

Integrating Ideas on Insertional Mutagenesis by Gene Transfer Vectors

3rd Stem Cell Clonality and Genotoxicity Retreat

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The 3rd Stem Cell Clonality and Genotoxicity Retreat was held 12–13 December 2006, directly following the 48th annual meeting of the American Hematological Society held in Orlando, Florida. This series of retreats was organized by David Williams, Chris Baum, and Christof von Kalle of Cincinnati Children's Hospital. The impetus was the incidence of insertional leukemogenesis arising in the French gene therapy trial treating X-linked severe combined immunodeficiency disease (X-SCID) in 2002. Prior to 2002, the risk of insertional mutagenesis from gene transfer vectors was a theoretical one that had not been followed up with rigorous proactive studies. Of course, this all changed after the adverse events in the French trial and a case of insertional mutagenesis discovered in an animal study earlier the same year by Chris Baum's group. In the following years, evidence was found for clonal dominance in hematopoietic stem cells transduced with retroviral vectors in both mice and macaques, and in patients with chronic granulomatous disease (CGD) treated with gene-corrected hematopoietic cells.

The previous retreat, held after the 8th annual ASGT meeting in St. Louis, Missouri, in June 2005, focused largely on defining factors that might have contributed to tumor development in the three affected children in the French trial (see ref. 1 for a report on that meeting). The nature of the transgene, the effect of insertion of

the transgene near the *LMO2* locus, and the large number of transduced cells delivered to these patients were all cited as possible contributing factors. There was much discussion of the potential benefits of self-inactivating (SIN) vectors to limit the possibility of long-terminal-repeat (LTR)-mediated activation of oncogenes. Lentiviral vectors were suggested as a possibly safer delivery system, and the benefits of insulator elements to shield local genes from the effects of an integrating vector were discussed.

In the intervening months there has been much progress on many of these issues and the 2006 retreat provided a forum for that new work. In addition, much more information is now available on the integration preferences of different vector systems and new studies in animals attempting to model insertional mutagenesis by gene transfer vectors. Nonviral systems for gene insertion, such as the *PiggyBac* transposon or engineered zinc-finger nucleases, are also being studied as alternatives to virus-based delivery systems, with the ultimate aim of identifying and targeting "safe" sites in the genome at which to insert corrective genes.

In the first session of the meeting, chaired by Brian Sorrentino, speakers discussed the insertion preferences of viruses and vectors. One of the more provocative talks did not deal with retroviral insertion but instead provided data showing that recombinant adeno-associated viruses

(AAVs) may also be able to contribute to tumor development following insertion into the genome. Previous studies by Mark Sands's group had shown that an AAV vector could apparently induce tumors in mucopolysaccharidosis II (MPSII) mice, although the workers were unable to recover proviral DNA from those tumors. Some had suggested that other factors had contributed to tumor formation or perhaps that the mouse model suffered an inherent susceptibility to tumor formation. In his talk, Dave Russell presented data showing that both normal mice and those with MPSVII develop hepatocellular carcinoma (HCC) after injection of an AAV vector expressing β -glucuronidase. AAV vector proviruses were isolated from tumor specimens from four normal mice. In each case, the provirus had inserted within a region of chromosome 12 that contains several imprinted transcripts, as well as numerous small nucleolar RNAs (snoRNAs) and microRNAs. Importantly, the workers found overexpression of adjacent chromosomal transcripts in tumor specimens—in particular a transcript containing snoRNAs. The findings not only implicate this locus in the development of HCC but also raise concerns that insertional mutagenesis may not be limited to retroviral vectors, which has important implications for many current human trials.

André Lieber showed that a helper-dependent adenovirus (Ad5/35)-AAV hybrid vector carrying the β -globin locus control region preferentially integrated into the chromosomal globin locus control region (LCR) in erythroid but not non-erythroid cells. He provided preliminary data showing tethering of the incoming Ad genome to the chromosomal LCR via specific chromatin elements. Potentially, this mechanism could provide a new approach to achieve targeted integration of vectors. Transposons and integrases have received much attention recently as potential gene-insertion vectors due at least to the perception that these elements could be safer, but of course efficient delivery of such vectors is still a problem. Mark Kay's group has recently described a hybrid vector combining the high transduction efficiency of an adenoviral vector with the integration machinery of the bacteriophage-derived integrase ϕ C31.

