FDA-NTP Studies of Insertional Mutagenesis:
Definitive Study A - LTR vs SIN Retrovirus

Revision Date: 01.02.06
(revisions from version of 12.02.05:

1. Clarified to collect small intestine, Peyer's Patch tissue, for DNA isolation for all animals.
2. Clarified to collect large intestine GALT tissue for DNA isolation ONLY if grossly abnormal.
3. Modified age of donor animals for receipt at CCHMC to assure optimal age of 2-3 months at time of bone marrow harvest.

Reason for Revision: Previous document was found inadequate in addressing detailed items as discussed in the Battelle meeting 22 Sep 05 and as identified in subsequent discussion among FDA, MHH and CCHMC scientists.

Objective

This study will compare the frequency of insertional mutations leading to development of hematopoietic malignancies/disorders produced by a gamma-retroviral vector with an LTR containing an intact U3 enhancer/promoter and a vector from which the U3 enhancer/ promoter has been deleted from the LTR. These vectors will carry a transgene coding for red fluorescent protein.

Test Articles

LTR-vector: pSF91dsRed2 PRE;
SIN-vector: pSRS11SFdsRed2 PRE
Control vector: empty vector particles.

Animals C57B/6J mice; male donors (CD45.2); females recipients (CD45.1)

Recipients: Four (4) to 6-week old C57B/6J mice (female CD45.1) shall be quarantine for 10-14 days and then maintained until they reach 12 weeks of age. Twelve (12) week old animals shall be assigned at random to treatment and control groups. All animals shall be housed individually, feed and water shall be freely available.

	Recipient Animals		Gender		Species		Test Groups	Total
Phase-1	50	x	1	x	1	x	4 =	200
Phase-2	100^	x	1	x	1	x	4 =	400
Control-1	50	x	1	x	1	x	1 =	50
Control-2	50^^	x	1	x	1	x	1 =	50
Total								700

* 2 vectors x 2-Infectious Titers (Low and High) = 4 groups
^ assumes 2-secondary recipients for every primary donor.
^^ assumes 2-secondary recipients for 50% of primary controls

Donors: Seven (7) to 8-week old C57B/6J mice (male CD45.2) shall be quarantined for minimally 4 days and then until they are sacrificed for bone marrow harvest. All animals shall be housed individually and feed and water shall be freely available.
This pilot study requires **150 donors** (calculations based on 1 donor per 2 recipients plus a 20% margin for cell loss).

Treatment

Isolation and transduction of donor bone marrow cells

Lin- cells will be isolated from bone marrow of syngeneic male donor mice and transduced ex vivo as described by Z. Li et al. (Exp. Hematol., 2003). In brief, following isolation Lin- cells will be cultured in serum-free media supplemented with IL-3, IL-11, SCF and Flt3L; transductions will be performed on vector-precoated 6-well plates on days 3 and 4 of culture. This results in a mixture of transduced and non-transduced cells which will be used to inject into recipient animals. The infectious titer will be adjusted based on pilot in vitro studies with the NTP vector preparations. The "Low Infectious Titer" for the supernatant is defined as the titer that results in >10% and <50% marking by flow analyses of the bulk culture 4 days after vector exposure. The "High Infectious Titer" is the infectious titer equivalent to 10X the "Low Infectious Titer".

Lin- cells isolated and expanded under conditions identical to those above but exposed to producer cell supernatant that contains empty vector particles will be used to inject control mice. The empty particle vector supernatant will be used at the same supernatant to medium ratio as used for the "High Infectious Titer" transductions.

This study will be conducted in two phases;

phase-1: following whole body irradiation utilizing Cs137 γ-radiation (conditions TBD), 50 female mice per group (primary recipients) will be injected with ex vivo cultured cells ($5x10^5$ cells/animal based on post transduction cell number)) isolated from the bone marrow of syngeneic donor males and transduced ex vivo at low or high infectious titer with the LTR or SIN vector. An aliquot of the cell pool used for injection shall be taken for flow cytometry to examine transgene expression as well as for plating in Methocult to determine vector presence in CFU-derived DNA via qPCR.

