GENE THERAPY FOR ATHABASCAN SCID

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DEDICATION

I dedicate this work to my family. To my father, James, and my mother, Lorraine, for stressing the importance of education and encouraging me to pursue my passion for science throughout my undergraduate and graduate studies. I would also like to thank my parents for inspiring me to set goals and to persistently apply a strong work ethic in order to achieve them, an action often reflected throughout their own lives. I would like to thank my brother, Trevor, for teaching me to never take life too seriously and that success can only truly be achieved by following the path carefully determined and set by oneself.

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ABSTRACT

Artemis is an endonuclease characterized as a key factor involved in both non-homologous end joining (NHEJ) and variable (diversity) joining (V(D)J) recombination. Mutations in the gene encoding Artemis result in a radiation-sensitive form of severe combined immunodeficiency (SCID) found at a high incidence in Athabascan-speaking Native Americans (SCID-A) and characterized by the absence of mature B and T lymphocytes. Early treatment is critical since otherwise the disease results in severe infections that ultimately lead to fatality at a young age. The current therapy for SCID-A is allogeneic hematopoeitic cell transplantation (HCT); however, HCT often results in incomplete reconstitution of B lymphocytes and may lead to complications such as graft versus host disease. Transplantation with genetically corrected autologous cells is an alternative approach that may provide improved treatment of SCID-A.

Lentiviral vectors pseudotyped with VSV-G are compelling candidate vectors for gene transfer considering their high transduction efficiency and capability to mediate gene transfer in non-dividing cells populations, such as quiescent hematopoietic stem cells. Accordingly, I developed several lentiviral vectors for the transduction of human Artemis cDNA into hematopoeitic cells for the correction of a murine model of SCID-A. Upon characterization of these vectors I found that Artemis over-expression results in a decrease in cell survival due to genomic DNA fragmentation, cell cycle arrest, and ultimately apoptosis. These data emphasize the importance of transgene regulation and demonstrate the necessity of establishing conditions that provide Artemis expression at a level

that is non-toxic yet sufficient to complement Artemis deficiency. To this end, I subsequently recovered and characterized the endogenous human Artemis promoter (APro) as a one-kilobase region located directly upstream of the human Artemis translational start site. APro conferred a moderate level of reporter gene expression *in vitro* and *in vivo*, including secondary mouse transplant recipients, thus demonstrating reliable expression after lentiviral gene transfer into hematopoeitic stem cells. Subsequently, I compared innate regulation of the human Artemis cDNA using its own endogenous promoter sequence to that of the strong EF1α and more moderate PGK promoter for the capacity to mediate correction of a murine model of Artemis deficiency presenting a B^- T^- phenotype and exhibiting no leakiness (mArt $^-/^-$). Transplantation with both APro-hArtemis and PGK-hArtemis transduced mArt $^-/^-$ marrow led to complete reconstitution of the immune compartment in the recipient animals. Beginning at 8 weeks post-transplant, the recipient animals had wild-type levels of $CD3^+CD4^+$ and $CD3^+CD8^+$ T lymphocytes and $B220^+NK1.1^-$ B lymphocytes, cell populations that are absent in mArt $^-/^-$ immunodeficient mice. However, transplantation with EF1α-hArtemis transduced marrow did not support immune reconstitution, suggestive of cytotoxic effects caused by Artemis over-expression. APro-hArtemis treated mice exhibited restored IgM and IgG responses against 4-hydroxyl-3-nitrophenylacetyl hapten conjugated-keyhole limpet hemocyanin as well as restored cellular immune function, as assessed by *in vitro* stimulation of isolated splenocytes with anti-CD3 or concanavalin A. These results demonstrate that the naturally regulated Artemis lentiviral vector effectively complemented

murine SCID-A, contributing to the development and advancement of gene transfer as a clinically relevant and feasible approach for treatment of SCID-A in humans.

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CHAPTER 1

INTRODUCTION

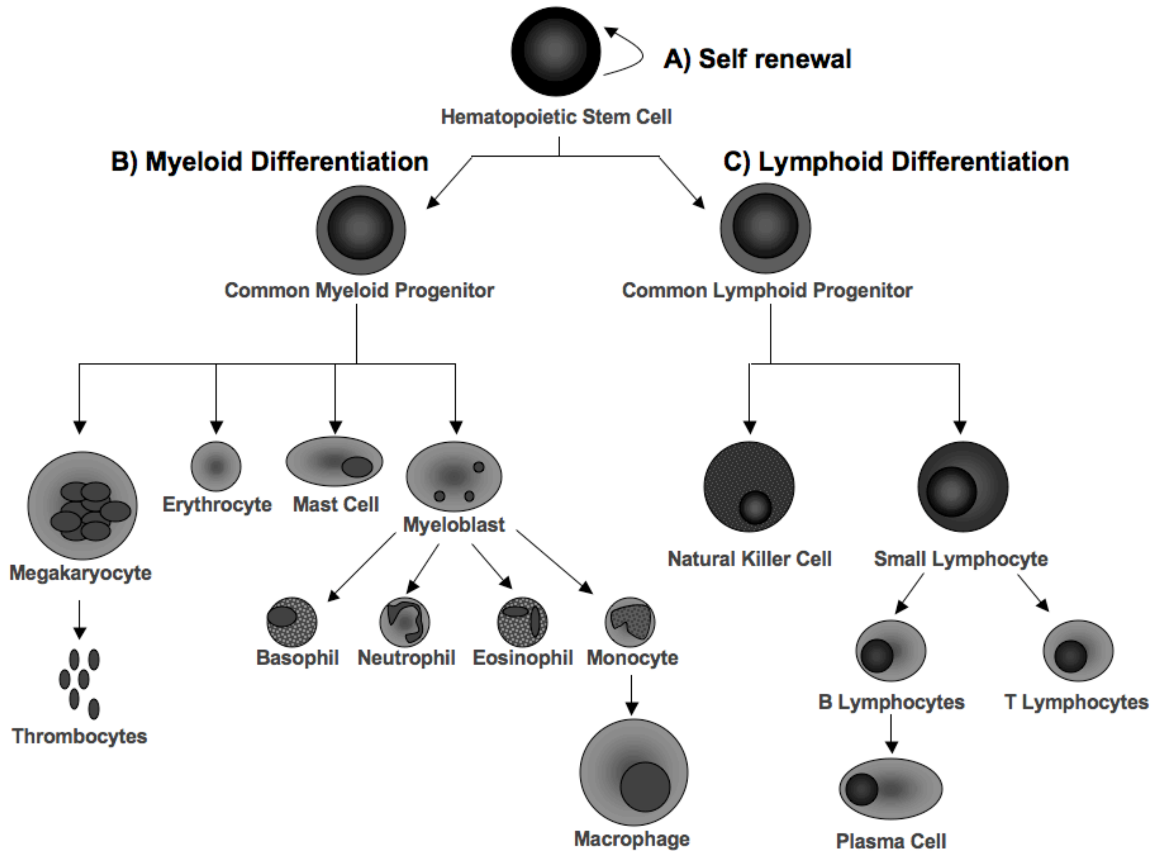
PRIMARY IMMUNODEFICIENCIES

DISCOVERY AND CHARACTERIZATION. A complete and functioning immune system is a highly regulated and integrated series of defenses mediated by several biological structures, cellular components, and chemical cascades. Immunodevelopment begins at hematopoiesis, which is the process of formation, development, and differentiation of whole blood components of both innate and adaptive immunity. During this process multipotent hematopoeitic stem cells (HSC) receive signals inducing both self-sustaining proliferation as well as delineation towards myeloid, or lymphoid differentiation and development (**Figure 1**). Genetic mutations occurring within a HSC will be carried through all lineages; consequently, if this genetic mutation disrupts one of several steps in the normal process of immunodevelopment, it is characterized as a primary immunodeficiency (PID), and will result in a perturbation of the immune system rendering the patient susceptible to frequently reoccurring infections.

PIDs were first identified upon the advent of using sulfonamides and antibiotics to treat infection. While antibiotics were successful in treating most pathogen-induced illness, a rare group of children emerged presenting with reoccurring infections (11). This immediately caught the attention of pediatrician Ogdeon Bruton, who identified an 8-year-old patient presenting with reoccurring pneumonia infections. Upon his attempt to locate antibody production against pneumococci, Bruton discovered that this patient completely lacked serum gamma immunoglobulin and termed this disorder agammaglobulinemia (12). Bruton addressed this disorder by administering subcutaneous immune human serum globulin; subsequently, the patient no longer experienced reoccurring

sepsis (12) (53). This discovery changed the world of medicine by extending the focus on infection beyond the culpability of a pathogen to that of proper immune function and the consequence of immune deficiency.

Figure 1: Hematopoiesis. Hematopoiesis encompasses the process of formation, development, and differentiation of whole blood cell components of both innate and adaptive immunity. During this process, multipotent hematopoeitic stem cells (HSC) encompass the potential to maintain A) self-sustaining proliferation as well as differentiation into two separate lineages; B) myeloid, or C) lymphoid. The myeloid lineage results from the differentiation of a common myeloid progenitor into a number of morphologically and functionally distinct cell types including subsets of megakaryocytes, erythrocytes, mast cells, granulocytes, monocytes, and macrophages. The lymphoid lineage arises from differentiation of a common lymphoid progenitor into B, T, and natural killer (NK) cells.


A) Self renewal
Hematopoietic Stem Cell
B) Myeloid Differentiation
C) Lymphoid Differentiation
Common Myeloid Progenitor
Common Lymphoid Progenitor
Erythrocyte
Mast Cell
Myeloblast
Megakaryocyte
Thrombocytes
Basophil
Neutrophil
Eosinophil
Monocyte
Macrophage
Natural Killer Cell
Small Lymphocyte
B Lymphocytes
T Lymphocytes
Plasma Cell

CLINICAL PRESENTATION AND PENETRANCE. Since the discovery of PIDs in the 1950s, the International Union of Immunological Societies Expert Committee on Primary Immunodeficiencies has classified over 120 genetic mutations that account for over 150 different PIDs, some of which are relatively common while others are quite rare (53). This organization gathers biannually to identify novel PIDs and classify these disorders with the ultimate goal of facilitating diagnosis and treatment (24).

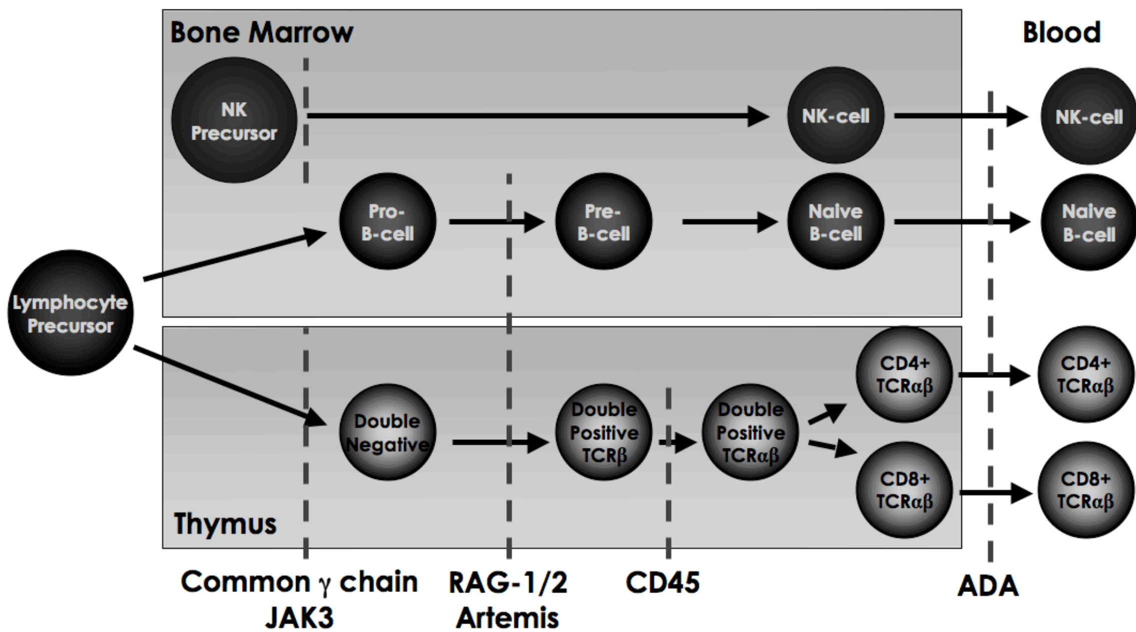
Considering that PIDs are an inherited, heterogeneous class of disorders affecting distinct components of both the innate and the adaptive immune system, these diseases can be subdivided into several classes that include phagocytic disorders and complement deficiencies as well as disorders of humoral immunity affecting B lymphocyte differentiation or antibody production and T lymphocyte defects (**Figure 1**) (53).

SCID emerges in patients presenting with T lymphocyte deficiency accompanied by defects within one or more immune sub-compartments. SCID may result from a variety of molecular defects ensuing in profound developmental or functional perturbations within the lymphocyte compartment as a whole. These defects include deficiencies of signaling molecules (common γ chain, IL-7 receptor α, and JAK-3), molecules regulating T-cell receptor and immunoglobulin rearrangement (Artemis, Cernunnos, DNA Ligase IV, DNA-PKcs, and RAG-1/2), or signaling through the pre-T cell receptor (CD3δ, CD3ε, CD3ζ, and CD45) (11, 20, 53) (**Figure 2**). Without treatment, SCID is fatal within the first year of life, which therefore necessitates early detection and intervention.

Children are routinely diagnosed with a PID within the first year of birth when the child presents with an increased susceptibility to infection, readily develops opportunistic infection, or displays an overall inability to thrive. Laboratory analysis is then undertaken to determine the presence or absence of immunoglobulin, evaluate cellular immunity, and assess complement function. If values are found to be outside the appropriate reference ranges, further studies are conducted to determine the genetic mutation and properly diagnose the primary immunodeficiency.

Figure 2: Genetic Mutations Resulting in Severe Combined Immunodeficiency. Several mutations resulting in severe combined immunodeficiency have been identified. A) Schematic representation of different genetic defects, which result in a halt at varying stages throughout immunodevelopment. Perturbation of T or B lymphocyte and NK cell development within the thymus or bone marrow, respectively, results in the absence of circulating lymphocytes. B) Each genetic deficiency results in the disruption of immunodevelopment and ultimately presents with the absence of circulating T lymphocytes, and the absence or presence of B lymphocytes and NK cells.

A) Genetic mutations arresting lymphoid development ultimately resulting in SCID



B) SCID deficiencies with associated lymphoid presentation and mechanisms

Deficiency	T Cells	B Cells	NK Cells	Mechanism
Common γ chain	-	+ defective	-	Defective cytokine-dependent developmental signaling
JAK-3	-	+ defective	-	Defective cytokine-dependent developmental signaling
Artemis	-	-	+	Defective V(D)J rearrangement
RAG-1/2	-	-	+	Defective V(D)J rearrangement
CD45	-	+ defective	+	Defective TCR signaling
ADA	-	+/- defective	-	Premature Lymphocyte Death

CURRENT THERAPY FOR PRIMARY IMMUNODEFICIENCIES

HEMATOPOIETIC STEM CELL TRANSPLANTATION. Given that inherited immunodeficiency results in an increased susceptible to infection, patients endure recurrent health tribulations that often develop into serious and debilitating illnesses. Therefore, the treatment for SCID is considered a pediatric emergency, and without treatment most children will die within the first year of life due to severe infections that result in a failure to thrive. The goal of therapy for a PID is to maximize the body's ability to fight off an infection and to reduce the risk of acquiring an infection from the environment.

After diagnosis, the first therapeutic measure is to minimize the potential for infection; precautions are taken by administering daily antibiotics as well as intravenous immunoglobulin. However, the overall goal is to augment the child's immune system so that it will obtain the ability to elicit an effective response against infection. The current standard of care for these patients is allogeneic hematopoietic stem cell transplantation (HSCT). HSCT replaces the child's abnormal blood-forming cells with healthy hematopoietic stem cells (HSC) from a bone marrow donation or cord blood unit. This process ultimately complements the immune deficiency by allowing donor HSCs to engraft and then differentiate and successfully repopulate a complete and functional immune system.

ATTENDANT RISKS OF CURRENT THERAPY. HSCT with a fully matched human leukocyte antigen (HLA) donor has proven to be highly successful,

approaching a survival rate of 90% at five years post-treatment, thus remaining the therapy of choice if an HLA-matched donor is available (56). However, in the absence of an HLA matched donor, haploidentical donors are typically employed. Transplantation outcomes from HLA-mismatched donors are remarkably inferior, carrying a survival rate of only 50 to 70% dependent on the disease background, and presenting several additional inherent risks (56).

One additional risk arising during haploidentical HLA-mismatched HSCT is graft versus host disease (GVHD), in which transplanted immunocompetent cells mount an immunologic response against the tissues of the recipient, as well as an inherent risk of opportunistic infection. Taking into account the immunocompromised nature of these patients, they lack the ability to counter attack any infiltrating donor T lymphocytes responsible for initiating GVHD as well as any pathogens encountered during the procedure.

HLA matching also plays a key role in HSC engraftment and leukocyte repopulation potential. Patients receiving a sibling HLA-matched HSCT exhibit rapid lymphocyte reconstitution, demonstrating marked expansion and proliferation of the T lymphocyte compartment (21). However, this outcome is strikingly different in patients receiving haploidentical HLA-mismatched HCST in that T lymphocyte repopulation is significantly delayed, rendering the patient profoundly immunodeficient months after transplant (21). Additionally, the immunodeficient phenotype plays a major role in determining the disease free reconstitution potential of haploidentical HLA-mismatched HSCT, particularly the presence or absence of B lymphocytes (B^+ or B^-, respectively) (8, 29). B^- SCID patients who received sibling HLA-matched HSCT demonstrated sustained

donor derived B lymphocyte reconstitution (21), whereas patients presenting with B^- SCID treated by HLA-mismatched HSCT failed to reconstitute their B lymphocyte compartment (8, 21, 29).

It has been demonstrated that applying proper preparative conditioning regimes prior to haploidentical HLA-mismatched HSCT can result in superior engraftment potential as well as repopulation of the B lymphocyte compartment to therapeutically relevant levels (8, 21). Several preparative conditioning regimens have been implemented, including immunosuppressive therapy such as horse antithymocyte globulin or myeloablative conditioning including total body irradiation or chemotherapeutic regimens such as cyclophosphamide, or busulfan (54). In patients presenting with B^- SCID, immunosuppressive preparative conditioning resulted in T lymphocyte repopulation but failed to reconstitute B lymphocyte populations, similar to the repopulation efficiency observed in patients not receiving preparative conditioning (54). Although myeloablative conditioning regimens are more likely to result in successful B lymphocyte reconstitution they are also associated with higher risks for morbidity and mortality (54). Due to these inherent risks and caveats, there is a great need for alternative therapeutic approaches for the treatment of PIDs in the absence of an HLA-matched donor.

GENE THERAPY FOR PRIMARY IMMUNODEFICIENCIES

A NOVEL APPROACH FOR THE TREATMENT OF PIDS. Recent results from clinical trials have demonstrated the effectiveness of transplantation using autologous HSC after *ex vivo* genetic correction by retroviral transduction for two

severe combined immunodeficiencies which are caused by genetic disruption of lymphocyte differentiation and survival (2, 16, 27). In these trials, the patients' hematopoietic stem cells were transduced *ex vivo* with retroviral vectors encoding a corrective product then infused back into the patient. Both of these studies reported long-term engraftment of corrected stem cells in the majority of patients, ultimately resulting in reconstitution of cellular and humoral immunity (2, 16, 27).

ADA SCID. Adenosine deaminase (ADA) deficiency is a rare inherited disorder of purine metabolism characterized by intracellular and extracellular accumulation of the toxic metabolites deoxyadenosine (dAdo) and deoxyadenosine triphosphate (dATP). This buildup results in deleterious effects on lymphocyte development: excess dATP inhibits ribonucleic reductase, an enzyme necessary for proper DNA replication and repair, induces premature lymphocyte cell death, and interferes with terminal deoxynucleotidyl transferase activity thereby limiting V(D)J recombination and antigen receptor diversity (6, 22, 36). These immunologic defects render patients impaired for T, B, and NK function as well as a variety of other systemic alterations including hepatic, skeletal, neurological, and behavioral anomalies (**Figure 2**) (1, 5, 32, 57). Although HSCT remains the mainstay of treatment, in the absence of an HLA-matched donor, enzyme replacement therapy (ERT) with PEGylated bovine ADA serves as an additional therapeutic option (2, 23). Administration of PEG-ADA may correct the metabolic defects and improves the clinical condition of these patients, however, it is limited by neutralizing antibodies rendering the

therapy unsuccessful in sustaining correction of the immunodeficient manifestation (18, 31, 41). This suggests the need for an alternative approach.

The first attempts to treat a PID by gene transfer were performed in the early 1990s where peripheral blood lymphocytes or hematopoeitic progenitor cells were transduced with a retroviral vector containing the adenosine deaminase (ADA) sequence and infused into ADA-deficient SCID patients (9). Although most patients presented with a persistent population of circulating gene-modified T lymphocytes, successful therapeutic response was limited by relatively low engraftment and gene transfer efficiency into long-lasting progenitor cell populations. Nevertheless, this trial demonstrated that gene therapy was a safe and feasible therapeutic approach towards correction of ADA-SCID [(23).

Major progress was accomplished after adoption of an improved retroviral gene transfer protocol into autologous CD34$^+$ HSCs which incorporated preparative conditioning administration of intravenous busulfan or melphalan prior to cell infusion, and withdrawal from PEG-ADA to promote a selective advantage towards ADA-transduced cell engraftment (4). Over 15 children lacking HLA-match donors, most of who had displayed an inadequate response to ERT or had failed previous transplantation attempts, have been enrolled in this experimental protocol since its initiation in 2000. Following treatment, the majority of the patients experienced long-term engraftment of genetically corrected HSC resulting in immune repopulation and restored immune function ultimately curing their immunodeficiency (2, 23, 61). Nearly 100% of circulating lymphocytes were ADA-transduced, confirming the selective

advantage of transduced cells in the absence of PEG-ADA. Clonal analysis revealed the presence of common integration sites across both myeloid and lymphoid lineages, demonstrating successful transduction and engraftment of multipotent hematopoeitic stem cells (2, 23, 61).

X-LINKED SCID. X-LINKED severe combined immunodeficiency results from a deficiency of the common gamma (γc) chain for several cytokine receptors (including IL-2, -4, -7, -9, -15, and -21) involved in lymphocyte signaling cascades responsible for growth and maturation. Deficiency of the common gamma chain results in an X-linked recessive condition of aberrant lymphocyte development presenting as a $B^{+}T^{-}NK^{-}$ phenotype (**Figure 2**). Although B lymphocytes are found in the circulation, they are rendered defective due to aberrant lymphocyte signaling and the absence of T lymphocyte mediated activation (19). Unlike ADA SCID, the only current therapy for X-linked SCID is HSCT. Despite high survival rates following identical HLA-matched HSCT, some patients continue to display reoccurring infection resultant from an aberrant humoral or cellular immune function (19). Patients lacking an adequately matched-HLA donor may also undergo HSCT but experience high mortality rates up to 50%, which increase with the addition of preparative conditioning as well as with age (56).

To date, two clinical trials based out of Paris and London have reported correction of X-linked SCID by gene therapy (16, 27). The British study utilized the conventional amphotrophic Moloney leukemia virus (MLV)- pseudotyped vector and the French group employed a gibbon ape leukemia virus (GALV)-pseudotyped vector. Both protocols were based on *ex vivo* retroviral transduction

of the common γ chain cDNA into autologous $CD34^+$ HSCs (2). Cells were harvested from patients lacking a matched HLA donor, transduced, and then infused back into the patient in the absence of preparative conditioning. Following gene transfer, 17 of the 20 treated patients benefited from the therapy displaying normal levels of T lymphocytes demonstrated by a normal response to antigen stimulation (17, 19). One year post-treatment, patients presented evidence for active thymopoiesis with a broadly diversified T cell receptor (TCR) repertoire (27). Additionally, patients demonstrated gene marking in virtually all circulating T and NK cells but to a lesser extent in B lymphocytes and myeloid cells, demonstrating successful gene transfer with a strong selective advantage for repopulation of gene corrected T and NK progenitors (17).

GENE THERAPY FOR OTHER PRIMARY IMMUNODEFICIENCIES.

Transplantation of genetically corrected autologous HSCs has emerged as a successful alternative approach for the correction of SCID. These trials have shown that gene therapy is an efficacious and effective treatment for a genetic deficiency, and this presents a compelling prospect for the correction of several other forms of inherited primary immunodeficiency. Moreover, development of several murine models of inherited immunodeficiencies displaying clinically relevant phenotypes similar to what is presented by human patients has contributed to preclinical advancement of gene therapy as a prospective treatment for several additional PIDs.

DNA DOUBLE STRAND BREAK REPAIR PROTEIN DEFICIENCIES: ARTEMIS AND RAG-1. Several preclinical studies have emerged since reports of both the ADA and X-linked SCID clinical trials. One class of deficiencies presenting as prime candidates for gene therapy are SCIDs associated with DNA double-strand break (DSB) repair proteins involved in NHEJ. NHEJ plays a vital role in adaptive immunity, which requires breaking, rearrangement, and rejoining of DNA sequences encoding immunoglobulin (Ig) genes and T cell receptor (TCR) genes (62, 68). This site-specific rearrangement process begins when the recombination activating gene (RAG) complex, comprised of RAG-1 and RAG-2, is recruited to recombination signal sequences (RSS) flanking each V, D, or J coding segment (55). The RAG complex introduces a nick adjacent to each RSS; the resulting 3′ hydroxyl group undergoes nucleophilic attack on the antiparallel DNA strand to form a hairpin structure at the coding ends (42, 58). The Artemis:DNA-Protein Kinase (DNA-PK) complex is recruited to this DNA hairpin configuration and endonucleolytically cleaves the coding end hairpin (40). The resulting DSB is processed and repaired through the NHEJ pathway (40).

Deficiencies of several key regulators of the NHEJ pathway have been reported, including the endonuclease Artemis as well as both components of the RAG complex. Clinical manifestation presents as a radiation sensitive form of severe combined immunodeficiency due to disruption of both DNA DSB repair and V(D)J recombination and is characterized by the inability to rearrange Ig and TCR genes, ultimately resulting in a loss of B and T lymphocytes (**Figure 2**).

Recently, two independent groups reported correction of a murine model of Artemis deficiency, each utilizing a lentiviral vector encoding human Artemis cDNA regulated by the PGK promoter for transduction and transplantation of hematopoeitic stem cells (7, 48). SCID-A animals receiving HSC transduced with PGK regulated human Artemis displayed repopulation of both B and T cell compartments. However, Mostoslavsky *et al*. reported that RAG-1 deficient animals receiving SCID-A HSCs transduced with either CMV or EF1α regulated human Artemis lentiviral vectors were incapable of to repopulating B and T cells, demonstrating a potential toxic effect upon Artemis over-expression ultimately necessitating further preclinical characterization of transgene expression regulation (7, 48).

Additionally, Lagreslel-Peyrou *et al.* reported long-term immune reconstitution of a murine model of RAG-1 deficiency following retroviral transduction of Sca1$^+$ cells with a vector encoding RAG-1 (35). They reported stable immune reconstitution with complete T lymphocyte repopulation accompanied by moderate, yet functional, B lymphocyte counts. High gene marking was detected in peripheral lymphoid organs with an obvious selective advantage for transduced cells over non-transduced cells (35). Interestingly immune reconstitution was only observed when a high transgene copy number could be detected, suggesting that RAG-1 may need to reach a particular threshold of expression to achieve functionality during lymphoid development.

T CELL RECEPTOR AND LYMPHOID SIGNALING DEFICIENCIES: CD45 AND JANUS KINASE 3. Additional classes of immunodeficiencies serving as

good candidates for gene therapy are SCIDs resulting from genetic disruptions due to aberrant lymphoid signaling. The T cell receptor (TCR) is a complex of integral membrane proteins initiating T lymphocyte activation in the presence of antigen. An effective immune response begins by TCR recognition of foreign antigens in the context of peptide-bound major histocompatibility complex (MHC) molecules (39). Engagement of the TCR initiates positive signal-enhancing and negative signal-attenuating cytokine cascades ultimately resulting in cytokine production, cellular proliferation, differentiation, and activation-induced cell death (39). TCR activation is regulated by various co-stimulatory receptors, such as CD45, that regulate TCR signaling by modulating tyrosine kinases ultimately antagonizing inhibitory peptides favoring T cell activation (43, 50). Deficiency of CD45 results in a $T^{-}B^{+}NK^{+}$ severe combined immunodeficiency in which thymocyte development, migration, and proliferation is blocked resulting in only a few circulating T lymphocytes with defective function which are unable to proliferate in the presence of mitogen activators (**Figure 2**) (14, 43). B lymphocyte maturation is not affected by CD45 deficiency, although these patients are unable to mount an appropriate proliferative IgM response (14).

Recently, a study by Virts *et al.* demonstrated immune restoration of a CD45 deficient mouse model generated to carry a CD45 minigene under the control of the human leukocyte function-associated antigen (LFA-1) promoter (63). CD45-deficient mice carrying the CD45 minigene exhibited lymphocyte-specific transgene expression resulting in normal thymocyte development, proliferation, and migration to the periphery. Additionally, T lymphocytes demonstrated wild type response to mitogen, and B cell anti-IgM response

against antigen was restored (63). These results demonstrate the potential effectiveness of a gene therapeutic approach towards the correction of CD45 deficient SCID, yet simultaneously emphasize the necessity for further study and development of a preclinical protocol utilizing vector mediated gene transfer of CD45 minigenes.

An additional factor involved in T cell activation is Janus Kinase 3 (JAK3), a non-receptor tyrosine kinase predominantly expressed on hematopoeitic cells and coupled with several cytokine receptors involved in signaling cascades responsible for lymphocyte growth and maturation. Mutations in JAK3 result in a perturbation of lymphocyte development clinically indistinguishable from X-linked SCID, which is not surprising considering the physical and functional links between JAK3 and the common γ chain. JAK3 deficiency is a SCID characterized by the absence of T lymphocytes and NK cells. Normal levels of circulating B lymphocytes are present, but defective in eliciting a proper immune response (**Figure 2**).

