Gene Therapy (2012), 1–7

www.nature.com/gt

REVIEW

# Lentiviral vectors and cardiovascular diseases: a genetic tool for manipulating cardiomyocyte differentiation and function

E Di Pasquale[1,2], MVG Latronico[3], GS Jotti[4] and G Condorelli[5]

Engineered recombinant viral vectors are a powerful tool for vehiculating genetic information into mammalian cells. Because of their ability to infect both dividing and non-dividing cells with high efficiency, lentiviral vectors have gained particular interest for basic research and preclinical studies in the cardiovascular field. We review here the major applications for lentiviral-vector technology in the cardiovascular field: we will discuss their use in trailing gene expression during the induction of differentiation, in protocols for the isolation of cardiac cells and in the tracking of cardiac cells after transplantation *in vivo*; we will also describe lentivirally-mediated gene delivery uses, such as the induction of a phenotype of interest in a target cell or the treatment of cardiovascular diseases. In addition, a section of the review will be dedicated to reprogramming approaches, focusing attention on the generation of pluripotent stem cells and on transdifferentiation, two emerging strategies for the production of cardiac myocytes from human cells and for the investigation of human diseases. Finally, in order to give a perspective on their future clinical use we will critically discuss advantages and disadvantages of lentivirus-based strategies for the treatment of cardiovascular diseases.
Gene Therapy advance online publication, 1 March 2012; doi:10.1038/gt.2012.19

**Keywords:** lentiviral vectors; heart; cardiomyocytes; iPS cells; reprogramming strategies; cell tracking

## INTRODUCTION

Bioengineered recombinant viruses are vehicles of choice for delivering genetic information into a target cell, being endowed with a sophisticated machinery that facilitates efficient cell entry, transport to the nucleus and expression of their genomic package in the infected cell. Adenoviral and adeno-associated vectors have been the preferred system for myocardial gene therapy applications for many years (and adeno-associated viruses are still used for myocardial gene delivery); however, their popularity has been diminished by immunogenicity issues, their innate tropism for some human tissues and their inability to integrate into the host genome, drawbacks that are reflected in inefficient gene delivery and a short duration of transgene expression.[1]

Lentiviral vectors (LVVs) can potentially overcame such limitations, and for this reason they are now widely used in biological research and gene therapy applications: on one hand, they resemble γ-retroviral vectors and so are able to stably integrate into the genome of the target cell, allowing for persistent expression of the gene of interest (the transgene); on the other hand, LVVs can transduce both replicating and non-replicating cells with high efficiency, making them suitable for use on terminally differentiated cells, such as neurons, hepatocytes and cardiomyocytes (CMCs).[2–6] These characteristics make LVVs a favourite choice for major applications in the cardiovascular field (Figure 1): they are routinely employed for dissecting the cardiovascular differentiation process, through their use in protocols for the purification of cardiac cells and for the tracking of gene expression *in vitro* and *in vivo*; in addition, they are used in the study of cardiovascular diseases and are being evaluated for their potential in gene transfer or gene correction strategies for therapeutic ends. Moreover, LVV-mediated transduction is instrumental in two recently developed methodologies, transdifferentiation and induced pluripotent stem (iPS) cell generation, processes that represent important milestones in the production of patient-specific cells for research on pathogenesis and for drug discovery.

## THE LVV SYSTEM

LVVs mainly derive from the HIV-1 virion. They are designed to be replication-defective by separating the *cis*- and *trans*-acting sequences of the HIV genome. This minimizes the risk of generating replication-competent recombinants and produces virus particles that are unable to continue to infect their host after they deliver their genetic content[7]. Since they were first described,[8] many improvements have been made to subsequent 'generations' of vectors in order to improve efficiency and biosafety. Today, the third generation system is used: this consists of three plasmids that are transfected together into a 'packaging' cell line (usually 293T cells) to produce lentiviral particles. These three plasmids are the packaging vector, encoding the genes required for replication under the control of a heterologous promoter; the envelope-encoding plasmid, carrying the envelope gene (usually from the vesicular stomatitis virus (VSV-G)) and the transfer vector, in which the transgene—which can be either a reporter, such as green fluorescence protein, or an exogenous gene for experimentation or therapeutic use—is cloned flanked by long terminal repeats and the Psi-sequence of HIV. The long terminal repeats are necessary for the integration of the transgene into the genome of the target cell, whereas the Psi-sequence acts as a signal sequence necessary for packaging RNA with the transgene into the virion. Recent progress in LVV development and production are reviewed elsewhere.[9,10]

[1]IRCCS Multimedica, Milan, Italy; [2]Institute of Genetic and Biomedic Research (IRGB) – UOS Milan, Milan, Italy; [3]IRCCS Istituto Clinico Humanitas, Rozzano, Italy; [4]Department of Public Health, University of Parma, Parma, Italy and [5]Department of Medicine, National Research Council of Italy, Milan, Italy
Correspondence: Professor G Condorelli, Department of Medicine, National Research Council of Italy, Via G. Fantoli 16/15, Milan 20138, Italy.
E-mail: gianluigi.condorelli@cnr.it
Received 31 October 2011; revised 12 January 2012; accepted 6 February 2012