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The ability of retroviral vectors to activate oncogenes after insertion has led to their use to screen for cancer-related genes (see ref. 2 for a review). Transposons have recently also been shown to be able to act as insertional mutagens and to cause hematopoietic cancer in mice. Adam Dupuy described a *Sleeping Beauty* (SB) transgenic approach to model carcinoma and to look for integrations in mice. Key findings were that the promoter used to drive expression of the SB transposase appeared to dictate the tissue compartment in which the tumor arose, and that a promoterless vector did not induce tumors. The system should be useful to screen for common integration sites (CISs), and may also be of use as a model system to screen for genotoxicity of particular vector designs or promoters.

Integration-site selection has emerged as a key determinant of the potential toxicity of an integrated vector. Integration of lentiviral vectors is known to be favored in active transcription units, but the molecular mechanisms responsible for targeting have not been fully clarified. Rick Bushman reported use of pyrophosphate sequencing to map 40,569 unique sites of HIV integration. Computational analysis showed that the integration sites were periodically distributed on the surface of nucleosomes, indicating favored integration into outward-facing DNA major grooves on nucleosomes *in vivo*. Analysis of integration-site positions in the densely annotated ENCODE regions revealed favored integration near transcription-associated histone modifications, including H3 acetylation, H4 acetylation, and H3 K4 methylation, but disfavored integration in regions of transcription-inhibiting modifications, which include H3 K27 trimethylation and DNA CpG methylation. The data indicated that epigenetic regulation contributes to integration-site selection.

Art Nienhuis described an innovative strategy to reproduce the retroviral insertion event that occurred in one of the patients from the French trial. Nienhuis's group used an rAAV vector to integrate an oncoretroviral LTR driving a green fluorescent protein (GFP)-expression cassette with flanking loxP sites into the first intron of the *LMO2* gene in a human lymphoblastic cell line. The workers found that the *LMO2* gene was strongly activated upon LTR insertion. Cre-recombinase-mediated

cassette exchange was used to replace the original LTR-GFP cassette with one flanked by the cHS4 insulator element, leading to a several-fold reduction in *LMO2* activity. These data are consistent with recent results demonstrating suppression of clonal dominance in Jurkat cells by addition of the cHS4 insulator to a lentiviral vector containing an oncoretroviral LTR. The LTR-GFP cassette was also replaced with a globin-gene regulatory cassette, which failed to activate the *LMO2* gene in lymphoid cells. This same cassette was shown, in separate experiments, to have the capacity to activate nearby genes in primary erythroid cells (Phillip Hargrove and Derek Persons, manuscript submitted). The results indicate that regulatory elements vary in their capacity for proto-oncogene activation, that insulator elements reduce that potential risk, and that regulatory elements exhibit tissue specificity with regard to the capacity for gene activation. Mike Themis wrapped up the session with an update on his studies showing oncogenesis following lentivirus gene transfer to fetal and neonatal mice.

The second session, chaired by Mark Kay, examined the incidence of clonal dominance and the pattern of vector integration in gene-modified hematopoietic stem cells. Cindy Dunbar updated her group's experience studying retroviral vector insertion events and consequences in the rhesus macaque autologous transplantation model. Nine animals with 14 inserts into the *mds1/evl1* locus continue to be followed, with no leukemic events to date. As presented in an oral session in the preceding ASH meeting, extending *ex vivo* expansion of transduced CD34⁺ cells to 10 days instead of 4 days resulted in an markedly increased representation of clones with *mds1/evl1* insertion contributing to the myeloid lineage long term, supporting the hypothesis that insertions activating this locus are immortalizing events that may convert short-term repopulating myeloid progenitors into long-term repopulating cells. She also presented data supporting the engraftment of lineage-restricted transduced myeloid and lymphoid progenitors for at least 6 months following transplantation in the non-human primate model.

Fulvio Mavilio presented an analysis of γ -retroviral and lentiviral vector integra-

tion in human cord blood- and bone marrow-derived CD34⁺ hematopoietic progenitors that had been transduced *in vitro* and analyzed without selection. Retroviral vectors showed a twofold higher propensity to target gene-dense regions, highly active genes, and promoter-proximal regions, although the biological relevance of such a change remains unclear. However, retroviral—but not lentiviral—integration occurs at high frequency (>20%) at hot spots that are significantly enriched in proto-oncogenes, cancer-associated CISs, and growth-controlling genes, suggesting that the clonal selection observed *in vivo* in humans and animal models may be at least favored by the high frequency of insertions with such characteristics. On the contrary, the lower propensity of lentiviral vectors for integration in potentially dangerous regions of the human genome may suggest a preferred safety profile for gene therapy applications. Interestingly, a network-based pathway analysis indicated that a significant number of genes targeted by γ -retroviral integration are functionally linked in regulatory networks, with genes involved in hematopoietic and immune system development targeted at particularly high frequency. The results suggest that the gene expression program of a cycling hematopoietic cell may be key in directing retroviral integration into the human genome.