Bunting *et al*. demonstrated lymphocyte repopulation in a murine model of JAK3 deficiency following retroviral transduction and transplantation of whole marrow with a vector encoding JAK3. The gene transfer was sufficient to restore peripheral T and B lymphocytes as well as increased plasma Ig levels (13). Additionally, mice receiving gene therapy were able to elicit a specific immune response against influenza-A virus, resulting in complete clearance of the virus within two weeks of exposure, demonstrating restoration of cellular and humoral immunity. As a whole, this work demonstrates the potential

effectiveness of a retroviral vector mediated gene therapeutic approach towards the correction of JAK3 deficient SCID.

INSERTIONAL MUTAGENESIS

RETROVIRAL VECTOR MEDIATED INSERTIONAL MUTAGENESIS. Despite the success achieved by *ex vivo* transduction with GALV and MLV pseudotyped gammaretroviral vectors encoding therapeutic genes, the occurrence of serious therapy related adverse events began to emerge. Between two and six years post-treatment of X-linked SCID by retroviral gene transfer, five out of 20 children developed a leukemia-like clonal T cell proliferative disorder. This disease appeared to be the result of vector-mediated cis-activation of host cellular oncogenes. The children were treated using chemotherapeutic strategies followed by haploidentical HSCT. Currently, four of the children remain in remission from the leukemia-like proliferative disorder but one child succumbed to complications from the bone marrow transplant (28, 33).

Following emergence of these leukemia-like conditions, retroviral insertion sites were mapped to several genes implicated in leukemogenesis including the LIM domain only 2 (LMO2), a transcription factor involved in hematopoiesis, BMI1 polycomb ring finger oncogene (BMI1) whose transcript is involved in transcriptional control, and the cyclin D2 (CCND2) gene encoding a cell cycle regulatory factor (28). These results presented an inherent risk associated with transduction of HSC by retroviral vectors; to this end, novel vector systems have been characterized.

SELF-INACTIVATING LENTIVIRAL VECTORS MAY REDUCE INSERTIONAL MUTAGENESIS. Gamma-retroviral insertional patterning studies following the emergence of the X-linked SCID trial adverse events revealed that these vectors have a preference to integrate into active genes; moreover, the strong enhancing elements located within the retroviral long terminal repeat (LTR) regions may cause aberrant trans-activation of neighboring genes (28, 33). Recent *in vitro* immortalization assays have supported these claims by demonstrating that the enhancer sequences embedded in the LTR regions of gamma-retroviral vectors are the major cause of cellular transformation upon transduction (45). Development of self-inactivating (SIN) replication-defective viral vectors for gene transfer may reduce the likelihood of insertional proto-oncogene activation and may present a safer integration patterns (15, 45). These SIN vectors have a deletion within the LTR U3 region containing the retroviral CAAT and TATA boxes necessary for transcription; thus, following reverse transcription, are inactivated and unable to direct transcription of neighboring genes (51). Cell clones transduced by SIN- retroviral vectors exhibit less likelihood to activate neighboring cellular genes, thereby reducing the potential for cis-activation mediated adverse events (51, 52).

Concurrent with the implementation of SIN retroviral vectors, experimentation utilizing lentiviral vectors for gene transfer began to emerge. Previously, classical Moloney murine leukemia virus based retroviral vectors had been widely used as gene transfer vectors of choice *in vitro* considering their reliability and efficiency of gene transfer in dividing cells (10). Additionally, modifications in vector design including differential pseudotyping with various

envelope proteins assisted in producing higher titer infectious vector stocks (10). However, successful application of these vectors *in vivo* has been limited by lack of transduction in non-dividing cells. Lentiviral vectors, on the other hand, are capable of mediating gene transfer in quiescent cells, rendering these vectors compelling candidates for gene transfer into slow-dividing cell populations such as hematopoietic stem cells (10).

Retroviral vectors integrate preferentially upstream of active genes, potentially resulting in aberrant gene regulation and adverse events (37, 66). A study by Cattoglio *et al.* demonstrated the preference of retroviral vectors to integrate into recurrent "hot spots" enriched in proto-oncogenes, cancer-associated common insertion sites, and growth-regulation genes (15). In contrast, lentiviral vectors display an integration pattern exhibiting a lower propensity for insertion into transcriptionally active sites, thus demonstrating a superior safety profile for gene therapy applications (15). Taken as a whole, implementation of both self-inactivation and lentiviral vector based technologies may generate a safer gene transfer vector for future gene therapy protocols.

TRANSGENE REGULATION

ABERRANT EXPRESSION OF THE CLINICAL TRANSGENE. Although insertional activation of the LMO2 oncogene was reported in three of the leukemic cases in the X-linked SCID trial described above, it has also been demonstrated that over-expression of the transgene, the common γ chain, induces cellular proliferation and thus may have contributed to the T

lymphocyte clonal outgrowth. For example, the over-expression of the cytokine receptor IL-7, which is composed of γc and IL-7Rα subunits, results in profound changes in lymphocyte development, and in some instances can result in the development of lymphoid tumors (44). Considering the common γ chain is an integral component of several cytokine receptors, it is not surprising that aberrant γc expression under the strong regulation of the gamma-retroviral LTR may affect the function of several different cytokine cascades and lymphocyte modulation pathways and ultimately stimulate abnormal cell growth.

ELEMENTS PROMOTING AND ENHANCING TRANSGENE EXPRESSION.

Considering that the common γ chain was transcriptionally regulated by the strong retroviral LTR in the X-linked SCID trial, temporal expression may be an important goal, especially in the context of gene therapy, for expression of a signaling molecule crucial for immune development and maturation. It is interesting to note the kinetic differences of lymphocyte repopulation subsequent to transplantation of retrovirally transduced autologous HSC displayed in both of the X-linked and ADA SCID trials. X-linked SCID patients receiving transduced HSC repopulated gene-marked lymphocytes at a more rapid rate than ADA SCID patients receiving similar treatment, suggesting that the strong, constitutive γc transgene expression mediated by the gamma-retroviral LTR may have caused inappropriate temporal expression at different stages of lymphocyte development, initiating uncharacteristic proliferation and filling of the lymphocyte compartment (3). Additionally, a study by Woods *et al.* demonstrated that ubiquitous over-expression of the common γ chain transgene

in the context of a murine model of X-lined SCID contributed to the development of T cell lymphomas as compared to animals treated with an analogous GFP control vector (65). Importantly, the observed adverse effect did not correlate with integration site, but to constitutive non-temporally regulated expression of the common γ chain transgene (65). Thus, constitutive transgene regulation may not only direct cytotoxic effects but may also confer inappropriate temporal expression resulting in aberrant cellular stimulation and repopulation. Together, these studies stress the importance of regulated transgene expression.

Cis-activation of neighboring cellular genes may occur upon SIN-lentiviral transduction, albeit to a lesser extant than that observed with gamma-retroviral transduction; however, the likelihood of neighboring gene activation by SIN-lentiviral vector transduction is highly dependent upon the vector's internal promoter regulating transgene expression (30). Supporting data by Modlich *et al* demonstrated that altering internal enhancer-promoter elements conveyed a greater impact on cytotoxicity than altering insertional patterns by utilizing lentiviral vectors in lieu of retroviral vectors. Moreover, they demonstrated that a lentiviral vector expressing the Wiskott-Aldrich Syndrome protein under the control of its endogenous promoter had no transforming potential (45). These results suggest that employing natural regulatory elements of therapeutic transgenes in the context of lentiviral vector mediated gene transfer may not only benefit temporal lymphocyte repopulation but may circumvent transgene mediated toxicity as well.

THE ROLE OF DISEASE BACKGROUND IN DETERMINING ADEQUATE LEVELS OF TRANSGENE EXPRESSION. Despite the occurrence of serious adverse events associated with retroviral mediated gene therapy, the overall survival displayed by these patients is superior to survival rates displayed by patients treated by HLA-mismatched haploidentical HSCT. With this in mind, gene therapy remains a viable approach for the treatment of PIDs. It is notable that despite over 15 patients treated for ADA-deficiency by retroviral gene therapy, no patients have presented with an adverse event in contrast to patients enrolled in the X-linked SCID trial. Several possibilities may account for this discrepancy. ADA is a constitutively expressed enzyme of purine metabolism; considering the non-autonomous nature of ADA enzymatic activity, cellular over-expression may actually provide a selective advantage for the engraftment and proliferation of transduced lymphocytes ultimately benefiting systemic detoxification. In contrast, the common γ chain is a cell-autonomous transmembrane protein involved in lymphocyte proliferation and activation, for which temporally dysregulated over-expression may result in proliferative T cell expansion, as discussed. Furthermore, the X-linked SCID disease background may constitute a predisposition to the accumulation of mutations, as demonstrated in two independent mouse models, which may ultimately support the progression of a leukemia-like syndrome (59, 60).

Transgene regulation must be carefully considered when employing gene transfer for the correction of other PIDs. These include deficiencies of DNA metabolizing enzymes such as the endonuclease Artemis or the recombination mediating RAG enzymes. We have shown that Artemis over-expression

following lentiviral transduction results in global genomic damage, arrest in cell cycle progression, and ultimately apoptosis (see Chapter 2) (49). Temporally unregulated over-expression of the recombination initiating RAG enzymes may result in similar toxic effects considering their lineage-restricted expression in the development of lymphoid progenitor populations as well as their enzymatic role in DNA cleavage induction. Furthermore, taking into account the aberrant lymphoproliferative effects observed upon over-expression of the common γ chain, attention to transgene regulation must also be employed when considering gene transfer for the correction of other lymphoid deficiencies, such as JAK3 deficiency. Recently, a report by Knoops *et al.* demonstrated cytokine-independent growth with constitutive STAT activation in cells over-expressing JAK3, suggestive of *in vitro* cell transformation (34). These observations taken as a whole challenge the field of clinical gene therapy to establish conditions that provide transgene expression levels that are non-toxic yet sufficient to correct the presented phenotype. This approach will be crucial for vector development in preclinical studies of gene therapy and for clinical application to human PIDs.

STATEMENT OF THESIS

Based on this background information, I propose that gene transfer may be utilized for the treatment of SCID caused by the absence of the Artemis protein (SCID-A). Artemis deficiency results from any one of several potential missense and splice mutations and is designated SCID-A due to a founder mutation occurring in Athabascan speaking Native Americans (38, 46, 47).

Artemis is a hairpin opening endonuclease involved in the NHEJ pathway. NHEJ is the major pathway by which multicellular eukaryotic organisms repair DSBs, including insults generated by alkylating agents and ionizing radiation. The NHEJ cascade begins when the Ku70/Ku80 heterodimer recognizes and binds to the ends of a DNA DSB. Upon DNA binding, the Ku heterodimer recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to the break site (64, 67). Since the ends generated at a DSB site are rarely compatible, DNA-PKcs subsequently recruits and binds Artemis, which acquires endonucleolytic activity recognizing 5′ and 3′ overhangs (40). After Artemis digests the DNA overhangs, the break is repaired and sealed by a heteromultimer of XLF, XRCC4, and DNA Ligase IV (25, 26).

NHEJ also plays a vital role in the repair of breaks generated by normal cellular processes such as metabolism and is essential for the correction of breaks generated by RAG-mediated rearrangement of Ig genes and TCR genes (62, 68) (see above, P. 11-12). Deficiency of Artemis interrupts the DNA end joining reaction mediating Ig and TCR gene rearrangements resulting in a radiosensitive $B^-T^-NK^+$ severe combined immunodeficiency. Humans lacking Artemis therefore cannot produce antibodies or mount a cellular immune response, and are thus afflicted with SCID. Since Artemis also contributes to NHEJ, Artemis deficient humans also exhibit extreme sensitivity to radiation.

Presently, Artemis deficiency can be treated by allogeneic HSCT; however, in the absence of an appropriately matched HLA donor, allotransplant for the correction of SCID-A carries the inherent risks of incomplete reconstitution of B cell function as well as an increased susceptibility to

complications such as graft failure and graft versus host disease, all of which are associated with a decreased survival rate (54). Genetic correction of autologous hematopoeitic stem cells could provide an alternative therapeutic approach for SCID-A, thus avoiding the complications associated with allogeneic HSCT.

To this end, I generated several lentiviral vectors expressing the human Artemis cDNA sequence using different promoters with the intention of complementing Artemis deficiency *in vitro* and *in vivo*. **CHAPTER 2** details my findings upon characterization of these vectors, and that transduction by a lentiviral vector in which Artemis is regulated by the strong elongation factor-1 alpha (EF1α) promoter resulted in a dose-dependent loss of cell viability that was not observed in cultures exposed to identical amounts of control vector. Furthermore, I found this toxic effect to be reproducible in cultures transfected with an identical DNA construct, ruling out the possibility of toxicity associated with lentiviral transduction rather than Artemis expression. I performed mechanistic studies to determine the underlying cause of Artemis mediated toxicity and found that Artemis over-expression was associated with an increase in DNA damage, G1 arrest of the cell cycle, and a relative increase in the proportion of apoptotic cells.

These results underscore the importance of regulating Artemis expression in transduced cell populations and present a novel challenge for effective correction of the B^-T^- SCID-A phenotype. Establishment of conditions that provide Artemis expression that is non-toxic and yet sufficient to correct the T^-B^- phenotype will be crucial for vector development in preclinical studies using Artemis-deficient mice and in clinical application to human SCID-A.

To address the concern of appropriate Artemis expression, I hypothesized that employing natural regulatory elements of the Artemis transgene, i.e. the endogenous human Artemis promoter, would abrogate Artemis mediated cytotoxicity yet complement the deficiency. In **CHAPTER 3**, I isolated a one kilobase region directly upstream of the human Artemis translational start site (APro) and characterized the potential of this sequence to mediate transgene expression.

The APro sequence was isolated by PCR amplification, then characterized and shown to support GFP and luciferase reporter gene expression *in vitro*. Interestingly, 5'RACE revealed evidence for multiple transcriptional start points and deletion analysis revealed the ability of multiple deletion constructs to promote expression *in vitro*. Additionally, *ex vivo* transduction of murine bone marrow with an APro-regulated GFP lentiviral vector resulted in GFP expression at a significantly reduced level in comparison with control mice transplanted with EF1α-GFP transduced marrow. Importantly, the human Artemis promoter supported GFP expression in all hematopoeitic lineages that persisted in secondary transplant recipients.

These results demonstrate the effectiveness of the human Artemis promoter in providing moderate and yet reliable levels of expression in hematopoeitic lineages. Importantly, these results thereby establish the usefulness of this promoter for the purpose of generating a clinical vector that provides Artemis expression at a non-toxic level that is nonetheless sufficient to correct the B^-T^- SCID-A phenotype. In **CHAPTER 4,** I employ innate regulation of the human Artemis cDNA using its own endogenous promoter sequence for

correction of a murine model of SCID-A (mArt $^{-}/^{-}$). A lentiviral vector containing these sequences (pOK/APro-hArtemis) was assembled, packaged by VSV-G pseudotyping, and then used to transduce mArt $^{-}/^{-}$ donor marrow with subsequent transplantation into mildly pre-conditioned (500 cGy, X-irradiation) mArt $^{-}/^{-}$ recipient mice. Recipient animals were evaluated on a monthly basis post-transplant, and beginning at 8 weeks were found to have normal levels of $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ T lymphocytes and $B220^{+}NK1.1^{-}$ B lymphocytes, thus providing evidence for immune reconstitution resulting from APro-hArtemis transduction of HSCs.

I also compared the efficacy of lentiviral vectors expressing human Artemis regulated by three different promoters of varying strength: EF1α, PGK, or APro (promoters are listed in order of decreasing strength). I found that the kinetics of lymphocyte repopulation differed substantially between these groups. Animals receiving EF1α–hArtemis transduced bone marrow were not repopulated in their B cell compartments and their T lymphocyte compartments were reduced relative to normal C57BL/6 animals, with the majority displaying an aberrant response to *in vivo* antigen challenge. Animals receiving either PGK-hArtemis or APro-hArtemis transduced marrow exhibited lymphocyte compartment reconstitution with functional B and T cells at a level equal to that of wild-type C57BL/6 animals.

Overall, these data attest to the importance of transgene regulation during genetic transfer as a therapeutic approach for SCID-A, with varying outcomes observed upon appropriately regulated versus aberrantly regulated transgene expression. Moreover, these results demonstrate effective complementation of a

murine model of SCID-A following transduction with a lentiviral encoding human Artemis regulated by its endogenous promoter, ultimately contributing to the development and advancement of gene transfer as a clinically relevant and feasible approach for treatment of SCID-A in humans.

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CHAPTER 2

CYTOTOXICITY ASSOCIATED WITH ARTEMIS OVER-EXPRESSION AFTER LENTIVIRAL VECTOR MEDIATED GENE TRANSFER

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Artemis is a hairpin opening endonuclease involved in nonhomologous end-joining and V(D)J recombination. A deficiency of Artemis results in radiation-sensitive severe combined immunodeficiency characterized by the complete absence of T and B cells due to an arrest at the receptor recombination stage. We have generated several lentiviral vectors designed for transduction of the Artemis sequence intending to complement the deficient phenotype. We found that transduction by a lentiviral vector in which Artemis was regulated by a strong EF1α promoter resulted in a dose-dependent loss of cell viability due to a perturbed cell cycle distribution and increased DNA damage and apoptotic cell frequency. This toxic response was not observed in cultures exposed to identical amounts of control vector. Loss of cell viability was also observed in cells transfected with an Artemis expression construct, indicating that toxicity is independent of lentiviral transduction. Reduced toxicity was observed when cells were transduced using a moderate-strength PGK promoter to regulate Artemis expression. These results present a novel challenge in the establishment of conditions that support Artemis expression at levels that are non-toxic yet sufficient to correct the T^-B^- phenotype, crucial for preclinical studies and clinical application of Artemis gene transfer in the treatment of human SCID-A.

INTRODUCTION

DNA double strand break (DSB) repair is essential for the maintenance of genomic stability. Non-homologous end joining (NHEJ) is the canonical pathway by which multicellular eukaryotic organisms repair DSBs, including insults generated by alkylating agents, ionizing radiation and breaks generated by normal cellular processes such as RAG mediated V(D)J recombination. The NHEJ cascade begins when the Ku70/Ku80 heterodimer recognizes and binds a DNA DSB. Upon DNA binding, the Ku heterodimer recruits DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to the break site (34, 37). Since the ends generated at a DSB site are rarely compatible, DNA-PKcs subsequently recruits and binds Artemis, which acquires endonucleolytic activity recognizing 5′ and 3′ overhangs (18). After Artemis digests the DNA overhangs, the break is repaired and sealed by a heteromultimer of XLF, XRCC4, and DNA Ligase IV (9, 10).

NHEJ also plays a vital role in the rearrangement of immunoglogulin (Ig) genes and T cell receptor (TCR) genes (33, 39). This site-specific rearrangement process begins when the RAG complex, comprised of RAG-1 and RAG-2, is recruited to recombination signal sequences (RSS) flanking each V (variable), D (diversity), or J (joining) coding segment (26). The RAG complex introduces a nick adjacent to each RSS; the resulting 3′ hydroxyl group undergoes nucleophilic attack on the antiparallel DNA strand to form a hairpin structure at the coding ends (19, 29). The Artemis:DNA-PK complex then endonucleolytically cleaves the coding end hairpin and the DSB is processed and repaired through the NHEJ pathway (18).

Deficiency of Artemis interrupts Ig and TCR gene rearrangement resulting in a radiosensitive $B^-T^-NK^+$ severe combined immunodeficiency, designated SCID-A due to the founder mutation occurring in Athabascan speaking Native Americans (16, 21, 22). Artemis deficiency can be treated by allogeneic hematopoeitic cell transplantation (HCT); however, allotransplant often results in incomplete reconstitution of B cell function and is also associated with complications such as graft failure and graft versus host disease (25).

Genetic correction of autologous hematopoeitic stem cells could provide an alternative therapeutic approach for SCID-A, thus avoiding the complications associated with allogeneic HCT. Recently, two independent groups have reported correction of a SCID-A murine model, each utilizing a lentiviral vector encoding human Artemis cDNA regulated by the PGK promoter for transduction and transplantation of hematopoeitic stem cells (4, 23). SCID-A animals receiving HSC transduced with a PGK regulated human Artemis displayed complete repopulation of both B and T cell compartments. However, Mostoslavsky *et al*. reported that RAG-1 deficient animals receiving SCID-A HSCs transduced with either CMV or EF1α regulated human Artemis lentiviral vectors were unable to repopulate B and T cells (4, 23).

Our laboratory has also generated several lentiviral vectors expressing the human Artemis cDNA sequence using different promoters with the intention of complementing Artemis deficiency *in vitro* and *in vivo*. Upon characterization of these vectors, we found that transduction by a lentiviral vector in which Artemis is regulated by the strong elongation factor-1 (EF1α) promoter resulted in a dose-dependent loss of cell viability that was not observed in cultures exposed to

identical amounts of control vector. This toxic effect was reproduced in cultures transfected with the identical DNA construct, ruling out the possibility of toxicity associated with lentiviral transduction rather than Artemis expression. Mechanistically, Artemis over-expression was associated with an increase in DNA damage as determined by comet assays, G1 arrest of the cell cycle, and a relative increase in the proportion of apoptotic cells.

These results underscore the importance of regulating Artemis expression in transduced cell populations and present a novel challenge for effective correction of the B^-T^- SCID-A phenotype. Establishment of conditions that provide Artemis expression that is non-toxic and yet sufficient to correct the T^-B^- phenotype will be crucial for vector development in preclinical studies using Artemis-deficient mice and in clinical application to human SCID-A.

RESULTS

Lentiviral transduction and expression of the Artemis gene. To establish effective conditions for restoring Artemis expression by gene transfer, lentiviral vectors containing the murine or human Artemis coding sequences were generated (**Figure 1**). Initial vector constructs were also engineered for expression of green fluorescent protein (GFP) to facilitate tracking of transduced cells and quantitation of viral vector stocks by flow cytometry. Lentiviral vectors were packaged by transfection in human 293T cells as described in Materials and Methods. Transduction of mouse NIH 3T3 cells was verified forty-eight hours after exposure to vector by flow cytometry for GFP expression and by real-time quantitative-PCR. Expression of Artemis protein was verified by western blot analysis (**Figure 2**).

We developed a hairpin-opening assay to evaluate Artemis activity in cell extracts (**Figure 2**). A 19 base oligodeoxynucleotide was designed to contain an internal stem-loop structure with a predicted melting temperature of 72° C. The hairpin oligodeoxynucleotide was synthesized with a FAM fluorophore at the 5′ terminus and a TAMRA quencher at the 3′ terminus. In hairpin configuration, FAM is quenched by TAMRA; however, upon hairpin cleavage the double-stranded molecule acquires a melting temperature of 20° C, with subsequent strand dissociation at room temperature, separation of FAM from TAMRA and fluorescence at 494 nm. Increasing FAM fluorescence was monitored in real time using a Biorad Realplex thermocycler as described in Materials and Methods.

Extracts were prepared from murine embryonic fibroblasts wild-type, heterozygous, and deficient for Artemis, and analyzed for hairpin-opening

activity by increasing FAM fluorescence. As expected, Artemis wild-type cell lysates displayed greater hairpin opening activity than the Artemis deficient cell lysates (**Figure 2**). Extracts from Artemis heterozygous cells exhibited an intermediate level of hairpin opening activity (**Figure 2**). A background of hairpin opening activity was also observed in Artemis deficient MEFs, most likely due to the presence of other nucleases in whole cell extracts.

Artemis deficient MEFs were then transduced with varying amounts of EF1α-mArtemis lentiviral vector (**Figure 2**). Cell lysates were assayed for hairpin-opening activity, and, as expected, transduction with the Artemis vector at a MOI = 1 restored hairpin-opening activity to wild-type levels ($p < .005$ vs Artemis null MEFs). Surprisingly, exposure to even greater amounts of the Artemis vector resulted in a reduced level of hairpin-opening activity in cell extracts ($p < .01$ for MOI = 10 vs MOI = 5) (**Figure 2**). One potential explanation for this result is that the higher levels of Artemis expression brought about by transduction at higher multiplicity may have been toxic, resulting in loss of viability among those cells expressing the highest level of Artemis. We therefore tested the cytotoxic effect of Artemis over-expression as described below.

Figure 1. Lentiviral vector constructs. Several lentiviral vectors were constructed for analysis and complementation of Artemis deficiency. A) Lentiviral vectors engineered to express both Artemis and green fluorescent protein (GFP) cDNA sequences for monitoring transduction. B) Lentiviral vectors constructed to express either the human or murine Artemis cDNA sequence from either EF1α or PGK promoters. C) Control lentiviral vectors that express either the puromycin resistance gene or GFP regulated by EF1α. CMV, cytomegalovirus early promoter/enhancer region; U3/U5/R, unique 3'/unique 5'/repeat regions of the HIV long terminal repeat; ψ, packaging signal; cPPT, central polypurine tract; WPRE, woodchuck post-transcriptional regulatory element; IRES, internal ribosome entry site. Arrows indicate sites and direction of transcript initiation.

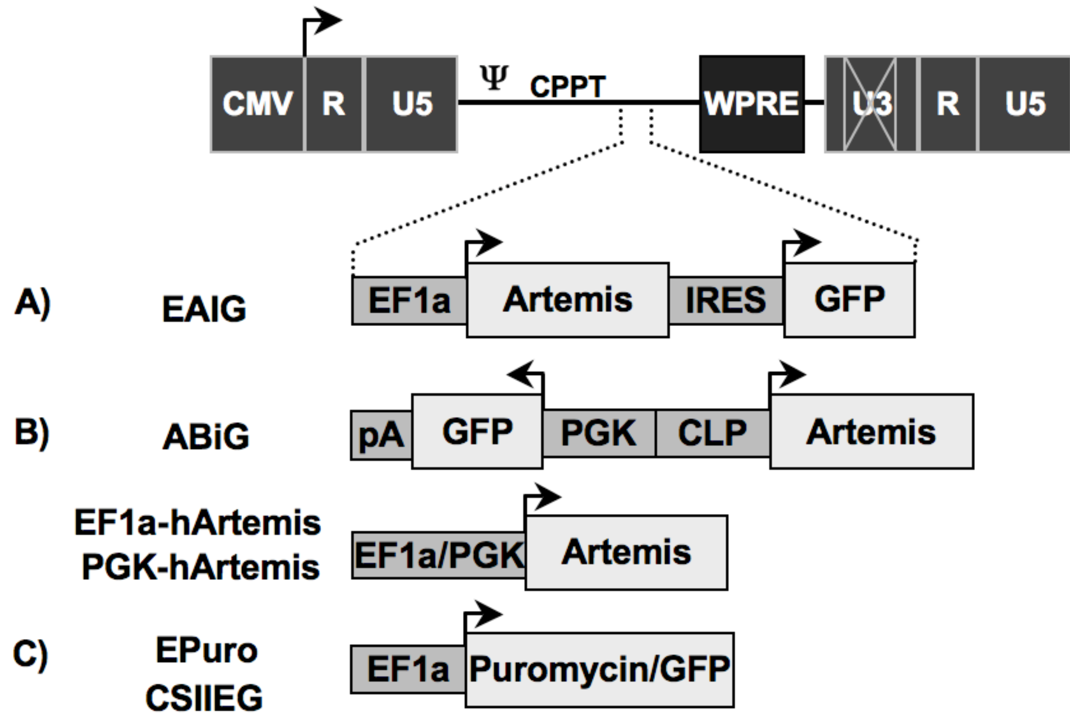
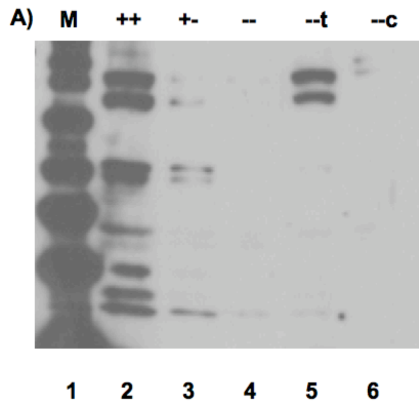

CMV
R
U5
Ψ
CPPT
WPRE
U3
R
U5
A)
EAIG
EF1a
Artemis
IRES
GFP
B)
ABiG
pA
GFP
PGK
CLP
Artemis
EF1a-hArtemis
PGK-hArtemis
EF1a/PGK
Artemis
C)
EPuro
CSIIEG
EF1a
Puromycin/GFP

Figure 2. Analysis of Artemis expression in transduced cell populations. A) Expression of Artemis protein was verified by western blot analysis (78 kDa) of nuclear extracts derived from tMEFSCIDA wild type (++), Artemis heterozygous (+-), Artemis deficient (--), and Artemis deficient MEFs transduced with the ABiG lentiviral vector (--t), or the control CSIIEG lentiviral vector (--c). B) Artemis activity was detected using a fluorescence hairpin-opening assay. Nuclear extracts generated from tMEFSCIDA cells wild-type (++), heterozygous (+-), and deficient (--) for Artemis as well as Artemis deficient MEFs transduced at increasing multiplicity with ABiG lentiviral vector were assayed for hairpin opening activity as described in Materials and Methods. 0 = no lysate; - = not transduced. Each value represents the mean of 3 replicates +/- S.D.


A)
M
++
+-
--
--t
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1
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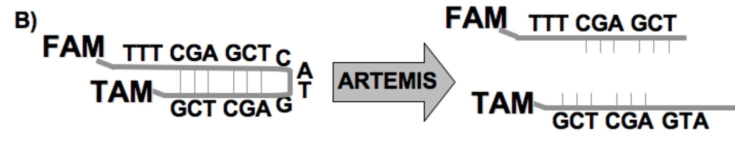
B)
FAM
TTT CGA GCT
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A
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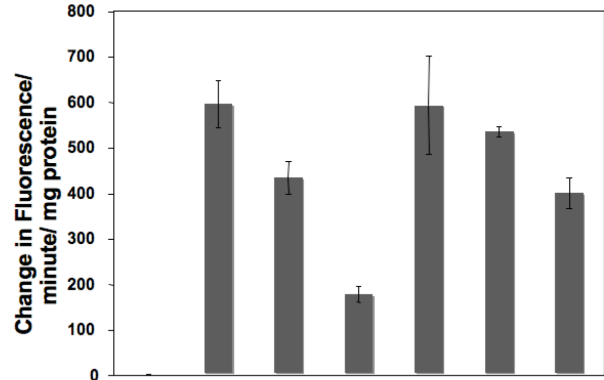
G
TAM
GCT CGA
ARTEMIS
FAM
TTT CGA GCT
TAM
GCT CGA GTA


Change in Fluorescence/ minute/ mg protein
800
700
600
500
400
300
200
100
0
Artemis Genotype: 0 ++ +- -- -- -- --
EAIG MOI: 0 - - - 1 5 10

Artemis-mediated growth inhibition. To test the possibility that over-expression of Artemis leads to a loss in cell viability, a dose response experiment was carried out in which 3T3 cells were transduced with varying amounts (MOI) of EAIG (Artemis) and CSIIEG (GFP control) lentiviral vectors. Integrant copy number was well controlled in this experiment because these vector preparations were also titered on 3T3 cells by quantitative PCR (see Materials and Methods). For five continuous days post-transduction, cell populations were assayed for viability by trypan blue exclusion using a Beckman Coulter Vi-CELL. Cultures transduced with CSIIEG remained viable independent of transduction at increasing MOI; however, a dose dependent decrease in cell survival was observed over time in cultures transduced with EAIG at increasing multiplicity ($p < .005$ for EAIG vs CSIIEG at both MOI = 3 and MOI = 10) (**Figure 3**).

