A publication of
The Williamsburg BioProcessing Foundation

July/August 2004

BioProcessing™ JOURNAL

Advances & Trends In Biological Product Development

Vol. 3 No. 4

www.bioprocessingjournal.com

The ACE System: *A Versatile Chromosome Engineering Technology with Applications for Gene-Based Cell Therapy*

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Current *in vivo* gene therapy (GT) approaches are beginning to demonstrate significant clinical and safety limitations that may ultimately reduce their therapeutic utility. In particular, the potential for systemic toxicity due to the gene transfer vector, the inability to administer multiple doses due to the antigenicity of the gene transfer vector, the prospect of insertional mutagenesis/oncogenesis during gene transfer, and the possibility of germ line transfer of the transgene are issues raising concern.[1–5] One promising alternative to gene therapy that mitigates these clinical and safety issues is gene-based cell therapy (GBCT), in which autologous cells are removed from a patient and modified *ex vivo* for a desired characteristic prior to reimplantation. By transferring the transgene *ex vivo*, many of the issues surrounding the *in vivo* use of the transfer vectors are reduced and issues surrounding germ line transfer can be practically eliminated.

As a result, GBCTs are rapidly emerging as a viable clinical strategy for the treatment of diseases that have become the typical targets of *in vivo* gene therapy (Table 1); i.e., cancer, infectious diseases, and metabolic and autoimmune disorders for which gene replacement, enhanced immunological responses, or administration of growth factors and metabolic regulators are believed to elicit a therapeutic or ameliorative effect. To this end, GBCT now represents approximately 29 percent of the ongoing gene therapy clinical trials, and all indications are that this percentage will continue to increase over the coming years.

Table 1. Clinical Trials in Gene-Based Cell Therapy*

Class	Vector	Indications				Total
		Cancers	Genetic Diseases	Infectious Diseases	Acquired Diseases	
Viral	Retrovirus	124	38	30	5	197
	Lentivirus	—	—	1	—	1
	Adenovirus	25	—	3	—	28
	AAV	2	—	—	—	2
Non-Viral	Naked DNA	22	1	—	1	24
	Formulated DNA	14	—	—	—	14
	Formulated RNA	10	—	—	—	10
Total		197	39	34	6	276

*From http://www.wiley.co.uk/genetherapy/clinical/ (Updated January 31, 2004)

Gene therapy and GBCT currently rely on similar gene delivery vehicles and reagents to introduce the transgene(s) (Table 2). As a result, gene transfer, regardless of whether it occurs *in vivo* or *ex vivo*, suffers from the limitations of low DNA carrying capacity, insertional mutagenesis/oncogenesis, short-term transgene expression, and/or delivery efficiency (Table 2). Specifically, integration of retroviral vectors into the host chromosome has led to variegated gene expression, insertional mutagenesis, and oncogenesis.[4,5] Recent concerns have been raised with adeno-associated virus (AAV) vectors regarding the presence of DNA vector sequences detected in semen samples from patients in a phase 1 clinical trial, and the association of AAV integration with chromosomal deletions and other rearrangements that frequently are located on human chromosome 19.[2,3] With respect to non-integrating vectors such as adenoviral vectors, the primary concern is transient transgene expression, which in the case of *in vivo* gene therapy, necessitates repeated administrations.[1,6] All viral methods have a limited DNA carrying capacity, which prevents transfer of multiple transgenes or long stretches of genomic DNA. Finally, non-viral methods are simply not robust enough from the perspective of DNA delivery and

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long-term transgene expression. Thus, there has been considerable effort to develop technologies for gene therapy and GBCT that are phenotypically and immunologically "inert," are maintained autonomously with the host cell genome, and can carry large "payloads" of DNA sequences for regulated stable transgene expression (e.g., locus control regions, tissue specific promoters, genomic sequences). In light of the historical *in vivo* gene therapy setbacks with viral vectors, it is critical to further incorporate relevant safety features (e.g., suicide genes, inducible promoters) into gene-modified delivery vehicles.[4,5,7] One technology that offers a potential solution to the above

