Gene therapy finds its niche

Cormac Sheridan

Gene therapy is finally poised to make a contribution to the treatment of debilitating, highly penetrant genetic diseases that have proved intractable to other regimens.

A growing body of evidence for the effectiveness of a select set of gene therapies in certain debilitating conditions is restoring the reputation of a field previously beset by heartbreaking mishaps and forsaken by investors. Positive human efficacy data in a lengthening list of inherited conditions, such as Leber's congenital amaurosis, X-linked adrenoleukodystrophy (ALD), β-thalassemia and severe combined immunodeficiency (SCID), previously intractable to treatment have contributed to a growing sense that the promise of gene therapy is—after several false starts—finally being realized. That optimism is tempered by a sense of pragmatism gained through the many disappointments and setbacks the sector has endured. Even so, despite several clinical development programs progressing to late-stage trials (**Table 1**), the gene therapy community will likely have to wait 12 months or more before a product gains regulatory approval in Europe, let alone the United States.

Walking before running

The development of virus-based methods for transforming mammalian cells in the 1960s, coupled with the advent of recombinant DNA technology in the 1970s, offered the prospect of genetic medicines that could compensate for errors in an individual's DNA sequence associated with disease[1]. The early debates on gene therapy, like those on genetic engineering, advocated caution—and further research into the underlying mechanisms—in advance of any human trials[2]. Despite the controversy, several pioneering groups moved gene therapies forward to replace various deficient proteins (such as arginase in children with hyperargininemia and hemoglobin in thalassemics) in the 1980s, which many would argue was premature or even foolhardy. The clinical setbacks that followed lent notoriety to gene therapy in the early days, which it has found difficult to overcome.

Necker Hospital for Sick Children

Gene therapy hotbed. Researchers at the Necker Hospital for Sick Children conducted some of the early clinical trials for severe combined immune deficiency.

By the beginning of the 1990s, an understanding of the genetic basis of many diseases, combined with advances in tools for manipulating and delivering DNA, raised hopes that a clinical breakthrough was imminent. Notwithstanding the rapid scientific progress that had been made up to that point, unrealistic expectations were raised by the media, investors and even some of the researchers pioneering treatments. But the trials that were then getting underway in SCID children failed to deliver clinically significant results. "There was overoptimism in the initial years of gene therapy, 20 years ago," says Boro Dropulic, founder and CSO of Lentigen (Gaithersburg, MD, USA). "At that time, none of the vectors was really optimized," he adds. "While we were in the middle of it, we were thinking this is not going to do us much good at all," says Deborah Gill, co-leader of Oxford University's gene medicine group, who was involved in some of the early gene therapy trials in cystic fibrosis.

Many of the clinical disappointments that followed were unsurprising, given the unavoidable and unanticipated roadblocks and challenges faced by any novel therapy at a nascent stage. However, the death of young trial volunteer Jesse Gelsinger in 1999 from vector-associated toxicity was a stark exception (**Box 1**). The episode, as the headline above one editorial at the time put it, marked "a loss of innocence" for the field[3]. It also primed critics for any subsequent problems—which did not take long to emerge. Shortly after Gelsinger's death, five cases of leukemia in SCID-X1 children receiving gene therapy treatment for replacing the interleukin-2 receptor γ chain gene raised further concerns about the safety and even the overall feasibility of gene therapy[4]. The retroviral vector used in the procedure, which was based on Moloney murine leukemia virus, contained an enhancer sequence that activated proto-oncogenes, which in turn led to T-cell proliferation.

Even so, the backlash was, in this instance, both premature and unwarranted. Although one of the children died, this less-than-perfect outcome was still superior to the existing treatment. "The overall mortality in these patients is 5%, which is much, much lower than the 25% risk in allogeneic bone marrow transplant," says Patrick Aubourg, of the University Paris Descartes. Moreover, the SCID-X1 trial was also successful from an efficacy standpoint. Alain Fischer, of the Necker Hospital for Sick Children (Paris), and colleagues recently reported that 18 of the 20 patients who were treated (including the four surviving patients who were treated for leukemia) remain alive after around ten years of follow-up. The immunodeficiency was corrected in 17 of them[5]. Although these two pivotal episodes had contrasting outcomes, each has helped to shape the research agenda for the past decade, particularly in terms of vector design and safety.

Viral vectors

From the outset of gene therapy, viral vectors have been the main conduit for transferring genes to human cells (**Table 2** and **Fig. 1**), although programs using nonviral vectors are in development as well (**Box 2** and **Fig. 2**). "Viruses

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Table 1 Selected gene therapy clinical trials