Controls shall receive injections of donor Lin- cells ($5x10^5$ cells/animal based on post transduction cell number) isolated and expanded under identical conditions but exposed to producer cell supernatant that does not contain infectious vector (cells transfected with gag/pol & env only).

Treated and control animals will then be maintained for 7 months. Animals diagnosed as leukemic or moribund will be terminated at the time of diagnosis and necropsied as described below.

phase-2: bone marrow will be isolated from each primary animal and injected into two irradiated (TBD, same conditions as in phase-1) secondary recipients. Twenty five (25) controls selected at random will receive the same treatment. These animals shall then be maintained 7 months. Animals diagnosed as leukemic will be terminated at the time of diagnosis and necropsied as described below.

Irradiation:

Irradiations shall be performed with an irradiator utilizing a Cs137 source. The irradiator must be capable of exposing at least 10 mice at a time and of achieving an exposure rate of sufficient intensity to minimize exposure time. All treated animals shall receive a whole body exposure (TBD).

Vector Exposure:

Within 12-24 hours following irradiation animals shall receive transduced cells by tail vein injection. There will be two treatment groups; one group will receive cells transduced at low multiplicity of infection and a second group will receive cells transduced at a high multiplicity of infection. Transduction and transplant will occur in 5 groups of 10 animals each for Low and High Infectious Titer LTR vector, Low and High Infectious Titer SIN vector and empty vector particle control diluted as required for the LTR supernatant for the High Infectious Titer transduction.

Observations and In Life Monitoring

General Observations: Animals shall be weighed individually on day one of test (immediately prior to irradiation for treated animals), after seven days, and at weekly intervals thereafter. Animals shall be observed two times daily, once in the early morning and once in the late afternoon at least six hours apart (before 10:00 AM and after 2:00 PM), including holidays and weekends, for moribundity and death. Observations shall be made twice daily for clinical signs of pharmacologic and toxicologic effects. Clinical signs shall be recorded daily by animal number.

Transgene Analysis: Blood samples (maximum volume 250 µl) shall be taken of each animal 6 weeks after transplantation and then in 6 week intervals and analyzed for transgene expression. Flow cytometry and qPCR shall alternate as indicated below. Specifically, the peripheral blood will be subjected to red blood cell lysis and either analyzed by (1) flow cytometry to determine the frequency of cells expressing dsRed2, CD11b (FITC-labeled) and CD45.2 (APC-labeled) and a lysis control panel of TG, CD45 (FITC labeled), CD45.2 (APC labeled) or (2) cellular DNA will be prepared for qPCR analysis.

Peripheral Blood sample
1. 6 weeks -> flow cytometry
2. 12 weeks -> qPCR
3. 18 weeks -> flow cytometry
4. 24 weeks -> qPCR
5. 30 weeks -> flow and qPCR, see necropsy

Leukemia Monitoring: An aliquot of the above blood samples shall be analyzed for differential blood count and full hematologic analysis if quantity is sufficient.

Any animal clearly diagnosed as leukemic on the basis of differential blood counts and/or exhibiting signs of moribundity shall be sacrificed and necropsied as described below.

Necropsy Procedure and Tissue Collection

Peripheral Blood, tissues and organs from necropsy will be processed as follows for all animals found to be moribund/dead and for all animals sacrificed at the end of the 30 week study:

Tissue	Processing	Purpose
Any tumor	-Formalin fixation (50 mg piece) -snap freeze (≤50 mg piece), -80C -place a 50 mg piece in Trizol, -80C Make single cell suspension and -stain for flow cytometry 1.TG, CD3, CD117(c-kit) 2.TG, CD4, CD8 3.TG, CD41, Ter119 4.TG, Gr-1, CD11b 5.TG, B220, IgM -Cryopreserve (>5x10e6cells/vial) -Cryopreserve (>10e6/vial)	Histopathology DNA isol, qPCR, LM-PCR*, southern blot RNA isolation Immunophenotyping and transgene expression Archive at NIEHS Marker rescue (RCR)
Peripheral Blood, collected by cardiac puncture (about 400 µl in EDTA and 150 µl w/o EDTA)	-perform blood smear -automated cell count -isolate&freeze 100 µl serum@-80 Perform red blood cell lysis and -stain for flow cytometry TG, CD11b, CD45.2	Cytohistology Hematopathology evaluation check for infections Immunophenotyping and transgene expression