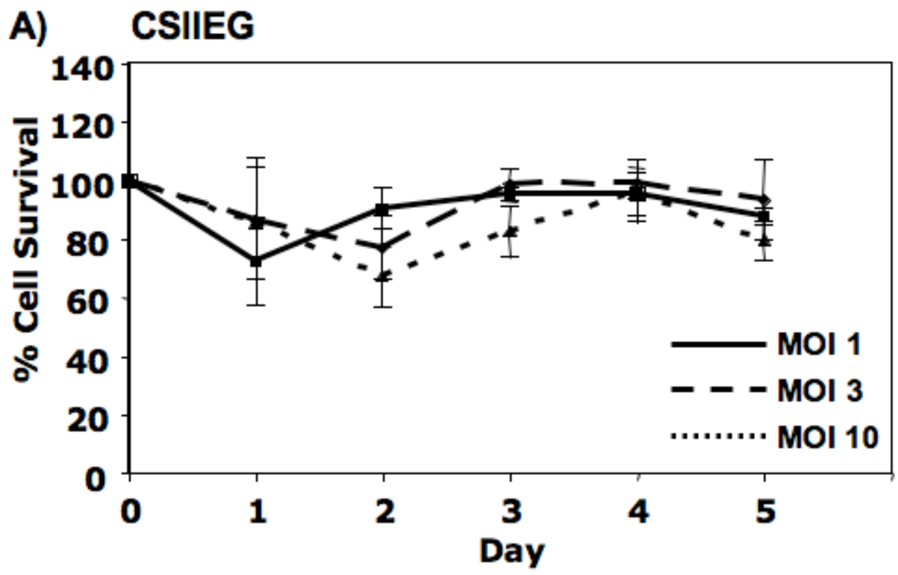
Decreased cell viability in the preceding experiment could have been the result of exposure to lentivirus vector at high multiplicity (MOI= 10) as well as Artemis over-expression. To verify that the observed loss of cell viability was due to Artemis over-expression, 293T cells were transfected with increasing amounts of plasmid DNAs EF1α-hArtemis or control EF1α-Puro (conferring resistance to puromycin), and cell viability was determined four days post-transfection by trypan blue exclusion (**Figure 4**). 293T cells were utilized to assess growth response following Artemis transfection due to their high transfection efficiency. Cultures transfected with increasing amounts of Ef1α-hArtemis plasmid exhibited a significant decrease in cell survival in comparison with cells transfected with increasing amounts of EF1α-Puromycin plasmid ($p < 0.001$), which had no effect on cell viability (**Figure 4**). These results confirm that the

loss of cell viability observed after lentiviral transduction was due to over-expression of Artemis rather than a toxic response to lentiviral transduction.

If growth inhibition results from a high level of Artemis expression in cells transduced with an Artemis expression vector, this predicts that transduction using a vector in which Artemis is regulated by a more moderate strength promoter will be less toxic. We therefore prepared a lentiviral vector in which the human Artemis gene was regulated by the phosphoglycerate kinase (PGK) promoter, and compared the effect of transduction with that of the EF1a regulated human Artemis vector. The PGK promoter has been documented to mediate gene expression at more moderate levels in comparison with stronger promoter systems in the liver (20, 35), and in several cell lines including 293T and 3T3 as well as $CD34^+$ cord blood progenitors (28, 31). Both EF1α and PGK regulated human Artemis vectors were used to transduce MEFs at varying multiplicities of infection; moreover, MEFs wild-type ($^{+/+}$), heterozygous ($^{+/-}$), and deficient ($^{-/-}$) for Artemis were utilized in this study to ensure that cytotoxicity associated with Artemis over-expression was not cell type or genotype specific (**Figure 5**). Cultures transduced with increasing amounts of the control vector EF1α-Puromycin retained cell viability, whereas cultures exposed to increasing amounts of EF1α-hArtemis displayed significant growth inhibition ($p < 0.001$ for all three genotypes) (**Figure 5**). Heterozygous MEFs transduced with increasing amounts of PGK-hArtemis displayed a less dramatic growth inhibition as compared to heterozygous MEFs transduced with EF1α-Puromycin vector ($p < .05$). Moreover, wild-type and Artemis deficient MEF cultures transduced with increasing amounts of PGK-hArtemis displayed growth curves statistically

indistinguishable from cultures transduced with the control EF1α-Puromycin vector. These data support the hypothesis that over-expression of Artemis leads to decreased cell growth. The endogenous level of Artemis expression (i.e. MEFs ++, +-, and -- for Artemis) did not appear to contribute towards cytotoxicity associated with over-expression of Artemis after transduction.

Figure 3. Cell survival response following Artemis transduction. Murine NIH 3T3 cells were transduced at increasing MOI using A) CSIIEG or B) EAIG lentiviral vectors as indicated. Cell survival was assessed over time, plotted as the percentage of cells surviving in control, untreated populations. Each value represents the mean of 3 replicates +/- S.D.


A)
CSIIEG
140
120
100
80
60
40
20
0
% Cell Survival
0
1
2
3
4
5
Day
MOI 1
MOI 3
MOI 10

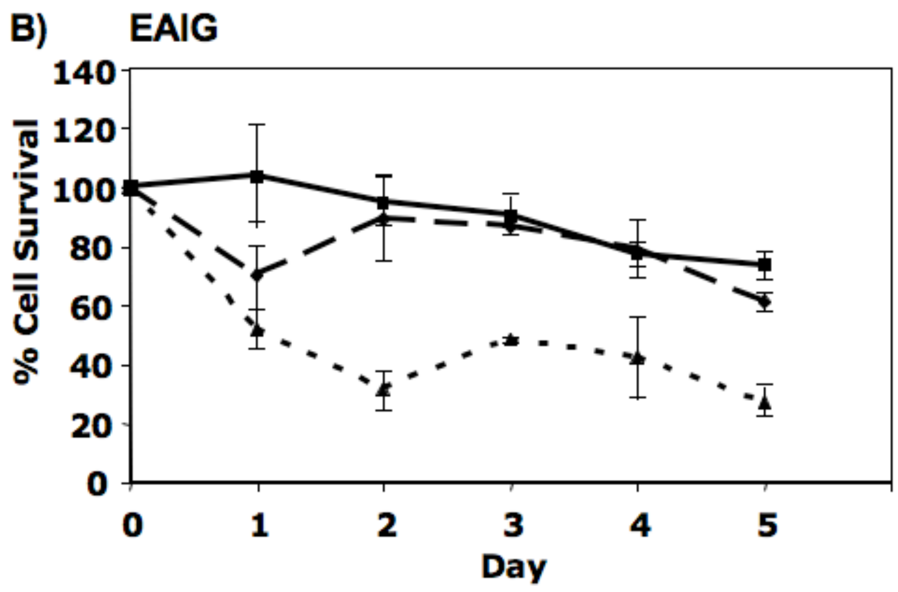

B)
EAIG
140
120
100
80
60
40
20
0
% Cell Survival
0
1
2
3
4
5
Day

Figure 4. Growth response following Artemis transfection. 293T cells were transfected with increasing amounts of the plasmids EF1α-hArtemis or EF1α-Puromycin using the DNA-calcium phosphate co-precipitation technique. Cell survival was assessed five days later by trypan blue exclusion, expressed here as the percentage of an untreated control cell population. Each value represents the mean of 3 replicates +/- S.D.

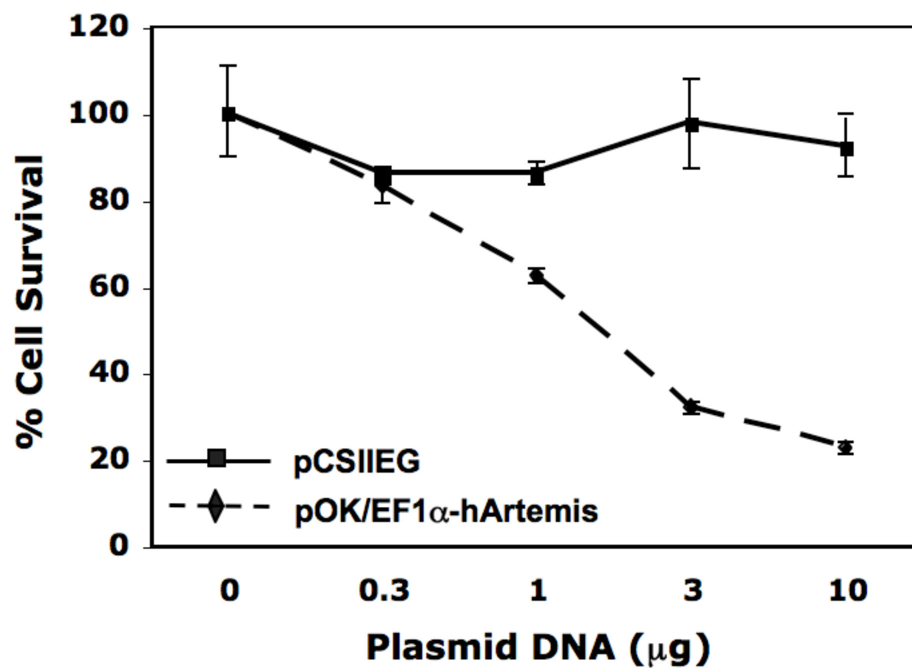
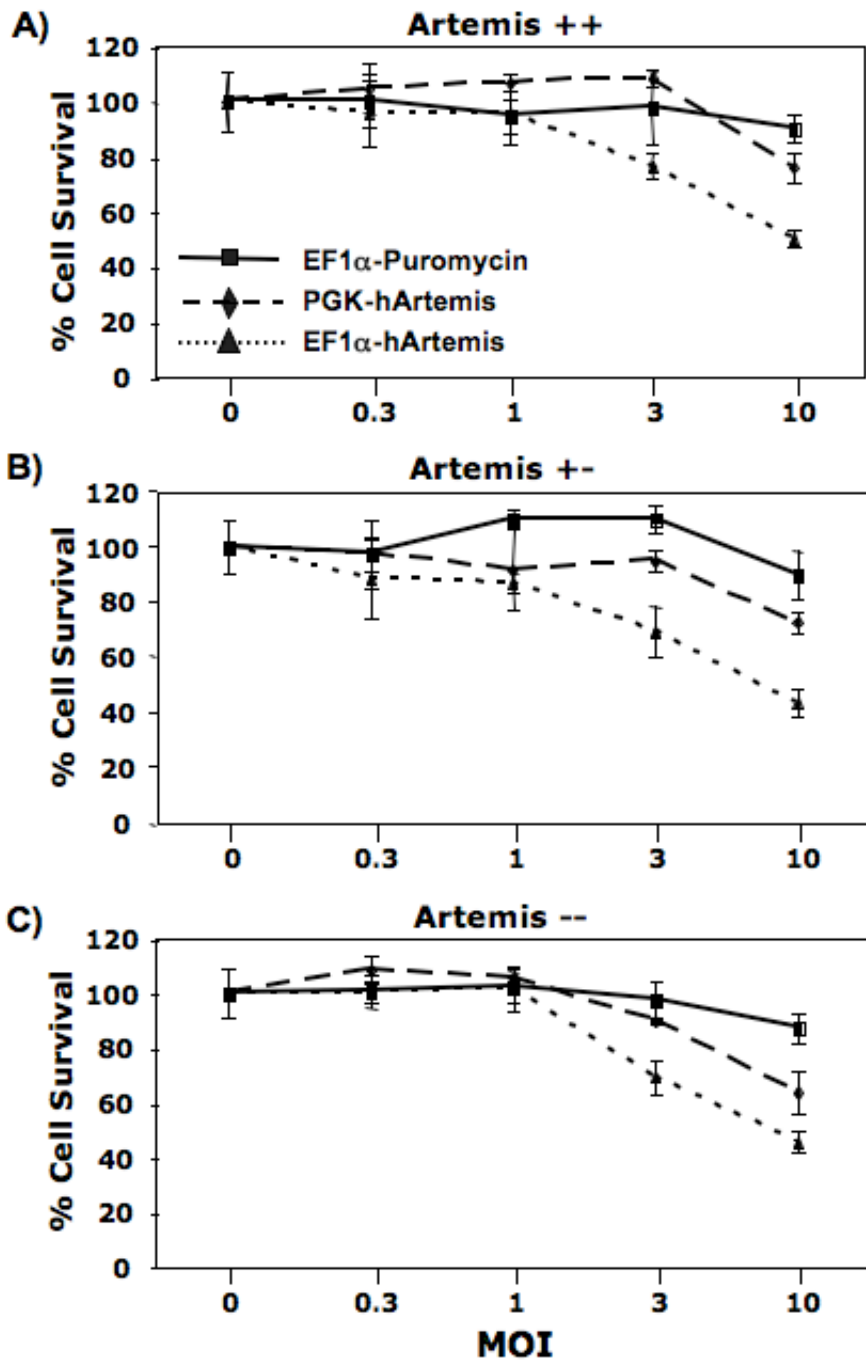

120
100
80
60
40
20
0
% Cell Survival
pCSIIEG
pOK/EF1α-hArtemis
0
0.3
1
3
10
Plasmid DNA (μg)

Figure 5. The effect of promoter strength on Artemis toxicity. The effect of promoter strength on Artemis toxicity was assayed by transducing A) tMEFSCIDA++, B) tMEFSCIDA+-, and C) tMEFSCIDA-- cells at increasing MOI with EF1α-hArtemis, PGK-hArtemis, and the control vector EF1α-Puromycin. To control for integrant copy number, side-by-side titering experiments indicated a consistent copy number between murine NIH 3T3 and tMEFSCIDA cell lines by quantitative PCR (see Materials and Methods) for all three vectors. Cell viability was assayed five days later by trypan blue exclusion, presented here as a percentage of an untreated cell population. Each value represents the mean of 3 replicates +/- S.D.


A)
Artemis ++
120
100
80
60
40
20
0
% Cell Survival
0
0.3
1
3
10
EF1α-Puromycin
PGK-hArtemis
EF1α-hArtemis
B)
Artemis +-
C)
Artemis --
MOI

***Cellular responses to Artemis over-expression**.* To further characterize the cytotoxicity associated with Artemis over-expression, we investigated several possible cellular responses. Because Artemis is an endonucleolytic DNA hairpin-opening enzyme, we tested for global genomic instability by a comet assay, conducted under alkaline conditions. The comet assay has been used extensively as an assessment of global DNA damage in cells subjected to nucleic acid damaging agents [Olive, 2006 #2]. Briefly, cells are exposed to damaging conditions, suspended in low melting agarose and lysed. Upon electrophoresis, highly fragmented DNA migrates more rapidly through the agarose matrix than the cell body, thus generating a smear that mimics the tail of a comet. Exposure of cells to increasing concentrations of damaging agents causes increasingly fragmented DNA, which can be directly correlated with a longer comet tail.

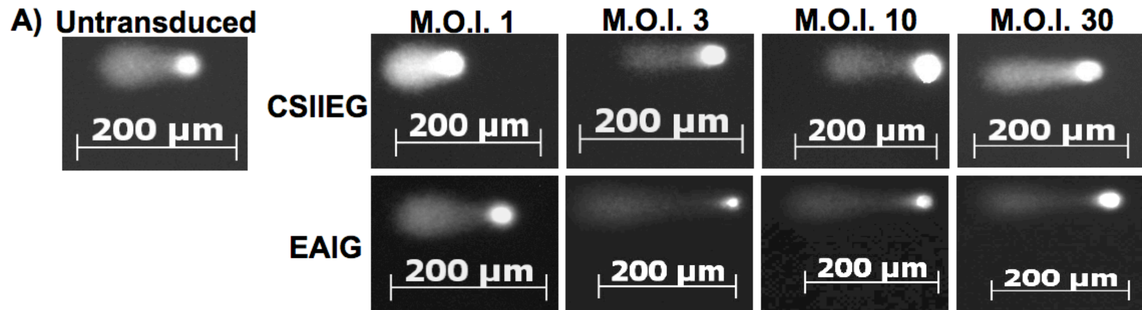
Murine embryonic fibroblasts deficient for Artemis were transduced at increasing MOI with either the control CSIIEG vector or with EAIG, which expresses both murine Artemis and GFP. Four days post-transduction, GFP positive cells from each transduced population were sorted by FACSDiva and subjected to a comet assay. GFP negative cells were also sorted from the EAIG transduced (MOI = 1) population to be used as a negative control. In cultures transduced with EAIG, we observed a significant increase in the comet tail length that was vector dose-dependent up to an MOI of 3 ($p < 0.001$ for EAIG vs CSIIEG transduced cells) (**Figure 6**). An increase in tail length was not observed in cultures exposed to identical amounts of control vector. These data demonstrate that the over-expression of Artemis resulted in an increase in total cellular DNA damage.

Because Artemis has been reported to play a role in cell cycle checkpoint (38), we conducted cell cycle analysis of 3T3 cells transduced with increasing amounts of EF1α-hArtemis vs EF1α-Puromycin. Cells were harvested 48 hours after transduction; nuclei were stained with propidium iodide and analyzed on a FACSCalibur using FlowJo software. Interestingly, after transduction with a greater amount of Artemis vector, there was an accumulation of cells in the G1 stage of the cell cycle (**Figure 7**). Cells over-expressing Artemis thus appear to arrest at the G1 phase checkpoint, preventing progression to DNA synthesis. When considered together with the results from the comet assay, these data indicate that Artemis over-expression may be inducing genomic DNA damage, resulting in cell cycle arrest at G1.

A potential outcome of Artemis-mediated DNA damage is the induction of an apoptotic response. To evaluate apoptosis in cells over-expressing Artemis, 3T3 cells were transduced with CSIIEG and EAIG lentiviral vectors at increasing MOI and after 20 hours were stained with Annexin V and 7-AAD as described in Materials and Methods. The GFP positive transduced cell population was gated and analyzed for Annexin V and 7-AAD positive cells. Both EAIG and control CSIIEG transduced cell populations exhibited similar levels of Annexin V single positive staining (**Figure 8**). However, the EIAG-transduced populations contained increased proportions of Annexin V/7-AAD double positive cells (i.e., cells undergoing apoptosis) ($p < 0.001$ for EAIG vs CSIIEG transduced cells). Additionally, EAIG-transduced populations contained significantly increased percentages of 7-AAD positive (Annexin-negative) cells (i.e., dead cells) ($p < 0.001$ for EAIG vs CSIIEG transduced cells). Along with our data from the comet

assay and cell cycle analyses, these results indicated that Artemis over-expression induces genomic damage that ultimately induces apoptosis and loss of cell viability.

Figure 6. Comet assay of genomic DNA damage. The comet assay was performed on tMEFSCIDA-- cells transduced with either CSIIEG or EAIG. Transduced cells were collected by GFP positive cell sorting and subjected to an alkaline comet assay as described in Materials and Methods. A) Comet tails were observed by fluorescent microscopy and B) mean tail length + S.D. (n=3) is plotted for -- MEF cells transduced at different multiplicities of EAIG and CSIIEG lentiviral vectors.


A) Untransduced
M.O.I. 1
M.O.I. 3
M.O.I. 10
M.O.I. 30
CSIIEG
EAIG
200 µm

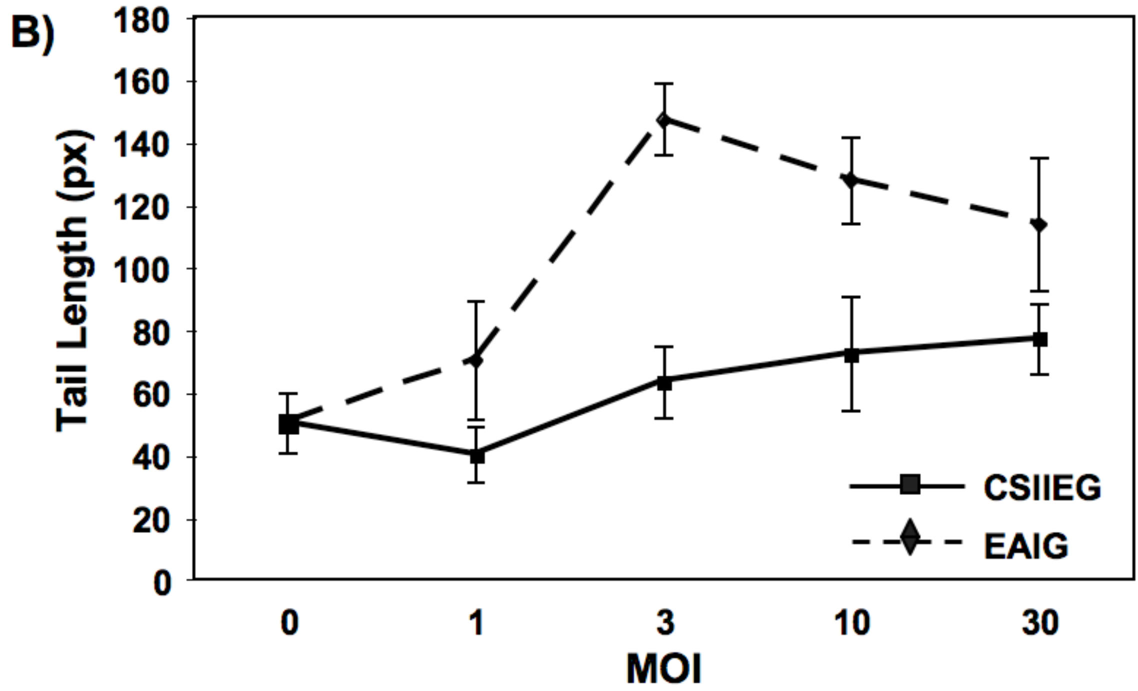
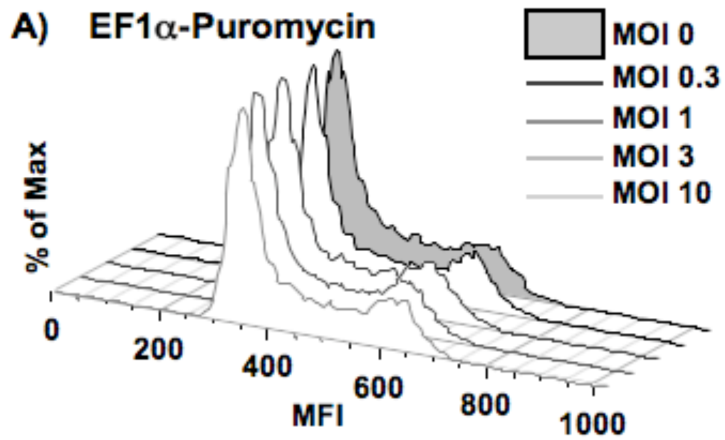
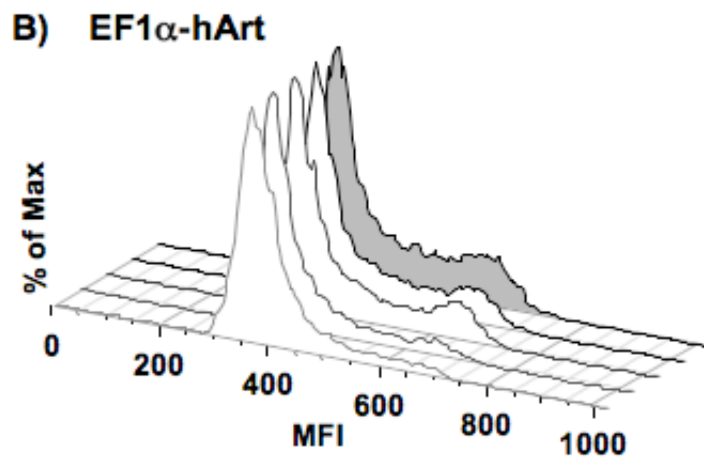

B)
Tail Length (px)
180
160
140
120
100
80
60
40
20
0
0
1
3
10
30
MOI
CSIIEG
EAIG

Figure 7. Effect of Artemis over-expression on cell cycle progression. NIH 3T3 cells were transduced with EF1α-Puromycin (A) or EF1α-hArtemis (B) at increasing MOI and then subjected to cell cycle analysis 48 hours later by flow cytometry using propidium iodide (see Materials and Methods). The percentage of cells in G1 of the cell cycle (C) was determined using FlowJo software. MFI= mean fluorescence intensity.

A) EF1α-Puromycin



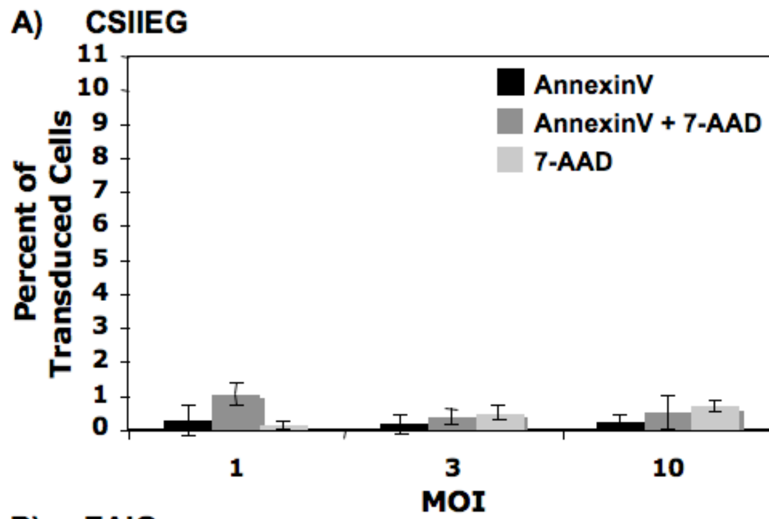
B) EF1α-hArt

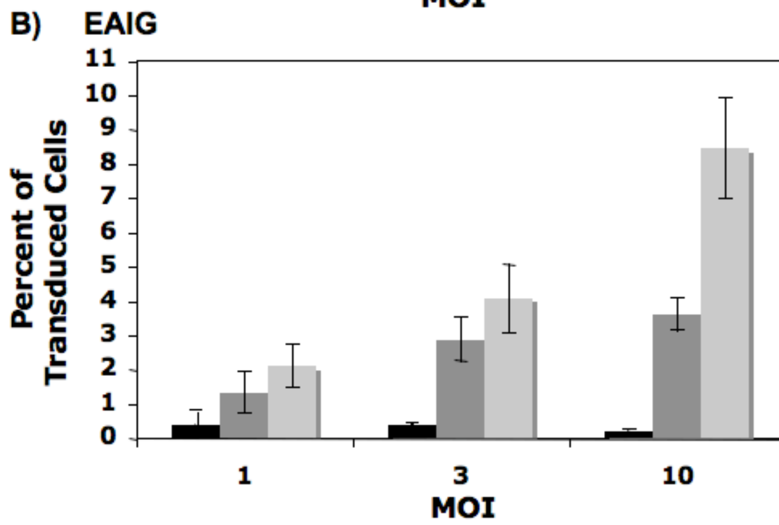


C) Percent of Cells in G1

MOI	EF1α- Puromycin	EF1α-hArtemis
0	53.8 (+/- 1.3)	
0.3	61.5 (+/- 5.9)	72.5 (+/- 4.8)
1	66.1 (+/- 1.3)	69.4 (+/- 0.7)
3	69.5 (+/- 4.5)	84.1 (+/- 5.3)
10	66.8 (+/-2.5)	78.4 (+/- 6.6)

Figure 8. Induction of apoptosis in Artemis-transduced cells. NIH 3T3 cells were transduced at increasing MOI with CSIIEG (A) or EAIG (B) lentiviral vectors and then 20 hours later stained with AnnexinV and 7-AAD. Transduced GFP positive cells were gated to determine AnnexinV binding and 7-AAD staining in each population, expressed here as the singly and doubly staining cells as a percentage of the whole GFP^+ cell population +/- S.D. (n=3).


A) CSIIEG
11
10
9
8
7
6
5
4
3
2
1
0
Percent of
Transduced Cells
AnnexinV
AnnexinV + 7-AAD
7-AAD
1
3
10
MOI


B) EAIG
11
10
9
8
7
6
5
4
3
2
1
0
Percent of
Transduced Cells
1
3
10
MOI

DISCUSSION

We found that Artemis over-expression by either transduction or transfection resulted in a dose-dependent cytotoxic response that was not observed in cultures exposed to identical amounts of control vector. Upon transduction with a lentiviral vector bearing an EF1α-regulated Artemis gene, we observed a direct correlation between over-expression and genomic DNA damage, arrest in the cell cycle at G1, and apoptosis. Interestingly, the observed toxicity was diminished when the strong EF1α promoter was replaced with a moderate strength PGK promoter, thus demonstrating the importance of restricting the level of Artemis expression.

Because of the inherent nucleolytic nature of Artemis, it is perhaps not surprising that increased expression levels may more nonspecific breaks, thus causing genomic damage sufficient to trigger cell cycle arrest and apoptosis. Mammalian cells respond to DNA damage by activating cell cycle checkpoints and if the damage is not sufficiently repaired, they arrest in their cell cycle progression, which ultimately results in apoptosis to prevent replication of severely damaged DNA (15) (14). With these observations in mind, it becomes evident that achieving clinically relevant levels of transgenic expression of Artemis may present a challenge.

A deficiency of Artemis results in SCID-A, which is characterized by the complete loss of B and T cell function coupled with radiation sensitivity (16, 21). Currently, hematopoeitic stem cell transplantation (HCT) is the most effective treatment of SCID-A; however, there are significant complications associated with HCT such as difficulty in identifying an HLA-matched donor, graft

rejection, and graft-vs-host disease (25). Additionally, initial studies reported that 11 of 12 SCID-A children treated by HSCT displayed T cell reconstitution but no B cell reconstitution (25). Because of the failure to reconstitute B cell immunity in SCID-A patients, there is a considerable need for novel therapies that improve upon HSCT.

