p=0.034. The T-cell lymphoma manifested itself as a gross thymic mass, which showed neoplastic cells with a CD3/B220 double positive phenotype, as well as larger CD4-/CD8+ blast-like cells, which were also predominant in the spleen. Vector integration site analysis showed lymphomic cells to be of clonal origin with similar integration site profiles being present in the thymuses, bone marrow cells, and spleens of individual mice. Dual transgene expression of IL2RG and LMO2 did not significantly accelerate lymphomagenesis. Our results suggest a re-evaluation of the current view that IL2RG has minimal oncogenic properties and offer caution that current gene therapy trials for XSCID may pose increased risks to patients than previously anticipated. Furthermore, these results may explain why leukemia has been seen with such prevalence in XSCID trials when gene therapy trials for other diseases have not shown any such side effects.

64. Rescue of B Cell Development in an Animal Model of X-Linked Agammaglobulinemia (XLA) Via B Lineage-Specific Lentiviral Gene Therapy

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X-linked agammaglobulinemia (XLA) is a human immunodeficiency caused by mutations in Bruton's tyrosine kinase (Btk); and characterized by a block in pre B-cell development leading to absence of serum immunoglobulin and recurrent bacterial infections. Using Btk and Tec double deficient (Btk/Tec-/-) mice as a model for XLA, we previously showed that onco-retroviral-mediated Btk gene transfer into hematopoietic stem cells (HSC) reconstituted in vivo Btk-dependent B-cell development and function (Yu et al. Blood 104(5): 1281-90).

In an effort to increase the safety of this approach, we developed a lentiviral system that incorporates a B lineage specific enhancer/promoter element, EμB29. Using SIN-lentiviral vectors expressing GFP, we observed that EμB29 consistently promoted 3-5 fold higher GFP expression in human B lineage cells derived from transduced HSC in vivo and in vitro (ASGT 2002 abstract #1302). We also evaluated this vector, CSOM- EμB29-GFP-WPRE, in lentiviral transgenic mice where it exhibited the highest GFP expression in peripheral B cells compared with all other hematopoietic lineages. Specifically, in more than 8 independent founder strains the MFI for GFP expression in B cells was 3 fold higher than that in T cells (p=0.0002).

Based upon these expression studies we generated EμB29-huBtk SIN-lentiviral vectors with or without the insulator element derived from the chicken β-globulin insulator (cHS4). Using both vectors to transduce Btk-/- DT40 B cells, followed by cloning by limiting dilution, we demonstrate Btk protein expression by intracellular staining and western blotting. All lines (including those clones containing single viral integrations) exhibited full rescue of Btk-dependent, B cell receptor (BCR)-mediated Ca^{2+} signaling. We next tested the capacity of these vectors to reconstitute Btk-dependent B-cell development and function in a cohort of Btk/Tec-/- mice. Marrow from 5-FU treated Btk/Tec -/- mice was harvested, cultured on fibronectin coated plates with growth factors (mIL-3,mIL-6, mSCF, mTPO and mFLT3ligand) and concentrated lentivirus ($2.3x10^7$pg/10^6 cells measured by p24 level). After 48h of in vitro culture, cells were transplanted into lethally or sublethally irradiated animals. Transplanted animals were serially evaluated for presence of B cells in the peripheral blood. B-cell numbers progressively increased with a significant difference as early as within 6 weeks in mice receiving transduced (16-18% $B220^+$ cells) vs. control marrow (8-9%; mock transduced). Further, mature B cells ($B220^+IgM^{low}IgD^{hi}$) represented 14-20% of total B cells in treated compared to <5% in control mice. Finally, mice receiving transduced cells exhibited a rescue of total serum IgM and IgG3 levels and responses to TI-II dependent immunization. In conclusion, our data demonstrate that EμB29-Btk SIN-lentiviral vector specifically promotes Btk expression in B lineage cells, and correction of the Btk-deficient phenotype both in vivo and in vitro.

