

Progress Toward Vector Design for Hematopoietic Stem Cell Gene Therapy

Robert G. Hawley*

Department of Hematopoiesis, Holland Laboratory, American Red Cross, 15601 Crabbs Branch Way, Rockville, MD 20855, USA

Abstract: Hematopoietic stem cells (HSCs) are attractive targets for gene therapy because of their capacity for self renewal and the wide systemic distribution of their progeny. Sustained expression of transgenes at clinically relevant levels in the progeny of HSCs would provide novel and potentially curative treatments for a wide range of inherited and acquired blood diseases. Recent improvements in retroviral transduction protocols have resulted in the first successful amelioration of a human hematologic disease—a form of severe combined immunodeficiency—by HSC gene transfer. However, continued advances in gene transfer technology are necessary if the inherent promise of HSC gene therapy is to be fully realized. Ongoing efforts are focused on modifying oncoretroviral vector designs and pseudotyping with alternative envelope proteins. In addition, because of their ability to transduce non-divided cells, safety-modified human immunodeficiency virus-1-based lentiviral vectors have emerged as promising tools for gene modification of HSCs, which reside primarily in the G₀/G₁ phase of the cell cycle. Irrespective of these advances, accumulated data indicate that stably integrated transgenes are frequently subject to position-effect variegation and extinction of expression. Therefore, the extent to which genetic control elements such as chromatin domain insulators and scaffold/matrix attachment regions in conjunction with posttranscriptional regulatory elements will result in enhanced probability and level of transgene expression is under active investigation. Collectively, these developments increase the likelihood that HSC gene transfer will ultimately become an effective therapeutic strategy.

INTRODUCTION

Gene therapy using hematopoietic stem cells (HSCs) as the target cell population has great potential to dramatically improve treatment for a variety of inherited hematologic and immune disorders, as well as acquired diseases like AIDS and cancer [Sorrentino and Nienhuis, 1999; Engel and Kohn, 1999]. The best candidates for HSC-directed gene therapy in the immediate future are monogenic diseases for which regulated, lineage-specific transgene expression is not required and moderate levels of transgene product will provide a clinical benefit. Possibilities include immunodeficiency syndromes [severe combined immuno-

deficiency (SCID) due to defects in signal transduction pathways, adenosine deaminase deficiency], disorders of phagocytic cells that result in recurrent life-threatening infections (chronic granulomatous disease, leukocyte adhesion deficiency), and metabolic storage diseases (Fabry disease, Gaucher disease, Hurler syndrome).

ADVANCED ONCORETROVIRAL VECTOR SYSTEMS

Although there were some encouraging exceptions, until recently the results of the majority of the human HSC gene therapy trials conducted with oncoretroviral vectors had been disappointing [Brenner *et al.*, 1993; Dunbar *et al.*, 1995; Bordignon *et al.*, 1995; Kohn *et al.*, 1995; Williams *et al.*, 2000], paralleling the limited success achieved in preclinical gene transfer

*Address correspondence to this author at the Department of Hematopoiesis, Holland Laboratory, American Red Cross, 15601 Crabbs Branch Way, Rockville, MD 20855, USA; Tel: 301-738-0424; Fax: 301-738-0444; Email: hawleyr@usa.redcross.org

studies in nonhuman primates and with human HSCs in xenogeneic transplant assays [Larochelle *et al.*, 1996; Donahue *et al.*, 1996]. However, new advances in oncoretroviral gene transduction methodology [Moritz and Williams, 1999] that have led to significant improvements in gene transfer efficiencies in animal transplant models have culminated of late in the successful treatment by gene therapy of four patients with X-linked SCID disease (SCID-X1) [Cavazzana-Calvo *et al.*, 2000]. This progress can be attributed in part to key modifications of earlier transduction protocols: the inclusion of flt3 ligand and thrombopoietin to cytokine cocktails, which help preserve the long-term hematopoietic repopulating ability of HSCs during the *ex vivo* transduction procedure [Dao *et al.*, 1997; Bhatia *et al.*, 1997; Luens *et al.*, 1998]; and the use of certain fibronectin fragments, which co-localize vector particles and cells while stimulating HSC survival and/or proliferation [Moritz *et al.*, 1994; Hanenberg *et al.*, 1996; Dao *et al.*, 1998]. Another development that is anticipated to contribute to improved clinical gene transfer efficacy is the generation of vector particles pseudotyped with envelope proteins from the gibbon ape leukemia virus (GALV) or the feline endogenous virus RD114, both of which appear to be more efficient than amphotropic vector particles for transduction of primate and human HSCs [Kiem *et al.*, 1998; Dorrell *et al.*, 2000; Porter *et al.*, 1996; Kelly *et al.*, 2000]. Efficient transduction of candidate human HSCs with oncoretroviral vectors pseudotyped with the vesicular stomatitis virus G (VSV-G) envelope protein has also been reported [Rebel *et al.*, 1999].

Despite the aforementioned marked improvements in HSC gene transfer efficiency, a second potential obstacle to effective gene therapy using HSCs, which has repeatedly been observed in preclinical studies with standard oncoretroviral vectors based on Moloney murine leukemia virus (MoMLV), is positional variegation and extinction of transgene expression [Fig. (1A)] [Williams *et al.*, 1986; Challita and Kohn, 1994; Lu *et al.*, 1996; Lange and Blankenstein, 1997; Robbins *et al.*, 1998; Cheng *et al.*, 1998; Halene *et al.*, 1999; Zentilin *et al.*, 2000]. Indeed, position effects and

transgene silencing have been found to occur even after *ex vivo* preselection of transgene-expressing HSCs [Pawliuk *et al.*, 1997; Barquinero *et al.*, 2000]. For this reason, it will be of great interest to learn whether the MoMLV-based MFG(B2) vector expressing a γ cytokine receptor transgene will be subject to transcriptional down-regulation during long-term follow up of the SCID-X1 patients who received transduced CD34⁺ bone marrow cells [Cavazzana-Calvo *et al.*, 2000]. The parental MFG vector was designed to achieve high level transgene expression by controlled splicing of vector transcripts, but transcription is driven by an unmodified MoMLV long terminal repeat (LTR) [Dranoff *et al.*, 1993; Krall *et al.*, 1996]. Using a serial bone marrow transplantation assay in lethally irradiated mice, Kohn and colleagues documented a high rate of MoMLV vector expression failure in secondary recipients concomitant with methylation of the LTR [Challita and Kohn, 1994]. Essentially identical frequencies of silencing were observed irrespective of whether the MFG vector backbone or another MoMLV-based vector backbone (N2) was employed [Krall and Kohn, 1996].

Non-function of the enhancer as well as negative regulatory factors that bind to the LTR and the 5'-untranslated region have been implicated as the control mechanisms responsible for restriction of expression of MoMLV-based vectors in murine embryonal carcinoma (EC) cells and embryonic stem (ES) cells [Flanagan *et al.*, 1989; Tsukiyama *et al.*, 1989; Petersen *et al.*, 1991; Prince and Rigby, 1991; Grez *et al.*, 1991; Flanagan *et al.*, 1992; Tsukiyama *et al.*, 1992; Kempler *et al.*, 1993; Yamauchi *et al.*, 1995]. The silencers identified include the CGCCATTTT and TCAAGGTCA elements at the 5' end of the LTR and the retroviral tRNA primer binding site, TGGGGGCTCGTCCGGGAT, in the 5'-untranslated region. The CGCCATTTT element, the core motif of an upstream conserved region present in over 90% of mammalian type C oncoretrovirus isolates (originally referred to as the negative control region), is bound by the YY-1 (Yin-Yang 1) repressor/activator, a member of the GLI-Krüppel zinc finger transcription factor family [Flanagan *et al.*, 1989; Flanagan *et al.*, 1992].

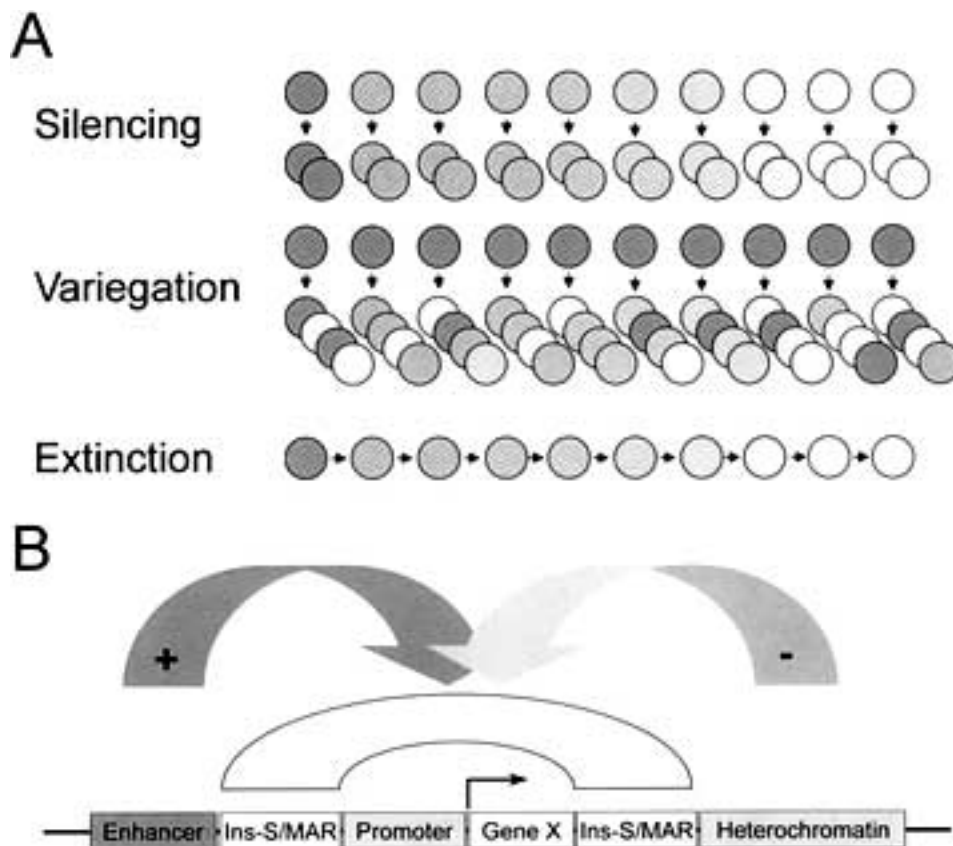


Fig. (1). Potential for insulators and S/MARs to protect against position effects. **(A)** Position effects due to the retroviral vector integration site are believed to contribute to transcriptional silencing of vectors shortly after integration, to expression variegation (where variable expression is observed within the clonal progeny of a target cell), and to extinction of expression (where expression is down-regulated with time). **(B)** Certain combinations of insulators (Ins) and S/MARs may confer position-independent transgene expression by blocking the negative effects of nearby heterochromatin (or, occasionally, the positive effects of enhancer elements) [Rivella and Sadelain, 1998; Emery and Stamatoyannopoulos, 1999].

