REVIEW: MEDICINE

Prospects for in Utero Human Gene Therapy

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Gene therapy for the treatment of disease in children and adults is being actively pursued at many medical centers. However, a number of genetic disorders result in irreversible damage to the fetus before birth. In these cases, as well as for those with genetic diseases who may benefit from therapy before symptoms are manifested, in utero gene therapy (IUGT) could be beneficial. Although some successes with in utero gene transfer have been reported in animals, significant questions remain to be answered before IUGT clinical trials would be acceptable. This review analyzes the state of the art and delineates the studies that still need to be performed before it would be appropriate to consider human IUGT.

Gene therapy, the treatment of disease by the transfer and appropriate expression of an exogenous normal gene into somatic cells of a patient, is the subject of intensive experimental and clinical investigation (*1*). Clinical success has been limited by low efficiency of gene transfer into the appropriate target cells and poor in vivo expression of the gene once transferred. All of the more than 400 approved clinical gene therapy protocols worldwide describe attempts to treat pediatric and adult patients after birth. Of the 313 U.S. protocols catalogued by the Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health (NIH) as of 14 June 1999, 40 (13%) are for the treatment of 15 different genetic diseases.

Rationale for Human in Utero Gene Therapy

The rationale for human IUGT is that it may allow the correction of some types of genetic diseases before the appearance of any clinical manifestations; in addition, gene transfer in the fetus offers a number of potential advantages over postnatal gene transfer (see below). For the neurologic genetic diseases (such as Tay-Sachs, Niemann-Pick, Lesch-Nyhan, Sandhoff, Leigh, many leukodystrophies, generalized gangliosidosis) that appear to produce irreversible damage during gestation, treatment before birth (perhaps early in pregnancy) may be required to allow the birth of a normal baby. Unfortunately, although these neurologic diseases may appear to be the logical targets for attempts at IUGT, it is not known how to efficiently and safely target brain tissue either in the adult or in the developing fetus. In some cases, gene transfer into blood cells, some of which will become microglial cells in the brain (*2*), may provide an approach for treatment. Initially, it will probably be necessary to target diseases that could be treatable by inserting a therapeutic gene into a more accessible cell type, specifically the hematopoietic stem or progenitor cell (HSC). Diseases that might be targeted in this way include immunologic diseases (for example, severe combined immunodeficiency), hematologic diseases (for example, thalassemias), and metabolic diseases (for example, osteopetrosis).

Although a broad range of cell types is being engineered in the various gene therapy protocols, the target that appears most promising for IUGT is the HSC because of the high proliferative potential and multilineage differentiation potential of HSCs for delivering the corrective gene to the patient. A number of viral and nonviral vectors have been used in gene therapy protocols (*1*). However, only retroviral vectors integrate efficiently into the target cell's genome and therefore insert the therapeutic gene permanently into the genetic make-up of the cell. For genetic diseases, where correction for the lifetime of the patient is desired, only retroviral vectors appear suitable at this time [for recent in utero studies with adenoviral vectors, see (*3*)]. A number of existing clinical protocols are based on ex vivo retroviral vector transduction of HSCs, followed by transplantation of the gene-engineered cells back into the patient (*4*). For a few diseases, such as severe combined immunodeficiency disease (SCID) caused by adenosine deaminase (ADA) deficiency, there is a positive pressure for the growth of gene-engineered blood cells (*5*). For most other diseases, however, the reengraftment of the transduced HSCs may require some form of marrow ablation therapy.

The primary disadvantage of IUGT is the potential risk of harm to both the fetus and the mother. Advances in prenatal diagnosis and molecular analysis now allow the identification of many congenital disorders by evaluation of trophoblastic tissue obtained by chorionic villus sampling (CVS) at 8 to 10 weeks of gestation. High-resolution ultrasound and midgestational interventional techniques have developed to the point that the manipulative techniques necessary to carry out second-trimester gene transfer at low risk to the mother and fetus are now available. However, there are a number of other risks inherent to in utero gene transfer, including possible interference with the developmental process from insertional mutagenesis and potential germ line gene transfer, which will be discussed below.

Advantages of IUGT

For a number of genetic diseases it may be advantageous to use IUGT rather than to wait until after birth to begin treatment. First, successful early treatment could preempt the appearance of any clinical manifestations of a disease. Second, gene transfer in the fetus is believed to be more efficient than in the more mature organism (see below), so that gene therapy should be easier to accomplish prenatally than postnatally.