Gene transfer is currently emerging as a feasible and realistic alternative to HCT for effective long-term treatment of primary immunodeficiencies. Two independent studies have reported correction of X-linked SCID by *ex vivo* transduction of $CD34^+$ hematopoietic stem cells using a retroviral vector expressing the common cytokine-receptor gamma chain (common γ chain) (5, 7, 11). Long-term engraftment of corrected stem cells was observed in the majority of patients, ultimately resulting in reconstitution of a functional lymphocyte compartment. Additionally, successful long-term treatment of adenosine deaminase (13) deficient SCID by gene transfer has been reported in which ten patients were infused with autologous $CD34^+$ marrow cells transduced *ex vivo* using an ADA expressing retroviral vector (2). After an average of four years post treatment, these patients have shown evidence of stable engraftment, differentiation of transduced cells, and immune reconstitution of functional T lymphocytes (2). Clinical improvement of patients in these trials for X-linked SCID and for ADA deficiency demonstrates the potential effectiveness of gene transfer in the treatment of primary immunodeficiencies in general, including SCID-A.

Cytotoxicity associated with over-expression of Artemis after lentiviral transduction presents a challenge in the development of gene transfer as a

therapeutic approach to correct SCID-A. While sufficient Artemis expression will be necessary in order to restore immune function, our results suggest expression levels that trigger a toxic response will need to be avoided. This supposition has recently been confirmed in two independent studies that have reported correction of a murine model of SCID-A by *ex vivo* lentiviral transduction of hematopoeitic stem cells (4, 23). In both of these studies, the lentiviral vectors successfully utilized for treatment contained the human Artemis cDNA regulated by the moderate strength PGK promoter. Optimally regulated Artemis expression would replicate endogenous conditions, so to this end we have recently isolated the human Artemis promoter region and are currently characterizing its expression *in vitro* and *in vivo* to be utilized as a potential regulator of Artemis in future preclinical studies (Multhaup M. et al, manuscript in preparation).

The importance of therapeutic gene regulation has recently been exemplified in the case of gene transfer therapy for X-linked SCID. To date, five out of twenty patients treated for X-linked SCID by gene transfer have developed clonal T cell outgrowth resulting in leukemia, from which one child has died (12) (13). Although insertional activation of the LMO2 oncogene was reported in three of the leukemic cases, over-expression of the common γ chain also likely contributed to these adverse events (12) (13). Over-expression of the common γ chain induces cellular proliferation that may have ultimately been responsible for the T lymphocyte clonal outgrowth (3).

Because allogeneic HSCT in the correction of SCID-A is sufficient for T cell repopulation but does not support repopulation of the B cell compartment (21),

there clearly is a need for improved therapy. Based on its emerging success in the treatment of primary immunodeficiencies such as ADA and X-linked SCIDs, gene transfer may also be developed for the effective treatment of Artemis deficient SCID. The results presented in this study, showing that over-expression of Artemis results in cytotoxicity, illustrate the need for regulated Artemis gene expression as a critical component to therapeutic vector design for gene therapy of SCID-A.

MATERIALS AND METHODS

Lentiviral vectors

Lentiviral vector constructs were generated by standard molecular techniques based on the pCSII and pCSIIEG lentiviral vectors, both of which have been described (1).

(i) pCSIIEG/EPuro. The *Streptomyces alboniger* puromycin-N-acetyl-transferase gene was PCR amplified from the pPUR plasmid (Clontech, Mountain View, CA) using the oligonucleotides: forward: 5' TCTGCTAGCCATGGCCGAGTACAAGCCC 3' and reverse: 5' GGCGACCGGTGGGGCACCGGGCTTGCGGG 3.' The amplified product was cleaved using *NheI* and *AgeI* (recognition sites underlined in the oligo sequence) and then ligated into pCSII-EF-MCS (1) to generate pCSIIEG/Puro.

(ii) pCSII/EAIG. The murine Artemis cDNA sequence was excised from pGEMT/mArt (17) with *NotI* and cloned into pCSII/EF1α-MCS (1) at the *NotI* site to generate pCSII/EF1α-mArtemis. The IRES-GFP sequence was excised from pCSII/CMV-I2G (8) with *NheI* and *XbaI* and cloned into the *XbaI* site of pCSII/EF1α-mArtemis to generate pCSII/EAIG.

(iii) pLL-ABiG. The CLP regulatory element was excised from pCpG-MCS (Invivogen, San Diego, CA, USA; http://www.invivogen.com) as an *Eco*RI-*Nhe*I fragment and cloned between *Eco*RI and *Spe*I of pKT2/CaL (36) replacing the CAGS promoter to generate pKT2/CLP-Luc. A 978 bp sequence containing the

511 bp human PGK promoter, a polylinker and a 442 bp rabbit beta-globin polyadenylation signal was excised from pKT2/PGK-pA by digestion with *Sma*I-*Eco*RV and inserted into the *Eco*RI (blunt) site of pKT2/CLP-Luc to generate pKT2/BiL. This vector was linearized with *Bgl*II to allow for insertion of an 1165 bp *Bam*HI-*Bam*HI fragment encoding a direct fusion between the blasticidin resistance gene (Bsd) and GFP (Bsd:GFP) to create pKT2/LuBiBG. This plasmid was then digested with *Nco*I, eliminating the Bsd coding sequence and reinserting the bidirectional promoter as an *Nco*I fragment in both orientations to produce pKT2/LuBiG. The murine Artemis coding sequence was isolated as a *Sac*II fragment from pGEM-T/mArt (17) and cloned into pKT2/LuBiG downstream of the PGK promoter between *Eco*RI (blunt) and *Eco*RV, replacing the luciferase coding sequence to generate pKT2/ABiG. The resulting sequence from 3′-Artemis-bidirectional promoter-GFP-5′ was isolated as a *Sal*I (blunt) - *Nhe*I fragment and inserted into pLentiLox (30) between *Pci*I (blunt) and *Spe*I to generate pLL-ABiG.

(iv) pOK/EF1α-hArtemis. The pOK/EF1α-MCS lentiviral vector was generated by restriction digest of pKT2/SE (6) with *PvuII* and *SwaI* to excise a minimal plasmid backbone containing the kanamycin resistance gene and the ColE1 origin of replication. pCSII/EF1α-MSC was digested with *ScaI* and *PmeI*, and then the LTR to LTR fragment was ligated into the minimal plasmid backbone. The human Artemis cDNA sequence was excised from pCMV-SCIDA (16), cloned into the *BssHI* restriction site of pSL301, excised with *BamHI* and cloned into pOK/EF1-MCS to generate pOK/EF1α-hArtemis.

(v) pOK/PGK-hArtemis. The human PGK promoter sequence was amplified by PCR from the pKT2/PGKi template (6) to include flanking *XmaI* restriction sites using oligonucleotides: forward: 5′- AATATTCCCGGGTACCGGGTAGGGG- 3′ and reverse: 5′- AATTTACCCGGGACGTCCACGTCCAGCTTTC- 3′ (recognition sites underlined) and cloned into pOK/EF1α-hArtemis at the *XmaI* restriction site, generated upon excision of the EF1α promoter sequence, to construct pOK/PGK-hArtemis.

Mammalian Cell Culture

Murine embryonic fibroblasts (Artemis wild type tMEFSCIDA^{++}, heterozygous tMEFSCIDA^{+-}, or deficient tMEFSCIDA^{-} (17)), HEK 293T, and murine NIH 3T3 tk^{-} cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic antimycotic at 37° C and 5% CO_2.

Preparation and Titering of Lentiviral Vectors

VSV-G pseudotyped vectors were generated by three plasmid co-transfection of HEK 293T cells as described (8, 40). Briefly, 24 hours pre-transfection, 1.4 X 10^7 HEK 293T cells were seeded into poly-L-lysine coated 15-cm^2 plates and cultured in DMEM supplemented with 1% penicillin streptomycin and 8% FBS at 37°C with 5% CO_2. Lentiviral vector plasmid constructs were co-transfected with pΔNRF to provide lentiviral structural and enzymatic proteins and pMD.G to provide vesicular stomatitis virus G (VSV-G) envelope protein. Twelve hours

post-transfection, the medium was replaced with DMEM supplemented with 4% FBS. Viral supernatants were collected 24, 36, and 48 hours post-transfection, pooled, and then concentrated 100-fold by centrifugation at 23,000 x g in a Sorvall RC5B centrifuge. Vector was resuspended in Iscove's modified Dulbecco's medium (IMDMEM). For quantitation of vector titers, NIH 3T3 tk^- cells were transduced with different amounts of vector in the presence of 8 μg/mL polybrene. Forty-eight hours later, the cells were harvested for flow cytometry to determine the percentage of cells expressing GFP. DNA was also extracted from the transduced cells for Taqman based real-time quantitative PCR utilizing a probe specific for the integrated lentiviral strong stop sequence or a probe for the GFP sequence (8). After titering each vector, copy number was determined in all of the cell lines utilized in this study (HEK 293T, NIH 3T3, tMEFSCIDA++, tMEFSCIDA+-, tMEFSCIDA--) and found to be comparable.

Western Blot analysis

tMEFSCIDA-- cells were transduced with Artemis or control GFP vector at a multiplicity of infection of 10. Forty-eight hours post-transduction, cells were sorted by FACSDiva and GFP positive cells were collected. Untransduced $tMEFSCIDA^{++}$, $tMEFSCIDA^{+-}$, $tMEFSCIDA^{--}$, and transduced $tMEFSCIDA^{--}$ cell cultures were subjected to nuclear extraction according to Schreiber *et al.* (32). Briefly, cells were harvested by trypsinization, washed with PBS and then resuspended in ice-cold lysis buffer [10mM HEPES pH 7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 0.5mM PMSF]. After a 15 minute incubation on ice, 10% Nonidet NP-40 was added, the lysate was vortexed vigorously and then

cleared by centrifugation for one minute at 16,000 x g. The supernatant (containing the cytoplasm) was discarded, and then the nuclei were resuspended in a second lysis buffer [20mM HEPES pH7.9, 0.4M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 1mM PMSF] and vigorously rocked at 4°C for 20 minutes. The nuclear extract was cleared by centrifugation for 5 minutes at 16,000 x g and the protein concentration determined by Bradford analysis as formulated by Bio-Rad. Nuclear lysates (25 μg) were boiled in the presence of 5X SDS loading buffer [300mM Tris pH 6.8, 25% glycerol, 20% beta-mercaptoethanol, 10% SDS, 0.02% bromophenol blue] for 5 minutes, electrophoresed through 10% Tris-HCl polyacrylamide-SDS gel, and transferred onto PVDF membrane using a Bio-Rad Trans-Blot® SD semi-dry system for 25 minutes at 12 volts. The membrane was washed in 1X Tris-buffered saline (1M Tris, pH7; 5M sodium chloride) + 0.05% Tween 20 (TBST), blocked for one hour with 5% milk (Bio-Rad) in 1X TBST, washed again with 1X TBST, and then incubated overnight at 4° C with a rabbit polyclonal anti-Artemis antibody (BioLegend) diluted 1:500 in 2.5% milk/1X TBST. After washing with 1X TBST, the membrane was incubated for one hour at 4° C with a secondary peroxidase-conjugated anti-rabbit IgG (whole molecule) (SantaCruz, Santa Cruz, California), diluted 1:3,000 in 2.5% milk/1X TBST. The signal was visualized using a SuperSignal West Pico Chemiluminescent Substrate Detection Kit (Thermo Fisher Scientific Inc., Rockford, Illinois).

<u>Hairpin Opening Assay</u>

Whole cell lysates were generated from MEF cells by freeze thawing four times and then clearing by centrifugation at 25,000 x g for 10 minutes in an Eppendorf

microcentrifuge.

Assays were conducted in a 96 well format. Each reaction consisted of 25 μg whole cell lysate and 300 nM hairpin substrate brought to a final volume of 100 μl in reaction buffer [25mM Tris (pH8), 50mM KCl, 10mM $MgCl_2$, 1mM DTT, 50ng/μl BSA, and 5mM ATP]. The hairpin substrate was generated as a 19 base oligodeoxynucleotide (5'-TTTCGAGCTCATGAGCTCG-3') modified at the 5' terminus by the fluorophore FAM and at the 3' terminus by the quencher TAMRA. At room temperature a double-stranded, stem-loop configuration is predicted for this sequence, with a Tm of 72°C; once cleaved, the resulting product acquires a Tm of 20°C and dissociates at room temperature, allowing for the release of FAM from TAMRA with ensuing fluorescence at 494 nm. FAM fluorescence was measured in real time at room temperature using a Biorad Realplex thermocycler. PCR Extract software was used to compile fluorescence readings once per minute for each reaction. Initial velocities were calculated by linear least squares and expressed as change in fluorescence/mg of protein/minute. The amount of substrate cleaved in moles per fluorescence unit was determined as the fraction of fluorescence assessed after complete degradation of the hairpin substrate by DNase. Initial velocities were then expressed as substrate cleaved in nmol/mg protein/minute.

Comet Assay

tMEFSCIDA$^{-/-}$ cells were transduced with lentiviral vectors and five days later sorted for the GFP^+ population by FACS Diva separation. The comet assay was performed under alkaline conditions as described by Olive and Banath (27).

Briefly, a single-cell suspension of each population was prepared and submerged in 1% low melting agarose at a concentration of 5 X 10^3 cells/mL. Each cell suspension was smeared onto a microscope slide pre-coated with a thin layer of 1% low melting agarose and allowed to gel for 5 minutes. Slides were then fully submerged into A1 alkaline lysis solution [1.2M NaCl, 100mM Na_2EDTA, 0.1% sodium lauryl sarcosinate, 0.26M NaOH, (pH>13)] overnight at 4°C in the dark. After lysis, slides were incubated with A2 solution [0.03M NaOH, 2mM Na_2EDTA, (pH~12.3)] at room temperature 3 times for 20 minutes each time. Slides were then placed in an electrophoresis chamber and fully submerged in fresh A2 solution. Electrophoresis was conducted for 25 minutes at 20 volts. Slides were removed from the electrophoresis chamber, neutralized by washing with distilled water, and incubated in staining solution [2.5 μg/ml propidium iodide in distilled water] for 20 minutes. Fluorescent comet images were collected for each population using a Zeiss Axioplan 2 imaging system (200 fold magnification). Images were analyzed using CometScore™ 1.5 software (http://www.autocomet.com/products_cometscore.php), assigning comet tail length in pixels.

Cell Cycle Analysis

3T3 cells transduced with EF1α- hArtemis or control vector EF1α-Puromycin were harvested 48 hours post-transduction and stained overnight with propidium iodide as described (24). Briefly, 0.5 X 10^6 transduced cells were harvested by trypsinization and pelleted at 377 x g. Cells were resuspended in hypotonic fluorochrome solution [50 μg/ml propidium iodide, 0.1% sodium

citrate, 0.1 Triton X-100 in distilled water] and incubated overnight at 4°C. Cell cycle distribution of transduced cell populations was determined by flow cytometry on a FACSCalibur with subsequent cell cycle analysis using FlowJo software.

Apoptosis

3T3 cells were transduced with CSIIEG and EAIG lentiviral vectors at increasing MOI. Twenty hours post-transduction, cells were harvested by trypsinization and subjected to Annexin V staining following the BioVision Annexin V-Biotin Apoptosis Detection Kit protocol (BioVision, Mountain View, California). Briefly, cells were resuspended in 1X binding buffer and incubated with Annexin V-Biotin at room temperature for five minutes. Cells were washed twice with binding buffer then incubated with avidin-PerCP (BD Biosciences, San Jose, California) at room temperature for fifteen minutes. 7-AAD was added to samples immediately before flow cytometry. The GFP positive transduced cell population was gated and analyzed for Annexin V (PerCP) and 7-AAD positive cells.

Statistical Analysis

Data were statistically evaluated either by unpaired students T-test (hairpin opening assays) or by ANOVA (all other analyses) using Prism 4 software (GraphPad Software, Inc., San Diego, CA), with $p < 0.05$ considered significant.

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CHAPTER 3

THE HUMAN ARTEMIS PROMOTER MEDIATES MODERATE AND CONSTITUTIVE GENE EXPRESSION BOTH *IN VITRO* AND *IN VIVO*

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Artemis is an endonucleolytic enzyme involved in nonhomologous double strand break repair and V(D)J recombination. Deficiency of Artemis results in a $B^- T^-$ radiosensitive severe combined immunodeficiency, which may potentially be treatable by Artemis gene transfer into hematopoietic stem cells. We recently found that over-expression of Artemis after transduction using a strong EF1α regulated lentiviral vector resulted in global DNA damage and increased apoptosis. To provide natural levels of Artemis expression, we isolated a one kilobase DNA sequence upstream of the human Artemis gene to recover the Artemis promoter (APro). The sequence includes numerous potential transcription factor binding sites, and several transcriptional start sites were mapped by 5′ rapid amplification of cDNA ends. APro and deletion constructs conferred significant reporter gene expression *in vitro* that was markedly reduced in comparison to EF1α regulated expression. *Ex vivo* lentiviral transduction of an APro-regulated green fluorescent protein (GFP) construct in mouse marrow supported GFP expression throughout hematopoeitic lineages in primary transplant recipients and was sustained in secondary recipients. The human Artemis promoter thus provides sustained and moderate levels of gene expression that will be of significant utility for the correction of SCID-A by gene transfer into hematopoeitic stem cells.

INTRODUCTION

Genomic stability is maintained by way of essential DNA double-strand break (DSB) repair mechanisms. Multicellular eukaryotic organisms ameliorate DSBs primarily by the canonical non-homologous end joining (NHEJ) pathway, which repairs genomic insults generated by both external damaging agents and by internal cellular processes.

The NHEJ cascade begins when the Ku70/Ku80 heterodimer recognizes and binds a DNA DSB. The catalytic subunit of DNA-dependent protein kinase catalytic subunit (DNA-PKcs) then complexes with the DNA bound Ku to form the DNA-PK holoenzyme (26, 28). The DNA-PK holoenzyme subsequently recruits and binds Artemis, which acquires endonucleolytic activity recognizing 5′ and 3′ overhangs (17). After Artemis digests the DNA overhangs, a heteromultimer of XLF, XRCC4, and DNA Ligase IV is recruited to the synaptic complex to repair and seal the break (10, 11).

NHEJ also plays a fundamental role in the rearrangement of immunoglogulin (Ig) genes and T cell receptor (TCR) genes (25, 29). This site-specific rearrangement process begins when the RAG-1 and RAG-2 complex is recruited to recombination signal sequences (RSS) flanking each V (variable), D (diversity), or J (joining) coding segment (21). The RAG complex introduces a nick adjacent to each RSS; the resulting 3′ hydroxyl group nucleophilically attacks the antiparallel DNA strand to form a hairpin structure at the coding ends (18, 22). The Artemis:DNA-PK complex then endonucleolytically cleaves the coding end hairpin and the DSB is processed and repaired through the NHEJ pathway (17).

Deficiency of Artemis disrupts both DNA double-strand break repair and V(D)J recombination, manifesting as a radiation sensitive form of severe combined immunodeficiency characterized by the inability to rearrange Ig and TCR genes, ultimately resulting in an absence of both B and T lymphocytes. Artemis deficiency is a primary immunodeficiency (PI) designated SCID-A due to the founder mutation occurring in Athabascan-speaking Native Americans resulting in a significantly high incidence in this population.

In general, PIs are treatable by allogeneic hematopoeitic stem cell transplantation (HSCT) if an appropriately matched donor can be identified, with an attendant risk of infection, graft rejection and graft-vs.-host disease associated with about 20% mortality worldwide. Accordingly, there is great interest in the development of more effective therapeutic approaches for this disease. Recent results from clinical trials have demonstrated the effectiveness of transplantation using autologous HSC after *ex vivo* genetic correction by retroviral transduction for two PI's, one caused by adenosine deaminase deficiency and the other caused by absence of the common gamma chain for several cytokine receptors (2, 6, 12). Both of theses studies reported long-term engraftment of corrected stem cells in the majority of patients, ultimately resulting in reconstitution of cellular and humoral immunity (2, 6, 12). These results demonstrate the effectiveness of gene transfer for the treatment of primary immunodeficiencies in general, potentially including SCID-A. However, we recently reported that over-expression of Artemis after lentiviral transduction is associated with cytotoxicity, inducing a halt in cell cycle progression, fragmentation of genomic DNA, and ultimately apoptosis (20). These results

highlight the importance of providing Artemis expression at a level that is non-toxic and yet sufficient to correct the T^-B^- phenotype in preclinical studies and in clinical application to human SCID-A.

To explore the potential of innate regulation using the endogenous human Artemis promoter, a one kilobase region directly upstream of the human Artemis translational start site was isolated by PCR amplification, characterized, and shown to support GFP and luciferase reporter gene expression *in vitro*. Interestingly, 5'RACE revealed evidence for multiple transcriptional start points and deletion analysis revealed the ability of multiple deletion constructs to promote expression *in vitro*. Additionally, *ex vivo* transduction of mouse bone marrow with an APro-regulated GFP lentiviral vector resulted in GFP expression at a significantly reduced level in comparison with control mice transplanted with EF1a-GFP transduced marrow. Importantly, the human Artemis promoter supported GFP expression in all hematopoeitic lineages that persisted in secondary transplant recipients. These results demonstrate the effectiveness of the human Artemis promoter in providing moderate and yet reliable levels of expression in hematopoeitic lineages, thereby establishing the usefulness of this promoter for expression of gene products for which over-expression may be detrimental, such as Artemis.

RESULTS

Isolation and sequence of the human Artemis promoter region. To identify the promoter of the human Artemis gene (APro), we evaluated the sequence of human chromosome 10p13 directly upstream of human Artemis translational start site. Several potential regulatory sites were identified within this region using the search engine TFSEARCH: Searching Transcription Factor Binding Sites (ver1.3) (14). This included several known hematopoeitic transcription factor binding sites such as GATA-1,2,3, AML1A and Lyf1 identified throughout the one kilobase sequence (**Figure 1**). Additionally, a CAAT box at -811 and a TATA box at -595 were identified (**Figure 1**): These motifs plus a potential 5′ splice site at base pair -502 (AAGGTTAG) from the translational start and several downstream 3′ splice sites predicted the possibility of an intron in the 5′ untranslated region of messages initiated at around -500 bp or further upstream.

Considering the observed sequence characteristics and potential regulatory elements, we recovered a one kilobase segment of human chromosome 10 genomic DNA located directly upstream of the human Artemis translational start site. The PCR product was cloned into TOPO vector PCR2.1, sequenced, and found to be identical to the published human genomic sequence (PubMed Reference: NT_077569).

Figure 1: Artemis promoter sequence. The one kilobase genomic region located directly upstream of the human Artemis translational start site was isolated by PCR amplification (nucleotide sequence shown). The region was subjected to an online transcription factor binding site search engine TFSEARCH. Several potential transcriptional regulation sites were identified and are labeled according to their position relative to the human Artemis translational start site. Nucleotides are in bold. Canonical sequences are underlined and displayed directly above the APro sequence. The positions of APro deletion constructs relative to the translational start site are shown boxed in dark grey. Transcriptional start sites within the APro region were identified by 5'RACE and are labeled and boxed in light grey.

```
                                          A/TGATAA/G
-1000 CAGAGAGCCGAAATCACGCCACTGCACTCCAGCCTGGGCGATAGAACTAGACCCCAACTGAATAAATAAATAAAATAAAGCGAAGTGTTCTGGGTTGTTT
                                          GATA-1,2,3 (-965)
                                                                                A/TGATAA/G
                         A/TGATAA/G                                                 ATTGCGCAAT
-900  GTGTTTAATTATACTAAGGAGATTTGATAGGAAGGAGCACAATAAATGTCTAGGTGATTATTTGGTTTATTGTAATACAATAAAGGAAATACAATAAAAG
                         GATA-1,3 (-880)                  GATA-1,2,3 (-849)  C/EBP (-843)          CAAT Box (-811)

-800  GTTTAACAAACCTTTTCATAAAGTTGCTTTGGTTGTTAAAAAATTTTAGACCTTTCCTGCCATTTTTCTTTTTTTAAGTACATCATTCTAACTTCCTAAA

-700  AGTATAGCTCAAAAAGGTGGGGGCCCAGGGGAGAGGCCAGCAGCGAAGGCATTATTGGGATCTTATTTGCCCCAAGATCACGCAGAGATGATTTAYYCAG

                 RTGAGnnnGC                                TGAGTCA
-600  TTTGTAGATTATATAAACGGAAGAGGGAATTAATAGTTCCTGAATCTACCATGAGTCAGAATACAAACTTTCTCATACAACCCTCACGGTAATCCCACAA
          TATA (-595)  NRF2 (-586)                         AP-1 (-551)
      -500 to ATG                                                                                    -405 (RACE #3)
-500  GGTTAGAAATTGGGAACAGAAGAGAGAGGGCGGGGAACTGAGCTCGGGTTTCAGGCTAGCTGCAGACAAGCAGGAAGCGGAACGAAGAATGATTTCTAAG

                              -374 to ATG YCACGTGY
                                                 YYTGGGAGU
-400  CGCCAGTTCCGCAGCCACTCACCTCGTCGGCTGGGGCCACCTGCTCTGGGAGTTTCGATTTCCCTTCCCGCGACTGCACCTCCACAGACATGGGCAACGC
                                         USF (-364)  Lyf1 (-356)

-300  CTTACCAGAGCAACACCTGTGTTTGTTGGGCGGGAATGAGCCTTGCACTGGGCAGGGCTCAGGGCCCATCGCGTGCAGCGAAGCGCGGGTGCTTTAAACC

                   -184 to ATG  A/TGATAA/G                                                    -108 (RACE #2)
-200  CAAGCAGCGGGCGCCTAGAACCCGACCGGATGCTCCTTGTCTTTGCCCCCGGTCTCCGGACTCCTTCTGATTGGACGTGGCTGCGTTCGGCCGCCCAATG
                                GATA-1 (-175)
                                RTGAGnnnGC
                        TTCCT/CC/AGAA                                       -32 (RACE #1)
      -100 to ATG          ACCGGAAGTR                                              TGTGGT           A/TGATAA/G
-100  GCGAGGCAGCGCGCGGCTTCCCGGAAGTGGCGGCGCGGTCAGGGCTGGCCTTGGCTTCAGCTGCGGTTTTGGGGTCCCGGACTCTGGGATCGGCGGCGCTATG
          STAT1 (-90)  Elk-1 (-83)  NRF-2 (-81)                                  AML1A (-32)      GATA-1 (-16)
```

NNNN= Transcription Factor Binding Site or TATA Box

N = Deletion Construct

N = 5' RACE Product

Gene expression mediated by the human Artemis promoter. A reporter construct was generated in which the one-kilobase APro sequence was inserted directly upstream of the firefly luciferase gene (pGL3Basic/APro). Considering the non-specific nature of Artemis expression, we introduced the APro reporter construct into readily transfectable HEK 293T cells, where the APro sequence supported luciferase expression at a magnitude of about 10^5-fold less than that of pKT2/Cal, a strong CAGS regulated firefly luciferase construct (**Figure 2A**). Luciferase expression mediated by a downstream 500 bp fragment (pGL3Basic/-500ATG) was equal to or greater than that mediated by the full-length 1 kb APro sequence. In contrast, luciferase expression mediated by an upstream 500 bp sequence (pGL3Basic/-1000-500_ATG) was indistinguishable from background (**Figure 2A**). These results identify the 500 nt region immediately upstream of the translational start site as the human Artemis promoter in HEK 293T cells.

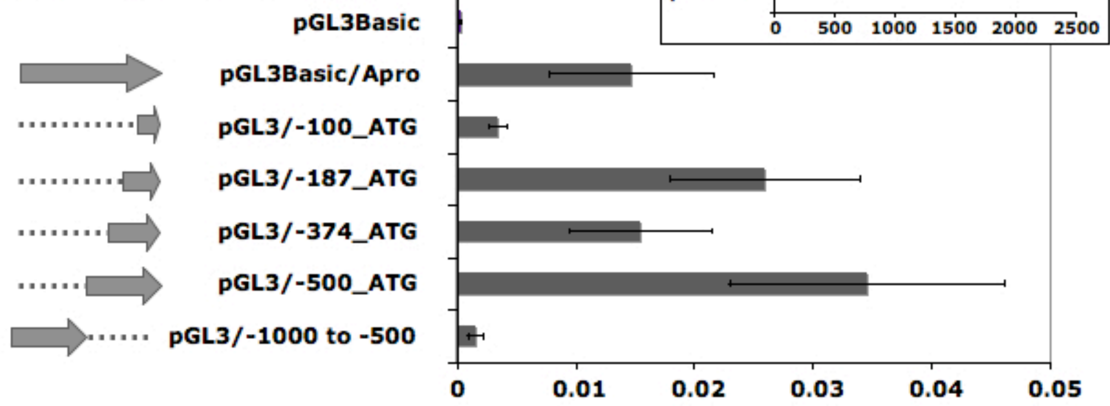
To functionally map those regions within the downstream 500 nt of the APro sequence that are important for gene expression, deletion constructs were generated based on the clustered location of TFSEARCH-identified transcription factor binding sites. The region from -374 to the Artemis translational start site, including USF and Lyf1 transcription factor binding sites (pGL3Basic/-374_ATG), was found to mediate only half the amount of luciferase expression conferred by both the full-length APro and -500_ATG constructs (**Figure 2**A). However, the region from -184 to the Artemis translational start site (pGL3Basic/-184_ATG) including a GATA-1 transcription factor binding site, supported luciferase expression at a level similar to that of the full-length APro

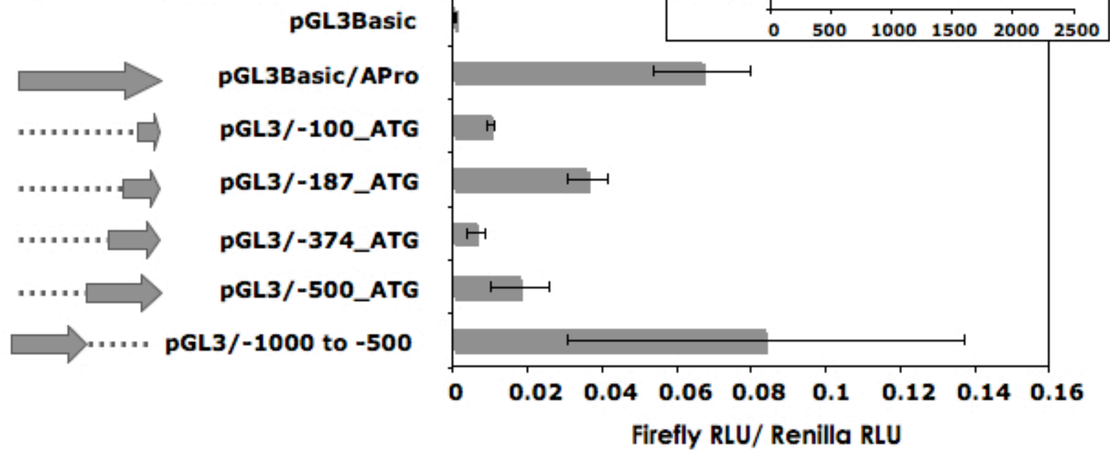
and -500_ATG sequences (**Figure 2A**). Finally, a fragment including nt -100 to the human Artemis translational start site, containing two major transcription factor binding site clusters (AML1A, GATA-1) and (STAT-1, Elk-1, NRF-2), conferred minimal luciferase activity comparable to background (**Figure 2A**). These results define a functional promoter region extending as little as 184 bp upstream of the translational start site of the human Artemis gene in HEK 293 cells.