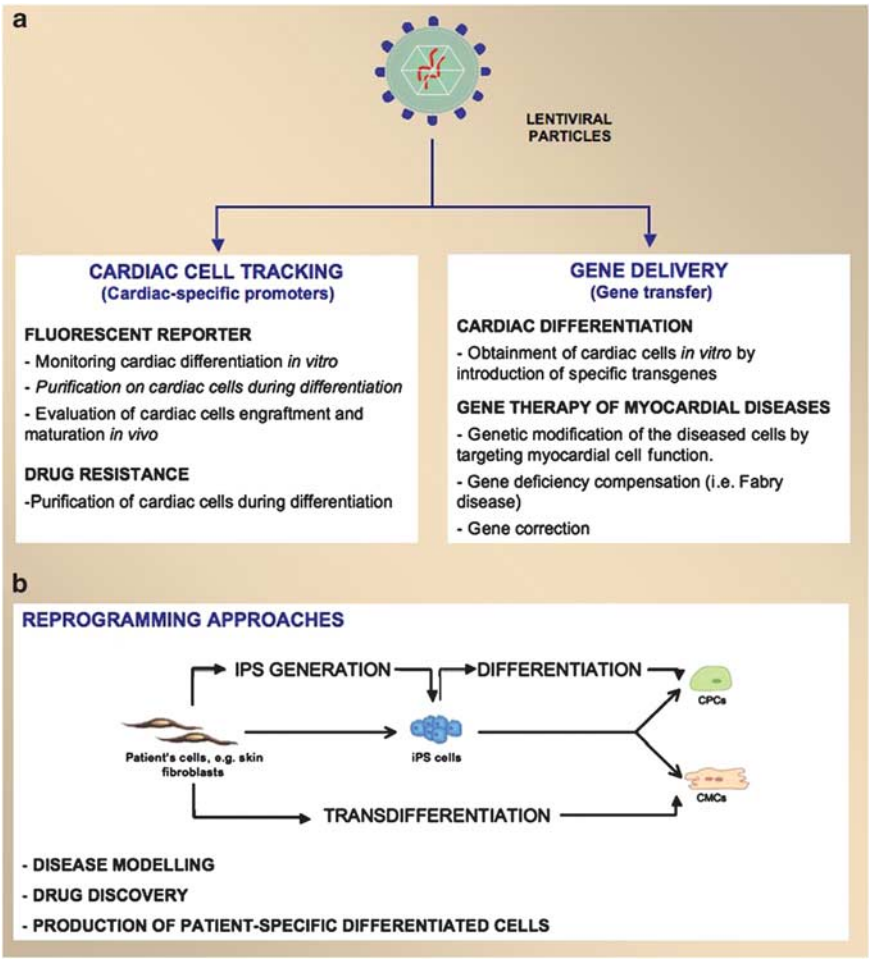



**Figure 1** LVV applications. (**a**) Schematic summary of the major applications of LVVs in the cardiovascular biology field. Cardiac-specific promoters: LVVs can be used as a tool for tracking cardiac cells during differentiation, identifying cardiac cells *in vivo* and purification of cardiac-specific cell populations during differentiation using either fluorescent reporter genes or proteins that confer specific resistance to drugs (left box); in addition, LVVs may be used for the delivery of specific genes to drive cardiac differentiation or for gene transfer/correction strategies for therapeutic ends (right box). (**b**) Reprogramming approaches and their use for dissecting and treating cardiovascular diseases: patient-specific CPCs and CMCs can be obtained from human skin fibroblasts either by generation and differentiation of iPS cells or by a direct transdifferentiation strategy. Major short-term applications include disease modelling and drug discovery; moreover, the possibility to produce patient-specific CPCs or CMCs is extremely interesting for future cell replacement therapy applications.

## LVVS AS A TOOL FOR TRACKING CARDIAC CELLS

Obtaining cardiac progenitor cells (CPCs) and mature CMCs is imperative for investigating the mechanisms underlying cardiovascular diseases and for developing regenerative-medicine applications. Because of their multilineage differentiation potential, pluripotent stem cells (PSCs), such as embryonic stem cells and iPS cells (*vide infra*), represent an ideal source from which to obtain CMCs: in fact, embryonic stem cells and iPS cells give rise to all the cell derivatives of the three germ layers (ectoderm, mesoderm and endoderm).[11–13] PSCs spontaneously differentiate into CMCs but, unfortunately, the efficiency of this process is extremely low (0.1–1%). Over the last few years, several methods have been proposed to improve the efficiency of this process;[14,15] however, inducing a cardiac fate is still extremely difficult, so easy and reliable approaches for the evaluation of differentiation strategies are needed.

In addition, having a defined cell population is important in obtaining meaningful results in basic science, and acquiring a highly pure cell population would be crucial for transplantation protocols, in order to prevent tumorigenesis associated with the presence of contaminating 'stem' cells. Towards these ends, sorting for specific surface antigens of the cell of interest is the best approach; however, no such antigens have been described for CMCs and only a limited number are available for CPCs.[16,17] Therefore, the creation of transgenic cell lines harbouring either a reporter gene or exhibiting antibiotic resistance under the control of a cardiac-restrictive promoter have been proposed; these methods have been demonstrated to be effective and easily applicable in mouse systems.[18–22] Unfortunately, the translation of these efforts to the clinic is not possible because in human cells, homologous recombination events are infrequent and cloning efficiency is extremely low.[23] As a result, alternative strategies are needed for obtaining stable, long-term gene expression in the human setting, and LVVs may play a part in this requirement. In fact, because they circumvent the limitations associated with the application of homologous recombination in humans, LVVs driving cardiac-specific expression can be used for monitoring cardiac induction methods, for evaluating the efficiency of these methods and to follow cardiac cell engraftment and maturation *in vivo*;[24] these vectors can also be employed for inducing PSCs towards the cardiac lineage and for purification of CMCs.

Various protocols have been optimized for the transduction of PSCs, and cardiac-specific systems have been developed for fluorescent tracking and drug-resistance selection of CMCs.[25,26] Our group has also given a contribution to this field: we have constructed a cardiac-specific LVV in which expression of the transgene is driven by a short fragment of the cardiac troponin I promoter (*TNNI3*) with a human cardiac α-actin enhancer (*hEnAct*).[27] Using an enhanced green fluorescence protein reporter, the TNNI3-LVV has been demonstrated to be effective in tracking cardiac lineage induction during differentiation in both mouse and human embryonic stem cells; moreover, the addition of *hEnAct* conferred a further increase in expression specificity to the TNNI3-LVV in human embryonic stem cells. In fact, a limiting factor of tissue-specific promoters is lower levels of transgene expression compared with more ubiquitous counterparts, such as human cytomegalovirus or phosphoglycerate kinase promoters; the identification of genomic sequences that enhance tissue-specific expression (called 'enhancers') overcomes this limitation.[28]

