1998)	Selection by G418 resistance is time consuming and time in culture may change cell characteristics, which can have a negative effect should selection by this method be used.
EGFP (and FPs)	Rapid detection and ability to select by fluorescence-activated cell and/or fluorescent microscopy (Hawley *et al.*, 2001; Persons *et al.*, 1997, 1998) Transgenic animals available	Possible immunogenicity (Morris *et al.*, 2004) High level of some FPs toxic (Hanazono *et al.*, 1997) Cell death by free radicals release after prolonged in vitro excitation DNA methylation effects (Liu *et al.*, 1999)
ΔLNGFR	Human protein, nonimmunogenic. Detectable by flow cytometry. Endogenous NGFR not present on hematopoietic cells Commercially available monoclonal antibodies for *ex vivo* immunoselection and detection Reported safe in human clinical trials targeted to T cells (Bonini *et al.*, 2003)	AML in murine studies using ΔNGFR- (Li *et al.*, 2002)
ΔCD19	Human protein Not immunogenic Detectable by flow cytometry. Commercially available monoclonal antibodies for *ex vivo* immunoselection and detection	Limited clinical experience
tCD34	Human protein Detectable by flow cytometry Commercially available monoclonal antibodies for *ex vivo* immunoselection and detection	Impact on homing and differentiation of transduced cells (Fehse *et al.*, 2000)
Drug-selectable FPs	Fluorescent-mediated detection and drug-mediated selection (Weber *et al.*, 2010)	Limited experience

EGFP, enhanced green fluorescent protein; FPs, fluorescent proteins; AML, acute myeloid leukemia; NGFR, nerve growth factor receptor.

modified T cells was safe and feasible. Lessons learned from these early marked TIL studies have informed all subsequent studies from Rosenberg and colleagues, culminating in more encouraging clinical results when immunoablation was given prior to infusion of lymphocytes genetically engineered to target tumor cells (Morgan *et al.*, 2006; Rosenberg and Dudley, 2009).

Donor-derived T cells have been used extensively in allogeneic stem cell transplantation to boost antitumor and antiviral immunity. Genetic marking of these cells has provided valuable information about their trafficking, homing, and efficacy. For example, Epstein Barr virus (EBV)-associated post transplant lymphoproliferative disorder (PTLD) occurs in up to 30% of patients undergoing T-cell–depleted stem cell transplantation. Infusion of donor-derived EBV-specific cytotoxic lymphocytes (CTLs) can successfully treat this complication, but the life-span and distribution of infused CTLs *in vivo* were unknown (Rooney *et al.*, 1995; Heslop and Rooney, 1997). In a series of studies from Heslop, Brenner, and Rooney's groups, EBV-specific donor CTLs were transduced with a *neo* marking vector and infused either preemptively for patients at very high risk of PTLD or as treatment in patients with established PTLD or Hodgkin disease linked to EBV (Heslop and Rooney, 1997; Bollard *et al.*, 2001). The most recent update reported 100% effectiveness of donor EBV-specific CTL infusions in preventing PTLD (101 patients), as well as a remission rate of higher than 80% when EBV-CTLs were used as therapy in 13 patients that had already developed EBV-related tumors following either hematopoietic or solid organ transplantation (Heslop *et al.*, 2010). Twenty-six patients reported in the long-term follow-up analysis received donor EBV-CTLs cells marked with the *neo* transgene, and these patients had similar clinical outcomes to those receiving unmarked CTLs, suggesting full functionality of the marked cells, despite undergoing transduction procedures and having integrated vector within their genome. Marked CTLs could be detected for up to 9 years in the transplant recipients, without evidence for clonal outgrowth or other adverse effects resulting from the presence of vector-containing cells. This series of careful clinical trials were a real tour de force that used gene marking to give further insights into the behavior of EBV-specific CTLs *in vivo* and provided invaluable information for further development of EBV-CTLs as a therapeutic option for the treatment or prevention of life-threatening EBV-related lymphoproliferative disease in transplant patients.

The efficacy of T-cell therapies for the treatment of cancer has been demonstrated by the complete and sustainable clinical tumor responses achieved in patients undergoing allogeneic HSCT (allo-HSCT) followed by infusions of donor T cells. Although donor T cells have been shown to be effective as both graft-versus-leukemia (GvL) and graft-versus-infection (GvI) therapies, the high risk of developing graft-versus-host disease (GvHD) still represents a major obstacle to the utilization of donor T-cell infusions after allo-HSCT.

To overcome this life-threatening complication, Bordignon *et al.* (1995) transduced donor T cells with a "suicide gene" to render them specifically sensitive to drug-induced killing, should deleterious severe GvHD occur following T-cell administration (Bonini *et al.*, 1997). The retroviral vector cleverly encoded both a suicide gene and a selectable cell surface marker gene, allowing *in vitro* selection of transduced cells before infusion into the patients. The marker was a truncated form of ΔNGFR. Endogenous NGFR is not present on hematopoietic cells, and the ΔNGFR form does not transduce any intracellular signals. It has several advantages, including the opportunity for positive selection via commercial immunoselection methods, selection without toxicity, in contrast to cells selected by growth in G418, and most importantly, transgene expression does not interfere with T-cell function (Bonini *et al.*, 2007). After *in vitro* selection for ΔNGFR-positive cells, the pure population of transduced donor lymphocytes can be infused into patients during or after allo-HSCT. Bordignon's group used the *Herpes simplex* virus thymidine kinase gene (*HSVtk)* as a suicide gene, allowing *in vivo* specific killing of vector-containing cells via administration the relatively nontoxic drug ganciclovir. Importantly, this strategy has proved to be effective in treating or to ameliorate GvHD in several Phase I/II clinical trials (Bonini *et al.*, 1997; Tiberghien *et al.*, 1997; Ciceri *et al.*, 2007; Qasim *et al.*, 2007).