Artemis plays a key role in lymphocyte development during the receptor V(D)J recombination stage. To assess the expression activity profile of the APro deletion constructs in lymphoid cell lines, Jurkat cells (an immortalized human T cell line) and BJAB cells (a human EBV-negative Burkitt-like B cell lymphoma) were electroporated using an Amaxa nucleofector and luciferase expression was assayed two days later. Surprisingly, Jurkat cells yielded only background levels of luciferase expression in all deletion construct transfection reactions (data not shown), suggesting that Artemis expression in mature T cells may be very moderate to null. Upon transfection into BJAB cells the full-length APro sequence as well as deletion constructs -374 and -184 displayed luciferase expression patterns similar to those seen in HEK 293T cells, i.e. the observed expression level was substantially less than that mediated by the CAGGS promoter, yet substantially above background (**Figure 2B**). However, the upstream APro construct -1000 to -500, containing the TFSearch-identified TATA box, generated the highest level of luciferase expression in BJAB cells (**Figure 2B**), in contrast to the background levels observed in 293T cells transfected with this construct (**Figure 2A**). These results may not be surprising, considering the numerous lymphoid specific transcription factor binding sites located in this

upstream region, suggesting the potential for two regions of the APro sequence with functional specificity in regulating Artemis gene expression.

Figure 2: Functional mapping of the human Artemis promoter *in vitro*. (a) HEK 293T cells and (b) BJAB lymphoid cells were transfected with reporter constructs consisting of firefly luciferase cDNA regulated by APro, an APro deletion construct, the CAGS promoter, or a promoterless control. Each cell population was co-transfected with a CMV regulated renilla firefly plasmid to control for transfection efficiency. Luciferase expression was assayed and plotted as the ratio of firefly RLU/ renilla RLU as described in Materials and Methods.


A) HEK 293T Transfection
pGL3Basic
pGL3Basic/Apro
pGL3/-100_ATG
pGL3/-187_ATG
pGL3/-374_ATG
pGL3/-500_ATG
pGL3/-1000 to -500
0
0.01
0.02
0.03
0.04
0.05
pKT2Cal
0
500
1000
1500
2000
2500


B) BJAB Transfection
pGL3Basic
pGL3Basic/APro
pGL3/-100_ATG
pGL3/-187_ATG
pGL3/-374_ATG
pGL3/-500_ATG
pGL3/-1000 to -500
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16
Firefly RLU/ Renilla RLU
pKT2Cal
0
500
1000
1500
2000
2500

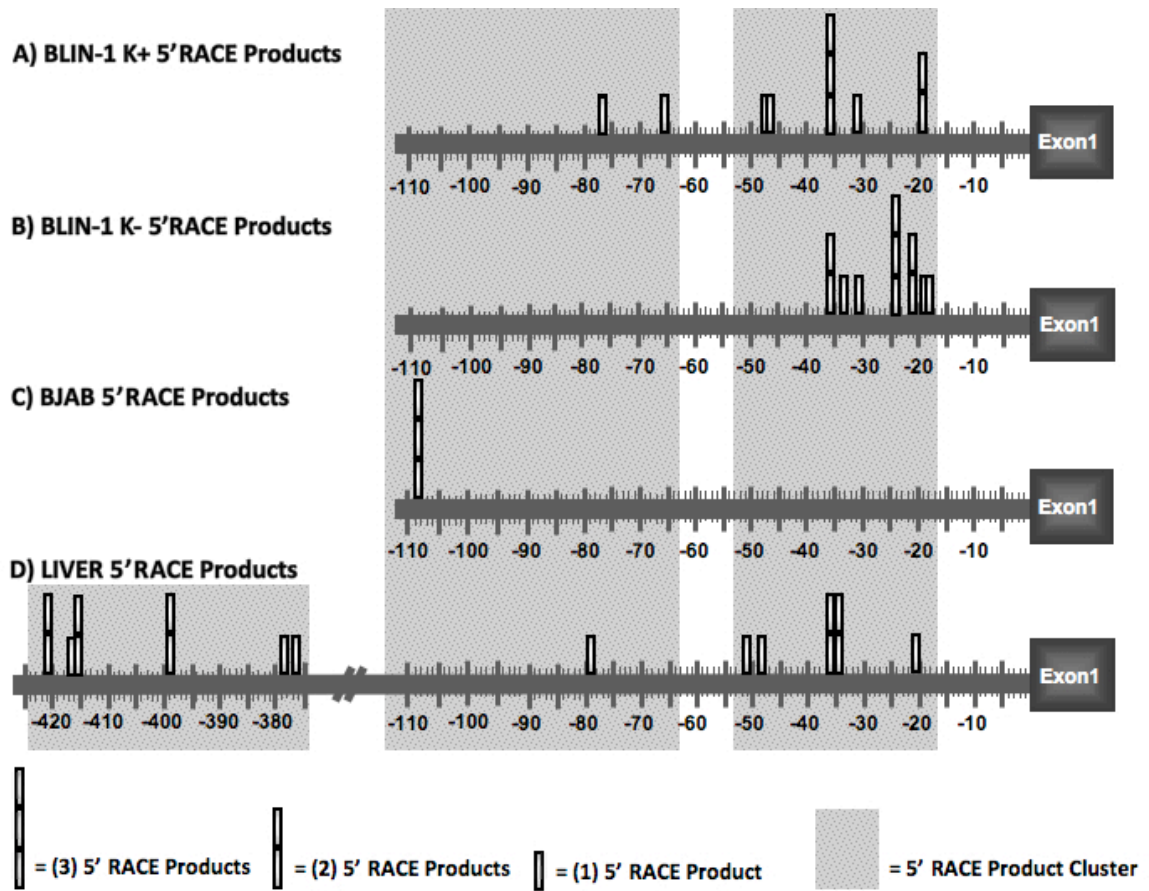
5′ RACE identifies multiple transcriptional start points. To determine the position of transcript initiation, 5′ rapid amplification of cDNA ends (RACE) was carried out as described in Materials and Methods using human liver RNA and cytoplasmic RNA extracted from BLIN-1 K^+, BLIN-1 K^-, BJAB, and Jurkat cell lines. BLIN-1 is a cell line derived from a human B cell lymphoma; BLIN-1 K^- is characterized as retaining the ability to continuously undergo V(D)J recombination whereas BLIN-1 K^+ is characterized as kappa light chain positive, therefore having completed V(D)J recombination. These cells are of interest not only due to their lymphocyte lineage, but also because of the anticipated requirement of Artemis for V(D)J recombination in BLIN-1 K^-. BLIN-1 K^+ serves as an internal control for the cessation of V(D)J recombination.

Several 5′ RACE products were identified that appeared to be grouped into multiple clusters (**Figure 3**). From these data, transcriptional start sites were designated as the average of all 5′ RACE products within each product cluster. Three transcriptional start points were identified from the liver RNA at base pairs -32, -80, and -405 relative to the human Artemis ATG. Two start sites were identified in RNA extracted from either of the BLIN-1 cell lines located at base pair -32 and -81 relative to the human Artemis ATG and one site was identified from the BJAB lymphoma line located at base pair -108 (**Figures 1 and 3**). No 5′RACE products were identified using RNA extracted from Jurkat cells.

Transcriptional start sites identified by 5′ RACE of liver RNA are consistent with APro deletion analysis in which transcriptional activity can be correlated with the luciferase expression profile. The full-length pGL3Basic/APro and deletion constructs pGL3Basic/APro-187_ATG and

pGL3Basic/APro-500_ATG, exhibiting the highest levels of luciferase activity, correspond with transcription start points located at base pairs -405 and -80 from the human Artemis ATG (**Figure 3**). Moreover, 5′ RACE products were not recovered using RNA extracted from Jurkat cells, consistent with the observed background level of expression mediated by APro regulated constructs upon transfection. However, we were also unable to generate upstream 5′ RACE products from BJAB RNA even though the APro construct -1000 to -500, containing the TFSearch-identified TATA box, conferred the highest level of luciferase expression in BJAB cells. Overall, these data suggest the potential for the presence of one promoter region located between base pair -500 and -405 from the human Artemis ATG, and a second promoter region exhibiting non-specific transcriptional initiation located between base pair -187 to -32 from the human Artemis ATG.

Figure 3: Transcriptional start sites in the Artemis promoter region mapped by 5′ RACE. 5′ RACE was performed on RNA generated from (a) BLIN K^+ cells, (b) BLIN K^- cells, (c) BJAB cells, and (d) human liver RNA. Each 5′ RACE product is displayed as a hatched bar positioned along an axis labeled with numbers representing nucleotide position upstream of the human Artemis translational start site. The number of stacked hatched bars represents the number of 5′ RACE products identified at that particular nucleotide position: 3, 2, and 1 stacked bars represent 3, 2, and 1 5′ RACE products, respectively. 5′ RACE products are grouped into 3 clusters and are boxed in light grey.


A) BLIN-1 K+ 5’RACE Products
B) BLIN-1 K- 5’RACE Products
C) BJAB 5’RACE Products
D) LIVER 5’RACE Products
Exon1
-110 -100 -90 -80 -70 -60 -50 -40 -30 -20 -10
-420 -410 -400 -390 -380
= (3) 5’ RACE Products
= (2) 5’ RACE Products
= (1) 5’ RACE Product
= 5’ RACE Product Cluster

The human Artemis promoter supports moderate but sustained levels of gene expression in vivo. Part of our motivation in characterizing the human Artemis promoter is so that we can incorporate it into vectors for APro regulated expression *in vivo*. We thus generated a lentiviral vector in which the GFP coding sequence is positioned immediately downstream of the full length APro sequence (CSII/AProGFP; **Figure 4**). Considering the sequence motifs contained in this region as well as expression profiles generated from deletion analysis, we determined that the full one kilobase APro sequence would be most appropriate to include in our vector design. Because this is a self-inactivating vector design, GFP expression in transduced cells is entirely dependent on expression mediated by the APro sequence. Vectors were generated as described in Materials and Methods, and vector titer was assessed by GFP expression analysis following transduction of NIH 3T3 cells. Flow cytometric analysis of NIH 3T3 cells transduced with CSII/AProGFP demonstrated that the CSII/AProGFP vector achieved a similar gene transfer frequency as the control CSIIEG vector, in which GFP is regulated by the strong EF1α promoter (**Figure 4**). However, the CSII/AProGFP transduced population exhibited a mean fluorescence intensity (MFI) of 21.5, significantly decreased in comparison with the CSIIEG MFI of 216 (**Figure 4**). These results demonstrate that the APro sequence confers a moderate level of expression compared to stronger promoters such as CAAGS or EF1α after transfection (**Figure 2A**) or after lentiviral transduction (**Figure 4**).

To test for APro mediated gene expression *in vivo*, whole bone marrow was harvested from C57BL/6 CD45.1 animals and transduced overnight with either CSII/AProGFP or the control vector CSIIEG at a multiplicity of infection of

10. The transduced marrow was transplanted into lethally-irradiated CD45.2 recipient animals, which were subsequently assayed monthly for donor engraftment and GFP expression by flow cytometry. Animals from both groups exhibited high level engraftment (~90%) with GFP expression persisting out to four months. Animals transplanted with CSII/AProGFP-transduced marrow expressed GFP in about 33% of the donor leukocyte compartment whereas animals transplanted with CSIIEG-transduced marrow maintained GFP expression in about 11% of the leukocyte compartment four weeks post-transplant (**Figure 5**). Interestingly, we found that CSII/AProGFP transduced cells in the peripheral blood exhibited a diminished level of GFP expression (MFI= 31.2) in comparison with the CSIIEG control (MFI= 2,869) (**Figure 5**), similar to the shift in MFI observed in transduced 3T3 cells.

Secondary transplantation was carried out by harvesting marrow from each primary recipient four months post-transplant and infusing each marrow sample into three recipient animals pre-conditioned with 800 Rads, Cs source. GFP expression was sustained in the secondary transplant recipients receiving either CSII/AProGFP or CSIIEG transduced marrow, demonstrating the capability of these vectors to maintain gene expression after transduction into primitive hematopoietic stem cells engrafted in the primary recipients (**Figure 5**). Because our intention is to ultimately use the human Artemis promoter to regulate gene expression for correction of SCID-A, we assayed for APro mediated expression in lymphocyte populations engrafted in secondary recipients. GFP expression was observed in both lymphoid and myeloid compartments, including B cells ($B220^{+}NK1.1^{-}$), T cells ($CD3e^{+}$), and myeloid cells

(CD11b) in animals that received marrow transduced with CSII/AProGFP (**Figure 5**). These results verify that the Artemis promoter provides a moderate level of expression in differentiated lymphoid cells after lentiviral transduction into long-term repopulating hematopoeitic stem cells.

Figure 4: APro mediates moderate levels of GFP expression after lentiviral transduction *in vitro*. Mouse 3T3 cells were transduced with increasing amounts (0.3 μl, 1 μl, or 3 μl) of either (a) an EF1α regulated GFP lentiviral vector (CSIIEG) or (b) an APro regulated lentiviral vector (APro-GFP) and analyzed by flow cytometry for GFP expression. Dot plot analysis reveals a similar transduction frequency for each vector; however CSIIEG transduced cells express GFP at an MFI significantly higher than APro-GFP transduced cell populations. (c) Histograms displaying the MFI exhibited by 3T3 cells transduced with 1000 μl of either CSIIEG or APro-GFP lentiviral supernatant reveal that cells transduced with CSIIEG exhibit an MFI of 216 while cells transduced with APro-GFP exhibit a much lower MFI of 21.5.

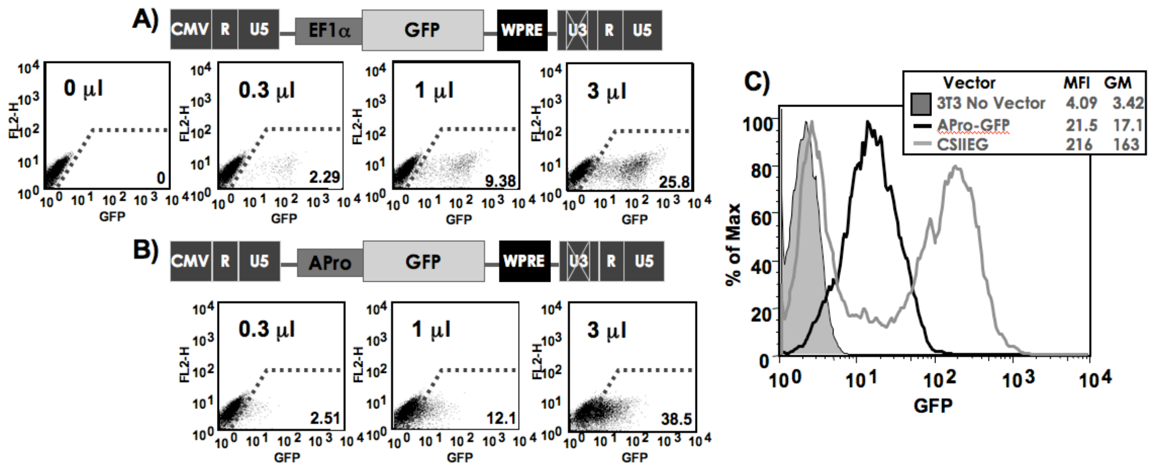
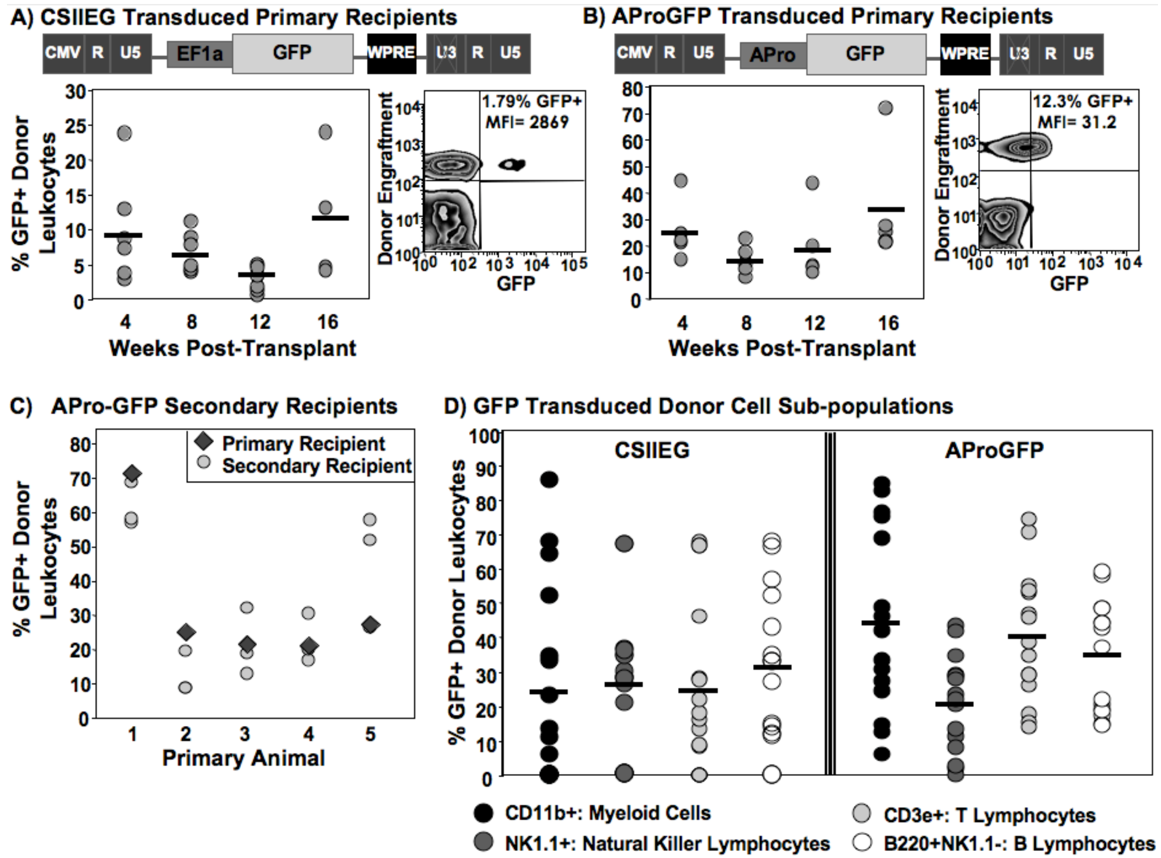

A)
CMV
R
U5
EF1α
GFP
WPRE
U3
R
U5
0 μl
0.3 μl
1 μl
3 μl
0
2.29
9.38
25.8
FL2-H
GFP
B)
CMV
R
U5
APro
GFP
WPRE
U3
R
U5
0.3 μl
1 μl
3 μl
2.51
12.1
38.5
C)
Vector MFI GM
3T3 No Vector 4.09 3.42
APro-GFP 21.5 17.1
CSIIEG 216 163
% of Max
GFP

Figure 5: APro mediated GFP expression *in vivo*. Donor CD45.1 C57BL/6 bone marrow was transduced with either (a) an EF1α regulated GFP lentiviral vector (CSIIEG) or (b) an APro regulated lentiviral vector (APro-GFP) and transplanted into preconditioned recipient CD45.2 C57BL/6 animals. GFP expression was monitored by flow cytometry over a 16 week time period and is indicated for individual animals, represented by open circles, with mean values represented as solid grey bars, both in units of percentage GFP positive cells in peripheral blood donor lymphocytes. (c) Four months post-primary transplant, animals were sacrificed and total bone marrow was infused into secondary C57BL/6 recipients. Four months post-secondary transplant, GFP expression mediated by the APro-GFP vector was observed to persist in secondary transplant recipients. (d) GFP expression mediated by the CSIIEG vector was observed in both myeloid and lymphoid lineages, whereas GFP expression mediated by the APro-GFP vector was mainly observed in the lymphoid lineage. Subpopulations are graphed as percentages of the total GFP^{+} transduced donor (CD45.1) population.


A) CSIIEG Transduced Primary Recipients
CMV R U5 EF1a GFP WPRE U3 R U5
% GFP+ Donor Leukocytes
Weeks Post-Transplant
Donor Engraftment
1.79% GFP+
MFI= 2869
GFP
B) AProGFP Transduced Primary Recipients
CMV R U5 APro GFP WPRE U3 R U5
Weeks Post-Transplant
12.3% GFP+
MFI= 31.2
C) APro-GFP Secondary Recipients
Primary Recipient
Secondary Recipient
Primary Animal
D) GFP Transduced Donor Cell Sub-populations
CSIIEG
AProGFP
% GFP+ Donor Leukocytes
CD11b+: Myeloid Cells
NK1.1+: Natural Killer Lymphocytes
CD3e+: T Lymphocytes
B220+NK1.1-: B Lymphocytes

DISCUSSION

We isolated a one-kilobase region of human genomic DNA directly upstream of the Artemis translational start site by PCR amplification (APro). An *in silico* transcription factor binding site analysis of this sequence uncovered several transcription factor binding sites, leading us to further characterize the genomic region for promoter activity. 5′ RACE analysis indicated multiple transcriptional start points in RNA extracted from liver and lymphoid cell lines, and deletion analysis revealed divergent expression patterns between 293T cells and the BJAB Burkett lymphoma-like cell line. Transfection studies *in vitro* revealed the potential for this sequence to regulate gene expression in multiple cell types; additionally, APro mediated expression at a level that was substantially lower than that mediated by the strong EF1α promoter. Furthermore, GFP expression regulated by APro was observed *in vivo* in mice transplanted with marrow that had been transduced with an APro-regulated lentiviral vector. Finally, expression was sustained in secondary transplant recipients in both myeloid and lymphoid lineages, establishing the effectiveness of APro to serve as a proficient promoter for gene expression within the hematopoeitic system.

The human Artemis promoter region identified through this study exhibits several characteristics similar to that observed for promoters of other NHEJ proteins. The downstream promoter region lying between –187 to the human Artemis ATG contains neither a TATA box nor a CCAAT box, motifs also absent in the promoters for DNA-PKcs (7), Ku70 (23) and other housekeeping genes. Lack of a distinct TATA box within this region of the human Artemis promoter sequence is associated with considerable variability in the location of

transcriptional start sites, as determined by 5′ RACE, analogous to variability in transcriptional initiation sites observed for other NHEJ messages such as DNA-PKcs in which at least 6 transcriptional start sites were observed (7). We also tested full-length APro and deletion constructs transfected into 293T cells for the effect of irradiation or exposure to the radiomimetic chemicals bleomycin and etoposide, but induced expression upon DNA damage was not observed (data not shown). Similarly, neither transcription nor translation of DNA-PKcs are induced upon DNA damage in either human or mouse cells (7, 16). These observations taken as a whole indicate the similarity of the human Artemis transcriptional regulation with that of other NHEJ components.

Gene transfer is emerging as a promising approach for treatment of genetic disorders. However, recently observed adverse events have called to attention the importance of regulating expression of therapeutic genes. Specifically, two independent studies have reported correction of X-linked SCID by *ex vivo* transduction of $CD34^+$ hematopoeitic stem cells using a retroviral vector expressing the interleukin common cytokine-receptor gamma chain (common γ chain) (6, 8, 12). Long-term engraftment of corrected stem cells was observed in the majority of patients, ultimately resulting in reconstitution of a functional lymphocyte compartment. To date, however, five out of twenty patients treated for X-linked SCID by gene transfer have developed clonal T cell outgrowths resulting in leukemia, from which one child has died (13) (15). Although insertional activation of the LMO2 oncogene was reported in three of the leukemic cases, over-expression of the common γ chain induces cellular proliferation and thus may have contributed to the T lymphocyte clonal

outgrowth (3, 15). More tightly regulated expression of the common γ chain may thus reduce the risk of oncogenesis resulting from common γ chain over-expression.

Achieving transgenic expression of human Artemis for the correction of SCID-A will be challenging considering previous results regarding Artemis over-expression. Recently, two independent groups reported correction of a murine model of SCID-A by lentiviral vector mediated gene transfer (4, 19). In both studies, SCID-A animals receiving HSC transduced with a lentiviral vector encoding a human Artemis cDNA regulated by the PGK promoter exhibited repopulation of both B and T lymphocyte compartments. However, Mostoslavsky *et al*. reported the inability of either CMV or EF1α regulated human Artemis lentiviral vectors to repopulate B and T cells in RAG-1 deficient animals receiving SCID-A HSCs transduced with either vector (4, 19). Considering the endonucleolytic nature of Artemis, these results lead us to consider whether Artemis over-expression might be inherently toxic. We subsequently characterized the effect of Artemis over-expression and found it to be associated with cytotoxicity ultimately resulting in a halt in cell cycle progression, fragmentation of genomic DNA, and apoptosis (20). These results emphasize the importance of providing Artemis expression at a level that is non-toxic and yet sufficient to correct the $T^{-}B^{-}$ phenotype in preclinical studies and in clinical application to human SCID-A.

Additionally, use of the human Artemis promoter to provide Artemis expression for gene therapy may be particularly important considering the evidence for multiple sites of transcription initiation. Our 5′ RACE results as well

as the *in vitro* deletion mapping presented in this study suggest the potential for multiple regulatory regions that comprise the endogenous Artemis promoter. This is consistent with Artemis' involvement in several distinct cellular functions such as NHEJ (17), V(D)J recombination (17), and apoptosis (5). It is tempting to speculate that Artemis expression may be spatially and/or temporally regulated per required function, similar to several other proteins demonstrated to be differentially regulated through transcript initiation at multiple promoter sites. For example, the murine α-amylase gene exhibits tissue specific expression regulated by two separate promoters (24). S1 nuclease mapping revealed that liver tissues yielded one minor α-amylase transcript whereas the parotid gland yielded two α-amylase transcripts including the minor transcript plus one additional major transcript; additionally, synthesis of each transcript was found to initiate directly downstream of TATA boxes identified by sequence analysis (24). Our data show two potential promoter regions regulating human Artemis expression, one containing a well-defined TATA box with the ability to regulate expression in B-lymphoid cells. The downstream promoter region is less defined in that it does not contain a TATA box, yet regulates expression in several other cell types. It will be of interest to study the relationship between Artemis function and tissue type expression to address the possibility that one region of the Artemis promoter sequence is necessary for expression during V(D)J recombination while the other region confers constitutive expression to support NHEJ.

Because over-expression of Artemis results in genome-wide damage and ultimately apoptosis, it is crucial to develop a clinical gene transfer vector in

which Artemis is expressed at a level that will correct the B^- T^- SCID-A phenotype yet evade cytotoxicity. The endogenous human Artemis promoter provides expression both *in vitro* and *in vivo* at levels significantly lower than that of well-characterized strong promoters such as CMV and EF1a. Further, when studied *in vivo*, the Artemis promoter regulated expression in several lymphoid cell populations, demonstrating the ability of APro to mediate expression in important hematopoeitic compartments after gene transfer into hematopoietic stem cells. The APro sequence thus has great potential as a regulator of therapeutic gene expression, including expression of the human Artemis gene for *in vivo* correction of human SCID-A. With this in mind, we have recently generated lentiviral vectors employing innate regulation of human Artemis cDNA using its own endogenous promoter sequence and have achieved successful *ex vivo* gene transfer resulting in functional lymphocyte repopulation of a murine model of SCID-A [Chapter 4]. Overall, these results suggest that providing innate Artemis expression via *ex vivo* lentiviral transduction into hematopoietic stem cells may serve as a clinically relevant and feasible treatment of human SCID-A.

MATERIALS AND METHODS

Isolation of the human Artemis promoter region.

A one-kilobase sequence directly upstream of the human Artemis translational start site was amplified from human genomic DNA (Roche, Indianapolis, IN) by PCR using forward primer 5'-ACCGGTCAGAGAGCCGAAATCACGCC-3' and reverse primer 5'-ACCGGTAGCGCCGCCGATCCCAGAGT-3' (flanked by *AgeI* restriction sites, underlined). Two rounds of PCR amplification were necessary to achieve a one-kilobase product (5 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 69°C, and 1.5 min at 72°C, with a final 10 min extension at 72ºC). The product was gel extracted (QIAGEN, Valencia, CA) and cloned into TOPO vector PCR2.1 to generate pCR2.1/APro. The resulting clone was sequenced and found to be identical to the published human chromosome 10 sequence one kilobase directly upstream of the human Artemis translational start site (PubMed Reference: NT_077569).

Plasmid Construction

i) APro regulated Luciferase Constructs. The full-length APro sequence was digested with *AgeI* and ligated into the *AgeI* site of pGL3Basic (Promega, Madison, WI) directly upstream of the firefly luciferase gene to generate pGL3/APro. Several APro deletion mutants were generated from the full-length APro sequence by PCR amplification. Primers utilized to amplify truncated APro sequences are illustrated in **Table 1**. PCR products containing various portions of the APro sequence were digested and cloned into the *AgeI* site of pGL3Basic.

ii) pCSII/AProGFP. A lentiviral vector plasmid designed for regulation of GFP by the full-length human Artemis promoter was generated from pCSIIEG (1). The CMV promoter was removed from pCSIIEG by *AgeI* digestion, and then the APro sequence was then ligated into the *AgeI* site directly upstream of the GFP gene to generate pCSII/APro-GFP.