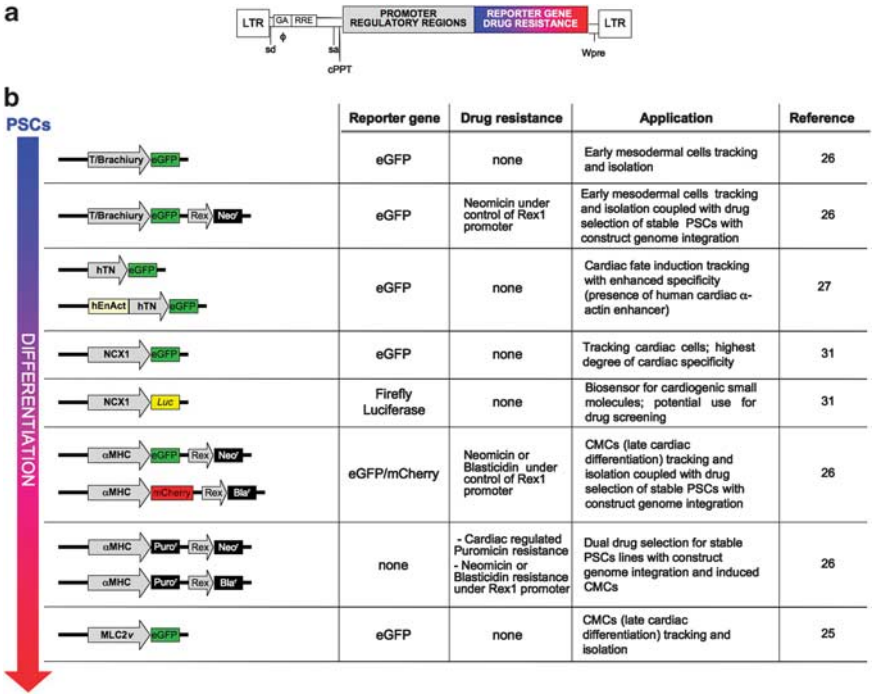
The efficacy and specificity of the hEnAct-TNNI3-LVV system has been confirmed by the determination of caffeine responsiveness in transduced cardiosphere-derived CPCs.[29] It is currently being used in our laboratory to set up improved differentiation protocols for PSCs (including iPS cells) and to determine the efficiency and reliability of methods for the induction of cardiac fate and for the isolation of CPCs and mature CMCs (Rizzi *et al*[30] and Di Pasquale *et al*., unpublished data).

Other systems have been described that employ promoters that are active earlier or later on along the differentiation track, that allow to follow cardiac differentiation induction in time and to select for specific CPCs or mature CMCs from a mixed bulk of differentiated cells.[26,31,32] A schematic representation of these cardiac-specific constructs is given in Figure 2.

In addition to these tracking methods, a specific cell population can be selected by controlled expression of genes that determine drug resistance; this strategy has been shown effective in mouse transgenic models.[19,22] Kita-Matsuo *et al*.[26] recently proposed vectors carrying T/Brachyury and alpha-myosin heavy chain (*αMHC*) promoters driving drug-resistance expression in early mesodermal cells and CMCs, respectively: this drug-selection protocol yielded 96% pure CMCs, which had a molecular profile and electrophysiological properties similar to those of human CMCs.

Altogether, these reports confirm the usefulness of cardiac-specific LVVs as a tool for monitoring cardiac cells and highlight their potential for isolating pure populations of CMCs or CPCs for replacement therapy of the damaged heart. Recent publications from Higuchi *et al*.[33] and Lee *et al*.[34] have described a cardiac-specific LVV for targeted gene therapy of Fabry disease (*vide infra*).

Despite the encouraging data obtained with the described cardiac-specific systems, expression leakage in non-cardiac cells has been reported. This emphasizes the need for continuous validation and refining of the LVV design to obtain more specific and finely regulated results. To this end, Barth *et al*.[32] proposed the use of the cardiac sodium-calcium exchanger (*NCX1*) promoter to mark cardiac cells more specifically: in fact, compared with promoters of sarcomeric genes (i.e., *myosin light chain* (*MLC*)*-2v*, *αMHC3*, *cardiac troponin*




| Reporter gene | Drug resistance | Application | Reference |
|---|---|---|---|
| eGFP | none | Early mesodermal cells tracking and isolation | 26 |
| eGFP | Neomicin under control of Rex1 promoter | Early mesodermal cells tracking and isolation coupled with drug selection of stable PSCs with construct genome integration | 26 |
| eGFP | none | Cardiac fate induction tracking with enhanced specificity (presence of human cardiac α-actin enhancer) | 27 |
| eGFP | none | Tracking cardiac cells; highest degree of cardiac specificity | 31 |
| Firefly Luciferase | none | Biosensor for cardiogenic small molecules; potential use for drug screening | 31 |
| eGFP/mCherry | Neomicin or Blasticidin under control of Rex1 promoter | CMCs (late cardiac differentiation) tracking and isolation coupled with drug selection of stable PSCs with construct genome integration | 26 |
| none | - Cardiac regulated Puromicin resistance<br>- Neomicin or Blasticidin resistance under Rex1 promoter | Dual drug selection for stable PSCs lines with construct genome integration and induced CMCs | 26 |
| eGFP | none | CMCs (late cardiac differentiation) tracking and isolation | 25 |

**Figure 2** Examples of cardiac-specific LVVs. (**a**) Schematic representation of a LVV construct, basically constituted by a reporter gene, or a gene conferring resistance to a specific drug, the expression of which is driven by a promoter of a gene specifically expressed either earlier or later on during the differentiation of PSCs towards CMCs, flanked by long terminal repeats (LTR). The vectors also include sequences necessary for the correct packaging of the viral genome (including the transgene). CPPT, nuclear import sequence; RRE, Rev-response element; sa, splice acceptor sites; sd, major splice donor site; Wpre, regulatory element of woodchuck hepatitis virus; ϕ, encapsidation signal including the 50bp portion of the gag gene (GA). (**b**) Summary of the characteristics and the potential applications of the cardiac-specific LVVs mentioned in this review. Bla$^r$, blasticidin resistance; eGFP, enhanced green fluorescent protein; hEnAct, human α-cardiac actin enhancer (850 bp); hTN, human cardiac troponin I 50 bp flanking region (hTNNI3-340 bp); mCherry, red fluorescent protein; MLC2*v*, myosin light chain 2 ventricular isoform; NCX1, cardiac sodium-calcium exchanger; Neo$^r$, neomycin resistance; Puro$^r$, puromicin resistance; αMHC, alpha-myosin heavy chain.