Transcriptional repression by YY-1 may be mediated by interaction with a chromatin modifying histone deacetylase complex targeted to methylated CpG (cytosine-guanine) dinucleotides within the promoter region [Yang *et al.*, 1996; Nan *et al.*, 1998; Jones *et al.*, 1998]. The TCAAGGTCA element is bound by a factor, originally termed embryonal LTR-binding protein (ELP), which was subsequently demonstrated to be an isoform of the murine SF-1 protein, an orphan member of the nuclear steroid receptor superfamily [Tsukiyama *et al.*, 1992; Ninomiya *et al.*, 1995]. Whereas ELP functions as a repressor in embryonal cells, alternatively spliced murine SF-1 transcripts encode positive regulators of gene expression in EC and steroidogenic cells [Ikeda *et al.*, 1993; Barnea and Bergman, 2000]. Wild-type MoMLV uses a cellular tRNA^{Pro} to prime

synthesis of the first (minus) strand strong stop DNA. The tRNA^{Pro} primer binding site region contains a silencer element that suppresses MoMLV expression in EC and ES cells, and directs the methylation of viral and adjacent cellular DNA [Petersen *et al.*, 1991; Berwin and Barklis, 1993]. A factor (factor A) that binds to a consensus GGRGGCTCGTYGGGAT sequence, which overlaps with 17 of the 18 nucleotides of the tRNA^{Pro} primer binding site, has been identified but not molecularly characterized [Kempler *et al.*, 1993; Yamauchi *et al.*, 1995]. Besides abrogation of these negative regulatory mechanisms, transcriptional inactivity of the MoMLV LTR in murine embryonal cells can be circumvented by insertion of an active enhancer region [Linney *et al.*, 1984].

The murine embryonic stem cell virus (MESV) created by Ostertag and colleagues contains modifications in several of the above *cis*-acting elements that allow effective transgene expression in murine EC and ES cells [Grez *et al.*, 1990]. Specifically, MESV harbors a point mutation in the LTR at position -345 with respect to the transcriptional start site that destroyed the ELP binding site [Hilberg *et al.*, 1987] and another point mutation in the enhancer region at position -166 that created a functional binding site for the transcription factor Sp1 (GGGCGG), which was shown to activate the MoMLV LTR in EC and ES cells [Grez *et al.*, 1991; Prince and Rigby, 1991]. The MESV vector also possesses a 5' untranslated region from the *dl587rev* virus containing a primer binding site for tRNA^{Gln} instead of tRNA^{Pro}, which removed the factor A repressor binding site [Colicelli and Goff, 1987]. Hypothesizing that mechanisms similar to those responsible for the restriction of expression of MoMLV-based vectors in undifferentiated murine EC and ES cells might be operative in HSCs, we derived the murine stem cell virus (MSCV) retroviral vector [see Fig. (2A)] from MESV and our HMB vector which had previously been shown to direct stable gene expression in these cell types from an internal murine phosphoglycerate kinase (*pgk*) promoter [Hawley *et al.*, 1992; Hawley *et al.*, 1989]. A number of investigators have confirmed that the MSCV retroviral vector system is highly efficient at expressing transgenes in murine HSCs [Sauvageau *et al.*, 1995; Yan *et al.*, 1996; Hawley *et al.*, 1996; Persons *et al.*, 1997; Pawliuk *et al.*, 1997]. Our group subsequently created safety-modified versions of the basic MSCV backbone for potential use in human HSC gene therapy protocols [Hawley *et al.*, 1994; Ally *et al.*, 1995; Ding *et al.*, 1996; Cheng *et al.*, 1997; Pawliuk *et al.*, 1999; Patel *et al.*, 2000; Kalberer *et al.*, 2000; Stewart *et al.*, 1999]. Preclinical studies have documented sustained MSCV-directed transgene expression following transplantation of transduced human CD34⁺ hematopoietic precursors into sublethally irradiated nonobese diabetic/SCID (NOD/SCID) mice and SCID-human (SCID-hu) chimeric mice [Conneally *et al.*, 1998; Cheng *et al.*, 1998; Novelli *et al.*, 1999; Dorrell *et al.*, 2000; Kaneko *et al.*, 2001; Guenechea *et al.*, 2001].

Stable *in vivo* expression from an MSCV-based vector in T and B lymphocytes for as long as 35 weeks was also observed following engraftment of rhesus macaques with transduced peripheral blood CD34⁺ cells [Donahue *et al.*, 2000]. On the basis of these results, the MSCV vector platform is being tested in two Phase I gene therapy trials at Indiana University School of Medicine. The intent of one trial is to insert the O⁶-methylguanine DNA methyltransferase (MGMT) gene into peripheral blood CD34⁺ cells transplanted into patients with brain tumors as a prelude to future therapeutic studies designed to increase the dose of the chemotherapy regimen [Williams, 1998]. The MGMT protein repairs DNA damage caused by the nitrosourea-type alkylating agents that are commonly used to treat this type of cancer. A major side effect limiting the use of these drugs is severe myelosuppression due to bone marrow sensitivity. Preclinical studies in mice suggest that overexpression of MGMT in hematopoietic precursor cells may provide multilineage protection against the chemotherapy-induced toxicity. The second trial involves patients with X-linked chronic granulomatous disease (X-CGD) [Smith *et al.*, 1997]. X-CGD patients have an inherited disease of host defense in which the generation of superoxide by the respiratory burst oxidase of phagocytic leukocytes is absent or markedly deficient. As a result, they are susceptible to life-threatening bacterial and fungal infections. The study addresses the safety of retroviral-mediated gene transfer of a functional version of the defective gene, gp91^{phox}, into peripheral blood CD34⁺ cells, with the eventual goal being to restore respiratory burst oxidase activity.

Other groups have also made MoMLV oncoretroviral vectors with mutant LTRs and variant 5'-untranslated regions [Challita *et al.*, 1995; Riviere *et al.*, 1995; Baum *et al.*, 1995]. Kohn and colleagues created the vector MND (myeloproliferative sarcoma virus [MP^SV] enhancer, negative control region deleted, dl587rev primer binding site substituted) and observed a higher frequency of expression in the reconstituted hematopoietic systems of mice compared with a standard MoMLV-based vector

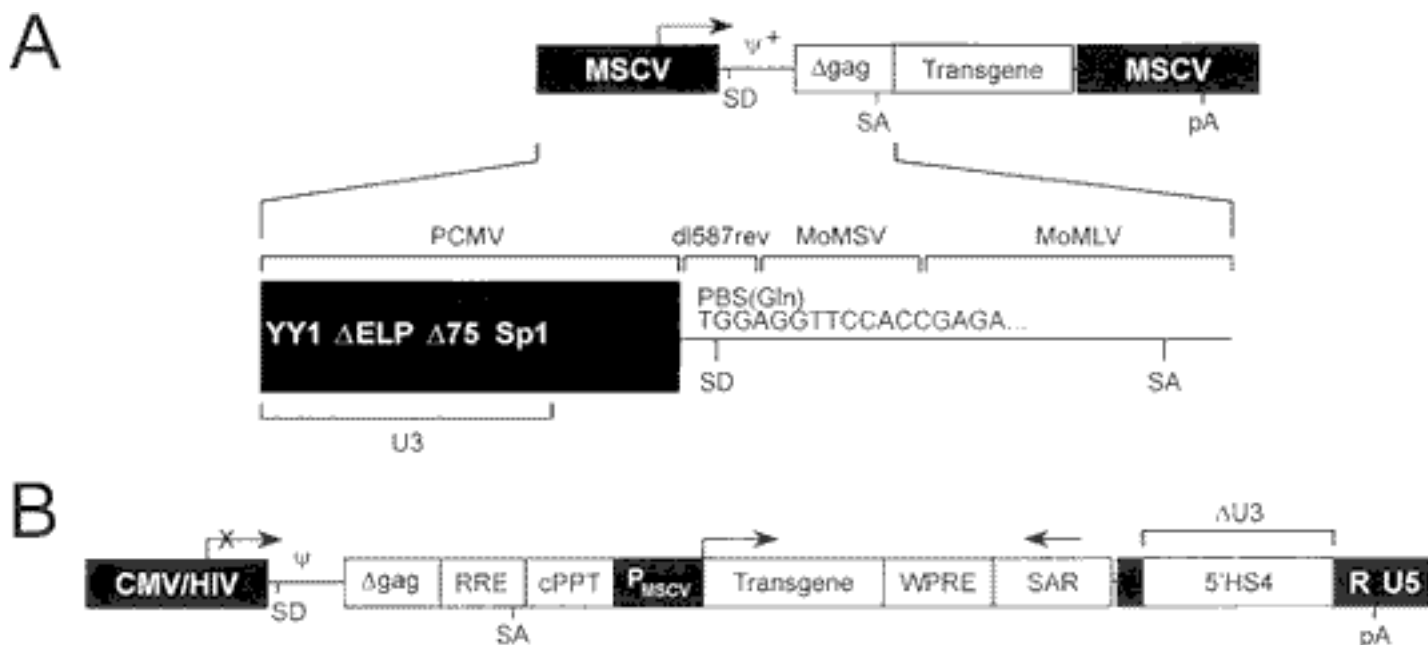


Fig. (2). Representative retroviral vectors used for HSC gene transfer. **(A)** MSCV oncoretroviral vector [Hawley *et al.*, 1994]. The region encompassing the 5' MESV/MSCV LTR and extended packaging signal (ψ^+) has been expanded to indicate the location of a number of features detailed in the text, including: the negative control region bound by the YY-1 repressor/activator; a mutated ELP binding site; deletion of one 75-bp direct repeat in the enhancer element ($\Delta 75$); a functional Sp1 binding site; and the primer binding site for tRNA^{Gln} from the *dl587rev* virus. The MESV/MSCV LTR was derived from the PCMV (PCC4 EC cell-passaged MPSY) retroviral mutant. The extended packaging signal consists of Moloney murine sarcoma virus (MoMSV) and MoMLV sequences, and harbors a mutated *gag* start codon. **(B)** SINF-MU3 HIV-1-based lentiviral vector [Ramezani *et al.*, 2000a; Ramezani *et al.*, 2000b]. The version shown is a self-inactivating vector containing a transgene expression cassette driven by the U3 region of a modified MESV/MSCV LTR (P_{MSCV}) in which the YY-1 binding site has been deleted. The backbone consists of: a chimeric cytomegalovirus immediate early region enhancer-promoter (CMV)/ HIV 5' LTR; a portion of the *gag* gene with a synthetic stop codon (Δgag); part of the *env* gene containing the RRE for nuclear export of vector RNAs; a central polypurine tract and an adjacent central termination sequence (cPPT) which creates a plus strand overlap (the central DNA flap) that acts as a *cis*-determinant for nuclear import of the HIV preintegration complex; the WPRE; the IFN-SAR in reverse orientation; and the 1.2-kb chicken γ -globin 5'HS4 insulator in the deleted U3 region of the 3' LTR, which is transferred to the 5' LTR after reverse transcription. Promoter start sites and directions of transcription are indicated. Other abbreviations: SD, splice donor; SA, splice acceptor; pA, polyadenylation site.