Table 2. Major Vectors Developed for Gene Therapy and Gene-Based Cell Therapy

Vector Type	Examples	Advantages	Disadvantages
Integrating RNA Viruses	Retrovirus	• Long-term expression • Low immunogenicity	• Potential insertional mutagenesis • Infects only dividing cells • Transduction efficiency ≈ 1% • Low insert capacity ≈ 8 Kbp
	Lentivirus	• Infects dividing & quiescent cells • Long-term expression • Low immunogenicity • Transduction efficiency > 40%	• Potential insertional mutagenesis • Low insert capacity ≈ 8 Kbp
	Foamy Virus	• Infects dividing & quiescent cells • Long-term expression • Low immunogenicity • Transduction efficiency > 85%	• Potential insertional mutagenesis • Low insert capacity ≈ 12 Kbp
Non-integrating RNA Viruses	Sindbis Virus, Semliki Forest Virus	• Infects dividing & quiescent cells • Non-integrating virus • High transient expression • Transduction efficiency ≈ 10%	• Low insert capacity ≈ 5 Kbp • Transient expression
DNA Viruses	AAV	• Infects dividing & quiescent cells • High transient expression • Low immunogenicity • Very low integration • Transduction efficiency > 95%	• Low insert capacity ≈ 4 Kbp • Requires helper viruses • Transient expression
	Adenovirus	• Infects dividing & quiescent cells • Non-integrating virus • High transient expression • Transduction efficiency > 90%	• Cellular immune response/ rejection • Transient expression • Low insert capacity ≈ 8 Kbp
	Herpes Simplex Virus	• Infects dividing & quiescent cells • Non-integrating virus • Good insert capacity ≈ 35 Kbp • Transduction efficiency > 90%	• Cellular immune response/ rejection • High to moderate cytotoxicity
Non-viral	Naked DNA, Complexed DNA	• Low immunogenicity • Good safety profile • Simple manufacturing	• Potential insertional mutagenesis • Transient expression • Stable transfection efficiencies < 0.1 – 1%

Table 3. Artificial Chromosome Technology for Gene-Based Cell Therapy

Type of Artificial Chromosomes	Average Size	Features	Limitations
Seeded Centromeres ("Bottom-Up")	3–10 Mbp	• Functional synthetic centromeres & telomeres	• *De novo* formation in target cells • No high yield isolation or efficient transfer • Loading transgene requires *de novo* formation
Fragmented Chromosomes ("Top-Down")	0.7 – 6 Mbp	• Natural centromeres • Natural or synthetic telomeres	• No high yield isolation or efficient transfer
Episomes (EBV-based)	0.7 – 0.9 Mbp	• High transgene copy number • Good transfection efficiency	• Target cells require EBV tumor antigen gene expression • No vector copy control
Marker Chromosomes	3 – 100 Mbp	• Occur naturally • Natural centromeres & telomeres	• No high yield isolation or efficient transfer
Satellite DNA-Based Artificial Chromosomes (SATACs)	60–360 Mbp	• Natural centromeres & telomeres • Isolated to a high yield & purity • Transferable	• Stable transformation efficiency < 0.1% • Loading transgene requires *de novo* amplification

ficial chromosome.

Mammalian Artificial Chromosomes

Mammalian artificial chromosomes (MACs) are the most promising among the categories of artificial chromosome technologies that are being evaluated as safer, more stable gene delivery vehicles for GBCT, and numerous recent reviews have been published.[8–19] Several approaches to generating human mammalian artificial chromosomes have been reported, as summarized in Table 3. In one approach — centromere seeding — artificial chromosomes are assembled *de novo* in cells from co-transfected DNAs that encode putative human centromeres, telomeres, and bacterial drug-resistant marker genes.[20–26] A second approach involves generating minichromosomes by fragmenting natural human chromosomes via telomere-directed breakage or by identifying naturally occurring fragmented human chromosomes.[27–38] Both approaches generate artificial chromosomes with functional centromeres and telomeres that are stably maintained alongside the host cell's chromosomes. A third approach creates artificial episomal chromosomes that require the use of the Epstein-Barr virus gene product, EBNA-1, to enable replication and persistence in lieu of functional centromeres and telomeres.[39–43] These vectors do not technically segregate as normal chromosomes, but actually "tether" to metaphase chromosomes during mitosis by the binding to EBNA-1 protein that in turn binds to the histone protein components of mammalian chromosomes.[44]

Although attractive as vectors for GBCT, all three of the above approaches possess practical and technical limitations. Most notable is the inability to isolate and purify centromere-seeded and fragmented-chromosome MACs. One technical option to deliver these MACs to patient cells is microcell mediated gene transfer (MMGT), but MMGT is not clinically feasible due to its tedious production of a heterogeneous population of microcells (endogenous chromosomes and minichromosomes) and the concomitant very low transfer

efficiencies (~10^{-7} – 10^{-6}) of the desired minichromosomes via microcell and host cell fusion. This lack of a clinically feasible MAC transfer procedure necessitates that these MACs be generated *de novo* in the desired target cell for both centromere seeding and chromosome fragmentation, which is an extremely inefficient process. Moreover, there is little to no predictable relationship between input DNA and *de novo* chromosomes' composition upon generation, making downstream characterization and quality control difficult. In addition, if a single subclone cannot be obtained and expanded to clinically relevant levels, the reimplanted GBCT product will contain a heterogeneous population of cells with respect to MAC composition and structure. With respect to centromere seeding, there is also the possibility that rearrangements and genomic integrations will occur when the transfected DNA fails to form new chromosomes. For the episomal vectors, there are safety concerns regarding the potential co-expression of a viral gene product (e.g., antigenicity), or gene dosage effects arising from variable copy numbers of the episomal chromosome. Therefore, for practical and clinically relevant application of artificial chromosome technologies to GBCTs, a dramatic improvement in generation and portability is needed. To this end, ACEs and the ACE System were envisioned.