*(cTn) I*), the *NCX1* promoter produced the lowest expression levels in vascular cells and fibroblasts. The authors also proposed the application of an NCX1-luciferase/green fluorescence protein system for the identification of cardiogenic molecules from small compound libraries, suggesting the potential of this technology in drug discovery applications for the identification of new therapeutic molecules active on relevant pathways.

As an alternative to the above, drug-inducible or conditional systems may represent valuable options and will be briefly discussed hereinafter.

## GENE DELIVERY BY LVVS: INDUCTION OF DIFFERENTIATION AND GENE THERAPY OF MYOCARDIAL DISEASES

The establishment of standardized protocols for the production of CPCs and CMCs would be advantageous for both basic research and preclinical sciences, facilitating molecular dissection of the events driving cardiac-fate specification on the one hand and acting as a way of obtaining a source of 'committed' cells suitable for replacement therapy of the failing heart on the other. LVV technology offers a promising means of delivering transgenes into cultured cardiac cells and the myocardium. The purposes of gene delivery are various and span from the induction of differentiation to the genetic modification of diseased cells for therapeutic reasons. Examples of LVV-based strategies are summarized in Table 1 and discussed below in detail.

The stable or regulated introduction of specific genes or regulatory sequences involved in cardiac-fate induction and CMC proliferation/survival has been demonstrated to improve the differentiation process and to act positively on cardiac function in animal models of myocardial diseases.[35–39] For example, LVV-mediated prodynorphin overexpression in mouse stem cells induces a remarkable enhancement of the expression of the two cardiac-promoting genes *GATA-4* and *Nkx2*.5, resulting in a dramatic increase of spontaneous beating activity.[38] In another study, Koyanagi *et al*.[37] provided evidence that Sox2 enhances the pluripotency of circulating mesangioblasts and facilitates their differentiation towards the cardiac lineage; in addition, a significant improvement of cardiac function was observed *in vivo* after transplantation of the *Sox2*-transduced cells in mice models of myocardial infarction. The positive effects on cardiac function was attributed mainly to the contribution of the *Sox2* injected cells to tissue regeneration, on account of their increased proliferation capacity and the gained pluripotency and developmental competence rather than to an effective role of Sox2 on cardiac function itself. A similar outcome may be reached by directly targeting molecular pathways regulating contractility,[40] calcium handling,[41] the response to oxygen changes[42] and others. To give an example, LVV-mediated intra-coronary delivery of *SERCA2*—which encodes the sarcoplasmic reticulum $Ca^{2+}$-ATPase pump that regulates CMC contraction and relaxation—has been shown to protect against cardiac remodelling and to improve functional parameters of the heart after myocardial infarction, resulting in a better survival rate of the treated mice; the effects observed *in vivo* are probably the results of molecular remodelling by SERCA2 expression.[39]

Similarly, gene therapy can make use of silencing approaches to downregulate the expression of genes leading to a certain disease phenotype: RNA interference technology targeting myotrophin, for example, has been shown to attenuate cardiac hypertrophy *in vitro* and *in vivo* through a mechanism involving the inhibition of the NF-κB signalling pathway.[36]

Regarding cardiovascular gene therapy, the potential targets are several, such as proteins involved in pathways regulating vascular, muscular and myocardial cell functions. Preclinical gene therapy studies have been attempted in many animal models of heart disease: the gene delivery strategies used have mostly employed adenoviruses and adeno-associated viruses to demonstrate the feasibility and the potential of gene therapy approaches for treating pathologies of the heart.[43–46] However, the use of these vectors has diminished because of immunogenicity issues, their inability to integrate into the host genome and their failure to maintain long-term expression. On the contrary, LVVs are capable of inducing prolonged expression of the transgene, and they do not trigger an inflammatory response, so they have low immunogenicity.

Depending on the specific experimental settings, the promoter driving transgene expression may be either constitutive, conditional

**Table 1 LVV-based gene delivery strategies for myocardial diseases**

| *Promoter type* | *Therapeutic gene* | *Species/biological model* | *Delivery* | *Biological/therapeutic effect* | *Reference* |
|---|---|---|---|---|---|
| *Differentiation induction* | | | | | |
| Constitutive (CMV) | Prodynorphin | Mouse ESC line (αMHC_Puro) | Cell infection | Cardiac fate induction and increased spontaneous beating activity | 38 |
| Constitutive (SFFV) | Sox2 | Human circulating mesangioblasts (cMAB) | Cell infection | Pluripotency enhancment and improvement of cMAB differentiation toward the cardiovascular lineages | 37 |
| | | Infarcted nude mice | Intra-muscular injection (of infected cMAB) | Improved cardiac function | |
| *Gene/cellular therapy* | | | | | |
| Constitutive (CMV) | Myotrophin-shRNA | Myotrophin transgenic mice | Direct heart injection | Attenuation of cardiac hypertrophy (cardiac mass reduction) | 36 |
| | SERCA | Rat ischemic heart failure model | Hypotermic intracoronary | Protection from cardiac remodelling after myocardial infarction with survival rate improvement | 39 |
| | HIF-1α | Mouse model of myocardial infarction | Bone marrow-derived HSC infection and intrafemoral injection in irradiated mice | Improved cardiac function after myocardial infarction | 35 |
| Cardiac-specific (either α-MHC, MLC2v or cTnT) | α-Gal | α-Gal deficient Fabry mice | Neonatal temporal vein injection | Decrease of the globotriaosylceramide (Gb3) accumulation in the heart | 34 |

Abbreviations: CMV, cytomegalovirus; cTnT, cardiac troponin T; HCS, haematopoietic stem cells; HIF-1α, hypoxia inducible factor-1alpha; MLC2v, myosin light chain; Puro, puromicin; SERCA, sarcoplasmic reticulum $Ca^{2+}$-ATPase; SFFV, spleen focus forming virus; sh, short hairpin; α-Gal, α-galactosidase A; α-MHC, alpha myosin heavy chain.
Summary of the most relevant LVV-based strategies used for basic research and preclinical studies of myocardial diseases through some relevant examples employed either for inducing cardiac differentiation or gene/cellular therapy applications.
Information about the therapeutic gene, the viral vector promoter type, the species and biological model systems used, the LVV delivery method and the biological or therapeutic effect are presented.

or tissue specific. Typically, strong and ubiquitous promoters, such as those of phosphoglycerate kinase and cytomegalovirus, have been used. However, the protracted and unrestricted expression of a transgene may potentially have deleterious side effects *in vivo*. Regulated conditional expression by tissue-specific, Cre-LoxP[47] and drug-inducible systems[48] have been suggested and developed for controlling transgene expression in space (cell-specific) and in time (inducible systems).

