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Recent Advances and Improvements in the Biosafety of Gene Therapy

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1. Introduction

1.1 Overview of gene therapy

Progress in understanding the cellular and molecular bases of human health and disease in recent decades has spawned research in the fields of regenerative medicine and gene therapy. These novel approaches to medical treatments offer new possibilities of mitigating, and even curing, a plethora of medical conditions ranging from rare inherited monogenic disorders, metabolic diseases, infections and even complex disorders such as cancer.

In a simplified form, gene therapy can be defined as any procedure aimed at genetically altering or modifying cells or tissues with exogenous genetic materials that encompasses RNA, DNA and even oligonucleotides. These molecules may be directly delivered, *in vivo* into patients, often with the goal of targeting particular tissues (or organs). Alternatively, patients' cells may be isolated, expanded and modified *ex vivo* before reimplantation into the same subject (figure 1).

Whilst gene therapy appears to be a relatively new concept in the field of biomedicine, the original conceptualization of treating diseases by genetic engineering dates back as early as the 1940s. Avery, MacLeod and McCarthy pioneered the notion and demonstrated that genes could be transferred within nucleic acids (Avery et al., 1944). Early visionary investigators such as Tatum (Tatum, 1966) envisioned " that viruses will be effectively used for man's benefit, in theoretical studies in somatic-cell genetics and possibly in genetic therapy..." And at the end of that same decade, the earliest experimentation of gene delivery in humans was carried out controversially by Rogers and colleagues, who explored the idea of using Shope papilloma virus to treat three patients with arginase deficiency (Wolff & Lederberg, 1994). The decades that followed witnessed tremendous advances in recombinant DNA technology and enabled the first approved human gene therapy clinical trial in 1990 for treating infants with adenosine deaminase deficiency (Blaese et al., 1995). By the turn of the millennium, almost 4000 patients had received gene therapy from more than 500 clinical trials worldwide (Scollay, 2001), albeit with varying and limited successes. Nonetheless, these trials were helpful in highlighting several aspects of gene therapy that demanded improvements and refinements to achieve meaningful therapeutic efficacy and patient safety.

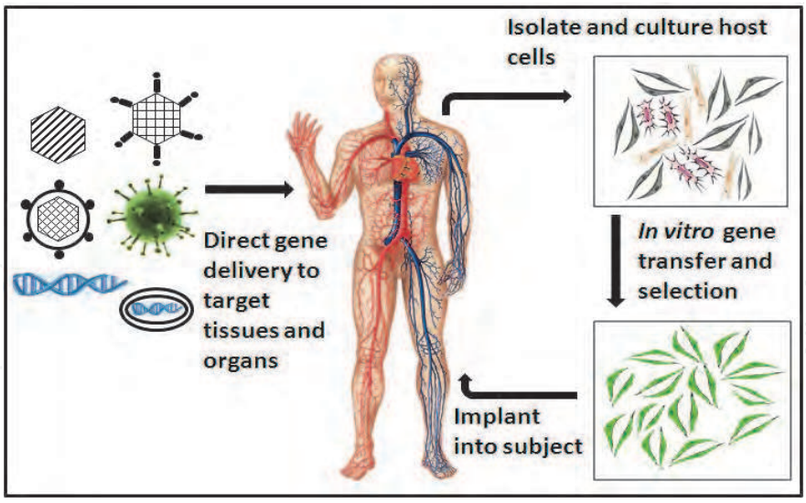

Fig. 1. Gene-based therapy. Left: *In vivo* administration of vector to modify cells in target organs or tissues directly. Right: *Ex vivo* modification of primary somatic cells that are reimplanted into the same subject (autologous cell therapy). Viral or non-viral vectors may be used to deliver transgenes.

Gene delivery can be achieved using either viral vectors or non-viral vectors. The latter may be episomally maintained or integrated into the host genome. To date, five main classes of viral vectors have been tested for clinical applications. These include retroviruses (RV), adenoviruses, adeno-associated viruses (AAV), lentiviruses and herpes simplex viruses (HSV) (Walther & Stein, 2000). Non-viral vectors most often utilize plasmid DNA which can be delivered into cells or tissues by physical methods such as electroporation, gene-gun bombardment, sonoporation, hydrodynamic injection or by chemical methods that utilize calcium phosphate, polymeric carriers, cell-penetrating peptides, cationic and anionic lipids (Niidome & Huang, 2002).

Gene therapy was initially conceptualized as ideal treatment for monogenic disorders such as adenosine deaminase, alpha-1-antitrypsin, ornithine transcarbamoylase and clotting factor (factors VIII and IX) deficiencies. These were considered ideal candidates as reconstitution of the missing protein in each case should alleviate or abolish the disease phenotype. The spectrum of gene therapy applications has now broadened considerably to every area of molecular medicine to include restoration of cellular and metabolic functions in various diseases, immuno-reconstitution of tumor cells in cancer immunotherapy, targeted cancer cell ablation in suicide gene therapy, treatment of infectious diseases, genetic manipulation, reprogramming of cancer and stem cell fate, reversing degenerative vascular and brain disorders, to name just a few.

Although gene therapy is conceptually appealing, the high hopes of translating such treatments into standard clinical practice has yet to be fulfilled, in part as initial enthusiasm from a few clinical successes have been marred by adverse, and even fatal, iatrogenic complications in a limited number of treated patients. Reactions to these sentinel events reiterate the need to understand and evaluate the genotoxic risks for any given gene therapy approach and for pertinent biosafety improvements to be incorporated into current treatment modalities. This chapter reviews current improvements to gene therapy with a focus on biosafety and highlights the essential advances and developments that could garner greater clinical acceptance for gene therapy applications.

1.2 Gene therapy clinical trials- successes and adverse outcomes

As of 2010, the Wiley Journal of Gene Medicine clinical trials database reported a total of 1644 gene therapy clinical trials, the majority (64.5%) of which were directed at cancer and related diseases (last accessed on 7th February 2011) (figure 2) . Given the greater depth of understanding of molecular virology, the broad tropism of viral vectors and their superior efficiencies of gene transfer, transgene delivery *via* viral vectors has been the favoured and most feasible option (67 % of all trials).

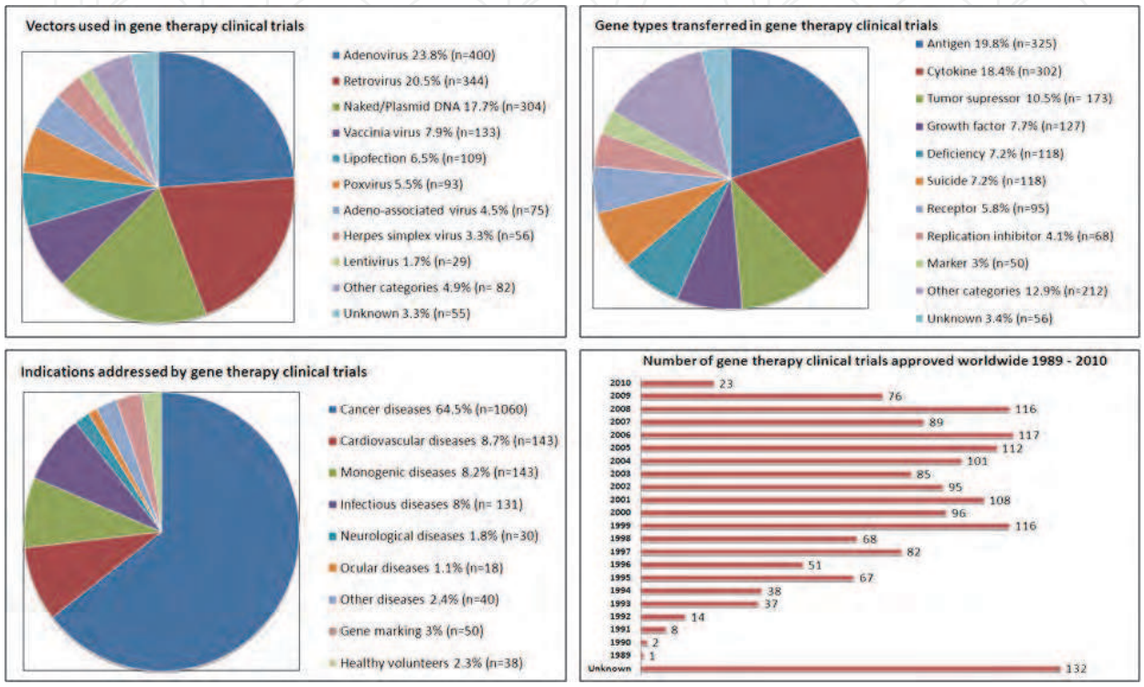

Fig. 2. Characteristics of clinical gene therapy trials. Categorisation of gene therapy trials according to indications, vectors used, gene types transferred and annual number of approved trials. (*Images reproduced from Journal of Gene Medicine clinical trials database (http://www.wiley.com/legacy/wileychi/genmed/clinical/)).*

Despite the impressive number of gene therapy trials, it is worth noting that only a small number of these trials reported clinically meaningful and long term outcomes. The first clinical success was for the treatment of X-linked severe combined immunodeficiency (SCID-X1) (Cavazzana-Calvo et al., 2000), a disease characterised by immature development of the immune system due to mutations in the interleukin-2 receptor common gamma chain gene (*IL2Rγ*). Nine of ten treated patients achieved long term immune reconstitution following implantation with gene modified hematopoietic stem cells and marked clinical improvement (Hacein-Bey-Abina et al., 2003). More success stories echoed from similar clinical trials in London, U.K., of the same disorder (Gaspar et al., 2004). In the years that followed, long term therapeutic efficacy was also reported in clinical trials for another form of SCID disorder due to adenosine deaminase deficiency (SCID-ADA) (Aiuti et al., 2009). In 2006, gene therapy scored more successes when impressive results were reported in two patients treated for X-linked chronic granulomatous disease (CGD) (Ott et al., 2006), caused by inactivating mutations of gp91^{phox} (*CYBB*) gene and characterised by neutrophil

dysfunction and recurrent serious infections. More recent and notable clinical success has been reported for gene therapy of Wiskott-Aldrich syndrome (Boztug et al., 2010), X-linked adrenoleukodystrophy (Cartier et al., 2009), Leber's congenital amaurosis and Parkinson's disease.

Although these impressive clinical outcomes provided incontrovertible proof-of-principle, it soon became evident that treatment benefits could occur in tandem with significant adverse effects when serious iatrogenic complications were reported in a small number of patients. The first gene therapy death was reported in 1999 from an ornithine transcarbamoylase trial conducted at the University of Pennsylvania. This was ascribed to a massive immune response to the adenoviral vector used in that trial (Raper et al., 2003). Gene therapy suffered the heaviest blows in the years 2003 to 2006, and attracted close scrutiny by regulatory authorities and the medical fraternity when five successfully treated SCID-X1 patients (from two different clinical trials) developed T-cell lymphoblastic leukemia, three to six years after treatment with autologous bone marrow-derived CD34+ hematopoietic cells transduced with a murine leukaemia virus (MLV) gammaretroviral vector to express the *IL2Rγ* gene (Howe et al., 2008; Hacein-Bey-Abina et al., 2008). Random integration of the MLV gamma retroviral vector that had strong enhancer elements in the long terminal repeat (LTR) regions resulted in the insertional activation of LIM domain only-2 (*LMO2*) proto-oncogene. This mutagenic event likely promoted clonal proliferation of T cells that culminated in acute lymphoblastic leukaemia. In a different trial in 2007, Targeted Genetics Corporation was forced to halt its gene therapy trial for rheumatoid arthritis involving intra-articular injection of an adenoviral vector expressing *tgAAC94*, following the death of a patient. In this case however, investigations by the US Food and Drug Administration (FDA) exonerated gene therapy as the direct cause of death (Frank et al., 2009), although there was evidence of vector-induced immune response; and the trials have since recommenced. The inherent risks of insertional mutagenesis by viral vectors surfaced again in another clinical trial in the year 2006 for treatment of CGD. Two adult CGD patients infused with granulocyte colony-stimulating factor (*G-CSF*)–mobilized peripheral blood CD34+ cells transduced with MLV gammaretroviral vector expressing $gp91^{phox}$ had markedly improved neutrophil functions and resistance to life threatening infections. Regrettably, both subjects later developed myelodysplasia and one subject died from this complication (Stein et al., 2010). Myelodysplasia probably developed from random integration of the gammaretroviral vector that activated the expression of a proto-oncogene, *MDS-EVI1*) (Stein et al., 2010). As of this writing, the most recent case of adverse gene therapy outcome, brought to light by the American Society of Gene and Cell therapy (http://www.asgct.org/media/news-releases/?c=505) affected one of ten Wiskott-Aldrich syndrome patients treated at the Hannover Medical School using a gammaretroviral vector similar to that used in the SCID-X1 trials. This patient was reported to have developed leukaemia. A comprehensive clinical evaluation of this adverse event is yet to be disclosed.

In summary, there is clear evidence that gene therapy can be clinically effective. Moreover, it offers the only treatment for certain serious life threatening diseases that are currently untreatable or poorly treated. An important issue that must be addressed if gene therapy is to mature from experimental treatment to standard of care is that of biosafety. The occurrence of serious iatrogenic outcomes, albeit uncommon, has brought into sharp focus the inherent risks of genetic modifications.

2. Biosafety considerations of gene therapy

2.1 Genotoxicity potential of gene therapy

The potential for genotoxicity in gene therapy is not unexpected. Initial studies investigating the integration site preferences of different viral vectors such as human immunodeficiency virus, avian sarcoma-leukosis virus and MLV gammaretrovirus, drew attention to the potential for insertional mutagenesis arising from random or quasi-random genomic integrations, aggravated by the marked propensity of these vectors to target transcription start sites and active genes (Mitchell et al., 2004). Even before reports of adverse events surfaced in clinical trials, a 2002 retroviral gene marking study in murine bone marrow cells already reported a high frequency of vector-induced hematopoietic disorders, including leukemia, caused in part by insertional activation of an oncogene (Li, Z. et al., 2002). Different strategies are being actively explored to reduce the genotoxic potential of current viral vectors. The main focus areas are to devise methods for: (a) appropriate tissue targeting of systemically delivered vectors, (b) disabling the capacity for generating replication competent viruses; (c) mitigating immune responses to vectors and/or transgene products; (d) avoiding germ-line modifications; (e) preventing unintended vector dissemination; and (f) directing the integration of transgenes into genomic safe harbors.

2.2 Insertional mutagenesis

Insertional mutagenesis refers to the induction of deleterious mutations to genes, promoters, enhancers or other regulatory elements that alter gene expression as a consequence of exogenous vector integration into the genome. Although a major concern of integrating vectors, even non-integrating vectors have a low but finite possibility of random genomic integration (Wang, Z. et al., 2004).

Prior to cases of gene therapy induced oncogenesis in recent clinical trials, the risk of malignant transformation from integrating vectors was considered theoretically plausible but unlikely to occur in practice. With hindsight, treatment-induced malignancies could have been predicted on the basis that as many as 1% of genes encoded in the genome are implicated in one or more forms of cancer (Futreal et al., 2004). Although oncogenesis is a process that requires multiple genetic hits, random integration of vectors into multiple genomic sites could sufficiently generate the right "cocktail" of aberrations in different oncogenes and/or tumor suppressor genes (Hanahan & Weinberg, 2000), Moreover, as the formerly regarded gene deserts are now known to be richly populated with different classes of non-protein-coding RNAs with key roles in cellular maintenance and cancer development (Farazi et al., 2011), evaluation of genotoxic risk of integration events requires extra caution.

Viral vectors do not integrate randomly but have a propensity for transcriptionally active units and transcription start sites in mammalian cells (Mitchell et al., 2004). Such studies have been instrumental in developing integration maps or profiles of the different viruses, highlighting their potential risks based on their propensity to integrate near transcription start sites, into active transcriptional units, close to oncogenes or tumor suppressor genes. Even disruptive integrations into other genes such as those necessary for cell survival or metabolism may be deleterious. Thus, insertional mutagenesis is a real risk that needs to be seriously addressed rather than being dismissed as inconsequential as was the attitude prior to reports of adverse gene therapy clinical outcomes.

Much has been learned about the molecular pathogenesis of oncogenesis associated with integrating viral vectors. MLV gammaretroviral vectors have a predilection for integrating

close to transcription start sites (Mitchell et al., 2004) and to perturb their expression possibly due to the strong enhancer effect inherent in the LTRs (Modlich et al., 2006). However, this effect alone may not be sufficient for complete oncogenic evolution as a clinical trial for SCID-ADA in ten patients treated with a similar MLV retroviral vector reported no untoward outcomes (median duration of follow-up of 4 years) (Aiuti et al., 2009). This has led to the speculation that other factors such as the nature of the expressed transgene (*IL2Rγ versus ADA*), the underlying disease, the cell types selected for transgenic modification and other patient-specific intrinsic factors could be necessary accessory factors to oncogenesis.

In contrast to retroviral vectors, no overt adverse events have been reported thus far from the use of other viral vectors such as lentiviral, adenoviral, HSV or AAV vectors. Some studies even suggest that lentiviral vectors pose significantly lower risks of insertional oncogenesis compared to retroviral vectors due to differences in their integration preferences (Montini et al., 2006). Generally, non-integrating vectors such as adenoviruses, recombinant AAV and HSV which are predominantly maintained as episomes are not considered to be mutagenic given their minute possibility of inducing rare random integrations in the genome. On the other hand, AAV which can integrate into the AAVS1 locus in the presence of viral proteins Rep68 or Rep78 (Smith, 2008), must be considered as having intermediate risks.

2.3 Tools for evaluating potential for genotoxicity

The reality of vector-induced oncogenesis need not be a fatal impediment to the goal of clinical gene therapy. Tools are now available to interrogate transgenically-modified cells *ex vivo* for undesirable genomic alterations and to evaluate tumorigenic potential. The ability to perform comprehensive biosafety assessments *ex vivo* before *in vivo* treatment could be a feasible approach to exploit the benefits of gene replacement while minimizing treatment risks to a clinically acceptable level.

A first step to genotoxicity analysis of any given modality would be to review databases for adverse outcomes encountered in past or ongoing clinical trials which can be accessed at several websites e.g. Wiley clinical trials database (http:// www.wiley.com/ legacy/ wileychi/genmed/clinical/), the US National Institutes of Health ClinicalTrials.gov (http://www.genetherapynet.com/clinicaltrialsgov.html) and Clinigene (http:// www.clinigene.eu/search-published-human-gene-therapy-clinical-trials-database/).

This section focuses on the biosafety assessment of *ex vivo* gene modified cells, with an emphasis on key features to monitor and molecular biology tools that aid the evaluation. The importance of bioinformatic tools in biosafety evaluation cannot be overemphasized. This section will also highlight useful programs, internet resources and databases.

2.4 Mapping genome integration sites

It is imperative to document integration events in gene modified cells, and prudent to do so even for episomal vectors that have a low probability of random integration (Stephen et al., 2008; Wang, Z. et al., 2004). Integration events are detailed with reference to their physical distance relative to promoter sites, transcription start sites, exons or introns, oncogenes, tumor suppressor genes, non-protein coding genes, CpG islands, repetitive elements and transcription factor and micro-RNA binding sites. Such integration profiles aid genotoxicity risk evaluation when comparing across vector types, modified cell types and the nature of transgenes.

