Introduction

Retroviral vectors represent both the first viral vector system used for clinical gene transfer, as well as one of the most widely used in clinical trials to date. The replication cycle of retroviruses (and retroviral vectors) involves transformation of the RNA genome to a double-stranded DNA that subsequently integrates into the genome of the target cells. Integration of retroviral vectors into the host cell genome makes them both attractive, as potential agents of gene therapy to obtain long-term gene expression, and potentially risky, as agents of insertional mutagenesis (alteration of the genome as a result of vector DNA insertion).

The potential for insertional mutagenesis leading to tumorigenesis has been shown to be more than theoretical in a clinical trial in France, where 3/11 children with X-linked Severe Combined Immunodeficiency Syndrome (X-SCID) have developed leukemia due to integration of the retroviral vector. In at least the first two children subsequent activation of a human oncogene, LMO-2, has been demonstrated to be causally linked to the development of leukemia ([1] details were discussed at the March, 2005, meeting of the CTGTAC, and transcripts are found at http://www.fda.gov/ohrms/dockets/ac/05/transcripts/2005-4093T2_02.htm). However, the clinical and laboratory results showing improved immune function in 10/11 children in this trial ([2, 3], and http://www.fda.gov/ohrms/dockets/ac/05/transcripts/2005-4093T2_02.htm) highlight the current dilemma for the clinical application of retroviral vectors: how to maximize the therapeutic benefit while reducing the risk to subjects. One approach being explored in the gene therapy community is to modify current retroviral vectors in a manner intended to reduce the risk of insertional tumorigenesis. (For example, modifying or deleting elements within the U3 enhancer of the LTR may provide one way to reduce activiation of adjacent genes with equal rates of insertion but different levels of tumorigenesis) As a consequence of these activities, there is now a need for development of a valid preclinical model to assess the risk of insertional mutagenesis and tumorigenesis, in order to allow the field of retroviral vector-mediated gene therapy to move forward along the critical path of product development.

To address the lack of a valid preclinical model, FDA has proposed a collaborative study administered through the National Toxicology Program (NTP). The NTP is a Congressionally funded program that performs GLP toxicology studies on products regulated and nominated by FDA (see Appendix 2). In collaboration with NTP, we plan to perform a study to assess the sensitivity and reproducibility of a mouse bone marrow transplantation model to predict the tumorigenic potential of traditional retroviral vectors, and those modified intentionally to reduce the risk of tumorigenesis. We are now seeking the advice and input of the CTGTAC on the attached study proposal (found in Appendix 1).

The goal of our study is consistent with advice from several bodies. The American Society of Gene Therapy published a series of reports on its website to review and summarize previous preclinical and clinical experiences with use of retroviral vectors (http://www.asgt.org/position_statements/report_042003/index.html). In the report titled, "Review of murine data on insertional tumorigenesis and predisposition to tumorigenesis following gene transfer in HSCs", the authors note that less than one-quarter of these animals are observed for longer than 6 months. Indeed, their survey of the published literature shows that of those mice studied in a bone marrow transplantation model, only 17% were observed for greater than 6 months or

transplanted into secondary recipients. In fact, only 5% of all bone marrow transplants were followed in secondary recipients for greater than 6 months. This survey led to the following draft recommendations in the ASGT report:

- "…authors should strive to follow cohorts of mice for at least one year…"
- "A proportion of treated animals should be maintained for at least one year…"
- "Preclinical animal studies should appropriately mimic the human trials."

Likewise, at the March, 2005, meeting of the CTGTAC, you advised the FDA that investigators in the field of gene therapy should be encouraged to explore alternative approaches, including new retroviral vector products to lessen risk, and that these new products should be adequately tested in relevant animal models, although the specific models were not discussed at the time (http://www.fda.gov/ohrms/dockets/ac/05/transcripts/2005-4093T2_02.htm). In addition, the UK Gene Therapy Advisory Committee recommended in their May, 2005 report: "Ideally, new vectors should be selected on the basis of improved safety in preclinical testing models…However, the current lack of validated systems remains a constraint to application of this principle." (http://www.advisorybodies.doh.gov.uk/genetics/gtac/FinalrecommendationsJune2005.pdf)

Rationale for and Summary of Proposed Study

FDA made the following assumptions in choosing what animal model should be chosen for this study:

- The model should mimic clinical use of retroviral vectors. Since over one-third of active INDs using retroviral vectors use ex vivo modification of hematopoietic stem cells (typically CD34+ cells) and these were the target cells used in the X-SCID clinical trial, we chose a bone marrow transplantation model for the study.
- The model should have a demonstrated rate of tumors directly attributable to the insertion of the retroviral vector.

