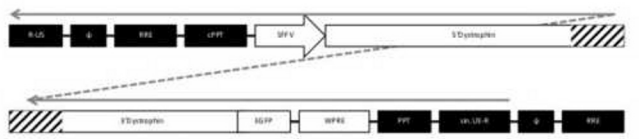

Figure 4: Schematic of recombination pathway for full-length dystrophin transfer. In the modified vector conformation, minus strand synthesis can only proceed from the 3' LTR of the 3' strand due to the lack of a 3'LTR on the 5' strand. For production of a provirus containing all essential elements, recombination has to occur within the homologous dystrophin region that exists in both strands (shaded box). This necessitates the production of full-length dystrophin in order for reverse transcription to synthesise a functional provirus.

528. Generation of a Cocal Envelope Packaging Cell Line for Robust Lentiviral Gene Transfer Into Hematopoietic Stem Cells and T Cells

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Lentiviral vectors (LVs) are routinely used for stable gene transfer and have demonstrated great promise in clinical hematopoietic stem cell and immune cell gene therapy. LVs are commonly pseudotyped with vesicular stomatitis virus envelope glycoprotein (VSV-G), which confers broad tropism to the vector and allows for vector concentration by centrifugation. However, the use of VSV-G has several limitations, such as susceptibility to inactivation by human serum complement making it unsuitable for in vivo delivery, and toxicity when constitutively expressed, which has impeded efforts to generate stable producer cell lines. With the goal to generate a self-inactivating LV packaging cell line, we stably expressed either the VSV-G or cocal envelope in HEK293T cells along with other required helper genes, 3rd generation gagpol and rev. Genes were sequentially introduced in cells by co-transfection with plasmids containing an antibiotic resistance gene. After selection, best producer clones were isolated by limiting dilution and were treated with the histone deacetylase inhibitor sodium butyrate to enhance vector titer. The resulting cocal packaging cell line produces 20 to 50-fold more infectious particles as compared to VSV-G expressing cells, reaching titers averaging 108 infectious units/mL upon concentration. We also extend on our previous studies (Trobridge, 2010) by showing more robust gene transfer for cocal-pseudotyped LVs as compared to VSV-G pseudotyped LVs in human (81% cocal vs. 45% VSV-G, MOI 5) and non-human primate (Macaca nemestrina) CD34+ HSPCs (45% cocal vs. 18% VSV-G, MOI 2x5) while preserving similar differentiation potential as determined by CFC assays. Cocal vectors also transduced human CD3+ blood cells more efficiently than VSV-G vectors (65% cocal vs. 42% VSV-G vs., MOI 3) and showed comparable transduction efficiency in non-human primate CD3+ cells. Using a competitive repopulation approach in the non-human primate model, we find increased gene marking in repopulating cells transduced with a cocal pseudotyped LVs compared to repopulating cells transduced with the same lentivirus backbone pseudotyped with VSV-G. We observed a dramatic difference in the granulocyte fraction where marking with the cocal vector reaches ~90% at 70 days post transplantation. Retroviral integration site analysis is currently underway to characterize the integration profile of cocal vs. VSV-G modified clones. Overall, our study suggests that the cocal envelope outperforms the VSV-G envelope in a stable lentivirus packaging cell line, which may prove useful in current gene therapy efforts to generate large-scale clinical grade vectors.

529. Novel LTR-1 Lentiviral Vectors Are Fully Functional Following the Removal of HIV-1 Gag-RRE Sequences

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Standard lentiviral vectors (LVs) require sequences from the wild-type virus (most commonly HIV-1) for effective packaging of vector genomes into viral particles. These sequences are preserved in reverse-transcribed proviral DNA, maintaining the packaging signal, major splice sites, the REV-response element (RRE) and CpG islands which amount to approximately 19% of HIV-1 genome in 3rd generation vectors. For gene therapy applications, these regions have been shown to be problematic, or pose potential risks. Vector integrations in proximity to host-cell genes have produced aberrant transcripts through genome-vector splicing. Transcriptional silencing of delivered transgenes can occur through methylation of viral CpG islands and the presence of the packaging signal allows remobilisation of vector genomes in cells expressing LV proteins which could be detrimental in HIV-positive patients.

In an attempt to reduce these risks and minimise HIV-1 sequence in the provirus, we have developed the novel LTR-1 vector in which the packaging sequences and RRE are located downstream of the 3' Long Terminal Repeat (LTR). This location means that the essential *cis*-elements are contained within the RNA genome for efficient processing and encapsidation, but eliminated from the delivered provirus following reverse transcription. This reduces the amount of HIV-1 genome in the integrated provirus to 4.9%. These vectors can be produced to high titre (>10^8 TU/ml by eGFP flow cytometry) and the proportion of eGFP positive cells is stable between 3 and 14 days post-transduction. Clonal analysis of plasmid rescue experiments confirm LTR-1 proviral sequences lack HIV packaging and RRE sequences following reverse transcription and sequencing confirms the expected structure. Unlike the 3rd generation vector, LTR-1 cannot be remobilised when transduced cells are transfected with HIV-1 packaging constructs. Investigations into safety improvements produced by the LTR-1 modifications are ongoing.

Live bioimaging experiments and immunohistochemistry confirm that LTR-1 also functions effectively *in vivo* when measured for up to 5 weeks post-injection: intracranial or intravenous injections into neonatal mice result in comparable expression levels to standard 3rd generation vectors in the brain and liver respectively.

We suggest that this LTR-1 configuration could have an improved safety profile for the next generation of retroviral gene therapy vectors.