Integration events within cells can be experimentally retrieved and identified by plasmid rescue, ligation mediated PCR (LM-PCR) (Laufs et al., 2003), inverse PCR (Silver & Keerikatte, 1989) or linear amplification mediated PCR (LAM-PCR)(Schmidt, M. et al., 2007). Sequence data can be analyzed for vector-flanking sequences by programs such as IntegrationSeq (Giordano et al., 2007) which may then be queried using programs such as NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/) or UCSC-BLAT (http:// www.genome.ucsc.edu/) to identify their genomic positions (figure 3). In recent years, several programs have been developed to automate the process of genome mapping. IntegrationMap (Giordano et al., 2007), SeqMap (Peters et al., 2008) and QuickMap (Appelt et al., 2009) are examples of web-based programs that are useful for annotating genome mapping information such as proximity to genes, neighbouring gene identity, exon/intron localization, distance from transcription start sites, repeat element localization and Gene Ontology functions. QuickMap (http://www.gtsg.org), most recently developed, provides a more comprehensive evaluation which includes information about proximity to oncogenes, pseudogenes, CpG islands, fragile sites, transcription factor and micro-RNA binding sites. Identity of potential cancer genes can be derived from lists compiled from the human cancer gene census (Futreal et al., 2004) or the retroviral tagged cancer gene database, RTCGD (http://rtcgd.abcc.ncifcrf.gov, mouse cancer genes). Another useful database with a comprehensive compilation of known oncogenes and tumor suppressor genes (Wang, G.P. et al., 2008) can be accessed at the following website (http:// microb230.med.upenn.edu/protocols/cancergenes.html) hosted by the University of Pennsylvania School of Medicine.

Another useful aspect of genomic profiling of integration sites is its application for the long-term monitoring of the clonality of *in vivo* implanted gene modified cells (Wang, G.P. et al., 2010). Integration profiles of gene modified cells determined pre-implantation can be periodically monitored post-implantation to detect the emergence of dominant clones. Deviation from a polyclonal pattern of growth could imply selection of a dominant clone of cells by virtue of a growth advantage or a greatly increased proliferation rate. This ought to alert close scrutiny for the likelihood of insertional oncogenesis. Gerrits et al. have recently demonstrated the use of tagged vectors with variable barcode signatures to track different clones *in vivo* (Gerrits et al., 2010). Such innovative techniques could be applied to enhance monitoring the clonality of implanted cells *in vivo* and increase the sensitivity of detecting potential oncogenic alterations.

2.5 Characterizing the modified genome

There are relevant concerns that integrating and non-integrating vectors can potentially alter the genomic architecture of cells. Copy number gains and deletions have been observed in transformed cancer cell-lines and to a lesser extent on cells treated with gene therapy vectors (Stephen et al., 2008). Recent advances to the array based technology have made it possible to study amplifications or deletions to the genome at very high resolutions with probes that span the genome on average at 2.5 kb intervals (Hester et al., 2009). As with most array based techniques, copy number analysis relies on a relatively homogeneous population of cells as events in a minor population of polyclonal cells may be masked or under-represented in the analysis that would otherwise highlight the effects that are observed in the dominant population of cells.

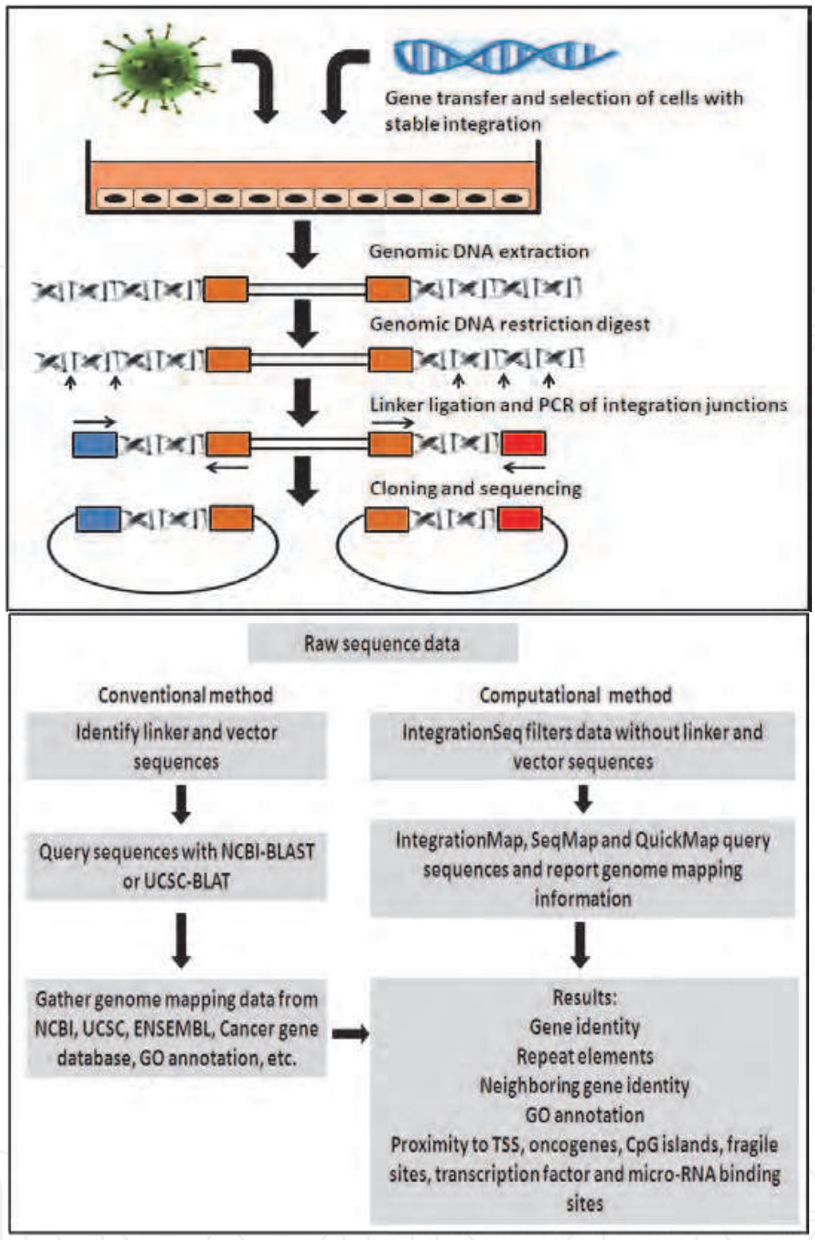

Fig. 3. Experimental recovery of integration events and computational analysis of integration site distribution in mammalian cell genomes. Top: Integration events in cells may be retrieved by digesting genomic DNA (with restriction enzymes that do not cleave within the vector sequences). Appropriate adapters are ligated to restriction fragments to serve as priming sites for PCR amplification of integration junctions which can then be cloned and sequenced. *(Adapted from Ciuffi et al., 2009.)* Bottom: Vector flanking raw sequence data may be selected with programs such as IntegrationSeq and queried using UCSC-BLAT or NCBI-BLAST to retrieve relevant genomic information. Computational programs such as IntegrationMap, SeqMap and QuickMap automate the process of genome mapping and provide the necessary genomic information required for biosafety assessment. (*Adapted from Peters et al., 2008.)*

Another serious genomic alteration that can be mediated by gene therapy vectors is chromosomal aneuploidy and/or gross structural abnormalities such as deletions and translocations, which are common hallmarks of transformed cells. Several studies have reported unexpected but rare cytogenetic abnormalities in cells treated with AAV (Miller, D.G. et al., 2005), retroviral vectors (Modlich, 2005) and non-viral vectors such as phiC31 phage integrase-mediated plasmid integration (Liu, J. et al., 2006). The inciting causes of such cytogenetic abnormalities are unclear, namely whether from direct effects of vector integration and repair or from recombination events secondary to vector integration.

Gross chromosomal rearrangements in gene modified cells can be evaluated by spectral karyotyping or multi-color FISH. However, karyotyping requires that a sufficient number of metaphases should be examined if rare rearrangements are not to be missed. Array based comparative genomic hybridization detects copy number abnormalities (deletions or amplifications) at high resolution, provided a fairly homogeneous cell population is analyzed. However, even high resolution copy number analysis could not be expected to detect aberrations in a rare subpopulation of cells. Genome sequencing to identify vector integration junctions can potentially identify translocations at high (nucleotide level) resolution provided junctional fragments can be confidently identified. However, this method (currently performed at relatively high cost) generates large datasets that require specialized bioinformatic analysis and awareness of technical artifacts (Koboldt et al, 2010). In conclusion, effective cytogenetic analysis should combine sequencing techniques (for integration site retrieval), multicolor karyotyping, whole genome copy number and possibly deep genome sequencing analysis as a complementary suite of techniques to completely characterize the chromosomes of gene modified cells.

2.6 Transcriptome and epigenome analysis

A necessary complement to genome analyses is to determine effects of gene transfer (however accomplished, but especially if the transgene is known to have integrated) on the transcriptome of gene modified cells. In this regard, it is worth noting that vector insertions are often accompanied by deletions of genomic regions (Miller, D.G. et al., 2002) that may in turn alter the epigenetic status of the cell if key histone proteins or histone modifying enzymes are affected. Thus it may also be relevant to determine effects of gene transfer on the epigenome.

Comparing the global transcriptomes of naïve and vector treated cells may help to identify genes whose expression are perturbed by vector treatment. Many technical platforms based on hybridization to gene-specific oligonucleotide probes are now available for genome-wide transcriptome analysis and, being unbiased, are the method of choice. Such data, in practice, reveals significantly altered gene expression mainly in the dominant cell population, though not necessarily in minor subpopulations. Ideally the transcriptome of a homogeneous or, preferably, clonal population of cells with a single known vector integration is more informative. The presence of multiple integration sites in a clonal population confounds attempts to distinguish effects attributable to any particular integration. Likewise, the study of a heterogeneous cell population would mask the transcriptional features of a minor subpopulation within a mixed culture. Therefore, microarray studies would yield useful information only when a sufficient number of clonal populations from different integration sites are characterized. Given that viral vectors mediate integrations into multiple sites, such clonal studies would be highly impractical. Clonal studies are especially important when integrations have been identified close to

oncogenes. Transcriptome analysis aims not only to identify individual genes with significantly altered expression but should also map individual aberrations to molecular pathways. There is a plethora of non-proprietary microarray analysis and bioinformatic software tools for data evaluation and analysis. For example, useful tools are hosted by groups such as the Gene Ontology (http://www.geneontology.org/GO.tools.microarray.shtml), Genomics and Bioinformatics Group from NIH (http://discover.nci.nih.gov/tools.jsp) and DAVID (http://david.abcc.ncifcrf.gov/home.jsp).

Epigenetic changes refer to the changes in the acetylation, methylation, sumoylation and phosphorylation patterns of histone proteins, which in turn may affect the dynamic chromatin architecture and determine the active or repressed status of genes. It also encompasses changes in CpG methylation status of DNA near promoter regions which may influence gene expression. Transgene integrations may directly attenuate gene expression, have a negative or positive effect on genes based on copy number aberrations of the genome or affect histone modifying enzymes which in turn may affect the epigenetic and gene expression status of cells. Global epigenetic status of cells are presently studied using a combination of global transcriptome analysis, cytosine methylation pattern, nucleosome positioning assay and chromatin immunoprecipitation (ChIP) based assays to determine transcription factor binding sites (Fazzari & Greally, 2010). The on-going human epigenome project (http://www.epigenome.org/) that aims to document the DNA methylation patterns of all human genes is likely to provide invaluable insights into the role of epigenetics in human diseases. However, the study of epigenetics is currently hampered by a lack of simple, high quality and high-throughput techniques. Technical advances should deepen knowledge of this important domain of human genetics.

2.7 *In vitro* and *in vivo* tumorigenicity studies

Transformed cells acquire altered phenotypes that can be detected under *in vitro* conditions to distinguish them from untransformed cells. Anchorage independent growth, loss of contact inhibition, resistance to apoptosis, increased proliferation rate and extended cell passaging are common characteristics of transformed cells.

Simple *in vitro* assays demonstrate the anchorage independent growth and increased proliferation rates of cells. The soft agar colony formation assay involves enumerating colonies (clonal propagation of cells) formed from individual cells in the absence of substrate adhesion. Anchorage independent cells typically form colonies while normal cells do not as they rely on surface attachment for proliferation. Assays that quantify incorporation of bromo-deoxyuridine (BrdU), reduction of tetrazolium compounds (e.g. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and colony formation are direct or indirect measures of cellular proliferation rates. Modlich and colleagues (Modlich et al., 2009) recently introduced another assay, termed the *in vitro* immortalization assay, which tests tumorigenic potential of virally transduced murine hematopoietic stem cells (HSCs) based on their replating capacity, thus obviating the need to use animal models.

Most *in vitro* biosafety assays seek to evaluate deviations from normal cellular characteristics. A more realistic evaluation of tumorigenicity would be to determine the potential to induce tumors *in vivo.* Two main models are used to evaluate the tumorigenic potential either of *ex vivo* modified cells or systemically delivered viral vectors. In the first model, gene modified human cells are implanted into immunocompromised mice that are known to support the engraftment of xenogeneic cells. It is helpful to know that different

strains of immunocompromised mice have different capacities to mount immune responses depending on which components of the immune system are still functional. Mouse strains that are most severely immunocompromised can be expected to have high sensitivity as tumorigenic hosts because low numbers of implanted cells will give rise to visible tumors. Such sensitive models are useful for the detection of rare populations of oncogenic cells in a heterogeneous population of otherwise untransformed cells. The absence of tumor formation should not immediately exonerate cells of their tumorigenic potential. It is essential to establish from immunohistology of the implantation sites or in the case of HSCs implantation, immunocytometric blood analysis that the implanted cells have indeed engrafted *in vivo* in animals that fail to form tumors. The second model is useful to evaluate the genotoxic potential of HSCs transduced with different gene therapy vectors. It is based on the transduction and transplantation of HSCs derived from a tumor-prone mouse model that lacks the tumor suppressor, cyclin dependent kinase inhibitor 2A (*cdkn2a*) gene (Montini et al., 2009). This assay thus evaluates tumorigenic risk in an already tumor-prone cell line and was used to compare the oncogenic potential of retroviral and lentiviral vectors, and to assess the benefits of introducing self-inactivating (SIN) long terminal repeats (LTR) in these vectors. However, a caveat is that due to the intrinsic oncogenic potential of the cdkn2-/- HSCs, the effects of subtle but relevant insertional mutagenic events may be masked or misinterpreted. Besides murine models, long-term studies can also be performed in pre-clinical animals such dogs and non-human primates (Kim, Y.J. et al., 2009) where the clonality of implanted cells can be dynamically monitored by documenting integration profiles of recovered cells to ascertain if dominant clones with clone-specific integration patterns have emerged.

3. Recent developments in biosafety enhancement of gene therapy

Comprehensive molecular studies of adverse outcomes of gene therapy trials have advanced our understanding of mechanisms that likely caused clinical complications. This has, in turn, spurred the development of safer vectors. In parallel, more sensitive experimental techniques for biosafety evaluations enable higher confidence in pre-clinical assessments of biosafety before treatments are implemented in clinical trials. This section reviews recent developments that enhance biosafety of gene therapy.

3.1 Improvements to viral vectors

Gene transfer *via* viral vectors remains the most prevalent choice in clinical trials of gene therapy. Knowledge of genotoxic risks that are inherent in viral life cycles and their biology have guided modifications aimed at improving the biosafety of viral vectors. The basic approaches are summarised in figure 4. They include the use of viral vectors that do not integrate or that do so with a more random and less selective integration spectrum, the inclusion of self-inactivating LTR elements and chromatin insulators to reduce neighborhood effects of integrated vectors on gene expression and the use of cell- or tissue-specific promoters for physiological and tissue-specific gene expression.

3.1.1 Replication defective vectors

Apart from certain oncolytic cancer gene therapies that use conditionally replicating viruses, most clinical applications rely on replication-incompetent viral vectors as virus replication *in*

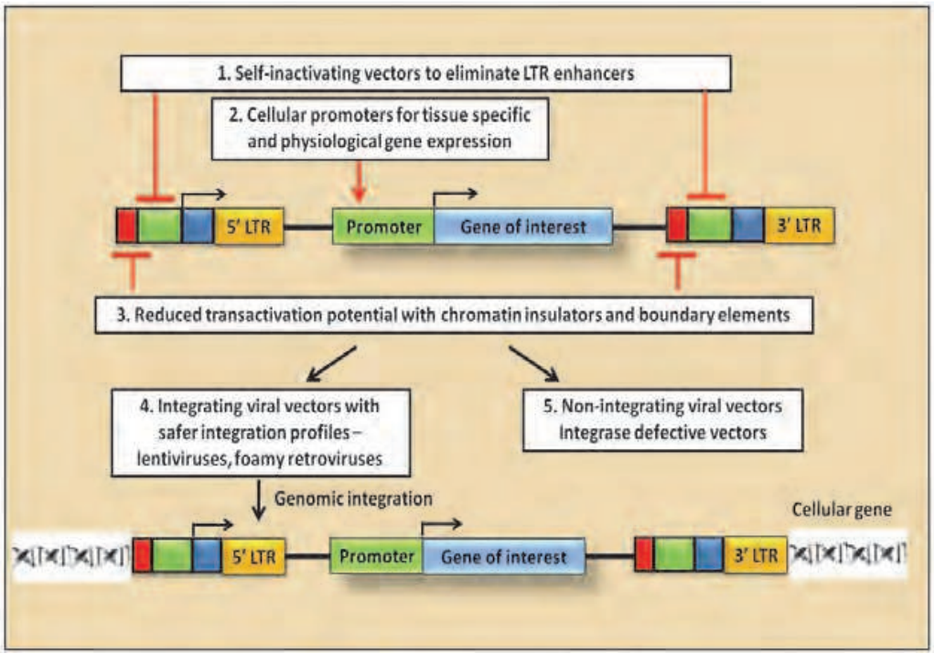

Fig. 4. Strategies to improve biosafety of integrating viral vectors. Integrating viral vectors have been shown to mediate transactivation of genes close to integration sites. This risk can be reduced by using self-inactivating vectors devoid of strong LTR enhancers, cellular promoters, incorporating chromatin insulators and other boundary elements and lastly by the selective use of viral vectors with intrinsically safer integration profiles. *(Adapted from Kohn and Candotti, 2009.)*

vivo could pose a serious health and mutagenic hazard to patients. Replication-defective vectors are generally designed to lack viral genes necessary for their replication and packaging and therefore need to be produced in helper cell lines which provide the necessary components (gag/pol, env and rev, as is the case for HIV-lentivirus) for their packaging *in trans*. Unintended homologous recombination between the replication-defective vector and packaging constructs or endogenous viral sequences in the human genome may potentially cause reversion to a replication-competent virus. Such a risk has been greatly mitigated through improved packaging constructs or packaging cell lines that have little or no homology to the vector that encodes the therapeutic gene to greatly minimise the likelihood of recombination.

Other avenues of improvements have been in the methods used to rigorously screen vector batches for contamination with replication competent viruses. Assays that detect recombination between gene transfer vector and helper vectors based on their known structures are available for detecting replication competent-AAV (Tenenbaum et al., 2003), replication competent adenovirus (Chuah et al., 2003) and replication competent retrovirus (Sinn et al., 2005). However, the development of accurate assays for RCL (replication competent lentivirus) detection has been more challenging due to the difficulty of predicting their genomic organizations. Nevertheless, recent assay developments such as the product-enhanced reverse transcriptase (PERT) assays (Sinn et al., 2005) and combination of p24 ELISA and psi-gag PCR (Cornetta et al., 2010) ought to increase the sensitivity and accuracy of screening for RCL contamination in clinical grades of viral stocks.

In summary, improvements to packaging cell-lines or helper constructs have minimized the risk of reversion to replication competency while improved screening methods should help prevent the unintentional clinical administration of viral vectors contaminated with replication competent viruses.