Company	Therapy	Indication	Phase of development
Retrovirus			
San Raffaele	ADA-SCID GT: $CD34^+$ cells transduced with Moloney murine leukemia virus carrying ADA gene	Primary immunodeficiencies	Phase 1/2
Neurologix	NLX-P101: GAD in virus injected into subthalmic nucleus of the brain	Parkinson's disease	Phase 2
Ribozyme Boulder, CO, USA	$CD34^+$ cells transduced with retrovirus vector with multiple ribozymes	Non-Hodgkin's lymphoma HIV/AIDS	Phase 2
Tocagen San Diego	Toca-511: replication competent retrovirus with prodrug activator cytosine deamidase gene injected into tumor	Glioma	Phase 1/2
Lentivirus			
Bluebird Bio	LentiGlobin: introduces globin gene into patient hematopoietic stem cells	β-thalassemia and sickle cell anemia	Phase 1/2
Lentigen	LG-740: T cells treated *ex vivo* with lentivirus with chimeric T-cell receptor gene	B-cell leukemia and lymphoma	Phase 1
Oxford BioMedica	ProSavin: lentivirus with three genes required for dopamine biosynthesis injected into striatum of brain	Parkinson's disease	Phase 1/2
Adenovirus			
Advantagene Auburndale, MA, USA	ADV-tk: replication-deficient adenovirus with HSV thymidine kinase gene injected into tumor during biopsy	Glioma Pancreatic cancer	Phase 1 Phase 1
Applied Genetic Technologies Alachua, FL, USA	rAAV1-CB-hAAT: AAV with alpha-1-antitrypsin gene	Alpha1-antitrypsin deficiency	Phase 2
	rAAV2-CB-human retinal pigment epithelium specific 65 dalton protein (RPE65)	Congenital amaurosis (blindness with mutation in RPE gene)	Phase 1/2
Amsterdam Molecular	AMT-101: adeno-associated virus with human lipoprotein lipase gene	LPL deficiency	Filed
Aventis Paris	Ad5CMV-p53	Head and neck cancer	Phase 2
Biogen	Adenoviral mediated interferon-β	Pleural mesothelioma Colon cancer, glioma	Phase 1 Phase 1/2
Ceregene San Diego	CERE-120: adeno-associated virus with neurotrophic factor, neurturin	Parkinson's disease	Phase 1/2
	Cere-110: adeno-associated virus with gene for nerve growth factor	Alzheimer's disease	Phase 1/2
Celladon La Jolla, CA, USA	SERCA-2a: sarcoplasmic reticulum Ca^{2+}ATPase gene with AAV vector	Congestive heart failure	Phase 1/2
Genzyme	AAV2-sFLT01: adeno-associated virus with anti-VEGF	Wet macular degeneration	Phase 1
GenVec	TNFerade: replication deficient adenovirus with TNF-a controlled by radiation-induced promoter	Esophageal cancer	Phase 2
Shenzhen SiBiono GeneTech	rAd-p53: replication deficient adenovirus encoding hu recombinant p53	Advanced thyroid tumors, oral, maxillofacial tumors	Phase 4
Targeted Genetics Seattle	tgAAG76: AAV with human RPE65	Congenital amaurosis (blindness with mutation in RPE gene)	Phase 1/2
	tgAAC94: AAV2 with TNF-a –IgG1 fusion gene	Arthritis	Phase 2 completed
Plasmid			
AnGes Tokyo	Hepatocyte growth factor-plasmid	Arterial disease	Phase 2
Genexine Seoul, Korea	GX-12: plasmid plus IL-12 mutant, given with HAART	HIV-AIDS	Phase 1
ScanCell Nottingham, UK	SCIB1: plasmid with tyrosine-related protein	Melanoma	Phase 1/2
Vical San Diego	Allovectin-7: plasmid with gene for HLA-B7 and b2microglobulin genes, injected into tumors	Melanoma	Phase 3
ViroMed Minnetonka, MN, USA	VM202: plasmid with two isoforms of hepatocyte growth factor, HGF728 and HGF 723	Limb ischemia Myocardial ischemia	Phase 2 Phase 1/2
Other			
Diamyd Medical Stockholm, Sweden	Nerve Targeting Drug Delivery System: HSV vector with enkaphalin administered intradermally	Pain	Phase 1
Epeius Biotechnologies San Marino, CA, USA	Rexin-G: nanoparticle delivering cyclin-G1 gene	Advanced pancreatic, metastatic breast, osteosarcoma, and soft tissue sarcoma	Phase 1/2
MultiGene Vascular Systems Nesher, Israel	Patient cells modified with four angiogenic genes	Peripheral artery disease	Phase 1/2

have developed a system to efficiently transport genetic information, so why not use it?" says Dropulic. "Over the past 20 years, we have now identified the parts of viruses that are important for gene delivery and the parts that are not necessary and are therefore deleted." Viral vectors fall into one of two main categories: integrating vectors, which insert themselves into the recipient's genome, and nonintegrating vectors, which usually (although not always) form an extrachromosomal genetic element. Integrating vectors, such as gamma-retroviral vectors and lentiviral vectors, are generally used to transfect actively dividing cells, as they are stably inherited. Nonintegrating vectors, such as adenoviral vectors and adeno-associated virus (AAV) vectors, can be used to transfect quiescent or slowly dividing cells, but they are quickly lost from cells that divide rapidly.

Other factors influence the choice of a particular vector, including its packaging capacity, its host range, its gene expression profile and its tendency to elicit immune responses, particularly important if repeated administration is needed. Some of these parameters can be adjusted. For example, host range can be altered by pseudotyping the vector with a heterologous protein that recognizes a different cell-surface receptor. AAVs encompass a range of serotypes, which offers built-in host-range diversity. "These serotypes are almost different systems," says Richard Snyder, of the University of Florida, in Gainesville. Moreover, they can be further manipulated by artificial means. "Even a slight mutation on the capsid can change the profile of the cells you can target," says Oxford's Gill. Tissue-specific promoters and enhancers can restrict gene expression to specific target cells or boost expression if it is too low. Luigi Naldini, director of the San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET; Milan), will shortly publish work on a method for fine-tuning vector expression using a microRNA-based regulatory element, which blocks expression in stem cells but permits it in progeny cells.