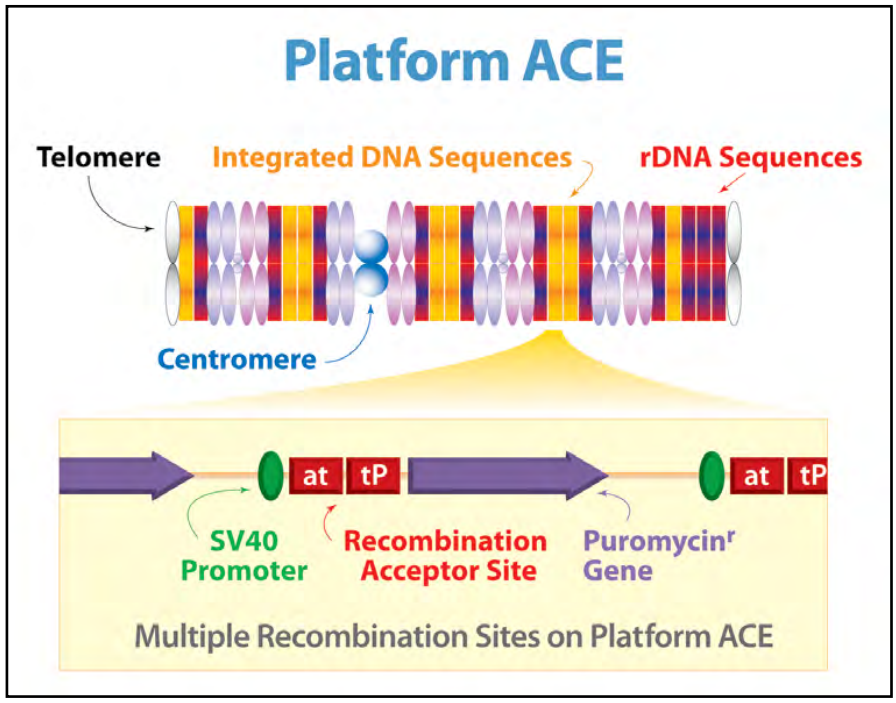

Figure 1. Schematic representation of Platform ACE. Platform ACE (depicted as a metaphase chromosome) encodes more than 50 copies of a recombination acceptor site cassette. Each acceptor site cassette encodes an SV40 promoter, *att*P recombination acceptor site, and the open reading frame of the puromycin resistance gene.

ACEs and the ACE System

ACEs. Hadlaczky and colleagues have developed a unique methodology to construct artificial chromosomes by the induction of large-scale amplifications of "satellite" sequences composing the pericentromeric heterochromatin (Table 3).[45,46] *De novo* centromeres and dicentric chromosomes were formed upon the integration of exogenous DNA sequences into regions of specific acrocentric mouse and human chromosomes that contain pericentromeric heterochromatin and the tandemly repeated ribosomal genes (rDNA). Ensuing breakage during mitosis generated new chromosomes ranging in size from 10 to 360 million base pairs.[47–49] These satellite-DNA based MACs are referred to as SATACs (Satellite DNA-based Artificial Chromosomes) or ACEs (formerly Artificial Chromosome Expression systems). This amplification mechanism has been successfully applied to rodent (mouse and hamster), human, and plant cells.

ACEs are attractive gene delivery vehicles for GBCT, given that they may be genetically engineered, characterized, purified, and easily transferred between cell types.[50] Briefly, transgenes have been targeted onto existing ACEs by homologous DNA recombination (unpublished data) or by incorporation of the transgene into *de novo* generated ACEs.[51] The transgene-loaded ACEs can be isolated by dual flow cytometry to high purities and yields, and have been transferred *in vitro* into a variety of mammalian cell lines and primary cells by cationic lipids and dendrimers.[52–55] Transgenic mice have also been generated by microinjecting purified ACEs into the pronuclei of fertilized oocytes.[56] The ACEs were stably maintained in these mice and passed through four generations of the germline.[57] All of the transgenic mice were healthy, robust, displayed no obvious abnormal phenotype, and manifested no neoplasms. These founders and their progeny offered the first indication that ACEs were stable, non-integrating, and non-deleterious *in vivo*, which are strong safety features for future clinical applications. Additional safety may be engineered into ACEs by the introduction of tissue specific promoters. As a proof-of-concept, a second line of ACE-transgenic mice was generated in which a therapeutic protein gene under the control of a mammary tissue specific promoter was expressed only in the mammary gland during lactation (unpublished data).