3.1.2 Self-inactivating vectors

Retroviral and lentiviral vectors have intrinsic promoter and enhancer activities in their LTR regions which have been implicated in the aberrant activation of neighboring genes. This potential to activate oncogenes, combined with other concurrent factors is the basis for insertional oncogenesis. Several studies have shown that the risks of insertional gene activation can be drastically reduced by deleting the strong internal promoter/enhancer elements in the U3 regions of the LTR of retroviral and lentiviral vectors. The concept of self-inactivating (SIN) vector requires the replacement of the U3 regions of the 5′ LTR with a heterologous promoter (such as CMV) and partial deletion of the U3 enhancer elements in the 3′LTR. The process of viral vector integration involves copying of the 3′LTR (now devoid of enhancer elements) to the 5′LTR and deletion of the 5′LTR-CMV promoter. The final result is a transcriptionally inert 5′LTR incapable of transactivating genes (Schäfer-Korting et al., 2010). Neoplastic transformation rates of bone marrow derived murine HSCs transduced with lentiviral and retroviral vectors with and without SIN elements have been compared (Montini et al., 2009; Modlich et al., 2009; Bosticardo et al., 2009). These studies demonstrated significantly reduced but not complete abrogation of oncogenicity (Bosticardo et al., 2009) of vectors bearing the SIN elements. These studies also highlighted that even with SIN vectors, insertional mutagenesis was still possible from internal enhancer/promoter elements and the choice of the internal promoter may reduce overt genotoxicity. Thus, although the development of SIN vectors has been an important step in improving biosafety, further refinements such as the use of cellular promoters devoid of enhancer elements and with decreased potential to induce activation of neighboring genes are necessary.

3.1.3 Chromatin insulators and cellular promoters

SIN vectors depend on an internal promoter to drive transgene expression. However, enhancer effects of the internal promoter in transactivating neighboring oncogenes has been a possible mechanism for neoplastic transformation associated with SIN vectors (Bosticardo et al., 2009; Modlich et al., 2009). The internal promoter of choice should ideally drive a high level of transgene expression without affecting the transcriptional status of neighboring genes. Thus, the use of moderately active internal promoters such as the phosphoglycerate kinase (PGK), elongation factor-1α (EF1α) or WAS promoter has been recommended to reduce the likelihood of neighborhood effects causing inadvertent gene activation (Zychlinski et al., 2008). The potential of vector-encoded promoters to transactivate host genes may be evaluated using *in vitro* assays (Weber & Cannon, 2007).

Another potential biosafety feature that has been explored is the inclusion of chromatin insulators to shield neighboring genes from the effects of vector-borne enhancers (Nienhuis et al., 2006). Insulator elements serve as barriers that separate transcriptionally active genomic regions (euchromatin) from heterochromatin and also prevent long-range interactions between enhancer/ regulatory elements and neighboring promoters, thereby reducing the risk of unintended transactivation of proximate genes. Insulator elements are typically cloned into the 3′ LTRs from where they are copied into the 5′LTRs following

genomic integration, thus flanking and isolating the transgene cassette. Zychlinski et al. reported moderately positive results with reduced transformation of murine HSCs when a 250 basepair (bp) core element of chicken hypersensitive site 4 (cHS4) insulator was incorporated into the viral vector (Zychlinski et al., 2008). More encouraging results of reduced transactivation were reported of a 77 bp element consisting of the β-globin 5′ HS4 insulator and a homologous region from human T-cell receptor BEAD-1 insulator (Ramezani et al., 2008). However, insulators may function in a tissue-specific manner or may be effective only against certain promoters/enhancers. Thus more work is needed to screen different insulator elements and test them in different cell types and with different promoter/enhancer configurations. Incorporation of strong polyadenylation signals in LTRs of vector is another option that has been explored to ensure the proper transcriptional termination of transgenes and to minimise the risk of internal promoters transcribing downstream coding sequences (Nienhuis et al., 2006). However, this carries the potential risk of premature transcript termination and transcriptional inactivation of neighbouring genes. Although the biosafety afforded by improved transcriptional termination is largely speculative, Schambach et al. did report encouraging results of improved transcriptional termination using upstream sequence elements in lentiviral and gammaretroviral SIN vectors (Schambach et al., 2007). Further evaluations in *in vitro* and *in vivo* models are necessary to determine if tightly regulated transcriptional termination translates to a reduced risk of insertional oncogenesis.

In summary, stringent selection of cellular promoters devoid of enhancer elements, inclusion of chromatin insulators and improved transcriptional termination should significantly enhance the biosafety of current SIN vectors.

3.1.4 Integrase defective vectors

Risks of insertional mutagenesis can be better managed if the frequency of genomic integration is reduced or if episomally maintained vectors are utilized. Virally encoded integrases bind to attachment regions (att) in the LTRs and mediate genomic integration of retro- and lentiviruses. Recent years have seen the developments of integrase defective vectors (IDV) which are generated either by mutating the viral integrase genes or att regions of the LTR (Sarkis et al., 2008). These altered vectors combine the efficient transduction capability of viruses with the higher biosafety of non-integrating vectors, although IDVs are not completely devoid of integration potential. Integrase defective retro- and lentiviral vectors have been developed, with the latter being more prevalent in use as lentiviral transduction is not limited to mitotic cells.

Integrase defective lentiviral vectors (IDLV) have been generated *via* mutations to integrase proteins at their catalytic domains, LTR-interacting N-terminal domains or non-specific DNA-binding C-terminal domains. Of these, the D64V and D116N mutations in the integrase catalytic domains have been more widely studied and reported to reduce residual integrase activity by 100- to 1000-fold compared with wild-type vectors (Apolonia et al., 2007) but with uncompromised transgene expression *in vivo* and about 2- to 10-fold decrease in expression *in vitro* (Apolonia et al., 2007). IDLV transduced cells can maintain stable transgene expression in the non-dividing state and may find useful applications in gene transfer to post-mitotic cells such as in muscle, liver and retina. They may also be useful in immuno gene therapy applications such as DNA vaccination where only transient expression of the transgene is required. Another application being explored is the use of IDLV as an episomal gene delivery technique for expressing site-specific integration factors such as zinc finger nucleases (ZFNs) (Lombardo et al., 2007), transposons (Vink et al., 2009)

and recombinases (Moldt et al., 2008). Lastly, IDLV may be developed as a safer alternative to non-integrating viral vectors such as AAV which are known to integrate quasi-randomly into the genome at low frequencies (Smith, 2008).

3.1.5 Novel and hybrid viral vectors

Ideal gene therapy vectors should be capable of accommodating large inserts and of efficient gene transfer in a broad range of cell types, while maintaining stable transgene expression with negligible genotoxicity. Much effort has been directed to designing and combining the positive traits of different viral vectors in a bid to refine therapeutic gene delivery.

As mentioned in the preceding section, IDVs combine the traits of broad tropism and efficient transduction of wild-type viruses with improved biosafety of episomal vectors. IDLV combined with Sleeping Beauty transposon/transposase, ZFNs and FLP/FRT recombinases are hybrid vectors tailored to integrate transgenes in a site-specific manner as directed by the transgene expressed proteins. Such a design also ensures that integrations are altered from the quasi-random pattern of the wild-type lentivirus to those directed by the coding proteins. Another example of a chimeric lentiviral vector with altered integration specificity was described by Gijsbers et al. (Gijsbers et al., 2010) who demonstrated retargeted integration specificity when artificial chromatin tethers were fused to a lentiviral-integrase interacting protein called lens epithelium-derived growth factor/p75 (LEDGF/p75). By replacing the DNA/chromatin interacting domain of LEDGF/p75 with heterochromatin 1β (*CBX1*), lentiviral integrations were retargeted to genomic loci bound by *CBX1*. This study also raises possibilities of designing both viral and non-viral vectors with LEDGF/p75 fusion proteins targeting safer genomic sites.

Hybrid HSV/AAV vectors have been constructed in an attempt to combine the large insert cloning capacity of non-integrating HSV vectors (up to 150 kb) with the site-specific integration property of AAV vectors into a genomic hotspot, the AAVS1 locus, and to a certain extent, randomly (De Oliveira & Fraefel, 2010). Other HSV-based hybrid vectors include the episomally maintained HSV/EBV and randomly integrating HSV/RV vectors.

Given the propensity of MLV gammaretroviral vectors to mediate insertional mutagenesis, vectors with safer integration profiles are being developed and investigated actively. For instance, the non-pathogenic foamy spumaretrovirus has shown promise in treating canine leukocyte deficiency without any reported adverse outcomes for up to 2 years (Bauer et al., 2008). These vectors also had a more favourable integration profile i.e. decreased frequency of integrating near oncogenes. While novel hybrid vectors appear promising, continued long-term efficacy and biosafety studies are necessary before they can be serious candidates for clinical gene therapy.

3.2 Improvements to integrating non-viral vectors

Non-viral vectors have found useful applications largely in the laboratory and pre-clinical settings but represent only 24% of all vectors used in clinical gene therapy trials. The fact that viruses have evolved over millennia to become effective infectious agents in humans understandably makes them superior in many aspects as gene transfer agents. The ultimate goal of designing synthetic non-viral vectors is to combine the positive traits of viruses without the negative traits of genotoxicity. Significant improvements have been made to methods of non-viral vector delivery (Conwell & Huang, 2005) with reported efficiencies that rival those achieved with viral transductions. Two classes of non-viral vectors may contribute to improved biosafety of gene therapy, namely episomally maintained vectors

and integrating vectors with safer integration profiles. The ultimate goal of an ideal gene therapy vector in the context of treating many genetic diseases would be to ensure durable and regulated transgene expression either from an autonomously replicating artificial chromosome/stable plasmid or from a limited number of transgenes integrated into safe harbors in the genome. This section will review the progress in the developments of non-viral integrating vectors with safer integration profiles.

3.2.1 Transposase and recombinase

Transposases and recombinases are two classes of site-specific genome modifying agents. These enzymes recognise and bind to short stretches of DNA sequences within the vector and in the genome to mediate the integration of exogenous vector DNA into the genome. Analysis of the integration spectrum of transposases and recombinases identified some that mediate quasi-random and sequence specific integrations into the genome, a distinct advantage over randomly integrating viral vectors. Transposases and recombinases are also less immunogenic (Yant et al., 2000), have lower enhancer/promoter activity (Walisko et al., 2008) and have fewer epigenetic effects at genomic integration sites (Zhu et al., 2010), relative to viral vectors. Given their capacity to function in mammalian cells, these non-viral integrating systems evoke exciting possibilities for development into safer alternatives than randomly integrating vector systems. Several different classes and strains of transposases and recombinases have been discovered and studied as gene therapy agents. One major limitation is their relatively relaxed stringency of site-specific integrations which again raises the spectre of insertional mutagenesis. Therefore, a major effort has been directed at developing non-virally targeted gene integration systems with improved specificity. A note of caution is the low risk of unintended integration of the transposase or recombinase, which could have deleterious effects on the genome. Such risks may be minimised or abrogated by using mRNA rather than DNA to deliver the recombineering proteins. The next sections highlight advances and developments of the more commonly used transposases and recombinases.

3.2.1.1 Transposase – Sleeping Beauty, Piggy Bac, Tol2

Sleeping Beauty (SB) transposon, derived from *tc1/mariner* superfamily, is one of the most widely investigated transposase systems to date. SB transposase mediates genomic integration of vector sequences flanked by 2 inverted terminal repeats (ITR) at both transposon ends, preferentially into TA dinucleotides located within DNA segments with increased local bendability (Geurts et al., 2006), via a "cut-and-paste" mechanism. Integrations are quasi-random , without any preference for transcriptionally active regions (Huang et al., 2010). Optimised SB has a transposition efficiency ranging from 2.5 to 17% (Ortiz-Urda et al., 2003). Stable transgene integration using this system has enabled long term transgene expression in a variety of mammalian cells and animal models (Izsvák et al., 2009). Owing to the randomness of integrations, SB systems have been used also in the genetic screening and identification of potential oncogenes in *in vitro* and *in vivo* models. It is worth reiterating that these SB systems are different from those used in gene therapy applications. SB systems used in oncogene screening and discovery are deliberately modified *via* incorporation of strong transcriptional enhancers and splice acceptor sites to be potently mutagenic (Collier et al., 2005). Thus far, the use of SB as a gene therapy agent in animal models has not been associated with any tumorigenesis (Ohlfest et al., 2005). Inherent limitations of the SB system include limited cloning capacity, inhibition of

transposition at high transposase concentrations and lack of targeting specificity of integrations.

Initial studies with naive SB system revealed their inherently low transposition efficiencies. Many modifications have since been introduced to create hyperactive versions of SB with increased transposition activity such as SB10 (Ivics et al., 1997), SB11 (Geurts et al., 2003) and SB100X (Mátés et al., 2009). The hyperactive SB100X, which was reported to have a 100-fold increased transposition activity, was discovered by high-throughput screening of mutants created by a PCR-based DNA shuffling strategy. Using these improved versions of SB, efficient transposition has been reported in a variety of human primary cells such as cord blood derived CD34+ hematopoietic progenitor cells (Xue et al., 2009) and primary T cells (Huang et al., 2010).

The issue of non-specific targeting by SB has been another prime focus of research aimed at inducing site-specific integration. An ideal modification would enable SB to direct transposition to a single pre-defined "safe harbor" in the genome. Skewing the random integration pattern of SB towards a more targeted profile would be hailed as an improvement. Several groups have attempted to do this by incorporating specific DNA-binding domains (DBD) either to the SB transposase (Yant et al., 2007), the transposon bearing the gene of interest (Ivics et al., 2007) or *via* a fused DBD-protein binding domain (PBD) that interacts with the transposase without modifying it (Ciuffi et al., 2006). The first strategy of fusing DBDs such as E2C (a synthetic zinc finger protein that recognizes an 18 bp target site in the 5'-untranslated region of the human *ERBB2* gene) and Gal-4 to the transposase has met with limited success. With the second strategy, Ivics and collaborators were able to demonstrate re-targeted integrations by incorporating a fusion of two DBDs to direct the transposon bearing the gene of interest to specific genomic sites where transposition could be mediated by the transposase (Ivics et al., 2007). The third strategy of utilizing a fusion of peptides to interact with the genomic locus of choice (*via* DBD) and the transposase (*via* PBD) without compromising transposase activity has also been reported by the same group (Ivics et al., 2007). However, it must be noted that none of these site targeting modifications has yet been successfully translated to human gene therapy applications, possibly due to the relatively poor efficiencies of re-targeting specificity.

The non-viral integrating SB system offers an alternative strategy for stably modifying cells for gene therapy applications. Their lack of propensity for integrating into active transcriptional units may make them safer than retroviral and lentiviral vectors. This has led to the idea of hybrid vectors that combine SB transposition with improved delivery by integrase defective lentiviruses (Vink et al., 2009). However, until effective solutions are developed to improve the specificity of integrations, the SB system may only have limited appeal for clinical gene therapy. The only human clinical trial (phase I/II, NIH-OBA no. 0804–922) utilizing the SB system is based on redirecting the specificity of T-cells by stable expression of CD19 specific-chimeric antigen receptors mediated by the SB11 transposase system (Hackett et al., 2010). However, caution should be exercised before more transposon-based systems are translated to clinical applications, especially in view of the unexpectedly high copy number of random integrations of transposase plasmid in human primary T cells (Huang et al., 2010).

Piggy Bac (PB) transposase, isolated from the cabbage looper moth (*Trichoplusia ni*) is another class of transposase which is active in human and murine cells (Ding et al., 2005). PB system has been effectively used to reprogramme induced pluripotent stem (iPS) cells (Woltjen et al., 2009) and to mutagenize mice for cancer gene discovery (Rad et al., 2010). PB

demonstrated higher transposase activity than SB11 and could also be modified to incorporate DBD without loss of transposase activity (Wu et al., 2006). Several improved versions of PB have been reported. Liang et al. (Liang et al., 2009) demonstrated increased chromosomal transposition with a codon optimised PB and, more recently, reported the development of a hyperactive PB with a 7-fold increase in integration activity and showed its application for generating murine iPS cells (Yusa et al., 2011).

The Tol2 transposon of the hobo/Activator/Tam3 (hAT) family of elements derived from the medaka fish (*Orizyas latipes*) is active in human cells (Grabundzija et al., 2010). Like PB, Tol2 also tolerates overproduction inhibition and unlike the SB system has a large cloning capacity (up to 18 kb). However, both PB and Tol2 systems have significantly increased integrations into transcription start sites (TSS), CpG islands, DNaseI hypersensitivity and were able to alter transcriptional levels of neighboring genes close to integration sites in human T cells (Huang et al., 2010). This suggests a greater risk of insertional mutagenesis compared with the SB system. In this respect, the PB and Tol2 transposases may be better suited for applications where high frequencies of mutagenesis are desired, such as cancer gene discovery in mice (Rad et al., 2010).

3.2.1.2 PhiC31 phage integrase

The *Streptomyces lividans* bacteriophage derived phiC31 integrase, belonging to another class of site-specific recombinases (SSR) known as serine recombinases , works through a "cut-and paste" mechanism to mediate unidirectional integration of an attB (34 bp bacterial attachment site) bearing vector sequence to attP (39 bp phage attachment site) or pseudo attP sequences found in mammalian genomes. Unlike the reversible cyclization recombination (Cre) recombinase/flippase (flp) systems, phiC31 integrase-mediated genomic integration results in irreversible insertion of vector sequences flanked by attL and attR sequences which are refractory to further recombination by the integrase. The phiC31 integrase system has been effectively employed in recombinase mediated cassette exchange (RMCE) studies to insert transgenes into pre-integrated wild-type attP sites and also, more importantly, for stable gene transfer into endogenous pseudo attP sites in mammalian genomes. Its property of mediating irreversible unidirectional site-specific recombination into a limited number of chromosomal sites in human cells spurred intense interest as a relatively safer method for stable gene transfer for clinical applications. PhiC31 integrase has been successfully employed both *in vitro* and *in vivo* to induce stable expression of therapeutic transgenes. Ortiz-Urda et al. demonstrated functional correction of type VII collagen deficiency and laminin V deficiency in skin samples from patients with recessive dystrophic epidermolysis bullosa and junctional epidermolysis, respectively (Ortiz-Urda et al., 2002; Ortiz-Urda et al., 2003), Thyagarajan et al. generated ES lines with stable transgene expression (Thyagarajan et al., 2008) and Ishikawa et al. showed the possibility of correcting X-linked SCID deficiency by expressing IL2 receptor gamma chain in T cell-lines from SCID-X1 patients (Ishikawa et al., 2006). Successful correction of deficiencies of fumarylacetoacetate hydrolase (Held et al., 2005), alpha-1-antitrypsin, factor IX (Olivares et al., 2002) and dystrophin (Bertoni et al., 2006) have also been demonstrated in murine models. Experimental data and bioinformatic analyses point to 370 actual and potential genomic sites for phiC31 integrase-mediated integrations (Chalberg et al., 2006). The limited number of potential sequence-specific integrations coupled with the potential for long term gene expression suggests that phiC31 integrase could be a safer alternative to randomly integrating vectors. However, several studies have raised the possibility that phiC31 integrase could induce infrequent chromosomal translocations (Liu et al., 2006), possibly by

promoting recombination between two endogenous pseudo attP sites in different chromosomes. Work done by our group (Sivalingam et al., 2010) suggests that the frequencies of chromosomal aberrations may differ in different cell types. Using spectral karyotyping, we observed translocations in only 4 of 300 metaphases of primary cells treated with phiC31 integrase, a frequency similar to the low background of chromosomal abnormalities reported in normal human somatic cells (Varella-Garcia et al., 2007). Moreover, chromosomal translocations have been observed *in vitro* in cells treated with vectors already approved for clinical trials such as the AAV vector (Miller et al., 2005), albeit without any pathological consequences *in vivo*. Concerns of potentially pathogenic chromosomal rearrangements have somewhat dampened interest in phiC31 integrase as an agent to be translated into clinical therapy. Although there is still a push to develop gene therapy vectors with impeccable safety profiles, our work suggests that phiC31 integrase has a relatively benign biosafety profile compared to randomly integrating retroviral and lentiviral vectors. Attempts to increase the site-specificity of phiC31 integrase include mutagenised versions of phiC31 integrase which display increased prevalence of integration at a pseudo attP site in chromosome 8p22 (Sclimenti et al., 2001) or other pseudo attP sites (Liesner et al., 2010), and versions with higher integration frequencies (Keravala et al., 2009). Thus, *ex vivo* gene therapy approaches utilising phiC31 integrase could be rendered even safer by using integrases with greater site-specificity and pre-screening gene modified cells, preferably with high-throughput methods, to exclude suspect cells and select cells with safe characteristics.