Adenoviral vectors and retroviral vectors based on Moloney murine leukemia virus featured prominently in early gene therapy trials, but there has been a movement away from both, after the death of Gelsinger (which was linked to the toxicity of the adenoviral vector used to introduce the ornithine transcarbamylase gene) and the leukemia cases in SCID-X1 patients (which were linked with activation of *LMO2*, an oncogene on chromosome 11, due to insertional mutagenesis associated with the murine leukemia viral vector). "There is a gravitation to lentiviral and adeno-associated viral vectors for various disease states," says Dropulic. Lentiviruses, including HIV, are also retroviruses, but their integration profiles have, so far, not given rise to the same concerns as those engendered by murine leukemia virus. The ideal solution for integrating vectors to avoid concerns about insertional oncogenesis would entail a site-specific insertion method that ensures both safety and high levels of transcription. Zinc finger nucleases, used in conjunction with homologous recombination, have the potential to offer this specificity, but it is not yet clear what—in different tissues—represents an optimal insertion site. "We call it the safe harbor issue," Naldini says. "You have to do a lot of empirical testing to identify a good spot."

Other viral vectors have applications in specific settings. For example, Diamyd Medical (Stockholm), which is working on several gene therapy approaches for pain, is using a replication-defective herpes simplex virus (HSV) vector for targeted delivery of transgenes to nerve cells, by intradermal injection. It exploits HSV's tropism for nerve tissue. "The effect is local. This isn't a systemic effect—you're treating pain, or whatever the neurological condition is, directly at the site of the condition," Darren Wolfe, CEO of Diamyd's US subsidiary, argued on a recent company webcast[6].

In cystic fibrosis, attaining sufficient expression of the cystic fibrosis transmembrane conductance regulator (CFTR) protein in patients has proven challenging for two reasons. Most viral vectors are unable to penetrate the heavy layer of mucus that coats patients' airways. Moreover, viral receptors on the epithelia lining the airways are mainly located at the cells' basolateral surface, which lowers the chances of successful pulmonary delivery of a viral vector. DNAVEC, of Tsukuba, Japan, has developed a nonintegrating recombinant vector system based on Sendai virus, which commonly infects the airway epithelia of animals. However, working with the UK Cystic Fibrosis Gene Therapy Consortium (which has members in Edinburgh, London and Oxford) they found that expression, although initially high, is transient—and repeated administrations are progressively less effective. The partners have recently described a replication-defective lentiviral vector system, based on a simian immunodeficiency virus, which has been pseudotyped with two Sendai virus envelope proteins, hemagglutinin-neuraminidase and fusion protein[7]. This combines the favorable expression profile of lentiviruses with the airway-tissue-targeting capabilities of Sendai viruses. The construct has shown promising activity in mice models, although further toxicology studies are needed before it can be moved into clinical trials.

In the clinic

The basic goal of gene therapy—safely achieving the stable expression of a gene of interest in the appropriate tissue—has not changed during the field's long development. However, an understanding of the underlying complexity has increased immeasurably. "We know how to design, construct and manufacture vectors," says Dropulic. "Manufacturing for commercial use is unfinished business." Making the transition from laboratory-scale, often academic, environments to robust, industrial production processes is particularly challenging for gene therapies intended for relatively large patient populations. Indications that have small patient populations and require only small doses, such as certain retinal disorders, can be adequately served by existing processes. But for conditions involving thousands of patients, most existing production processes are inadequate. It's an aspect of gene therapy that has received insufficient investment to date, given the field's commercial immaturity.

Next year, Genethon (Evry, France), a not-for-profit research organization funded mainly by the French Muscular Dystrophy Association, will commence operations at what it claims will be the world's largest facility dedicated to gene therapy process development and production. The €28 ($37) million unit, called Genethon BioProd, will have four independent production suites, each of which will house four 200-liter bioreactors. "We are going to be able to manufacture over 20 batches of AAV or lentiviral GMP [good manufacturing practices]-grade material per year," says Genethon CEO Frederic Revah. Industrial capacity in the area is sparse. Some contract manufacturers that have expertise in viral vaccine production can offer limited services, Revah says, but they lack an end-to-end appreciation of gene therapy. "If we don't do it, nobody's going to do it," he says. "You don't see large pharmaceutical companies in gene therapy for rare diseases, apart from Genzyme."

Bluebird Bio (formerly Genetix Pharmaceuticals; Cambridge, MA, USA) will be the first external player to tap into this new infrastructure. The company is commercializing programs in ALD, an ultra-rare disorder, and β-thalassemia, which, though classified as a rare disease, affects tens of thousands of patients. An order-of-magnitude increase in the efficiency of the company's current production process is needed to serve the β-thalassemia patient population, says Bluebird Bio CEO Nick Leschly. "It is a dramatic improvement that will be required—and a significant investment to get us there." Building that capacity in-house was not justified at the company's current stage of development. But partnering options were also limited. "There are only a few right now that are experienced in this regard," Leschly.

Scaling up production is not the only outstanding challenge, however. Further optimiza-

tion of gene delivery protocols and developing a better understanding of how specific vector constructs work in specific diseases remain key issues as well, Dropulic notes. Two recent regulatory setbacks can be viewed within this context: Houston-based Introgen Therapeutics' Advexin (contusugene ladenovec; a recombinant, E1-deleted serotype 5 adenoviral vector encoding the p53 tumor suppressor) treatment for head and neck cancer; and London-based Ark Therapeutics' Cerepro (sitimagene ceradenovec; a recombinant advenoviral vector lacking E1 and part of the E3 region, encoding the HSV gene for thymidine kinase) for malignant glioma. In 2008, the former therapy was refused approval by the US Food and Drug Administration (FDA), despite being taken through to approval in China by Sibiono GenTech of Schenzen in 2004 (it remains the only gene therapy to achieve regulatory approval anywhere). The latter therapy was turned down by the European Medicines Agency (EMA) in 2009. Each was a clinical failure rather than an outright technological flop. "It's very expensive to develop these drugs, and people try to get in [to regulatory review] as soon as they can, and sometimes it's too early," says Jeff Ostrove, president and CEO of Ceregene, of San Diego.