The ACE System. The ACE System was developed to efficiently, rapidly, and reproducibly engineer existing ACEs rather than having to continuously generate ACEs *de novo*. This novel system incorporates features of the mechanism used by bacteriophage lambda (λ) to integrate itself into the host chromosome of *E. coli*.

The λ-phage genome carries a specific integration site, *att*P, which is partially homologous to a smaller site on the bacterial chromosome, *att*B. The phage

also encodes a site-specific integrase, *Int*, that catalyzes recombination between the *att*P and *att*B sites, thereby inserting the λ-DNA into the host chromosome. For the integration process to occur in nature, a number of protein factors encoded by the host bacterium are also required.

Components of the λ-*Int* process have been modified and adapted to create a proprietary integration system for ACEs. It is designed so that the *att*P site resides on the ACE, the *att*B site is adjacent to the DNA sequence to be integrated on the ACE, and *Int* protein is co-delivered on a separate DNA plasmid. In addition, the gene encoding the *Int* protein has been site-specifically engineered to produce a novel recombinase (ACE Integrase) that catalyzes integration in the absence of any *E. coli* host factors. Integration is essentially unidirectional in mammalian cells, as the λ-phage excision protein *Xis* is not present.

A murine derived ACE containing multiple *att*P sites (>50), referred to as the Platform ACE, was generated (Fig. 1) (unpublished). The Platform ACE shares all the characteristics of ACEs described previously, including their inherent stability, and ease of purification and transferability to a variety of cell lines and cell types. As such, the ACE System can be readily established in virtually any mammalian cell through transfer of the Platform ACE. Another significant advantage of having a single universal Platform ACE is that it can be extensively characterized with respect to composition and organization, thereby providing a consistent molecular framework for downstream applications. Not only does this feature offer the ability to control the quality of resultant ACEs, but it also provides an inherent reproducibility and reliability to the system, as the same molecular context can be systematically targeted for transgene integration and expression.

Each recombination acceptor site cassette on the Platform ACE consists of an *att*P site flanked by an SV40 promoter at the 5'-position and an open reading frame sequence encoding the puromycin resistance (puromycin^R) gene at the 3'-position (which confers puromycin resistance to cells carrying the Platform ACE)(Fig. 1). The ACE Targeting Vector (ATV) was designed to systematically transfer onto or "load" the Platform ACE with either a single or multiple transgenes. Each of the ATVs encodes an *att*B site upstream of a promoterless secondary drug-selectable marker gene (e.g., zeocin^R, blasticidin^R, neomycin^R, or hygromycin^R), which becomes activated when the ATV integrates correctly via recombination between the *att*B and *att*P sites (Fig. 2). The combination of the multiple *att*P sites and the "unidirectional" ACE Integrase enables multiple loadings (during a single transfection) or sequential loadings (via multiple transfections) with the ATVs. We have not observed any ATV excision from the Platform ACE, which is not surprising, as the ACE Integrase requires the bacterial *Xis* protein to catalyze excision.