3.2.2 Targeted gene integration

Although transposases and SSRs integrate vectors non-randomly, some have questioned if these systems are truly sequence-specific or merely quasi-random as these systems are known to mediate integrations into degenerate sequences with very little homology to wild-type sequences. The terms site-directed or targeted gene integration could be used to describe modifications that are intended to direct integration to specific genomic regions recognised by the modifying agent which is usually a DNA-binding protein (DBP). Altering or skewing the integration preference of SSRs towards a particular locus is an apparent advantage as it reduces the risk of integrations into unfavourable and/or unsafe genomic regions. Gene targeting can be mediated by DNA-protein interactions or DNA-base pairing interactions. Naturally occurring DNA-binding proteins such as zinc finger proteins (ZFP) or viral peptides such as Rep have been deployed to favor DNA-protein interactions defined by their inherent specificities.

Several strategies have been proposed to achieve targeting specificity with DBPs. One approach is to tether a DBP to a recombinase by direct fusion or protein-protein interactions. This has the theoretical effect of enhancing local concentrations of the SSR at sites specified by the DBP and could more effectively restrict integration activity to a specific genomic region of choice. Care should be taken to ensure that the tethered SSR is not adversely compromised functionally. Another less frequently investigated approach relies on binding of the DBP to the vector sequence as a means of targeting vector sequences to the locus of interest (Izsvák et al., 2010). This section will review examples of targeted gene integration.

3.2.2.1 Targeting via DNA binding proteins

A classical example of targeted gene integration is observed with the AAV system which has been reported to mediate 70 to 85% of integrations into the AAVS1 site in human

chromosome 19q13.3. Site-specific integration of AAV is attributed to viral Rep proteins (Rep68/Rep78) that recognize Rep binding elements in the inverted terminal repeats (ITRs) of AAV and in the AAVS1 site (Jang et al., 2005). This has led to the development of non-viral gene targeting using vector sequences flanked by AAV ITRs that can be recognised, nicked and integrated into AAVS1 sites by Rep proteins expressed in *trans* (Pieroni et al., 1998). Philpott and collaborators (Philpott et al., 2002) reported that a 138 bp P5 integration efficiency element within the ITR was sufficient for efficient Rep binding. More recently Feng et al. (Feng et al., 2006) demonstrated that efficient RBE binding and AAVS1 targeted integration could be achieved with vector sequences flanked by a 16 bp fragment within the ITR (RBEitr). Rep based non-viral systems mediate AAVS1-specific integrations in *in vitro* clonal cultures at frequencies ranging from 12 to 60% (Howden et al., 2008; Pieroni et al., 1998). On this basis, these systems have been tested and shown to operate *in vivo* (Liu, R. et al., 2010). In this sense, Rep protein may be regarded as a DBP that redirects vector sequences to a targeted genomic locus, notwithstanding the possibility for random integrations simultaneously. The persistent potential for random gene integrations coupled with the need for antibiotic selections to isolate cells with the desired targeted integrations and the relatively low targeting efficiencies are possible reasons why this strategy has not garnered much interest.

Several groups have explored the possibility of combining the integration mechanisms of transposons, HIV-1 integrase, phage integrase or SSRs with the desired DNA binding specificities of DBPs. Early gene targeting studies relied on the use of a handful of well studied naturally occurring DBPs such as yeast Gal4 (binds upstream activating sequences), *Escherichia coli* Lex A (binds to Lex A operator sequence) (Katz et al., 1996), phage λ repressor (binds phage λ operator sites) (Bushman, 1994) and murine transcription factors such as Zif268 (Bushman & Miller, 1997). Although Gal4, lex A and λ repressor proteins were instrumental in demonstrating the feasibility of targeted gene integrations *in vitro*, they were not adaptable to clinical applications as they lack physiological binding sites in the human genome. However, they have been used to bind vector sequences bearing their recognition elements and, fused with other endogenous DBPs, can be engineered to recognise elements in the human genome (Ivics et al., 2007). Other naturally occurring cellular DBPs such as scaffold attachment factor (SAF) (Ivics et al., 2007) and LEDGF (Ciuffi et al., 2006) also bind to several human genomic regions (without precise sequence recognition) and facilitate integration *in vitro*. Recent work by Gijsbers and collaborators showed the potential for redirecting lentiviral integrations into transcriptionally inactive regions by modifying the natural LEDGF/p75-viral integrase interactions (Gijsbers et al., 2010). Such retargeting strategies could potentially be adapted to engineer hybrid viral vectors with safer integration characteristics compared to current generations of viral vectors.

Amongst transcription factors, the zinc finger proteins (ZFP) are an especially favored class of DBPs, given that the human genome codes for an estimated 4500 ZFPs. An inherent limitation of naturally occurring ZFPs is their tendency to recognize short DNA sequences which may be present at many sites in the genome. This prompted engineering artificial ZFPs that could be tailored to bind to unique genomic sites. Advances in protein structure elucidation and high-throughput techniques for studying DNA-protein interactions have ushered in new possibilities of creating user-defined custom ZFPs to target specific loci in the human genome. Great expectations of the practical utility of customized ZFPs has spawned commercial investment in this technology which is the business platform of

Sangamo Biosciences which focuses on designing novel customized synthetic ZFPs for use as modulators of transcriptional control and as gene targeting agents in combination with nucleases (zinc finger nucleases). These artificial ZFPs could potentially retarget the integration spectrum of SSRs or viral integrases to enhance their biosafety.

Although tethering DBPs to recombinases and transposases has enriched targeted gene integrations, such chimeric systems continue to suffer from the disadvantage of non-directed integrations owing to residual activity of the recombinase/transposase and its inherent specificity. The holy grail of gene targeting is integration only at a single user defined safe harbour without incurring the disruptive consequences of insertional mutagenesis. This ideal may now be within reach with the advent of synthetic ZFPs. The combination of such synthetic ZFPs with existing recombinases and transposase has not yet been sufficiently evaluated. Recent years have also seen the development of other gene targeting systems based on homologous recombination which promise highly accurate gene integration but whose effectiveness has yet to be proven.

3.2.3 Site-specific homologous recombination

The transgene integration strategies discussed thus far rely on the activity of an enzyme or protein to direct and mediate the integration of vector DNA into the genome randomly or with limited specificities. Another highly site-specific strategy that has been utilized for many years to create transgenic cells and animals with targeted genome modifications exploits endogenous repair mechanisms of host cells to execute homologous recombination, thereby incorporating exogenous DNA into specific genomic sites. Effective homologous recombination requires transgenic DNA to be flanked by sequences homologous to the genomic sequences into which they are to be integrated. These exogenous DNA sequences are templates in the process of homologous recombination and are subsequently replicated along with the genomic locus during host cell divisions. The basal frequency of homologous recombination involving exogenous DNA is very low, occurring in 1 out of 10^5 - 10^7 treated cells. It was discovered that this frequency can be enhanced 1000-fold by creating site-specific nicks in the genome (Rouet et al., 1994), thereby stimulating DNA repair at these sites. DNA is repaired by one of two main mechanisms i.e. non-homologous end joining or homologous recombination, although variations of these mechanisms are also possible. Error prone non-homologous end joining results in genomic DNA repair without transgene integrations while homologous recombination may result in site-specific integration of the transgene into the desired locus. In the context of gene therapy, the prospect of exploiting homologous recombination is appealing as it holds the potential for targeted gene repair and precise transgene integration into safe genomic loci. A patient's cells could in theory be modified *ex vivo* to correct disease-causing mutations or to integrate a transgene for long term expression of a deficient or defective protein before being reimplanted into the same patients (autologous cell therapy). Recent advances exploring such strategies will be discussed in this section.

3.2.3.1 Meganucleases

A more efficient and reproducible strategy for gene editing or integration that has been the focus of recent research is the use of highly site-specific endonucleases to induce double stranded DNA breaks into specific genomic sites in order to stimulate deletions via non-homologous end joining or homologous recombination of exogenously delivered DNA into these sites. Three main classes of engineered endonucleases have emerged: zinc finger

nucleases which are chimeras of ZFP and catalytic domain of *Fok* I restriction enzyme; chemical endonucleases which consist of chemical or peptidic cleavers fused with DNA recognising polymers; and meganucleases (homing endonucleases) which are capable of recognizing and cleaving target DNA sequences, usually 14 - 40 bp in length. HO endonuclease which mediates mating type switch in *Saccharomyces cerevisiae,* I-CreI and I-SceI meganucleases are examples of naturally occurring homing endonucleases. However, the applications of naturally occurring meganucleases have been limited either by the lack of recognition sites or by the presence of more than a single site in the human genome. The LAGLIDADG family of meganucleases includes I-SceI and I-CreI which are the largest and best characterised meganucleases, and are active as monomers or homodimers. Their catalytic cleavage centres are embedded within the DNA-binding domains and thus making non-specific cleavage very unlikely. Elucidation of the protein structures of endonucleases such as SceI and CreI have accelerated engineering of meganuclease variants with unique genomic recognition sites. Most effort have been directed to developing I-CreI and I-SceI variants with unique specificities and reduced off-target cleavage activity. Thus far two engineered meganucleases cleaving unique genomic loci in the human *XPC* (Arnould et al., 2007) and *Rag1* genes (Grizot et al., 2009) have been reported. Other improvements have been to engineer variant CreI (naturally homodimeric) meganuclease to function as obligate heterodimers (Fajardo-Sanchez et al., 2008) or as single-chain derivatives (Li et al., 2009). Computational approaches (Ashworth et al., 2006) have integrated structural and high throughput screening data to identify the cleavage properties of 18000 engineered meganucleases, based mostly on CreI meganuclease (Galetto et al., 2009).

Thus far, homologous recombination of transgenes with meganucleases has been demonstrated in only a few cell types and a comprehensive evaluation of their genotoxicity potentials has not been reported. The future development of engineered variants that collectively offer a wide spectrum of unique integration sites may be useful but will need careful evaluation. At present, there is a need to engineer endonucleases having user-defined specificities. This requirement may be more readily fulfilled with zinc finger nuclease technology given the potentially broader spectrum of genome-specific ZFPs that can be custom engineered.

3.2.3.2 Zinc finger nucleases

Zinc finger nucleases (ZFNs), first designed by Chandrasekaran and collaborators (Kim et al., 1996), are artificial chimeras composed of a tandem array of DNA-binding zinc finger proteins fused with the catalytic subunit of the non-specific *Fok*I restriction endonuclease *via* a short linker peptide. Naturally occurring zinc finger transcription factors, such as the murine Zif268 or human SP1, provide the framework in which each Cys2-His2 zinc finger that specifically recognises a 3-bp DNA sequence can be replaced to generate a novel ZFP capable in theory of binding to unique genomic sequences. Such polydactyl ZFPs have been assembled by modular assembly (Segal et al., 2003) by which individual zinc fingers are combined in a modular fashion to form a tandem array designed to recognize a selected DNA sequence. Another strategy is oligomerised pool engineering (OPEN) that takes into account the context dependence of sequence recognition and binding of each individual zinc finger as it may be influenced by its neighboring fingers (Maeder et al., 2008). Most recently, Sander and collaborators introduced yet another approach that takes into account context dependence of ZFPs. Termed the context dependent assembly (CoDA), this method involves first identifying two pairs of efficient ZFPs as identified by bacterial-2 hybrid assays. A 3-

finger ZFP array is next assembled using, as the central zinc finger, the finger that was common between the two pairs (Sander et al., 2011). ZFNs are designed as pairs to bind to adjacent nucleotide sequences on opposite strands. Their binding and localization at the intended locus induces dimerization and activation of *Fok*I endonuclease activity which induces double stranded DNA breaks at these specific sites. DNA breaks are repaired either by non-homologous end joining or by homology directed repair (presumably *via* synthesis dependent strand annealing). Initial concerns regarding the potential toxicity of off-target cleavage mediated by homodimers of *Fok*I catalytic monomers has been addressed by *Fok*I variants engineered to function as obligate heterodimers (Miller et al., 2007; Szczepek et al., 2007).

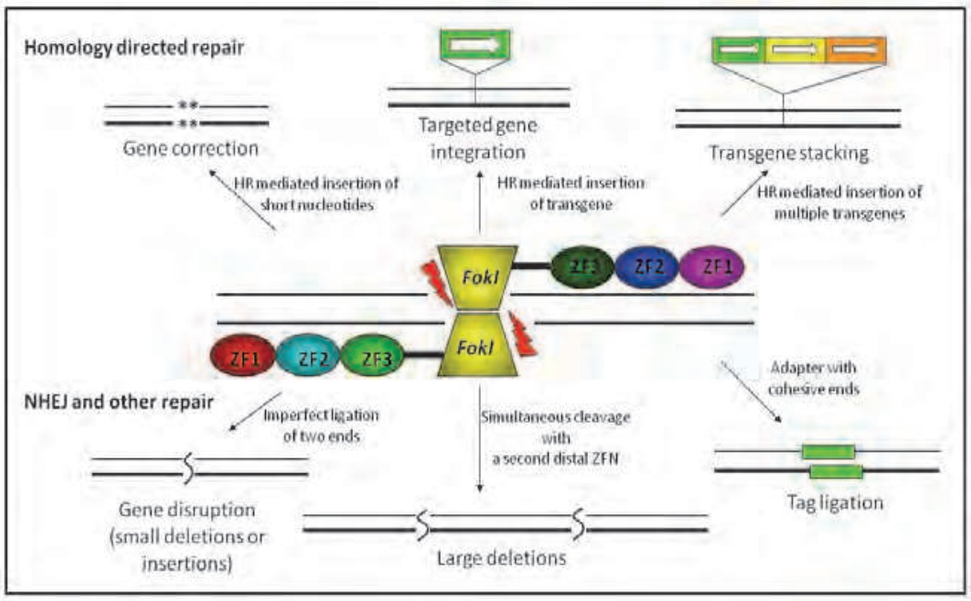

Fig. 6. Genome editing with ZFNs. Site-specific cleavage of genomic DNA by ZFNs can be repaired by homology-directed repair to correct or induce point mutations and to insert single or multiple transgenes (in the presence of donor molecule). Repair by NHEJ results in gene disruption *via* small insertions and/or deletions. Site-specific insertion of molecular tags and large genomic deletions may also be achieved with ZFN mediated cleavage of genomic DNA. (*Adapted from Urnov et al., 2010.*)

Two major applications of ZFNs to gene therapy are permanent gene disruption by insertions/deletions events during the error-prone non-homologous end joining repair or site-specific transgene insertion *via* repair by homologous recombination or homology-directed repair (figure 6). Since the turn of the millennium, ZFN technology has been harnessed to demonstrate feasibility of targeted gene corrections, transgene insertions and gene disruptions, in addition to pioneering a new approach for deriving transgenic plants and animals. ZFN technology has been used to derive transgenic crops with improved traits by mutagenesis of genes or targeted integration of herbicide resistance genes in species such as *Arabidopsis thaliana*, tobacco and *Zea mays* (Shukla et al., 2009; Townsend et al., 2009) and to derive specific gene knock-outs strains of mice and rats (Rémy et al., 2010). Given the ability to permanently disrupt specific genes, ZFNs have proved useful for elucidating gene functions during embryogenesis and development. Heritable targeted gene disruption has

been demonstrated in human embryonic stem cells, *Danio rerio* and *Drosophilia* (Hockemeyer et al., 2009; McCammon & Amacher, 2010; Carroll et al., 2010). ZFN-mediated gene knock-out has been effectively employed to disrupt the *CCR5* locus in human hematopoietic stem cells as a possible therapeutic strategy to confer resistance to HIV-1 infection by adoptive cell therapy *in vivo* (Perez et al., 2008). This has led to the use of ZFN-modified T cells in three phase I human clinical trials - for glioblastoma (NCT01082926) and HIV-1 treatment (NCT00842634, NCT01044654) (Urnov et al., 2010). Targeted disruption of several other genes such as *Bax* and *Bak* has also been demonstrated in human cells (Cost et al., 2010). The ability to correct genetic mutations by base substitution and the theoretical potential for exquisitely precise site-specific gene insertions has opened a plethora of possibilities for gene therapy applications. Porteus and Baltimore first reported the possibilities of targeted ZFN mediated genome editing in human somatic cells with gene correction of a pre-integrated GFP reporter gene (Porteus & Baltimore, 2003). Work by Urnov et al. has also been influential in demonstrating efficient correction of a *IL2Rγ* gene mutation in human cells, pointing to the prospect of future therapy for SCID-X1 (Urnov et al., 2005). Others have shown the feasibility of integrating exogenous DNA up to 8 kb in size (Moehle et al., 2007), and into other human genomic sites such as *PIGA, PPP1R12C and POU5F1* in primary cells such as mesenchymal stromal cells (Benabdallah et al., 2010), cord blood derived CD34+ HSCs (Lombardo et al., 2007), embryonic stem cells and iPS cells (Hockemeyer et al., 2009).

A current limitation of ZFN technology for site-directed transgene insertion is concern about unintended genomic modifications and possible biological hazards therefrom. Although several groups have demonstrated that the likelihood of off-target genomic modifications is low, there has been no comprehensive genome-wide analysis to date to rigorously support these claims. Potential off-target interactions of ZFNs must be evaluated by genome-wide techniques such as CHIP-based methods combined with deep sequencing in order to detect rare integration events. Long term monitoring of ZFN-modified cells is essential, using small and large animal models to assess fully any potential genotoxicity. The current efficiency of targeted gene insertion using ZFNs is still relatively low and may not warrant its broad application in human gene therapy. This awaits more specific ZFNs with robust and efficient targeted genome modification activity. Several useful resources are currently available in the public domain to aid the design, construction and testing of specific ZFNs. Helpful information and software tools pertaining to ZFN design and construction as well as a collection of ZFN plasmids and reagents for constructing and testing ZFNs are readily available to the research community at The Zinc Finger Consortium (http://www.zincfingers.org). Information on individual C2H2 zinc fingers and engineered zinc finger arrays have been compiled into databases such as the Zinc Finger Database (ZiFDB; http://bindr.gdcb.iastate.edu/ZiFDB) (Fu et al., 2009). Web-based resources such as Zinc Finger Targeter (ZiFiT; http://bindr.gdcb.iastate.edu/ZiFiT/) (Sander et al., 2010) and more recently ZFNGenome (http://bindr.gdcb.iastate.edu/ZFNGenome) (Reyon et al., 2011) provide excellent tools to aid the identification of potential ZF binding sites in user supplied target regions. They include software that calculate strengths of predicted ZFNs to be engineered by modular assembly or the OPEN method and also give information regarding potential off-target binding sites. Furthermore, Sangamo Biosciences and several other groups have described assays to evaluate the functional specificities of user-designed ZFNs Recent improvements to ZFNs have also focused on deriving *Fok*I variants with increased cleavage activities, in an attempt to increase the rate of genome modifications

(Guo et al., 2010). Higher ZFN cleavage activity possibly due to increased protein stability was also achieved by conditioning cells to transient mild hypothermia (Doyon et al., 2010). We need better understanding of the factors that influence the efficiency of intracellular homologous recombination and how these can be exploited to obtain higher gene targeting efficiencies. More work is needed to identify and test safe harbors in the human genome and to design ZFNs targeting them. Lastly, improvements to vector designs such as CpG-free vectors, the use of suitable physiological promoters, codon-optimised transgenes and incorporation of relevant insulator and enhancer elements would be pertinent to achieve durable transgene expression and minimise risks of insertional gene mishaps.