Third, immunological naïveté and the permissive environment of the early gestational fetus should allow acceptance of cells and vector without the need for immunosuppression or myeloablation. In early immunologic development, before thymic processing of mature lymphocytes, the fetus appears to be largely tolerant of foreign antigens. Exposure to foreign antigens during this period often results in sustained tolerance, which can become permanent if the presence of the antigen is maintained (*6*); transplantation of allogeneic or xenogeneic HSCs results in the creation of permanent chimeras (*7*). Cellular tolerance appears to be secondary to clonal deletion of reactive lymphocytes in the thymus, whereas the mechanism of B-lymphocyte tolerance (peripheral tolerance) appears to involve both clonal deletion and clonal suppression (*8*). The end result is an immune system that is specifically tolerant of foreign antigenic sources. The possible development of tolerance to the vector and gene product (*9*) may permit postnatal treatment of the patient, if required, with relative safety.

The developing fetal hematopoietic system provides additional advantages that can help circumvent some of the major difficulties encountered with postnatal treatment (*10*). The naturally occurring transition in the primary site of hematopoiesis from yolk sac to liver and spleen and finally to bone mar-

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row during ontogeny is accomplished by the migration of HSCs from one site (liver) to another (bone marrow) via the circulation (*11*). Fetal circulation can serve as a source of large numbers of HSCs for IUGT that are destined to populate the developing bone marrow system of the fetus. Furthermore, the availability of bone marrow spaces for homing and engraftment of HSCs in the fetus allows for the engraftment of transduced HSCs without the need for cytoablation of the patient's own marrow, thus avoiding the risks associated with this procedure (*10*). That significant HSC engraftment can occur in unprepared fetuses has been demonstrated in large animal species (*12*) and humans (*13*). Finally, fetal HSCs (as well as many other cell types) are rapidly dividing in order to provide cells to the growing organism so that they are much better targets for retrovirally mediated gene transfer than adult HSCs.

Experience from in Utero Transplantation of HSCs

Although clinical application of in utero transplantation of HSCs is in its early stages of development, there has been success in the treatment of fetal patients with immunodeficiency disorders (*14*). Knowledge gained from in utero HSC transplantation can be used to increase the efficacy and safety of IUGT using gene-engineered HSCs.

The scientific foundation for in utero HSC transplantation is based on a number of experiments-of-nature, as well as laboratory animal studies. Permanent hematopoietic chimerism, with specific transplantation tolerance to skin and organ transplants from their siblings, has been observed in normal dizygotic cattle twins with shared placental circulation (*15*). In cattle with mannosidosis, hematopoietic chimerism results in the cross-correction of the genetic defect (*16*). Experiments-of-nature resulting in hematopoietic chimerism have also been observed in multiple gestations in a number of species, including primates (*17*) and humans (*18*). Experiments designed to reproduce this phenomenon by the early gestational transplantation of allogeneic cells in mice, goats, sheep, and monkeys (*19*) have shown that long-term multilineage chimerism can be achieved across major histocompatibility barriers without evidence of rejection or the need for immunosuppression. The anemia in W/W mice and the immunodeficiency in SCID mice can be successfully treated by in utero HSC transplantation (*20*).

The Present: IUGT Studies in Animals

Long-term persistence and expression of vector-encoded genes after in utero transfer in small and large animal studies have provided evidence that IUGT might present a viable approach for the treatment of genetic disorders. Two IUGT approaches have been evaluated in small and large animals: gene-engineered cell transfer has been examined in sheep and monkeys, and direct injection of vector into the fetus has been studied in rodents and sheep (Fig. 1).