Hans-Peter Kiem's group has explored chemoprotection of gene-modified cells with mutant forms of methylguanine methyltransferase (MGMT) so as to increase the efficacy of clinical hematopoietic stem cell gene therapy in the canine model. The therapeutic benefits of MGMT gene-modified cells are due to two factors: (1) *in vivo* selection following transplantation to increase the level of cells that carry MGMT and a therapeutic transgene for genetic diseases or inhibitory transgenes for acquired diseases such as AIDS and (2) hematopoietic protection from chemotherapy agents such as BCNU and temozolomide (TMZ) during treatment of malignant diseases. Kiem presented data establishing the durability of MGMT gene-modified cells and also presented evidence that true hematopoietic stem cells were transduced. The data further demonstrated that MGMT gene-modified cells repopulated a primary recipient and

subsequently survived multiple rounds of *in vivo* selection while maintaining engraftment and repopulation potential in a secondary recipient.

The third session, chaired by Dave Williams, examined the latest developments in vector design. Mark Kay discussed recent work from his lab that uncovered a different type of vector toxicity—in this case likely due to the overloading of the endogenous miRNA processing system following delivery of an AAV vector that expresses an shRNA targeting a region of the hepatitis B genome in a mouse model of that disease. Kay stressed that overcoming such problems will require better vector design incorporating weaker promoters driving expression of shRNAs, so as to better titrate the expression of the shRNA such that it is within a therapeutic range but does not overwhelm the endogenous RNA processing machinery.

Punam Malik discussed the role of lentiviral *cis*-elements that impart high titer and expression to relatively simple expression cassettes, such as GFP, and the large, relatively complex expression cassette such as the β -globin gene and the locus control region. She further discussed the role of chromatin insulator elements in reducing clonal variegation and improved expression of lentiviral vectors in secondary and tertiary mice. The latter, combined with the enhancer-blocking effects of chromatin insulators presented by Nienhuis, underscores the importance of chromatin insulators in vector safety and design for gene therapy hemoglobinopathies, in which a high-level, consistent expression is necessary for a therapeutic effect.

Jack Lenz presented a test of the ability of an insulator element to block insertional activation of oncogenes and tumorigenesis by a replication-competent γ -retrovirus in mice. Enhancer-blocking insulators offer the hope of preventing strong enhancer elements in a gene therapy vector from activating promoters of host oncogenes. A single copy of the FII fragment of the chicken hypersensitive site 4 (cHS4) insulator was inserted near the end of the LTR of a T-cell lymphomagenic murine leukemia virus. The insulator was unable to block activation of multiple oncogenes and caused no significant reduction in lymphomagenesis. Although the use of a single cHS4 FII insulator is probably insufficient to improve

the safety of insertional gene therapy vectors, Lenz suggested that the use of multiple copies of the insulator in tandem might be more effective. The important aspect of the experimental approach used was that it examined oncogene activation on a genome-wide basis in aggressive, metastatic, virally induced tumors.

The fourth session, chaired by Hans-Peter Kiem, provided an update of ongoing clinical trials in gene therapy. David Williams reported on an initial patient enrolled in a phase I gene therapy trial for children with high-risk brain tumors using a 6-benzylguanine (6-BG) resistant mutant of MGMT. The trial, developed from two previous platform trials, attempts intrapatient dose escalation of TMZ in combination with 6-BG to improve therapy in brain tumors and test the hypothesis that transduced hematopoietic stem cells can be selected in humans. The first patient showed excellent marking of *in vitro* assayed CD34-derived colonies and NOD/SCID repopulating cells, but failed to show significant marking or evidence of selection *in vivo* in the patient. The trial is ongoing with amendments to enhance preservation of stem cell functions during *ex vivo* manipulation of target cell populations.