65. Long-Term Effects of Hematopoietic Stem Cell Gene Therapy in the Murine Model of Wiskott-Aldrich Syndrome: Persistence of Functional Correction of T Cells and Lack of Malignant Transformation

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Wiskott-Aldrich syndrome (WAS) is a severe X-linked immunodeficiency characterized by recurrent infections, thrombocytopenia, eczema and increased risk of autoimmune disorders and lymphomas. Hematopoietic stem cell (HSC) transplantation from HLA-identical sibling donors is a resolutive treatment, but it is available only for a minority of patients. Transplantation of genetically corrected autologous HSC could represent an alternative treatment, potentially applicable to all patients. In a murine model of WAS (*WAS*$^{-/-}$), we recently demonstrated correction of the T cell defect 4 months after lentiviral vector-mediated gene therapy [Dupré, Marangoni, et al. Hum Gene Ther. 2006, 17]. The aim of the present study was to investigate the long-term efficacy and safety of our gene therapy approach in *WAS*$^{-/-}$ mice.

Transduction of *WAS*$^{-/-}$ HSC was performed with two lentiviral vectors encoding human WASP under the control of either the human PGK promoter (pW) or the full-length (1.6Kb) human WAS autologous promoter (wW). Transduced HSC were transplanted into non-lethally irradiated *WAS*$^{-/-}$ mice. Mice were sacrificed either 7 months or 16 months after gene therapy. WASP expression was detected in approximately 50% and 40% of splenic T cells from mice treated with pW and wW, respectively. These expression levels were sufficient to fully correct TCR-driven proliferation and IL-2 production. Interestingly, the percentage of WASP-expressing cells was higher in FSC^{hi} and in $CD44^+$ T cells, as compared to other T cell subsets. This finding suggests a selective advantage for gene corrected cells within activated and memory T cells. Additionally, WASP expression was detected in T cells, B cells and granulocytes isolated from peripheral blood, as well as in bone marrow $CD45^+$ cells.

The safety of the gene therapy treatment was evaluated by hemogram and histopathologic analysis of thymus, spleen, lymph nodes and bone marrow from gene therapy treated mice. In parallel, untransplanted age-matched *WAS*$^{-/-}$ and wild-type mice were tested as controls. Normal organ architecture and histology together with the absence of leukemias or lymphomas could be demonstrated in the gene therapy treated mice.

In conclusion, we provide evidence of engraftment of WASP-expressing cells and correction of T cell defects without toxicity, up to 16 months after HSC gene therapy. Experiments aimed at investigating whether *WAS* gene therapy can correct the defects of platelets, B cells and dendritic cells, and restore normal *in vivo* immune response to pathogens are ongoing. Results from these studies will contribute to the design of a clinical trial for Wiskott-Aldrich Syndrome.

66. Gene Transfer-Mediated Expression of Murine Factor VIIa Improves Clot Formation in a Mouse Model for Bernard-Soulier Syndrome

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Moderate to severe bleeding occurs in Bernard-Soulier syndrome (BSS) patients due to a rare inherited dysfunction of their platelet GPIbα/β-IX-V receptor, a four-gene encoded protein complex. Defective receptor disrupts the interaction between platelets and vascular wall and form abnormal macrothrombocytopenia, in addition to causing impaired thrombin generation and defective platelet plug at the tissue injury site. While platelet transfusion is the conventional therapy, treatment with a bolus injection of recombinant human factor VIIa (rhVIIa, NovoSeven®) offers an alternative strategy especially when alloimmunization develops against administered platelets. At pharmacological plasma levels, rhVIIa led to cessation of mild and severe bleeding episodes in a number of BSS patients (Ozelo et al., Ann Hematol, 84(12), 2005 and Almeida et al, Br J Haematol, 121(3) 2003). However, the short half-life of the protein and its high expense remain as two major disadvantages of a rhVIIa regimen. We here report a gene therapy-based approach resulting in continuous expression of activated murine factor VII (mVIIa) in the plasma of a mouse model for BSS. Male BSS mice were tail-injected with three doses of AAV8-mVIIa vector ranging from 0.3 to 4.8e12 vg/mouse. The liver specific expression of mVIIa resulted in a dose-dependent increase in plasma mVIIa levels and shortening of prothrombin time (PT) with a plateau at four weeks after injection. At mVIIa levels >500 ng/ml (n=5), there was a significant increase in the rate of initial and progressing clot formation in whole blood measured by a thromboelastogram (ROTEM®) compared to untreated age- and gender-matched control mice (n=5); CT (266±44 vs 387±59, P<0.01), CFT (125±19 vs. 222±76, P<0.03) and a angle (66.4±3.6 vs. 52.7±9.5, P<0.02), respectively. Moreover, tail clip assay also showed a dose-dependent decrease in blood loss, which was lower in mice receiving doses of 1.2 (n=12) and 4.8e12 (n=5) vg/mouse; OD measurement of hemoglobin released after lysis of RBC (1.0±0.5, P<0.005 and 0.4±0.3, P<5e-5) in the two doses vs. (1.5±0.1) in untreated controls (n=5), respectively. Higher thrombin-anti thrombin (TAT) levels (49±36 vs 12±7, P<0.05) in the highest dose vs. control, respectively, reflected the increased overall thrombin generation upon VIIa treatment. Our data suggest that the increase in both the amount and rate of thrombin generation, resulting from continuous expression of mVIIa, could contribute to a better fibrin clot and partially correct the abnormal hemostasis in BSS mice. Whereas the mechanism of VIIa action is independent of the genetic defect, a gene approach to compensate for the ineffectiveness of receptor components largely depends on the affected gene(s), hence narrowing down the number of already rare recipients. Moreover, our approach could be expanded to include other bleeding diatheses due to more common platelet defects such as Glanzmann thrombasthenia, especially with failure of transfusion therapy.