Approximately 6 months before the first child in the French trial was reported to have developed leukemia, the first preclinical study to demonstrate tumorigenesis by retroviral vectors in the absence of replication competent retrovirus was published in Science by Li, et al [4]. Retroviral vectors were used to *ex vivo* modify murine hematopoietic stem cells that were then transplanted into irradiated syngeneic recipients. These "primary" recipients were observed for 6 months, and then their bone marrow was harvested and used to transplant a second set of irradiated syngeneic "secondary" recipient mice that were also observed for 6 months. 6/10 secondary recipient mice developed leukemias that were subsequently shown to contain monoclonal sites of integration of the retroviral vector into a known murine oncogene, *Evi-1*. The integration correlated with activation of gene expression of *Evi-1*, and is thought to have contributed to the leukemogenesis [4]. Since the initial publication of this work, these authors have extended these studies to demonstrate that the model is reproducible, detects leukemias in a transgene-independent manner (i.e., vectors with dsRED2, a neutral transgene, result in leukemias) and can be used to detect dose-dependent effects [5].

The above-referenced studies have used small group sizes (typically, n=5-10), sufficient for an academic study, but not sufficient to allow for distinguishing between a low incidence of tumorigenesis and a truly negative result. For example, if a modified retroviral vector reduces the risk from 60% to 5%, a 5% incidence rate of tumors may not be detected in a group size of 5-10 animals, and results would be observed as negative. Therefore, there is a need to extend this model further to be able to achieve confidence that the tumor incidence is very low for a novel vector, and to determine the range of sensitivity of detecting vector-mediated tumorigenesis, by using much larger sample sizes. In consultation with a statistician, we have determined that in order to achieve 90% confidence in a negative result, sample sizes of at least 40 animals per group would be required. A study of this size is too large for a single institution to perform, such as an academic investigator, however, through the National Toxicology Program, we have developed a collaborative approach to performing this study, including FDA scientists, academic investigators, and NIH scientists associated with NTP.

The study on retroviral vector leukemogenesis is the first NTP study of a complex biologic product. The study will be performed at two contract laboratory sites:

- The first site has extensive expertise in retroviral vector manufacture and ex vivo transduction of hematopoietic stem cells. This contract site will manufacture the retroviral vectors, quality control test the vectors, and perform the bone marrow isolations and ex vivo transductions.
- The second site has experience doing large-scale GLP studies that include immunologic and molecular biology analytical endpoints. This contract laboratory will perform the bone marrow infusions into irradiated recipient mice, follow these animals for signs of disease, perform the secondary transplants, and perform necropsies and analyses of tissues at the end of the study.

The detailed study proposal is found in **Appendix 1**. Briefly, the study will be performed in two parts. Initially, a pilot study will be performed that uses small group sizes and short duration to determine whether the operators at these two contract labs can successfully perform all the procedures to ensure a high rate of stem cell engraftment and reconstitution of the hematopoietic system (for example, one variable that will differ from the previously performed studies is the transportation of the transduced cells from Cincinnati to Columbus). The second phase will be a large-scale study in group sizes of 50 animals per treatment group. The following control groups will be used: a mock vector-transduced negative control and a positive control that receives cells transduced with an intact LTR-containing retroviral vector (pRSF91dsRed2pre*). A similar vector (with the exception of the PRE element) was used previously and shown to result in a detectable rate of leukemia (1/7 recipients) [5]. We will assess the frequency of vector positive cells (transduction rate) as well as leukemias and lymphomas in these two groups against the experimental group of animals that will receive a modified retroviral vector, termed a SIN vector because as it is produced, it generates a vector that "self-inactivates", because it contains a deletion of the U3 enhancer element from the LTR. The vector we will be using will have the U3 enhancer repositioned into an internal position adjacent to the dsRed2 cDNA open reading frame (pSRS11.SFdsRed2pre*), in order to assess the impact of the U3 element on tumorigenesis in terms of its placement within the vector structure. Retroviral vectors with analogous vector structures result in comparable levels of transduction of murine hematopoietic stem cells [6]. Each vector will be tested at high and low