Glybera (alipogene tiparvovec; an AAV vector encoding the human lipoprotein lipase gene), which Amsterdam Molecular Therapeutics (The Netherlands) has developed to combat lipoprotein lipase deficiency (LPLD), is gene therapy's current standard bearer. Amsterdam Molecular Therapeutics filed for approval with the EMA in January 2010, and it hopes to receive a decision by around the third-quarter of 2011. That decision will have a resonance that will extend beyond the immediate confines of the company and the LPLD patient community. A third rebuff, after the Introgen and Ark rejections, would cast a pall over the sector, just as it is beginning to develop a modest level of momentum. Amsterdam management is optimistic, however, that the therapy will succeed where those that have gone before it have failed. CEO Jörn Aldag observes that new technologies typically ride an initial wave of hype and enthusiasm, but then hit a trough, as the practical difficulties of translating innovation into clinically useful therapies become apparent. "In gene therapy, I would say, we are through that trough," he says. "Now people are seeing that gene therapy can live up to its promise."

LPLD, an ultra-rare condition, with a prevalence of about one or two cases per million, disrupts lipid metabolism, resulting in very high levels of blood triglycerides. Over half of the 22 patients enrolled in pivotal studies of

Box 1 Gene therapy in the clinic: the highs and the lows

• **1970.** Before the advent of recombinant DNA tools, Stanfield Rogers and colleagues at Oak Ridge National Laboratory, Oak Ridge, Tennessee, undertake a rudimentary and unsuccessful attempt at gene therapy. They administered wild-type Shope papillomavirus to two severely handicapped young girls with the nitrogen metabolism disorder hyperargininemia. However, the procedure was based on a mistaken assumption—that the virus expressed an arginase enzyme, which would correct the girls' genetic deficiency[12].

• **1980. Martin Cline** of the University of California, Los Angeles (UCLA), conducts the first gene therapy trial involving recombinant DNA. Bone marrow cells from two patients, in Italy and Israel, who had the inherited blood disorder β-thalassemia, which causes insufficient hemoglobin levels, were isolated and transformed with the human β-globin gene. A viral thymidine kinase gene, intended to boost the transformed cells' ability to replicate, was also included in the vector. It subsequently emerged that the five review committees that assessed the trial had not been informed that the protocol involved the delivery of recombinant DNA[13]. Cline was later found to be in breach of federal regulations on human experimentation and National Institutes of Health (NIH) guidelines on recombinant DNA research. He was sanctioned by the NIH.

• **1990. Michael Blaese**, French Anderson and colleagues at the NIH perform the first approved gene therapy trial in patients. It involved retroviral-mediated transfer of the gene encoding ADA into the T cells of two children with severe combined immunodeficiency (SCID)[14]. One patient, Ashanti DeSilva, exhibited a temporary response, although she continued on enzyme replacement therapy. The response was far more limited in the second patient.

• **1992. Claudio Bordignon**, Fulvio Mavillo and colleagues at HSR-TIGET begin the first human gene therapy trial involving genetically modified stem cells, in two infants with SCID. The protocol involved the co-administration of autologous peripheral blood lymphocytes and hematopoietic stem cells, each of which had undergone retroviral-mediated transfer of the ADA gene. It led to both short-term and long-term reconstitution of the subjects' immune system and correction of growth failure, although they required ongoing enzyme replacement therapy as well[15].

• **1999. Jesse Gelsinger**, an 18 year old with a relatively mild form of the nitrogen metabolism disorder ornithine transcarbamylase (OT) deficiency, is the first person to die on a gene therapy trial because of vector-associated toxicity. He experienced a severe inflammatory response after undergoing an infusion to the liver of an adenoviral vector carrying the gene encoding OT. He then suffered lung failure followed by multiple organ failure. The subsequent investigation into the dose-escalating phase 1 study at the Institute for Human Gene Therapy (IHGT), at the University of Pennsylvania, Philadelphia, uncovered protocol violations and failures to report previous adverse events. Gelsinger's liver status immediately before receiving the vector, according to some critics, ought to have ruled him out of the study. James Wilson, IHGT director and lead investigator on the trial, was suspended from clinical research for five years, whereas two colleagues received lesser sanctions. The University of Pennsylvania and the Children's National Medical Center, in Washington, DC, which was a partner in the trial, subsequently paid fines of over $500,000 each[16]. The case uncovered widespread underreporting of adverse events in other gene therapy trials.

• **2000. Alain Fisher** and Marina Cavazzana-Calvo at the Necker Hospital for Sick Children reported a dramatic clinical improvement in two children with X-linked SCID (SCID-X1), a genetic disorder characterized by the failure of T-cells and natural killer cells to differentiate. The patients' bone marrow cells were modified by transfer of the gene encoding the interleukin-2

Glybera met the primary endpoint of achieving a 40% reduction in triglycerides levels. The overall average for the study population was just shy of that mark, at 39%. A subsequent study, says Amsterdam's CSO Sander van Deventer, has demonstrated that the effect of Glybera on large lipoprotein structures called chylomicrons, which play a role in transporting lipids, is more significant. These particles block small blood vessels and lead to pancreatitis, a life-threatening inflammation of the pancreas that is the main clinical complication in LPLD. "We've shown a stunning effect on chylomicrons," van Deventer says.