As mentioned, the use of cardiac-restrictive LVVs has been recently proposed as a therapeutic approach for treating Fabry disease, a disorder caused by a deficiency of α-galactosidase A activity, that leads to the accumulation of globotriaosylceramide (Gb3) in various tissues, including the heart.[34] The authors created LVVs harbouring myocardial-specific promoters (α-*MHC*, *MLC* or *cTn*) to drive α-galactosidase A expression and used them to treat mouse models of Fabry disease: their results showed that the strategy was effective on the cardiac manifestation of the disease, decreasing accumulation of Gb3 in the heart, and indicated the potential application of such technology to other progressive pathologies of the heart.

## REPROGRAMMING STRATEGIES: A PEEK AT DISEASE MECHANISMS WHILE WAITING FOR A CURE

The employment of LVVs has recently made it possible to reprogramme somatic cells so that they become pluripotent. This is done through the introduction of a number of so called pluripotent genes—*Oct4*, *Sox2*, *Klf4*, *cMyc*, *Lin28* and *Nanog*—into the host genome; the resulting cells have been termed iPS cells.[49–51] iPS cells have been derived from many different adult somatic cell categories originating from several species, including humans, and these have been demonstrated to give rise to all cells types present in the body. Reviews from the Jaenisch[52] and Hochedlinger[53] laboratories give a comprehensive overview of this topic.

In virtue of their distinctive features, iPS cells offer a unique opportunity to derive patient-specific, differentiated cells and to create new *in vitro* model systems that facilitate not only the investigation of human diseases but also the screening of new therapeutic molecules.[54] iPS cells are also extremely exciting for future cell replacement therapy applications: the creation of such cells would overcome many of the immunological limitations currently hampering the therapeutic use of other PSCs and their derivatives.

The effectiveness of iPS cells has recently been tested in several diseases, including congenital cardiovascular disorders:[55,56] iPS cells have been employed in the investigation of a monogenic cardiac disorder (i.e., long QT syndrome) and pathologies where cardiac defects are part of a complex phenotype (i.e., Leopard and Timothy syndromes).[57–59] These reports confirmed that patient-specific iPS cells can give rise to differentiated CMCs that possess the main functional and morphological aberration typical of the disease *in vivo*.

To date, heterologous systems and transgenic models have been used to investigate disease mechanisms. However, CMCs possess characteristics and electrophysiological properties that differ among species. Consequently, deriving a species-specific cell-based system would be extremely advantageous for the investigation of cardiovascular disease mechanisms in a precise human context. It is worth pointing out that iPS-derived CMCs respond to specific pharmacological treatments (i.e., adrenergic stimulation, β-blockers and roscovitine), strongly indicating the feasibility of iPS cell technology for drug discovery applications and for testing patient-specific therapies.

Gene correction has also become possible because of the advances made in site-specific homologous recombination strategies using the LVV-based zinc finger nucleases. Zinc finger nucleases are synthetic, sequence-specific nucleases consisting of a customized zinc-finger DNA-binding domain engineered to bind to a specific DNA sequence, and a non-specific DNA endonuclease cleavage domain (Fok1): these engineered zinc finger nucleases are able to introduce site-specific, double-strand breaks in DNA and to drive targeted manipulation at genomic loci. The induced breaks stimulate the endogenous homologous recombination machinery, allowing the introduction of an exogenous DNA 'repair template'.[60] Physiological phenotypic traits can thus be rescued by the reversion of the genetic alteration leading to the disease. This represents a step forward in the combination of gene- and cell-replacement therapies.

Because they give rise to derivatives of all three germ layers, iPS cells may also constitute an inexhaustible source of cells for replacement therapy: in fact, CPCs, CMCs, smooth muscle cells and endothelial cells have all been obtained from iPS cells. However, tumorigenic issues related to the random integration of the viral genome used to introduce reprogramming and the presence of contaminant stem cells within the pool of differentiated cells for transplantation still limit the use of iPS cells for therapeutic applications. To overcome this, non-integrating approaches for reprogramming based on either excisable LVVs or non-viral systems (e.g., synthetic RNA, proteins and plasmids) have been proposed. Unfortunately, these techniques are not without difficulties: the excisable LVV approach requires sequencing of the whole genome to verify correct excision, and apart from the RNA-based approach—which, however, is technically challenging—non-viral systems are inefficient.[53] Additionally, these approaches do not overcome the issue related to tumorigenesis associated with cell contamination. A more recent step forward in cellular reprogramming techniques may be more decisive in overcoming these difficulties: it seems that it is possible to induce one somatic cell type to become another without first reprogramming it into a pluripotent state. This process, referred to as 'transdifferentiation', is induced by the ectopic expression of cell-specific factors using LVVs. To date, fibroblasts have been successfully converted into neuronal cells[61] and multilineage blood progenitors.[62] CMC-like cells have also been obtained by ectopic expression of the cardiac-specific genes *Gata4*, *Mef2c* and *Tbx5*.[63] Even though further studies are necessary to determine the molecular and functional properties of the converted cells, transdifferentiation may represent a valid alternative for the generation of patient-specific differentiated cells and the production of CMCs for modelling and therapy of cardiovascular disease.