Concerns regarding the ΔNGFR marker gene arose when Li *et al.* (2002) reported that a mouse developed acute myeloid leukemia (AML) after receiving bone marrow transduced with a retroviral vector containing ΔNGFR, which correlated with an integration of the provirus into the murine *Evi1* proto-oncogene. Some contribution of the ΔNGFR marker itself to the process, due to some residual potential signaling activity, was hypothesized. However, with time and further investigation it became apparent that this was a rare outcome because no malignant transformations were observed in other studies (Bonini *et al.*, 2003) that used retro- or lentivirus vectors expressing ΔNGFR in transduction of human lymphocytes or murine HSCs. Conversely it became apparent that any integrating gammaretroviral vector, regardless of the transgene, could activate Evi1 and result in adverse outcomes, as described by Metais and Dunbar (2008). Several clinical trials in aggregate including 110 patients, targeting either T lymphocytes or HSCs, with vectors containing ΔNGFR, have been conducted to date, with no reported major adverse effects or development of malignancies (Recchia *et al.*, 2006). Other groups investigated alternative positive selectable marker gene–suicide gene combinations, including a truncated CD34 protein cloned as a fusion with the *HSVtk* gene in either retro- (Fehse *et al.*, 2002) or lentiviral vectors (Qasim, 2007), allowing rapid positive selection of transduced human T lymphocytes via standard CD34 cell selection devices. In a murine model of GvHD, the fusion protein tCD34/*HSVtk* was suitable for selection of transduced cells and also for monitoring the gene-marking level in peripheral blood (Rettig *et al.*, 2004). Although the use of a fusion gene comprising the selectable marker and the gene of interest offers the advantage of equimolar production of a protein responsible for both functions, unexpected consequences can occur. In a recent paper Bennour *et al.* (2008) reported an unusual loss of functionality of the fusion protein tCD34/c*HSVtk* arose from deletions in *HSVtk* that yielded ganciclovir-resistant transduced cells but left intact expression of the $tCD34^+$ surface marker.

A truncated, nonsignaling CD19 (ΔCD19) surface marker was also tested recently for *in vitro* selection and enrichment of donor-positive T cells transduced with the human inducible caspase 9 (iCasp-9) suicide gene (Tey *et al.*, 2007). The

CD19 marker allows rapid *in vivo* tracing of positive T cells after infusion; Di Stasia *et al.* (2010) recently presented the preliminary results of a Phase I clinical trial (CASPALLO). The authors reported that two patients in the trial developed grade I/II GvHD, and that administration of the specific chemical inducer of dimerization (AP1903) that activates iCasp9 and results in apoptosis was effective in eliminating more than 90% of alloreactive T cells after allo-HSCT, completely resolving the skin and liver manifestations of acute GvHD within the first 24 hr. This approach has a number of potential advantages compared to the *HSVtk* suicide gene system. The iCasp-9 suicide gene product is unlikely to be immunogenic, since it consists almost wholly of an endogenous human gene product, in contrast to *HSVtk*, a completely foreign viral protein. Inclusion of HSVtk also precludes use of ganciclovir to treat CMV post-transplantation, without ablation of donor T cells, and thus undesirable impairment of antitumor and anti-infectious immunity, in the absence of GvHD.

Marking HSC/Progenitors Grafts

Source of relapse post autologous transplantation

In some of the initial human clinical trials, gene-marking vectors were used in an attempt to investigate the source of relapse in patients undergoing high-dose chemotherapy and autologous stem cell rescue for a number of malignancies. Brenner and co-workers carried out a series of studies using retroviral vectors carrying the *neo* gene to transduce autologous bone marrow from patients with AML and neuroblastoma (Rill *et al.*, 1992; Brenner *et al.*, 1993a). Tracking the *neo* transgene in malignant cells by PCR or colony forming unit (CFU) selection with G418, they found that some pediatric patients with AML relapsed with gene-marked blasts (Brenner *et al.*, 1993b). In similar gene-marking studies, adults with chronic myeloid leukemia (Deisseroth *et al.*, 1994) who relapsed after receiving autologous HSCT tested positive for the marker gene in bcr-abl positive marrow CFU. These pioneering studies provided absolutely definitive evidence, perhaps not surprisingly, that at least one source of relapse following autologous transplantation can be the graft itself.

However, other trials involving cancer patients did not show evidence of marked cells after transplantation with autologous *neo*-transduced grafts. A considerable number of adult patients with multiple myeloma, breast cancer (Alici *et al.*, 2007; Dunbar *et al.*, 1995; Emmons *et al.*, 1997), follicular lymphoma (Bachier *et al.*, 1999), AML (Cornetta *et al.*, 1992), and also pediatric patients with AML (Heslop *et al.*, 1996) whose underlying malignancy relapsed did not demonstrate marked tumor at the time of relapse. However, lack of detectable marked relapse is much less informative than the converse, since explanations for relapse without detectable marked cells includes lack of transduction of tumor cells *ex vivo*, due to specific features of the tumor cells, versus lack of tumor cells contaminating the graft, and overall poor transduction and engraftment due to suboptimal conditions for *ex vivo* manipulation of the cells.

Genetic marking/tracking studies

As previously reviewed, retroviral transduction conditions worked out using small animal and *in vitro* models in the early 1990s did not translate into successful stable engraftment with genetically modified cells in early human clinical trials (Donahue and Dunbar, 2001; Hu and Dunbar, 2002). Preliminary *in vivo* rodent studies showed potentially clinically relevant levels of engraftment (Ferrari *et al.*, 1991) and evidence of an intrinsic advantage on survival and growth of the transduced cells, which aided engraftment into the host without the need of myeloablation (Ferrari *et al.*, 1992). So, in the early-to-mid 1990s, several groups of European and American investigators performed the first gene therapy clinical trials on humans with adenosine deaminase deficient severe combined immunodeficiency (ADA-SCID), using T cells (Blaese *et al.*, 1995), bone marrow (Bordignon *et al.*, 1995; Hoogerbrugge *et al.*, 1996), or cord blood $CD34^+$ cells as targets of transduction (Kohn *et al.*, 1995). Although one of the studies demonstrated a selective survival advantage of genetically modified T cells after gene transfer, detectable gene-marked lymphocytes in peripheral blood were 0.1–0.01% (Kohn *et al.*, 1995), far below clinical benefit. Thus, the patients continued polyethylene glycol-modified adenosine deaminase (PEG-ADA) enzyme replacement treatment. Similar results were obtained in initial attempts to correct chronic granulomatous disease and Gaucher disease, in which the therapeutic gene does not confer a selective advantage for either survival or growth to the gene-modified cells. In a Phase I clinical trial for X-linked chronic granulomatous disease (X-CGD), the investigators incorporated flt3-ligand and granulocyte-macrophage colony-stimulating factor and utilized fibronectin in a 4-day *in vitro* transduction protocol. The patients underwent multiple cycles of mobilization and infusion of transduced cells without preconditioning. Nine months after transplantation, the levels of gene-corrected neutrophils were 0.06%–0.2%, which is below the desired 5%–10% required for therapeutic effects (Malech *et al.*, 1997). In patients with Gaucher disease, there was low gene marking, little or no expression, and no disease correction following transplantation of retrovirally transduced autologous HPCs (Dunbar *et al.*, 1998).

