Journal of Cellular Biochemistry Supplement 38:55–64 (2002)

Hematopoietic Stem Cell Gene Therapy for Inherited Bone Marrow Disorders: Past Accomplishments and Continued Challenges

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Abstract From the time that the genes encoding the defective proteins were cloned for a number of inherited diseases, it became a goal to correct those conditions by restoring the normal gene and thereby, its product. For the inherited disorders affecting the blood and its progenitor cells, the hematopoietic stem cells were the ideal target cells for gene transfer, because the normal gene would then be transferred to all of the progeny cells, theoretically for the lifetime of the recipient. However, the tasks of isolating the hematopoietic stem cells, introducing the new genes in such a manner as to preserve engraftment of the manipulated cells, and achieving long-term gene expression, have not been straightforward in the clinical trial setting, although there has been moderate success for cells in vitro, and in murine studies. With the report of clinical efficacy of gene transfer in children with X-linked severe combined immunodeficiency disease, the dream of clinical gene transfer to hematopoietic cells has become a reality. But there are still significant impediments remaining for a number of diseases. The innovations of introduction of synthetic receptors that confer growth advantage, the use of lentiviral vectors with increased stem cell transduction efficiency, and the addition of modified promoter/enhancer sequences to augment and preserve gene expression may bring wider success to gene therapy clinical trials for bone marrow disorders in the near future. J. Cell. Biochem. Suppl. 38: 55–64, 2002.

Key words: genetic diseases; retrovirus; lentivirus; hemoglobin; glucocerebrosidase

The hematopoietic stem cell has been an elusive target for gene transfer for over a decade. The difficulties include the following: (1) to reproducibly define and isolate true, multipotent, self-renewing stem cells a priori; (2) to maintain the properties of the stem cell after ex vivo manipulation; and (3) to preserve the ability to engraft stem cells that are manipulated ex vivo. In addition, the same challenges that have existed for gene transfer to all target cells also remain challenges for hematopoietic stem cells, including identification of ideal vectors, defining ideal promoter/enhancer strategies, achieving position independent expression for integrated transgenes, maintaining long-term transgene expression, achieving persistence of the transduced cell population, and circumventing immune recognition of the transduced cell population or the transgene product.

GENE THERAPY FOR INHERITED DISORDERS

Gene therapy approaches are currently being attempted for a number of genetic diseases, including familial hypercholesterolemia, Gaucher disease, severe combined immunodeficiency disease [adenosine deaminase (ADA) deficiency], hemophilia, and cystic fibrosis. Much effort has been devoted to optimizing gene transfer to hematopoietic stem cells for inherited disease of the bone marrow. In many cases, murine bone marrow cells have been used as the target cells for human gene transduction and murine recipients for transplantation of the transduced cells, including retroviral vectors containing the genes encoding human adenosine deaminase [Riviere et al., 1995], human

Grant sponsor: National Heart, Lung and Blood Institute; Grant number: R01 HL 63184.

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Received 18 December 2001; Accepted 8 January 2002
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jcb.10131

DNA repair protein O^6-methylguanine DNA methyltransferase [Moritz et al., 1995), human glucocerebrosidase [Nolta et al., 1990; Nimgaonkar et al., 1995a], dihydrofolate reductase [Corey et al., 1990], glucose 6-phosphate dehydrogenase [Rovira et al., 2000], human multidrug resistance [Podda et al., 1992; Hanania and Deisseroth, 1994; Hanania et al., 1995; Sorrentino et al., 1995], and others. These murine models have demonstrated successful transduction and functional expression. However, there has also been difficulty in achieving long term engraftment of transduced cells in non-human primates and humans. This failure has been attributed to the inability of retroviruses to transduce the relatively quiescent hematopoietic stem cells, a proposed defect in the engraftment capability of cycling cells, or a loss of the maintenance of multilineage potential and self-renewing ability because of the ex vivo manipulations used to optimize retroviral integration.

ISOLATION OF HEMATOPOIETIC PROGENITOR CELLS

The hematopoietic stem cell is the pluripotent cell capable of both self-renewal and generation of all of the mature blood cells. It is unable to be defined by unique morphology or a single characteristic surface marker, although a few antigens or receptors may be promising. The stem cells can only be proven retrospectively after long term blood cell recovery of all lineages can be established post transplant of myeloablated recipients. One theoretical mechanism for curing a genetic disease affecting cells of hematopoietic origin would be to introduce the gene into the hematopoietic stem cells. If this is performed ex vivo, then the cells must be capable of engraftment and the newly introduced transgene be capable of both mRNA and protein expression.