Table 1. Oligonucleotide sequences used during PCR amplification. Forward[a] and reverse[b] oligonucleotide sequences, each flanked by the AgeI sequence (underlined), utilized to amplify APro deletion sequences. (b) Forward[c] and reverse[d] oligonucleotide sequences utilized during 5′ RACE to identify human Artemis transcriptional start sites.

A) Deletion Sequence	Forward Primer 5′-3′[a]	Reverse Primer 5′-3′[b]
-100 to ATG	ACCGGTGCGAGGCAGCGCGCGGCTTCCCGGA	ACCGGTAGCGCCGCCGATCCCAGAGT
-184 to ATG	ACCGGTGCCTAGAACCCGACCGGATGCTCCT	ACCGGTAGCGCCGCCGATCCCAGAGT
-374 to ATG	ACCGGTGTCGGCTGGGGCCACCTGCTCTGGG	ACCGGTAGCGCCGCCGATCCCAGAGT
-500 to ATG	ACCGGTTCCCACAAGGTTAGAAATTG	ACCGGTAGCGCCGCCGATCCCAGAGT
-1000 to -500	ACCGGTCAGAGAGCCGAAATCACGCC	ACCGGTGTGGATTACCGTGAGGGTT GTATG
B) Reaction	**Forward Primer 5′-3′[c]**	**Reverse Primer 5′-3′[d]**
Reverse Transcription	No Forward Primer	CCTTGGGATGCCGGCATGCATGGAT
Amplification of RACE Products	CGACTGGAGCACGAGGACACTGA GeneRacer Forward Primer (Invitrogen, Carlsbad, CA)	GGATGTCTTTGACTCTGCCCCCGGA

[a]Forward and [b]reverse oligonucleotide sequences, each flanked by the *AgeI* sequence (underlined), utilized to amplify APro deletion sequences. [c]Forward and [d]reverse oligonucleotide sequences utilized during 5′RACE to identify human Artemis transcriptional start sites.

Mammalian Cell Culture and Transfections

HEK 293T, and murine NIH 3T3 tk^- cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and 1% antibiotic antimycotic at 37ºC and 5% CO_2. HEK 293T cells were transfected with 2 ug of pGL3/APro or derivative deletion constructs using the DNA calcium phosphate co-precipitation technique as described (30). Briefly, 2.5M $CaCl_2$ was added to the luciferase expression plasmid DNA mixture to a final concentration of 2 ug firefly luciferase expressing construct per transfection. An equal volume of 2 X BBS [0.5 M BES, 150 mM sodium phosphate dibasic, 2.8 M sodium chloride; pH 7.05] was added to the DNA-calcium mixture in a drop-wise fashion and mixed thoroughly. The $CaCl_2$-DNA-BBS solution was added to each well containing 5 X 10^4 HEK 293T cells and incubated at 37ºC with 3% CO_2. Transfection efficiency was controlled by co-transfection with 0.5 ug of phRL-CMV (Promega, Madison, WI); assay results are expressed as firefly RLU/renilla RLU. pKT2/Cal, a firefly luciferase construct regulated by the strong CAGS promoter (27), was utilized as a luciferase positive control, and the promoter-less pGL3Basic (Promega, Madison, WI) was used as a luciferase background control.

BJAB, BLIN-1 K^+ and BLIN-1 K^- cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and 1% antibiotic antimycotic at 37°C with 5% CO_2 and transfected utilizing an Amaxa Nucleofector Device (Lonza, Cologne, Germany). Cells were harvested, washed, and resuspended in Amaxa supplemented nucleofection buffer C. 5 X 10^5 cells were mixed with 5 ug of pGL3Basic/APro or derivative deletion construct plus 0.5 ug of phRL-CMV and then electroporated at the program setting of X-001.

Immediately following nucleofection, cells were transferred to pre-warmed culture medium and incubated at 37ºC with 3% CO_2.

Forty-eight hours post-transfection, cells were collected and analyzed for firefly and renilla luciferase expression via Promega's Dual Luciferase Reporter Assay System. Briefly, cells were harvested by trypsinization, washed with 1 X PBS buffer and resuspended in Passive Lysis Buffer. After a 5-minute incubation at RT, the lysate was vortexed vigorously and then cleared by centrifugation for two minutes at 13,200 x g, collecting the supernatant. A small aliquot (20 ul) of lysate supernatant was incubated with Promega firefly luciferase substrate, then subsequently incubated with Stop and Glo renilla luciferase substrate, assaying for luminescence in a Lumat LB 9507 luminometer.

5′ RACE Identification of Transcriptional Start Points

5′ RACE was carried out using the 5′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen, Carlsbad, CA), Version 2.0 kit and reagents, according to the manufacturer's instructions. Primers utilized for the 5′ RACE protocol are listed in **Table 1**. RACE products were isolated by gel electrophoresis, extracted, cloned into TOPO vector PCR2.1, and sequenced. Vector NTI Software (Invitrogen, Carlsbad, CA) was used for sequence analysis.

Preparation and Titering of Lentiviral Vectors

VSV-G pseudotyped lentiviral vectors were generated as described (9, 30). Briefly, 24 hours pre-transfection, 1.4 X 10^7 HEK 293T cells were seeded into poly-L-lysine coated 15-cm^2 plates and cultured in DMEM supplemented with

1% penicillin streptomycin and 8% FBS at 37°C with 5% CO_2. Lentiviral vector plasmid constructs were co-transfected with pΔNRF to provide lentiviral structural and enzymatic proteins and pMD.G to provide vesicular stomatitis virus G (VSV-G) envelope protein. Twelve hours post-transfection, the medium was replaced using DMEM supplemented with 4% FBS. Viral supernatants were collected 24, 36, and 48 hours post-transfection, pooled, and then concentrated 100-fold by centrifugation at 23,000 x g in a Sorval RC5B centrifuge. Vector was resuspended in Iscove's modified Dulbecco's medium (IMDMEM), aliquoted, and stored at -80°C for future use. For quantitation of vector titers, NIH 3T3 tk^- cells were transduced with varying amounts of vector in the presence of 8μg/mL polybrene. Forty-eight hours post-transfection, cells were harvested for flow cytometric analysis of GFP expression to determine the percentage of cells transduced. DNA was also extracted from the transduced cells and subjected to quantitative PCR as previously described, utilizing a probe specific for the integrated lentiviral strong stop sequence or a probe for the GFP sequence as a measure of lentiviral vector titer (9).

Lentiviral Transduction and Transplantation of Mouse Bone Marrow

All procedures were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee. CD45.2 and CD45.1 C57Bl/6 mice were obtained from the National Cancer Institute (Frederick, MD) and provided food and water *ad libitum*. Bone marrow was flushed from the long bones of the hind limbs of donor mice into DMEM supplemented with 10 U/ml heparin, 10% FBS, and 1% PenStrep antibiotic/antimycotic. Red blood cells were lysed using

ammonium chloride hemolysis buffer [0.8% NH_4Cl with 0.1 mM EDTA] (StemCell Technologies, Vancouver, BC, Canada), washed with 1 X PBS, and then triturated into a single cell suspension in transduction medium [complete StemPro-34 SFM media with supplement, 2mM L-glutamine, 1% PenStrep (all from Invitrogen, Carlsbad, CA), 100 ng/ml murine IL-3, 100 ng/mL murine IL-6, 100 ng/mL murine TPO, 100 ng/mL murine SCF (all cytokines from R&D Systems, Minneapolis, MN) and 8 ug/ml polybrene (Sigma-Aldrich, St. Louis, MO)]. The marrow was transduced twice, once immediately following marrow harvest and a second time 20 hours after the first exposure. Transduced cells were harvested, washed, counted, and prepared as a single-cell suspension in IMDMEM. Equal numbers of bone marrow cells were injected via lateral tail vein into sublethally irradiated (800 Rads, Cesium source) congenic recipients. For secondary transplants, marrow samples were collected individually from primary recipients as described above, isolating the nucleated fraction and transplanting 5×10^6 cells into each of three secondary irradiated (800 rads) recipients.

FACS Analysis

Blood was collected via cheek puncture and assayed for GFP expression in specific hematopoietic compartments by flow cytometry. Whole blood was treated with ammonium chloride hemolysis buffer (0.8% NH_4Cl with 0.1 mM EDTA) (StemCell Technologies, Vancouver, BC, Canada), washed, and then pelleted leukocytes were resuspended in staining buffer [1 X PBS plus 1% FBS and 0.002% sodium azide and fluorochrome-conjugated monoclonal antibodies

for identification of hematopoeitic compartment] (all antibodies utilized in flow cytometric analysis were purchased from eBiosciences, San Diego, CA). GFP expression as well as immunophenotype analysis was conducted using monoclonal mouse anti-bodies against CD45.1, CD45.2, B220 (B lymphocytes), CD3e (T lymphocytes), NK1.1 (Natural Killer cells), Gr-1 (myeloid lineages) and assayed on an LSRII instrument. Data were collected using CellQuest Pro (BD Biosciences) and analyzed using FlowJo (Tree Star, Inc., Achland, OR) software.

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CHAPTER 4

ROLE OF TRANSGENE REGULATION IN *EX VIVO* LENTIVIRAL CORRECTION OF ARTEMIS DEFICIENCY

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Artemis is an endonuclease characterized as a key factor involved in both non-homologous end joining and V(D)J recombination. Mutations in the gene encoding Artemis result in a radiation-sensitive form of severe combined immunodeficiency characterized by the absence of mature B and T lymphocytes. Early treatment is critical since the disease results in severe infections ultimately leading to fatality at a young age. The current therapy for SCID-A is allogeneic hematopoeitic cell transplantation (HCT); however, HCT often results in incomplete reconstitution of B lymphocytes and may lead to complications such as graft versus host disease. Transplantation with genetically corrected autologous cells is an alternative approach that may provide improved treatment of SCID-A. We have previously described that there is cytotoxicity associated with Artemis over-expression, demonstrating the necessity of establishing conditions that provide Artemis expression at a level that is non-toxic yet sufficient to complement the Artemis deficiency. We then recovered the endogenous human Artemis promoter (APro) as a one-kilobase region located directly upstream of the human Artemis translational start site, demonstrating that this sequence confers a moderate level of reporter gene expression *in vitro* and *in vivo* after lentiviral gene transfer into hematopoeitic stem cells (HSCs). In this study, we tested the effectiveness of the human Artemis promoter in comparison with the strong human EF1α promoter and the moderate-strength human phosphoglycerate kinase promoter to regulate expression of the Artemis coding sequence for correction of a murine model of SCID-A (mArt $^{-}/^{-}$). Lentiviral vectors containing these sequences were employed to transduce

mArt $^{-}/^{-}$ donor marrow with subsequent transplantation into mildly pre-conditioned mArt $^{-}/^{-}$ recipient mice. Recipient animals were evaluated on a monthly basis post-transplant, and beginning at 8 weeks animals transduced with either APro or PGK regulated human Artemis were found to have normal levels of $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ T lymphocytes and $B220^{+}NK1.1^{-}$ B lymphocytes, thus providing evidence for immune reconstitution. Additionally, *in vivo* challenge and splenocyte stimulation studies of animals receiving APro-hArtemis transduced marrow revealed that the emergent lymphocyte populations were indeed functional. In contrast, animals engrafted with marrow transduced using the EF1α regulated human Artemis vector displayed negligible levels of $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ T lymphocytes and no $B220^{+}NK1.1^{-}$ B lymphocytes were observed; of these animals, only a few presented a lymphocyte compartment with limited function. These results demonstrate the importance of regulated Artemis expression and that the naturally regulated Artemis lentiviral vector, APro-hArtemis, can effectively complement murine SCID-A, ultimately contributing to the development and advancement of gene transfer as a clinically relevant and feasible approach for treatment of SCID-A in humans.

INTRODUCTION

Primary immunodeficiencies (PIDs) are an inherited group of disorders characterized by the genetic disruption of one of several steps in the normal process of immunodevelopment, ultimately rendering the patient highly susceptible to frequently reoccurring infections (8). In general, these diseases can be treated by allogeneic hematopoietic stem cell transplantation (HSCT) if an appropriately matched donor is available. However, this procedure carries an attendant risk of infection, graft rejection, graft-versus-host disease and 20% mortality (27). Due to these inherent risks, there is a great need for alternative therapeutic approaches for the treatment of this disease.

Recent results from clinical trials have demonstrated the effectiveness of transplantation using autologous hematopoietic stem cells (HSCs) that have been genetically corrected for treatment of two PIDs, one caused by adenosine deaminase (ADA) deficiency and the other caused by absence of the common gamma chain (common γ chain) for several cytokine receptors (2, 10, 16). In these trials, the patients' own hematopoietic stem cells were transduced with retroviral vectors encoding either the normal ADA or the common γ chain gene to complement the genetic deficiency. In both clinical trials, the patients were successfully cured of their SCID (2, 10, 16). Thus gene transfer is an effective therapeutic approach for the correction of PIDs and provides a compelling strategy for the correction of several additional forms of inherited PIDs.

One such disorder that may benefit from gene therapy is a severe combined immunodeficiency caused by the absence of the Artemis protein (SCID-A). Artemis is a hairpin-opening, endonucleolytic enzyme involved in the

non-homologous end-joining (NHEJ) DNA double-strand break (DSB) repair pathway (23). NHEJ is the primary mechanism utilized by eukaryotes to repair genomic insults generated by both external damaging agents and by normal cellular processes such as rearrangement of immunoglobulin (Ig) genes and T cell receptor (TCR) genes mediated by the V(D)J recombination pathway (14, 21, 28). A deficiency of Artemis disrupts both DNA DSB repair and V(D)J recombination, and manifests as a radiation sensitive form of severe combined immunodeficiency characterized by the inability to rearrange Ig and TCR genes, ultimately resulting in a loss of B and T lymphocytes (22, 23).

Recently, two independent groups have reported the correction of murine models of SCID-A by transplantation of genetically modified HSC (5, 24). In both studies, Artemis deficient animals receiving HSC transduced with a lentiviral vector encoding a human Artemis cDNA regulated by the PGK promoter resulted in the reconstitution of B and T lymphocyte compartments (5, 24). However, Mostoslavsky *et al*. reported the lack of lymphoid reconstitution in RAG-1 deficient animals receiving SCID-A HSCs transduced with lentiviral vectors encoding human Artemis regulated by the stronger CMV or EF1α promoters (24). Subsequent to these findings, we demonstrated that over-expression of Artemis via lentiviral transduction is associated with cytotoxicity, a halt in cell cycle progression, and fragmentation of genomic DNA ultimately resulting in apoptosis (25). Taken as a whole, these results bring to light the importance of providing Artemis expression at a level that is non-toxic and yet sufficient to correct the SCID-A T^-B^- phenotype. Accordingly, we sought to employ innate regulation of the human Artemis gene under its own endogenous

promoter sequence. Previously, we reported isolation and characterization of the human Artemis promoter (APro) as a moderate strength regulatory region located one kilobase directly upstream of the human Artemis translational start site [Chapter 2]. The APro sequence supported GFP and luciferase reporter gene expression *in vitro* [Chapter 2]. Additionally, *ex vivo* transduction of murine bone marrow with an APro-regulated GFP lentiviral vector resulted in GFP expression at a significantly reduced level in comparison with control mice transplanted with EF1α-GFP transduced marrow [Chapter 2]. Importantly, the human Artemis promoter supported GFP expression in all hematopoeitic lineages that persisted in secondary transplant recipients, ultimately establishing the usefulness of this promoter for the purpose of generating a clinical vector that provides Artemis expression [Chapter 2].

In this study, we aimed to completely rescue a murine model of SCID-A by lentiviral transduction and transplantation of Artemis deficient (mArt $^{-}/^{-}$) whole bone marrow with a vector encoding human Artemis cDNA regulated by its own innate 1 kb human Artemis promoter sequence (APro-hArtemis). Additionally, the correction potential of APro regulated human Artemis was compared to that of lentiviral vectors expressing human Artemis regulated by two other promoters of varying strength, the strong EF1α promoter and the more moderate PGK promoter. Lentiviral vectors containing these sequences (APro-hArtemis, PGK-hArtemis, EF1α-hArtemis) were assembled and used to transduce mArt $^{-}/^{-}$ donor marrow with subsequent transplantation into mArt $^{-}/^{-}$ recipient mice. Recipient animals were evaluated on a monthly basis post-transplant for the presence of donor engraftment and lymphocyte repopulation.

All animals displayed donor engraftment and lentiviral gene marking within circulating peripheral blood cells. Moreover, the majority of animals displayed successful lymphoid reconstitution. Interestingly, however, the success and the kinetics driving lymphocyte repopulation appeared to be promoter dependent. Both APro-hArtemis and PGK-hArtemis treated animal groups engrafted at high rates and presented normal levels of $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ T lymphocytes and $B220^{+}NK1.1^{-}$ B lymphocytes beginning at 8 weeks post infusion, providing evidence for immune reconstitution. On the contrary, EF1α-hArtemis treated animals demonstrated lower engraftment potential, were unable to repopulate their B lymphoid compartment, and presented with abnormally low T lymphocyte counts. Additionally, SCID-A animals receiving APro-hArtemis transduced marrow responded to *in vivo* antigen challenge and *in vitro* splenocyte mitogen stimulation as proficiently as wild-type mice, demonstrating an appropriately restored and functional immune compartment. Animals receiving EF1α-hArtemis transduced marrow, on the other hand, displayed an incomplete response to antigen challenge in which the majority of animals were able to generate antigen specific IgM following *in vivo* challenge but lacked the ability to class switch and display antigen-specific IgG.

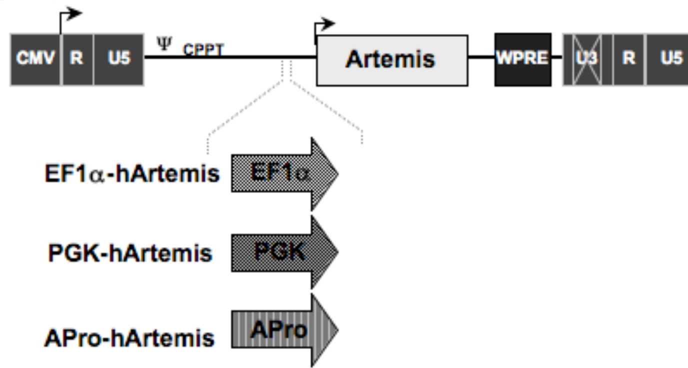
Overall, these data demonstrate the importance of transgene regulation during genetic transfer as a therapeutic approach for SCID-A by presenting outcomes obtained from both appropriately regulated, as well as aberrantly regulated, transgene expression. The incomplete lymphoid reconstitution and immune response demonstrated by the EF1α-hArtemis treated animals highlights the importance of Artemis regulation, signifying that over-expression

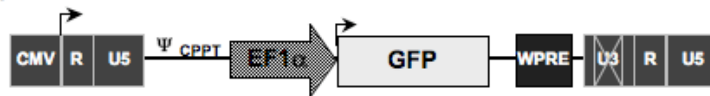
results in the malfunction of both donor engraftment and lymphoid reconstitution ultimately resulting in the failure to successfully complement SCID-A. Meanwhile, innate regulation of human Artemis by its endogenous promoter resulted in expression levels that effectively complemented the Artemis deficient phenotype, ultimately demonstrating restoration of a functional immune compartment. Taken as a whole, these data contribute to the development and advancement of gene transfer as a clinically relevant and feasible approach for treatment of SCID-A in humans.

RESULTS

Lentiviral transduction and expression of the Artemis gene. A lentiviral vector was generated to express human Artemis under transcriptional regulation of its endogenous promoter (APro) (**Figure 1**). To determine the effect of varying levels of Artemis expression on the potential for correction of SCID-A by lentiviral transduction, lentiviral vectors containing the human Artemis coding sequence regulated by the strong EF1α promoter and the more moderate PGK promoter were also generated (**Figure 1**). Lentiviral vectors were packaged by transfection in human 293T cells as described in Materials and Methods and titered by real-time quantitative PCR analysis of DNA extracted from mouse NIH 3T3 cells 48 hours after exposure to vector.

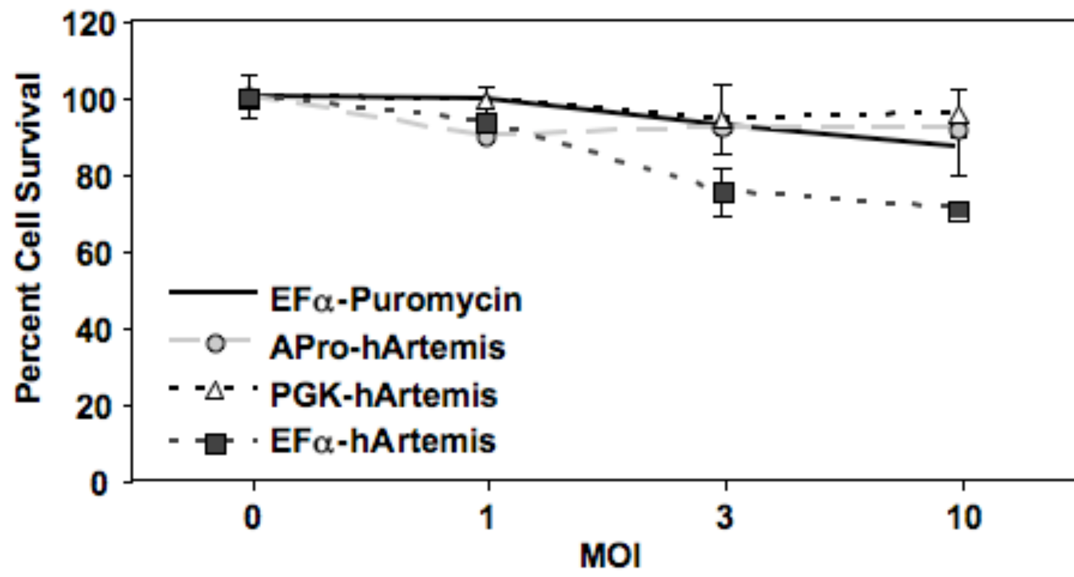
Figure 1: Lentiviral Vector Constructs. Several lentiviral vectors were constructed for analysis and complementation of Artemis deficiency. A) Experimental lentiviral vectors were engineered to express the human Artemis coding sequence under transcriptional regulation of either the human elongation factor 1 alpha (EF1α), phosphoglycerate kinase (PGK), or the one kilobase endogenous human Artemis promoter (APro). B) Lentiviral vectors serving as a transduction controls constructed to expresses either green fluorescent protein (CSIIEG) or puromycin resistance (CSII/E-Puro) regulated by EF1α. Abbreviations: CMV, cytomegalovirus early promoter/ enhancer region; U3/U5/R, unique 3'/ unique 5'/ repeat regions of the HIV long terminal repeat; ψ, packaging signal; cPPT, central polypurine tract; WPRE, woodchuck post-transcriptional regulatory element. Arrows indicate sites and direction of transcript initiation.


A) Experimental Lentiviral Vectors
CMV
R
U5
Ψ CPPT
Artemis
WPRE
U3
R
U5
EF1α-hArtemis
EF1α
PGK-hArtemis
PGK
APro-hArtemis
APro


B) Control Lentiviral Vector- CSIIEG
CMV
R
U5
Ψ CPPT
EF1α
GFP
WPRE
U3
R
U5

Innate regulation of human Artemis circumvents toxicity. Previously, we have reported that over-expression of human Artemis results in apoptosis due to DNA fragmentation and a halt at G1 in the cell cycle (25). We subsequently characterized the 1 kb APro segment as a weak promoter both *in vitro* and *in vivo*. To determine if APro regulation of human Artemis obviates cytotoxicity, dose-dependent cell survival was assessed in 3T3 cells five days post-transduction with increasing multiplicities of infection (MOI) of APro, PGK, and EF1α regulated human Artemis lentiviral vectors, with an EF1α regulated Puromycin vector serving as a transduction control (**Figure 2**). Vectors were generated as described in Materials and Methods, and vector titer was assessed by qPCR analysis of strong stop sequence integration following transduction of NIH 3T3 cells. Cultures transduced with both EF1α-Puromycin and PGK-hArtemis remained viable independent of transduction at increasing MOI; however, a dose dependent decrease in cell survival was observed over time in cultures transduced with EF1α-hArtemis at increasing multiplicity (**Figure 2**). Importantly, cultures transduced with the innately regulated APro-hArtemis remained viable, independent of an increase in MOI, demonstrating that innate regulation of human Artemis obviates a cytotoxic response (**Figure 2**).

Figure 2: Artemis Regulation by APro Avoids Cytotoxicity. Murine NIH 3T3 cells were transduced at increasing MOI using CSII/E-Puro, EF1α-hArtemis, PGK-hArtemis, or APro-hArtemis lentiviral vectors as indicated. Cell survival was assessed five days post-transduction by MTT assay and plotted as the percentage of cells surviving in control, untreated populations. Each value represents the mean of 3 replicates.


120
100
80
60
40
20
0
Percent Cell Survival
EFα-Puromycin
APro-hArtemis
PGK-hArtemis
EFα-hArtemis
0
1
3
10
MOI

Successful engraftment following lentiviral transduction of the human Artemis coding sequence. To evaluate the effectiveness of APro regulated human Artemis versus PGK or EF1α regulated human Artemis in the correction of murine SCID-A, whole bone marrow was harvested from donor CD45.1 SCID-A animals and transduced twice (once overnight and once 20 hours post harvest) with either APro-hArtemis, PGK-hArtemis, or EF1α-hArtemis at a MOI of 30 based on qPCR analysis (**Figure 3**). Control transduction and transplantation experiments were performed in which either C57BL/6 or SCID-A whole marrow was transduced twice with the control vector CSIIEG at a multiplicity of infection of 10 based on FACS analysis (**Figure 3**). The transduced marrow was subsequently transplanted into CD45.2 recipient SCID-A animals pre-conditioned with 500 Rads (X-irradiation). Animals were monitored monthly for donor engraftment by flow cytometric analysis as well as for gene marking by qPCR analysis of DNA extracted from PBMC for integrated lentiviral strong stop sequence.

Beginning at four weeks post-transplant, donor cell populations emerged and were observed in all recipient animal groups by 8 weeks post-transplant (**Figure 4**). Interestingly, animals transplanted with PGK/hArt or EF1α/hArt transduced marrow engrafted at a rate slower (77.7% and 87.4%, respectively) than animals transplanted with APro/hArt (95.1%) (**Figure 4**). Further, in the majority of both PGK-hArtemis and APro-hArtemis treated animals the lymphocyte compartment was repopulated to wild type levels whereas EF1α-hArtemis treated animals remained lymphopenic (**Figure 4**). Control SCID-A donor marrow engrafted at very low levels, remaining below the wild type lymphocyte range for the duration of the 16-week time course, whereas wild

type C57BL/6 marrow engrafted to wild type levels by 8 weeks post-transplantation and completely filled the lymphocyte compartment (**Figure 4**).

Transduction and gene marking was determined by qPCR analysis of the integrated lentiviral strong stop sequence in peripheral blood 16 weeks post-transplantation. All groups were found to have significant levels of gene marking above background levels presented by normal SCID-A and C57BL/6 animals (0.02 and 0.01% respectively) (**Figure 4**). Notably, APro-hArtemis treated animals had the greatest levels of marking presenting with 6.8% strong stop marking within peripheral blood while EF1α-hArtemis and PGK-hArtemis animals only demonstrated 2.7% and 2.0% gene marking, respectively (**Figure 4**). Additionally, the control group in which SCID-A animals were transplanted with CSIIEG-transduced wild-type marrow displayed only 0.6% gene marking, demonstrating that transduction with an Artemis-encoding vector is required for the observed selective advantage in engraftment and repopulation (**Figure 4**).

Figure 3: Bone Marrow Transduction and Transplantation. A) Total bone marrow was harvested from murine SCID-A or control C57BL/6 donor animals (expressing the CD45.1 congenic marker) treated with 5-fluorouracil (50 mg/kg and 150 mg/kg, respectively) two days prior to harvest. B) Marrow was manipulated into a single-cell suspension and then transduced twice, once overnight and once 20 hours post-harvest in the presence of retronectin, stem cell factor (100 ng/ml), thrombopoietin (100 ng/ml), murine recombinant IL-3 (100 ng/ml), murine recombinant IL-6 (100 ng/ml), and polybrene (8 μg/ml). C) Recipient SCID-A animals (expressing the CD45.2 congenic marker) received preparative conditioning of 500 Rads (X-irradiation source) four hour prior to infusion of transduced marrow via tail vein injection.