## FINAL CONSIDERATIONS

It has been consistently demonstrated over the past decade that LVVs represent a feasible and effective strategy for cardiac gene detection and transfer *in vitro* and *in vivo*. The capacity of LVVs to maintain efficient, stable, long-term expression of the transgene, their ability to transduce non-dividing cells and their low immunogenicity have made LVVs one the most suitable tools for gene therapy applications, especially in the cardiovascular field. In fact, LLVs are being increasingly tested for applications in preclinical studies.[34–37,39,64–66] LVVs have also been used successfully for reprogramming strategies that generate iPS cells and for the transdifferentiation of somatic cells. However, safety issue concerns are still restraining their use in the clinical setting: indeed, LVVs do have the potential drawback of causing insertional mutagenesis through the random integration of DNA into the host genome, leading to the aberrant expression of important genes and to tumorigenesis.[67,68]

Preclinical *in vivo* studies have highlighted several limitations of gene therapy applications for human cardiovascular diseases: the most relevant of these regard the route of administration (LVV delivery is

reviewed by Ly *et al.*[69]), transduction efficiency and specificity, and, most importantly, the obtainment of a functional and synchronous engraftment. In fact, arrhythmic events are often associated with cell replacement therapy of injured myocardium because homogeneous transduction of the entire myocardium is not possible and transduced cells are not uniformly distributed; engrafted cells can also fail to synchronize with the rhythm of the recipient heart. Additionally, perfusion of LVVs into the heart may result in the transduction of aspecific cells, and systemic spread may occur causing the appearance of adverse side effects. As mentioned above, the development of more finely regulated and specific systems may overcome some of these issues: a system in which transgene expression is restricted to a given cell type and can be reversibly induced with an antibiotic is probably the best approach. Another strategy that may be interesting to consider regards the alteration of the viral envelope so that it becomes specific for a certain cell type: LVVs are usually pseudotyped with a VSV-G envelope, a glycoprotein that interacts with a ubiquitous receptor and that confers the viral particles a broad host-cell range. The tropism of LVVs may be controlled by using alternative glycoproteins that preferentially interact with specific cell types or by genetic modification of the viral surface (this topic has been reviewed by Bouard *et al.*[70]).

Nevertheless, LVVs are currently being assessed in clinical trials for their use in several non-cardiac human diseases; completion of these studies will allow us to better evaluate the efficacy of this strategy and to get a perspective of the wider therapeutic value of LVVs. These trials are being carried out on patients with Parkinson's disease, β-thalassaemia and other haematopoietic disorders.[71–73] Overall, the results are promising so far. However, in one β-thalassaemic patient treated with an LVV-based β-globin therapy, clonal dominance of haematopoietic stem cells has been reported.[74] Even though isolated, this occurrence has renewed the need for the development of improved systems to strengthen the safety of LVV technology. Technical improvements made in the design of viral vectors to produce site-specific insertion and the use of inducible, cell-specific systems may help to reach this goal. It is noteworthy that genotoxicity is closely related to the type of transduced cell and appears to affect primarily stem cells rather than somatic cells;[75] for this reason, when an LVV is constructed for therapy, vector design, purity and transduction methods should be carefully evaluated in the appropriate cellular context.

The studies published so far strongly support the feasibility of the application of LVV-based strategies to treat patients with cardiovascular diseases. Before this becomes a reality, however, the safety and specificity of the vector systems available require further development.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

This work was supported by Grants from the CARIPLO Foundation (# 2010-0768) and the Italian Ministry of Health (to GC). EDP was supported by the Superpig Program project co-financed by the Lombardy Region through the 'Fund for promoting institutional agreements'.

1 Lyon AR, Sato M, Hajjar RJ, Samulski RJ, Harding SE. Gene therapy: targeting the myocardium. *Heart* 2008; **94**: 89–99.
2 Blomer U, Gruh I, Witschel H, Haverich A, Martin U. Shuttle of lentiviral vectors via transplanted cells *in vivo*. *Gene Therapy* 2005; **12**: 67–74.
3 Bonci D, Cittadini A, Latronico MV, Borello U, Aycock JK, Drusco A *et al.* Advanced' generation lentiviruses as efficient vectors for cardiomyocyte gene transduction *in vitro* and *in vivo*. *Gene Therapy* 2003; **10**: 630–636.
4 Naldini L. Lentiviruses as gene transfer agents for delivery to non-dividing cells. *Curr Opin Biotechnol* 1998; **9**: 457–463.