The gene-marking trials carried out in patients undergoing autologous transplantation for cancer had dual purposes, first to investigate the source of relapse, as detailed earlier, and second to explore gene transfer efficiency and persistence in humans. Even with full myeloablation, patients had levels of marked circulating blood cells significantly less than 1% long-term, if marking could be detected at all (Cornetta *et al.*, 1992; Brenner *et al.*, 1993a; Dunbar *et al.*, 1995; Emmons *et al.*, 1997). However, these studies did unequivocally demonstrate the autologous mobilized peripheral blood $CD34^+$ cells contained long-term repopulating stem cell activity, since very long-term marking from these transduced cells was documented.

Despite the discouraging results of these first attempts, they highlighted the need to improve methods for more effective retroviral gene transfer into true long term repopulating HSCs. These early trials also underscored that some sort of conditioning was very likely necessary to improve engraftment of gene-modified cells. A number of preclinical studies in the late 1990s and early 2000s, using *neo* or GFP marker genes, provided invaluable insights on culture conditions necessary for improving gene transfer into repopulating cells of primates and humans. The use of optimized cytokine cocktails, such as stem cell factor, Flt-3

ligand, and thrombopoietin, together with shortening the *ex vivo* culturing time, enabled increased transduction of more primitive HSCs endowed with long-term repopulating ability (Dunbar *et al.*, 1996; Tisdale *et al.*, 1998). Coating culture flasks with a specific fragment of human recombinant fibronectin to co-localize retroviral particles and $CD34^+$ cells produced demonstrable improvements in the efficiency of transduction into rhesus and human HSCs. Gene-marking levels with potential clinical utility were observed in studies using these optimized conditions (Hanenberg *et al.*, 1996; Dao *et al.*, 1998; Kiem *et al.*, 1998). Additionally, gene marker studies in large-animal models helped address the effectiveness of submyeloablative conditioning (320–500 cGy radiation) prior to HSCT. Persistence of as much as 15% of gene-marked leukocytes was observed in some animals for as long as 33 weeks after transplantation, suggesting that the gene correction may be achievable while reducing transplant-related toxicities (Huhn *et al.*, 1999; Rosenzweig *et al.*, 1999). These and other preclinical studies set the stage for the second generation of human gene therapy trials for inherited immunodeficiencies (Cavazzana-Calvo *et al.*, 2000; Aiuti *et al.*, 2002, 2009; Gaspar *et al.*, 2004; Thrasher *et al.*, 2005; Gaspar *et al.*, 2006; Ott *et al.*, 2006; Boztug *et al.*, 2010), which have been discussed extensively in the literature.

Gene-marking studies in large animals have also been of great interest to study the properties of hematopoietic stem and progenitor cells *in vivo*, offering insights into clinically relevant processes that are difficult to explore directly in any other way. For instance, clonal analysis of granulocyte insertions has resulted in estimates for the frequency and cycling characteristics of primate long-term repopulating cells of the bone marrow. These estimates match those obtained from measurement of telomere attrition (Shepherd *et al.*, 2007). They have also provided direct evidence for short- and long-term repopulating clones, analogous to the distinct populations identified in murine studies (Schmidt *et al.*, 2002). Furthermore, the behavior of individual stem and progenitor cell clones could be tracked following nonmyeloablative conditioning or chronic cytokine therapy (Kuramoto *et al.*, 2004; Laukkanen *et al.*, 2005).

Drug resistance genes for in vivo *selection*

Inclusion of drug resistance genes as *in vivo* selectable markers can increase engraftment of genetically modified HSCs following exposure to drugs that are toxic to unmodified cells in the graft. Such *in vivo* selectable markers include the genes for multi-drug resistance (MDR-1), dihydrofolate reductase (DHFR), and O6-methylguanine-DNA methyltransferase (MGMT). Preclinical studies in mice, and then more importantly in dogs and nonhuman primates that underwent HSCT with MGMTP140K gene-modified $CD34^+$ cells following submyeloablative conditioning have shown impressive enhancement of *in vivo* gene-marking levels following treatment with O6-benzylguanine and 1,3-bis-(2-chloroethyl)-1-nitrosourea or tremozolomide (Gerull *et al.*, 2007; Beard *et al.*, 2010), along with eventual protection from the myelosuppressive effects of repeated cycles of these chemotherapeutic agents. However, two other groups did not find this approach efficient or nontoxic in an alternative primate model (Larochelle *et al.*, 2009). Based in the somewhat promising preclinical findings, phase I/II clinical trials in glioblastoma are ongoing, with the goal of allowing nontoxic 1,3-bis-(2-chloroethyl)-1-nitrosourea dose escalation.

Insights into genotoxicity from gene-marking trials

Studies performed prior to the availability of the human and murine genome sequences and without high-throughput methodologies suggested that retroviral integration was random, and that thus insertional mutagenesis was likely to be an exceedingly rare event in the absence of replication-competent vectors and required activation of multiple genes (Baum *et al.*, 2003). However, the unexpected occurrence of insertional oncogenesis in human clinical trials for primary immunodeficiencies (Hacein-Bey-Abina *et al.*, 2003; Ott *et al.*, 2006; Howe *et al.*, 2008) and in animal models (Li *et al.*, 2002; Kustikova *et al.*, 2005; Modlich *et al.*, 2005) firmly indicated the need to re-examine and modify gene transfer vectors to avoid this serious adverse consequence of gene therapy using integrating vectors. While HIV vectors integrate within genes, MLV integrates within 5 kb of transcriptional start sites, in particular in actively transcribed genes. The integration preferences of gene transfer vectors have been discussed in several recent review papers (Nienhuis *et al.*, 2006; Baum, 2007). The targeted coding regions likely depend on the genes that are highly expressed in the transduced cell and murine serial transplantation models using retrovirus encoding marker genes showed viral insertions in or near genes committed to self-renewal or survival of stem cells (Kustikova *et al.*, 2005). In addition, in the rhesus macaques transplantation studies using MLV vectors containing solely marker genes, one animal demonstrated clonal dominance with integrations found in an anti-apoptotic gene (*BCLA2*) shortly after transplantation. Importantly, the clone remained quiescent for 5 years, then re-emerged, with progression to an overt myeloid leukemia (Seggewiss *et al.*, 2006). These studies indicate the importance of constantly monitoring viral integration profiles and the value of *in vitro* assays (Modlich *et al.*, 2006) as well as *in vivo* tumor-prone animal models (Montini *et al.*, 2006), which may be of considerable utility in designing approaches to prevent oncogenesis in future clinical studies.