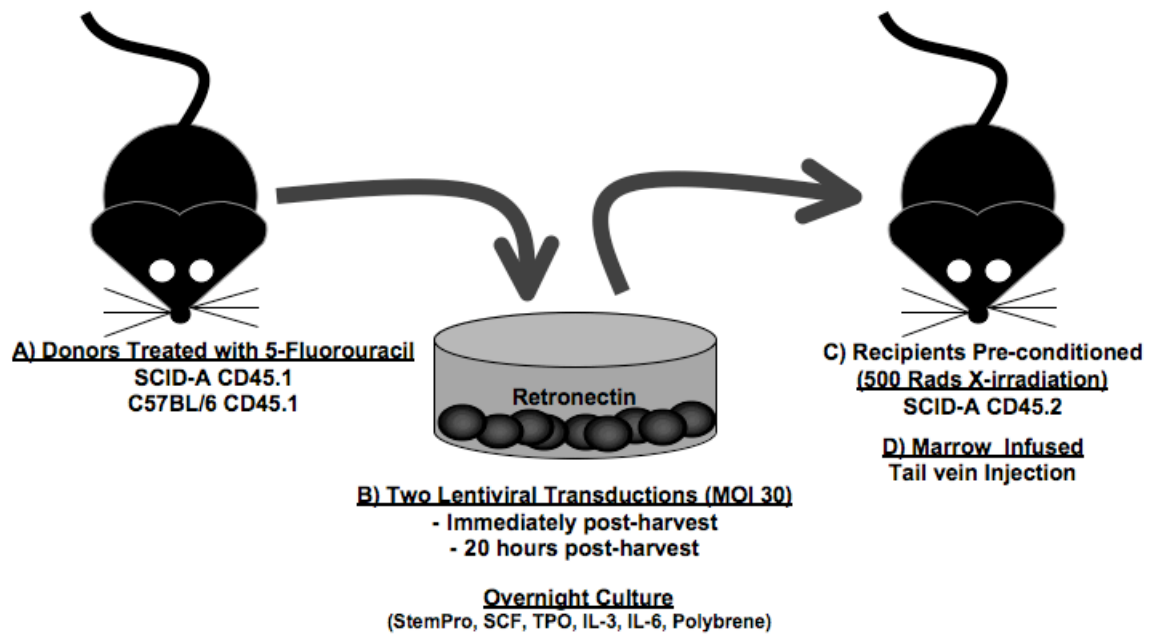
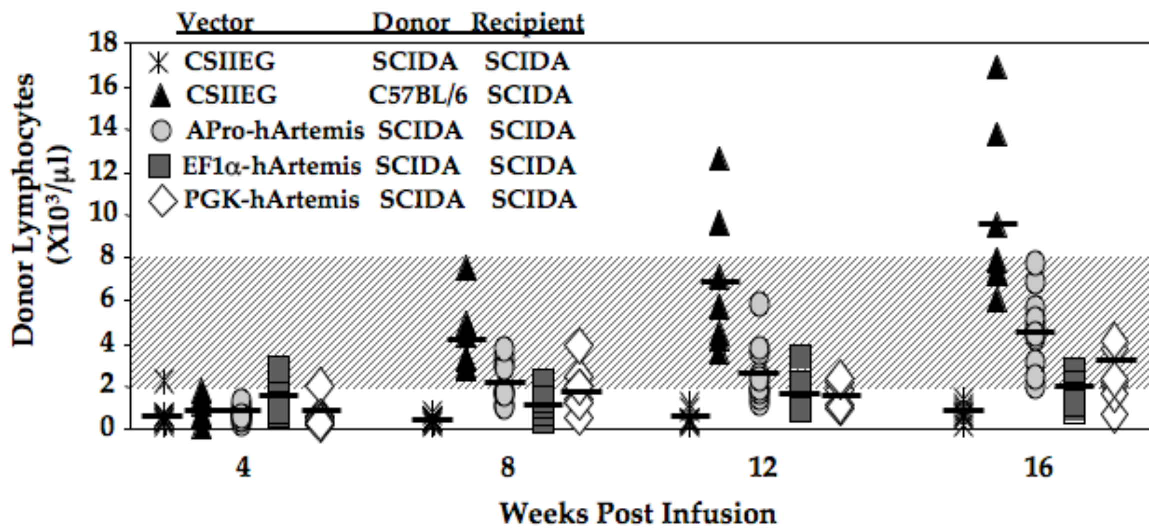

A) Donors Treated with 5-Fluorouracil
SCID-A CD45.1
C57BL/6 CD45.1
Retronectin
B) Two Lentiviral Transductions (MOI 30)
- Immediately post-harvest
- 20 hours post-harvest
Overnight Culture
(StemPro, SCF, TPO, IL-3, IL-6, Polybrene)
C) Recipients Pre-conditioned
(500 Rads X-irradiation)
SCID-A CD45.2
D) Marrow Infused
Tail vein Injection

Figure 4: Time Course of Donor Engraftment and Gene Marking. A) Following infusion of transduced donor marrow, peripheral blood was collected over a period of 16 weeks to monitor donor lymphoid engraftment and repopulation, and plotted as the number of donor lymphocytes (X 10^3) per μl. B) Total lymphocyte number exhibited by animals 16 weeks post transplantation was determined by Hemavet analysis of whole blood and compared to levels observed in wild type C57BL/6 and SCID-A animals. Sixteen weeks post transplantation, donor engraftment was determined by flow cytometric analysis and is displayed as the percentage of total circulating lymphocytes. Quantitative PCR for the lentiviral strong stop sequence was used to determine lentiviral integration in peripheral blood collected 14 weeks post transplantation and is presented as percent strong stop within peripheral blood mononucleocytes.

A) Donor Lymphocyte Engraftment Over-Time



B) Week 16 Post Infusion

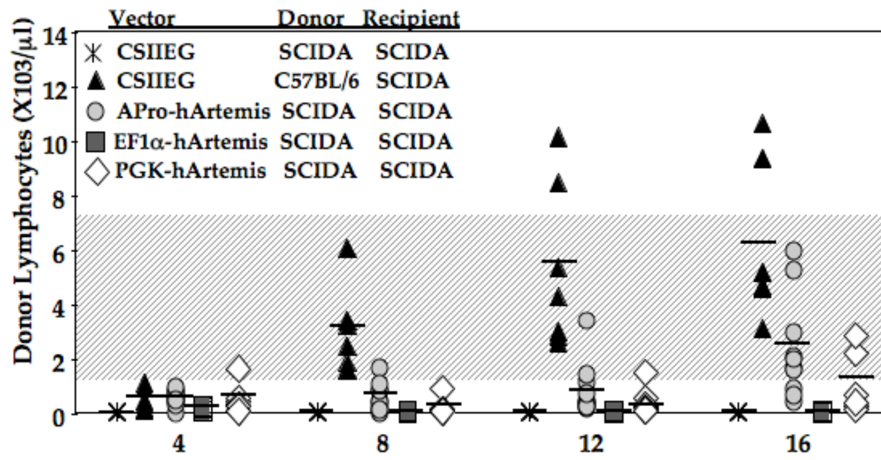
Vector	Donor	Recipient	Total Lymphocytes ($X10^3/\mu l$)	Donor Engraftment (%)	Lentiviral Integration (% Strong Stop)
none	none	C57BL/6	6.5 (+/- 1.2)	-	0.01 (+/- 0.01)
none	none	SCIDA	0.5 (+/- 0.3)	-	0.02 (+/- 0.02)
CSIIEG	C57BL/6	SCIDA	10.1 (+/- 4.1)	96.6 (+/- 2.5)	0.6 (+/- 0.3)
CSIIEG	SCIDA	SCIDA	1.4 (+/- 0.8)	55.7 (+/- 28.7)	3.2 (+/- 2.5)
APro-hArtemis	SCIDA	SCIDA	4.9 (+/- 2.0)	95.1 (+/- 7.7)	6.8 (+/- 4.4)
PGK-hArtemis	SCIDA	SCIDA	2.9 (+/- 1.2)	77.7 (+/- 12.3)	2.0 (+/- 1.7)
EF1α-hArtemis	SCIDA	SCIDA	1.9 (+/- 0.7)	87.4 (+/- 12.9)	2.7 (+/- 2.5)

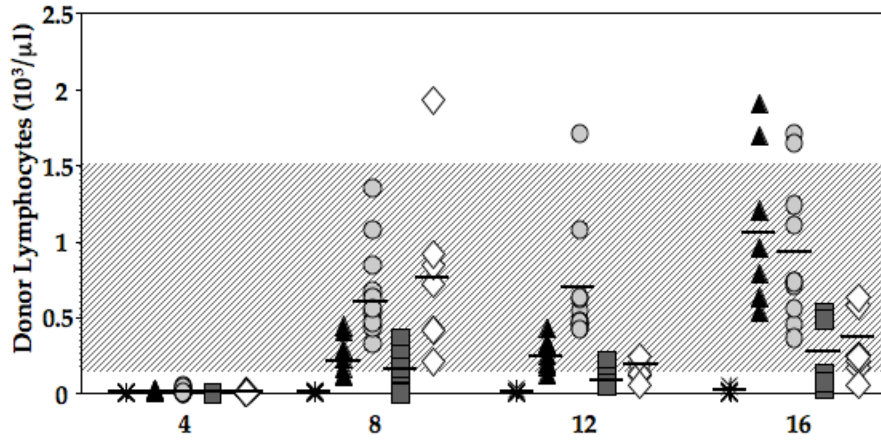
Complete immune reconstitution following ex vivo gene transfer of APro regulated human Artemis. Following bone marrow transplantation, recipient animals were monitored for reconstitution of lymphoid sub-compartments by flow cytometric analysis. The B lymphocyte compartment, assayed by the presence of B220^{+}NK1.1^{-} cells, emerged in all groups receiving Artemis transduced marrow, and persisted to wild type levels in groups receiving both PGK and APro regulated human Artemis (**Figure 5A**). Although animals transplanted with EF1α transduced marrow exhibited a similar B lymphocyte emergence at 4 weeks post transplant, this population did not persist to 8 weeks post transplant and did not reappear throughout the 16-week time-course of the experiment (**Figure 5A**). Interestingly, the majority of EF1α-hArtemis treated animals did show near wild type levels of both CD3^{+}CD4^{+} helper and CD3^{+}CD8^{+} cytotoxic T lymphocytes as did PGK-hArtemis treated animals (**Figure 5B and C**). Moreover, APro-hArtemis treated animals displayed complete reconstitution of both CD3^{+}CD4^{+} and CD3^{+}CD8^{+} T lymphocyte compartments to wild type range (**Figure 5B and C**). Control groups receiving CSIIEG transduced C57BL/6 or SCIDA marrow displayed lymphocyte repopulations as expected in that animals transplanted with CSIIEG transduced C57BL/6 marrow displayed complete B and T lymphocyte repopulation while animals transplanted with CSIIEG transduced SCIDA marrow displayed background levels of lymphocytes (**Figure 5A-C**).

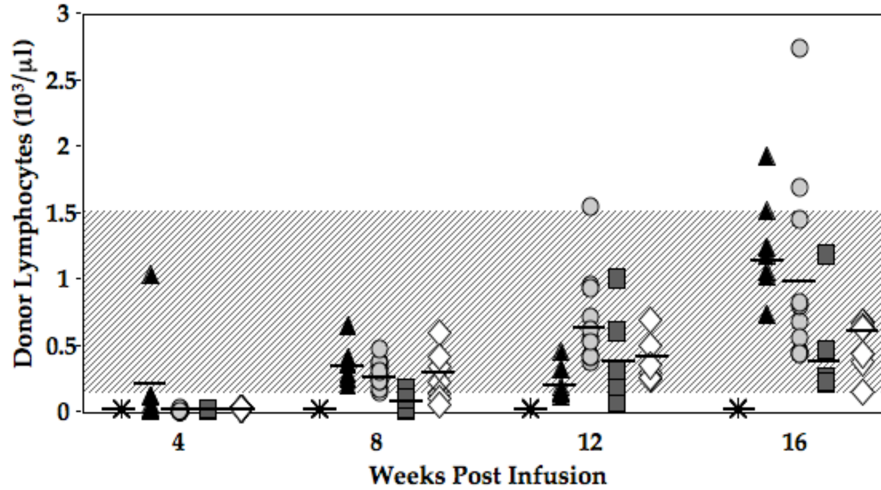
Upon sacrifice, whole blood was drawn from APro-hArtemis treated animals as well as C57BL/6 control animals, sorted for IgM^{+} and CD3^{+} populations and assayed for vector integration by qPCR analysis of the strong

stop sequence. APro-hArtemis treated animals displayed a strong stop copy number per lymphocyte of 2.1 +/- 1.8 (data not shown) indicating that all resulting B and T lymphocytes were generated from successfully transduced pre-lymphoid targets containing at least one lentiviral integrant.

Figure 5: Repopulation of Circulating Lymphocytes Over Time. Peripheral blood was collected over a period of 16 weeks post transplantation for analysis of lymphocyte populations. A) Circulating B lymphocytes ($B220^+NK1.1^-$), B) Helper T lymphocytes ($CD3^+CD4^+$), and C) Cytotoxic T lymphocytes ($CD3^+CD8^+$) are plotted over time as number of lymphocytes ($X\ 10^3$) per µl within the donor lymphocyte population. Percentages were obtained by flow cytometry and cell counts were obtained by Hemavet analysis of whole blood.


A) B Lymphocytes: B220+NK1.1-
Vector
Donor
Recipient
CSIIEG
SCIDA
SCIDA
CSIIEG
C57BL/6
SCIDA
APro-hArtemis
SCIDA
SCIDA
EF1α-hArtemis
SCIDA
SCIDA
PGK-hArtemis
SCIDA
SCIDA
Donor Lymphocytes (X103/µl)
0
2
4
6
8
10
12
14
4
8
12
16


B) Helper T Lymphocytes: CD3+CD4+
Donor Lymphocytes (10³/µl)
0
0.5
1
1.5
2
2.5
4
8
12
16


C) Cytotoxic T Lymphocytes: CD3+CD8+
Donor Lymphocytes (10³/µl)
0
0.5
1
1.5
2
2.5
3
4
8
12
16
Weeks Post Infusion

Innate regulation of Artemis results in a functional lymphoid compartment whereas regulation of human Artemis by EF1α results in an aberrant immune response*.* Our results demonstrating lymphoid reconstitution in PGK-hArtemis treated mice are consistent with what was previously observed by Mostoslavksi *et al* (24). However, it was imperative to determine the functionality of the observed lymphocyte populations in the APro-hArtemis treated mice as well as the incompletely reconstituted EF1α-hArtemis treated animals. Both groups of animals were challenged *in vivo* by injection of 4-hydroxy-3-nitrophenylacetyl hapten conjugated Keyhole Limpet hemocyanin (NP-KLH). Animals were administered NP-KLH and then boosted 5 weeks later. One week after the boost injection, serum was collected and analyzed by enzyme-linked immunosorbent assay (ELISA) for the presence of anti-KLH IgM and IgG as compared to naïve sera collected pre challenge. Both groups of animals mounted a significant IgM response against NP-KLH as compared to wild-type animals (**Figure 6**). Additionally, APro-hArtemis treated animals exhibited effective class-switch and generated an IgG response against the antigen, demonstrating reconstitution of a functionally responsive lymphoid population (**Figure 6**). However, EF1α-hArtemis treated animals were unable to generate anti-NP-KLH IgG, demonstrating a failure to class switch and ultimately an aberrant immune response against antigen challenge (**Figure 6**).

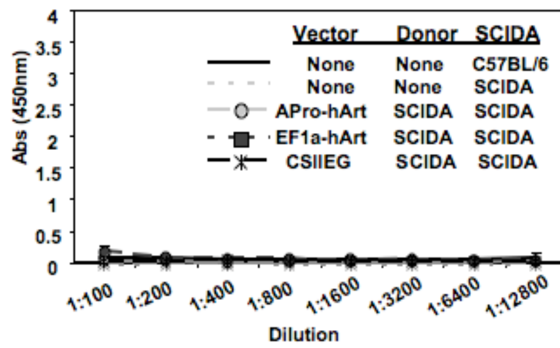
Following the *in vivo* NP-KLH challenge, APro-hArtemis treated animals were sacrificed for further immunophenotyping. All animals demonstrated wild type levels of $B220^{+}IgM^{+}$ B lymphocytes and $CD3^{+}CD4^{+}$, $CD3^{+}CD8^{+}$ T lymphocytes in peripheral primary lymphoid organs: bone marrow, lymph

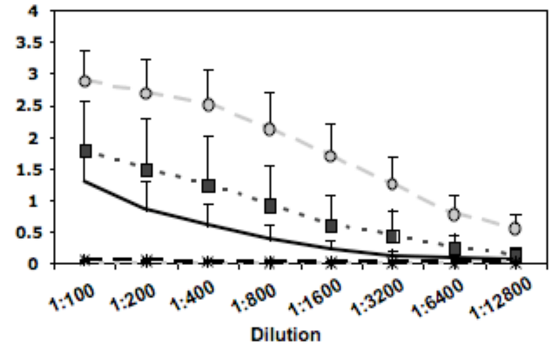
nodes, spleen, and thymus (**Figure 7**). Additionally, immune maturation observed in the bone marrow and thymus of APro-hArtemis treated animals was found to be comparable to wild type. B lymphocytes observed in APro-hArtemis treated bone marrow included cells in the pro-B ($B220^{+}IgM^{-}$), as well as immature B lymphocyte stage ($B220^{+}IgM^{+}$), comparable to wild-type C57BL/6 animals, whereas untreated SCIDA animals displayed a characteristic arrest in B lymphocyte development at the pro-B stage ($B220^{+}IgM^{-}$) (**Figure 7**). APro-hArtemis treated animals also displayed developmental patterning of single positive ($CD4^{+}$ or $CD8^{+}$) as well as double positive ($CD4^{+}CD8^{+}$) T lymphocytes within the thymus at levels comparable to wild type untreated C57BL/6 control animals, while untreated SCIDA animals displayed background levels of these populations (**Figure 7**). As a whole, these data demonstrate that *ex vivo* lentiviral transduction with an endogenously regulated human Artemis vector results in adequate development of B and T lymphocytes within the bone marrow and thymus respectively, but also that these lymphocytes attain the ability to regenerate sub-populations within primary lymphoid organs.

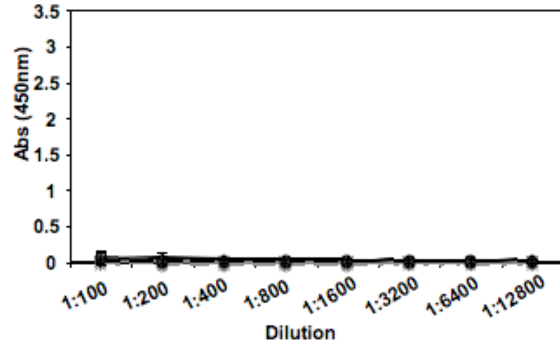
Proliferative responses to mitogen stimulation of splenocytes from APro-hArtemis treated animals were determined. Spleens were harvested from animals upon sacrifice and manipulated into single cell suspensions. Cells were cultured in the presence of either anti-CD3 or concanavalin-A and then evaluated for proliferative response 48 hours post-stimulation by a MTT assay. Splenocytes from APro-hArtemis treated animals exhibited a proliferative index to both stimuli that was similar to that of wild type C57BL/6 wild type animals, while there was essentially no proliferative response in splenocytes from Artemis

deficient control animals (**Figure 8**). These results further emphasize the functional capacity of lymphocytes reconstituted in animals treated by *ex vivo* lentiviral transduction with the APro-hArtemis lentiviral vector.

Figure 6: Functional *in vivo* Immune Response. APro-hArtemis and EF1α-hArtemis treated animals as well as control C57BL/6 and SCID-A untreated animals were challenged with NP-KLH and boosted five weeks post initial challenge. One week after the final boost, sera were collected and analyzed by ELISA for the presence of A) IgM and B) IgG elicited against NP-KLH as compared to naïve sera collected pre challenge. Ig levels are plotted as absorbance (450 nm) versus serum dilution.


A.1) Naïve Sera: IgM Pre-Challenge
Vector Donor SCIDA
None None C57BL/6
None None SCIDA
APro-hArt SCIDA SCIDA
EF1a-hArt SCIDA SCIDA
CSIIEG SCIDA SCIDA
Abs (450nm)
Dilution
1:100 1:200 1:400 1:800 1:1600 1:3200 1:6400 1:12800


A.2) Immunized Sera: IgM Post-Challenge
Dilution
1:100 1:200 1:400 1:800 1:1600 1:3200 1:6400 1:12800


B.1) Naïve Sera: IgG Pre-Challenge
Abs (450nm)
Dilution
1:100 1:200 1:400 1:800 1:1600 1:3200 1:6400 1:12800

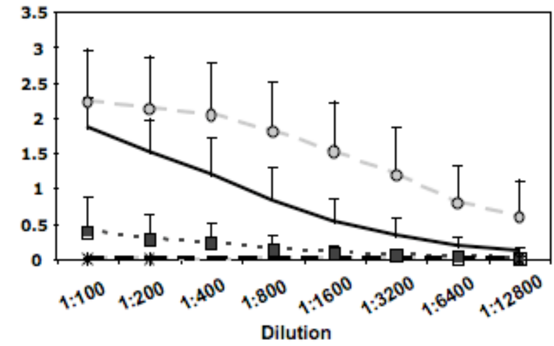
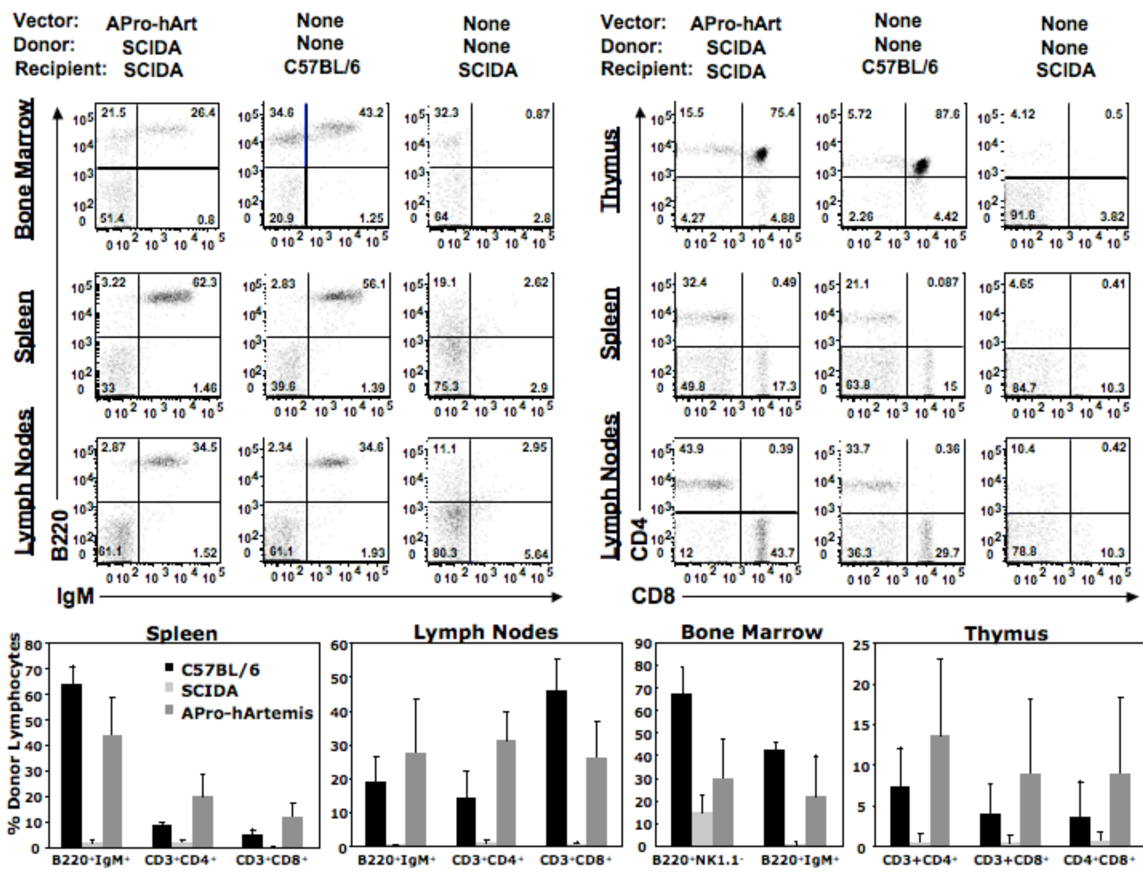
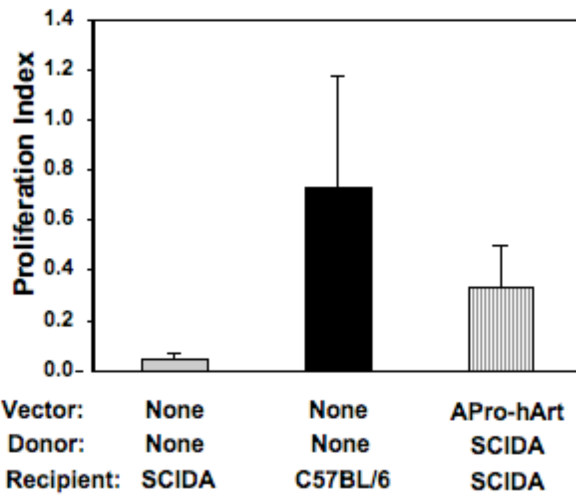

B.2) Immunized Sera: IgG Post-Challenge
Dilution
1:100 1:200 1:400 1:800 1:1600 1:3200 1:6400 1:12800

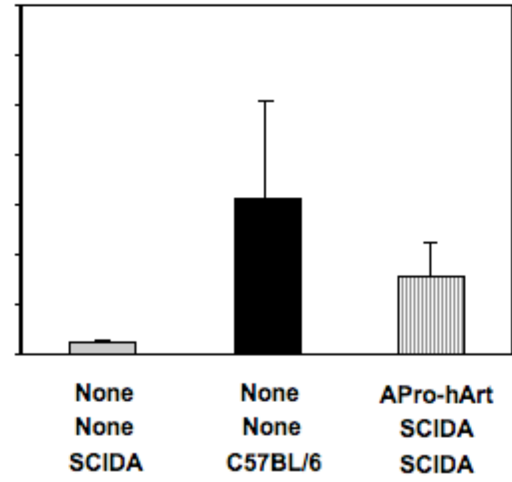
Figure 7: Repopulation Within Lymphoid Organs. Upon sacrifice, primary lymphoid organs were harvested, manipulated into single cell suspension, then analyzed by flow cytometry for the presence of B, Helper T, and Cytotoxic T donor lymphocyte populations of APro-hArtemis treated animals, control C57BL/6, and control untreated SCID-A animals. A) Bone marrow, spleen, and lymph nodes were analyzed for the presence of $B220^{+}IgM^{+}$ B lymphocytes in both APro-hArtemis treated and wild type C57BL/6 animals. B) Cytometric analysis of the $CD3^{+}$ donor lymphocyte compartment within the thymus, spleen, and lymph nodes detected the presence of helper T cells ($CD4^{+}$), cytotoxic T cells ($CD8^{+}$), and double positive T lymphocytes ($CD4^{+}CD8^{+}$) in APro-hArtemis treated and wild type C57BL/6 animals. C) Cumulative results for the presence of B ($B220^{+}IgM^{+}$), helper T ($CD3^{+}CD4^{+}$), and cytotoxic T ($CD3^{+}CD4^{+}$) lymphocytes in spleen and lymph nodes. Lymphocyte development was monitored in bone marrow ($B220^{+}NK1.1^{-}$ pro-B lymphocytes, and $B220^{+}IgM^{+}$ immature B-lymphocytes) and thymus ($CD3^{+}CD4^{+}$ helper T-lymphocytes, $CD3^{+}CD8^{+}$ Cytotoxic T-lymphocytes, $CD4^{+}CD8^{+}$ double positive T lymphocytes). Results are plotted as a percentage of total lymphocytes in control C57BL/6 animals and control untreated SCID-A animals and as a percentage of total donor lymphocytes in APro-hArtemis treated animals.



Vector: APro-hArt
Donor: SCIDA
Recipient: SCIDA
None
None
C57BL/6
None
None
SCIDA
Bone Marrow
Spleen
Lymph Nodes
Thymus
B220
IgM
CD4
CD8
Spleen
Lymph Nodes
Bone Marrow
Thymus
% Donor Lymphocytes
C57BL/6
SCIDA
APro-hArtemis
B220+IgM+
CD3+CD4+
CD3+CD8+
B220+NK1.1-
CD4+CD8+

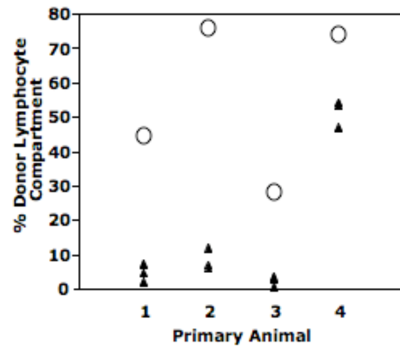
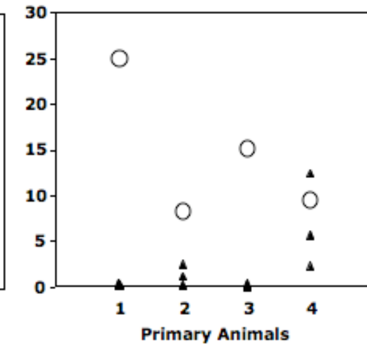
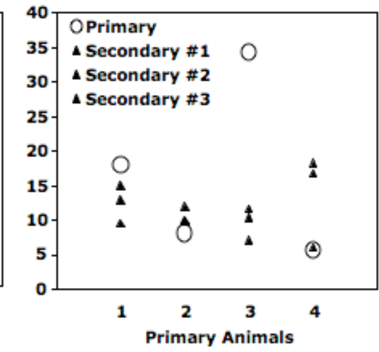
Figure 8: Response to Mitogen Stimulation. Upon sacrifice of APro-hArtemis C57BL/6 control, and untreated SCID-A control animals, spleens were harvested, manipulated into single cell suspensions, and then plated in the presence of increasing amounts of either anti-CD3 or concanavalin-A mitogen. Proliferative indices were calculated by dividing the absorbance acquired in the presence of mitogen by absorbance acquired from samples without mitogen stimulation.


A) Anti-CD3 Proliferative Response
Proliferation Index
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Vector: None None APro-hArt
Donor: None None SCIDA
Recipient: SCIDA C57BL/6 SCIDA


B) Concanavalin-A Proliferative Response
None None APro-hArt
None None SCIDA
SCIDA C57BL/6 SCIDA

APro retains the capacity to regulate gene expression after secondary transplantation. Secondary transplantation was carried out by harvesting marrow from four CD45.1 APro-hArtemis primary recipients six months post-transplant and infusing each marrow sample into three pre-conditioned CD45.2 C57BL/6 recipient animals (800 Rads, x-irradiation). Donor marrow successfully engrafted, allowing for detection of both B and T donor lymphocyte compartments derived from transduced cells in marrow samples harvested from primary recipients. The sustainability of these lymphoid compartments after engraftment in secondary transplant recipients demonstrates the capability of APro-hArtemis to maintain gene expression after transduction into primitive hematopoietic stem cells engrafted in the primary recipients (**Figure 9**). These results verify that the Artemis promoter provides transgene expression in differentiated lymphoid cells after lentiviral transduction into long-term repopulating hematopoeitic stem cells at levels moderate enough to evade cytotoxicity yet adequate to provide long-term correction of the SCIDA B^-T^- phenotype.

Figure 9: Lymphoid Reconstitution After Secondary Transplantation of APro-hArtemis Transduced Marrow. Bone marrow was collected individually from APro-hArtemis treated primary recipients and infused into each of three secondary irradiated (800 Rads, X-irradiation source) C57BL/6 recipient animals. Peripheral blood was collected at monthly time points post transplantation. A) B lymphocyte ($B220^{+}NK1.1^{-}$) as well as B) helper T ($CD3^{+}CD4^{+}$) and C) cytotoxic T ($CD3^{+}CD8^{+}$) lymphocyte repopulations were found to persist in secondary transplant recipients, plotted as a percentage of the donor lymphocyte compartment. Filled triangles represent lymphocyte percentages in secondary transplant recipients 12 weeks post transplant while open circles represent lymphocyte percentages in respective primary donors at week 16 post primary transplantation.