5 Naldini L, Blomer U, Gage FH, Trono D, Verma IM. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci USA* 1996; **93**: 11382–11388.
6 Sakoda T, Kasahara N, Hamamori Y, Kedes L. A high-titer lentiviral production system mediates efficient transduction of differentiated cells including beating cardiac myocytes. *J Mol Cell Cardiol* 1999; **31**: 2037–2047.
7 Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D *et al.* A third-generation lentivirus vector with a conditional packaging system. *J Virol* 1998; **72**: 8463–8471.
8 Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH *et al. In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 1996; **272**: 263–267.
9 Matrai J, Chuah MK, VandenDriessche T. Recent advances in lentiviral vector development and applications. *Mol Ther* 2010; **18**: 477–490.
10 Sinn PL, Sauter SL, McCray Jr PB. Gene therapy progress and prospects: development of improved lentiviral and retroviral vectors—design, biosafety, and production. *Gene Therapy* 2005; **12**: 1089–1098.
11 Gai H, Leung EL, Costantino PD, Aguila JR, Nguyen DM, Fink LM *et al.* Generation and characterization of functional cardiomyocytes using induced pluripotent stem cells derived from human fibroblasts. *Cell Biol Int* 2009; **33**: 1184–1193.
12 Mauritz C, Schwanke K, Reppel M, Neef S, Katsirntaki K, Maier LS *et al.* Generation of functional murine cardiac myocytes from induced pluripotent stem cells. *Circulation* 2008; **118**: 507–517.
13 Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* 1998; **282**: 1145–1147.
14 Laflamme MA, Murry CE. Heart regeneration. *Nature* 2011; **473**: 326–335.
15 Murry CE, Keller G. Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. *Cell* 2008; **132**: 661–680.
16 Leschik J, Stefanovic S, Brinon B, Puceat M. Cardiac commitment of primate embryonic stem cells. *Nat Protoc* 2008; **3**: 1381–1387.
17 Yang L, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M *et al.* Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature* 2008; **453**: 524–528.
18 Hidaka K, Lee JK, Kim HS, Ihm CH, Iio A, Ogawa M *et al.* Chamber-specific differentiation of Nkx2.5-positive cardiac precursor cells from murine embryonic stem cells. *FASEB J* 2003; **17**: 740–742.
19 Klug MG, Soonpaa MH, Koh GY, Field LJ. Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts. *J Clin Invest* 1996; **98**: 216–224.
20 Kolossov E, Fleischmann BK, Liu Q, Bloch W, Viatchenko-Karpinski S, Manzke O *et al.* Functional characteristics of ES cell-derived cardiac precursor cells identified by tissue-specific expression of the green fluorescent protein. *J Cell Biol* 1998; **143**: 2045–2056.
21 Muller M, Fleischmann BK, Selbert S, Ji GJ, Endl E, Middeler G *et al.* Selection of ventricular-like cardiomyocytes from ES cells *in vitro*. *FASEB J* 2000; **14**: 2540–2548.
22 Schroeder M, Niebruegge S, Werner A, Willbold E, Burg M, Ruediger M *et al.* Differentiation and lineage selection of mouse embryonic stem cells in a stirred bench scale bioreactor with automated process control. *Biotechnol Bioeng* 2005; **92**: 920–933.
23 Zwaka TP, Thomson JA. Homologous recombination in human embryonic stem cells. *Nat Biotechnol* 2003; **21**: 319–321.
24 Bai X, Yan Y, Coleman M, Wu G, Rabinovich B, Seidensticker M *et al.* Tracking long-term survival of intramyocardially delivered human adipose tissue-derived stem cells using bioluminescence imaging. *Mol Imaging Biol* 2011; **13**: 633–645.
25 Huber I, Itzhaki I, Caspi O, Arbel G, Tzukerman M, Gepstein A *et al.* Identification and selection of cardiomyocytes during human embryonic stem cell differentiation. *FASEB J* 2007; **21**: 2551–2563.
26 Kita-Matsuo H, Barcova M, Prigozhina N, Salomonis N, Wei K, Jacot JG *et al.* Lentiviral vectors and protocols for creation of stable hESC lines for fluorescent tracking and drug resistance selection of cardiomyocytes. *PLoS One* 2009; **4**: e5046.
27 Gallo P, Grimaldi S, Latronico MV, Bonci D, Pagliuca A, Ausoni S *et al.* A lentiviral vector with a short troponin-I promoter for tracking cardiomyocyte differentiation of human embryonic stem cells. *Gene Therapy* 2008; **15**: 161–170.
28 Gruh I, Wunderlich S, Winkler M, Schwanke K, Heinke J, Blomer U *et al.* Human CMV immediate-early enhancer: a useful tool to enhance cell-type-specific expression from lentiviral vectors. *J Gene Med* 2008; **10**: 21–32.
29 Altomare C, Barile L, Marangoni S, Rocchetti M, Alemanni M, Mostacciuolo G *et al.* Caffeine-induced Ca(2+) signaling as an index of cardiac progenitor cells differentiation. *Basic Res Cardiol* 2010; **105**: 737–749.
30 Rizzi R, Di Pasquale E, Portararo P, Papait R, Cattaneo P, Latronico MV *et al.* Post-natal cardiomyocytes can generate iPS cells with an enhanced capacity toward cardiomyogenic re-differentation. *Cell Death Differ* 2012; e-pub ahead of print 20 January 2012; doi: 10.1038/cdd.2011.205.
31 Bai X, Pinkernell K, Song YH, Nabzdyk C, Reiser J, Alt E. Genetically selected stem cells from human adipose tissue express cardiac markers. *Biochem Biophys Res Commun* 2007; **353**: 665–671.
32 Barth AS, Kizana E, Smith RR, Terrovitis J, Dong P, Leppo MK *et al.* Lentiviral vectors bearing the cardiac promoter of the Na+-Ca2+ exchanger report cardiogenic differentiation in stem cells. *Mol Ther* 2008; **16**: 957–964.