[Halene *et al.*, 1999]. Like the MESV/MSCV LTR, the MND LTR contains an activating Sp1 binding site in the enhancer region due to a point mutation at position -166. It differs slightly from the former in that the ELP-binding site has been retained while the YY-1 binding site has been deleted. The MPSV/MND LTR enhancer is generally stronger than the MESV/MSCV LTR enhancer, which is from PCC4 EC-cell passaged MPSV and contains only one direct repeat element [Hilberg *et al.*, 1987; Baum *et al.*, 1995]; however, there is some evidence to suggest that it may not perform as well as the MESV/MSCV LTR enhancer in HSCs [Pawliuk *et al.*, 1997; Beck-

Engeser *et al.*, 1991; Kaneko *et al.*, 2001]. Ostertag and colleagues have gone on to combine the U3 regions of the LTRs from other retroviruses with the ES cell-permissive *dl587rev* primer binding site to create hybrid retroviral vectors which they postulate may express better in HSCs than the MSCV or MND vectors [Baum *et al.*, 1995; Baum *et al.*, 1996]. Their FMEV vector backbone, incorporating the U3 region from the polycythemic strain of the spleen focus-forming virus, is especially noteworthy in that it contains an altered enhancer core and Sp1-activating mutations that disrupted the ELP-binding site [Baum *et al.*, 1997]. Xenogeneic transplant

experiments in NOD/SCID mice of human CD34⁺ cells transduced with GALV-pseudotyped FMEV-derived vectors have demonstrated persistence of transgene-expressing cells for up to 120 days post-engraftment [van Hennik *et al.*, 1998; Barquinero *et al.*, 2000]. Finally, it should be noted that the version of the MFG vector used in the clinical gene therapy trial for SCID-X1 contains a modified primer binding site (B2 mutation, a G to A transition at position +160), which Mulligan and colleagues previously reported led to significantly improved transgene expression in murine bone marrow transplant recipients [Cavazzana-Calvo *et al.*, 2000; Riviere *et al.*, 1995].

MULTIPLY ATTENUATED LENTIVIRAL VECTOR SYSTEMS

Because of their ability to transduce non-divided cells (although not necessarily those which are truly quiescent) [Follenzi *et al.*, 2000; Sirven *et al.*, 2000; Zennou *et al.*, 2000], lentiviral vectors are promising tools for *ex vivo* genetic modification of HSCs, which reside almost exclusively in the G₀/G₁ phase of the cell cycle [Cheshier *et al.*, 1999; Gothot *et al.*, 1998]. VSV-G-pseudotyped, human immunodeficiency virus-1 (HIV-1)-based vectors have been shown to readily introduce exogenous genes into human hematopoietic progenitors detectable in clonal culture assays [Akkinia *et al.*, 1996; Reiser *et al.*, 1996; Uchida *et al.*, 1998; Case *et al.*, 1999; Sutton *et al.*, 1999] as well as into more primitive precursors capable of regenerating lymphomyelopoiesis in NOD/SCID mice [Miyoshi *et al.*, 1999; Guenechea *et al.*, 2000; Ramezani *et al.*, 2000b; Woods *et al.*, 2000] and thymopoiesis in the SCID-hu thymus-liver mouse model [An *et al.*, 2000]. Current generation lentiviral vectors contain a 400-bp deletion of enhancer and promoter sequences in the U3 region of the 3' HIV-1 LTR which, after transfer to the 5' LTR by reverse transcription, yields a 'self-inactivated' proviral form incapable of synthesizing vector transcripts [Zufferey *et al.*, 1998; Dull *et al.*, 1998]. In addition to improving the biosafety of lentiviral-mediated gene delivery, removal of these LTR sequences eliminates the possibility that they might negatively influence the transcriptional

activity of the internal promoter driving transgene expression [Rosen *et al.*, 1985; Miyoshi *et al.*, 1998]. While the utility of HIV-1-based lentiviral vectors for gene transfer into human HSC subsets has been established, the majority of the lentiviral vectors presently in use are not optimized for transgene expression in HSCs. This is because the internal promoter typically utilized, the human cytomegalovirus immediate early enhancer-promoter [Boshart *et al.*, 1985] does not perform well in hematopoietic cells [Baskar *et al.*, 1996; Miyoshi *et al.*, 1999; An *et al.*, 2000]. For this reason, HIV-1-based lentiviral vectors carrying other internal promoters, such as the MESV/MSCV LTR, have recently been constructed by a number of groups and are being evaluated in HSC gene transfer studies in the context of a central DNA flap that acts as a *cis*-determinant of HIV-1 DNA nuclear import [Chang *et al.*, 1999; Follenzi *et al.*, 2000; Ramezani *et al.*, 2000a; Ramezani *et al.*, 2000b; Salmon *et al.*, 2000; Sirven *et al.*, 2000; Woods *et al.*, 2000; Zennou *et al.*, 2000].

CHROMATIN ORGANIZATION AND POSITION EFFECT VARIATION

Even though MSCV-, MND- and FMEV-type oncoretroviral vectors can overcome many of the limitations of MoMLV-based vectors, loss of transgene expression in HSCs still occurs with these backbones [Halene *et al.*, 1999; Zentilin *et al.*, 2000; Pawliuk *et al.*, 1997; Barquinero *et al.*, 2000]. Position effects due to the retroviral vector integration site are believed to contribute to transcriptional silencing of vectors shortly after integration, to expression variegation (where variable expression is observed within the clonal progeny of a target cell), and to extinction of expression (where expression is down-regulated with time) [Fig. (1A)] [Rivella and Sadelain, 1998; Emery and Stamatoyannopoulos, 1999]. There is considerable evidence that transcriptionally active genes in the eukaryotic genome are organized into independent domains. The domain organization is believed to be brought about by regions that function to attach the DNA to a skeleton of protein cross-ties called the nuclear scaffold (in metaphase) or nuclear matrix (in interphase) so-

called scaffold or matrix attachment regions (S/MARs) forming chromatin loops [Bode and Maass, 1988; Garrard, 1990]. Not all S/MARs form boundaries or 'insulators' between domains, however, since some of them reside within the introns of genes [Cockerill and Garrard, 1986]; conversely, elements exist that serve as domain boundaries without having S/MAR activity [Chung *et al.*, 1993; Chung *et al.*, 1997].

Chromatin Insulators

The first example of insulator elements that can serve as domain boundaries were the constitutive DNase I hypersensitive *scs* and *scs'* sites of *Drosophila*, which marked the chromatin boundaries of a heat shock locus and could protect an inserted exogenous gene from both negative and positive endogenous influences [Udvardy *et al.*, 1985]. Insulator elements have also been identified in vertebrates based on enhancer blocking activity. The best characterized vertebrate insulator is a 1.2-kb fragment containing the chicken γ -globin 5' constitutive DNase I hypersensitive site (5'HS4) present in all tissues [Chung *et al.*, 1993; Chung *et al.*, 1997]. Felsenfeld and colleagues first showed that the 1.2-kb chicken γ -globin 5'HS4-containing fragment blocked the activating effects of a nearby γ -globin locus control region when assayed in the human erythroid cell line K562. These investigators further demonstrated that the 5'HS4 element functions as an *scs*-like insulator in *Drosophila*, protecting a stably introduced *white* minigene from position effects. The 5'HS4 element has also been shown to prevent position effect variegation in transgenic mice [Wang *et al.*, 1997]. In other work from the Felsenfeld laboratory, transgene methylation and histone deacetylation associated with extinction of transgene expression were prevented by flanking the reporter construct with 5'HS4 insulators [Pikaart *et al.*, 1998]. A GC-rich core element of the 5'HS4 insulator has been mapped to a 250-bp fragment that contains binding motifs for Sp1 but no significant promoter activity [Chung *et al.*, 1997]. More recently, a 42-bp sequence within the core has been identified that is both necessary and sufficient for the enhancer blocking activity of the

insulator [Bell *et al.*, 1999]. The 42-bp sequence is recognized by CTCF (CCCTC-binding factor), a highly conserved and ubiquitous DNA-binding protein implicated in both transcriptional silencing and activation [Bell *et al.*, 1999].