An ideal gene-based treatment for some monogenic disorders would be to derive self-renewing cells expressing a corrected version of the defective gene *via* site-specific integration in a safe genomic locus. Such gene modified cells could be exhaustively evaluated for their genotoxic potential *ex vivo* before being administered into patients. Given the lexicon of site-specific ZFNs that is being developed, this could be a real possibility in the near future with ZFN-modified stem cells.

3.3 Episomal vectors

One of the most apparent advantages of extra-chromosomal vectors as gene transfer agents is the greatly decreased risk of insertional mutagenesis compared to integrating vectors. Episomal plasmids can be maintained at high copy number, have potentially higher transgene expression levels and are less likely to suffer transgene silencing or positional variegation effects associated with genomic integrations.

The essential characteristics of extra-chromosomal vectors are episomal maintenance, autonomous replication and segregation into daughter cells. Episomal vectors can be categorised as either viral based if they rely on viral origins of replication and other virally encoded proteins for replication and partitioning into daughter cells, or chromosome based, if they depend on elements derived from the human genome (figure 7). Examples of viral based episomal vectors include those based on plasmid replicons of viruses such as simian virus 40 (SV40), bovine papillomavirus (BPV) and Epstein Barr virus (EBV) or those based on plasmid replicons carrying limited viral components such as oriP/EBV nuclear antigen 1 (EBNA1). Chromosome based episomal vectors include the scaffold/matrix attachment region (S/MAR) based pEPI vectors and artificial chromosomes.

3.3.1 Viral based episomal vectors

Concerns relating to the oncogenic transforming properties of polyoma viruses such as SV40 and the restricted mode of replication afforded by BPV have deterred the use of such viral replicon based vectors. The EBNA/oriP episomal vector has been one of the more commonly used viral based episomal vector systems tested for gene therapy. It relies on the EBV origin of replication (oriP) and the trans-acting factor, EBNA-1, for episomal replication within eukaryotic cells. The origin of replication consists of the dyad symmetry (DS) element and the family of repeats (FR) elements which serve as binding sites for EBNA-1. Interaction of EBNA-1 with DS elements mediates episomal replication while interaction with FR elements ensures nuclear retention of oriP bearing plasmids. Long term transgene expression has been demonstrated with the use of EBNA/oriP plasmids both *in vitro* and *in vivo* (Saeki et al., 1998). A major drawback is the concern that EBNA-1 may have oncogenic effects (Schulz & Cordes, 2009) although this has been questioned by a single report that

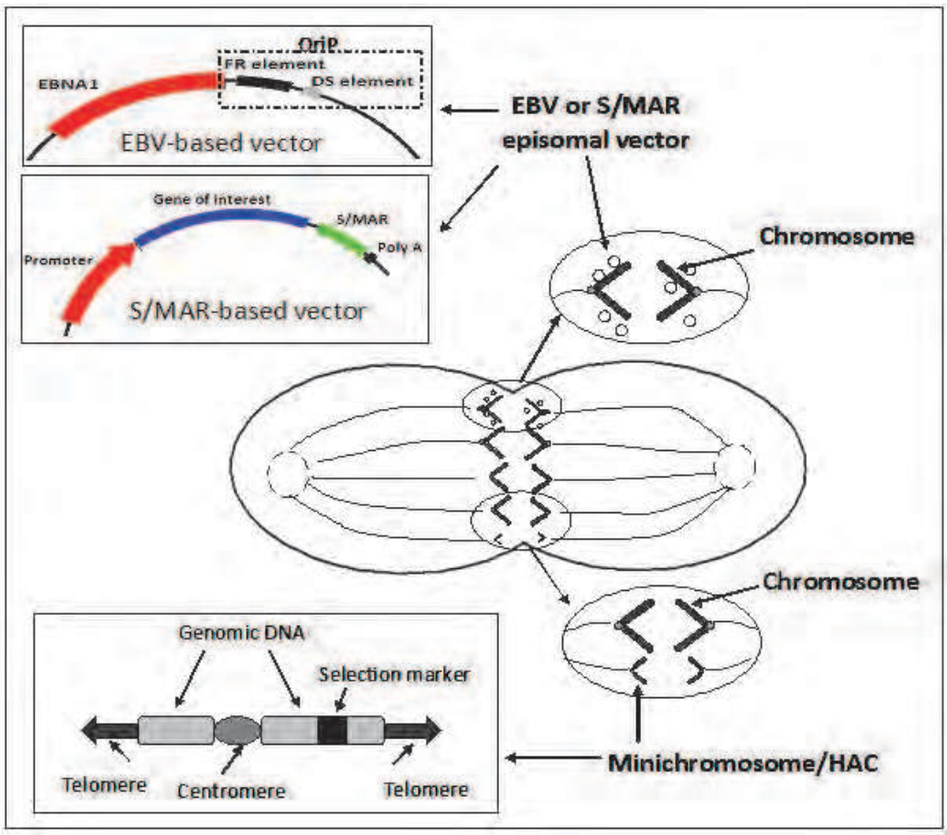

Fig. 7. Episomal vectors and artificial chromosomes. EBV-based episomal vectors require EBNA-1 and oriP elements for nuclear retention and episomal replication while S/MAR elements support the same functions in chromosome-based pEPI vectors. Nuclear retention for both types of vectors is mediated by tethering to subnuclear structures and transcriptionally active sites in the chromosomes. Mini-chromosomes or artificial chromosomes bearing selective genomic regions, centromeres, telomeres and selection marker can be assembled *de novo* and have been shown to segregate into daughter cells during mitosis. (*Redrawn from Lufino et al., 2008.*)

EBNA-1 does not activate cellular genes (Kang et al., 2001). Nevertheless, this prompted development of safer episomal vectors devoid of EBNA-1/oriP elements. Based on the known functions of DS/FR elements and EBNA-1, several groups investigated the functional features of episomal vectors substituted for these viral elements. Gerhardj et al. showed that episomal replication was possible even when the EBNA-1/oriP DS elements were replaced with small eukaryotic sequences (Gerhardt et al., 2006). Recently, Thomae and collaborators reported episomal replication and retention of an episomal plasmid (pCon) in which the DS elements were replaced with tet-operator sites and EBNA-1 replaced with a fusion protein comprised of the high mobility group protein, HGMA1a, which is known to interact with the cellular replication machinery (Thomae et al., 2008).These studies have shown the feasibility of developing episomal vectors devoid of EBNA-1/oriP elements but have yet to find favor as efficient and safer alternatives to existing EBNA-1/oriP episomal vectors. With their ultimate intended use in human clinical applications in view, the trend has now shifted to developing episomally replicating vectors

exclusively composed of functional eukaryotic chromosomal elements devoid of foreign or viral elements.

3.3.2 Chromosome based episomal vectors

Three main approaches have been adopted in attempts to develop chromosome based episomal vectors: plasmid vectors with mammalian origins of replication, plasmid vectors with chromosomal S/MAR and mammalian artificial chromosomes.

Attempts to derive autonomously replicating plasmids by incorporating mammalian origins of replication had previously met with limited success, leading to speculation that epigenetic factors control the activation of mammalian origins of replication (Jackson et al., 2006).

The idea of deriving episomally maintained plasmids by incorporating S/MAR was based on the assumptions that mammalian replication origins are bound to the nuclear scaffold or matrix before the onset of DNA replication. S/MAR sequences function by tethering DNA to subnuclear structures and transcriptionally active sites in the chromosomes through the interaction with nuclear matrix protein scaffold attachment factor A (SAF-A) (Stehle et al., 2007) and are necessary for organization of chromatin loops that define the boundaries of chromatin domains (Jackson et al., 2006). The best characterised S/MAR based vectors are the pEPI vectors, which carry a 2kb- S/MAR element derived from human β-interferon gene cluster. These vectors are maintained at copy numbers of less than 10 per cell, replicate once per cell cycle and have been shown to be maintained episomally for several hundred generations in cell lines (Papapetrou et al., 2005), primary cells and animal models. Episomal replication is stringently dependent on transcription upstream from and into the 2kb-S/MAR element present within the plasmid during, but not after, episome establishment. Early generation S/MAR-based pEPI vectors have significant limitations associated with low rates of nuclear establishment, unintended genomic integrations, intrinsic vector instability, limited cloning capacity and loss of vector in dividing cells in the absence of initial selection pressure (Papapetrou et al., 2005).

Several improvements have since been made to pEPI vectors. Hasse and collaborators reported higher and persistent transgene expression *in vitro* and *in vivo* when they modified the pEPI vector to contain 60% reduced CpG DNA motifs and replaced the CMV promoter with EF1-alpha promoter (Haase et al., 2010). Another study looked into splitting pEPI vectors into a "mini-plasmid" containing prokaryotic vector sequences and a "mini-circle vector" comprised only of eukaryotic sequences including the transgene and a minimised S/MAR (Broll et al., 2010). The reduced size of S/MAR element allowed efficient and complete read-through of transcripts into the S/MAR regions and was associated with improved mRNA processing and higher expression levels. Furthermore, the use of plasmids devoid of prokaryotic sequences avoided the problems commonly associated with prokaryotic expression vectors such as heterochromatin formation, gene silencing and eventual loss from host cells (Haase et al., 2010).

Theoretically, artificial chromosomes would fit the role of ideal episomal vectors, given its very large cloning capacity, superior mitotic and meiotic stability and efficient segregation into daughter cells. Initial work on *Saccharomyces cervisiae* led to the discovery of yeast autonomously replicating sequences and subsequently, to yeast centromeres and telomeres which eventually enabled construction of yeast artificial chromosomes. This defined the path for constructing human artificial chromosomes (HACs) after human centromeres, telomeres and origins of replication had been identified and isolated as essential features for

extra-chromosomal replication and retention. HACs can be constructed by a tedious process using a top-down or bottom-up approach. In the former, whole chromosomes in live cells are truncated by irradiation or telomere fragmentation into minichromosomes. The bottom-up approach assembles artificial chromosomes from isolated functional elements i.e. centromeres, telomeres and replication origins. Transgenes of interest are usually cloned into these artificial chromosomes by recombination using the Cre-recombinase/loxP or FLP/FRT systems (Kotzamanis et al., 2005). HAC and minichromosomes are delivered into cells by microcell mediated chromosome transfer (MMCT) or micro-injection. Several groups have demonstrated long-term transgene expression in several cell types modified with HACs and minichromosomes (Grimes et al., 2001). The major disadvantages of HAC are the difficulty of constructing and producing them and the low efficiency of intracellular delivery, given their very large size compared to other gene transfer vectors. Nevertheless, successful gene transfer of a 245 kb BAC vector has been demonstrated even with non-viral vectors based on a "LID vector" design comprised of lipofectin (L), integrin-targeting peptide (I) and DNA of interest (D), with efficiencies ranging from 10 -15% in 293 and MRC-5v2 cells. HACS are most efficiently delivered by MMCT. The efficiency of delivery and integrity of delivered chromosomes can be improved by the use of polycations such as poly-L-lysine and poly-ethylenimine. Viral delivery systems for extra-chromosomal vectors include vectors with EBV and CMV based amplicons, adenoviruses and HSV vectors.

Another class of gene transfer vectors capable of accommodating large genomic segments are the high-capacity extra-chromosomal vectors. These vectors drive the expression of genes of interest from a genomic DNA locus of extensive size that could be expected to be superior as well as to have greater fidelity of physiological control owing to the combined effects of regulatory elements, non-coding regions, chromatin opening elements and native promoters compared to cDNA expression from minimal promoters. Recent improvements in extra-chromosomal vectors include the development of large capacity F-factor based bacterial artificial chromosomes and P1-derived artificial chromosomes that incorporate EBV retentions systems (oriP and EBNA). Recent studies have also shown stable transgene expression from BAC vectors coupled with S/MAR elements (Lufino et al., 2008).

Episomal non-viral vectors represent a class of vectors that could function as efficient and safe gene therapy agents for persistent long term expression not only in *ex vivo* modified cells but also *in vivo*. The exciting possibility of utilizing them with adult and embryonic stem cells for *ex vivo* gene therapy warrants investigation. However, as with other gene transfer techniques, caution must be exercised to rigorously estimate the small but troubling risk of potentially random vector insertion (Wang et al., 2004).

3.4 Suicide genes as safety mechanisms for treatment modalities

The benefits of gene therapy for life threatening diseases for which there is currently no effective treatment justify their continued evaluation in clinical trials despite the known risks of iatrogenic complications. It is clear from the preceding sections that most research efforts have been directed at enhancing the biosafety of gene therapy vectors. An additional strategy to intervene and reverse adverse vector effects is to include secondary safety mechanisms capable of rapidly triggering the selective elimination of rogue transgenic cells. Suicide gene therapy or gene-directed enzyme prodrug therapy relies on the expression of transgene products from "suicide genes" that convert inactive prodrugs into cytotoxic drugs, thus selectively eliminating transgenic cells that express the suicide gene. Several

suicide genes such as herpes simplex virus 1 thymidine kinase (HSV-TK), bacterial cytosine deaminase (CD), bacterial carboxypeptidase-G2 (CPDG2), purine nucleoside phosphorylase (PNP) and nitroreductase (NR) and their cognate prodrugs have been tested for their efficacy as agents of selective cell destruction (Denny, 2003). Problems such as suicide gene silencing, incomplete elimination of targeted cells, cytotoxicity to non-gene expressing cells and immune response to suicide genes have reduced the efficacy of such approaches. Continued improvements to existing suicide genes and prodrugs as well as development of novel genes capable of selective elimination of cells with reduced cytotoxicity to normal cells are necessary improvements to suicide gene therapy for clinical applications. Recent developments in suicide gene therapy strategies will be briefly discussed in this section.

3.4.1 HSV thymidine kinase

The HSV-TK suicide gene and its prodrug, gancyclovir (GCV) is one of the most extensively studied and the only clinically validated suicide gene/prodrug system. HSV-TK phosphorylates the non-toxic acyclic analogs of deoxyguanosine such as GCV and acyclovir (ACV) into a toxic form that becomes incorporated into DNA. This leads to eventual cell death by inhibiting DNA synthesis and disrupting DNA replication in sensitive cells. The use of HSV-TK has found broad applications *in vitro* as negative selection in homologous recombination studies and has been successfully used in phase I-II clinical trials for prevention of graft versus host disease following allogeneic stem cell transplantation (Lupo-Stanghellini et al., 2010). It has also been investigated extensively in cancer gene therapy to eliminate tumor cells. An on-going phase III clinical trial by Ark Therapeutics (www.arktherapeutics.com) is evaluating HSV-TK combined with surgery and chemotherapy in patients with high grade gliomas (cited by Preuß et al., 2010). However, there are certain disadvantages of the HSV-TK/GCV system. These include GCV toxicity at clinical doses, insensitivity of HSV-TK expressing cells to GCV due to inactive spliced HSV-TK variants (Garin et al., 2001), cellular toxicity of high levels of HSV-TK that phosphorylate endogenous thymidine (Balzarini et al., 2006) and the inherent immunogenicity of viral epitopes presented by HSV-TK protein (Berger et al., 2006). Several improvements have been made to improve the performance of HSV-TK such as reduced splice variants (Chalmers et al., 2001), improved GCV sensitivity (Black et al., 2001) and decreased affinity for endogenous thymidine (Balzarini et al., 2006). Notable HSV-TK variants with improved sensitivity to GCV include the SR39 (Black et al., 2001) and Q7530A (Mercer et al., 2002) mutants. Splice corrected versions of HSV-TK (scHSV-TK) have been derived by mutating internal splice sites within wild-type HSV-TK gene to prevent the emergence of GCV-resistant cells expressing inactive HSV-TK splice variants (Chalmers et al., 2001). Another recent development is the use of a codon-optimized HSV-TK A168H mutant, TK007 which causes faster and more robust GCV mediated killing of cells while having less non-specific cytotoxicity (Preuß et al., 2010) due to the reduced affinity for endogenous thymidine. These improved versions of HSV-TK could function effectively as benign suicide genes that would be activated to selectively eliminate implanted gene modified cells in the event of a serious adverse complication e.g. oncogenic transformation. However, outstanding issues such as immunogenicity of HSV-TK and the possibility of immune-mediated rejection of gene modified cells reiterate the need to investigate other novel human-based and possibly non-immunogenic suicide genes as better alternatives.

3.4.2 Suicide genes in development

The immunogenic nature of non-mammalian suicide genes such as HSV-TK and cytosine deaminase and the unintended immune mediated elimination of suicide gene expressing cells has prompted the search for novel human and/or non-immunogenic genes able to function as suicide genes. A human T-cell surface antigen, CD20, was one of the first human suicide genes to be investigated for its capacity to eliminate CD20 expressing T-cells using anti-CD20 antibodies. The CD20/anti CD20 mAb may be suitable for use in gene modified HSCs but requires high cellular expression of CD20 antigen and may also deplete cells normally expressing CD20 (Lupo-Stanghellini et al., 2010). Other systems that could be useful include the FK-506 binding protein (FKBP-FAS)/AP20187, AP1903 dimerization system that relies on the selective induction of apoptosis by expressing proapoptotic Fas-ligand molecules intracellularly, to be activated by non-toxic chemically induced dimerization of the FKBP-FAS molecules. Another notable non-immunogenic system (iCasp9) relies on activating apoptosis in selected cells by fusing the death domains of Caspase-9 with FKBP elements, which can be induced to dimerize and activate apoptosis (Tey et al., 2007). This system is currently being evaluated in an on-going clinical trial for graft versus host disease (cited by Lupo-Stanghellini et al., 2010).

In summary, the incorporation of safety switches in the form of suicide genes to eliminate gene modified cells would be essential and beneficial features in future clinical gene therapy. Ongoing efforts to develop suicide genes with increased prodrug sensitivity and reduced unintended toxicity, as well as exploring novel systems to selectively induce cell death ought to be helpful adjuncts to improve the biosafety of human gene therapy – currently mainly in clinical trials.

4. Challenges and future prospects

Although there have been major innovations and improvements to gene therapy in the past decade, the key challenges of sustained efficacy, biosafety and immunogenicity remain important challenges that need to be dealt with. Several early clinical trials have emphasized the primary need for increased transduction efficiencies and durable expression of delivered transgenes to achieve clinically meaningful treatment efficacy. Viral vectors now have significantly improved tissue specificity and transduction efficiencies. Delivery methods of non-viral vectors have also significantly advanced to attain near-comparable efficiencies. The use of *ex vivo* modified stem cells with self-renewing capacity *in vivo* may overcome the constraints of utilizing nondividing cells for *ex vi*vo gene therapy for selected diseases. Continuous improvements are being integrated into vector designs to enable durable transgene expressions and minimise transgene silencing *in vivo*. Biosafety concerns of immunogenicity and insertional mutagenesis, although uncommon, are nonetheless barriers to clinical acceptance and there are ongoing concerted efforts to address these problems. It is important to ensure that improvised or novel vectors (viral or non-viral) are comprehensively tested and evaluated for their genotoxic potential. Absence of evidence is not evidence of absence of genotoxic risk. Genotoxicity risks should be evaluated using a range of tools to address interrogate cells at multiple levels i.e. transcriptome, genome, epigenome and chromosomes. High-throughput screening methods are highly desirable to increase the sensitivity and accuracy of characterization. Currently, integrating vectors are favoured choice given their ability to mediate high levels of and long-term transgene expression. The caveat is the random or quasi-random nature of gene integrations mediated by most currently used integrating vectors. The risks of insertional mutagenesis may be

minimized by using episomally maintained vectors and by gene targeting strategies, ideally by targeted gene addition into a safe and unique genomic locus. At present such alternative strategies have not achieved sufficient efficacy to be translated into clinical applications. Continued efforts and greater resources therefore need to be channelled into developing episomal vectors and site-specific integrating vectors.