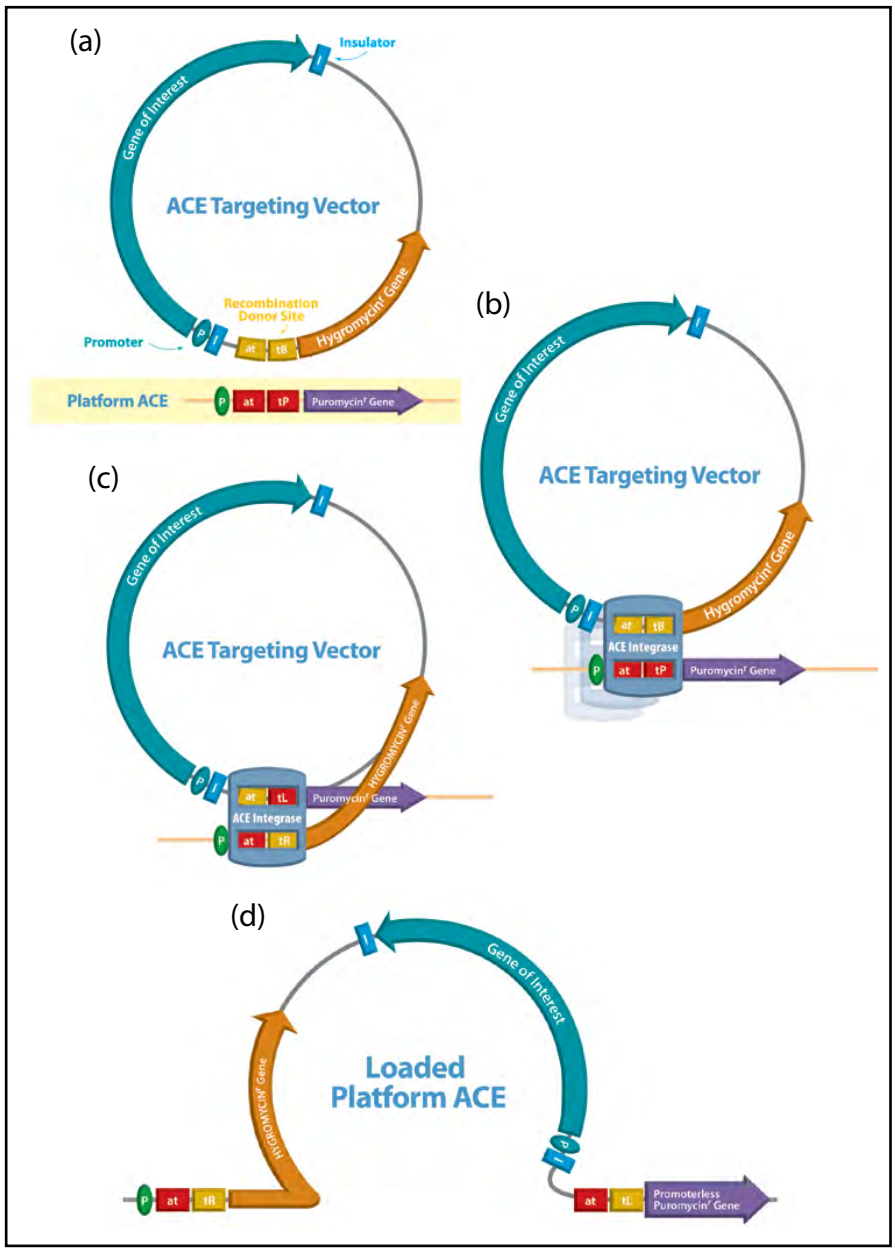

Figure 2. Site-Specific Loading of Platform ACE. The Platform ACE cell line is co-transfected with the ACE Targeting Vector (ATV) and ACE Integrase plasmid. (a) The ATV encodes an *att*B donor site and the promoterless hygromycin resistance gene. (b) The ACE Integrase protein binds at the aligned *att*P and *att*B sites, and (c) catalyzes recombination between the ATV and the acceptor cassette. Precise DNA recombination activates the hygromycin resistance gene, and (d) enables the identification of desired recombinants after hygromycin selection.

Engineering. Genetically modifying a Platform ACE using the ACE System is accomplished simply by co-transfecting an ATV (which is loaded with the transgene) and a plasmid encoding the ACE Integrase into a Platform ACE host cell line. Transfection, isolation of clones, and subsequent verification of transgene loading can be completed within eight to 12 weeks. Typically, more than 80 percent of the drug-resistant clones contain accurately loaded ACEs (a 240-fold enrichment compared to homologous recombination). High levels of gene expression have been measured for ACEs loaded with monoclonal antibodies (>30 pg/cell•day), for erythropoietin (epo) (>800 IU/10^6 cells•day), and for fluorescent proteins (red and green, unpublished data). In addition, we have sequentially loaded a Platform ACE with an ATV encoding the humanized *Renilla* GFP (hrGFP-ATV), followed by an epo-ATV, generating a hrGFP-epo-ACE for *ex vivo* studies (unpublished data). Moreover, ACEs have tremendous carrying capacities and have been able to carry payloads exceeding 1.25 Mbp (unpublished data).

Isolation. A powerful feature of the Platform ACE is that it can be isolated efficiently from the host chromosomes by dual high-speed flow cytometry. As mentioned above, this allows a single ACE to be consistently transferred between cell types. In order to isolate the ACEs, cells are blocked in metaphase and mechanically ruptured to release condensed chromosomes prior to flow sorting. Hoechst 33258 and chromomycin-A3 bind preferentially to AT- and GC-basepairs, respectively. Platform ACEs are composed of more than 350,000 copies of the AT-rich 234-bp mouse major satellite sequences, and hence bind more Hoechst 33258 and less chromomycin-A3 than the endogenous host cell chromosomes. The dual-stained ACEs are readily distinguished and separated from the host chromosomes at sort rates exceeding one million ACEs/hour/sorter and at purities exceeding 99 percent. Establishing these parameters engendered a pilot production facility focused on the large-scale isolation of ACEs for applications in biopharmaceutical production, transgenesis, and gene-based cell therapy.[52]

Delivery & Transfer. The Platform ACE and ACEs can be readily delivered and transferred to a variety of cells and cell types through transfection, microinjection, and microcell mediated chromosome transfer (MMCT). ACEs have been microinjected into the pronuclei of fertilized oocytes, generating transgenic mice and bovine blastocysts at an efficiency, for mice, consistent with conventional murine transgenesis.[56–58] ACEs have also been introduced into various mammalian cells (e.g., rodent, human, bovine) by MMCT, although only at transfer efficiencies from 10^{-7} to 10^{-5}.[59] Implementing a rapid and reproducible iododeoxyuridine labeling and detection method for ACEs has facilitated efficient ACE transfer to both primary cells and cell lines via cationic lipids and dendrimers.[54] ACE transfection efficiencies in the range of 10^{-3} to 10^{-2} are routinely attained using commercially available cationic reagents and employing simple formulation protocols.[53–55]