Several groups have examined the ability of the 5'HS4 element to protect oncoretroviral vectors from position effects. Stamatoyannopoulos and colleagues incorporated the 1.2-kb 5'HS4 fragment in a 5' 3' orientation within the 3' LTR of an MSCV-based vector [Emery and Stamatoyannopoulos, 1999; Emery *et al.*, 2000]. During reverse transcription, the 5'HS4 element is copied to the 5' LTR and thus flanks the vector sequences upon integration. In a murine bone marrow transplant model, the 5'HS4 element was found to result in ~7-fold increases in the probability of expression from the MESV/MSCV LTR and the internal *pgk* promoter compared with an uninsulated vector when assayed 8 months post-transplant [Emery *et al.*, 2000]. Nienhuis and colleagues similarly observed that inclusion of the 5'HS4 element in the MESV/MSCV 3' LTR improved human γ -globin gene expression from a chimeric HS40 enhancer/ γ -globin promoter in differentiated murine erythroleukemia cells [Persons *et al.*, 2000]. The Stamatoyannopoulos group also reported that 5'HS4 insulating activity appears to be somewhat orientation-independent; decreased transgene expression and lack of insulation were observed for particular vector configurations, perhaps due to topological constraints and/or distance effects [Yannaki *et al.*, 2000]. The findings are consistent with other work which demonstrated that the 5'HS4 insulator could exert negative effects on promoter activity in some instances and that the insulating activity of the element was not dominant in all contexts [Walters *et al.*, 1999]. In this regard, it is important to note that two flanking copies of the 5'HS4 element have usually been used to prevent position effects in long-term cell culture studies and in transgenic *Drosophila* and mice [Chung *et al.*, 1993; Wang *et al.*, 1997; Pikaart *et al.*, 1998]. Full insulator activity in the enhancer blocking assay also required two copies of the 5'HS4 element [Chung *et al.*, 1993; Chung *et al.*, 1997]. In accord with these results, Sadelain and colleagues found that

when the 1.2-kb 5'HS4 fragment was inserted into the 3' LTR of an MFG-based vector, it increased the probability of transgene expression and decreased the level of de novo methylation of the retroviral LTR in erythroleukemia cells but could not prevent MFG LTR methylation and vector silencing in ES cells [Rivella *et al.*, 2000]. Notably, the 5'HS4 element did not appear to be functioning as a boundary element at the silenced integration sites since there was no evidence of DNase I hypersensitivity.

Scaffold/Matrix Attachment Regions

DNase I hypersensitive sites are frequently found in regions of DNA containing S/MARs [Gasser *et al.*, 1989]. As noted above, S/MARs are DNA elements, which are operationally defined by their *in vitro* affinity for the nuclear scaffold or matrix, that appear to mediate several functions in the eukaryotic genome including delimiting the topological borders between chromatin domains and augmenting gene expression. The 1.2-kb chicken γ -globin 5'HS4 insulator does not appear to have S/MAR activity [Chung *et al.*, 1993; Chung *et al.*, 1997]. In contrast to the core region of the 5'HS4 element, which is GC-rich (~70%) with a high density of CpG dinucleotides (reminiscent of a CpG 'island'), S/MARs are highly AT rich (>70%) [Boulikas, 1993]. Although S/MAR elements share some common structural features (i.e., stress-induced base-unpairing regions [Benham *et al.*, 1997]), no consensus sequence has been identified. Since they can be distinguished from enhancers by inactivity in transient expression experiments, it has been proposed that S/MARs may support the targeting of enhancers to the nuclear matrix providing for their proximity to transcribed units or limiting the action of an enhancer to the domain in which it participates. Another hypothesis is that S/MARs function as dominant activators to establish local access of transcription factors to enhancer/promoter sequences within a domain [Jenuwein *et al.*, 1997]. Whatever the mechanism, certain S/MARs have been demonstrated to stimulate position-independent transgene expression in stable transfection experiments and in preimplantation transgenic mice [Stief *et al.*, 1989;

Klehr *et al.*, 1991; McKnight *et al.*, 1992; Thompson *et al.*, 1999].

Two groups have investigated whether insertion of the S/MAR believed to define the upstream border of the human interferon- γ locus into various oncoretroviral vectors could result in enhanced transgene expression. Plavec and colleagues reported that incorporation of a 0.8-kb fragment of the human interferon- γ S/MAR (IFN-SAR) resulted in 2- to 10-fold improved transgene expression in human primary CD8⁺ T cells, prevented loss of transgene expression during *in vitro* differentiation of CD34⁺ cells into monocyte-macrophages (enhancing transgene expression 3- to 6-fold) and increased transgene expression in CD4⁺ T cells generated from retrovirally transduced human CD34⁺ cells engrafted into a SCID-hu thymus-liver mouse model [Agarwal *et al.*, 1998; Auten *et al.*, 1999]. Interestingly, these investigators observed an enhancing effect when the IFN-SAR was inserted into the 3' LTR or just upstream of the 3' LTR only when the element was inserted in the reverse orientation with respect to its orientation in the human interferon- γ locus. In the direct orientation, transgene expression was found to be lower than with the parental vector. Context-dependent augmentation of retroviral-mediated transgene expression by the IFN-SAR had been observed previously by Bode and colleagues [Schubeler *et al.*, 1996]. They showed that when the same IFN-SAR fragment was inserted into the 3' LTR or just upstream of the 3' LTR in an MPSV-based retroviral vector, enhancement of transgene expression occurred when the distance between the IFN-SAR and the transcriptional start site was about 4 kb. However, at distances below 2.5 kb, transcription from the retroviral LTR was essentially shut off. In more recent studies, Plavec and colleagues have documented that the IFN-SAR in the reverse orientation can confer copy number-dependent transgene expression on an MoMLV-derived retroviral vector and can prevent de novo methylation of the 5' LTR in a human T-cell line [Dang *et al.*, 2000]. It is notable that out of seven additional S/MARs tested by these investigators, only one other (undisclosed) S/MAR apparently exhibited retroviral vector enhancing activity

[Plavec *et al.*, 1999], indicating that S/MAR activity was not predictive of a positive effect on transgene expression. In view of the suggestion that eukaryotic cells utilize two strategies for chromatin opening, CpG islands and S/MARs [Zhao *et al.*, 1993], it will be of interest to determine whether the combination of the chicken β -globin 5'HS4 insulator and the human IFN-SAR in an oncoretroviral or lentiviral vector platform will provide a superior protective effect in HSCs [Fig. (1B)].

POSTTRANSCRIPTIONAL REGULATORY ELEMENTS AND CONSTITUTIVE TRANSPORT ELEMENTS

Most cellular mRNAs in eukaryotes are expressed as primary transcripts containing introns that must be removed by splicing prior to mRNA export and translation [Luo and Reed, 1999; Zhou *et al.*, 2000]. By comparison, retroviruses have evolved mechanisms that allow the nuclear export and cytoplasmic accumulation of unspliced as well as spliced RNAs. This bypass of the requirement for splicing is accomplished by specific interactions between *cis*-acting RNA transport elements and corresponding viral or cellular factors [Gruter *et al.*, 1998]. For example, MoMLV contains a nucleocytoplasmic transport element within the *gag* open reading frame, originally identified as the 'extended packaging signal' [King *et al.*, 1998]. In the case of lentiviruses like HIV-1, nuclear export is accomplished by the interaction of a virally encoded protein, Rev, with a *cis*-acting RNA element, the Rev-responsive element (RRE). The simian type D retroviruses, Mason-Pfizer monkey virus and the simian retroviruses type 1 and 2, contain autonomous RNA export signals termed constitutive transport elements, which can largely replace the HIV-1 Rev-RRE system [Bray *et al.*, 1994; Ernst *et al.*, 1997; Mautino *et al.*, 2000]. Other elements, referred to as posttranscriptional regulatory elements, are present in hepatitis B virus and woodchuck hepatitis virus [Huang and Yen, 1994; Donello *et al.*, 1998]. Recent studies have demonstrated that these elements can augment the expression of transgenes delivered by retroviral and lentiviral vectors [Gasmi *et al.*,

1999; Zufferey *et al.*, 1999; Donahue *et al.*, 2000; Kalberer *et al.*, 2000; Mautino *et al.*, 2000]. The woodchuck posttranscriptional regulatory element (WPRE) appears to be particularly effective when added to retroviral vectors, outperforming the hepatitis B virus posttranscriptional regulatory element and the Mason-Pfizer monkey virus constitutive transport element, improving transgene expression by as much as 10-fold [Zufferey *et al.*, 1999; Jiang *et al.*, 1999; Schambach *et al.*, 2000]. The increased activity of the WPRE appears to be related to an ancillary capability of the element to enhance 3'-end processing and polyadenylation [Loeb *et al.*, 2000]. Recent results have confirmed that the WPRE can markedly improve transgene expression from certain lentiviral transfer backbones in hematopoietic cells [Ramezani *et al.*, 2000b]. Notably, however, inclusion of the WPRE in lentiviral vectors carrying the human elongation factor 1 (EF1) promoter and the composite CAG promoter (consisting of the cytomegalovirus immediate early enhancer and the chicken β -actin promoter) only nominally influenced the levels of transgene expression that could be achieved from these intron-containing transcriptional units. The findings are congruent with the notion that there is substantial overlap in the functional activity of the WPRE and the splicing of introns [Huang *et al.*, 1999]—a concerted process that is tightly linked to 3'-end formation, polyadenylation and cytoplasmic export of mRNAs [Minvielle-Sebastia and Keller, 1999]—and provide a basis for the rational design of optimized lentiviral delivery vehicles for human HSC gene transfer applications.

CONCLUDING REMARKS

In conclusion, the accumulated data suggest that the use of modified LTRs in conjunction with alternative envelope proteins will result in improved oncoretroviral-mediated transfer of functional transgenes into human HSCs. On the other hand, with a wild-type genome size of 9.7 kb and the ability to package more than 11 kb of viral RNA [Trono and Baltimore, 1990], self-inactivating HIV-1-based lentiviral vectors can more readily accommodate additional chromatin and posttranscriptional genetic control elements

than MoMLV-based oncoretroviral vectors. This type of retroviral vector may therefore overcome the remaining obstacles preventing widespread successful translation of HSC gene transfer methodology to the clinic. Studies in NOD/SCID mice transplanted with candidate human HSCs transduced with next generation HIV-1-derived lentiviral vectors incorporating the features discussed in this review [Fig. (2B)] are currently underway [Ramezani *et al.*, 2000a; Ramezani *et al.*, 2000b]. Positive outcome of these investigations is expected to provide the foundation for therapeutic HSC gene transfer in humans.

