Rec'd from Rick Irwin 12/22/05/updated to include comments LR 01.02.06

FDA-NTP Studies of Insertional Mutagenesis: Pilot Study

Revision Date: 01.02.06
Reason for Revision: To include comments from Rick Irwin/Chris Baum of 12/22/05 communication.

1. Do histopathology evaluation on only the spleen, thymus, lymph notes, bone marrow, liver, kidney lungs, heart, ovary and small intestine.
2. Deleted isolation of DNA from lungs and intestine.
3. Modified age of donor animals for receipt at CCHMC to assure optimal age of 2-3 months at time of bone marrow harvest.
4. Specifically included the quantitative PCR for copy number for all animals, as referenced in text and necropsy detail table, in the "Summary of Tissue Analyses" table

Objective:
This 12 week (?) pilot study will serve to establish and evaluate methodology and procedures that will be employed in larger and longer term studies (Definitive Studies).

Test Article:
LTR-containing vector based on the murine leukemia virus (MLV) and carrying a transgene for red fluorescent protein (dsRed2). Similar SIN vector and empty particle control supernatant. Vector is from lot prepared for the Definitive Study and will minimally have been passed research level certification assays for sterility, mycoplasma, endotoxin and RCR.

Animals:
C57B/6J mice; male donors (CD45.2), female recipients (CD45.1; BoyJ). All animals will be provided by Battelle and shall be Pseudomonas free.

Donors: **Donors will be housed and processed at CCHMC.** 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 (NTP 2000) and water shall be freely available. This pilot study requires **36 donors** (calculations based on 1 donor per 2 recipients plus a 20% margin for cell loss**.**

Transduced cell will be transported from CCHMC to Batelle Labs for injection into irradiated recipients.

Recipients: Four (4) to 6-week old C57B/6J mice (female CD45.1; BoyJ) 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	
Treatment (LTR)*	15	x	1	x	1	x	1	=	15	+ 5 @ CCHMC
Treatment (SIN)*	15	x	1	x	1	x	1	=	15	+ 5 @ CCHMC
Controls (Empty)*	20	x	1	x	1	x	1	=	20	
Total									50	+ 10 = **60 Recipients**

*Animal Study transduction/transplant groupings are as follows:

Group	Vector	Battelle Animals	CCHMC Animals
1	LTR	15	5
1	Empty Particle	10	0
2	SIN	15	5
2	Empty Particle	10	0

Treatment:

Irradiation (Battelle):

Irradiations shall be performed with an irradiator utilizing a Cs 137 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 groups of animals shall receive a whole body exposure (between 9 and 12 Gy, exact conditions to be defined prior to study initiation).

Vector Exposure:

Lin- cells will be isolated from bone marrow of syngeneic donor mice and transduced ex vivo as described by Z. Li et al. (Exp. Hematol., 2003) at CCHMC. 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 using Fibronectin fragment CH296 on days 3 and 4 of culture. 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”. It is expected that the “High Infectious Titer” will result in >80% marking. The Pilot Study utilizes the “High Infectious Titer” for all transductions. . An aliquot of the cell pool used for injection (not to exceed $5x10^5$ cells) 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.

Mice will be injected 12-24 h after irradiation at a dose of $5x10^5$ cells (cell number is as obtained at the end of transduction)/animal.

Controls shall receive injections of $5X10^5$ cells isolated and expanded under identical conditions but exposed to producer cell supernatant that contains empty vector particles. The empty particle vector supernatant will be used at the same supernatant to medium ratio as used for the "High Infectious Titer" transductions.

Observations:

Animals shall be weighed individually on day one on 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.

Evaluation of Peripheral Blood:

Six (6) weeks after injection: A blood sample (up to 75ul) shall be withdrawn from the retro-orbital sinus of all treated and control mice and evaluated as described below. It is anticipated that leukocyte counts will not be high at this point the evaluations have been designed to minimize the volume of blood collected. The laboratory will provide in advance specific methods/protocols/procedures for the above. PCR diagnostics will be performed at week 12.