To date, no adverse effects have been reported when T cells were the targets for gene transfer. Interestingly, the evaluation of vector insertion sites *in vitro* and after *in vivo* infusion of herpes tk-transduced T cells in the clinical allogeneic transplantation trials demonstrated that integrations mostly landed close to genes that were prone to down-regulate T-cell functions. However, this did not lead to abnormal immune responses in the transplanted patients (Recchia *et al.*, 2006). The lack of malignant transformation was also demonstrated in a murine study in which T cells and HSCs were genetically modified with gammaretrovirus vectors that expressed T-cell oncogenes. While the enforced expression of *LMO2*, *TCL1*, or *TrkA* into HSCs resulted in T-cell leukemia/lymphoma in the transplanted animals, the infusion of similar genetically modified T cells was well tolerated (Newrzela *et al.*, 2008).

Further gene therapy studies in which the occurrence of insertional oncogenesis as adverse effects was not observed include the ADA-SCID trials (Cassani *et al.*, 2009). In contrast to the SCID-X1 trial, in all the ADA-SCID patients treated with gene transfer, the long-term analysis of integration sites

revealed a polyclonal pattern along with a lack of *in vivo* skewing for risky insertions. The fact that the therapeutic gene confers only selective survival to the transduced HSCs and does not participate in proliferation signaling within the cell can account for a lower risk of participating in oncogene activation (Kohn, 2008; Aiuti *et al.*, 2009).

Consequences of Genotoxicity Results

The combination of the adverse events in X-SCID and then the X-CGD clinical trials targeting HSCs, in addition to the clear evidence for nonrandom integration patterns for both retroviruses and lentiviruses in cell lines and primary cells stimulated intense further investigation into integration profiles for various vector systems and new vector designs predicted to reduce the likelihood of insertional activation of proto-oncogenes. Mitchell *et al.* (2004) showed that avian sarcoma leukosis virus (ASLV) has little or no bias toward integration into active genes and does not favor integration near transcription start sites. Hu *et al.* (2007) demonstrated the feasibility of obtaining efficient long-term engraftment in rhesus macaques $CD34^+$ cells transduced with an ASLV-derived RCAS (Replication Competent ALV long-terminal repeat [LTR] with a Splice Acceptor) vector and GFP as a gene marker. Furthermore, the authors analyzed approximately 300 insertions and showed that the vector integrations did not favor gene-rich regions, transcription start sites, or CpG islands (Hu *et al.*, 2008), demonstrating that ASLV may be a safer candidate for gene transfer into human HSCs compared with MLV. The ASLV viral enhancer is only active in avian cells, making constitutive activation of adjacent proto-oncogenes less likely following insertion of ASLV vectors into mammalian genomes. Self-inactivating (SIN) vectors have the strong viral promoter/enhancer deleted from the proviral LTR, and instead transgene expression is driven from an internal tissue-specific or constitutive promoter. This design has been employed almost universally with lentiviral vectors, and more recently there has been a resurgence of interest in SIN MLV vectors. SIN vector design has been shown to greatly reduce the risk of insertional leukemogenesis (Gabriel *et al.*, 2009) in the murine *in vivo* model. MLV SIN gammaretrovirus vector with *neo* and cellular phosphoglycerate kinase as an internal promoter was evaluated in an *in vitro* immortalization assay using murine HSCs and demonstrated lower level of myeloid immortalization than a non-SIN vector (Bosticardo *et al.*, 2009). Certainly, MLV SIN vectors are not completely devoid of insertional oncogenesis risk, and SIN vectors completely lacking enhancer activity in the LTR or internally may be required.

Lentiviral vectors derived from the human immunodeficiency virus (HIV) or simian immunodeficiency virus (SIV) are also attractive tools for improving safety in gene therapy. Their lack of potent enhancer sequences in the modified LTRs reduces the risk of activating neighboring genes. Recent studies demonstrate that vectors derived from lentiviruses will be useful for the correction of genetic blood diseases (Hayakawa *et al.*, 2009). The inclusion of physiological promoters in SIN vectors, such as enlongation factor-1α, also significantly reduces the risk of insertional transformation when evaluated in recently established serial replating assays that detect insertional transformation (Zychlinski *et al.*, 2008). Hanawa *et al.* (2004) recently reported high gene-marking levels and long-term polyclonal reconstitution of hematopoiesis in rhesus macaques after transplantation of mobilized $CD34^+$ cells transduced with SIV vectors and GFP as a gene marker. Follow-up of these animals now for almost 5 years has shown no common integration sites in known proto-oncogenes, highly polyclonal hematopoiesis, and very stable levels of vector-containing cells and transgene expression (Kim *et al.*, 2009). Several HSC-targeted therapy trials using HIV-derived lentiviral vectors were recently reported or are in progress. Two patients with adrenoleukodystrophy who lacked a histocompatible bone marrow donor received infusions of HSCs transduced with the therapeutic *ALD* gene following myeloablative conditioning. Importantly, at 36 months post-transplantation both patients had highly polyclonal reconstitution of hematopoiesis and normal levels of ALD protein, which appeared to retard the progressive cerebral demyelization process (Cartier *et al.*, 2009). Since the ALD protein is very unlikely to impact the behavior of HSCs and progenitor cells, giving neither a positive nor a negative growth advantage, these patients, in addition to the nonhuman primates discussed earlier, will provide very valuable information on the dynamics of *in vivo* hematopoiesis over time, besides the very encouraging clinical outcomes regarding the underlying disease. The recent report of a clonal expansion from integration of lentivirus vector in the DNA-binding protein HMGA2 (high mobility group AT-hook 2) gene in a patient treated with gene therapy for thalassemia imposing, once more, further caution (Cavazzana-Calvo *et al.*).