Tissue	**Processing**	**Purpose**
	TG, CD45.2, CD45 (common) [important control in case rbc lysis wasn't complete] -isolate DNA	 qPCR
Bone marrow, isolated from femurs	-perform cytospin -automated cell count -Flow cytometry (10e6 cells) 1.TG, Sca-1, CD45.2 2.TG, CD117(c-kit), CD3 3.TG, Gr-1, CD11b 4.TG, B220, Ter119 -Freezing cell pellet (>5x10e6 cells) -Cryopreserve >10e7 cells -Cryopreserve remaining cells >5x10e6 /vial	Cytohistology Hematopathology Immunophenotyping and transgene expression DNA isol, qPCR, (LM-PCR*), Eco env PCR Secondary transplant Archive at NIEHS
Bone marrow, sternum	Formalin Fixation	Histopathology
Spleen	-determine weight -Formalin Fixation (50 mg piece) Make Single cell suspension and -automated cell count -stain for flow cytometry 1.TG, CD3, CD117(c-kit) 2.TG, CD4, CD8 3.TG, CD41, Ter119 4.TG, Gr-1, CD11b 5.TG, B220, IgM -freeze cell pellet (>5x10e6 cells, if enlarged, freeze >3x10e7) -Cryopreserve remaining cells, >5x10e6 /vial	 Histopathology Hematopathology Immunophenotyping and transgene expression DNA isolation, qPCR and eventually Southern Blot Preserve viable cells
Thymus	-determine weight -Formalin fixation of one lobe Make Single cell suspension 2nd lobe, get cell count. -stain for flow cytometry 1.TG, CD4, CD8 1.TG, CD3, CD45.2 -freeze cell pellet (>5x10e6 cells) -Cryopreserve remaining cells, >5x10e6 /vial	 Histopathology Immunophenotyping and transgene expression DNA isolation Preserve viable cells

Tissue	**Processing**	**Purpose**
Lymph nodes (mandibular, mesenteric and peripheral)	-Formalin fixation If enlarged: Prepare single cell suspension and -stain for flow cytometry 1. TG, CD3, B220 or CD19 2. TG, CD4, CD8 -freeze cell pellet (5x10e5 cells)	Histopathology Immunophenotyping and transgene expression DNA isolation
Liver	-determine weight -Formalin fixation (50 mg pieces) (2 sections including left lateral lobe and median lobe) -snap freeze a 50 mg piece If enlarged: Prepare single cell suspension and -stain for flow cytometry 1. TG, CD3, B220 2. TG, Gr-1, CD11b	 Histopathology DNA isolation Immunophenotyping and transgene expression
Kidney	-determine weight (right kidney) -Formalin fixation (50 mg piece) -snap freeze remainder	 Histopathology DNA isolation
Lungs (and mainstem bronchi)	-Determine weight -Formalin fixation (50 mg piece) -snap freeze remainder	 Histopathology DNA isolation
Brain	-Formalin fixation (3 sections including frontal cortex and basal ganglia, parietal and thalamus, and cerebellum and pons) -snap freeze 50 mg piece	Histopathology DNA isolation
Ovary	-Formalin fixation (≤50 mg piece) -snap freeze 50 mg piece	Histopathology DNA isolation
Heart (and aorta)	-determine weight -Formalin fixation (half) -snap freeze (half)	 Histopathology DNA isolation
Small intestine	-Formalin fixation (2 cm) -snap freeze (2 cm) (Peyer Patch Tissue)	Histopathology DNA isolation
Adrenal glands	Formalin Fix	Histopathology
Clitoral glands	Formalin Fix	Histopathology
Esophagus	Formalin Fix	Histopathology
Eyes	Formalin Fix	Histopathology
cartilage plate, articular cartilage	Formalin Fix	Histopathology