Adrian Thrasher provided an update on the treatment of 10 children with X-SCID by gene transfer to bone marrow CD34⁺ cells using a gibbon ape leukemia virus–pseudotyped retroviral vector. No serious adverse effects following infusion of genetically modified cells have been observed. All patients developed substantial immunity, with a normally complex T-cell spectratype, although in one patient recovery was partial. Clinical response has been excellent, and all are leading normal lives at home. Thrasher's group has also treated one adult patient with failing immunity following allogeneic transplantation for classic X-SCID in infancy. Although gene transfer was successful, they did not observe improvement in immunological parameters more than a year after infusion of cells, suggesting that there are age-related restrictions to the efficacy of stem cell or gene therapy that may reflect inability to retrieve thymic function. In another study, three children with adenosine deaminase (ADA)-deficient SCID were treated using non-myeloablative conditioning with melphalan after discontinuation of PEG-ADA, resulting in good immune recovery in two

of the patients. Levels of multilineage gene marking indicated that significant stem cell engraftment occurred.

Alessandro Aiuti presented an update on his ADA-SCID gene therapy clinical trial. All the children treated with retrovirally transduced CD34⁺ cells are well and no adverse events related to gene transfer have been observed, with the longest follow-up at 6 years after gene therapy. A comparison of the insertion sites retrieved *in vivo* from patients with those identified in transduced CD34⁺ cells before infusion showed no skewing in the profile of genome distributions or in the genes targeted by the vector. Of note, shared integration sites were observed among multiple hematopoietic lineages, with polyclonal integrations in patients' T cells and oligoclonal integrations in myeloid cells.

Manuel Grez presented an update of the CGD clinical trial. He reported that one patient (P1) died 27 months after gene therapy, from severe sepsis that led to multiorgan failure and acute respiratory syndrome. Although gene marking was still high at day +820, the level of superoxide production by gene-marked cells declined markedly with time, suggesting vector silencing due to methylation of the viral LTRs. P2 and P3 are in good condition, although the level of superoxide production by gene-modified cells is also low in both patients at 31 and 19 months, respectively.

The fifth session included talks by Carolyn Wilson of the FDA and her European counterpart, Klaus Cichutek, on the issue of regulatory oversight and long-term follow-up of gene therapy clinical trials. Wilson reviewed the recently revised publication of the FDA's "Guidance for Industry: Gene Therapy Clinical Trials—Observing Subjects for Delayed Adverse Events" (available at <http://www.fda.gov/cber/gdlns/gtclin.htm>). Dr. Wilson reviewed the scientific bases for concerns regarding long-term risks associated with gene therapy clinical trials, as well as the specific decision tree provided in the guidance for a sponsor to use to determine whether long-term observations should be performed in a particular clinical trial. Finally, she reviewed the special considerations in the guidance for the clinical use of integrating vectors, especially when used to transduce target cells with high replicative capacity and long survival.

The final session, chaired by Punam Malik, discussed ways of preventing vector-associated leukemia. James DeGregori discussed work from his lab that supports a hypothesis that the risk of leukemogenesis increases with age. Age is the single most important prognostic factor in the development of many cancers, including chronic myelogenous leukemia. The major reason for this is thought to be the progressive accumulation of oncogenic mutations with age. DeGregori proposes that the functional status, or fitness, of the hematopoietic compartment substantially impacts the evolutionary outcome of cells with an oncogenic mutation. Using mouse bone marrow transplantation models, they have shown that the selective advantage conferred by the Bcr-Abl translocation is much stronger in a functionally impaired, aged stem cell environment as compared with a younger stem cell pool. Because young progenitors can prevent Bcr-Abl-mediated leukemias initiated in old progenitors, increased leukemogenesis in the aged background seems to result largely from reduced competitiveness. Similarly, chemotherapeutic or genetic impairment of cellular replication in mice also promotes Bcr-Abl-dependent leukemogenesis. Thus, reduced cellular fitness within hematopoietic progenitor cell pools can select for adaptive oncogenic events (such as Bcr-Abl) and thereby promote leukemia, demonstrating the importance of healthy competition in the prevention of cancer.

Chris Baum next discussed work from his lab demonstrating the generation of clonal dominance by retroviral vector insertions in the vicinity of proto-oncogenes or other signaling genes during both normal and malignant murine hematopoiesis. With the aim of developing rational approaches to prevent this risk, Baum's group has designed experimental systems based on "wild-type" C57BL6/J mice. They have also developed a cell-based *in vitro* assay that shows a good correlation with results obtained *in vivo*. In the *in vitro* model, SIN γ -retroviral vectors with a human cellular promoter were much less transforming than SIN or LTR vectors using retroviral enhancer-promoters. The hope is that a combination of rational vector design with additional strategies of cell purification and culture modifications might reduce or

eliminate the risk of insertional transformation in clinical trials.