Peripheral blood 6 week sample will be evaluated as follows:

Purpose	**Method: Battelle**(50 mice)	**Method: CCHMC**(10 mice)
Hematologic Evaluation	Erythrocyte count	Erythrocyte count
	Hemoglobin	Hemoglobin
	Leukocyte count	Leukocyte count
	Leukocyte differential	Leukocyte differential
	Reticulocyte count	
	Platelet count	Platelet count
	Morphologic assessment of erythrocytes, leukocytes and platelets	Morphologic assessment of erythrocytes, leukocytes and platelets
Transgene Expression	Flow Cytometry for red fluorescence	Flow Cytometry for red fluorescence
Donor Chimerism	Flow Cytometry Immuno	Flow Cytometry Immuno

	staining for CD45.2-APC versus CD45 common FITC	staining for CD45.2-APC versus CD45 common FITC
Myeloid Lineage Differentiation	Flow Cytometry Immuno staining for CD11b-FITC versus CD45.2-APC	Flow Cytometry Immuno staining for CD11b-FITC versus CD45.2-APC

Organ and Tissue Collection Necropsy Procedure at 12 weeks after injection or if found to be moribund/dead on observation

Peripheral Blood, tissues and organs from necropsy will be processed as follows:

Tissue	**Processing**	**Purpose**	**Battelle** 50 Mice	**CCHMC** 10 Mice
Any tumor (not expected in the pilot study)	-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)	Yes	Yes
Peripheral Blood, collected by cardiac puncture (about 400 ml)	-perform blood smear -automated cell count -isolate&freeze 100 ml serum@-80 Perform red blood cell lysis and -stain for flow cytometry TG, CD11b, CD45.2 TG, CD45.2, CD45 (common) [important control in case rbc lysis wasn't complete] -isolate DNA	Cytohistology Hematopathology check for infections Immunophenotyping and transgene expression qPCR	Yes	Yes

Tissue	Processing	Purpose	Battelle 50 Mice	CCHMC 10 Mice
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 remaining cells >5x10e6 /vial	Cytohistology Hematopathology Immunophenotyping and transgene expression DNA isol, qPCR, (LM-PCR*), Eco env PCR Archive at NIEHS	Yes	Yes
Bone marrow, sternum	Formalin Fixation	Histopathology	Yes	Yes
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	Yes	Yes
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	Histopathology Immunophenotyping and transgene expression DNA isolation Preserve viable cells	Yes	Yes

Tissue	Processing	Purpose	Battelle 50 Mice	CCHMC 10 Mice
	cells, >5x10e6 /vial			
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	Yes	Yes
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	Yes	Yes
Kidney	-determine weight (right kidney) -Formalin fixation (50 mg pieces) -snap freeze remainder	Histopathology DNA isolation	Yes	Yes
Lungs (and mainstem bronchi)	-Determine weight -Formalin fixation (50 mg piece) -snap freeze 50 mg piece	Histopathology	Yes	Yes
Ovary	-Formalin fixation (≤50 mg piece) -snap freeze 50 mg piece	Histopathology DNA isolation	Yes	No
Heart (and aorta)	-determine weight -Formalin fixation (half) -snap freeze (half)	Histopathology DNA isolation	Yes	No
Small intestine	-Formalin fixation (2 cm) -snap freeze (2 cm)	Histopathology	Yes	No

Abbreviations: TG = transgene expression (red fluorescence)
Procedural Note: Monoclonal antibodies must be labeled with FITC and APC.

* for animals with ≥10% vector marking and all tumor tissues

Summary of Tissue Analyses

Analysis	Tissue Source	Animals			Time Point(s)	Projected Total Assays done by	
		Test		Control			
		Tumor	Non-tumor			Battelle	CCHMC
PB panels Blood Counts Cytospins Flow Panels	PB	y	y	y	6 wks 12 wks	100	20
Necropsy Panels	As defined	y	y	y	12 wks	50	10
RCR Eco env	PB	y	y	y	12 wks	0	60
LM	BM	y	y	2 randomly chosen	12 weeks	0	42
Q-PCR – Bulk PB copy number	PB	y	y	y	12 wks	0	60