The sweet spot

For Bluebird Bio's Leschly, gene therapy in severe genetic disease is where the greatest opportunity lies right now. Disease biology and clinical trial endpoints in monogenic disorders are, he says, more clear-cut than those in more complex conditions, such as cancer. "It's complicated, but it's clear what you need to do to affect the disease," he says. Technological and scientific advances over the past decade and a half in designing vectors and understanding the safety and the biology of gene therapy, coupled with promising clinical data and awakening investor interest have created "a perfect storm" he says, illustrated by the company's $35 million funding round last March. The recent, eye-catching entry into the field by London-based GlaxoSmithKline (GSK, Brentford, UK), which has entered a broad alliance in the area of *ex vivo* gene therapy of hematopoietic stem cells with HSR-TIGET, further underlines the message. "That means more smart people. That means another gas station on the street," Leschly says.

The GSK alliance covers seven indications in all, including clinical-stage programs in Wiskott-Aldrich syndrome, an X-linked immune disorder characterized by low blood-platelet levels, and metachromatic leukodystrophy, a fatal condition marked by a progressive deterioration of both physical and intellectual abilities. The most advanced program concerns SCID arising from ADA deficiency, a rare and fatal condition characterized by recurrent infections and by the toxic accumulation of purine metabolites. Alessandro Aiuti, of HSR-TIGET, and colleagues reported last year that five of ten children who underwent the procedure (because they lacked matched donors for bone marrow transplantation) exhibited normal immune functions after a median of four years of follow-up. The other five had significantly improved immune functions[8]. "In my view, the ADA-SCID therapy is curative, possibly even better than bone marrow transplant, which is the current standard of care," says HSR-TIGET director Naldini.

receptor gamma chain, encoded by a murine retroviral vector[17]. It was hailed as the first clear-cut success in the field. Twenty children in all received this treatment, but five subsequently developed leukemia, one of whom died, after the activation of proto-oncogenes promoting T-cell proliferation by an enhancer sequence encoded by the vector.

• **2003. Shenzhen SiBiono GenTech** (Shenzhen, China) gains approval in China for treating head and neck cancer with Gendicine, a modified adenovirus vector encoding the p53 tumor suppressor gene. Sunway Biotech (Shanghai) gained approval two years later for H101, which is based on Onyx-15, a recombinant oncolytic adenovirus originally developed by Onyx Pharmaceuticals (Emeryville, CA, USA), which targets p53-deficient tumor cells. Western critics have questioned the two approvals, due to a lack of available information on the two therapies[18].

• **2003. Carl June**, of the University of Pennsylvania, Boro Dropulic, then of Virxsys (Gaithersburg, MD, USA), and colleagues start the first human trial involving a lentiviral vector[19]. The phase 1 study, in HIV patients who had failed antiviral therapy, assessed the safety of a conditionally replicating HIV 1–derived vector expressing an antisense sequence against the HIV-1 envelope gene.

• **2008. Introgen Therapeutics** (Austin, TX, USA) files the first biologics license application for a gene therapy with the FDA, for Advexin (contusugene ladenovec), a modified adenovirus vector carrying the p53 tumor suppressor gene. Although the FDA originally granted Advexin a fast-track designation in head and neck cancer, the agency refused to accept the application for review, citing incompleteness. The company filed for bankruptcy protection shortly afterward.

• **2008. Ark Therapeutics** files for European approval of Cerepro (sitimagene ceradenovec) in malignant glioma, but a year later the Committee for Human Medicinal Products handed down a negative opinion, citing a negative risk-benefit profile, due to insufficient efficacy and risks of hemiparesis (slight paralysis on one side) and seizures. Cerepro consisted of the HSV thymidine kinase (tk) gene, encoded by a replication-deficient adenoviral vector lacking the E1 and E3 regions. It was injected into the brain immediately after surgical removal of the tumor. Subsequent administration of the prodrug ganciclovir resulted in the production of a toxic metabolite that prevents DNA replication in dividing cells.

• **2009. Jean Bennett**, of the University of Pennsylvania, in Philadelphia, and colleagues report that an eight-year-old boy with Leber's congenital amaurosis attained normal eyesight after AAV-mediated transfer of a gene encoding the retinal pigment epithelium-specific 65 kDa protein (RPE65). The degenerative disorder causes severe vision loss at birth or in early childhood and normally leads to total blindness during adulthood. All participants in the 12-patient study, whose ages ranged from 8 to 44 years, gained some improvement in eyesight, although the youngest obtained the greatest benefit[20].

• **2010. Amsterdam Molecular Therapeutics** files a marketing authorization application in Europe for Glybera (alipogene tiparvovec) in lipoprotein lipase deficiency, a genetic condition characterized by high levels of blood triglycerides. It can lead to regular debilitating and even fatal attacks of pancreatitis. The LPL gene, encoded by an AAV vector, is administered by means of multiple subcutaneous injections to the upper thighs during a single outpatient procedure.

• **2010. Philippe Leboulch**, of the University Paris Descartes, and colleagues report that a young adult patient with a severe form of β-thalassemia no longer required monthly blood transfusions after *ex vivo* modification of his bone marrow cells with a self-inactivating lentiviral vector expressing the β-globin gene[21]. Analysis of the transformed population of red blood cells indicated that most of the initial benefit arose from a partially dominant clone, in which the vector insertion activated *HMGA2*, a gene associated with the formation of malignant and benign tumors. However, its dominance appeared to decline over time.