The traditional methods for isolating the population of blood or bone marrow cells containing human stem cells rely on expression of the CD34 antigen. The CD34 antigen is a 115-kDa surface membrane glycoprotein that is expressed by both maturing hematopoietic cells [Civin, 1990] and endothelial cells [Fina et al., 1990]. It has also recently been identified as one of the potential ligands for L-selectin [Baumhueter et al., 1994]. Approximately 1–3% of bone marrow cells and 0.2% of unstimulated peripheral blood mononuclear cells bear the CD34 antigen. The numbers of $CD34^+$ cells in the peripheral blood can be augmented by "mobilization" procedures, which consist of growth factors or chemotherapy or a combination of both. The growth factors include G-CSF, GM-CSF, or SCF. The chemotherapy approaches include high dose cyclophosphamide or combination chemotherapy. Cells enriched for CD34 by immunoabsorption techniques are capable of restoring blood cell recovery after myeloablative chemotherapy [Berenson et al., 1991].

One major problem with CD34 selection is that only a small subset of $CD34^+$ cells fulfill the definition of stem cells, able to self-renew indefinitely and give rise to all types of blood cell lineages. There is no single precise marker for the hematopoietic stem cell; in fact, CD34 may not be the best marker for the stem cell. Certain other characteristics can be identified on primitive hematopoietic stem cells: $CD38^-$, $CD33^-$, $CD71^-$, HLA-DR^-, Rhodamine123^{lo}, Hoechst33342^{lo}, Thy-1^{lo}, lin^-, $CD45RA^{lo}$. However, all of these features are exclusions. With regard to gene therapy approaches in stem cells, there is a need to reduce the population to include only the highly purified stem cells, motivated by the fact that far less vector would be required if smaller cell numbers could be utilized to maintain an adequate vector to cell ratio for transduction.

Another obstacle is that true stem cells can only be proven retrospectively, when long-term bone marrow repopulating ability is achieved. Several assay systems are available to document the existence of stem cells, but all of these methods only give retrospective indication of the presence of stem cells. A number of in vitro surrogate assays have been defined for hematopoietic stem cells, including long-term bone marrow culture-initiating cells (LTC-ICs) [Petzer et al., 1996], high-proliferative potential colony forming cells (HPP-CFCs) [McNiece et al., 1989], and cobblestone area forming cells (CAFCs) [van der Loo and Ploemacher, 1995]. Moreover, in vivo xenograft assays have also been developed to define human hematopoietic stem cells, including the SCID repopulating cell (SRC) [Bhatia et al., 1997; Conneally et al., 1997] capable of repopulating immunodeficient mice, and the human cells capable of growth and differentiation in fetal sheep [Zanjani et al., 1994], which are believed to define cells with long term in vivo repopulating ability and

therefore approximate more closely the capabilities of true stem cells.

The definition of hematopoietic stem cell has become even more complex, with the recent findings that neural stem cells [Bjornson et al., 1999] or adult muscle stem cells [Jackson et al., 1999] are capable of giving rise to hematopoiesis as well. Thus, it will be exceedingly difficult to isolate highly purified stem cells a priori to serve as targets for gene transfer, or to prove that the transferred gene is present in a stem cell, except after long term reconstitution and assays for gene expression.

GENE TRANSFER FOR GLUCOCEREBROSIDASE

An example of an inherited condition for which the preclinical studies in vivo in mice, and in vitro in human cells predicted success in the clinical trial is Gaucher disease. Several groups of investigators were successful in transducing murine hematopoietic progenitors [Nolta et al., 1990; Nimgaonkar et al., 1995a], fibroblasts [Aran et al., 1996], and myoblasts [Liu et al., 1998] with retroviruses bearing the gene for glucocerebrosidase. Irradiated myeloablated murine recipients [Correll et al., 1992; Ohashi et al., 1992; Krall et al., 1994] have been successfully reconstituted with bone marrow cells transduced with a retrovirus containing the cDNA for human glucocerebrosidase, and demonstrated expression of the human enzyme. In addition, bone marrow cells from nonablated murine syngeneic recipients of transduced cells exhibited expression of the human transgene [Schiffman et al., 1995].