Gene-engineered cell transfer approach. An autologous retroviral vector and HSC transplantation protocol, but without myeloablation, was used to introduce the bacterial neomycin resistance (Neo^R) gene into sheep fetuses (*21*). Circulating mononuclear cells obtained from sheep fetuses by exchange transfusion at about 100 days of gestation (term: 145 days) were incubated with a retroviral vector and reinfused intravenously. Of the ten recipients that could be evaluated, six were positive for G418-resistant progenitor cells [colony-forming units (CFUs)-Mix, granulocyte-macrophage CFUs (GM-CFUs), erythrocyte burst-forming units (BFU-Es) and erythroid CFUs (CFU-Es)] (*22*). Vector DNA sequences were present in both the blood and the bone marrow of two animals. In addition, the presence of neomycin phosphotransferase (NPT) activity was documented in the bone marrow of another animal. That the protocol resulted in the transfer of the Neo^R gene into the pluripotent HSCs was demonstrated by the continued presence of G418-resistant progenitors in two of the animals studied for 43 and 59 months after birth (at concentrations ranging from $<1\%$ to approximately 10% G418-resistant colonies). Lambs born to in utero–treated ewes expressing the Neo^R gene did not exhibit any drug-resistant hematopoietic progenitor (*21*).

Retroviral vectors were also used in an in utero transplantation protocol of gene-engineered HSCs to transfer the bacterial Neo^R gene into two cynomolgus and five rhesus monkeys (*22*). An autologous protocol similar to that used in sheep fetuses, but without exchange transfusion, was used in recipients at a gestational age between 113 and 144 days (term: 165 days). Although the volume of blood from each fetus was relatively small (1.3 to 3.0 ml per fetus), three out of the six animals that could be evaluated exhibited low numbers (less than 1%) of G418-resistant progenitors on several occasions after birth (*22*).

Overall, the gene-engineered HSC transplantation approach in sheep and monkeys resulted in the long-term transfer and expression of the Neo^R gene, albeit at low efficiency, into hematopoietic cells. The amount of transfer and expression of a therapeutic gene would need to be significantly improved before a cellular gene transfer protocol would be clinically effective. However, the advantages of this protocol are that it could be applied in cases where late diagnosis prevents early intervention. In addition, the transfer would be limited to hematopoietic cells, thus avoiding any possible inadvertent transfer into other cells, including germ line cells. Nonetheless, in addition to low efficacy, this approach suffers from other disadvantages. First, it requires multiple manipulations of the fetus, which increases the risk of injury. Second, the percentage of gene-engineered cells in the treated fetus is inherently limited by the fact that transduction is restricted to the number of cells removed, which represents a relatively small fraction of the total HSC pool in the fetus. In addition, the procedure is of necessity performed late in gestation when the fetus is less receptive to HSC engraftment, thereby further reducing the number of transduced HSCs that find the appropriate niche in hematopoietic sites for proper function. Third, the older fetus is immunocompetent. To circumvent these difficulties and to achieve more efficient transgene transfer, a direct vector protocol for IUGT has been developed.

Direct injection of vector approach. Most genetic disorders in humans can now be diagnosed very early in gestation. It is thus possible to treat these patients as early as 13 to 15 weeks (that is, at the beginning of the

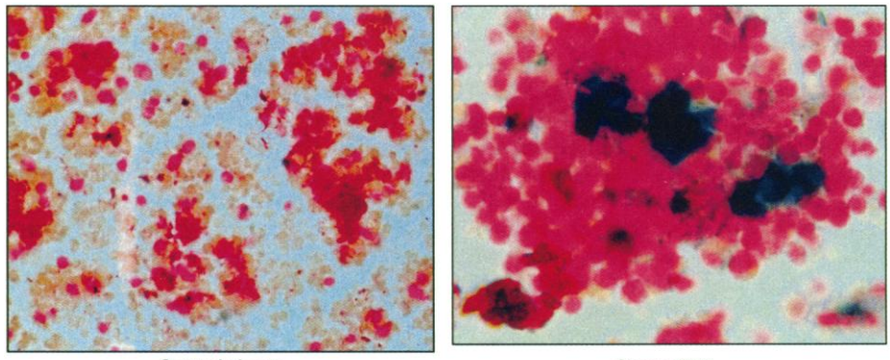

Fig. 1. Expression of LacZ gene in bone marrow of sheep 690 about 27 months after in utero gene transfer. Only cells with nuclear fast red stain were observed in bone marrow of control (age-matched) sheep, whereas significant X-gal blue-stained cells were observed in bone marrow of treated sheep.

second trimester). Although the size of the fetus at these early gestational ages readily permits the injection of materials, it is not possible to obtain sufficient amounts of blood or other HSC-containing tissues for gene transfer purposes. A direct vector approach can be performed early in the second trimester at a preimmune stage of development (thereby avoiding immune responses to the vector or it product) and the cells of the fetus are rapidly dividing, but all the major organ systems have developed, thereby lessening the danger of a congenital anomaly produced by an inserted vector. Moreover, in theory, a high percentage of HSCs could be exposed to the vector with the possibility that a greater fraction of these cells may become transduced.