REFERENCES

- Agarwal, M., Austin, T.W., Morel, F., Chen, J., Bohnlein, E., and Plavec, I. (1998) Scaffold attachment region-mediated enhancement of retroviral vector expression in primary T cells. *J. Virol.*, **72**: 3720-3728.
- Akkina, R.K., Walton, R.M., Chen, M.L., Li, Q.-X., Planelles, V., and Chen, I.S.Y. (1996) High-efficiency gene transfer into CD34⁺ cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G. *J. Virol.*, **70**: 2581-2585.
- Ally, B.A., Hawley, T.S., Kundig, T.M., Oehen, S.U., Pircher, H., Hawley, R.G., and Ohashi, P.S. (1995) Prevention of autoimmune disease by retroviral-mediated gene therapy. *J. Immunol.*, **155**: 5404-5408.
- An, D.S., Wersto, R.P., Agricola, B.A., Metzger, M.E., Lu, S., Amado, R.G., Chen, I.S.Y., and Donahue, R.E. (2000) Marking and gene expression by a lentivirus vector in transplanted human and nonhuman primate CD34⁺ cells. *J. Virol.*, **74**: 1286-1295.
- Auten, J., Agarwal, M., Chen, J., Sutton, R., and Plavec, I. (1999) Effect of scaffold attachment region on transgene expression in retrovirus vector-transduced primary T cells and macrophages. *Hum. Gene Ther.*, **10**: 1389-1399.
- Barnea, E. and Bergman, Y. (2000) Synergy of SF1 and RAR in activation of Oct-3/4 promoter. *J. Biol. Chem.*, **275**: 6608-6619.
- Barquinero, J., Segovia, J.C., Ramirez, M., Limon, A., Guenechea, G., Puig, T., Briones, J., Garcia, J., and Bueren, J.A. (2000) Efficient transduction of human hematopoietic repopulating cells generating stable engraftment of transgene-expressing cells in NOD/SCID mice. *Blood*, **95**: 3085-3093.
- Baskar, J.F., Smith, P.P., Nilaver, G., Jupp, R.A., Hoffmann, S., Pepper, N.J., Tenney, D.J., Colberg-Poley, A.M., Ghazal, P., and Nelson, J.A. (1996) The enhancer domain of the human major immediate-early promoter determines cell type-specific expression in transgenic mice. *J. Virol.*, **70**: 3207-3214.
- Baum, C., Eckert, H.-G., Stocking, C., and Ostertag, W. (1996) Activity of Friend mink cell focus-forming retroviruses during myelo-erythroid hematopoiesis. *Exp. Hematol.*, **24**: 364-370.
- Baum, C., Hegewisch-Becker, S., Eckert, H.-G., Stocking, C., and Ostertag, W. (1995) Novel retroviral vectors for efficient expression of the multidrug resistance (*mdr-1*) gene in early hematopoietic cells. *J. Virol.*, **69**: 7541-7547.
- Baum, C., Itoh, K., Meyer, J., Laker, C., Ito, Y., and Ostertag, W. (1997) The potent enhancer activity of the polycythemic strain of spleen focus-forming virus in hematopoietic cells is governed by a binding site for Sp1 in the upstream control region and by a unique enhancer core motif, creating an exclusive target for PEBP/CBF. *J. Virol.*, **71**: 6323-6331.
- Beck-Engeser, G., Stocking, C., Just, U., Albritton, L., Dexter, M., Spooncer, E., and Ostertag, W. (1991) Retroviral vectors related to the myeloproliferative sarcoma virus allow efficient expression in hematopoietic stem and precursor cell lines, but retroviral infection is reduced in more primitive cells. *Hum. Gene Ther.*, **2**: 61-70.
- Bell, A.C., West, A.G., and Felsenfeld, G. (1999) The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. *Cell*, **98**: 387-396.
- Benham, C., Kohwi-Shigematsu, T., and Bode, J. (1997) Stress-induced duplex DNA destabilization in scaffold/matrix attachment regions. *J. Mol. Biol.*, **274**: 181-196.
- Berwin, B. and Barklis, E. (1993) Retrovirus-mediated insertion of expressed and non-expressed genes at identical chromosomal locations. *Nucl. Acids Res.*, **21**: 2399-2407.
- Bhatia, M., Bonnet, D., Kapp, U., Wang, J.C.Y., Murdoch, B., and Dick, J.E. (1997) Quantitative analysis reveals expansion of human hematopoietic repopulating cells after short-term *ex vivo* culture. *J. Exp. Med.*, **186**: 619-624.
- Bode, J. and Maass, K. (1988) Chromatin domain surrounding the human interferon- γ gene as defined by scaffold-attached regions. *Biochemistry*, **27**: 4706-4711.
- Bordignon, C., Notarangelo, L.D., Nobili, N., Ferrari, G., Casorati, G., Panina, P., Mazzolari, E., Maggioni, D., Rossi, C., Servida, P., Ugazio, A.G., and Mavilio, F. (1995) Gene therapy in peripheral blood lymphocytes and bone marrow for ADA(-) immunodeficient patients. *Science*, **270**: 470-475.

- Boshart, M., Weber, F., Jahn, G., Dorsch-Hasler, K., Fleckenstein, B., and Schaffner, W. (1985) A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell*, **41**: 521-530.
- Boulikas, T. (1993) Nature of DNA sequences at the attachment regions of genes to nuclear matrix. *J. Cell. Biochem.*, **52**: 14-22.
- Bray, M., Prasad, S., Dubay, J.W., Hunter, E., Jeang, K.T., Rekosh, D., and Hammarskjold, M.L. (1994) A small element from the Mason-Pfizer monkey virus genome makes human immunodeficiency virus type 1 expression and replication Rev-independent. *Proc. Natl. Acad. Sci. USA*, **91**: 1256-1260.
- Brenner, M.K., Rill, D.R., Holladay, M.S., Heslop, H.E., Moen, R.C., Buschle, M., Krance, R.A., Santana, V.M., Anderson, W.F., and Ihle, J.N. (1993) Gene marking to determine whether autologous marrow infusion restores long-term haemopoiesis in cancer patients. *Lancet*, **342**: 1134-1137.
- Case, S.S., Price, M.A., Jordan, C.T., Yu, X.J., Wang, L., Kohn, D.B., and Crooks, G.M. (1999) Stable transduction of quiescent CD34⁺CD38⁻ human hematopoietic cells by HIV-1-based lentiviral vectors. *Proc. Natl. Acad. Sci. USA*, **96**: 2988-2993.
- Cavazzana-Calvo, M., Hacein-Bey, S., de Saint Basile, G., Gross, F., Yvon, E., Nussbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J.-L., Bousso, P., le Deist, F., and Fischer, A. (2000) Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science*, **288**: 669-672.
- Challita, P.-M. and Kohn, D.B. (1994) Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation *in vivo*. *Proc. Natl. Acad. Sci. USA*, **91**: 2567-2571.
- Challita, P.-M., Skelton, D., El-Khoueiry, A., Yu, X.-J., Weinberg, K., and Kohn, D.B. (1995) Multiple modifications in *cis* elements of the long terminal repeat of retroviral vectors lead to increased expression and decreased DNA methylation in embryonic carcinoma cells. *J. Virol.*, **69**: 748-755.
- Chang, L.-J., Urlacher, V., Iwakuma, T., Cui, Y., and Zucali, J. (1999) Efficacy and safety analyses of a recombinant human immunodeficiency virus type 1 derived vector system. *Gene Ther.*, **6**: 715-728.
- Cheng, L., Du, C., Lavau, C., Chen, S., Tong, J., Chen, B.P., Scollay, R., Hawley, R.G., and Hill, B. (1998) Sustained gene expression in retrovirally transduced, engrafting human hematopoietic stem cells and their lympho-myeloid progeny. *Blood*, **92**: 83-92.
- Cheng, L., Du, C., Murray, D., Tong, X., Zhang, Y.A., Chen, B.P., and Hawley, R.G. (1997) A GFP reporter system to assess gene transfer and expression in viable human hematopoietic progenitors. *Gene Ther.*, **4**: 1013-1022.
- Cheshier, S.H., Morrison, S.J., Liao, X., and Weissman, I.L. (1999) *In vivo* proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA*, **96**: 3120-3125.
- Chung, J.H., Bell, A.C., and Felsenfeld, G. (1997) Characterization of the chicken γ -globin insulator. *Proc. Natl. Acad. Sci. USA*, **94**: 575-580.
- Chung, J.H., Whiteley, M., and Felsenfeld, G. (1993) A 5' element of the chicken γ -globin domain serves as an insulator in human erythroid cells and protects against position effect in *Drosophila*. *Cell*, **74**: 505-514.
- Cockerill, P.N. and Garrard, W.T. (1986) Chromosomal loop anchorage of the kappa immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. *Cell*, **44**: 273-282.
- Colicelli, J. and Goff, S.P. (1987) Isolation of a recombinant murine leukemia virus utilizing a new primer tRNA. *J. Virol.*, **57**: 37-45.
- Conneally, E., Eaves, C.J., and Humphries, R.K. (1998) Efficient retroviral-mediated gene transfer to human cord blood stem cells with *in vivo* repopulating potential. *Blood*, **91**: 3487-3493.
- Dang, Q., Auten, J., and Plavec, I. (2000) Human beta interferon scaffold attachment region inhibits *de novo* methylation and confers long-term, copy number-dependent expression to a retroviral vector. *J. Virol.*, **74**: 2671-2678.
- Dao, M.A., Hannum, C.H., Kohn, D.B., and Nolte, J.A. (1997) FLT3 ligand preserves the ability of human CD34⁺ progenitors to sustain long-term hematopoiesis in immunodeficient mice after *ex vivo* retroviral-mediated transduction. *Blood*, **89**: 446-456.
- Dao, M.A., Hashino, K., Kato, I., and Nolte, 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.
- Ding, C., Kume, A., Bjorgvinsdottir, H., Hawley, R.G., Pech, N., and Dinauer, M.C. (1996) High level reconstitution of respiratory burst activity in a human X-linked chronic granulomatous disease (X-CGD) cell line and correction of murine X-CGD bone marrow cells by retroviral-mediated gene transfer of human gp91^{phox}. *Blood*, **88**: 1834-1840.
- Donahue, R.E., Byrne, E.R., Thomas, T.E., Kirby, M.R., Agricola, B.A., Sellers, S.E., Gaudernack, G., Karlsson, S., and Lansdorp, P.M. (1996) Transplantation and gene transfer

of the human glucocerebrosidase gene into immunoselected primate CD34⁺Thy-1⁺ cells. *Blood*, **88**: 4166-4172.

Donahue, R.E., Wersto, R.P., Allay, J.A., Agricola, B.A., Metzger, M.E., Nienhuis, A.W., Persons, D.A., and Sorrentino, B.P. (2000) High levels of lymphoid expression of enhanced green fluorescent protein in nonhuman primates transplanted with cytokine-mobilized peripheral blood CD34⁺ cells. *Blood*, **95**: 445-452.