Human cord blood cells [Mannion-Henderson et al., 1995] and mobilized peripheral blood or bone marrow $CD34^+$ cells [Xu et al., 1994; Nimgaonkar et al., 1995b; Xu et al., 1995] from Gaucher patients were successfully transduced with the retroviral vector containing the glucocerebrosidase cDNA and demonstrated increased enzyme expression.

With the preclinical murine and human studies completed, three gene therapy clinical trials [Barranger et al., 1997; Schuening, 1997; Dunbar et al., 1998] were initiated for Gaucher disease, and a total of ten patients have been studied thus far. The trial by Dunbar et al. [1998] consisted of three adult type I Gaucher patients, for which peripheral blood stem cells were used for two patients, and bone marrow for the third. One patient's cells were transduced on stroma, and two on stroma with cytokines. The initial transduction efficiencies were 1–10%, but after infusion, there was a very low level of corrected cells, and the cells could only be detected for 1–3 months. The trial by Schuening and co-workers [Schuening, 1997] also consisted of three adult type I patients. G-CSF mobilized $CD34^+$ cells were transduced in long-term culture, and the transduction efficiency was 0.01–0.1%. An immediate two-fold increase in enzyme level was detected, but there was no engraftment of transduced cells. The trial by Barranger et al. [1997] studied four type I patients, who underwent G-CSF mobilization of peripheral blood stem cells, and the $CD34^+$ cells were transduced by centrifugation. The average transduction efficiency was 20%, and the enzyme activity of the transduced cells increased ten-fold. The peripheral blood leukocytes from the recipients showed marking with the transgene by polymerase chain reaction. The first of the treated subjects sustained a rise in the enzyme level of peripheral blood leukocytes to the 80% of the normal level, and was able to be tapered off of the enzyme replacement therapy over 9 months, but subsequently needed to go back on enzyme infusions after 27 months [Novelli et al., 2000]. Thus, all of the attempts to utilize the procedures that had been highly successful in murine recipients and secondary transplants, as well as in vitro in human cells, were not successful in achieving long term enzyme expression in adult human patients affected by the the enzyme deficiency.

GENE TRANSFER FOR GLOBIN

Another gene that has remained challenging in terms of achieving adequate expression in vivo is globin. Defects in hemoglobin lead to both thalassemia and sickle cell disease, in addition to other disorders, and affect millions worldwide. Initially, there was poor expression, that was improved by the incorporation of sequences from the locus control region. However, another problem arose, that of frequent gene rearrangements. With modifications to eliminate cryptic splice sites, expression of human beta globin was achieved in a mouse transplant model at 5 and 20% of the endogenous murine globin at 6 and 8 months post-transplant, and this expression was preserved in irradiated secondary

recipients [Raftopoulos et al., 1997]. With the innovation of a lentiviral vector containing large segments of the locus control region including HS2, HS3, and HS4 and the human beta globin gene, up to 13% of the total hemoglobin was comprised of human beta globin chains in normal mice, and 17–24% of the hemoglobin in mice with heterozygous β^0 thalassemia [May et al., 2000]. The thalassemic mice exhibited improved hemoglobin and hematocrit levels, reduced reticulocyte counts, and improvement of the morphology on the peripheral blood smear. This is consistent with the prediction of the model of chimeric β-thalassemic mice, either transplanted with different levels of normal cells or bred with transgenic mice expressing different levels of human globins, that clinical improvement in thalassemia would occur if 20% of the hematopoietic stem cells contained a gene that was expressed at 15% or higher of the endogenous α-globin level of expression [Persons et al., 2001]. In addition, another lentiviral vector containing the central polypurine tract and the rev/rre RNA export elements from HIV, carrying either the human beta globin or a hybrid A-gamma/beta globin gene under the control of the human beta globin promoter linked to a fragment encompassing the HS2, 3, and 4 sites of the human beta globin LCR was used to transduce bone marrow cells transplanted into histocompatible recipients after myeloablative irradiation [Pawliuk et al., 2001]. The recipient mice of the cells transduced with the beta globin vector exhibited 10% human beta globin chains in the peripheral blood two months post transplant.