Injection of retroviral supernatant into the liver of fetal rats (*23*) demonstrated that fetal liver HSCs are a suitable target for IUGT. These in situ transduced HSCs ultimately homed to the nascent fetal marrow and persisted for the life span of the adult animals (>20 months), producing gene-marked progeny of multiple hematopoietic lineages. However, the direct injection into the fetal liver led to a high rate of mortality. In shorter term studies, similar fetal gene transfer strategies have been used to achieve the transfer of exogenous genes into skin (*24*), pulmonary epithelium (*25*), hepatocytes (*26*), and heart (*27*), among others (*28*).

In sheep, long-term persistence and expression of the Neo^R gene in hematopoietic cells was demonstrated after direct injection of vector into the peritoneal cavity of preimmune fetuses (*29*). The studies in sheep also begin to address some of the maternal and fetal safety issues. Long-term (>5 years) evaluation of these animals revealed the following: (i) The injections resulted in the transduction of the long-term engrafting HSCs as demonstrated both by the persistence and multilineage expression of the transgene in primary recipients for >5 years, and by secondary transplant studies in which HSCs from primary animals engrafted and exhibited transgene activity in secondary recipients. (ii) The level of transgene expression in hematopoietic cells was associated with the titer of the vectors used (ranging from 0.2% to 2.8% NPT-positive cells in peripheral blood smears 2 to 3 years after transplantation for low-titer vector, to 4.8% to 12.0% NPT-positive cells in peripheral blood smears 2 to 3 years after transplantation for high-titer vector). (iii) Of the 27 pregnant ewes, 19 were examined for possible transfer of the transgene into the mother. Barely detectable amounts of proviral DNA were detected in the circulation of five ewes soon after fetal treatment; however, its presence was transient, and no proviral DNA was detected 3 months after fetal treatment. (iv) Vector sequences were present in all tissues of the primary animals analyzed, including the reproductive organs. However, no pathology was noted in any of the organs, and, as is detailed below, breeding experiments and analysis of purified sperm from the three rams having semen positive for proviral DNA indicated that no vector sequences were present in the sperm. The semen samples were positive because of vector integration into nongermline cells (*29*).

Exogenous genes were detected in the brain of several animals that were euthanized at various time points during the studies. The presence of proviral DNA in the brain may have been the result of entry of the vector into the central nervous system with subsequent transduction of the nervous tissue, or presence of the vector may reflect the migration of transduced hematopoietic microglial precursors into the brain during development. Regardless of the mechanism, however, the ability to deliver exogenous genes to the brain in utero may offer the possibility of treating storage diseases that affect the central nervous system.

In Utero Gene Therapy Preproposals

It is not clear which genetic diseases would be the best initial candidates for IUGT. As a minimum, the disease should be life-threatening or cause significant disability either during gestation or in early infancy, and it should be probable that, based on the pathophysiology of the disease, treatment in utero would be beneficial and would not produce any significant risks to the fetus or the mother.

In July 1998, we submitted two preproposals for IUGT to the NIH RAC. The submission was to provide concrete examples so that a discussion of the scientific, medical, ethical, and social issues generated by this new technology could be initiated (hence they were "preproposals," not formal submissions for approval). In the submission we made clear that considerably more data would be necessary before human application would be appropriate. These preproposals, and more particularly the generic issues they raised, were extensively discussed at the 27 and 28 September 1998 RAC meeting, the Third Gene Therapy Policy Conference on 7 and 8 January 1999, and again at the 11 and 12 March 1999 RAC meeting. An extensive report is being prepared by the RAC (*30*) that examines each of the issues in depth and concludes that in utero gene therapy will be an important treatment option but that considerable additional data from animal studies are necessary before it would be appropriate to attempt a clinical trial. In addition, there continues to be an ongoing discussion in the scientific literature [for example (*31*)].