The unique capability to transfer loaded Platform ACEs can be exploited in numerous ways; in particular, to audition host cells for advantageous characteristics or properties (e.g., increased gene expression, protein secretion, or if desired, post-translational modification). As an example, a Platform ACE (carried in a CHO cell line) was loaded with both heavy and light chain immunoglobulin genes of a monoclonal antibody (MAb), generating a MAb-ACE. The resultant MAb-ACE production cell line was shown to secrete the MAb at a specific productivity of 12 pg/cell•day. The MAb-ACEs were subsequently isolated and transferred into the parental CHO cell line (without the Platform ACE). The MAb-ACE remained functional and the expression levels of the transfected "daughter" cell lines were not statistically different from the original production cell line (11.4, 13.8, 12.5 pg/cell•day, respectively). In addition, the isolated MAb-ACE was transferred to a different CHO strain, with the resultant clones secreting the MAb at 55 pg/cell•day. Similarly, different cell types (e.g., HSCs, MSCs, myoblasts) may be auditioned to identify target delivery cells with desirable qualities for gene-based cell therapy indications.

Gene-Based Cell Therapy

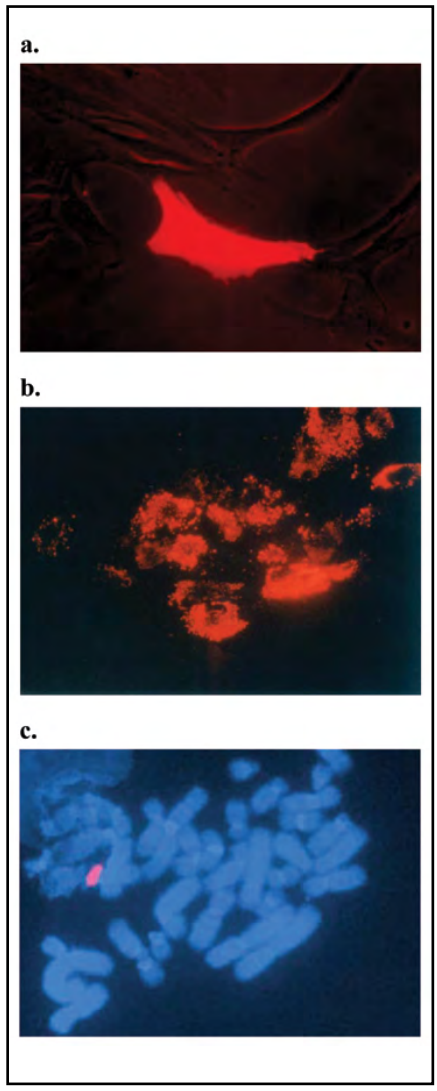

Figure 3. ACE Delivery to Human Adult Stem Cells. An ACE encoding the red fluorescent protein (RFP) gene was generated (RFP-ACE). Human mesenchymal stem cells (hMSCs) derived from bone marrow were transfected with purified RFP-ACEs by standard techniques (see text). (a) A transfected hMSC expressing RFP. (b) RFP expression from transfected hMSCs two weeks after chemically induced differentiation into adipocytes. The globular appearance is due to the accumulation of RFP in the oil drops within the adipocyte. (c) Fluorescent *in situ* hybridization of a transfected hMSC. A metaphase spread was hybridized with an ACE-specific DNA probe and detected with a rhodamine reagent (red signal). The ACE is intact and maintained as an autonomous chromosome.

We are currently applying the ACE System technology to human mesenchymal stem cells (hMSCs), which are appealing cell moieties due to their ease of purification from bone marrow, and their ability to expand without loss of differentiation potential.[60] In addition, they are candidates for "universal donor" cells as they have been shown to suppress T-cell lymphocyte proliferation and do not present alloantigens.[61–63] For our initial studies, ACEs encoding red fluorescent protein (RFP) genes were generated and introduced into hMSCs using commercially available transfection reagents.[55] Not only did the transfected cells express RFP, but they also maintained RFP expression when differentiated along adipogenic and osteogenic lineages (Fig. 3). Although preliminary, the data suggests that ACEs and their expression products can be maintained for prolonged periods in culture (>2 weeks), and that the presence of an ACE does not diminish the multi-potential differentiation capacity of these stem cells.[55]

Simultaneously, we have demonstrated that a loaded Platform ACE encoding a blood hormone protein could elicit a therapeutic response in a small animal model. The gene encoding human epo, a hematopoietic growth hormone involved in the stimulation of red blood cell production, was loaded onto the Platform ACEs carried in both LMTK$^-$ and CHO cells.[64] When these cells were implanted in immunodeficient mice, they secreted therapeutic quantities of epo that produced statistically significant elevations in hematocrits (P <0.05; Table 4).