Taken together, these data reflect a mixed message: gammaretrovirus vectors in which the transgene is not a growth/survival-promoting factor may have a relatively low but still appreciable risk for inducing clonal dominance and eventual leukemia in patients treated with HSC-directed gene therapy. As soon as the first two children with X-SCID developed leukemia linked to insertional mutagenesis, human gene-marking trials became unacceptable almost overnight, with the realization that risk was not minimal and could not be justified, given that there was no chance for clinical benefit. The further development and validation of predictive animal models including human–murine xenografts, dogs, and nonhuman primates obviated the need to carry out additional nontherapeutic marking studies in patients. Instead, carefully chosen clinical trials are being designed to further test putatively safer vectors in situations with some hope for clinical benefit.

Nonviral Methods of Gene Transfer

Physical-chemical methods have been used with great success to enable, enhance, or localize gene delivery. These nonviral methods of gene transfer into primary human T cells (Tahvanainen *et al.*, 2006; Magg *et al.*, 2009) and HSCs (Sumiyoshi *et al.*, 2009) are currently being developed and tested in a growing number of *in vitro* studies using gene markers. Some of the methods have shown good transfection efficiency and expression levels. However, most of these methods result in significant loss of viability of primary cells, particularly if functional assays are performed, such as proliferation *in vitro* or engraftment *in vivo*. Using optimized electroporation for delivering a dual-expression vector into the nucleus Magg *et al.* (2009) achieved successful stable

transfection of primary human T cells expressing the gene of interest and ΔNGFR as a gene marker. This enabled rapid and efficient positive selection by cell sorting. Another group (Hackett *et al.*, 2010) has recently combined electroporation with the Sleeping Beauty (SB) transposase/transposon DNA plasmid system to introduce a CD19-specific chimeric antigen receptor to redirect the specificity of human T cells. By improving the efficiency of electroporation and integration by iterative adaptations of the SB system, this potentially safer nonviral approach is moving towards clinical trials. Two recent reports document the potential for the SB system even in delivering genes to $CD34^+$ HSCs (Sumiyoshi *et al.*, 2009; Xue *et al.*, 2009). Although the efficiency of gene transfer is below that achieved with viral vectors, the potential for these approaches warrant further studies to optimize and evaluate the safety of this novel strategy in the future.

Conclusions

Gene-marking studies constitute the earliest application of gene transfer to HSCs and T cells. Since the 1990s, the contribution of gene-marking studies to experimental and clinical hematology has been remarkable, providing invaluable translational data to the fields of HSCT, T-cell immunotherapy, and gene therapy. Pioneering marking studies in large animals greatly aided in the optimization of conditions for gene transfer to hematopoietic stem and progenitor cells, furthered the expression of therapeutic genes to clinically relevant levels, and provided the foundation for the second generation of human gene therapy trials. More recently, the knowledge gained from *in vitro* and *in vivo* marking studies designed to assess the risks of the genotoxicity phenomena associated to gene transfer have been translated to the development of safer viral vectors for gene delivery into HSCs. In addition, nonviral methods of gene delivery are currently being evaluated in studies using marker genes. Taken together, the contribution of gene marking to the continued development of these cell and gene therapies provides considerable hope for improving the effectiveness and safety of these applications for malignant and nonmalignant hematologic diseases in the near future.

References

Aiuti, A., Cattaneo, F., Galimberti, S., *et al.* (2009). Gene therapy for immunodeficiency due to adenosine deaminase deficiency. N. Engl. J. Med. 360, 447–458.

Aiuti, A., Slavin, S., Aker, M., *et al.* (2002). Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. Science 296, 2410–2413.

Alici, E., Bjorkstrand, B., Treschow, A., *et al.* (2007). Long-term follow-up of gene-marked $CD34^+$ cells after autologous stem cell transplantation for multiple myeloma. Cancer Gene Ther. 14, 227–232.

Bachier, C.R., Giles, R.E., Ellerson, D., *et al.* (1999). Hematopoietic retroviral gene marking in patients with follicular non-Hodgkin's lymphoma. Leuk. Lymphoma 32, 279–288.

Baum, C. (2007). Insertional mutagenesis in gene therapy and stem cell biology. Curr. Opin. Hematol. 14, 337–342.

Baum, C., Dullmann, J., Li, Z., *et al.* (2003). Side effects of retroviral gene transfer into hematopoietic stem cells. Blood 101, 2099–2114.

Beard, B.C., Trobridge, G.D., Ironside, C., *et al.* (2010). Efficient and stable MGMT-mediated selection of long-term repopulating stem cells in nonhuman primates. J. Clin. Invest. 120, 2345–2354.

Bennour, E., Ferrand, C., Remy-Martin, J.P., *et al.* (2008). Abnormal expression of only the CD34 part of a transgenic CD34/herpes simplex virus-thymidine kinase fusion protein is associated with ganciclovir resistance. Hum. Gene Ther. 19, 699–709.

Blaese, R.M., Culver, K.W., Miller, A.D., *et al.* (1995). T lymphocyte-directed gene therapy for ADA-SCID: initial trial results after 4 years. Science 270, 475–480.

Bollard, C.M., Heslop, H.E., and Brenner, M.K. (2001). Gene-marking studies of hematopoietic cells. Int. J. Hematol. 73, 14–22.

Bonini, C., Bondanza, A., Perna, S.K., *et al.* (2007). The suicide gene therapy challenge: how to improve a successful gene therapy approach. Mol. Ther. 15, 1248–1252.

Bonini, C., Ferrari, G., Verzeletti, S., *et al.* (1997). HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. Science 276, 1719–1724.

Bonini, C., Grez, M., Traversari, C., *et al.* (2003). Safety of retroviral gene marking with a truncated NGF receptor. Nat. Med. 9, 367–369.

Bordignon, C., Notarangelo, L.D., Nobili, N., *et al.* (1995). Gene therapy in peripheral blood lymphocytes and bone marrow for ADA-immunodeficient patients. Science 270, 470–475.

Bosticardo, M., Ghosh, A., Du, Y., *et al.* (2009). Self-inactivating retroviral vector-mediated gene transfer induces oncogene activation and immortalization of primary murine bone marrow cells. Mol. Ther. 17, 1910–1918.

Boztug, K., Schmidt, M., Schwarzer, A., *et al.* (2010). Stem-cell gene therapy for the Wiskott-Aldrich syndrome. N. Engl. J. Med. 363, 1918–1927.

Brenner, M.K., Rill, D.R., Holladay, M.S., *et al.* (1993a). Gene marking to determine whether autologous marrow infusion restores long-term haemopoiesis in cancer patients. Lancet 342, 1134–1137.