Another innovation for globin gene transfer was the use of the cHS4 chromatin insulator that can protect retroviral vectors from promoter-enhancer interactions and positional effects. This 1.2-kb fragment that contains the cHS4 DNase-I hypersensitive site that constitutes the boundary of the chicken beta globin locus behaves as an insulator, was able to protect a retroviral vector from position effects [Emery et al., 2000]. Moreover, it can decrease the level of de novo methylation of the 5′LTR believed to be responsible for the silencing of retroviral vectors, and increase the probability that the integrated proviruses will express [Rivella et al., 2000]. When applied to a retroviral vector containing a truncated β-globin promoter linked to a modified γ-globin cassette, the fraction of vector-containing red cells expressing γ-globin increased from less than 5% to nearly 50% in mice transplanted with transduced marrow, by reducing gene silencing [Emery et al., 2001].

A number of studies have been conducted to utilize alternative erythroid promoters to drive globin expression. For example, the human ankyrin promoter can effectively enhance gene expression and confer erythroid specificity, not only for the ankyrin cDNA in a retroviral vector [Dooner et al., 2000], but also for γ-globin in a retroviral vector [Sabatino et al., 2000a]. In addition, the ankyrin promoter linked to the γ-globin gene also conferred position independence in transgenic mice [Sabatino et al., 2000b].

Alternatives to attempts to restore hemoglobin expression by transferring a therapeutic gene to hematopoietic stem cells include use of antisense oligonucleotides to aberrant splice sites in β-globin pre-mRNA to restore correct splicing and production of significant levels of hemoglobin A in erythroid progenitors isolated from patients with thalassemia [Lacerra et al., 2000], or transplant of normal bone marrow cells transduced with a mutant O6-methyl guanine methyltransferase gene and subsequent treatment in a murine model of severe beta thalassemia with O^6-benzylguanine and temezolomide resulted in up to 90% chimerism of normal red cells and improved mean hemoglobin from 9.6 to 11 g/dl [Persons et al., 2001a].

CYTOKINES INDUCE A DEFECT IN LONG-TERM ENGRAFTMENT

Manipulations that induce cycling of cells, critical to the success of retroviral integration, such as cytokines or 5-flurouracil appear to have a reversible, negative effect on engraftment. 5-FU treatment of murine bone marrow results in a dramatic impairment in engraftment in the nonmyeloablated mouse [Stewart et al., 1993] that is reversible [Ramshaw et al., 1995]. A 48 h incubation in a combination of cytokines, IL-3, IL-6, IL-11, and SCF, which expands progenitor cells, virtually abolishes long-term engraftment of male BALB/c bone marrow in nonmyeloablated female hosts [Peters et al., 1995], and results in a loss of competitive repopulation ability in irradiated hosts [Peters et al., 1996]. Engraftment was

studied for marrow cells treated for varying time intervals with these cytokines to determine that there were marked fluctuations in engraftment, with low points in the late S/early G_2 phases of cell cycle and at 32 h and 44–48 h of cytokine incubation, and preservation of engraftment at earlier and later time points [Habibian et al., 1998]. In addition, there was a loss $CD34^+$ cells to repopulate NOD/scid mice with cell cycle progression from G_0 to G_1, or when $CD34^+$ cells in G_0 were incubated in a combination of IL-3, SCF, and Flt3 ligand for 36 h to cause progression to G_1, but before cell division [Gothot et al., 1998]. Other investigators found that the ability of retrovirally transduced human $CD34^+$ cells to repopulate immunodeficient beige/nude/xid mice was dependent on adhesion to fibronectin [Dao et al., 1998], either stroma or recombinant fibronectin peptide CH-296. Another group found that a particular schedule of incubation of human $CD34^+$ cells for 6 days in one of two cytokine combinations, IL-6, TPO, flt3/flk2 ligand, and SCF or IL-6, SCF, IL-3, flt 3/flk2 ligand, G-CSF, with additions of retrovirus on the 3^{rd}, 4^{th}, and 5^{th} days on fibronectin-coated plates, resulted in engraftment in nearly all NOD-scid mice, $> 90\%$ transduction efficiency of LTC-IC, and a high level of transgene expression [Hennemann et al., 1999]. Recombinant fibronectin peptide CH-296 was previously shown to enhance retroviral mediated gene transfer, presumably by colocalizing the virus and target cell in geometric configuration favorable for infection [Hanenberg et al., 1996; Kiem et al., 1998], and also enhanced lentiral mediated gene transfer to human $CD34^+$ cells with inhibition of apoptosis and stimulation of cell division in mobilized peripheral blood $CD34^+$ cells [Donahue et al., 2001]. Thus, human cells also fail to engraft in NOD-scid animals after cytokine incubation in suspension, but did successfully engraft when fibronectin coated tissue culture plates were used. This failure of engraftment with growth stimulatory cytokines could have profound implications for stem cell expansion and retroviral mediated gene transfer. Since cytokines have become a routine part of protocols for gene therapy because they enhance retroviral transduction efficiency and integration, the precise timing of the cytokine incubation or extracellular matrix anchor may be critical to the engraftment of transduced hematopoietic progenitors.