Table 2 Gene therapy vectors

	Adenovirus	AAV	Retrovirus/lentivirus	Herpesvirus
Family	Adenoviridae	Parvoviridae	Retroviridae	Herpesviridae
Genome	dsDNA	ssDNA	ssRNA+	dsDNA
Infection/tropism	Dividing and nondividing cells	Dividing and nondividing cells	Dividing cells	Dividing and nondividing cells
Host genome interaction	Nonintegrating	Nonintegrating	Integrating	Nonintegrating
Transgene expression	Transient	Potential long lasting	Long lasting	Potential long lasting
Packaging capacity	7.5 kb	4.5 kb	8 kb	>30 kb

dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.
Source: Gene Therapy Net <http://www.genetherapynet.com/viral-vectors.html>

Previous attempts to treat ADA-SCID through gene therapy were only partially successful and required continued enzyme replacement therapy as well. (Replacement therapy alone is not entirely successful either, as over the long term, its benefits can fade). Aiuti's team added a nonmyeloablative preconditioning step, which suppressed but did not wipe out patients' immune systems, to ensure that engraftment of the transduced stem cells could take place. Problems with leukemia did not arise, even though the protocol employed a retroviral vector similar to that used in the University Paris Descartes SCID-X1 trial, which suggests that the vector alone was not responsible for the complications seen in Paris.

The other programs in GSK's alliance with HSR-TIGET are based on a human immunodeficiency virus (HIV)-derived lentiviral vector platform, which has its roots in the seminal lentiviral vector development work that Naldini did at the Salk Institute, in La Jolla, California, with Inder Verma and Didier Trono[9]. Up until then, the ability to induce long-term expression of transgenes using retroviruses was limited to dividing cells, as vectors derived from the widely used murine leukemia virus required breakdown of the nuclear membrane before they could integrate into the host genome. After HIV—and other lentiviruses—enter the cell, a pre-integration complex forms, containing the viral genome packaged with viral and host proteins forms. This enters the nucleus through the nuclear pore complex. "At that time, the idea was really to expand on the gammaretroviral vector, to target more cells," Naldini recalls. "The capacity to infect nondividing cells was the major rationale."

To extend the vector's host cell range, as HIV infects only lymphocytes and macrophages, it was pseudotyped using the envelope protein from vesicular stomatitis virus. To ensure that the vector is incapable of replicating in a human host, it was pared down to less than 10% of its genome. What remains comprises flanking sequences that border the transgene, to facilitate packaging into the vector during assembly. Even though it was not understood at the time, Naldini says, this precaution resulted in a safer integration profile than that of murine retroviral vectors. "It was a bonus I would say," he notes. It offers a second unforeseen benefit as well. "It tends to express more robustly the gene you deliver."

Nevertheless, inadvertent activation or inactivation of genes involved in processes such as oncogenesis or tumor suppression continues to be a safety concern with all integrating vectors. Clonal analysis methods, based on deep sequencing techniques, allow researchers to profile mixed populations of transfected cells in patients over time to detect any signs of clonal dominance that could point to the emergence of uncontrolled cell growth. Achieving highly efficient gene transfer, resulting in polyclonal populations of transfected cells with vector insertions at different sites, seems to reduce this risk, Naldini says. "It looks like the more [variation] you have *in vitro*, the better you are *in vivo*."

The ALD gene therapy program at the Saint-Vincent de Paul Hospital in Paris, which is led by Aubourg, recently demonstrated this principle. After successful gene therapy in two boys with ALD, a fatal, demyelinating condition, the clonal distribution of transfected CD34+ cells appeared to be unbiased up to 30 months later. "We did not find any evidence of clonal dominance," Aubourg says. Further confirmation, over longer time frames and in larger numbers will be necessary, however.

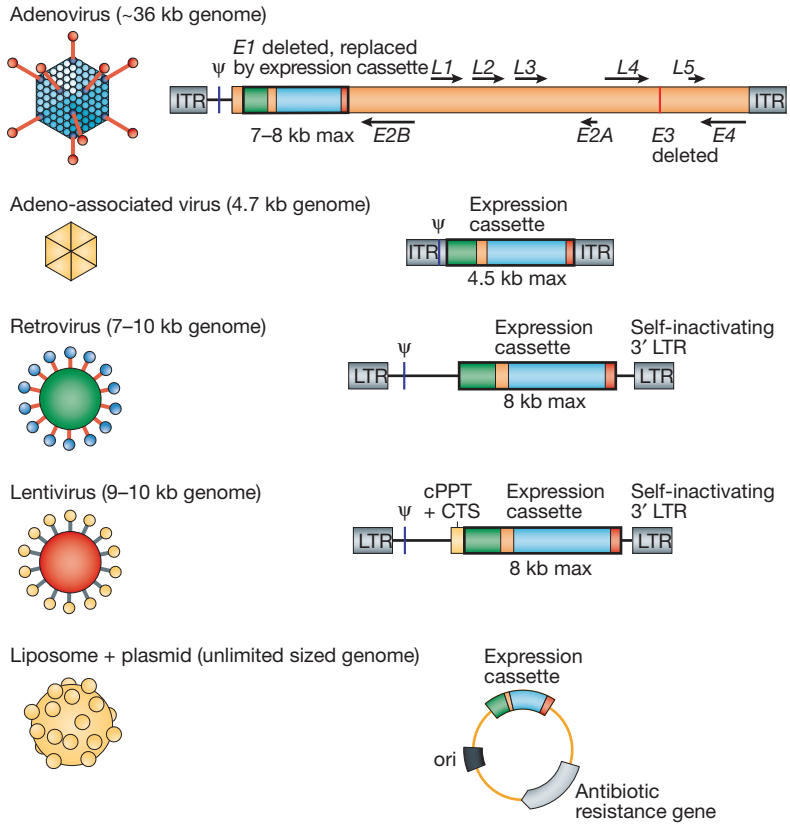

Figure 1 Mechanism of adenovirus-associated vector gene delivery. (Modified from O'Connor, T.P. & Crystal, R.G. *Nat. Rev. Genet.* **7**, 261–276 (2006).)