Whilst gene therapy aims primarily to correct inherent deficiencies in cells and organ systems, the emerging field of regenerative medicine offers the prospect of producing replacements for diseased or defective cells. Since Yamanaka and collaborators (Takahashi & Yamanaka, 2006) demonstrated the ability to convert somatic cells, such as fibroblasts, into induced pluripotent stem (iPS) cells by combined expression of *Oct4, Sox2, Klf4* and *c-Myc*, there has been a flurry of reports on the ability of other combinations of transcription factors and safer reprogramming methods to attain similar outcomes. In view of concerns of the need for genomic integration of the transcription factor genes for continued expression, others have adapted the use of episomal vectors, RNA and even peptide versions of the transcription factors to generate iPS cells. iPS cells have the potential to be differentiated into cells of the endoderm, ectoderm and mesoderm and could prove to be useful for treating diseases where replacement with fully functional surrogate cells or regenerating stem cells is a therapeutic option. Thus the field of regenerative medicine is an exciting field that could rival or complement present forms of gene and cell-based therapy in future.

5. Conclusion

Beginning in the 1960s, convergent advances in human genetics and recombinant DNA technology spawned the seductively compelling notion of gene therapy to cure, or at least, ameliorate diseases caused by defective genes. Almost half a century later, the initial enthusiasm and euphoria have been greatly tempered by the sober recognition that while gene therapy is simple in concept, it is highly complex and challenging in execution. The early promises of human gene therapy raised unrealistically high expectations that gene medicine was round the corner. Compounded by well publicised serious iatrogenic complications from a small number of clinical trials, a pall quickly descended on the field from the late 1990s that led many investigators to flee from a field of research that came to be perceived as both unfeasible and unfundable.

Gene therapy has now emerged from a much needed phase of reflection and correction. There is clear evidence that appropriately selected monogenic and acquired diseases can benefit from gene-based therapy. Notwithstanding that there remains a risk to certain viral vectors, the decision to reinitiate gene therapy trials for SCID-X1 (NCT01129544) is acknowledgement of what gene therapy may offer to diseases that are currently difficult to treat effectively or at reasonable cost. Failures of gene therapy should not discredit the field but ought to be opportunities to deepen scientific understanding of the complex processes demanded for therapeutic success. Safety is a key consideration, particularly with respect to genotoxicity. The confluence of autologous cell therapy with conventional gene therapy appears to be a promising approach. Cells that are first modified *ex vivo* lend themselves readily to comprehensive biosafety assessments that are not feasible with conventional *in vivo* gene therapy. The ability to thoroughly characterize cells for the desired phenotype, and for genotoxicity and other risks before *in vivo* implantation or administration should go some way to making such novel treatments safe.

(The authors were unable to cite all relevant publications owing to page limitations.)

6. References

Aiuti, A., Cattaneo, F., Galimberti, S., Benninghoff, U., Cassani, B., Callegaro, L., Scaramuzza, S., Andolfi, G., Mirolo, M. & Brigida, I. (2009). Gene therapy for immunodeficiency due to adenosine deaminase deficiency. *N Engl J Med*, Vol.360, No.5, pp. 447-458, ISSN 0028-4793

Apolonia, L., Waddington, S. N., Fernandes, C., Ward, N. J., Bouma, G., Blundell, M. P., Thrasher, A. J., Collins, M. K. & Philpott, N. J. (2007). Stable gene transfer to muscle using non-integrating lentiviral vectors. *Mol Ther*, Vol.15, No.11, pp. 1947-1954, ISSN 1525-0016

Appelt, J. U., Giordano, F. A., Ecker, M., Roeder, I., Grund, N., Hotz-Wagenblatt, A., Opelz, G., Zeller, W. J., Allgayer, H., Fruehauf, S. & Laufs, S. (2009). QuickMap: a public tool for large-scale gene therapy vector insertion site mapping and analysis. *Gene Ther*, Vol.16, No.7, pp. 885-893, ISSN 0969-7128

Arnould, S., Perez, C., Cabaniols, J. P., Smith, J., Gouble, A., Grizot, S., Epinat, J. C., Duclert, A., Duchateau, P. & Pâques, F. (2007). Engineered I-CreI derivatives cleaving sequences from the human XPC gene can induce highly efficient gene correction in mammalian cells. *J Mol Biol*, Vol.371, No.1, pp. 49-65, ISSN 0022-2836

Ashworth, J., Havranek, J. J., Duarte, C. M., Sussman, D., Monnat, R. J., Stoddard, B. L. & Baker, D. (2006). Computational redesign of endonuclease DNA binding and cleavage specificity. *Nature*, Vol.441, No.7093, pp. 656-659, ISSN 0028-0836

Avery, O. T., Macleod, C. M. & McCarty, M. (1944). Studies on the chemical nature of the substance inducing transformation of pneumococcal types: Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. *J Exp Med*, Vol.79, No.2, pp. 137-158, ISSN 0022-1007

Balzarini, J., Liekens, S., Solaroli, N., El Omari, K., Stammers, D. K. & Karlsson, A. (2006). Engineering of a single conserved amino acid residue of herpes simplex virus type 1 thymidine kinase allows a predominant shift from pyrimidine to purine nucleoside phosphorylation. *J Biol Chem*, Vol.281, No.28, pp. 19273-19279, ISSN 0021-9258

Bauer, T. R., Allen, J. M., Hai, M., Tuschong, L. M., Khan, I. F., Olson, E. M., Adler, R. L., Burkholder, T. H., Gu, Y. C., Russell, D. W. & Hickstein, D. D. (2008). Successful treatment of canine leukocyte adhesion deficiency by foamy virus vectors. *Nat Med*, Vol.14, No.1, pp. 93-97, ISSN 1078-8956

Benabdallah, B. F., Allard, E., Yao, S., Friedman, G., Gregory, P. D., Eliopoulos, N., Fradette, J., Spees, J. L., Haddad, E., Holmes, M. C. & Beauséjour, C. M. (2010). Targeted gene addition to human mesenchymal stromal cells as a cell-based plasma-soluble protein delivery platform. *Cytotherapy*, Vol.12, No.3, pp. 394-399, ISSN 1465-3249

Berger, C., Flowers, M. E., Warren, E. H. & Riddell, S. R. (2006). Analysis of transgene-specific immune responses that limit the in vivo persistence of adoptively transferred HSV-TK-modified donor T cells after allogeneic hematopoietic cell transplantation. *Blood*, Vol.107, No.6, pp. 2294-2302, ISSN 0006-4971

Bertoni, C., Jarrahian, S., Wheeler, T. M., Li, Y., Olivares, E. C., Calos, M. P. & Rando, T. A. (2006). Enhancement of plasmid-mediated gene therapy for muscular dystrophy by directed plasmid integration. *Proc Natl Acad Sci U S A*, Vol.103, No.2, pp. 419-424, ISSN 0027-8424

Black, M. E., Kokoris, M. S. & Sabo, P. (2001). Herpes simplex virus-1 thymidine kinase mutants created by semi-random sequence mutagenesis improve prodrug-mediated tumor cell killing. *Cancer Res*, Vol.61, No.7, pp. 3022-3026, ISSN 0008-5472

Blaese, R. M., Culver, K. W., Miller, A. D., Carter, C. S., Fleisher, T., Clerici, M., Shearer, G., Chang, L., Chiang, Y., Tolstoshev, P., Greenblatt, J. J., Rosenberg, S. A., Klein, H., Berger, M., Mullen, C. A., Ramsey, W. J., Muul, L., Morgan, R. A. & Anderson, W. F. (1995). T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. *Science*, Vol.270, No.5235, pp. 475-480, ISSN 0036-8075

Bosticardo, M., Ghosh, A., Du, Y., Jenkins, N. A., Copeland, N. G. & Candotti, F. (2009). Self-inactivating retroviral vector-mediated gene transfer induces oncogene activation and immortalization of primary murine bone marrow cells. *Mol Ther*, Vol.17, No.11, pp. 1910-1918, ISSN 1525-0016

Boztug, K., Schmidt, M., Schwarzer, A., Banerjee, P. P., Díez, I. A., Dewey, R. A., Böhm, M., Nowrouzi, A., Ball, C. R., Glimm, H., Naundorf, S., Kühlcke, K., Blasczyk, R., Kondratenko, I., Maródi, L., Orange, J. S., von Kalle, C. & Klein, C. (2010). Stem-cell gene therapy for the Wiskott-Aldrich syndrome. *N Engl J Med*, Vol.363, No.20, pp. 1918-1927, ISSN 0028-4793

Broll, S., Oumard, A., Hahn, K., Schambach, A. & Bode, J. (2010). Minicircle performance depending on S/MAR-nuclear matrix interactions. *J Mol Biol*, Vol.395, No.5, pp. 950-965, ISSN 0022-2836

Bushman, F. D. (1994). Tethering human immunodeficiency virus 1 integrase to a DNA site directs integration to nearby sequences. *Proc Natl Acad Sci U S A*, Vol.91, No.20, pp. 9233-9237, ISSN 0027-8424

Bushman, F. D. & Miller, M. D. (1997). Tethering human immunodeficiency virus type 1 preintegration complexes to target DNA promotes integration at nearby sites. *J Virol*, Vol.71, No.1, pp. 458-464, ISSN 0022-538X

Carroll, D., Beumer, K. J. & Trautman, J. K. (2010). High-efficiency gene targeting in Drosophila with zinc finger nucleases. *Methods Mol Biol*, Vol.649, pp. 271-280, ISSN 1064-3745

Cartier, N., Hacein-Bey-Abina, S., Bartholomae, C. C., Veres, G., Schmidt, M., Kutschera, I., Vidaud, M., Abel, U., Dal-Cortivo, L., Caccavelli, L., Mahlaoui, N., Kiermer, V., Mittelstaedt, D., Bellesme, C., Lahlou, N., Lefrere, F.,

Blanche, S., Audit, M., Payen, E., Leboulch, P., l'Homme, B., Bougneres, P., Von Kalle, C., Fischer, A., Cavazzana-Calvo, M. & Aubourg, P. (2009). Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science*, Vol.326, No.5954, pp. 818-823, ISSN 0036-8075

Cavazzana-Calvo, M., Hacein-Bey, S., de Saint Basile, G., Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J. L., Bousso, P., Deist, F. L. & Fischer, A. (2000). Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science*, Vol.288, No.5466, pp. 669-672, ISSN 0036-8075

Chalberg, T. W., Portlock, J. L., Olivares, E. C., Thyagarajan, B., Kirby, P. J., Hillman, R. T., Hoelters, J. & Calos, M. P. (2006). Integration specificity of phage phiC31 integrase in the human genome. *J Mol Biol*, Vol.357, No.1, pp. 28-48, ISSN 0022-2836

Chalmers, D., Ferrand, C., Apperley, J. F., Melo, J. V., Ebeling, S., Newton, I., Duperrier, A., Hagenbeek, A., Garrett, E., Tiberghien, P. & Garin, M. (2001). Elimination of the truncated message from the herpes simplex virus thymidine kinase suicide gene. *Mol Ther*, Vol.4, No.2, pp. 146-148, ISSN 1525-0016

Chuah, M. K. L., Collen, D. & VandenDriessche, T. (2003). Biosafety of adenoviral vectors. Curr Gene Ther, Vol.3, No.6, pp. 527-543, ISSN 1566-5232

Ciuffi, A., Diamond, T. L., Hwang, Y., Marshall, H. M. & Bushman, F. D. (2006). Modulating target site selection during human immunodeficiency virus DNA integration in vitro with an engineered tethering factor. *Hum Gene Ther*, Vol.17, No.9, pp. 960-967, ISSN 1043-0342

Ciuffi, A., Ronen, K., Brady, T., Malani, N., Wang, G., Berry, C. C. & Bushman, F. D. (2009). Methods for integration site distribution analyses in animal cell genomes. Methods, Vol.47, No.4, pp. 261-268, ISSN 1046-2023

Collier, L. S., Carlson, C. M., Ravimohan, S., Dupuy, A. J. & Largaespada, D. A. (2005). Cancer gene discovery in solid tumours using transposon-based somatic mutagenesis in the mouse. *Nature*, Vol.436, No.7048, pp. 272-276, ISSN 0028-0836

Conwell, C. C. & Huang, L. (2005). Recent advances in non-viral gene delivery. *Adv Genet*, Vol.53, pp. 3-18, ISSN 0065-2660

Cornetta, K., Yao, J., Jasti, A., Koop, S., Douglas, M., Hsu, D., Couture, L. A., Hawkins, T. & Duffy, L. (2010). Replication-competent Lentivirus Analysis of Clinical Grade Vector Products. *Mol Ther*, ISSN 1525-0016

Cost, G. J., Freyvert, Y., Vafiadis, A., Santiago, Y., Miller, J. C., Rebar, E., Collingwood, T. N., Snowden, A. & Gregory, P. D. (2010). BAK and BAX deletion using zinc-finger nucleases yields apoptosis-resistant CHO cells. *Biotechnol Bioeng*, Vol.105, No.2, pp. 330-340, ISSN 0006-3592

De Oliveira, A. & Fraefel, C. (2010). Herpes simplex virus type 1/adeno-associated virus hybrid vectors. *The open virology journal*, Vol.4, pp. 109-122

Denny, W. A. (2003). Prodrugs for Gene-Directed Enzyme-Prodrug Therapy (Suicide Gene Therapy). *Journal of biomedicine & biotechnology*, Vol.2003, No.1, pp. 48-70, ISSN 1110-7243

Ding, S., Wu, X., Li, G., Han, M., Zhuang, Y. & Xu, T. (2005). Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice. *Cell*, Vol.122, No.3, pp. 473-483, ISSN 0092-8674

Doyon, Y., Choi, V. M., Xia, D. F., Vo, T. D., Gregory, P. D. & Holmes, M. C. (2010). Transient cold shock enhances zinc-finger nuclease-mediated gene disruption. *Nat Methods*, Vol.7, No.6, pp. 459-460, ISSN 1548-7091

Fajardo-Sanchez, E., Stricher, F., Pâques, F., Isalan, M. & Serrano, L. (2008). Computer design of obligate heterodimer meganucleases allows efficient cutting of custom DNA sequences. *Nucleic Acids Res*, Vol.36, No.7, pp. 2163-2173, ISSN 0305-1048

Farazi, T. A., Spitzer, J. I., Morozov, P. & Tuschl, T. (2011). miRNAs in human cancer. J Pathol, Vol.223, No.2, pp. 102-115, ISSN 0022-3417

Fazzari, M. J. & Greally, J. M. (2010). Introduction to epigenomics and epigenome-wide analysis. *Methods Mol Biol*, Vol.620, pp. 243-265, ISSN 1064-3745

Feng, D., Chen, J., Yue, Y., Zhu, H., Xue, J. & Jia, W. W. (2006). A 16bp Rep binding element is sufficient for mediating Rep-dependent integration into AAVS1. *J Mol Biol*, Vol.358, No.1, pp. 38-45, ISSN 0022-2836

Frank, K., Hogarth, D., Miller, J., Mandal, S., Mease, P., Samulski, R., Weisgerber, G. & Hart, J. (2009). Investigation of the cause of death in a gene-therapy trial. *N Engl J Med*, Vol.361, No.2, pp. 161-169, ISSN 0028-4793

Fu, F., Sander, J. D., Maeder, M., Thibodeau-Beganny, S., Joung, J. K., Dobbs, D., Miller, L. & Voytas, D. F. (2009). Zinc Finger Database (ZiFDB): a repository for information on

C2H2 zinc fingers and engineered zinc-finger arrays. *Nucleic Acids Res*, Vol.37, No.Database issue, pp. D279-D283, ISSN 0305-1048

Futreal, P. A., Coin, L., Marshall, M., Down, T., Hubbard, T., Wooster, R., Rahman, N. & Stratton, M. R. (2004). A census of human cancer genes. *Nat Rev Cancer*, Vol.4, No.3, pp. 177-183, ISSN 1474-175X

Gaspar, H., Parsley, K., Howe, S., King, D., Gilmour, K., Sinclair, J., Brouns, G., Schmidt, M., Vonkalle, C. & Barington, T. (2004). Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *The Lancet*, Vol.364, No.9452, pp. 2181-2187, ISSN 01406736

Garin, M. I., Garrett, E., Tiberghien, P., Apperley, J. F., Chalmers, D., Melo, J. V. & Ferrand, C. (2001). Molecular mechanism for ganciclovir resistance in human T lymphocytes transduced with retroviral vectors carrying the herpes simplex virus thymidine kinase gene. *Blood*, Vol.97, No.1, pp. 122-129, ISSN 0006-4971

Gerhardt, J., Jafar, S., Spindler, M. P., Ott, E. & Schepers, A. (2006). Identification of new human origins of DNA replication by an origin-trapping assay. *Mol Cell Biol*, Vol.26, No.20, pp. 7731-7746, ISSN 0270-7306

Gerrits, A., Dykstra, B., Kalmykowa, O. J., Klauke, K., Verovskaya, E., Broekhuis, M. J. C., de Haan, G. & Bystrykh, L. V. (2010). Cellular barcoding tool for clonal analysis in the hematopoietic system. *Blood*, Vol.115, No.13, pp. 2610-2618, ISSN 0006-4971

Geurts, A. M., Hackett, C. S., Bell, J. B., Bergemann, T. L., Collier, L. S., Carlson, C. M., Largaespada, D. A. & Hackett, P. B. (2006). Structure-based prediction of insertion-site preferences of transposons into chromosomes. *Nucleic Acids Res*, Vol.34, No.9, pp. 2803-2811, ISSN 0305-1048

Geurts, A. M., Yang, Y., Clark, K. J., Liu, G., Cui, Z., Dupuy, A. J., Bell, J. B., Largaespada, D. A. & Hackett, P. B. (2003). Gene transfer into genomes of human cells by the sleeping beauty transposon system. *Mol Ther*, Vol.8, No.1, pp. 108-117, ISSN 1525-0016

Gijsbers, R., Ronen, K., Vets, S., Malani, N., De Rijck, J., McNeely, M., Bushman, F. D. & Debyser, Z. (2010). LEDGF hybrids efficiently retarget lentiviral integration into heterochromatin. *Mol Ther*, Vol.18, No.3, pp. 552-560, ISSN 1525-0016

Giordano, F. A., Hotz-Wagenblatt, A., Lauterborn, D., Appelt, J. U., Fellenberg, K., Nagy, K. Z., Zeller, W. J., Suhai, S., Fruehauf, S. & Laufs, S. (2007). New bioinformatic strategies to rapidly characterize retroviral integration sites of gene therapy vectors. *Methods Inf Med*, Vol.46, No.5, pp. 542-547, ISSN 0026-1270

Grabundzija, I., Irgang, M., Mátés, L., Belay, E., Matrai, J., Gogol-Döring, A., Kawakami, K., Chen, W., Ruiz, P., Chuah, M. K. L., VandenDriessche, T., Izsvák, Z. & Ivics, Z. (2010). Comparative analysis of transposable element vector systems in human cells. *Mol Ther*, Vol.18, No.6, pp. 1200-1209, ISSN 1525-0016

Grimes, B. R., Schindelhauer, D., McGill, N. I., Ross, A., Ebersole, T. A. & Cooke, H. J. (2001). Stable gene expression from a mammalian artificial chromosome. *EMBO Rep*, Vol.2, No.10, pp. 910-914, ISSN 1469-221X

Grizot, S., Smith, J., Daboussi, F., Prieto, J., Redondo, P., Merino, N., Villate, M., Thomas, S., Lemaire, L., Montoya, G., Blanco, F. J., Pâques, F. & Duchateau, P. (2009). Efficient targeting of a SCID gene by an engineered single-chain homing endonuclease. *Nucleic Acids Res*, Vol.37, No.16, pp. 5405-5419, ISSN 0305-1048

Guo, J., Gaj, T. & Barbas, C. F. (2010). Directed evolution of an enhanced and highly efficient FokI cleavage domain for zinc finger nucleases. *J Mol Biol*, Vol.400, No.1, pp. 96-107, ISSN 0022-2836

Haase, R., Argyros, O., Wong, S. P., Harbottle, R. P., Lipps, H. J., Ogris, M., Magnusson, T., Vizoso Pinto, M. G., Haas, J. & Baiker, A. (2010). pEPito: a significantly improved non-viral episomal expression vector for mammalian cells. *BMC Biotechnol*, Vol.10, pp. 20