Tissue	Processing	Purpose
articular surface	Formalin Fix	Histopathology
Gallbladder (mouse)	Formalin Fix	Histopathology
Gross lesions	Formalin Fix	Histopathology
Harderian glands	Formalin Fix	Histopathology
Intestine, large (cecum, colon, rectum)	Formalin Fix Snap Freeze GALT Tissue if grossly abnormal	Histopathology DNA
Mammary gland with adjacent skin	Formalin Fix	Histopathology
Muscle, thigh (if neuromuscular signs were present)	Formalin Fix	Histopathology
Nasal cavity and nasal turbinates (3 sections)	Formalin Fix	Histopathology
Pancreas	Formalin Fix	Histopathology
Parathyroid glands	Formalin Fix	Histopathology
Pituitary gland	Formalin Fix	Histopathology
Preputial glands	Formalin Fix	Histopathology
Salivary glands	Formalin Fix	Histopathology
Seminal vesicle	Formalin Fix	Histopathology
Spinal cord and sciatic nerve (if neurologic signs were present)	Formalin Fix	Histopathology

Abbreviations: TG = transgene expression (red fluorescence)
Procedural Note: Monoclonal antibodies must be labeled with FITC and APC.
* for all animals with ≥10% vector marking and all tumor tissues

Secondary Transplant of Bone Marrow From Asymtomatic Primary Recipients:

Bone marrow from each primary recipient (taken at necropsy as described above) will be transplanted into two (2) irradiated secondary recipients. These animals shall be maintained for 7 months or until diagnosed as leukemic and then necropsied as described above.

Summary of Tissue Analyses
(Note: Details for the Projected Total Assays based on estimated frequency of tumor occurrence follows.)

Analysis	Tissue Source	Animals Test		Animals Control		Time Point(s)	Projected Total Assays done by	
		Tumor	Non-tumor	Tumor	Non-Tumor		Battelle	CCHMC
PB panels Blood Counts Cytospins	PB	y	y	y	y	6 wks 12 wks 18 wks 24 wks 30 wks	3500	0
Flow Panels	PB	y	y	y	y	6 wks 18 wks 30 wks	3x700 = 2100	0
Necropsy Panels	As defined	y	y	y	y	necr	700	0
RCR Eco env	PB	y	y	y	y	necr		700
RCR Marker Rescue	Tumor	y	n	y	n	necr		187 = 178 +9 controls
LM	Tumor or BM	y*	y*	y*	2 randomly chosen	0		210 (phase 1) 420 (phase 2)
Southern Blot for ecotropic	Tumor	y	n	y	n	necr		187 = 178+9 controls
Southern Blot for polytropic	Tumor	y	n	y	n	necr		187 = 178+9 controls
Archive DNA for Southern Blot	Tumor or BM	y+	y+	y+	y+	necr		To be determined

For detecting vector, 2 enzymes needed = 2 lanes per sample								retrospectively and quoted separately as cost per analysis with total number of analyses TBD.
Real time PCR for detecting vector copy number	Tumor and 3xPB	y	y	y	y	necr (tumor) wks 12, 24, 30 (PB)		tumor: 187=178 +9 PB: 3x700 = 2100

y* only if real-time PCR shows > 10% marking and for all tumor tissue.
Y+ Sufficient tumor tissue will be retained for DNA to do a genomic Southern Blot if determined to be required retrospectively.

Predicted Tumor Incidence by Phase, Vector, and Infectious Titer

Tumor animals phase-1	
50 animals LTR High Infectious Titer = 50% predicted tumor incidence	25
50 animals SIN High Infectious Titer = 25% predicted tumor incidence	12-13
100 animals Low Infectious Titer = 4% predicted tumor incidence	4
50 control animals = 4% predicted tumor incidence	2
Total tumor animals phase-1	**44**
Tumor animals phase-2	
100 animals LTR High Infectious Titer (2 x 25 with tumors from phase-1 and 25 with “new“ tumors)	75
100 animals SIN High Infectious Titer (2x12 with tumors from phase-1 and 13 with “new“ tumors)	37
200 animals Low High Infectious Titer (2x4 with tumors from phase-1 and 8 with “new“ tumors)	16
50 control animals (2x2 with tumors from phase-1 and 2 with “new” tumors)	6
Total tumor animals phase-2	**134**

Total tumor animals Phase-1+ Phase 2	**178**