Therapies for two diseases were proposed for this preliminary review: (i) treatment of ADA-deficient SCID by direct intraperitoneal injection of a retroviral vector carrying a normal ADA gene into a 13- to 15-week human fetus, and (ii) treatment of homozygous α-thalassemia by transfusion of gene-engineered autologous HSCs at 18 to 20 weeks of gestation. These two examples were chosen, not because they are necessarily the most appropriate initial diseases for IUGT, but because they satisfied an important technical requirement related to in vivo gene expression as discussed below.

The efficiency of current gene therapy protocols is low owing to two factors: the low level of gene transfer into target cells in vivo, and the fact that, in many cases, even when an exogenous gene is initially expressing in target cells in vivo, the gene shuts down in days or weeks (*1*). This loss of gene expression can be from an immune response or from transcriptional silencing (for example, by methylation of the regulatory sequences). Even though the mechanism of silencing is unknown, it may be that the cell can recognize that the regulatory sequences present in the retroviral vectors currently in clinical use are of viral origin and are not the natural endogenous regulatory sequences that the target cell would normally employ. In order to construct retroviral vectors carrying only human regulatory sequences, it is necessary to select human genes for which the regulatory sequences are known. Only two human genes have been studied adequately in this regard: ADA and globin. Therefore, ADA deficiency and a fetal globin deficiency (α-thalassemia) were the diseases chosen for initial study. These two diseases will be used to illustrate some of the issues discussed below.

Questions to Be Answered Concerning Efficacy

The primary question is: Can IUGT successfully treat a disease? Several factors are involved. Can enough genes be transferred into the appropriate target cells by any approach? Will expression from the transferred gene be at the correct level, and for an adequate duration, and will it be regulated appropriately for the disease treated? Will the gene product be eliminated by the immune system or by some other mechanism?

As IUGT has not been attempted in any large animal disease model, the data to address whether or not there could be sufficient gene transfer to successfully treat a disease does not yet exist. The factors that need to be considered will vary with each disease. To use the preproposals as examples: direct injection of an ADA vector into a 13- to 15-week fetus suffering from ADA-deficient SCID may very well result in sufficient gene transfer into HSCs to be therapeutic since corrected cells would have a positive growth

selection and, based on the fact that there are individuals with only 10% the normal concentration of ADA who are symptom-free, 10% ADA-positive blood cells should be therapeutic. However, in the case of homozygous α-thalassemia, it is unlikely that sufficient HSCs could be engineered ex vivo, using present techniques, to be curative. This conclusion is based on the amount of blood that could be safely removed from a mid–second trimester fetus, the small number of ex vivo HSCs genes transferred, and the efficiency of engraftment of HSCs (as determined from transplantation studies with HSCs that are not gene-engineered). Recent studies examining gene transfer into third-trimester fetal baboon HSCs (*32*) are beginning to provide the quantitative data that will be necessary to determine when human IUGT will be appropriate.

Safety Issues

The mother. The additional risk created by adding a gene transfer component to standard obstetrical procedures should be minimal for the mother. However, there can be unique risks such as accidental transfer of vector into the maternal blood stream. On the basis of the studies in sheep (*29*), this risk is probably very low; the amount of transfer into ewes was minimal and transient. Nonetheless, there is evidence that placental tissue is readily infected with a variety of retroviruses (*33*) and that the placenta allows the transfer of retroviral-like particles from the mother to the fetus (*34*). Carefully designed animal studies are needed to fully address this question, especially in cases where multiple injections of high-titer vector may be contemplated.

Of greater significance is the possible harm to the mother if a fetus with a lethal disease, like homozygous α-thalassemia, is only partially treated. The thalassemic fetus usually dies during the third trimester, producing toxicity for the mother. These fetuses are ordinarily aborted at 24 weeks for the protection of the mother. A serious situation would exist if a fetus were to be kept alive by IUGT but only in critical condition, thereby inducing ongoing toxicity in the mother and, perhaps, the birth of a severely ill, destined-to-die infant. The in utero approach must result in sufficient gene expression to ensure that the affected fetus is significantly benefited. Experiments in large animal models are needed to provide quantitative assessments of the amount and duration of transgene expression.