Hacein-Bey-Abina, S. (2003). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science*, Vol.302, No.5644, pp. 415-419, ISSN 0036-8075

Hacein-Bey-Abina, S., Garrigue, A., Wang, G. P., Soulier, J., Lim, A., Morillon, E., Clappier, E., Caccavelli, L., Delabesse, E., Beldjord, K., Asnafi, V., MacIntyre, E., Dal Cortivo, L., Radford, I., Brousse, N., Sigaux, F., Moshous, D., Hauer, J., Borkhardt, A., Belohradsky, B. H., Wintergerst, U., Velez, M. C., Leiva, L., Sorensen, R., Wulffraat, N., Blanche, S., Bushman, F. D., Fischer, A. & Cavazzana-Calvo, M. (2008). Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest*, Vol.118, No.9, pp. 3132-3142, ISSN 0021-9738

Hackett, P. B., Largaespada, D. A. & Cooper, L. J. (2010). A transposon and transposase system for human application. (1). *Mol Ther*, Vol.18, No.4, pp. 674-683, ISSN 1525-0016

Hanahan, D. & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell*, Vol.100, No.1, pp. 57-70, ISSN 0092-8674

Held, P. K., Olivares, E. C., Aguilar, C. P., Finegold, M., Calos, M. P. & Grompe, M. (2005). In vivo correction of murine hereditary tyrosinemia type I by phiC31 integrase-mediated gene delivery. *Mol Ther*, Vol.11, No.3, pp. 399-408, ISSN 1525-0016

Hester, S. D., Reid, L., Nowak, N., Jones, W. D., Parker, J. S., Knudtson, K., Ward, W., Tiesman, J. & Denslow, N. D. (2009). Comparison of comparative genomic hybridization technologies across microarray platforms. *J Biomol Tech*, Vol.20, No.2, pp. 135-151, ISSN 1524-0215

Hockemeyer, D., Soldner, F., Beard, C., Gao, Q., Mitalipova, M., DeKelver, R. C., Katibah, G. E., Amora, R., Boydston, E. A., Zeitler, B., Meng, X., Miller, J. C., Zhang, L., Rebar, E. J., Gregory, P. D., Urnov, F. D. & Jaenisch, R. (2009). Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. (1). *Nat Biotechnol*, Vol.27, No.9, pp. 851-857, ISSN 1087-0156

Howden, S. E., Voullaire, L. & Vadolas, J. (2008). The transient expression of mRNA coding for Rep protein from AAV facilitates targeted plasmid integration. *J Gene Med*, Vol.10, No.1, pp. 42-50, ISSN 1099-498X

Howe, S. J., Mansour, M. R., Schwarzwaelder, K., Bartholomae, C., Hubank, M., Kempski, H., Brugman, M. H., Pike-Overzet, K., Chatters, S. J., de Ridder, D., Gilmour, K. C., Adams, S., Thornhill, S. I., Parsley, K. L., Staal, F. J. T., Gale, R. E., Linch, D. C., Bayford, J., Brown, L., Quaye, M., Kinnon, C., Ancliff, P., Webb, D. K., Schmidt, M., von Kalle, C., Gaspar, H. B. & Thrasher, A. J. (2008). Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J Clin Invest*, Vol.118, No.9, pp. 3143-3150, ISSN 0021-9738

Huang, X., Guo, H., Tammana, S., Jung, Y. C., Mellgren, E., Bassi, P., Cao, Q., Tu, Z. J., Kim, Y. C., Ekker, S. C., Wu, X., Wang, S. M. & Zhou, X. (2010). Gene transfer efficiency and genome-wide integration profiling of Sleeping Beauty, Tol2, and piggyBac

transposons in human primary T cells. *Mol Ther*, Vol.18, No.10, pp. 1803-1813, ISSN 1525-0016

Huang, X., Haley, K., Wong, M., Guo, H., Lu, C., Wilber, A. & Zhou, X. (2010). Unexpectedly high copy number of random integration but low frequency of persistent expression of the Sleeping Beauty transposase after trans delivery in primary human T cells. *Hum Gene Ther*, Vol.21, No.11, pp. 1577-1590, ISSN 1043-0342

Ishikawa, Y., Tanaka, N., Murakami, K., Uchiyama, T., Kumaki, S., Tsuchiya, S., Kugoh, H., Oshimura, M., Calos, M. P. & Sugamura, K. (2006). Phage phiC31 integrase-mediated genomic integration of the common cytokine receptor gamma chain in human T-cell lines. *J Gene Med*, Vol.8, No.5, pp. 646-653, ISSN 1099-498X

Ivics, Z., Katzer, A., Stüwe, E. E., Fiedler, D., Knespel, S. & Izsvák, Z. (2007). Targeted Sleeping Beauty transposition in human cells. *Mol Ther*, Vol.15, No.6, pp. 1137-1144, ISSN 1525-0016

Ivics, Z., Hackett, P. B., Plasterk, R. H. & Izsvák, Z. (1997). Molecular reconstruction of Sleeping Beauty, a Tc1-like transposon from fish, and its transposition in human cells. *Cell*, Vol.91, No.4, pp. 501-510, ISSN 0092-8674

Izsvák, Z., Chuah, M. K. L., Vandendriessche, T. & Ivics, Z. (2009). Efficient stable gene transfer into human cells by the Sleeping Beauty transposon vectors. *Methods*, Vol.49, No.3, pp. 287-297, ISSN 1046-2023

Izsvák, Z., Hackett, P. B., Cooper, L. J. & Ivics, Z. (2010). Translating Sleeping Beauty transposition into cellular therapies: victories and challenges. *Bioessays*, Vol.32, No.9, pp. 756-767, ISSN 0265-9247

Jackson, D. A., Juranek, S. & Lipps, H. J. (2006). Designing nonviral vectors for efficient gene transfer and long-term gene expression. *Mol Ther*, Vol.14, No.5, pp. 613-626, ISSN 1525-0016

Jang, M. Y., Yarborough, O. H., Conyers, G. B., McPhie, P. & Owens, R. A. (2005). Stable secondary structure near the nicking site for adeno-associated virus type 2 Rep proteins on human chromosome 19. *J Virol*, Vol.79, No.6, pp. 3544-3556, ISSN 0022-538X

Kang, M. S., Hung, S. C. & Kieff, E. (2001). Epstein-Barr virus nuclear antigen 1 activates transcription from episomal but not integrated DNA and does not alter lymphocyte growth. *Proc Natl Acad Sci U S A*, Vol.98, No.26, pp. 15233-15238, ISSN 0027-8424

Katz, R. A., Merkel, G. & Skalka, A. M. (1996). Targeting of retroviral integrase by fusion to a heterologous DNA binding domain: in vitro activities and incorporation of a fusion protein into viral particles. *Virology*, Vol.217, No.1, pp. 178-190, ISSN 0042-6822

Keravala, A., Lee, S., Thyagarajan, B., Olivares, E. C., Gabrovsky, V. E., Woodard, L. E. & Calos, M. P. (2009). Mutational derivatives of PhiC31 integrase with increased efficiency and specificity. *Mol Ther*, Vol.17, No.1, pp. 112-120, ISSN 1525-0016

Kim, Y. G., Cha, J. & Chandrasegaran, S. (1996). Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proc Natl Acad Sci U S A*, Vol.93, No.3, pp. 1156-1160, ISSN 0027-8424

Kim, Y. J., Kim, Y. S., Larochelle, A., Renaud, G., Wolfsberg, T. G., Adler, R., Donahue, R. E., Hematti, P., Hong, B. K., Roayaei, J., Akagi, K., Riberdy, J. M., Nienhuis, A. W., Dunbar, C. E. & Persons, D. A. (2009). Sustained high-level polyclonal hematopoietic marking and transgene expression 4 years after autologous transplantation of rhesus macaques with SIV lentiviral vector-transduced CD34+ cells. *Blood*, Vol.113, No.22, pp. 5434-5443, ISSN 0006-4971

Koboldt, D. C., Ding, L., Mardis, E. R. & Wilson, R. K. (2010). Challenges of sequencing human genomes. Brief Bioinform, Vol.11, No.5, pp. 484-498, ISSN 1467-5463

Kohn, D. B. & Candotti, F. (2009). Gene therapy fulfilling its promise. N Engl J Med, Vol.360, No.5, pp. 518-521, ISSN 0028-4793

Kotzamanis, G., Cheung, W., Abdulrazzak, H., Perez-Luz, S., Howe, S., Cooke, H. & Huxley, C. (2005). Construction of human artificial chromosome vectors by recombineering. *Gene*, Vol.351, pp. 29-38, ISSN 0378-1119

Laufs, S., Gentner, B., Nagy, K. Z., Jauch, A., Benner, A., Naundorf, S., Kuehlcke, K., Schiedlmeier, B., Ho, A. D., Zeller, W. J. & Fruehauf, S. (2003). Retroviral vector integration occurs in preferred genomic targets of human bone marrow-repopulating cells. *Blood*, Vol.101, No.6, pp. 2191-2198, ISSN 0006-4971

Li, Z., Dullmann, J., Schiedlmeier, B., Schmidt, M., von Kalle, C., Meyer, J., Forster, M., Stocking, C., Wahlers, A. & Frank, O. (2002). Murine leukemia induced by retroviral gene marking. *Science*, Vol.296, No.5567, pp. 497, ISSN 0036-8075

Li, H., Pellenz, S., Ulge, U., Stoddard, B. L. & Monnat, R. J. (2009). Generation of single-chain LAGLIDADG homing endonucleases from native homodimeric precursor proteins. *Nucleic Acids Res*, Vol.37, No.5, pp. 1650-1662, ISSN 0305-1048

Liang, Q., Kong, J., Stalker, J. & Bradley, A. (2009). Chromosomal mobilization and reintegration of Sleeping Beauty and PiggyBac transposons. *Genesis*, Vol.47, No.6, pp. 404-408, ISSN 1526-954X

Liesner, R., Zhang, W., Noske, N. & Ehrhardt, A. (2010). Critical amino acid residues within the φC31 integrase DNA-binding domain affect recombination activities in mammalian cells. *Hum Gene Ther*, Vol.21, No.9, pp. 1104-1118, ISSN 1043-0342

Liu, J., Jeppesen, I., Nielsen, K. & Jensen, T. G. (2006). Phi c31 integrase induces chromosomal aberrations in primary human fibroblasts. *Gene Ther*, Vol.13, No.15, pp. 1188-1190, ISSN 0969-7128

Liu, R., Li, Y., Hu, R., Jin, T., Deng, S., Liang, W., Zhang, N., Chen, J., Prud'homme, G. J., Jia, W. W., Ma, D. & Wang, Q. (2010). A site-specific genomic integration strategy for sustained expression of glucagon-like peptide-1 in mouse muscle for controlling energy homeostasis. *Biochem Biophys Res Commun*, Vol.403, No.2, pp. 172-177, ISSN 0006-291X

Lombardo, A., Genovese, P., Beausejour, C. M., Colleoni, S., Lee, Y. L., Kim, K. A., Ando, D., Urnov, F. D., Galli, C., Gregory, P. D., Holmes, M. C. & Naldini, L. (2007). Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. (1). *Nat Biotechnol*, Vol.25, No.11, pp. 1298-1306, ISSN 1087-0156

Lufino, M. M. P., Edser, P. A. H. & Wade-Martins, R. (2008). Advances in high-capacity extrachromosomal vector technology: episomal maintenance, vector delivery, and transgene expression. *Mol Ther*, Vol.16, No.9, pp. 1525-1538, ISSN 1525-0016

Lupo-Stanghellini, M., Provasi, E., Bondanza, A., Ciceri, F., Bordignon, C. & Bonini, C. (2010). Clinical impact of suicide gene therapy in allogeneic hematopoietic stem cell transplantation. *Hum Gene Ther*, Vol.21, No.3, pp. 241-250, ISSN 1043-0342

Maeder, M. L., Thibodeau-Beganny, S., Osiak, A., Wright, D. A., Anthony, R. M., Eichtinger, M., Jiang, T., Foley, J. E., Winfrey, R. J., Townsend, J. A., Unger-Wallace, E., Sander, J. D., Müller-Lerch, F., Fu, F., Pearlberg, J., Göbel, C., Dassie, J. P., Pruett-Miller, S. M., Porteus, M. H., Sgroi, D. C., Iafrate, A. J., Dobbs, D., McCray, P. B., Cathomen, T., Voytas, D. F. & Joung, J. K. (2008). Rapid "open-source" engineering of

customized zinc-finger nucleases for highly efficient gene modification. *Mol Cell*, Vol.31, No.2, pp. 294-301, ISSN 1097-2765

Mátés, L., Chuah, M. K. L., Belay, E., Jerchow, B., Manoj, N., Acosta-Sanchez, A., Grzela, D. P., Schmitt, A., Becker, K., Matrai, J., Ma, L., Samara-Kuko, E., Gysemans, C., Pryputniewicz, D., Miskey, C., Fletcher, B., VandenDriessche, T., Ivics, Z. & Izsvák, Z. (2009). Molecular evolution of a novel hyperactive Sleeping Beauty transposase enables robust stable gene transfer in vertebrates. *Nat Genet*, Vol.41, No.6, pp. 753-761, ISSN 1061-4036

McCammon, J. M. & Amacher, S. L. (2010). Using zinc finger nucleases for efficient and heritable gene disruption in zebrafish. *Methods Mol Biol*, Vol.649, pp. 281-298, ISSN 1064-3745

Mercer, K. E., Ahn, C. E., Coke, A., Compadre, C. M. & Drake, R. R. (2002). Mutation of herpesvirus thymidine kinase to generate ganciclovir-specific kinases for use in cancer gene therapies. *Protein Eng*, Vol.15, No.11, pp. 903-911, ISSN 0269-2139

Miller, D. G., Rutledge, E. A. & Russell, D. W. (2002). Chromosomal effects of adeno-associated virus vector integration. *Nat Genet*, Vol.30, No.2, pp. 147-148, ISSN 1061-4036

Miller, D. G., Trobridge, G. D., Petek, L. M., Jacobs, M. A., Kaul, R. & Russell, D. W. (2005). Large-scale analysis of adeno-associated virus vector integration sites in normal human cells. *J Virol*, Vol.79, No.17, pp. 11434-11442, ISSN 0022-538X

Miller, J. C., Holmes, M. C., Wang, J., Guschin, D. Y., Lee, Y. L., Rupniewski, I., Beausejour, C. M., Waite, A. J., Wang, N. S., Kim, K. A., Gregory, P. D., Pabo, C. O. & Rebar, E. J. (2007). An improved zinc-finger nuclease architecture for highly specific genome editing. *Nat Biotechnol*, Vol.25, No.7, pp. 778-785, ISSN 1087-0156

Mitchell, R. S., Beitzel, B. F., Schroder, A. R. W., Shinn, P., Chen, H., Berry, C. C., Ecker, J. R. & Bushman, F. D. (2004). Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. (1). *PLoS Biol*, Vol.2, No.8, pp. E234, ISSN 1544-9173

Modlich, U. (2005). Leukemias following retroviral transfer of multidrug resistance 1 (MDR1) are driven by combinatorial insertional mutagenesis. *Blood*, Vol.105, No.11, pp. 4235-4246, ISSN 0006-4971

Modlich, U., Bohne, J., Schmidt, M., von Kalle, C., Knöss, S., Schambach, A. & Baum, C. (2006). Cell-culture assays reveal the importance of retroviral vector design for insertional genotoxicity. *Blood*, Vol.108, No.8, pp. 2545-2553, ISSN 0006-4971

Modlich, U., Navarro, S., Zychlinski, D., Maetzig, T., Knoess, S., Brugman, M. H., Schambach, A., Charrier, S., Galy, A., Thrasher, A. J., Bueren, J. & Baum, C. (2009). Insertional transformation of hematopoietic cells by self-inactivating lentiviral and gammaretroviral vectors. (1). *Mol Ther*, Vol.17, No.11, pp. 1919-1928, ISSN 1525-0016

Moehle, E. A., Rock, J. M., Lee, Y. L., Jouvenot, Y., DeKelver, R. C., Gregory, P. D., Urnov, F. D. & Holmes, M. C. (2007). Targeted gene addition into a specified location in the human genome using designed zinc finger nucleases. *Proceedings of the National Academy of Sciences*, Vol.104, No.9, pp. 3055-3060, ISSN 0027-8424

Moldt, B., Staunstrup, N. H., Jakobsen, M., Yáñez-Muñoz, R. J. & Mikkelsen, J. G. (2008). Genomic insertion of lentiviral DNA circles directed by the yeast Flp recombinase. *BMC Biotechnol*, Vol.8, pp. 60

Montini, E., Cesana, D., Schmidt, M., Sanvito, F., Bartholomae, C., Ranzani, M., Benedicenti, F., Sergi, L., Ambrosi, A. & Ponzoni, M. (2009). The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a

mouse model of HSC gene therapy. *J Clin Invest*, Vol.119, No.4, pp. 964-975, ISSN 0021-9738

Montini, E., Cesana, D., Schmidt, M., Sanvito, F., Ponzoni, M., Bartholomae, C., Sergi Sergi, L., Benedicenti, F., Ambrosi, A., Di Serio, C., Doglioni, C., von Kalle, C. & Naldini, L. (2006). Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. *Nat Biotechnol*, Vol.24, No.6, pp. 687-696, ISSN 1087-0156

Nienhuis, A. W., Dunbar, C. E. & Sorrentino, B. P. (2006). Genotoxicity of retroviral integration in hematopoietic cells. *Mol Ther*, Vol.13, No.6, pp. 1031-1049, ISSN 1525-0016

Niidome, T. & Huang, L. (2002). Gene therapy progress and prospects: nonviral vectors. *Gene Ther*, Vol.9, No.24, pp. 1647-1652, ISSN 0969-7128

Ohlfest, J. R., Frandsen, J. L., Fritz, S., Lobitz, P. D., Perkinson, S. G., Clark, K. J., Nelsestuen, G., Key, N. S., McIvor, R. S., Hackett, P. B. & Largaespada, D. A. (2005). Phenotypic correction and long-term expression of factor VIII in hemophilic mice by immunotolerization and nonviral gene transfer using the Sleeping Beauty transposon system. *Blood*, Vol.105, No.7, pp. 2691-2698, ISSN 0006-4971

Olivares, E. C., Hollis, R. P., Chalberg, T. W., Meuse, L., Kay, M. A. & Calos, M. P. (2002). Site-specific genomic integration produces therapeutic Factor IX levels in mice. *Nat Biotechnol*, Vol.20, No.11, pp. 1124-1128, ISSN 1087-0156

Ortiz-Urda, S., Lin, Q., Yant, S., Keene, D., Kay, M. & Khavari, P. (2003). Sustainable correction of junctional epidermolysis bullosa via transposon-mediated nonviral gene transfer. (1). *Gene Ther*, Vol.10, No.13, pp. 1099-1104, ISSN 0969-7128

Ortiz-Urda, S., Thyagarajan, B., Keene, D. R., Lin, Q., Fang, M., Calos, M. P. & Khavari, P. A. (2002). Stable nonviral genetic correction of inherited human skin disease. *Nat Med*, Vol.8, No.10, pp. 1166-1170, ISSN 1078-8956

Ott, M. G., Schmidt, M., Schwarzwaelder, K., Stein, S., Siler, U., Koehl, U., Glimm, H., Kühlcke, K., Schilz, A., Kunkel, H., Naundorf, S., Brinkmann, A., Deichmann, A., Fischer, M., Ball, C., Pilz, I., Dunbar, C., Du, Y., Jenkins, N. A., Copeland, N. G., Lüthi, U., Hassan, M., Thrasher, A. J., Hoelzer, D., von Kalle, C., Seger, R. & Grez, M. (2006). Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nat Med*, Vol.12, No.4, pp. 401-409, ISSN 1078-8956