Tackling complex indications

In complex, multifactorial diseases, the picture is less optimistic at present, particularly after the recent, unexpected phase 3 failure of Temusi (riferminogen pecaplasmid), a plasmid encoding fibroblast growth factor 1, which Sanofi-Aventis (Paris) was developing in critical limb ischemia (CLI). In the so-called Tamaris trial, which recruited 525 patients, Temusi, which is intended to boost the growth of new blood vessels, actually performed worse than placebo on both amputation rates and on death. Expectations surrounding this study had been quite high. "The previous data was pretty positive," says Timothy Henry, of the Minneapolis Heart Institute Foundation. Temusi is based on plasmid technology developed by San Diego–based Vical. Another Vical licensee, AnGes (Osaka, Japan), recently withdrew an application it had filed with the Japanese authorities for Collategene (beperminogene perplasmid), a plasmid encoding hepatocyte growth factor, which is also aimed at CLI patients. The company decided to include data from a forthcoming 560-patient global phase 3 trial in the application.

Henry notes that even though the scientific basis underlying gene therapy in therapeutic angiogenesis was considered sound, trial design remains a problem in both CLI and a related indication, chronic angina and says, "The major challenge in pushing this whole field forward is we do not have an ideal endpoint." Measuring improvements in blood supply, the main goal of gene therapy, is difficult, he says. Moreover, amputation arises from multiple factors, and its frequency may not offer a dependable endpoint. "Amputation rates have varied from trial to trial, so if your event rate is too low you can't make a difference," he says. The event rates in the two trials of Temusi varied considerably. Amputation or death occurred within six months in around half of the patients in the phase 2 study, whereas only one-third of patients in the phase 3 study had a similar outcome after a full year.

For, Ceregene, one of three companies with a clinical-stage gene therapy program in Parkinson's disease, the challenge is not only sparse knowledge of the complex biology underlying disease progression and pathology but also the issue of tissue targeting. Data from a phase 2 trial indicate that its product Cere-120, an AAV vector encoding the neurotrophic growth factor neurturin, did not demonstrate sufficient efficacy at 12 months—as required by the primary endpoint—when delivered to the putamen of the brains of Parkinson's patients. "The primary endpoint was disappointing. The 18-month data showed we had statistically significant improvement, though the majority of the effects were not as great as we'd hoped," Ostrove says. The procedure was also superior to sham surgery in all 22 secondary endpoints studied. However, antibody staining of the brains of two patients who died of unrelated causes indicated that only about 15% of the cells of the substantia nigra—where Parkinson's causes the most damage—had taken up Cere-120. "We were hoping for 50%. We expected the transport [of the vector] from the putamen down to the nigra, and in the patients we did not see that," Ostrove says. Subsequent research indicated that the transport mechanism linking the two regions is compromised in Parkinson's patients, he says[10]. The company has now started a 60-patient phase 2b study, with a larger dose of Cere-120, which targets both the putamen and the substantia nigra.

Scaling up

As gene therapy starts to come of age as a clinical science, it is also undergoing a parallel process of maturation in terms of industrial

Box 2 Nonviral vectors

Nonviral gene delivery methods, although a minority pursuit, continue to be explored in certain settings, particularly where viral delivery remains a problem or where repeat administrations increase the risk of an immune response to a viral antigen. Approaches encompass both naked plasmids, which can be administered by intramuscular injection or with the help of assistive technologies, such as electroporation or propulsion using gene guns, and plasmids complexed with liposome nanoparticles into lipoplexes. Aerosolizing plasmid DNA, to enable delivery through inhalation, has also been accomplished, says Gill, whose group is exploring both viral and nonviral approaches to gene delivery in cystic fibrosis. "Lots of viruses, including lentivirus, are more challenging. That's going to be tricky," she says. Lipid-based nanoparticles offer advantages in being less immunogenic than their viral counterparts. "They are synthetic, and they can be designed in such a way as to be poorly antigenic," says Leaf Huang, chair of the school of pharmacy at the University of North Carolina, in Chapel Hill, North Carolina.

Gill and colleagues, for example, have designed a plasmid that lacks any CpG motifs to avoid triggering innate immune responses associated with Toll-like receptor 9 signaling. Studies in a mouse model showed that it delivered sustained levels of gene expression, without any accompanying lung inflammation, whereas previous work had shown that even a single CpG occurrence was enough to trigger a reaction[22]. A phase 1 trial involving a single dose of the CFTR-expressing plasmid, pGM169, in complex with a cationic lipid GL67A, is currently underway; a multi-dose, one-year trial is due to start next year. Huang has focused on adapting nonviral vectors so they can evade digestion by macrophages before entering their target cells. His group has developed a method of stabilizing lipid membranes so that high concentrations of polyethylene glycol (PEG) can be added to the surface of a lipid particle. "That protects the particles from opsonization and uptake by the reticuloendothelial system," he says. The resulting particle can be targeted toward specific cell types by the attachment of different ligands to the PEG chain.

Lipid particles or nanoparticles enter the cell by an endocytic mechanism. Various physicochemical methods have been developed to optimize the escape of their nucleic acid payloads from the endosomes formed during this process and into the cytoplasm. Huang's group deploys a calcium phosphate precipitate in the core of the nanoparticle, which, on encountering the acidic pH of the endosome, causes the particle to de-assemble and the endosome to burst. Others have developed stable nucleic acid–lipid particles based on cationic liposomes, which bind with anionic lipids present in the endosome membrane and cause it to rupture. Robert Langer and Daniel Anderson at Massachusetts Institute of Technology (Cambridge, MA, USA) have used a combinatorial approach to develop a library of 'lipidoids' or lipid-like delivery molecules that can be produced easily and rapidly[23]. These have demonstrated high levels of efficiency across a range of cell types.