Currently, we are building on the successes of these preliminary experiments and conducting longer term *in vivo* proof-of-principle studies using epo-loaded ACEs and hMSCs. Two ATVs were engineered to encode the hrGFP and the human epo genes, respectively, and sequentially loaded onto the Platform ACE, generating a hrGFP-epo-ACE. These ACEs were isolated to 99% purity by flow cytometry and then transferred into hMSCs using cationic reagents. The transfected hMSC population was enriched to 20% GFP+ cells by flow cytometry, which in turn secreted epo in the range of 50–100 IU/10^{6}cells•day. The population of extrapolated 100% GFP+ cells would attain levels of epo expression (250–500 IU/10^{6}cells•day) which are comparable to those attained with multiple cycles of epo-retroviral transductions in MSCs (100–700 IU/10^{6}cells•day).[65] Currently, we are implanting these epo-expressing hMSCs into NOD/SCID mice and will monitor hematocrits over extended periods of time.

Although cells with self-renewing or expansion capabilities (>30 population doublings) work well with the current ACE transfer efficiencies and potential drug selectable human marker genes (e.g., MDR-1, methylguanine methyltransferase, cytidine deaminase), there is a need to develop synergistic technologies that do not require substantial enrichment steps. Therefore, we have begun internal development efforts to attain stable ACE transfection efficiencies comparable to viral transduction levels (>90%). Once established, we envision that ACEs can be transferred to cells with a limited expansion capacity, thereby opening up additional clinical and therapeutic opportunities.

Applications. Monogenic and acquired diseases are the primary candi-

Table 4. *In vivo* Therapeutic Responses with ACE-Modified Cell Lines

Cells Implanted into SCID mice	Hematocrits		*P* value
	Control (Platform ACE)	Experimental (Platform ACE + Epo gene)	
LMTK$^-$ (mouse)	51.2 ± 1.4 % (n = 6)	65.2 ± 1.5 % (n = 5)	0.00000005
CHO (hamster)	49.0 ± 1.7 % (n= 4)	65.1 ± 1.1 % (n = 2)	0.0008

10^6 ACE-Modified cells were implanted subcutaneously into SCID mice. Hematocrits were determined at 2 weeks (LMTK$^-$ cells) and 3 weeks (CHO cells) post-implantation.

Table 5. Potential Applications for ACE System Technology

Indications/Applications	Examples		
	Disease	Target Cells	Genes
Lysosomal storage disorders	Sandhoff	NSCs, MSCs	Hexosaminidase B
	Krabbe	NSCs, MSCs	Galactosylceramidase
	Gaucher	MSCs, HSCs	β-Glucosidase
	Hurler	MSCs, HSCs	α-L-Iduronidase
Hematological diseases	Hemophilia A	MSCs, HSCs	FVIII
	Hemophilia B	MSCs, HSCs	FIX
	β-Thalassemia	HSCs	β-globin
	Anemia	MSCs, HSCs	Erythropoietin
Immunodeficiency diseases	X-linked SCID	HSCs	γc cytokine receptor
	Adenosine deaminase def.	HSCs	Adenosine deaminase
	Chronic granulomatous disease	HSCs	gp91phox
Other genetic diseases	Osteogenesis imperfecta	MSCs	Type I collagen
	Leukocyte adhesion deficiency	HSCs	CD18
	Familial hypercholesterolemia	Hepatocytes	LDLR
	Gyrate atrophy	Keratinocytes	Ornithine aminotransferase
	Rheumatoid arthritis	Synoviocytes	IL-1, TNFR
	Parkinson's disease	NSCs	Glial-derived neurotrophic factor
	Alzheimer's disease	NSCs	Nerve growth factor
	Myocardial ischemia	Endothelial cells	VEGF
Cancer	Melanoma	TILs	TNF
		Auto. fibroblasts	Cytokines: IL-1, -2, -6, -12, TNF
		Allo. tumor cells	Cytokines: GM-CSF
		Auto. tumor cells	Cytokines: GM-CSF
		Dendritic cells	Cytokines: IL-1, -2, -6, -12, TNF
			Co-stimulatory: CD80, CD86
	Melanoma, prostate	Dendritic cells	Tumor-associated antigens
		Allogeneic cells	Cytokines + co-stim. molecules
Infectious diseases	HBV, CMV, EBV	T cells, APCs	Viral core proteins
	HIV	HSCs, CTLs	Multiple genes
Enhancing cell therapies & chemotherapy	Leukemia	MSCs	Genes for HSC expansion
		HSCs	MDR-1, MGMT, CD
Imprinting stem cells	Cancer; regenerative medicine	HSCs, MSCs	Genes that control differentiation
Evaluating stem cell fate		HSCs	HOXB4, β-catenin; GFP