The fetus. For direct intraperitoneal injection of vector in the 13- to 15-week fetus, the risks of the procedure are low because of the technological advances that have been made in obstetrical techniques. For gene-engineered HSC transplantation, the risks would be similar to those for HSC transplantation, except that autologous HSCs would be used, thereby adding the risk of the initial collection. By week 18, fetal blood collection in experienced hands is low risk. Experience with in utero treatment for Rh disease would indicate a 1 to 3.5% risk factor (*35*). Nonetheless, even though the extensive obstetric experiences with CVS and fetal transfusion can be informative, the special circumstances of removal of blood from the fetus and its reinjection into the fetus will require additional studies in human-sized animals to determine risks.

In addition, there are the as-yet-unknown risks associated with insertional mutagenesis that could lead to possible interference with developmental processes or to tumor formation. This concern is particularly acute with the direct injection approach, because there would be widespread distribution of the vector throughout the fetus. When it becomes possible to target vector to specific cell types, then this concern will lessen. These problems have not been apparent when in utero gene transfer occurred after the first trimester in the small and large animals studied, but more data are needed to determine the actual amount of risk.

Inadvertent germ line alteration. There is no biological basis to indicate that the cellular approach to IUGT would result in germ line alterations, because only transduced cells, and no retroviral vector, are given to the fetus. However, the possibility that the direct vector injection protocol may inadvertently lead to the alteration of the germ line cannot be ignored. Studies in mice and sheep have thus far failed to indicate any detectable transfer of exogenous genes into germ line cells when in utero gene transfer occurs after the first trimester. In a carefully designed study, Ye *et al.* (*36*) evaluated 578 offspring of matings in which either one or both parent mice were injected with a high dose of vector (in this case, an adenoviral vector). The dose of the vector was sufficient to affect 80% of the hepatocytes with a small amount of dissemination to ovaries and testes in 94% of the animals. No evidence of germ line transmission was seen. In sheep, breeding studies (in which either one or both parents expressed in utero–administered transgene activity) produced 10 lambs. None of the offspring exhibited proviral DNA. In addition, although ejaculates were on occasion positive by polymer chain reaction (PCR) analysis, further PCR analysis of the purified sperm obtained from seven treated rams on a total of 21 separate occasions demonstrated no proviral DNA (*29*).

The limited breeding and sperm purification studies in sheep, and the studies in mice, did not suggest germ line cell involvement, but considerable more data need to be obtained in small and large animal studies to establish the overall incidence of germ line transmission and whether the degree of risk may be acceptable.

Ethical Implications

The experimental nature of IUGT dictates that an ethical framework be established, from which patients can be counseled and clinical decisions made (*37*). There are well-established criteria for fetal therapy that can also serve to facilitate the process for IUGT (*38*). The ethical considerations unique for IUGT have been discussed well by Fletcher (*39*).

Conclusions

The IUGT approach is promising not only for the treatment of genetic diseases that produce fetal damage before birth, but also as an additional treatment procedure that may offer advantages over postnatal therapy. However, despite the existence of encouraging data in small and large animals, the question remains whether an IUGT protocol can be developed that will provide sufficient gene transfer in vivo to be effective in the treatment of a genetic disease. Once questions concerning the appropriate disease, the delivery procedure, efficacy, safety, and germ line transmission are successfully resolved by further animal experiments, then it would be appropriate to conduct clinical trials using IUGT.

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[4] **Prolonged Production of NADPH Oxidase-Corrected Granulocytes after Gene Therapy of Chronic Granulomatous Disease**

Harry L. Malech; Phillip B. Maples; Narda Whiting-Theobald; Gilda F. Linton; Sudhir Sekhsaria; Sarah J. Vowells; Fei Li; Judi A. Miller; Ellen DeCarlo; Steven M. Holland; Susan F. Leitman; Charles S. Carter; Robert E. Butz; Elizabeth J. Read; Thomas A. Fleisher; Richard D. Schneiderman; Dennis E. Van Epps; S. Kaye Spratt; Christopher A. Maack; Joseph A. Rokovich; Lawrence K. Cohen; John I. Gallin

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http://links.jstor.org/sici?sici=0027-8424%2819971028%2994%3A22%3C12133%3APPONOG%3E2.0.CO%3B2-C

[4] **Results of MDR-1 Vector Modification Trial Indicate that Granulocyte/Macrophage Colony-Forming Unit Cells Do not Contribute to Posttransplant Hematopoietic Recovery Following Intensive Systemic Therapy**

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