A) B Lymphocytes: B220+NK1.1-
% Donor Lymphocyte Compartment
80
70
60
50
40
30
20
10
0
1
2
3
4
Primary Animal

B) T Lymphocytes: CD3+CD4+
30
25
20
15
10
5
0
1
2
3
4
Primary Animals

C) T Lymphocytes: CD3+CD8+
Primary
Secondary #1
Secondary #2
Secondary #3
40
35
30
25
20
15
10
5
0
1
2
3
4
Primary Animals

DISCUSSION

We have effectively corrected a murine model of SCID-A by lentiviral gene transfer with a naturally regulated human Artemis vector. We demonstrate that innate regulation of human Artemis by its endogenous promoter resulted in expression levels that effectively complemented the Artemis deficient phenotype; ultimately solving the problem of cytotoxicity related Artemis over-expression exemplified by animals receiving EF1α-hArtemis transduced marrow. These animals exhibited repopulation of the T lymphocyte compartment but an absence of circulating B lymphocytes. Additionally, they presented an aberrant response to NP-KLH antigen representative of an incomplete immune compartment. SCID-A animals treated with either PGK-hArtemis or APro-hArtemis lentiviral vectors provided complete reconstitution of both B and T lymphocyte compartments. Moreover, APro-hArtemis treated animals mounted *in vivo* immune responses against NP-KLH antigen and displayed an *in vitro* mitogen stimulation of splenocytes similar to that of wild type animals, thus demonstrating the effectiveness of lentiviral transduction using the natural human Artemis promoter for correction of SCID-A.

Advancements in the field of gene transfer have promoted its emergence as a promising approach for treatment of genetic disorders, exemplified by recent results from clinical trials demonstrating the effectiveness of transplantation using autologous HSC after *ex vivo* genetic correction by retroviral transduction for two severe combined immunodeficiencies caused by genetic disruption of adenosine deaminase and the common γ chain receptor (1, 10). Both of these studies reported long-term engraftment of corrected stem cells

in the majority of patients, ultimately resulting in reconstitution of cellular and humoral immunity. However, two independent studies have reported adverse events following *ex vivo* genetic correction of X-linked SCID by retroviral transfer of the common cytokine-receptor gamma chain (common γ chain) to $CD34^+$ hematopoietic stem cells (10, 12, 16). Although long-term engraftment of corrected stem cells was observed in the majority of patients, which ultimately resulted in the reconstitution of a functional lymphocyte compartment, five out of twenty patients have developed clonal T cell outgrowth resulting in a leukemia-like syndrome (17, 18). Although oncogenic insertional activation was reported in three of the leukemic cases it has also recently been demonstrated that over-expression of the common γ chain induces cellular proliferation and may have ultimately contributed to the T lymphocyte clonal outgrowth (4, 17, 18). Controlling expression of the common γ chain may reduce the risk of oncogenesis resulting from aberrant over-expression.

Achieving transgenic expression of human Artemis for the correction of SCID-A may present similar challenges, considering previous results regarding cytotoxicity associated with Artemis over-expression. Two independent groups recently demonstrated correction of a murine model of Artemis deficiency by lentiviral vector mediated gene transfer (5, 24). In both studies, SCID-A animals were transplanted with HSC transduced with a lentiviral vector containing the human Artemis cDNA regulated by the human PGK promoter, resulting in successful reconstitution of both B and T lymphocyte compartments. However, Mostoslavsky *et al*. reported that transplantation with HSC after transduction with vectors containing CMV or EF1α to regulate human Artemis failed to

repopulate B and T cells in RAG-1 deficient animals (24). We subsequently found that over-expression of human Artemis upon lentiviral transduction results in genomic shearing, cell cycle arrest, and apoptosis (25). Considering the endonucleolytic nature of Artemis (7, 9, 11, 19) these results are not surprising, yet they emphasize the importance of providing Artemis expression at a level that is non-toxic and yet sufficient to correct the $T^{-}B^{-}$ phenotype in preclinical studies and in clinical application to human SCID-A. We then hypothesized that use of the endogenous promoter would provide close to natural levels of human Artemis expression that would avoid toxicity associated with Artemis over-expression.

We subsequently reported the isolation and characterization of the human Artemis promoter (APro) as a 1 kb region directly upstream of the human Artemis translational start site [Chapter 3]. This sequence effectively mediated gene expression at levels substantially lower than that mediated by the strong EF1α promoter. Additionally, mice transplanted with C57BL/6 marrow transduced with an APro regulated GFP vector exhibited GFP expression in both myeloid and lymphoid lineages that was sustained in secondary transplantation recipients. Here we report that transduction using a lentiviral vector in which human Artemis regulated by APro did not adversely affect cell survival in comparison to populations transduced with a control Puromycin vector. Taken as a whole, these data establish the effectiveness of APro to serve as a proficient promoter for gene expression in the hematopoeitic system, specifically for the regulation of human Artemis expression and *ex vivo* lentiviral vector mediated complementation of SCID-A.

Our current study highlights the importance of Artemis transgene regulation in the context of *ex vivo* lentiviral vector mediated complementation of SCID-A. Consistent with previous reports, we demonstrate that Artemis expression regulated by the moderate-strength PGK promoter results in lymphoid reconstitution (5, 24). Furthermore, transplantation of SCID-A marrow transduced with an APro regulated human Artemis lentiviral vector resulted in complete immune reconstitution yielding functional B and T lymphocyte compartments and demonstrating appropriate immune response to both *in vivo* antigen and *in vitro* mitogen stimulation. Additionally, we report that when the strong EF1α promoter mediated Artemis expression, we observed aberrant immune reconstitution with only T lymphocytes detected in the circulating peripheral blood. Furthermore, these animals demonstrated an incomplete immune response to *in vivo* antigen challenge, mounting an IgM response against NP-KLH comparable to wild type mice but unable to generate an efficient IgG response. Collectively, these data suggest that Artemis over-expression may be effecting improper B lymphocyte development. Especially considering that a strong IgM response accompanied by the inability to mount an IgG response to *in vivo* antigen challenge is characteristic of a more transitional thymic-independent B lymphocyte response as opposed to a thymic-dependent B lymphocyte response (20). It will be of interest to compare transitional versus immature B lymphocyte populations reconstituting the bone marrow and spleen of EF1α-hArtemis treated animals as compared to wild type.

Although we observed lymphoid repopulation in SCID-A mice treated with either PGK or APro regulated human Artemis it is important to note that

natural regulation as opposed to a constitutive promoter to provide Artemis expression for gene therapy may be particularly important; especially considering our previously reported evidence for multiple regulatory regions within the natural Artemis promoter. Our 5′ RACE results as well as *in vitro* deletion mapping studies (Chapter 3) suggest the potential for multiple regulatory regions that comprise the Artemis endogenous promoter. Additionally, a differential pattern of transgene expression regulation was observed between HEK 293T cells and the B lymphoid BJAB cell line (Chapter 3), which is not surprising considering the numerous lymphoid specific transcription factor binding sites located in within the upstream region. These findings are consistent with Artemis' involvement in several distinct cellular functions such as non-homologous end joining (19), V(D)J recombination (19), and apoptosis (9); and is suggestive that Artemis expression may be spatially and/or temporally regulated per required function. Thus, natural regulation of the Artemis transgene may be crucial for proper temporal expression.

One particular concern in HSC therapy (allogeneic or genetically modified HSC) for SCID-A is the preparatory conditioning of patients prior to cellular infusion (3, 6, 13). For our studies in SCID-A mice, we found that 500 cGy preconditioning was required to allow engraftment of transduced SCID-A HSC. We also conducted transduction and transplantation studies in which SCID-A recipient animals were preconditioned at lower doses of irradiation (100, 200, or 300 Rads). Engraftment was achieved in all groups but lymphocyte repopulation was unsuccessful (data not shown). It was only when animals were preconditioned with 500 rads that successful immune reconstitution was

accomplished. Similarly, preparative conditioning is necessary for successful repopulation of the B lymphocyte compartment for SCID-A patients undergoing HLA-matched donor marrow transplant (6), (26). However, due to the inherent radiation sensitivity of Artemis deficient patients (22, 26), establishing a tolerable, yet effective, preparative conditioning regime is essential. In addition to total body irradiation, other preparative regimens reported for SCID-A patients undergoing HSC transplantation have included horse antithymocyte globulin (AGT), cyclophosphamide, or busulfan cytoreduction (26). Immunosuppressive preconditioning resulted in T lymphocyte repopulation but failed to reconstitute B lymphocyte populations, similar to what has been observed in patients transplanted without preparative conditioning (26). Although myeloablative conditioning regimens are more likely to result in successful B lymphocyte reconstitution, they are also associated with a higher risk of morbidity and mortality, particularly for radiation-sensitive SCID-A (22, 26). SCID-A patients given myeloablative preconditioning displayed growth defects, a failure to develop secondary teeth, and more severe effects such as restrictive lung disease and alveolar hemorrhages; thus, novel preparative measures are necessary (26). One novel and promising alternative to myeloablation is administration of anti-CD34 globulin. Whereas antithymocyte globulin pre-conditioning targets only developmentally halted pro T lymphocyte progenitor populations in SCID-A recipients, anti-CD34 globulin will target all hematopoietic stem cell and progenitor populations, ultimately creating a microenvironment conducive to HSC engraftment and B lymphocyte reconstitution.

Overall, our results highlight the importance of transgene regulation during *ex vivo* complementation of SCID-A, underscoring the requisite of providing levels of Artemis expression that avoid cytotoxicity yet allow for successful reconstitution of the lymphoid lineage. We provide an example of how Artemis over-expression results in aberrant lymphoid reconstitution and incomplete immune response, and corroborate previously reported results demonstrating complete correction using a moderately regulated Artemis vector (5, 24). Moreover, we report the complete reconstitution of a functional lymphoid population in SCID-A animals by *ex vivo* lentiviral gene transfer using a vector in which human Artemis expression was regulated by its natural promoter. These results suggest the provision of natural levels of Artemis expression via *ex vivo* lentiviral transduction into hematopoietic stem cells will serve as a clinically relevant and feasible treatment of human SCID-A.

MATERIALS AND METHODS

Lentiviral Plasmid Construction

pOK/APro-hArtemis. A lentiviral vector containing the human Artemis gene regulated by the full length APro sequence was constructed by standard molecular technique based on the pOK lentiviral vector (25). The EF1α promoter was excised from pOK/EF1αhArtemis by *AgeI* restriction digest yielding pOK/hArtemis. The APro fragment was excised from pCR2.1/APro, previously described [Chapter 2], by restriction digest with *AgeI* and ligated into the *AgeI* site of pOK/hArtemis directly upstream of the human Artemis gene to generate pOK/AProhArtemis.

Mammalian Cell Culture

HEK 293T, and murine NIH 3T3 tk^- cells were cultured and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and 1% antibiotic antimycotic at 37° C and 5% CO_2.

Preparation and Titering of Lentiviral Vectors

VSV-G pseudotyped lentiviral vectors were generated as described (15, 30). Briefly, 24 hours pre-transfection, 1.4 X 10^7 HEK 293T cells were seeded into poly-L-lysine coated 15-cm^2 plates and cultured in DMEM supplemented with 1% penicillin streptomycin and 8% FBS and incubated at 37°C with 5% CO_2. Lentiviral vector plasmid constructs were co-transfected with pΔNRF, encoding lentiviral structural and enzymatic proteins, and pMD.G, encoding vesicular stomatitis virus G (VSV-G) envelope protein, and placed at 37°C with 10% CO_2.

Twelve hours post-transfection, the medium was replaced using DMEM supplemented with 4% FBS and cells were incubated at 37°C with 5% CO_2. Viral supernatants were collected 24, 36, and 48 hours post-transfection, pooled, and then concentrated 100-fold by centrifugation at 23,000 x g in a Sorval RC5B centrifuge. Lentiviral vectors were resuspended in Iscove's modified Dulbecco's medium (IMDMEM), aliquoted, and stored at -80°C for future transduction.

For quantitation of vector titers, NIH 3T3 tk^- cells were transduced with increasing volumes of vector in the presence of 8 μg/mL polybrene. Forty-eight hours post-transfection, DNA was extracted from the transduced cells and subjected to quantitative PCR as described, utilizing a probe specific for the integrated lentiviral strong stop sequence or a probe for the GFP sequence as a measure of lentiviral vector titer (15). Additionally, cells transduced with GFP encoding lentiviral vectors were harvested for flow cytometric analysis of GFP expression to determine the percentage of cells transduced and determine functional vector titer.

<u>Lentiviral Transduction and Transplantation of Murine Whole Bone Marrow</u>

All procedures were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee.

A murine model of Artemis deficiency backcrossed onto C57BL/6 background and exhibiting no leakiness was obtained from the laboratory of Dr. Morton Cowan (29) and further bred onto both CD45.1 and CD45.2 congenic markers. CD45.2 and CD45.1 C57BL/6 mice were obtained from the National

Cancer Institute (Frederick, MD). Animals were provided food and water *ad libitum*.

Bone marrow was flushed from the long bones of the hind limbs of donor mice into DMEM supplemented with 10 U/ml heparin, 10% FBS, and 1% PenStrep antibiotic/antimycotic. Red blood cells were lysed using ammonium chloride hemolysis buffer [0.8% NH_4Cl with 0.1 mM EDTA] (StemCell Technologies, Vancouver, BC, Canada), washed with 1 X PBS, and then rendered into a single cell suspension in transduction medium [complete StemPro-34 SFM media with supplement (Invitrogen, Carlsbad, CA), 2mM L-glutamine (Invitrogen, Carlsbad, CA), 1% PenStrep (Invitrogen, Carlsbad, CA), 100 ng/ml murine IL-3, 100 ng/mL murine IL-6, 100 ng/mL murine TPO, 100 ng/mL murine SCF (cytokines all from R&D Systems, Minneapolis, MN) and 8 ug/ml polybrene (Sigma-Aldrich, St. Louis, MO)]. The marrow was subjected to lentiviral transduction twice- once immediately following marrow harvest and a second time 20 hours after the initial exposure. Transduced cells were harvested, washed, counted, and prepared as a single cell suspension in IMDMEM. Transduced bone marrow cells were injected via lateral tail vein into sublethally irradiated (500 Rads, X-irradiation source) congenic recipients.

For secondary transplantation, marrow samples were collected individually from primary recipients as described above and infused into each of three secondary irradiated C57BL/6 (800 Rads, X-irradiation source) recipients.

FACS Analysis

Blood was collected via cheek puncture and assayed for repopulation of the lymphoid compartment by flow cytometry. Whole blood was treated with ammonium chloride hemolysis buffer [0.8% NH_4Cl with 0.1 mM EDTA] (StemCell Technologies, Vancouver, BC, Canada), washed, and then pelleted leukocytes were resuspended in staining buffer [1 X PBS plus 1% FBS and 0.002% sodium azide and fluorochrome-conjugated monoclonal antibodies for identification of hematopoeitic compartment] (all antibodies utilized in flow cytometric analysis were purchased from eBiosciences, San Diego, CA). Immunophenotype analysis, as well as GFP expression for CSIIEG transduced control animals, was conducted using monoclonal mouse antibodies against CD45.1, CD45.2, B220 (B lymphocytes), CD3e (T lymphocytes), CD4 (helper T lymphocytes), CD8 (cytotoxic T lymphocytes), NK1.1 (Natural Killer cells), Gr-1 and Cd11b (myeloid lineages) and assayed on an LSRII instrument. Data were collected using CellQuest Pro (BD Biosciences) and analyzed using FlowJo (Tree Star, Inc., Achland, OR) software.

in vivo KLH Challenge and ELISA

Naïve sera were collected from treated animals as well as control SCID-A and C57BL/6 mice prior to immunization: blood was drawn via submandibular vein puncture and serum was collected by centrifugation, aliquoted, and stored at -20°C until needed for ELISA. Mice were immunized by intraperitoneal injection of 100 μg NP-KLH (4-hydroxy-3-Nitrophenylacetyl hapten conjugated Keyhole Limpet Hemocyanin) (Biosearch Technologies, Novato, CA). Five weeks

subsequent to initial immunization, mice were boosted with an additional intraperitoneal injection of 100 μg of NP-KLH. One week following the boost immunization, post-immune sera was collected via submandibular vein puncture, aliquoted, and stored at -20°C until analyzed by an enzyme-linked immunosorbent assay (ELISA).

The presence of IgM and IgG was detected by ELISA in both naïve and post-challenge sera from treated animals as well as SCID-A and C57BL/6 control animals. Briefly, Microtiter plates (Nunc, Rochester, NY) were coated with 0.5 μg NP-BSA (4-hydroxy-3-nitrophenylacetyl hapten conjugated bovine serum albumin) (Biosearch Technologies, Novato, CA) in coating buffer [0.05M carbonate-bicarbonate, pH 9.6] and incubated overnight at 4°C. Coating solution was aspirated from the plate and each well washed three times with wash solution [50mM Tris Buffered Saline, pH 8.0, 0.05% Tween 20]. Plates were subsequently incubated with blocking solution [50mM TBS, pH 8.0, 1% BSA] for 30minutes at room temperature and then washed three times. Serum samples were diluted 1:100 in sample diluent buffer [50mM TBS, pH 8.0, 1% BSA, 0.05% Tween 20], then plated at serial dilutions to 1:12,800 and allowed to incubate for one hour at room temperature. Following serum incubation, the wells were washed five times and then supplemented with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted sample diluent buffer (1:100,000 dilution of anti-IgM-HRP and 1:150,000 dilution anti-IgG-HRP; both antibodies from Bethyl Laboratories, Montgomery, TX). After one hour, the plates were washed five times and then supplemented with HRP enzymatic reaction components using the TMB 20Component Microwell Peroxidase Substrate Kit

(Kirkegaard & Perry, Gaithersburg, MD). Reaction products quantified using a Biotech SpectraMax plate reader at 450 nm.

Splenocyte Stimulation

Spleens were harvested from mice upon sacrifice and brought to a single cell suspension in RPMI + 10% FBS. The spleen cell suspension was treated with ammonium chloride hemolysis buffer [0.8% NH_4Cl with 0.1 mM EDTA] (StemCell Technologies, Vancouver, BC, Canada) washed twice in RPMI + 10% FBS and resuspended in complete RPMI Medium (RPMI media, 1% PenStrep and 10% FBS). Microtiter plates were pretreated with anti-CD3 prior to splenocyte harvest: antibody was diluted in 1XPBS to concentrations of 0, 2, 5, and 10 μg/mL, added to wells in triplicate, and then incubated at 37°C for four hours. Wells were washed twice with 1 X PBS and 4 x 10^5 splenocytes were added to each well and allowed to incubate at 37°C + 5% CO_2 for 48 hours. For Concanavalin A stimulation, splenocytes were plated at 5 x 10^5 cells per well. The plate was supplemented with Concanavalin-A (Amersham Biosciences, Piscataway, NJ) diluted in complete RPMI medium to yield final concentrations of 0, 2.5, 5, and 10 μg/mL, and then incubated at 37°C + 5% CO_2 for 48 hours. Following incubation, proliferation was assayed utilizing the CellTiter 96 Non-Radioactive Cell Proliferation Assay (MTT) kit (Promega, Madison, WI) and quantified using a Biotech SpectraMax plate reader at 570 nm. Results were reported as a proliferation index, calculated by dividing the absorbance observed in the presence of mitogen by absorbance observed for samples incubated without mitogen.

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CHAPTER 5

CONCLUSIONS

This work has focused on the pre-clinical development of a gene transfer strategy for the correction of Artemis deficient severe combined immunodeficiency (SCID). Allogeneic hematopoietic stem cell transplantation current serves as the standard of care, but is associated with several inherent risks in the absence of an HLA-matched donor. These risks include increased rates of morbidity and mortality of up to 50%, increased susceptibility for graft versus host disease, a delay in T lymphocyte repopulation, and a failure to reconstitute the B lymphocyte compartment (5, 13, 14). Accordingly, these inherent caveats necessitate an alternative therapeutic approach for the treatment of Artemis deficient SCID in the absence of an HLA-matched donor.

Recent results from clinical trials have established the effectiveness of autologous HSC transplantation following *ex vivo* genetic correction by retroviral transduction for both adenosine deaminase (ADA) deficient SCID and X-linked SCID caused by a disruption in the common γ chain coding sequence (1, 3, 6). In both of these trials, the patients' HSC were harvested, transduced *ex vivo* with retroviral vectors encoding the corrective gene product, and then intravenously infused back into the patient. Both studies reported successful long-term engraftment of gene marked, corrected stem cells ultimately resulting in reconstitution of cellular and humoral immunity (1, 3, 6). The success demonstrated in these trials revealed that *ex vivo* gene transfer is an effective treatment for genetic deficiency, and thus gene transfer emerges as a compelling therapy for the correction of other forms of SCID.

However, recent clinical observations following *ex vivo* retroviral gene transfer of the common γ chain have also drawn attention to the importance of

transgene regulation. Following *ex vivo* retroviral gene therapy for common γ chain deficiency, five out of 20 treated children developed a leukemia-like clonal T cell proliferative disorder (7, 8). Initially, this disease appeared to be the result of vector-mediated cis-activation of host oncogenes implicated in leukemogenesis (7). However, recent findings have demonstrated that over-expression of the common γ chain induces aberrant cellular effects, including clonal expansion and cellular proliferation, and thus appears to have contributed to the T lymphocyte clonal outgrowth (7, 8). Additionally, **Chapter 2** reports an unanticipated observation that emerged upon characterization of Artemis encoding lentiviral vectors; transduction by a vector in which Artemis is regulated by the strong elongation factor-1 (EF1α) promoter resulted in a dose-dependent decrease in cell survival not observed in cell cultures exposed to equal amounts of control vector (11). Mechanistic studies revealed that Artemis over-expression mediated cytotoxicity associated with an increase in genomic DNA damage and a halt in cell cycle progression at G1 ultimately resulting in a relative increase in the proportion of apoptotic cells as compared to cultures treated with control vector.

Because of the nucleolytic, enzymatic role Artemis plays in NHEJ, it is perhaps foreseeable that increased expression levels may produce nonspecific breaks, ultimately resulting in genomic damage sufficient to cause cell cycle arrest and cell death. Cell cycle checkpoints initiated by DNA damage activate inherent cellular DNA damage repair pathways and if the damage is not sufficiently repaired, arrest in cell cycle progression ultimately results in apoptosis to avoid replication of severely damaged DNA (10, 9). Together, these

observations present a novel challenge for effective complementation of genetic deficiencies and bring to light the importance of applying more stringent control of transgene regulation in future studies involving gene transfer. The goal of gene transfer light the importance of regulating human Artemis transgene expression and present a novel challenge for effective correction of SCID-A by gene transfer; to achieve an adequate level of Artemis expression that is non-toxic and yet sufficient to correct the T^-B^- phenotype

Considering the challenges presented by Artemis over-expression, we hypothesized that using natural elements of the human Artemis gene, i.e. its own promoter, would provide effective levels of transgene expression for correction of SCID-A. **Chapter 3** illustrates the isolation and characterization of endogenous human Artemis promoter (APro). This sequence exhibited several characteristics similar to that observed for promoters of other NHEJ proteins: it contains neither a TATA box nor a CCAAT box and neither transcription nor translation are induced upon DNA damage. Additionally, 5'RACE results as well as *in vitro* deletion mapping presented in this study suggest the potential for multiple regulatory regions. Importantly, APro was shown to support reporter gene expression *in vitro* and *in vivo*. Moreover, *ex vivo* transduction of murine bone marrow with an APro-regulated GFP lentiviral vector resulted in GFP expression at a significantly reduced level in comparison to mice transplanted with EF1α-GFP transduced marrow, a result that we expected of the endogenous promoter, since Artemis over-expression results in toxicity. Notably, APro supported GFP expression in all peripheral hematopoeitic populations, demonstrating the

proficiency of the endogenous human Artemis promoter to regulate gene expression in hematopoeitic lineages.

The results presented in **CHAPTER 3** as a whole, establish the usefulness of the natural human Artemis promoter for the purpose of generating a clinical vector that provides Artemis expression at a non-toxic level that is nonetheless sufficient to correct the B^-T^- SCID-A phenotype. Subsequently, this promoter was employed to provide innate regulation of the human Artemis cDNA in the context of a lentiviral vector for correction of a murine model of SCID-A (mArt $^-/^-$) and results are presented in **CHAPTER 4**. A lentiviral vector containing these sequences (pOK/APro-hArtemis) was assembled and utilized to transduce mArt $^-/^-$ donor marrow with subsequent transplantation into mArt $^-/^-$ recipient mice. Following transplant, recipient animals were found to have normal levels of functional T and B lymphocytes, therefore demonstrating immune reconstitution resulting from APro-hArtemis transduction of HSCs.

The results reported in **Chapter 4** present a solution to the problem of cytotoxicity associated with Artemis over-expression, exemplified by mArt $^-/^-$ animals treated with a lentiviral vector encoding human Artemis regulated by the strong EF1α promoter. Animals receiving EF1α–hArtemis transduced bone marrow exhibited repopulation of the T lymphocyte compartment but an absence of circulating B lymphocytes. Moreover, the incomplete lymphoid repertoire demonstrated aberrant immune function representative of incomplete B lymphocyte development. Considering the Artemis over-expression associated toxicity and cell cycle arrest data presented in **Chapter 3**, it may be speculated that Artemis over-expression by EF1α results in a cytotoxic effect interfering with

proper B lymphocyte development, ultimately resulting in the arrest of B lymphocyte differentiation in a primitive transitional state. The presence of T lymphocytes was unexpected and calls for further study addressing the functionality of this compartment. Mitogen stimulation of splenocytes harvested from mArt $^{-}/^{-}$ animals treated by EF1α-hArtemis will be performed to determine T lymphocyte activation and proliferation potential.

Overall, the results presented in **Chapter 4** represent encouraging pre-clinical data demonstrating effective complementation of a murine model of SCID-A following transduction with a lentiviral vector providing natural regulation of human Artemis under its endogenous promoter. Not only does APro regulation of the Artemis transgene obviate toxicity, natural regulation as opposed to regulation by a constitutive promoter may be particularly important for providing Artemis expression for gene therapy as supported by the observations presented in **Chapter 3**. Our 5′ RACE results provided evidence for multiple regulatory regions that comprise the Artemis endogenous promoter. Additionally, *in vitro* deletion mapping studies revealed a divergent pattern of transgene expression between HEK 293T cells and the B lymphoid BJAB cell line. These findings are consistent with Artemis' involvement in several distinct cellular functions such as non-homologous end joining (19), V(D)J recombination (19), and apoptosis (9); and is suggestive that Artemis expression may be spatially and/or temporally regulated per required function. Thus, natural regulation of the Artemis transgene may be crucial for proper temporal expression.

Our work thus far presents encouraging pre-clinical data for the correction of human SCID-A; however, there are many concerns that must be addressed when moving forward therapeutically such as vector design, vector safety analysis, dose escalation, and clinical protocol design. The APro regulated human Artemis vector reported in **Chapter 4** was assembled with intention for use in a clinical therapy setting. The construct was engineered on a minimal plasmid backbone consisting of only the ColE1 origin of replication and the kanamycin resistance gene (4, 11). We used the kanamycin resistance gene because materials generated from cultures in which a β-lactamase gene is used as a selection marker are discouraged in clinical studies (12). The lentiviral construct includes self-inactivating LTR technology along with the cytomegalovirus (CMV) promoter to regulate transcription of the full-length lentiviral vector genome. Self-inactivation disrupts the natural lentiviral LTR, as discussed in **Chapter 1**, thus theoretically preventing generation of replication competent virus. Nonetheless, testing for replication competent lentivirus (RCL) is an imperative pre-clinical step to rule out the possibility of contamination at any point during the production procedure or following patient exposure (12).

Selection of clinical dose to be employed is also of concern, especially considering the cytotoxicity associated with Artemis over-expression. **Chapter 4** presents relevant pre-clinical transgene activity data demonstrating outcomes following transduction with a lentiviral vector regulated by a strong promoter (EF1α), a more moderate promoter (PGK), and its own endogenous promoter (APro). We hypothesize that the APro regulated human Artemis vector (APro-hArtemis) provides natural levels of human Artemis expression following

lentiviral transduction into mArt $^{-}/^{-}$ bone marrow. It will be of interest to quantitate the levels of human Artemis expression in circulating B and T lymphocytes isolated from APro-hArtemis treated animals as compared to endogenous levels of human Artemis present in lymphocytes extracted from wild-type mice and from mice treated with EF1α-hArtemis lentiviral vectors.

An additional clinical concern associated with HSCT therapy for SCID-A is the preparatory conditioning of patients preceding cellular infusion. Our studies in Artemis deficient mice indicate that 500 cGy preconditioning was both adequate and necessary to allow engraftment of transduced HSCs. Considering the radiation-sensitive nature of these animals, we conducted transduction and transplantation studies in which SCID-A recipient animals were preconditioned at lower doses of irradiation (100, 200, or 300 Rads). Although engraftment was achieved in all groups, lymphocyte repopulation was unsuccessful. Similarly, preparative conditioning is necessary for successful B lymphocyte reconstitution in SCID-A patients undergoing HSCT (2, 13). However, due to the inherent radiation sensitivity of Artemis deficient patients, establishing a tolerable, yet effective, preparative conditioning regimen is essential and thus necessitates future study. Several preparative procedures have been reported for SCID-A patients undergoing allogeneic HSC transplantation, including total body irradiation, horse antithymocyte globulin, cyclophosphamide, and busulfan cytoreduction. Immunosuppressive preconditioning using horse antithymocyte globulin resulted in T lymphocyte repopulation but failed to reconstitute B lymphocyte populations, similar to what has been observed in patients transplanted without prior preparative conditioning (13). Although

myeloablative conditioning regimens, such as total body irradiation and chemotherapeutic therapies, are more likely to result in successful B lymphocyte reconstitution, they are also associated with a higher risk of morbidity and mortality, particularly for radiation-sensitive SCID-A (13). One novel and promising alternative to myeloablation is the administration of anti-CD34 globulin prior to HSCT infusion. Whereas antithymocyte globulin pre-conditioning targets only developmentally halted pro T lymphocyte progenitor populations residing within the bone marrow of in SCID-A recipients, anti-CD34 globulin will target all hematopoietic stem cell and progenitor populations, ultimately creating a microenvironment conducive to HSC engraftment and B lymphocyte reconstitution. Future studies exploring alternative preparative conditioning regimens would ultimately advance the field of allogeneic and genetically modified autologous HSCT for the treatment of Artemis deficient SCID.

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