APCs = Antigen presenting cells
CTLs = Cytotoxic T lymphocytes
HSCs = Hematopoietic stem cells
MSCs = Mesenchymal stem cells
NSCs = Neural stem cells
TILs = Tumor infiltrating lymphocytes
X-linked SCID = X-linked severe combined immunodeficiency

dates for ACE-modified gene-based cell therapy (Table 5). Lysosomal storage disorders are a group of more than 40 diseases that are caused by the pronounced deficiency of one or more lysosomal enzymes.[66–68] This enzymatic deficiency leads to an accumulation of undegraded substrate in the lysosome, which in turn can lead to various cellular and tissue damage, subsequent organ failure, and even premature death. In addition, monogenic hematological diseases (hemophilias A and B, hemoglobinopathies, and anemia) along with immunodeficiency diseases (X-linked severe combined immunodeficiency, adenosine deaminase deficiency, and chronic granulomatous diseases) are suitable clinical targets. Acquired diseases amenable to GBCT include autoimmune (e.g., rheumatoid arthritis), neurodegenerative (e.g., Parkinson's, Alzheimer's), and vascular (e.g., myocardial ischemia) diseases.

For monogenic and acquired diseases, we currently envision a strategy for GBCT, in which autologous stem cells will be isolated, transfected with ATV-loaded Platform ACEs, expanded, characterized for release, and re-implanted in the patient. Currently, we have several collaborations with academic laboratories engaged in investigating the potential of ACE-modified gene-based cell therapy to treat selected monogenic diseases.

Immunotherapy also offers an opportunity for the application of the ACE System technology, particularly the capability of transferring payloads of multiple genes to appropriate cell targets (Table 5). Cellular vaccines consisting of autologous fibroblasts, autologous tumor cells, allogeneic tumor cells, or mixtures of cells may be genetically modified with ACEs encoding multiple cytokines, co-stimulatory molecules, and tumor-associated antigens (TAAs) to increase efficacy. Dendritic cells may be genetically modified to become more effective antigen presenting cells (APCs) by the simultaneous expression of cytokines, co-stimulatory molecules, and as appropriate, TAAs or viral associated antigens. It is even possible to modify allogeneic or xenogeneic cells (e.g., fibroblasts) to generate artificial APCs.[69] For adoptive cellular immunotherapy, epitope-specific CTLs may be more efficiently activated and expanded *ex vivo* from the interactions with APCs modified with ACEs encoding co-stimulatory molecules and TAAs or viral proteins (e.g., CMV, HIV, HBV).

ACE System technology may also be used to enhance cell therapies and chemotherapy (Table 5). Autologous MSCs may be targeted with genes that enhance HSC expansion *in vivo* after chemotherapy or radiotherapy. Additionally, HSCs may be modified with human genes that encode drug resistance to chemotherapeutic agents: methylguanine methyltransferase, multiple drug resistance, dihydrofolate reductase, and cytidine deaminase. Using human encoded genes will eliminate the potential for a patient's immune system to expunge ACE-modified cells.[70]

Summary

The ACE System is a versatile and unique technology that enables the rapid "loading" of transgenes onto a transportable artificial chromosome — the Platform ACE. These Platform ACEs contain natural centromeres and telomeres that provide autonomous maintenance through excellent mitotic/meiotic stability along with tight control in the copy number of the ACE. The Platform ACEs' large payload capacities (>1.25 Mbp) and multiple integration acceptor sites impart novel capabilities to drive transgene expression to high levels (by loading multiple ATVs) while simultaneously allowing for the incorporation of additional transgenes. Moreover, the "unidirectional" integration feature of the ACE Integrase permits sequential and multiple loadings onto the Platform ACE without the loss of previously integrated DNA sequences. Additionally, these efficiently loaded Platform ACEs may be isolated to highly purified yields, and subsequently transferred to a plethora of primary cells and cell lines utilizing commercially available reagents.

The ACE System offers compelling advantages over current gene delivery technologies for GBCT, including increased safety, engineering versatility, and unique portability. We are exploiting these advantages in preliminary proof-of-principle experiments for GBCT using the ACE System. Our current focus is on transfecting loaded-ACEs into adult stem cells, particularly mesenchymal stem cells, that will in turn be implanted into animal models of lysosomal storage disorders. These studies will generate seminal data for the development of ACE System technology, and will hopefully usher in novel approaches for gene-based cell therapy indications using artificial chromosomes. We believe that the combination of the ACE System technology with the multi-potent, self-renewing properties of autologous human adult stem cells will provide a powerful therapeutic and clinical strategy for numerous intractable diseases.

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