33 Higuchi K, Yoshimitsu M, Fan X, Guo X, Rasaiah VI, Yen J *et al.* Alpha-galactosidase A-Tat fusion enhances storage reduction in hearts and kidneys of Fabry mice. *Mol Med* 2010; **16**: 216–221.
34 Lee CJ, Fan X, Guo X, Medin JA. Promoter-specific lentivectors for long-term, cardiac-directed therapy of Fabry disease. *J Cardiol* 2011; **57**: 115–122.
35 Dong F, Khalil M, Kiedrowski M, O'Connor C, Petrovic E, Zhou X *et al.* Critical role for leukocyte hypoxia inducible factor-1alpha expression in post-myocardial infarction left ventricular remodeling. *Circ Res* 2010; **106**: 601–610.
36 Gupta S, Maitra R, Young D, Gupta A, Sen S. Silencing the myotrophin gene by RNA interference leads to the regression of cardiac hypertrophy. *Am J Physiol Heart Circ Physiol* 2009; **297**: H627–H636.
37 Koyanagi M, Iwasaki M, Rupp S, Tedesco FS, Yoon CH, Boeckel JN *et al.* Sox2 transduction enhances cardiovascular repair capacity of blood-derived mesoangioblasts. *Circ Res* 2010; **106**: 1290–1302.
38 Maioli M, Asara Y, Pintus A, Ninniri S, Bettuzzi S, Scaltriti M *et al.* Creating prodynorphin-expressing stem cells alerted for a high-throughput of cardiogenic commitment. *Regen Med* 2007; **2**: 193–202.
39 Niwano K, Arai M, Koitabashi N, Watanabe A, Ikeda Y, Miyoshi H *et al.* Lentiviral vector-mediated SERCA2 gene transfer protects against heart failure and left ventricular remodeling after myocardial infarction in rats. *Mol Ther* 2008; **16**: 1026–1032.
40 Port JD, Bristow MR. beta-Adrenergic receptors, transgenic mice, and pharmacological model systems. *Mol Pharmacol* 2001; **60**: 629–631.
41 Hoshijima M. Gene therapy targeted at calcium handling as an approach to the treatment of heart failure. *Pharmacol Ther* 2005; **105**: 211–228.
42 Eckle T, Kohler D, Lehmann R, El Kasmi K, Eltzschig HK. Hypoxia-inducible factor-1 is central to cardioprotection: a new paradigm for ischemic preconditioning. *Circulation* 2008; **118**: 166–175.
43 del Monte F, Harding SE, Dec GW, Gwathmey JK, Hajjar RJ. Targeting phospholamban by gene transfer in human heart failure. *Circulation* 2002; **105**: 904–907.
44 del Monte F, Harding SE, Schmidt U, Matsui T, Kang ZB, Dec GW *et al.* Restoration of contractile function in isolated cardiomyocytes from failing human hearts by gene transfer of SERCA2a. *Circulation* 1999; **100**: 2308–2311.
45 Maurice JP, Hata JA, Shah AS, White DC, McDonald PH, Dolber PC *et al.* Enhancement of cardiac function after adenoviral-mediated *in vivo* intracoronary beta2-adrenergic receptor gene delivery. *J Clin Invest* 1999; **104**: 21–29.
46 Shah AS, Lilly RE, Kypson AP, Tai O, Hata JA, Pippen A *et al.* Intracoronary adenovirus-mediated delivery and overexpression of the beta(2)-adrenergic receptor in the heart : prospects for molecular ventricular assistance. *Circulation* 2000; **101**: 408–414.
47 Heine HL, Leong HS, Rossi FM, McManus BM, Podor TJ. Strategies of conditional gene expression in myocardium: an overview. *Methods Mol Med* 2005; **112**: 109–154.
48 Vigna E, Cavalieri S, Ailles L, Geuna M, Loew R, Bujard H *et al.* Robust and efficient regulation of transgene expression *in vivo* by improved tetracycline-dependent lentiviral vectors. *Mol Ther* 2002; **5**: 252–261.
49 Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; **131**: 861–872.
50 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; **126**: 663–676.
51 Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science* 2007; **318**: 1917–1920.
52 Hanna JH, Saha K, Jaenisch R. Pluripotency and cellular reprogramming: facts, hypotheses, unresolved issues. *Cell* 2010; **143**: 508–525.
53 Stadtfeld M, Hochedlinger K. Induced pluripotency: history, mechanisms, and applications. *Genes Dev* 2010; **24**: 2239–2263.
54 Yamanaka S. A fresh look at iPS cells. *Cell* 2009; **137**: 13–17.
55 Josowitz R, Carvajal-Vergara X, Lemischka IR, Gelb BD. Induced pluripotent stem cell-derived cardiomyocytes as models for genetic cardiovascular disorders. *Curr Opin Cardiol* 2011; **26**: 223–229.
56 Park IH, Arora N, Huo H, Maherali N, Ahfeldt T, Shimamura A *et al.* Disease-specific induced pluripotent stem cells. *Cell* 2008; **134**: 877–886.
57 Carvajal-Vergara X, Sevilla A, D'Souza SL, Ang YS, Schaniel C, Lee DF *et al.* Patient-specific induced pluripotent stem-cell-derived models of LEOPARD syndrome. *Nature* 2010; **465**: 808–812.
58 Moretti A, Bellin M, Welling A, Jung CB, Lam JT, Bott-Flugel L *et al.* Patient-specific induced pluripotent stem-cell models for long-QT syndrome. *N Engl J Med* 2010; **363**: 1397–1409.
59 Yazawa M, Hsueh B, Jia X, Pasca AM, Bernstein JA, Hallmayer J *et al.* Using induced pluripotent stem cells to investigate cardiac phenotypes in Timothy syndrome. *Nature* 2011; **471**: 230–234.
60 Zou J, Maeder ML, Mali P, Pruett-Miller SM, Thibodeau-Beganny S, Chou BK *et al.* Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. *Cell Stem Cell* 2009; **5**: 97–110.
61 Vierbuchen T, Ostermeier A, Pang ZP, Kokubu Y, Sudhof TC, Wernig M. Direct conversion of fibroblasts to functional neurons by defined factors. *Nature* 2010; **463**: 1035–1041.
62 Szabo E, Rampalli S, Risueno RM, Schnerch A, Mitchell R, Fiebig-Comyn A *et al.* Direct conversion of human fibroblasts to multilineage blood progenitors. *Nature* 2010; **468**: 521–526.
63 Ieda M, Fu JD, Delgado-Olguin P, Vedantham V, Hayashi Y, Bruneau BG *et al.* Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell* 2010; **142**: 375–386.
64 Jacome A, Navarro S, Rio P, Yanez RM, Gonzalez-Murillo A, Lozano ML *et al.* Lentiviral-mediated genetic correction of hematopoietic and mesenchymal progenitor cells from Fanconi anemia patients. *Mol Ther* 2009; **17**: 1083–1092.
65 Marangoni F, Bosticardo M, Charrier S, Draghici E, Locci M, Scaramuzza S *et al.* Evidence for long-term efficacy and safety of gene therapy for Wiskott-Aldrich syndrome in preclinical models. *Mol Ther* 2009; **17**: 1073–1082.
66 Zhao H, Pestina TI, Nasimuzzaman M, Mehta P, Hargrove PW, Persons DA. Amelioration of murine beta-thalassemia through drug selection of hematopoietic stem cells transduced with a lentiviral vector encoding both gamma-globin and the MGMT drug-resistance gene. *Blood* 2009; **113**: 5747–5756.
67 Connolly JB. Lentiviruses in gene therapy clinical research. *Gene Therapy* 2002; **9**: 1730–1734.
68 Woods NB, Muessig A, Schmidt M, Flygare J, Olsson K, Salmon P *et al.* Lentiviral vector transduction of NOD/SCID repopulating cells results in multiple vector integrations per transduced cell: risk of insertional mutagenesis. *Blood* 2003; **101**: 1284–1289.
69 Ly H, Kawase Y, Yoneyama R, Hajjar RJ. Gene therapy in the treatment of heart failure. *Physiology (Bethesda)* 2007; **22**: 81–96.
70 Bouard D, Alazard-Dany D, Cosset FL. Viral vectors: from virology to transgene expression. *Br J Pharmacol* 2009; **157**: 153–165.
71 Bank A, Dorazio R, Leboulch P. A phase I/II clinical trial of beta-globin gene therapy for beta-thalassemia. *Ann NY Acad Sci* 2005; **1054**: 308–316.
72 Cartier N, Aubourg P. Hematopoietic stem cell gene therapy in Hurler syndrome, globoid cell leukodystrophy, metachromatic leukodystrophy and X-adrenoleukodystrophy. *Curr Opin Mol Ther* 2008; **10**: 471–478.
73 Isacson O, Kordower JH. Future of cell and gene therapies for Parkinson's disease. *Ann Neurol* 2008; **64** (Suppl 2): S122–S138.
74 Kaiser J. Gene therapy. Beta-thalassemia treatment succeeds, with a caveat. *Science* 2009; **326**: 1468–1469.
75 Dropulic B. Lentiviral vectors: their molecular design, safety, and use in laboratory and preclinical research. *Hum Gene Ther* 2011; **22**: 649–657.