OVERCOMING OBSTACLES

In order to circumvent the difficulty in obtaining a high proportion of cycling stem cells while preserving the ability to self-renew and maintain multilineage potential, many investigators have been utilizing lentiviral vectors which have the advantage of infecting non-dividing cells [May et al., 2000]. The original hypothesis was that the lentiviral vectors were more efficient than the Moloney murine leukemia viruses at transducing non-dividing cells. In fact, although a lentiviral vector was shown to be more efficient at transducing growth inhibited cell lines, when compared directly in a co-transduction assay, both VSV-G pseudotyped lentivirus and Moloney murine leukemia virus vectors transduced at low frequency in the absence of cytokines, but with greater efficiency after incubation in IL-3, IL-6, and SCF for 96 h [Barrette et al., 2000b]. Thus, it is believed that entry into cell cycle, although not actual cell division, is necessary for efficient transduction with lentiviral vectors.

Another intervention to enhance transduction efficiency is to circumvent the problem of low level of expression of the Pit 2 amphotropic retroviral receptor on hematopoietic stem cells is the use of alternative envelope proteins. These include using the gibbon ape leukemia virus pseudotype, the vesicular stomatitis virus G protein, or the RD114 pseudotype. The gp70 envelope protein of the gibbon ape leukemia virus binds to another receptor, Pit-1, which is believed to have a higher level of expression than Pit-2 on human hematopoietic stem cells and can transduce primate cells more efficiently than amphotropic retroviral vectors [Kiem et al., 1997]. Pseudotyping retroviruses with VSV-G [Burns et al., 1993] permits concentration of the virus to high titer, and permits a mode of entry by binding to cell surface glycolipids rather than requiring the specific retroviral receptors, and efficiently transduces hematopoietic stem cells [Barrette et al., 2000a]. A dog model was used to demonstrate persistence of transgene bearing white blood cells at 11–23 months after infusion of CD34-enriched bone marrow cells transduced with RD114-pseudotype vectors [Goerner et al., 2001] bearing the envelope protein of the endogenous feline virus, RD114.

Another crucial consideration is the ability to achieve a competitive advantage for the trans-

duced cell population. A number of approaches have been utilized, including bicistronic vectors that include a drug resistance gene, achieving a natural advantage by virtue of improving the survival of genetically defective cells by transducing the cells with the normal gene and restoring normal function (e.g., −SCID), or by using modified growth factor receptors to enhance the prolieration of the cells bearing the transgene. Examples of bicistronic vectors with drug resistance genes that can be used to select transduced cells in vitro include a bicistronic vector with the multidrug resistance gene, *MDR-1*, and human glucocerebrosidase [Aran et al., 1996], or a selectable marker, CD24, the human small cell surface antigen and human glucocerebrosidase [Migita et al., 1995]. Examples of how replacement of defective genes with their normal counterparts results in a competitive advantage includes the adenosine deaminase gene [Kohn et al., 1998], for which there is a survival advantage for the gene-corrected T-lymphocytes. Another clever approach is to confer a reversible growth advantage by using the signaling domain of the thrombopoietin receptor, mpl, fused to the binding site for a nontoxic synthetic drug, chemical inducer of dimerization, such that the presence of the synthetic drug activates the signaling domain of the growth factor receptor, and results in expansion of the transduced cells [Richard et al., 2000]. In addition, use of a hypersensitive truncated erythropoietin receptor mutant allowed amplification of the genetically modified cells by virtue of endogenous erythropoietin [Persons et al., 2001b].