Brenner, M.K., Rill, D.R., Moen, R.C., *et al.* (1993b). Gene-marking to trace origin of relapse after autologous bone-marrow transplantation. Lancet 341, 85–86.

Cartier, N., Hacein-Bey-Abina, S., Bartholomae, C.C., *et al.* (2009). Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. Science 326, 818–823.

Cassani, B., Montini, E., Maruggi, G., *et al.* (2009). Integration of retroviral vectors induces minor changes in the transcriptional activity of T cells from ADA-SCID patients treated with gene therapy. Blood 114, 3546–3556.

Cavazzana-Calvo, M., Hacein-Bey, S., de Saint Basile, G., *et al.* (2000). Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science 288, 669–672.

Cavazzana-Calvo, M., Payen, E., Negre, O., *et al.* (2010). Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. Nature 467, 318–322.

Ciceri, F., Bonini, C., Marktel, S., *et al.* (2007). Antitumor effects of HSV-TK-engineered donor lymphocytes after allogeneic stem-cell transplantation. Blood 109, 4698–4707.

Cornetta, K., Tricot, G., Broun, E.R., *et al.* (1992). Retroviral-mediated gene transfer of bone marrow cells during autologous bone marrow transplantation for acute leukemia. Hum. Gene Ther. 3, 305–318.

Dao, M.A., Hashino, K., Kato, I., and Nolta, J.A. (1998). Adhesion to fibronectin maintains regenerative capacity during ex

vivo culture and transduction of human hematopoietic stem and progenitor cells. Blood 92, 4612–4621.

Deisseroth, A.B., Zu, Z., Claxton, D., *et al.* (1994). Genetic marking shows that Ph+ cells present in autologous transplants of chronic myelogenous leukemia (CML) contribute to relapse after autologous bone marrow in CML. Blood 83, 3068–3076.

Dick, J.E., Magli, M.C., Huszar, D., *et al.* (1985). Introduction of a selectable gene into primitive stem cells capable of long-term reconstitution of the hemopoietic system of W/Wv mice. Cell 42, 71–79.

Di Stasia, A., Tey, S.K., Fujita, Y., *et al.* (2010). CASPALLO: Phase I clinical trial of allodepleted T cells transduced with inducible caspase 9 suicide gene after haploidentical stem cell transplantation. Mol. Ther. 18, Abs. 288.

Donahue, R.E., and Dunbar, C.E. (2001). Update on the use of nonhuman primate models for preclinical testing of gene therapy approaches targeting hematopoietic cells. Hum. Gene Ther. 12, 607–617.

Dunbar, C.E., Cottler-Fox, M., O'Shaughnessy, J.A., *et al.* (1995). Retrovirally marked CD34-enriched peripheral blood and bone marrow cells contribute to long-term engraftment after autologous transplantation. Blood 85, 3048–3057.

Dunbar, C.E., Kohn, D.B., Schiffmann, R., *et al.* (1998). Retroviral transfer of the glucocerebrosidase gene into $CD34^+$ cells from patients with Gaucher disease: in vivo detection of transduced cells without myeloablation. Hum. Gene Ther. 9, 2629–40.

Dunbar, C.E., Seidel, N.E., Doren, S., *et al.* (1996). Improved retroviral gene transfer into murine and Rhesus peripheral blood or bone marrow repopulating cells primed in vivo with stem cell factor and granulocyte colony-stimulating factor. Proc. Natl. Acad. Sci. U. S. A. 93, 11871–11876.

Emmons, R.V., Doren, S., Zujewski, J., *et al.* (1997). Retroviral gene transduction of adult peripheral blood or marrow-derived $CD34^+$ cells for six hours without growth factors or on autologous stroma does not improve marking efficiency assessed in vivo. Blood 89, 4040–4046.

Fehse, B., Kustikova, O.S., Li, Z., *et al.* (2002). A novel 'sort-suicide' fusion gene vector for T cell manipulation. Gene Ther. 9, 1633–1638.

Fehse, B., Richters, A., Putimtseva-Scharf, K., *et al.* (2000). CD34 splice variant: an attractive marker for selection of gene-modified cells. Mol. Ther. 1, 448–456.

Ferrari, G., Rossini, S., Giavazzi, R., *et al.* (1991). An in vivo model of somatic cell gene therapy for human severe combined immunodeficiency. Science 251, 1363–1366.

Ferrari, G., Rossini, S., Nobili, N., *et al.* (1992). Transfer of the ADA gene into human ADA-deficient T lymphocytes reconstitutes specific immune functions. Blood 80, 1120–1124.

Gabriel, R., Eckenberg, R., Paruzynski, A., *et al.* (2009). Comprehensive genomic access to vector integration in clinical gene therapy. Nat. Med. 15, 1431–1436.

Gaspar, H.B., Bjorkegren, E., Parsley, K., *et al.* (2006). Successful reconstitution of immunity in ADA-SCID by stem cell gene therapy following cessation of PEG-ADA and use of mild preconditioning. Mol. Ther. 14, 505–513.

Gaspar, H.B., Parsley, K.L., Howe, S., *et al.* (2004). Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. Lancet 364, 2181–2187.

Gerrits, A., Dykstra, B., Kalmykowa, O.J., *et al.* (2010). Cellular barcoding tool for clonal analysis in the hematopoietic system. Blood 115, 2610–2618.

Gerull, S., Beard, B.C., Peterson, L.J., *et al.* (2007). In vivo selection and chemoprotection after drug resistance gene therapy in a nonmyeloablative allogeneic transplantation setting in dogs. Hum. Gene Ther. 18, 451–456.

Hacein-Bey-Abina, S., von Kalle, C., Schmidt, M., *et al.* (2003). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science 302, 415–419.

Hackett, P.B., Largaespada, D.A., and Cooper, L.J. (2010). A transposon and transposase system for human application. Mol. Ther. 18, 674–683.

Hanawa, H., Hematti, P., Keyvanfar, K., *et al.* (2004). Efficient gene transfer into rhesus repopulating hematopoietic stem cells using a simian immunodeficiency virus-based lentiviral vector system. Blood 103, 4062–4069.

Hanazono, Y., Yu, J.M., Dunbar, C.E., and Emmons, R.V. (1997). Green fluorescent protein retroviral vectors: low titer and high recombination frequency suggest a selective disadvantage. Hum. Gene Ther. 8, 1313–1319.