530. Development of New Lentiviral Vectors With a Reduced Splicing Interference Potential and a Safer In Vivo Genotoxic Profile

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The excellent therapeutic potential of self-inactivating (SIN) lentiviral vectors (LV) has been demonstrated in pre-clinical studies

and clinical trials. However, weaker mechanisms of insertional mutagenesis could endanger their clinical applications. Systemic vector injection into newborn tumor-prone Cdkn2a-/- and Cdkn2a+/- mice, conducted in our previous work, demonstrated that SINLVs harboring strong or moderate enhancer/promoters in internal position caused acceleration in hematopoietic tumor onset with respect to control mice. Integration sites analyses of vector-induced tumor showed that oncogene activations or tumor suppressor inactivation by LV integrations occur by combining mechanisms of transcript truncation, induction of aberrant splicing and/or enhancer-mediated overexpression of cellular transcription units. Although oncogene activation may be reduced by the use of self-inactivating design, moderate cellular promoters and insulator sequences how to reduce genotoxic splicing-capture events and aberrant transcript formation triggered by vector integration is still unclear.

From this and a previous study, we identified the LV sequences most frequently involved in chimeric transcript formation. In our rationale, these LV sequences could be tagged by sequences complementary to microRNAs (mirT sequence) active in hematopoietic cells in order to allow selective degradation, through the miRNA pathway, of vector-mediated aberrantly spliced transcripts. Hence, we specifically designed SIN LVs harboring mirT sequences recognized by mir223 and mir142-3p (that are expressed in hematopoietic lineages) within the SIN LTR (mirsT-LTR LV) or in the vector backbone and outside the gene expression cassette (mirT LV). We then assessed the genotoxicity of the SIN LVs harboring mirT sequences by taking advantage of our in vivo models. Interestingly, injection of mirsT-LTR LV (N=73) and mirT LV (N=73) in Cdkn2a-/- mice did not caused any significant acceleration in hematopoietic tumor onset compared to control un-injected mice (N=40). Similar results have been obtained after injection in Cdkn2a+/- mice (N=28 for mirsT-LTR LV, N=26 for mirT LV and N=34 un-injected mice). We are currently performing integration site analyses in Cdkn2a-/- and Cdkn2a+/- treated mice to dissect if and how the integrated mirsT-LTR LV and mirT LV proviral genome interacts with the surrounding cellular genome.

Overall, these studies show that this new advanced design lentiviral vectors completely abrogated residual vector genotoxicity in highly sensitive mouse models and could represent the vector design of choice in future gene therapy applications.

531. Intravenous Delivery of Toca 511 Gene Therapy in Combination with 5-Fluorocytosine for Intratumoral Production of 5-Fluorouracil in a Colon Cancer Metastasis Model

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Despite advances in screening, colorectal cancer (CRC) remains the fourth most commonly diagnosed cancer and the second leading cause of cancer death for both men and women in the US. Approximately one half of patients with CRC develop liver metastases (mCRC). The standard treatment for mCRC is 5-fluorouracil (5-FU) based combination chemotherapy. 5-FU combination chemotherapy has extended the median survival of these patients from 6 to >20 months.

We are pursuing a unique investigational approach to treat cancer via in situ production of 5-FU. Toca 511 (vocimagene amiretrorepvec), a retroviral replicating vector (RRV), selectively replicates and spreads in malignant cells and encodes an optimized yeast cytosine deaminase (CD) gene. Within infected cells, the CD enzyme is expressed and converts 5-FC (flucytosine, an orally available anti-fungal drug) to the anti-cancer drug 5-FU. Both a direct cytotoxic effect and an extended immunotherapeutic effect have been reported using this approach.

Toca 511, in conjunction with subsequent oral extended-release 5-fluorocytosine (Toca FC), is currently under investigation in patients with recurrent high grade glioma. In these studies, Toca 511 is delivered either intratumorally (NCT01156584), by injection into the surgical resection bed (NCT01470794), or intravenously (NCT01985256) in subjects scheduled for subsequent resection. We tested the suitability of the intravenous approach for the treatment of mCRC in a mouse syngeneic liver metastasis model. CT-26-luciferase colon carcinoma cells were delivered via intrasplenic injection producing multiple tumor foci within the liver. Intravenous delivery of RRV resulted in expression of the vector encoded transgene in tumor foci but not in adjacent normal liver tissue. Intravenous delivery of Toca 511 followed by courses of 5-FC resulted in shrinkage or elimination of tumor foci and improved survival in this model of mCRC. The data is supportive of future clinical trials of intravenous Toca 511 followed by cycles of Toca FC in metastatic CRC.

532. Foamy Viral Vector Integration Sites in SCID-Repopulating Cells After MGMTP140K-Mediated In Vivo Selection

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Foamy virus (FV) vectors are promising for hematopoietic stem cell (HSC) gene therapy but preclinical data on the clonal composition of FV vector transduced human repopulating cells is needed. Human $CD34^+$ human cord blood cells were transduced with an FV vector encoding a methylguanine methyltransferase (MGMT) P140K transgene, transplanted into immunodeficient NOD/SCID $IL2Rg^{null}$ (NSG) mice, and selected in vivo for gene-modified cells. The retroviral insertion site (RIS) profile of repopulating clones was examined using modified genomic sequencing PCR (MGS-PCR). We observed polyclonal repopulation with no evidence of clonal dominance even with the use of a strong internal spleen focus forming virus (SFFV) promoter known to be genotoxic. However we did observe that highly captured sites were found more often near proto-oncogenes than less frequently captured sites (Figure 1). Our data supports the use of FV vectors with MGMTP140K for HSC gene therapy, but also suggests additional safety features should be developed and evaluated.