Another potential problem related to the murine Moloney leukemia (MuMoLV) virus backbone is that there is failure of long term gene expression in hematopoietic cells because of suppression of gene expression due to its regulatory regions. The development of the spleen focus forming virus based vector, SFFV, or the murine embryonic stem cell virus (MESV = MSCV), for which the natural targets of the viruses are hematopoietic cells, may successfully overcome this problem. The FMEV backbone has the LTRs from the spleen focus forming virus and the leader sequence from the murine embryonic stem cell virus The FMEV based vector achieves high level expression in hematopoietic cells, superior to that obtained using MuMoLV vectors [Hildinger et al., 1998; Tsuji et al., 2000]. Addition of the human interferon beta scaffold attachment region (SAR) to retroviral backbones, including MSCV, increased the level of transgene expression in hematopoietic cells derived from transduced stem cells engrafted in NOD-scid mice [Murray et al., 2000].

HUMAN GENE THERAPY TRIALS TO DATE

As of September 2001, a total of 3,464 patients have been enrolled on a total of 596 gene therapy trials worldwide [Journal of Gene Medicine website, 2001]. The majority of the protocols have been for patients with cancer, 63.1%, followed by inherited disease, 12.6%, and then infectious disease, 6.4% [Journal of Gene Medicine website, 2001]. Retroviruses have been used primarily, for 35.8% of the trials, followed by adenovirus for 27.7% of the trials. Bone marrow transplantation has been utilized for 7.4% of the trials, for which 276 patients have been enrolled. Of these protocols involving bone marrow transplantation, there has been only limited success in terms of being able to demonstrate clinical benefit, or even persistent transgene presence or expression. One of the greatest problems has been the ability to achieve engraftment of transduced hematopoietic cells after transplant, so that even if there appears to be successful transduction after the period of ex vivo manipulation, there are negligible transduced cells present after intravenous infusion, and these are soon lost.

THE BEGINNING OF SUCCESS IN HUMAN HEMATOPOIETIC STEM CELL GENE THERAPY TRIALS

The most impressive example of success in a clinical gene therapy trial was achieved in children with X-linked severe combined immunodeficiency disease (SCID-Xl), who have defects in the gene encoding the γc cytokine receptor and therefore, a block in T and NK cell differentiation [Cavazzana-Calvo et al., 2000]. The $CD34^+$ cells isolated by immunomagnetic beads were incubated in serum-free media with cytokines in gas-permeable bags coated with CH-296 fibronectin peptide and retroviral supernatant. The washed cells were then reinfused in the patients. The children showed improved T-lymphocyte and natural killer cell counts and responses to immunization.

A second human study that demonstrated persistence of cells bearing the transgene at 12

months post infusion was the transfer of the multidrug resistance gene MDR-1 to $CD34^+$ selected peripheral blood progenitor cells from patients with germ cell tumors [Abonour et al., 2000]. These patients received oral etoposide post transplant of the transduced cells, which may have aided in applying selective pressure in favor of cells expressing the transgene.

SUMMARY

Thus, there have been major advances in the experimental application of gene transfer to inherited bone marrow disorders, and the beginning of the fruition of these efforts is evident. The results with retroviral vectors has been enhanced with vector modifications that confer growth advantage and reduce gene silencing in vivo. Lentiviral vectors have demonstrated extraordinary success in murine models, but there have been reservations about using these vectors in the clinic, although new generations of vectors that are not pathogenic for humans, such as the equine infectious anemia virus [Yamada et al., 2001] could hold significant promise. Persistence of transduced cells has been observed in humans at at least one year, and there has been a clinical effect in a handful of patients with X-linked severe combined immunodeficiency disease [Cavazzana-Calvo et al., 2000]. The continued challenge will be to develop this methodology to the point, at which it can be broadly applied to large scale clinical trials, and eventually, result in long term amelioration of the chronic debilitating symptoms of some inherited disorders, reduce the need for lifelong, high cost infusions of recombinant proteins, eliminate the need for complex medical management of complications, and in some cases, rescue patients from fatal genetic defects for which there is no current successful therapy available.

ACKNOWLEDGMENT

This publication was made possible by Grant No. R01 HL 63184 from the National Heart, Lung and Blood Institute. Its contents are solely the responsibility of the author and do not necessarily represent the official views of the awarding agency.

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