Hanenberg, H., Xiao, X.L., Dilloo, D., *et al.* (1996). Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. Nat. Med. 2, 876–882.

Hawley, T.S., Telford, W.G., and Hawley, R.G. (2001). "Rainbow" reporters for multispectral marking and lineage analysis of hematopoietic stem cells. Stem Cells 19, 118–124.

Hayakawa, J., Ueda, T., Lisowski, L., *et al.* (2009). Transient in vivo beta-globin production after lentiviral gene transfer to hematopoietic stem cells in the nonhuman primate. Hum. Gene Ther. 20, 563–572.

Heslop, H.E., Brenner, M.K., Krance, R.A., *et al.* (1996). Use of double marking with retroviral vectors to determine rate of reconstitution of untreated and cytokine expanded $CD34^+$ selected marrow cells in patients undergoing autologous bone marrow transplantation. Hum. Gene Ther. 7, 655–667.

Heslop, H.E., and Rooney, C.M. (1997). Adoptive cellular immunotherapy for EBV lymphoproliferative disease. Immunol. Rev. 157, 217–222.

Heslop, H.E., Slobod, K.S., Pule, M.A., *et al.* (2010). Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. Blood 115, 925–935.

Hoogerbrugge, P.M., van Beusechem, V.W., Fischer, A., *et al.* (1996). Bone marrow gene transfer in three patients with adenosine deaminase deficiency. Gene Ther. 3, 179–183.

Howe, S.J., Mansour, M.R., Schwarzwaelder, K., *et al.* (2008). Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. J. Clin. Invest. 118, 3143–3150.

Hu, J., and Dunbar, C.E. (2002). Update on hematopoietic stem cell gene transfer using non-human primate models. Curr. Opin. Mol. Ther. 4, 482–490.

Hu, J., Ferris, A., Larochelle, A., *et al.* (2007). Transduction of rhesus macaque hematopoietic stem and progenitor cells with avian sarcoma and leukosis virus vectors. Hum. Gene Ther. 18, 691–700.

Hu, J., Renaud, G., Gomes, T.J., *et al.* (2008). Reduced genotoxicity of avian sarcoma leukosis virus vectors in rhesus long-term repopulating cells compared to standard murine retrovirus vectors. Mol. Ther. 16, 1617–1623.

Huhn, R.D., Tisdale, J.F., Agricola, B., *et al.* (1999). Retroviral marking and transplantation of rhesus hematopoietic cells by nonmyeloablative conditioning. Hum. Gene Ther. 10, 1783–1790.

Kiem, H.P., Andrews, R.G., Morris, J., *et al.* (1998). Improved gene transfer into baboon marrow repopulating cells using recombinant human fibronectin fragment CH-296 in combi-

nation with interleukin-6, stem cell factor, FLT-3 ligand, and megakaryocyte growth and development factor. Blood 92, 1878–1886.

Kim, Y.J., Kim, Y.S., Larochelle, A., *et al.* (2009). Sustained high-level polyclonal hematopoietic marking and transgene expression 4 years after autologous transplantation of rhesus macaques with SIV lentiviral vector-transduced $CD34^+$ cells. Blood 113, 5434–5443.

Kohn, D.B. (2008). Gene therapy for childhood immunological diseases. Bone Marrow Transplant 41, 199–205.

Kohn, D.B., Weinberg, K.I., Nolta, J.A., *et al.* (1995). Engraftment of gene-modified umbilical cord blood cells in neonates with adenosine deaminase deficiency. Nat. Med. 1, 1017–1023.

Kuramoto, K., Follmann, D.A., Hematti, P., *et al.* (2004). Effect of chronic cytokine therapy on clonal dynamics in nonhuman primates. Blood 103, 4070–4077.

Kustikova, O., Fehse, B., Modlich, U., *et al.* (2005). Clonal dominance of hematopoietic stem cells triggered by retroviral gene marking. Science 308, 1171–1174.

Larochelle, A., Choi, U., Shou, Y., *et al.* (2009). In vivo selection of hematopoietic progenitor cells and temozolomide dose intensification in rhesus macaques through lentiviral transduction with a drug resistance gene. J. Clin. Invest. 119, 1952–1963.

Laukkanen, M.O., Kuramoto, K., Calmels, B., *et al.* (2005). Low-dose total body irradiation causes clonal fluctuation of primate hematopoietic stem and progenitor cells. Blood 105, 1010–1015.

Li, Z., Dullmann, J., Schiedlmeier, B., *et al.* (2002). Murine leukemia induced by retroviral gene marking. Science 296, 497.

Liu, H.S., Jan, M.S., Chou, C.K., *et al.* (1999). Is green fluorescent protein toxic to the living cells? Biochem. Biophys. Res. Commun. 260, 712–717.

Magg, T., Hartrampf, S., and Albert, M.H. (2009). Stable nonviral gene transfer into primary human T cells. Hum. Gene Ther. 20, 989–998.

Malech, H.L., Maples, P.B., Whiting-Theobald, N., *et al.* (1997). Prolonged production of NADPH oxidase-corrected granulocytes after gene therapy of chronic granulomatous disease. Proc. Natl. Acad. Sci. U. S. A. 94, 12133–12138.

Metais, J.Y., and Dunbar, C.E. (2008). The MDS1-EVI1 gene complex as a retrovirus integration site: impact on behavior of hematopoietic cells and implications for gene therapy. Mol. Ther. 16, 439–449.

Mitchell, R.S., Beitzel, B.F., Schroder, A.R., *et al.* (2004). Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. PLoS Biol. 2, E234.

Modlich, U., Kustikova, O.S., Schmidt, M., *et al.* (2005). Leukemias following retroviral transfer of multidrug resistance 1 (MDR1) are driven by combinatorial insertional mutagenesis. Blood 105, 4235–4246.

Modlich, U., Bohne, J., Schmidt, M., *et al.* (2006). Cell-culture assays reveal the importance of retroviral vector design for insertional genotoxicity. Blood 108, 2545–2553.

Montini, E., Cesana, D., Schmidt, M., *et al.* (2006). Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. Nat. Biotechnol. 24, 687–696.

Morgan, R.A., Dudley, M.E., Wunderlich, J.R., *et al.* (2006). Cancer regression in patients after transfer of genetically engineered lymphocytes. Science 314, 126–129.