Difficulties with the next step continue to hamper the use of nonviral approaches in classic gene therapy, however. "The biggest problem with nonviral vectors is the passage of the DNA across the nuclear membrane into the nucleus. That has been a rate-limiting step, and very few advances have been made in the past five to six years," says Huang. For that reason, most researchers working on nonviral delivery methods have concentrated their efforts on small interfering RNA, which needs only to reach the cytoplasm to function. According to Gill, in cystic fibrosis, at least, the issue of nuclear transport is still subordinate to the problem of ensuring efficient nucleic acid delivery to the target cells. "I think there is huge scope for the improvement of plasmid vectors, and currently we are only just scratching the surface," she notes.

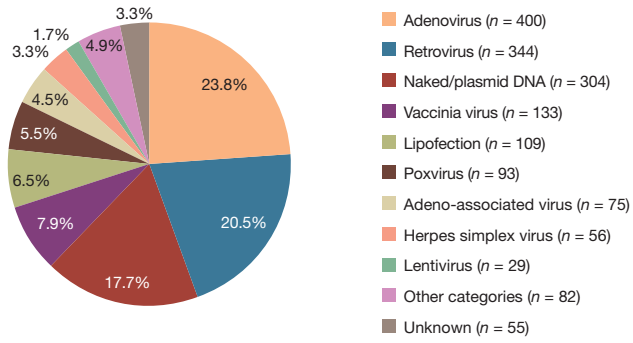

Figure 2 Breakdown of vectors used in gene therapy trials. (Source: *The Journal of Gene Medicine*, Wiley and Sons).

manufacture. It's an area, according to Amsterdam Molecular's van Deventer, that has not received enough attention. "People have underestimated for a long time simply how important manufacturing is. People did the same with antibodies in around 1995," he says. This oversight has, he says, hampered the development of the area. "One important reason gene therapy never took off is the quality of product has not been great," he says. "The quality of the product can determine the efficacy of therapy and can make a 10- to 100-fold difference in [transgene] expression." Indeed, AMT's main competitive advantage, he says, lies in its AAV GMP production platform, which has at this stage completed "well over 100" production runs. "Our release specs are very tight," he says. The batch production system employs a baculovirus expression system in insect cells, which are grown in suspension. "You need to have a soluble cell system," van Deventer says. Adherent cell systems, he says, cannot scale up. In AMT's process, the insect cells are co-infected by three baculovirus constructs, encoding, respectively, the therapeutic protein, the AAV replication (Rep) and packaging proteins, and the AAV capsid protein. "A lot of the technology is in the quality of the capsid and the efficiency of packaging," van Deventer says. "That is determined by Rep."

A further milestone in the maturation of AAV vector technology was reached this year with the publication of a reference standard for AAV serotype 2 (AAV2), the most commonly used AAV vector[11]. "It was recognized pretty early—I would say almost ten years ago—that people using AAV as a vector were administering and reporting doses that weren't standardized," says University of Florida's Snyder, who coordinated the effort. That meant the results obtained by different groups using similar AAV2 vectors were not directly comparable. Even with tightly standardized protocols and reagents, the various laboratories involved in the standard-setting effort still obtained differing results. "We're as clear as we can be, I think, in knowing the true titer value of that reference standard," Snyder says. "It's a much better starting position than not having the reference standard."

With lentiviral vectors, optimizing process development remains an unfinished task. "One way to improve the efficiency of transducing hematopoietic stem cells is to improve the quality of the lentiviral vector—and that is a major issue," says Aubourg. Only around one in a thousand vector particles is successfully loaded with the therapeutic gene, he says. Most are empty—mirroring the natural biological process of virus assembly—and compete with active vectors to gain entry to target cells. This has direct clinical consequences. In the ALD trial, demyelination continued for over a year after the gene therapy procedure, until the population of transduced cells became large enough to start producing the target protein, an ATP-binding cassette transporter, in the required amounts. More efficient transduction would lead to faster engraftment and arrest patients' deterioration more rapidly, Aubourg says. Multiple factors influence the efficiency of packaging, including the packaging cell line employed and the culture conditions. Improving it is an empirical process of trial and error, he says.

Lentiviruses are budding viruses, says Stuart Naylor, CSO of Oxford BioMedica (Oxford, UK), which makes downstream processing and purification difficult, as membrane components have to be removed reliably and consistently. Adenoviral vectors were easier to work with. "They were hardy, robust creatures, which were able to survive harsh downstream processes," he says. Oxford BioMedica is currently focused on converting the production process for its lentivector platform from a batch to a continuous system to scale up for larger clinical trials of its ProSavin gene therapy for Parkinson's disease, which encodes the three enzymes required for dopamine synthesis, tyrosine hydroxylase, aromatic amino acid dopa decarboxylase and GTP cyclohydrolase 1. The platform is "good to go" for small phase 1 or phase 2 studies, says Naylor. However, scaling up simply by adding on extra cell culture vessels is not feasible, as a disproportionately large fraction of the finished product would then be consumed during quality testing.

Facing reality

The divergence of opinion on gene therapy may be rooted in its slow emergence and its continuing immaturity. Its novelty has, arguably, been more of a burden than a blessing. "For some reason, we've always expected too much from gene therapy," says Naldini. "Where we've had success, it's been remarkable." Notwithstanding all of the controversy that has attended its development, gene therapy, like any other therapeutic intervention, will ultimately be judged on a risk-benefit basis, even if that type of calculation has often been absent in the assessments that have been made so far. "Scientists in particular have to remember we are only doing medicine, which is an art, not a science—and not a perfect art," Aubourg says. More clinical data required for a sound assessment of where the field's true potential lies are becoming available. In the next decade, therefore, expectations surrounding gene therapy may finally be matched by its performance.

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