67. The Chicken Hypersensitive Site (cHS4) Insulator Element Reduces Position Effects Thereby Increasing Expression from Globin Lentiviral Vectors

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Lentiviral vectors (LV) carrying the human β-globin gene (hβ) and locus control region (LCR) have changed the field of gene therapy for hemoglobinopathies. However, their random integration into host cells results in variable hβ expression from chromatin position effects. We analyzed the role of a 1.2 kb chicken β-globin locus hypersensitive site 4 insulator element (cHS4) in a self-inactivating (SIN) LV. The BGM vector (carrying hβ/LCR) was compared to an analogous vector BGMI, with cHS4 such that it flanks the provirus upon integration. Both vectors additionally carried the mutant P140K methyl guanine methyl transferase ($MGMT^{P140K}$) cDNA to enrich for genetically modified cells. First, murine erythroleukemia (MEL) cells were transduced at <5% transduction efficiency to generate single-copy clones and transduced clones identified by PCR ('Unselected' clones) and analyzed for hβ expression by FACS and RNase Protection Assay (RPA). 'Unselected' $BGMI^+$-MEL clones had a higher proportion of hβ+ cells (68±3%) compared to BGM^+ clones (36±9%, n=24; P<0.001) and a reduced coefficient of variation (CV) of hβ expression: 168±20 vs. 327±64 in BGM (P<0.02). RPA showed a 2-fold increase in hβ/total mα-globin mRNA: BGMI 45±6% vs. BGM 21±7%, n=24, P<0.01. Next, MEL cells were selected with BG/BCNU and single-copy clones isolated ('Selected' clones). 'Selected' $BGMI^+$-MEL clones also had a higher proportion of hβ+ cells compared to BGM+ clones (80±15% vs. 72±20%, n=86, P<0.03), with reduced CV (140±2.8 vs. 170±13, P<0.01) and higher hβ-mRNA ($BGMI^+$ 83.6%±40 vs. 49.6%±25, n=24, P<0.02). Two additional vectors termed BG and BGI (with cHS4) were constructed with the hβ/LCR but no $MGMT^{P140K}$ cassette. RPA showed that BGI+ MEL pools expressed 85% hβ/total mα-globin/vector copy as compared to 38% from BG. BG and BGI single copy MEL clones showed similar results. In i*n vivo* studies lethally irradiated normal mice were transplanted with BGM and BGMI-transduced thalassemia hematopoietic stem cells (MOI 20). Engraftment and vector copy number in both groups were similar (66%±15% vs. 68%±10%, and 0.21 vs. 0.17 in BGMI and BGM groups, respectively, n=12). While, 18±4% of RBC expressed hβ in the BGMI group, only 4%±1.5% expressed hβ in the BGM group (P<0.001). There was a 4-fold increase in chimeric hemoglobin (mα-hβ) in BGMI mice (13%±4% vs. 3%±2% in BGM mice, P<0.001). Secondary colony forming units-spleen (CFU-S) derived from these mice showed increased numbers of hβ+ cells in BGMI (21%±4% vs. 8%±4% BGM, n=30, P<0.03) with reduced CV (698±91 vs. 987±99 in BGM, P<0.04). Taken together, 'insulated' SIN-hβ/LCR LV increased the probability of expression of integrants and reduced chromatin position effects, resulting in consistent and higher expression of hβ. The enhancer blocking effect of the cHS4, although not tested here, would further improve the bio-safety of these lineage-specific, SIN LV.