Donello, J.E., Loeb, J.E., and Hope, T.J. (1998) Woodchuck hepatitis virus contains a tripartite posttranscriptional regulatory element. *J. Virol.*, **72**: 5085-5092.

Dorrell, C., Gan, O.I., Pereira, D.S., Hawley, R.G., and Dick, J.E. (2000) Expansion of human cord blood CD34⁺CD38⁻ cells in *ex vivo* culture during retroviral transduction without a corresponding increase in SCID repopulating cell (SRC) frequency: dissociation of SRC phenotype and function. *Blood*, **95**: 102-110.

Dranoff, G., Jaffee, E., Lazenby, A., Golumbek, P., Levitsky, H., Brose, K., Jackson, V., Hamada, H., Pardoll, D., and Mulligan, R.C. (1993) Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. USA*, **90**: 3539-3543.

Dull, T., Zufferey, R., Kelly, M., Mandel, R.J., Nguyen, M., Trono, D., and Naldini, L. (1998) A third-generation lentivirus vector with a conditional packaging system. *J. Virol.*, **72**: 8463-8471.

Dunbar, C.E., Cottler-Fox, M., O'Shaughnessy, J.A., Doren, S., Carter, C., Berenson, R., Brown, S., Moen, R.C., Greenblatt, J., Stewart, F.M., Leitman, S.F., Wilson, W.H., Cowan, K., Young, N.S., and Nienhuis, A.W. (1995) Retrovirally marked CD34-enriched peripheral blood and bone marrow cells contribute to long-term engraftment after autologous transplantation. *Blood*, **85**: 3048-3057.

Emery, D.W. and Stamatoyannopoulos, G. (1999) Stem cell gene therapy for the α -chain hemoglobinopathies. Problems and progress. *Ann. N.Y. Acad. Sci.*, **872**: 94-107.

Emery, D.W., Yannaki, E., Tubb, J., and Stamatoyannopoulos, G. (2000) A chromatin insulator protects retrovirus vectors from chromosomal position effects. *Proc. Natl. Acad. Sci. USA*, **97**: 9150-9155.

Engel, B.C. and Kohn, D.B. (1999) Stem cell directed gene therapy. *Frontiers in Bioscience*, **4**: e26-33.

Ernst, R.K., Bray, M., Rekosh, D., and Hammarskjold, M.-L. (1997) A structured retroviral RNA element that mediates nucleocytoplasmic export of intron-containing RNA. *Mol. Cell. Biol.*, **17**: 135-144.

Flanagan, J.R., Becker, K.G., Ennist, D.L., Gleason, S.L., Driggers, P.H., Levi, B.-Z., Apella, E., and Ozato, K. (1992) Cloning of a negative transcription factor that binds to the upstream conserved region of Moloney murine leukemia virus. *Mol. Cell. Biol.*, **12**: 38-44.

Flanagan, J.R., Krieg, A.M., Max, E.E., and Khan, A.S. (1989) Negative control region at the 5' end of murine leukemia virus long terminal repeats. *Mol. Cell Biol.*, **9**: 739-746.

Follenzi, A., Ailles, L.E., Bakovic, S., Geuna, M., and Naldini, L. (2000) Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat. Genet.*, **25**: 217-222.

Garrard, W.T. (1990) Chromosomal loop organization in eukaryotic genomes. In: *Nucleic Acids and Molecular Biology*, Eds. Eckstein, F. & Lilley, D.M.J. Springer-Verlag, Berlin, pp163-175.

Gasmi, M., Glynn, J., Jin, M.-J., Jolly, D.J., Yee, J.-K., and Chen, S.-T. (1999) Requirements for efficient production and transduction of human immunodeficiency virus type 1-based vectors. *J. Virol.*, **73**: 1828-1834.

Gasser, S.M., Amati, B.B., Cardenas, M.E., and Hofmann, J.F. (1989) Studies on scaffold attachment sites and their relation to genome function. *Int. Rev. Cytol.*, **119**: 57-96.

Gothot, A., van der Loo, J.C.M., Clapp, D.W., and Srour, E.F. (1998) Cell cycle-related changes in repopulating capacity of human mobilized peripheral blood CD34⁺ cells in non-obese diabetic/severe combined immune-deficient mice. *Blood*, **92**: 2641-2649.

Grez, M., Akgün, E., Hilberg, F., and Ostertag, W. (1990) Embryonic stem cell virus, a recombinant murine retrovirus with expression in embryonic stem cells. *Proc. Natl. Acad. Sci. USA*, **87**: 9202-9206.

Grez, M., Zornig, M., Nowock, J., and Ziegler, M. (1991) A single point mutation activates the Moloney murine leukemia virus long terminal repeat in embryonal stem cells. *J. Virol.*, **65**: 4691-4698.

Gruter, P., Taberero, C., von Kobbe, C., Schmitt, C., Saavedra, C., Bachi, A., Wilm, M., Felber, B.K., and Izaurralde, E. (1998) TAP, the human homolog of Mex67p, mediates CTE-dependent RNA export from the nucleus. *Mol. Cell*, **1**: 649-659.

Guenechea, G., Gan, O.I., Dorrell, C., and Dick, J.E. (2001) Distinct classes of human stem cells that differ in proliferative and self-renewal potential. *Nat. Immunol.*, **2**: 75-82.

Guenechea, G., Gan, O.I., Inamitsu, T., Dorrell, C., Pereira, D.S., Kelly, M., Naldini, L., and Dick, J.E. (2000) Transduction of human CD34⁺CD38⁻ bone marrow and cord

blood-derived SCID-repopulating cells with third-generation lentiviral vectors. *Mol. Ther.*, **1**: 566-573.

Halene, S., Wang, L., Cooper, R.M., Bockstoe, D.C., Robbins, P.B., and Kohn, D.B. (1999) Improved expression in hematopoietic and lymphoid cells in mice after transplantation of bone marrow transduced with a modified retroviral vector. *Blood*, **94**: 3349-3357.

Hanenberg, H., Xiao, X.L., Dillo, D., Hashino, K., Kato, I., and Williams, D.A. (1996) Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nat. Med.*, **2**: 876-882.

Hawley, R.G., Fong, A.Z.C., Burns, B.F., and Hawley, T.S. (1992) Transplantable myeloproliferative disease induced in mice by an interleukin 6 retrovirus. *J. Exp. Med.*, **176**: 1149-1163.

Hawley, R.G., Hawley, T.S., Fong, A.Z.C., Quinto, C., Collins, M., Leonard, J.P., and Goldman, S.J. (1996) Thrombopoietic potential and serial repopulating ability of murine hematopoietic stem cells constitutively expressing interleukin-11. *Proc. Natl. Acad. Sci. USA*, **93**: 10297-10302.

Hawley, R.G., Lieu, F.H.L., Fong, A.Z.C., and Hawley, T.S. (1994) Versatile retroviral vectors for potential use in gene therapy. *Gene Ther.*, **1**: 136-138.

Hawley, T.S., Sabourin, L.A., and Hawley, R.G. (1989) Comparative analysis of retroviral vector expression in mouse embryonal carcinoma cells. *Plasmid*, **22**: 120-131.

Hilberg, F., Stocking, C., Ostertag, W., and Grez, M. (1987) Functional analysis of a retroviral host-range mutant: altered long terminal repeat sequences allow expression in embryonal carcinoma cells. *Proc. Natl. Acad. Sci. USA*, **84**: 5232-5236.

Huang, Y., Wimler, K.M., and Carmichael, G.G. (1999) Intronless mRNA transport elements may affect multiple steps of pre-mRNA processing. *EMBO J.*, **18**: 1642-1652.

Huang, Z.M. and Yen, T.S. (1994) Hepatitis B virus RNA element that facilitates accumulation of surface gene transcripts in the cytoplasm. *J. Virol.*, **68**: 3193-3199.

Ikeda, Y., Lala, D.S., Luo, X., Kim, E., Moisan, M.-P., and Parker, K.L. (1993) Characterization of the mouse FTZ-F1 gene, which encodes a key regulator of steroid hydroxylase gene expression. *Mol. Endocrinol.*, **7**: 852-860.

Jenuwein, T., Forrester, W.C., Fernandez-Herrero, L.A., Laible, G., Dull, M., and Grosschedl, R. (1997) Extension of chromatin accessibility by nuclear matrix attachment regions. *Nature*, **385**: 269-272.

Jiang, G., Perelman, N., Mathias, L., Pepper, K., Xu, D., and Malik, P. (1999) The woodchuck hepatitis virus post-

transcriptional regulatory element can greatly increase titers and expression from Moloney based retroviral vectors and improve expression of intron-less mRNA of the normally intron-dependent globin mRNA. In: American Society of Gene Therapy, 2nd Annual Meeting, 142a.

Jones, P.L., Veenstra, G.J., Wade, P.A., Vermaak, D., Kass, S.U., Landsberger, N., Strouboulis, J., and Wolffe, A.P. (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat. Genet.*, **19**: 187-191.

Kalberer, C.P., Pawliuk, R., Imren, S., Bachelot, T., Takekoshi, K.J., Fabry, M., Eaves, C.J., London, I.M., Humphries, R.K., and Leboulch, P. (2000) Preselection of retrovirally transduced bone marrow avoids subsequent stem cell gene silencing and age-dependent extinction of expression of human β -globin in engrafted mice. *Proc. Natl. Acad. Sci. USA*, **97**: 5411-5415.

Kaneko, S., Onodera, M., Fujiki, Y., Nagasawa, T., and Nakauchi, H. (2001) Simplified retroviral vector GCsap with murine stem cell virus long terminal repeat allows high and continued expression of enhanced green fluorescent protein by human hematopoietic progenitors engrafted in nonobese diabetic/severe combined immunodeficient mice. *Hum. Gene Ther.*, **12**: 35-44.

Kelly, P.F., Vandergriff, J., Nathwani, A., Nienhuis, A.W., and Vanin, E.F. (2000) Highly efficient gene transfer into cord blood nonobese diabetic/severe combined immunodeficiency repopulating cells by oncoretroviral vector particles pseudotyped with the feline endogenous retrovirus (RD114) envelope protein. *Blood*, **96**: 1206-1214.

Kempler, G., Freitag, B., Berwin, B., Nanassy, O., and Barklis, E. (1993) Characterization of the Moloney murine leukemia virus stem cell-specific repressor binding site. *Virology*, **193**: 690-699.