Gerard Wagemaker presented work analyzing integration sites over time in bone marrow cells in primary and secondary recipient mice that received bone marrow cells transduced with a murine leukemia virus retroviral vector. The results showed that 81% of the retrovirus integrations occurred near genes expressed in mouse hematopoietic stem cells and that, in addition, the probability of integration was strongly correlated with the expression levels of the surrounding genes. They also found that genes upregulated by growth-factor stimulation did not display a higher frequency of retroviral insertion than other expressed genes. An initial network-based analysis demonstrated that virus integrations clustered near genes involved in cancer, cell cycling, signal transduction, connective tissue development and function and, in line with the targeted stem cells, hematopoietic development and function. Thus, retroviral vector integrations in cells capable of long-term hematopoietic reconstitution occur preferentially near highly expressed genes with specific functions in immature hematopoietic stem cells.

Brian Sorrentino described a new model system that recapitulated many key aspects of insertional oncogenesis that were observed in the French X-SCID trial. This mouse model was null both for the γ c gene and for the *Arf* tumor-suppressor gene. These animals displayed the X-SCID phenotype and were highly tumor prone by virtue of the *Arf* mutation. Bone marrow cells from these mice were transduced with an oncoretroviral vector expressing the γ c gene that was very similar to the vector used in the French trial. Upon transplantation into irradiated, wild-type recipients, approximately 90% of the mice developed T-cell lymphomas by 52 weeks. Integration-site analyses of DNA from tumor samples revealed multiple instances of insertions near or within known oncogenes. In some cases, identical insertion events were noted in multiple tumors from different mice, indicating that outgrowth of a single clone had occurred *in vitro* during the transduction phase. When the γ c vector was used to transduce bone marrow cells from mice that were wild-type for the γ c allele, but that contained the

Arf mutation, significantly lower rates of tumor formation were noted. This result indicates that the X-SCID background conferred increased risk for tumor formation, possibly due to alterations in the bone marrow cell population of X-SCID mice. This observation suggests that the risk for insertional mutagenesis in X-SCID is elevated and that this risk may be lower in gene therapy approaches for other disorders. This model is now being used to test the relative transformation rates of safety-modified vectors, including lentiviral vectors that express the γ c gene from internal cellular promoters, and vectors that contain chromatin insulators.

In the final talk of the retreat, Luigi Naldini described a recently published *in vivo* genotoxicity assay, based on transplantation of tumor-prone *Cdkn2a*^{-/-} hematopoietic stem cells, which are very sensitive to genotoxic stress, treated with retroviral and lentiviral vectors. Retroviral vector treatment significantly accelerated tumor onset in the transplanted mice. Proto-oncogenes were targeted at high frequency in the cells before transplantation, and integrations at these sites were further enriched in the tumors and associated with earlier mortality. On the other hand, lentiviral vector treatment did not cause tumor acceleration and did not target preferentially any gene class. This model is now being used to dissect the role of different genetic elements on vector insertional oncogenesis and to understand how significantly they influence the vector safety profile. Preliminary data indicate that transcriptionally active LTRs, rather than promoter strength, are the major risk factor in insertional oncogenesis. Naldini also presented data on a new technological platform based on integrase-defective lentiviral vectors and zinc-finger nucleases that allows the correction of point mutations at selected genes by exploiting the homologous recombination pathway. A major advantage of this approach is that it restores not only the function but also the endogenous expression control of the affected gene. Moreover, he showed that a similar platform could be used to precisely integrate transgenes into a desired location of the genome at high efficiency and feasibility in a variety of cells, thus alleviating the concerns associated with random insertional mutagenesis.

Taken together, the data presented at the retreat provided increasing insight into both the mechanisms and context dependence of the transformation of cells due to insertional mutagenesis by gene transfer vectors. It is of interest that there have been no clonal proliferations in the X-SCID gene therapy trial in London, despite the fact that the sequence of the integrated vector is nearly identical to that used in the French trial in which insertional leukemogenesis

has now been observed in a fourth patient. This latter observation supports further the idea that other protocol-specific factors contributed to the development of transformed cell clones in the French trial. While it is clear that many gene transfer vectors can be mutagenic, the number of cases of tumor development in patients and in animal models remains comparatively low. Clearly, this demonstrates that a better understanding of the confounding factors

that predispose a cell to transformation following insertional mutagenesis will be key to learning how to minimize even further the arguably low risk of insertional oncogenesis in future gene transfer trials.

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