infectious doses for transduction to also assess the impact of vector dose, and transduction efficiency will be measured. Schematic diagrams of the two retroviral vectors that will be used in this study are shown in Figure 1**. Appendix 1** also includes a list of the safety and characterization assays that will be used to quality control the vectors used in this study – the tests closely parallel what would be required for a clinical trial.

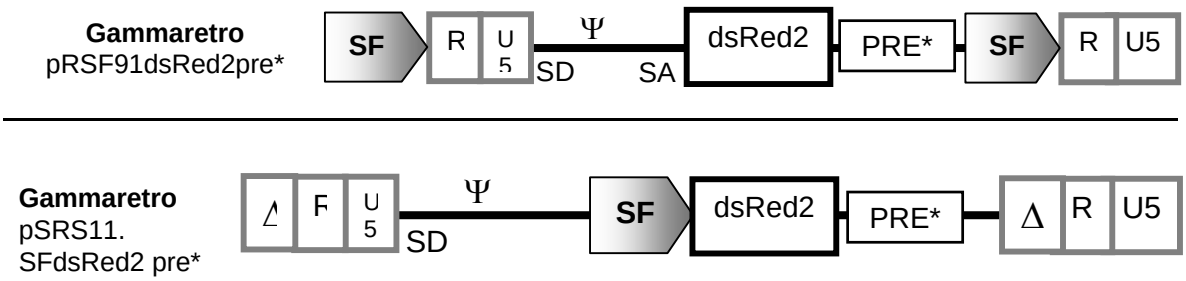

Figure 1: Schematic diagram of key elements within the two retroviral vectors planned for use in this study. SF, U3 enhancer-promoter is from Spleen Focus-Forming Virus. dsRed2 is a marker gene isolated from a reef coral that encodes a protein which fluoresces red (at wavelength 583 nm) [7], R and U5 are elements within the LTR that mediate transcription initiation and poly-adenylation. Δ, represents a deletion of the U3 enhancer-promoter element (including the TATA box). SD and SA, are splice donor and acceptor sites, respectively. Ψ, or psi element, represents the packaging motif on the vector RNA. PRE*, is the Wood-Chuck post-transcriptional regulatory element, modified to not include the pX open reading frame.

Goals of Current Study and for the Future

The current study proposal is designed to meet the following goals:

- Develop and assess the sensitivity of a preclinical model for assessing the risk of retroviral vector-mediated insertional tumorigenesis
- Assess the effect of vector dose
- Assess the effect of deleting U3 from the LTR

The FDA has the opportunity to extend this partnership with the NTP to include other modifications, such as insertion of insulator elements, use of lentivirus vectors, etc. In addition, long-term, we may explore other animal models. For example, a recent publication from Themis and colleagues demonstrated that *in utero* administration of certain lentivirus vectors resulted in liver tumors in over 60% of progeny mice in some cases [8]. This study suggests that *in utero* administration may provide a sensitive method of detecting tumorigenic events associated with lentivirus vectors that may be undetectable in other animal models. Other investigators are exploring use of various tumor suppressor knock-out models as ways to increase the sensitivity of detecting retroviral vector-mediated insertional tumorigenesis (summary of meeting where these models were discussed: [9]). FDA and NTP will need to stay abreast of these types of developments in order to make an informed choice about additional models that would be useful for safety assessment of retroviral and lentiviral vectors in the future.

Therefore, we would also like the CTGTAC to comment on any models they are aware of that may be worthy of consideration for future investigation through the NTP.

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** Selected references found in **Appendix 3**