Papapetrou, E. P., Zoumbos, N. C. & Athanassiadou, A. (2005). Genetic modification of hematopoietic stem cells with nonviral systems: past progress and future prospects. *Gene Ther*, Vol.12 Suppl 1, pp. S118-S130, ISSN 0969-7128

Perez, E. E., Wang, J., Miller, J. C., Jouvenot, Y., Kim, K. A., Liu, O., Wang, N., Lee, G., Bartsevich, V. V., Lee, Y. L., Guschin, D. Y., Rupniewski, I., Waite, A. J., Carpenito, C., Carroll, R. G., S Orange, J., Urnov, F. D., Rebar, E. J., Ando, D., Gregory, P. D., Riley, J. L., Holmes, M. C. & June, C. H. (2008). Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. *Nat Biotechnol*, Vol.26, No.7, pp. 808-816, ISSN 1087-0156

Peters, B., Dirscherl, S., Dantzer, J., Nowacki, J., Cross, S., Li, X., Cornetta, K., Dinauer, M. C. & Mooney, S. D. (2008). Automated analysis of viral integration sites in gene therapy research using the SeqMap web resource. *Gene Ther*, Vol.15, No.18, pp. 1294-1298, ISSN 0969-7128

Philpott, N. J., Gomos, J., Berns, K. I. & Falck-Pedersen, E. (2002). A p5 integration efficiency element mediates Rep-dependent integration into AAVS1 at chromosome 19. *Proc Natl Acad Sci U S A*, Vol.99, No.19, pp. 12381-12385, ISSN 0027-8424

Pieroni, L., Fipaldini, C., Monciotti, A., Cimini, D., Sgura, A., Fattori, E., Epifano, O., Cortese, R., Palombo, F. & La Monica, N. (1998). Targeted integration of adeno-associated virus-derived plasmids in transfected human cells. *Virology*, Vol.249, No.2, pp. 249-259, ISSN 0042-6822

Porteus, M. H. & Baltimore, D. (2003). Chimeric nucleases stimulate gene targeting in human cells. *Science*, Vol.300, No.5620, pp. 763, ISSN 0036-8075

Preuß, E., Treschow, A., Newrzela, S., Brücher, D., Weber, K., Felldin, U., Alici, E., Gahrton, G., von Laer, D., Dilber, M. S. & Fehse, B. (2010). TK.007: A novel, codon-optimized HSVtk(A168H) mutant for suicide gene therapy. *Hum Gene Ther*, Vol.21, No.8, pp. 929-941, ISSN 1043-0342

Rad, R., Rad, L., Wang, W., Cadinanos, J., Vassiliou, G., Rice, S., Campos, L. S., Yusa, K., Banerjee, R., Li, M. A., de la Rosa, J., Strong, A., Lu, D., Ellis, P., Conte, N., Yang, F. T., Liu, P. & Bradley, A. (2010). PiggyBac transposon mutagenesis: a tool for cancer gene discovery in mice. *Science*, Vol.330, No.6007, pp. 1104-1107, ISSN 0036-8075

Ramezani, A., Hawley, T. S. & Hawley, R. G. (2008). Combinatorial incorporation of enhancer-blocking components of the chicken beta-globin 5'HS4 and human T-cell receptor alpha/delta BEAD-1 insulators in self-inactivating retroviral vectors reduces their genotoxic potential. *Stem Cells*, Vol.26, No.12, pp. 3257-3266, ISSN 1066-5099

Raper, S. E., Chirmule, N., Lee, F. S., Wivel, N. A., Bagg, A., Gao, G. P., Wilson, J. M. & Batshaw, M. L. (2003). Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab*, Vol.80, No.1-2, pp. 148-158, ISSN 1096-7192

Reyon, D., Kirkpatrick, J. R., Sander, J. D., Zhang, F., Voytas, D. F., Joung, J. K., Dobbs, D. & Coffman, C. R. (2011). ZFNGenome: A comprehensive resource for locating zinc finger nuclease target sites in model organisms. *BMC Genomics*, Vol.12, No.1, pp. 83

Rouet, P., Smih, F. & Jasin, M. (1994). Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. *Proc Natl Acad Sci U S A*, Vol.91, No.13, pp. 6064-6068, ISSN 0027-8424

Rémy, S., Tesson, L., Ménoret, S., Usal, C., Scharenberg, A. M. & Anegon, I. (2010). Zinc-finger nucleases: a powerful tool for genetic engineering of animals. *Transgenic Res*, Vol.19, No.3, pp. 363-371, ISSN 0962-8819

Saeki, Y., Wataya-Kaneda, M., Tanaka, K. & Kaneda, Y. (1998). Sustained transgene expression in vitro and in vivo using an Epstein-Barr virus replicon vector system combined with HVJ liposomes. *Gene Ther*, Vol.5, No.8, pp. 1031-1037, ISSN 0969-7128

Sander, J. D., Dahlborg, E. J., Goodwin, M. J., Cade, L., Zhang, F., Cifuentes, D., Curtin, S. J., Blackburn, J. S., Thibodeau-Beganny, S., Qi, Y., Pierick, C. J., Hoffman, E., Maeder, M. L., Khayter, C., Reyon, D., Dobbs, D., Langenau, D. M., Stupar, R. M., Giraldez, A. J., Voytas, D. F., Peterson, R. T., Yeh, J. R. J. & Joung, J. K. (2011). Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). Nat Methods, Vol.8, No.1, pp. 67-69, ISSN 1548-7091

Sander, J. D., Maeder, M. L., Reyon, D., Voytas, D. F., Joung, J. K. & Dobbs, D. (2010). ZiFiT (Zinc Finger Targeter): an updated zinc finger engineering tool. *Nucleic Acids Res*, Vol.38, No.Web Server issue, pp. W462-W468, ISSN 0305-1048

Sarkis, C., Philippe, S., Mallet, J. & Serguera, C. (2008). Non-integrating lentiviral vectors. *Curr Gene Ther*, Vol.8, No.6, pp. 430-437, ISSN 1566-5232

Schambach, A., Galla, M., Maetzig, T., Loew, R. & Baum, C. (2007). Improving transcriptional termination of self-inactivating gamma-retroviral and lentiviral vectors. *Mol Ther*, Vol.15, No.6, pp. 1167-1173, ISSN 1525-0016

Schmidt, M., Schwarzwaelder, K., Bartholomae, C., Zaoui, K., Ball, C., Pilz, I., Braun, S., Glimm, H. & von Kalle, C. (2007). High-resolution insertion-site analysis by linear amplification-mediated PCR (LAM-PCR). *Nat Methods*, Vol.4, No.12, pp. 1051-1057, ISSN 1548-7091

Schulz, T. F. & Cordes, S. (2009). Is the Epstein-Barr virus EBNA-1 protein an oncogen? *Proc Natl Acad Sci U S A*, Vol.106, No.7, pp. 2091-2092, ISSN 0027-8424

Schäfer-Korting, M., Heilbronn, R. & Weger, S. (2010). Viral vectors for gene transfer: current status of gene therapeutics. *Handb Exp Pharmacol*, No.197, pp. 143-170, ISSN 0171-2004

Sclimenti, C. R., Thyagarajan, B. & Calos, M. P. (2001). Directed evolution of a recombinase for improved genomic integration at a native human sequence. *Nucleic Acids Res*, Vol.29, No.24, pp. 5044-5051, ISSN 0305-1048

Scollay, R. (2001). Gene therapy: a brief overview of the past, present, and future. *Ann N Y Acad Sci*, Vol.953, pp. 26-30, ISSN 0077-8923

Segal, D. J., Beerli, R. R., Blancafort, P., Dreier, B., Effertz, K., Huber, A., Koksch, B., Lund, C. V., Magnenat, L., Valente, D. & Barbas, C. F. (2003). Evaluation of a modular strategy for the construction of novel polydactyl zinc finger DNA-binding proteins. *Biochemistry*, Vol.42, No.7, pp. 2137-2148, ISSN 0006-2960

Shukla, V. K., Doyon, Y., Miller, J. C., DeKelver, R. C., Moehle, E. A., Worden, S. E., Mitchell, J. C., Arnold, N. L., Gopalan, S., Meng, X., Choi, V. M., Rock, J. M., Wu, Y. Y., Katibah, G. E., Zhifang, G., McCaskill, D., Simpson, M. A., Blakeslee, B., Greenwalt, S. A., Butler, H. J., Hinkley, S. J., Zhang, L., Rebar, E. J., Gregory, P. D. & Urnov, F. D. (2009). Precise genome modification in the crop species Zea mays using zinc-finger nucleases. *Nature*, Vol.459, No.7245, pp. 437-441, ISSN 0028-0836

Silver, J. & Keerikatte, V. (1989). Novel use of polymerase chain reaction to amplify cellular DNA adjacent to an integrated provirus. *J Virol*, Vol.63, No.5, pp. 1924-1928, ISSN 0022-538X

Sinn, P. L., Sauter, S. L. & McCray, P. B. (2005). Gene therapy progress and prospects: development of improved lentiviral and retroviral vectors--design, biosafety, and production. *Gene Ther*, Vol.12, No.14, pp. 1089-1098, ISSN 0969-7128

Sivalingam, J., Krishnan, S., Ng, W. H., Lee, S. S., Phan, T. T. & Kon, O. L. (2010). Biosafety assessment of site-directed transgene integration in human umbilical cord-lining cells. *Mol Ther*, Vol.18, No.7, pp. 1346-1356, ISSN 1525-0016

Smith, R. H. (2008). Adeno-associated virus integration: virus versus vector. *Gene Ther*, Vol.15, No.11, pp. 817-822, ISSN 0969-7128

Stehle, I. M., Postberg, J., Rupprecht, S., Cremer, T., Jackson, D. A. & Lipps, H. J. (2007). Establishment and mitotic stability of an extra-chromosomal mammalian replicon. *BMC Cell Biol*, Vol.8, pp. 33, ISSN 1471-2121

Stein, S., Ott, M. G., Schultze-Strasser, S., Jauch, A., Burwinkel, B., Kinner, A., Schmidt, M., Krämer, A., Schwäble, J., Glimm, H., Koehl, U., Preiss, C., Ball, C., Martin, H., Göhring, G., Schwarzwaelder, K., Hofmann, W. K.,Karakaya, K., Tchatchou, S., Yang, R., Reinecke, P., Kühlcke, K., Schlegelberger, B., Thrasher, A. J., Hoelzer, D., Seger, R., von Kalle, C. & Grez, M. (2010). Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. *Nat Med*, Vol.16, No.2, pp. 198-204, ISSN 1078-8956

Stephen, S. L., Sivanandam, V. G. & Kochanek, S. (2008). Homologous and heterologous recombination between adenovirus vector DNA and chromosomal DNA. *J Gene Med*, Vol.10, No.11, pp. 1176-1189, ISSN 1099-498X

Szczepek, M., Brondani, V., Büchel, J., Serrano, L., Segal, D. J. & Cathomen, T. (2007). Structure-based redesign of the dimerization interface reduces the toxicity of zinc-finger nucleases. *Nat Biotechnol*, Vol.25, No.7, pp. 786-793, ISSN 1087-0156

Takahashi, K. & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, Vol.126, No.4, pp. 663-676, ISSN 0092-8674

Tatum, E. L. (1966). Molecular biology, nucleic acids, and the future of medicine. *Perspect Biol Med*, Vol.10, No.1, pp. 19-32, ISSN 0031-5982

Tenenbaum, L., Lehtonen, E. & Monahan, P. E. (2003). Evaluation of risks related to the use of adeno-associated virus-based vectors. Curr Gene Ther, Vol.3, No.6, pp. 545-565, ISSN 1566-5232

Tey, S. K., Dotti, G., Rooney, C. M., Heslop, H. E. & Brenner, M. K. (2007). Inducible caspase 9 suicide gene to improve the safety of allodepleted T cells after haploidentical stem cell transplantation. *Biol Blood Marrow Transplant*, Vol.13, No.8, pp. 913-924, ISSN 1083-8791

Thomae, A. W., Pich, D., Brocher, J., Spindler, M. P., Berens, C., Hock, R., Hammerschmidt, W. & Schepers, A. (2008). Interaction between HMGA1a and the origin recognition complex creates site-specific replication origins. *Proc Natl Acad Sci U S A*, Vol.105, No.5, pp. 1692-1697, ISSN 0027-8424

Thyagarajan, B., Liu, Y., Shin, S., Lakshmipathy, U., Scheyhing, K., Xue, H., Ellerström, C., Strehl, R., Hyllner, J., Rao, M. S. & Chesnut, J. D. (2008). Creation of engineered human embryonic stem cell lines using phiC31 integrase. *Stem Cells*, Vol.26, No.1, pp. 119-126, ISSN 1066-5099

Townsend, J. A., Wright, D. A., Winfrey, R. J., Fu, F., Maeder, M. L., Joung, J. K. & Voytas, D. F. (2009). High-frequency modification of plant genes using engineered zinc-finger nucleases. *Nature*, Vol.459, No.7245, pp. 442-445, ISSN 0028-0836

Urnov, F. D., Rebar, E. J., Holmes, M. C., Zhang, H. S. & Gregory, P. D. (2010). Genome editing with engineered zinc finger nucleases. *Nature Reviews Genetics*, Vol.11, No.9, pp. 636-646, ISSN 1471-0056

Urnov, F. D., Miller, J. C., Lee, Y. L., Beausejour, C. M., Rock, J. M., Augustus, S., Jamieson, A. C., Porteus, M. H., Gregory, P. D. & Holmes, M. C. (2005). Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature*, Vol.435, No.7042, pp. 646-651, ISSN 0028-0836

Varella-Garcia, M., Chen, L., Powell, R. L., Hirsch, F. R., Kennedy, T. C., Keith, R., Miller, Y. E., Mitchell, J. D. & Franklin, W. A. (2007). Spectral karyotyping detects chromosome damage in bronchial cells of smokers and patients with cancer. *Am J Respir Crit Care Med*, Vol.176, No.5, pp. 505-512, ISSN 1073-449X

Vasquez, K. M., Wang, G., Havre, P. A. & Glazer, P. M. (1999). Chromosomal mutations induced by triplex-forming oligonucleotides in mammalian cells. *Nucleic Acids Res*, Vol.27, No.4, pp. 1176-1181, ISSN 0305-1048

Vink, C. A., Gaspar, H. B., Gabriel, R., Schmidt, M., McIvor, R. S., Thrasher, A. J. & Qasim, W. (2009). Sleeping beauty transposition from nonintegrating lentivirus. *Mol Ther*, Vol.17, No.7, pp. 1197-1204, ISSN 1525-0016

Walisko, O., Schorn, A., Rolfs, F., Devaraj, A., Miskey, C., Izsvák, Z. & Ivics, Z. (2008). Transcriptional activities of the Sleeping Beauty transposon and shielding its genetic cargo with insulators. *Mol Ther*, Vol.16, No.2, pp. 359-369, ISSN 1525-0016

Walther, W. & Stein, U. (2000). Viral vectors for gene transfer: a review of their use in the treatment of human diseases. *Drugs*, Vol.60, No.2, pp. 249-271, ISSN 0012-6667

Wang, G. P., Berry, C. C., Malani, N., Leboulch, P., Fischer, A., Hacein-Bey-Abina, S., Cavazzana-Calvo, M. & Bushman, F. D. (2010). Dynamics of gene-modified progenitor cells analyzed by tracking retroviral integration sites in a human SCID-X1 gene therapy trial. *Blood*, Vol.115, No.22, pp. 4356-4366, ISSN 0006-4971

Wang, G. P., Garrigue, A., Ciuffi, A., Ronen, K., Leipzig, J., Berry, C., Lagresle-Peyrou, C., Benjelloun, F., Hacein-Bey-Abina, S., Fischer, A., Cavazzana-Calvo, M. & Bushman, F. D. (2008). DNA bar coding and pyrosequencing to analyze adverse events in therapeutic gene transfer. *Nucleic Acids Res*, Vol.36, No.9, pp. e49, ISSN 0305-1048

Wang, Z., Troilo, P. J., Wang, X., Griffiths, T. G., Pacchione, S. J., Barnum, A. B., Harper, L. B., Pauley, C. J., Niu, Z., Denisova, L., Follmer, T. T., Rizzuto, G., Ciliberto, G., Fattori, E., Monica, N. L., Manam, S. & Ledwith, B. J. (2004). Detection of integration of plasmid DNA into host genomic DNA following intramuscular injection and electroporation. *Gene Ther*, Vol.11, No.8, pp. 711-721, ISSN 0969-7128

Weber, E. L. & Cannon, P. M. (2007). Promoter choice for retroviral vectors: transcriptional strength versus trans-activation potential. *Hum Gene Ther*, Vol.18, No.9, pp. 849-860, ISSN 1043-0342

Wolff, J. & Lederberg, J. (1994). An early history of gene transfer and therapy. *Hum Gene Ther*, Vol.5, No.4, pp. 469-480, ISSN 1043-0342

Woltjen, K., Michael, I. P., Mohseni, P., Desai, R., Mileikovsky, M., Hämäläinen, R., Cowling, R., Wang, W., Liu, P., Gertsenstein, M., Kaji, K., Sung, H. K. & Nagy, A. (2009). piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature*, Vol.458, No.7239, pp. 766-770, ISSN 0028-0836

Wu, S. C. Y., Meir, Y. J. J., Coates, C. J., Handler, A. M., Pelczar, P., Moisyadi, S. & Kaminski, J. M. (2006). piggyBac is a flexible and highly active transposon as compared to sleeping beauty, Tol2, and Mos1 in mammalian cells. *Proc Natl Acad Sci U S A*, Vol.103, No.41, pp. 15008-15013, ISSN 0027-8424

Xue, X., Huang, X., Nodland, S. E., Mátés, L., Ma, L., Izsvák, Z., Ivics, Z., LeBien, T. W., McIvor, R. S., Wagner, J. E. & Zhou, X. (2009). Stable gene transfer and expression in cord blood-derived CD34+ hematopoietic stem and progenitor cells by a hyperactive Sleeping Beauty transposon system. *Blood*, Vol.114, No.7, pp. 1319-1330, ISSN 0006-4971

Yant, S. R., Meuse, L., Chiu, W., Ivics, Z., Izsvak, Z. & Kay, M. A. (2000). Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system. *Nat Genet*, Vol.25, No.1, pp. 35-41, ISSN 1061-4036

Yant, S. R., Huang, Y., Akache, B. & Kay, M. A. (2007). Site-directed transposon integration in human cells. *Nucleic Acids Res*, Vol.35, No.7, pp. e50, ISSN 0305-1048

Yusa, K., Zhou, L., Li, M. A., Bradley, A. & Craig, N. L. (2011). A hyperactive piggyBac transposase for mammalian applications. *Proc Natl Acad Sci U S A*, Vol.108, No.4, pp. 1531-1536, ISSN 0027-8424

Zhu, J., Park, C. W., Sjeklocha, L., Kren, B. T. & Steer, C. J. (2010). High-level genomic integration, epigenetic changes, and expression of sleeping beauty transgene. *Biochemistry*, Vol.49, No.7, pp. 1507-1521, ISSN 0006-2960

Zychlinski, D., Schambach, A., Modlich, U., Maetzig, T., Meyer, J., Grassman, E., Mishra, A. & Baum, C. (2008). Physiological promoters reduce the genotoxic risk of integrating gene vectors. *Mol Ther*, Vol.16, No.4, pp. 718-725, ISSN 1525-0016

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The aim of this book is to cover key aspects of existing problems in the field of development and future perspectives in gene therapy. Contributions consist of basic and translational research, as well as clinical experiences, and they outline functional mechanisms, predictive approaches, patient-related studies and upcoming challenges in this stimulating but also controversial field of gene therapy research. This source will make our doctors become comfortable with the common problems of gene therapy and inspire others to delve a bit more deeply into a topic of interest.

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