Kiem, H.-P., Andrews, R.G., Morris, J., Peterson, L., Heyward, S., Allen, J.M., Rasko, J.E.J., Potter, J., and Miller, A.D. (1998) Improved gene transfer into baboon marrow repopulating cells using recombinant human fibronectin fragment CH-296 in combination with interleukin-6, stem cell factor, Flt-3 ligand, and megakaryocyte growth and development factor. *Blood*, **92**: 1878-1886.

King, J.A., Bridger, J.M., Gounari, F., Lichter, P., Schulz, T.F., Schirmacher, V., and Khazaie, K. (1998) The extended packaging sequence of MoMLV contains a constitutive mRNA nuclear export function. *FEBS Lett.*, **434**: 367-371.

Klehr, D., Maass, K., and Bode, J. (1991) Scaffold-attached regions from the human interferon domain can be used to enhance the stable expression of genes under the control of various promoters. *Biochemistry*, **30**: 1264-1270.

- Kohn, D.B., Weinberg, K.I., Nolta, J.A., Heiss, L.N., Lenarsky, C., Crooks, G.M., Hanley, M.E., Annett, G., Brooks, J.S., el-Khoureyi, A., Lawrence, K., Wells, S., Moen, R.C., Bastian, J., Williams-Herman, D.E., Elder, M., Wara, D., Bowen, T., Hershfield, M.S., Mullen, C.A., Blaese, R.M., and Parkman, R. (1995) Engraftment of gene-modified umbilical cord blood cells in neonates with adenosine deaminase deficiency. *Nat. Med.*, **1**: 1017-1023.
- Krall, W. and Kohn, D.B. (1996) Expression levels by retroviral vectors based upon the N2 and the MFG backbones. *Gene Ther.*, **3**: 365
- Krall, W.J., Skelton, D.C., Yu, X.-J., Riviere, I., Lehn, P., Mulligan, R.C., and Kohn, D.B. (1996) Increased levels of spliced RNA account for augmented expression from the MFG retroviral vector in hematopoietic cells. *Gene Ther.*, **3**: 37-48.
- Lange, C. and Blankenstein, T. (1997) Loss of retroviral gene expression in bone marrow reconstituted mice correlates with down-regulation of gene expression in long-term culture initiating cells. *Gene Ther.*, **4**: 303-308.
- Larochelle, A., Vormoor, J., Hannenberg, H., Wang, J.C.Y., Bhatia, M., Lapidot, T., Moritz, T., Murdoch, B., Xiao, X.L., Kato, I., Williams, D.A., and Dick, J.E. (1996) Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy. *Nat. Med.*, **2**: 1329-1337.
- Linney, E., Davis, B., Overhauser, J., Chao, E., and Fan, H. (1984) Non-function of a Moloney murine leukaemia virus regulatory sequence in F9 embryonal carcinoma cells. *Nature*, **308**: 470-472.
- Loeb, J., Harris, M., and Hope, T. (2000) The woodchuck hepatitis virus posttranscriptional regulatory element increases transgene expression by enhancing the 3'-end metabolism of mRNAs. *Mol. Ther.*, **1**: S142.
- Lu, M., Zhang, N., Maruyama, M., Hawley, R.G., and Ho, A.D. (1996) Retrovirus-mediated gene expression in hematopoietic cells correlates inversely with growth factor stimulation. *Hum. Gene Ther.*, **7**: 2263-2271.
- Luens, K.M., Travis, M.A., Chen, B.P., Hill, B.L., Scollay, R., and Murray, L.J. (1998) Thrombopoietin, kit ligand, and flk2/flt3 ligand together induce increased numbers of primitive hematopoietic progenitors from human CD34⁺Thy-1⁺Lin⁻ cells with preserved ability to engraft SCID-hu bone. *Blood*, **91**: 1206-1215.
- Luo, M.J. and Reed, R. (1999) Splicing is required for rapid and efficient mRNA export in metazoans. *Proc. Natl. Acad. Sci. USA*, **96**: 14937-14942.
- Mautino, M.R., Keiser, N., and Morgan, R.A. (2000) Improved titers of HIV-based lentiviral vectors using the SRV-1 constitutive transport element. *Gene Ther.*, **7**: 1421-1424.
- McKnight, R.A., Shamay, A., Sankaran, L., Wall, R.J., and Hennighausen, L. (1992) Matrix-attachment regions can impart position-independent regulation of a tissue-specific gene in transgenic mice. *Proc. Natl. Acad. Sci. USA*, **89**: 6943-6947.
- Minvielle-Sebastia, L. and Keller, W. (1999) mRNA polyadenylation and its coupling to other RNA processing reactions and to transcription. *Curr. Opin. Cell Biol.*, **11**: 352-357.
- Miyoshi, H., Blomer, U., Takahashi, M., Gage, F.H., and Verma, I.M. (1998) Development of a self-inactivating lentivirus vector. *J. Virol.*, **72**: 8150-8157.
- Miyoshi, H., Smith, K.A., Mosier, D.E., Verma, I.M., and Torbett, B.E. (1999) Transduction of human CD34⁺ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science*, **283**: 682-686.
- Moritz, T., Patel, V.P., and Williams, D.A. (1994) Bone marrow extracellular matrix molecules improve gene transfer into human hematopoietic cells via retroviral vectors. *J. Clin. Invest.*, **93**: 1451-1457.
- Moritz, T. and Williams, D.A. (1999) Methods for gene transfer: genetic manipulation of hematopoietic stem cells. In: Hematopoietic Cell Transplantation, Eds. Thomas, E.D., Blume, K.G., & Forman, S.J. Blackwell Science Inc., Malden, pp79-88.
- Nan, X., Ng, H.H., Johnson, C.A., Laherty, C.D., Turner, B.M., Eisenman, R.N., and Bird, A. (1998) Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature*, **393**: 386-389.
- Ninomiya, Y., Okada, M., Kotomura, N., Suzuki, K., Tsukiyama, T., and Niwa, O. (1995) Genomic organization and isoforms of the mouse ELP gene. *J. Biochem.*, **118**: 380-389.
- Novelli, E.M., Cheng, L., Yang, Y., Leung, W., Ramirez, M., Tanavde, V., Enger, C., and Civin, C.I. (1999) *Ex vivo* culture of cord blood CD34⁺ cells expands progenitor cell numbers, preserves engraftment capacity in nonobese diabetic/severe combined immunodeficient mice, and enhances retroviral transduction efficiency. *Hum. Gene Ther.*, **10**: 2927-2940.
- Patel, D.H., Allay, J.A., Belt, J.A., and Sorrentino, B.P. (2000) Retroviral transfer of the hENT2 nucleoside transporter cDNA confers broad-spectrum antifolate resistance in murine bone marrow cells. *Blood*, **95**: 2356-2363.

- Pawliuk, R., Bachelot, T., Wise, R.J., Mathews-Roth, M.M., and Leboulch, P. (1999) Long-term cure of the photosensitivity of murine erythropoietic protoporphyria by preselective gene therapy. *Nat. Med.*, **5**: 768-773.
- Pawliuk, R., Eaves, C.J., and Humphries, R.K. (1997) Sustained high-level reconstitution of the hematopoietic system by preselected hematopoietic cells expressing a transduced cell-surface antigen. *Hum. Gene Ther.*, **8**: 1595-1604.
- Persons, D., Allay, E., Hargrove, P., and Nienhuis, A.W. (2000) Recombinant globin retroviral vectors incorporating the chicken HS4 insulator element or the DHFR drug-resistance gene for *in vivo* selection enhance the probability of globin transgene expression. *Mol. Ther.*, **1**: S143.
- Persons, D.A., Allay, J.A., Allay, E.R., Smeyne, R.J., Ashmun, R.A., Sorrentino, B.P., and Nienhuis, A.W. (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.
- Petersen, R., Kempler, G., and Barklis, E. (1991) A stem cell specific silencer in the primer-binding site of a retrovirus. *Mol. Cell. Biol.*, **11**: 1214-1221.
- Pikaart, M.J., Recillas-Targa, F., and Felsenfeld, G. (1998) Loss of transcriptional activity of a transgene is accompanied by DNA methylation and histone deacetylation and is prevented by insulators. *Genes Dev.*, **12**: 2852-2862.
- Plavec, I., Agarwal, M., Moss, K., and Austin, T.W. (1999) Scaffold-attachment region-encoding retroviral vectors for improved expression in human hematopoietic cells. In: American Society of Gene Therapy, 2nd Annual Meeting, 239a.
- Porter, C.D., Collins, M.K.L., Taylor, C.S., Parkar, M.H., Cosset, F.-L., Weiss, R.A., and Takeuchi, Y. (1996) Comparison of efficacy of infection of human gene therapy target cells via four different retroviral receptors. *Hum. Gene Ther.*, **7**: 913-919.
- Prince, V.E. and Rigby, P.W.J. (1991) Derivatives of Moloney murine sarcoma virus capable of being transcribed in embryonal carcinoma stem cells have gained a functional Sp1 binding site. *J. Virol.*, **65**: 1803-1811.
- Ramezani, A., Hawley, T.S., and Hawley, R.G. (2000a) Lentiviral vector design for long-term transgene expression in human hematopoietic stem cells. *Mol. Ther.*, **1**: S138.
- Ramezani, A., Hawley, T.S., and Hawley, R.G. (2000b) Lentiviral vectors for enhanced gene expression in human hematopoietic cells. *Mol. Ther.*, **2**: 458-469.
- Rebel, V.I., Tanaka, M., Lee, J.-S., Hartnett, S., Pulsipher, M., Nathan, D.G., Mulligan, R.C., and Sieff, C.A. (1999) One-day *ex vivo* culture allows effective gene transfer into human nonobese diabetic/severe combined immune-deficient repopulating cells using high-titer vesicular stomatitis virus G protein pseudotyped retrovirus. *Blood*, **93**: 2217-2224.
- Reiser, J., Harmison, G., Kluepfel-Stahl, S., Brady, R.O., Karlsson, S., and Schubert, M. (1996) Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. *Proc. Natl. Acad. Sci. USA*, **93**: 15266-15271.
- Rivella, S., Callegari, J.A., May, C., Tan, C.W., and Sadelain, M. (2000) The cHS4 insulator increases the probability of retroviral expression at random chromosomal integration sites. *J. Virol.*, **74**: 4679-4687.
- Rivella, S. and Sadelain, M. (1998) Genetic treatment of severe hemoglobinopathies: the combat against transgene variegation and transgene silencing. *Semin. Hematol.*, **35**: 112-125.
- Riviere, I., Brose, K., and Mulligan, R.C. (1995) Effects of retroviral vector design on expression of human adenosine deaminase in murine bone marrow transplant recipients engrafted with genetically modified cells. *Proc. Natl. Acad. Sci. USA*, **92**: 6733-6737.
- Robbins, P.B., Skelton, D.C., Yu, X.-J., Halene, S., Leonard, E.H., and Kohn, D.B. (1998) Consistent, persistent expression from modified retroviral vectors in murine hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA*, **95**: 10182-10187.
- Rosen, C.A., Sodroski, J.G., and Haseltine, W.A. (1985) The location of *cis*-acting regulatory sequences in the human T cell lymphotropic virus type III (HTLV-III/LAV) long terminal repeat. *Cell*, **41**: 813-823.
- Salmon, P., Kindler, V., Ducrey, O., Chapuis, B., Zubler, R.H., and Trono, D. (2000) High-level transgene expression in human hematopoietic progenitors and differentiated blood lineages after transduction with improved lentiviral vectors. *Blood*, **96**: 3392-3398.
- Sauvageau, G., Thorsteinsdottir, U., Eaves, C.J., Lawrence, H.J., Largman, C., Lansdorp, P.M., and Humphries, R.K. (1995) Overexpression of *HOXB4* in hematopoietic cells causes the selective expansion of more primitive populations *in vitro* and *in vivo*. *Genes Dev.*, **9**: 1753-1765.
- Schambach, A., Wodrich, H., Hildinger, M., Bohne, J., Krausslich, H.-G., and Baum, C. (2000) Context dependence of different modules for posttranscriptional enhancement of gene expression from retroviral vectors. *Mol. Ther.*, **2**: 435-445.
- Schubeler, D., Mielke, C., Maass, K., and Bode, J. (1996) Scaffold/matrix-attached regions act upon transcription in a context-dependent manner. *Biochemistry*, **35**: 11160-11169.