Morris, J.C., Conerly, M., Thomasson, B., *et al.* (2004). Induction of cytotoxic T-lymphocyte responses to enhanced green and yellow fluorescent proteins after myeloablative conditioning. Blood 103, 492–499.

Newrzela, S., Cornils, K., Li, Z., *et al.* (2008). Resistance of mature T cells to oncogene transformation. Blood 112, 2278–2286.

Nienhuis, A.W., Dunbar, C.E., and Sorrentino, B.P. (2006). Genotoxicity of retroviral integration in hematopoietic cells. Mol. Ther. 13, 1031–1049.

Ott, M.G., Schmidt, M., Schwarzwaelder, K., *et al.* (2006). Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. Nat. Med. 12, 401–409.

Persons, D.A., Allay, J.A., Allay, E.R., *et al.* (1997). Retroviral-mediated transfer of the green fluorescent protein gene into murine hematopoietic cells facilitates scoring and selection of transduced progenitors in vitro and identification of genetically modified cells in vivo. Blood 90, 1777–1786.

Persons, D.A., Allay, J.A., Riberdy, J.M., *et al.* (1998). Use of the green fluorescent protein as a marker to identify and track genetically modified hematopoietic cells. Nat. Med. 4, 1201–1205.

Qasim, W., Mackey, T., Sinclair, J., *et al.* (2007). Lentiviral vectors for T-cell suicide gene therapy: preservation of T-cell effector function after cytokine-mediated transduction. Mol. Ther. 15, 355–360.

Recchia, A., Bonini, C., Magnani, Z., *et al.* (2006). Retroviral vector integration deregulates gene expression but has no consequence on the biology and function of transplanted T cells. Proc. Natl. Acad. Sci. U. S. A. 103, 1457–1462.

Rettig, M.P., Ritchey, J.K., Prior, J.L., *et al.* (2004). Kinetics of in vivo elimination of suicide gene-expressing T cells affects engraftment, graft-versus-host disease, and graft-versus-leukemia after allogeneic bone marrow transplantation. J. Immunol. 173, 3620–3630.

Rill, D.R., Moen, R.C., Buschle, M., *et al.* (1992). An approach for the analysis of relapse and marrow reconstitution after autologous marrow transplantation using retrovirus-mediated gene transfer. Blood 79, 2694–2700.

Rooney, C.M., Smith, C.A., Ng, C.Y., *et al.* (1995). Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. Lancet 345, 9–13.

Rosenberg, S.A., and Dudley, M.E. (2009). Adoptive cell therapy for the treatment of patients with metastatic melanoma. Curr. Opin. Immunol. 21, 233–240.

Rosenberg, S.A., Aebersold, P., Cornetta, K., *et al.* (1990). Gene transfer into humans–immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. N. Engl. J. Med. 323, 570–578.

Rosenzweig, M., Macvittie, T.J., Harper, D., *et al.* (1999). Efficient and durable gene marking of hematopoietic progenitor cells in nonhuman primates after nonablative conditioning. Blood 94, 2271–2286.

Schmidt, M., Zickler, P., Hoffmann, G., *et al.* (2002). Polyclonal long-term repopulating stem cell clones in a primate model. Blood 100, 2737–2743.

Seggewiss, R., Pittaluga, S., Adler, R.L., *et al.* (2006). Acute myeloid leukemia is associated with retroviral gene transfer to hematopoietic progenitor cells in a rhesus macaque. Blood 107, 3865–3867.

Shepherd, B.E., Kiem, H.P., Lansdorp, P.M., *et al.* (2007). Hematopoietic stem-cell behavior in nonhuman primates. Blood 110, 1806–1813.

Sumiyoshi, T., Holt, N.G., Hollis, R.P., *et al.* (2009). Stable transgene expression in primitive human $CD34^+$ hematopoietic stem/progenitor cells, using the Sleeping Beauty transposon system. Hum. Gene Ther. 20, 1607–1626.

Tahvanainen, J., Pykalainen, M., Kallonen, T., *et al.* (2006). Enrichment of nucleofected primary human CD4+ T cells: a novel and efficient method for studying gene function and role in human primary T helper cell differentiation. J. Immunol. Methods 310, 30–39.

Tey, S.K., Dotti, G., Rooney, C.M., *et al.* (2007). Inducible caspase 9 suicide gene to improve the safety of allodepleted T cells after haploidentical stem cell transplantation. Biol. Blood Marrow Transplant. 13, 913–924.

Thrasher, A.J., Hacein-Bey-Abina, S., Gaspar, H.B., *et al.* (2005). Failure of SCID-X1 gene therapy in older patients. Blood 105, 4255–4257.

Tiberghien, P., Cahn, J.Y., Brion, A., *et al.* (1997). Use of donor T-lymphocytes expressing herpes-simplex thymidine kinase in allogeneic bone marrow transplantation: a phase I-II study. Hum. Gene Ther. 8, 615–624.

Tisdale, J.F., Hanazono, Y., Sellers, S.E., *et al.* (1998). Ex vivo expansion of genetically marked rhesus peripheral blood progenitor cells results in diminished long-term repopulating ability. Blood 92, 1131–1141.

Weber, K., Mock, U., Petrowitz, B., *et al.* (2010). Lentiviral gene ontology (LeGO) vectors equipped with novel drug-selectable fluorescent proteins: new building blocks for cell marking and multi-gene analysis. Gene Ther. 17, 511–520.

Wu, T., Bloom, M.L., Yu, J.M., *et al.* (1998). Murine bone marrow expressing the neomycin resistance gene has no competitive disadvantage assessed in vivo. Hum. Gene Ther. 9, 1157–1164.

Xue, X., Huang, X., Nodland, S.E., *et al.* (2009). Stable gene transfer and expression in cord blood-derived $CD34^+$ hematopoietic stem and progenitor cells by a hyperactive Sleeping Beauty transposon system. Blood 114, 1319–1330.

Zychlinski, D., Schambach, A., Modlich, U., *et al.* (2008). Physiological promoters reduce the genotoxic risk of integrating gene vectors. Mol. Ther. [Epub ahead of print Feb. 19.]

Address correspondence to:
Dr. Cynthia E. Dunbar
CRC-Building 10, Room 4E-5132
National Institutes of Health
10 Center Drive
Bethesda, MD 20852

E-mail: dunbarc@nhlbi.nih.gov

Received for publication December 16, 2010;
accepted after revision January 23, 2011.

Published online: January 24, 2011.