- Sirven, A., Pflumio, F., Zennou, V., Titeux, M., Vainchenker, W., Coulombel, L., Dubart-Kupperschmitt, A., and Charneau, P. (2000) The human immunodeficiency virus type-1 central DNA flap is a crucial determinant for lentiviral vector nuclear import and gene transduction of human hematopoietic stem cells. *Blood*, **96**: 4103-4110.
- Smith, F.O., Dinauer, M.C., Williams, D.A., Cornetta, K., Curnutte, J., Hawley, R.G., Lazaridis, E., and Holbrook, E. (1997) Human Gene Marker/Therapy Clinical Protocols. Recombinant DNA Advisory Committee Protocol #9706-196. Fibronectin-assisted, retroviral-mediated transduction of CD34⁺ peripheral blood cells with gp91phox in patients with X-linked chronic granulomatous disease: a phase I study (7/21/97). *Hum. Gene Ther.*, **8**: 2301-2338.
- Sorrentino, B.P. and Nienhuis, A.W. (1999) The hematopoietic system as a target for gene therapy. In: *The Development of Human Gene Therapy*, Ed. Friedmann, T. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp351-426.
- Stewart, A.K., Dube, I.D., and Hawley, R.G. (1999) Gene marking and the biology of hematopoietic cell transfer in human clinical trials. In: *Blood Cell Biochemistry*, Vol. 8, Hematopoiesis and Gene Therapy, Eds. Fairbairn, L.J. & Testa, N. Kluwer Academic/Plenum Publishers, New York, pp243-268.
- Stief, A., Winter, D.M., Stratling, W.H., and Sippel, A.E. (1989) A nuclear DNA attachment element mediates elevated and position-independent gene activity. *Nature*, **341**: 343-345.
- Sutton, R.E., Reitsma, M.J., Uchida, N., and Brown, P.O. (1999) Transduction of human progenitor hematopoietic stem cells by human immunodeficiency virus type 1-based vectors is cell cycle dependent. *J. Virol.*, **73**: 3649-3660.
- Thompson, E.M., Christians, E., Stinnakre, M.-G., and Renard, J.-P. (1999) Scaffold attachment regions stimulate HSP70.1 expression in mouse preimplantation embryos but not in differentiated tissues. *Mol. Cell. Biol.*, **14**: 4694-4703.
- Trono, D. and Baltimore, D. (1990) A human cell factor is essential for HIV-1 Rev action. *EMBO J.*, **9**: 4155-4160.
- Tsukiyama, T., Niwa, O., and Yokoro, K. (1989) Mechanism of suppression of the long terminal repeat of Moloney leukemia virus in mouse embryonal carcinoma cells. *Mol. Cell. Biol.*, **9**: 4670-4676.
- Tsukiyama, T., Ueda, H., Hirose, S., and Niwa, O. (1992) Embryonal long terminal repeat-binding protein is a murine homolog of FTZ-F1, a member of the steroid receptor superfamily. *Mol. Cell. Biol.*, **12**: 1286-1291.
- Uchida, N., Sutton, R.E., Frieria, A.M., He, D., Reitsma, M.J., Chang, W.C., Veres, G., Scollay, R., and Weissman, I.L. (1998) HIV, but not murine leukemia virus, vectors mediated high efficiency gene transfer into freshly isolated G₀/G₁ human hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA*, **95**: 11939-11944.
- Udvardy, A., Maine, E., and Schedl, P. (1985) The 87A7 chromomere: identification of novel chromatin structures flanking the heat shock locus that may define the boundaries of higher order domains. *J. Mol. Biol.*, **185**: 341-358.
- van Hennik, P.B., Verstegen, M.M.V., Bierhuizen, M.F.A., Limon, A., Wognum, A.W., Cancelas, J.A., Barquinero, J., Ploemacher, R.E., and Wagemaker, G. (1998) Highly efficient transduction of the green fluorescent protein gene in human umbilical cord blood stem cells capable of cobblestone formation in long-term cultures and multilineage engraftment of immunodeficient mice. *Blood*, **92**: 4013-4022.
- Walters, M.C., Fiering, S., Bouhassira, E.E., Scalzo, D., Goeke, S., Magis, W., Garrick, D., Whitelaw, E., and Martin, D.I.K. (1999) The chicken γ -globin 5'HS4 boundary element blocks enhancer-mediated suppression of silencing. *Mol. Cell. Biol.*, **19**: 3714-3726.
- Wang, Y., DeMayo, F.J., Tsai, S.Y., and O'Malley, B.W. (1997) Ligand-inducible and liver-specific target gene expression in transgenic mice. *Nat. Biotech.*, **15**: 239-243.
- Williams, D.A. (1998) Recombinant DNA Advisory Committee Protocol #9701-173: A pilot study of dose intensified procarbazine, CCNU, Vincristine (PCV) for poor prognosis pediatric and adult brain tumors utilizing fibronectin-assisted, retroviral-mediated modification of CD34⁺ peripheral blood cells with O⁵-methylguanine DNA methyltransferase. *Hum. Gene Ther.*, **9**: 913.
- Williams, D.A., Nienhuis, A.W., Hawley, R.G., and Smith, F.O. (2000) Gene Therapy 2000. In: *Hematology 2000*, The American Society of Hematology Education Program Book, Ed. Schechter, G. W.B. Saunders Company, Philadelphia, pp97-114.
- Williams, D.A., Orkin, S.H., and Mulligan, R.C. (1986) Retrovirus-mediated transfer of human adenosine deaminase gene sequences into cells in culture and into murine hematopoietic cells *in vivo*. *Proc. Natl. Acad. Sci. USA*, **83**: 2566-2570.
- Woods, N.-B., Fahlman, C., Mikkola, H., Hamaguchi, I., Olsson, K., Zufferey, R., Jacobsen, S.E., Trono, D., and Karlsson, S. (2000) Lentiviral gene transfer into primary and secondary NOD/SCID repopulating cells. *Blood*, **96**: 3725-3733.
- Yamauchi, M., Freitag, B., Khan, C., Berwin, B., and Barklis, E. (1995) Stem cell factor binding to retrovirus primer binding site silencers. *J. Virol.*, **69**: 1142-1149.
- Yan, X.-Q., Lacey, D., Hill, D., Chen, Y., Fletcher, F., Hawley, R.G., and McNiece, I.K. (1996) A model of

myelofibrosis and osteosclerosis in mice induced by overexpressing thrombopoietin (mpl ligand): reversal of disease by bone marrow transplantation. *Blood*, **88**: 402-409.

Yang, W.-M., Inouye, C., Zeng, Y., Bearss, D., and Seto, E. (1996) Transcriptional repression by YY1 is mediated by interaction with a mammalian homolog of the yeast global regulator RPD3. *Proc. Natl. Acad. Sci. USA*, **93**: 12845-12850.

Yannaki, E., Emery, D.W., Tubb, J., and Stamatoyannopoulos, G. (2000) Topological constraint governing the use of a chicken HS4 insulator in retrovirus vectors. *Mol. Ther.*, **1**: S138.

Zennou, V., Petit, C., Guetard, D., Nerhbass, U., Montagnier, L., and Charneau, P. (2000) HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell*, **101**: 173-185.

Zentilin, L., Qin, G., Tafuro, S., Dinauer, M.D., Baum, C., and Giacca, M. (2000) Variegation of retroviral vector gene expression in myeloid cells. *Gene Ther.*, **7**: 153-166.

Zhao, K., Kas, E., Gonzalez, E., and Laemmli, U.K. (1993) SAR-dependent mobilization of histone H1 by HMG-I/Y *in vitro*: HMG-I/Y is enriched in H1-depleted chromatin. *EMBO J.*, **12**: 3237-3247.

Zhou, Z., Luo, M., Straesser, K., Katahira, J., Hurt, E., and Reed, R. (2000) The protein Aly links pre-messenger-RNA splicing to nuclear export in metazoans. *Nature*, **407**: 401-405.

Zufferey, R., Donello, J.E., Trono, D., and Hope, T.J. (1999) Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J. Virol.*, **73**: 2886-2892.

Zufferey, R., Dull, T., Mandel, R.J., Bukovsky, A., Quiroz, D., Naldini, L., and Trono, D. (1998) Self-inactivating lentivirus vector for safe and efficient *in vivo* gene delivery. *J. Virol.*, **72**: 9873-9880.