4. USE OF GENETICALLY MODIFIED STEM CELLS IN EXPERIMENTAL GENE THERAPIES

*by Thomas P. Zwaka**

INTRODUCTION

Gene therapy is a novel therapeutic branch of modern medicine. Its emergence is a direct consequence of the revolution heralded by the introduction of recombinant DNA methodology in the 1970s. Gene therapy is still highly experimental, but has the potential to become an important treatment regimen. In principle, it allows the transfer of genetic information into patient tissues and organs. Consequently, diseased genes can be eliminated or their normal functions rescued. Furthermore, the procedure allows the addition of new functions to cells, such as the production of immune system mediator proteins that help to combat cancer and other diseases.

Originally, monogenic inherited diseases (those caused by inherited single gene defects), such as cystic fibrosis, were considered primary targets for gene therapy. For instance, in pioneering studies on the correction of adenosine deaminase deficiency, a lymphocyte-associated severe combined immunodeficiency (SCID), was attempted.[1] Although no modulation of immune function was observed, data from this study, together with other early clinical trials, demonstrated the potential feasibility of gene transfer approaches as effective therapeutic strategies. The first successful clinical trials using gene therapy to treat a monogenic disorder involved a different type of SCID, caused by mutation of an X chromosome-linked lymphocyte growth factor receptor.[2]

While the positive therapeutic outcome was celebrated as a breakthrough for gene therapy, a serious drawback subsequently became evident. By February 2005, three children out of seventeen who had been successfully treated for X-linked SCID developed leukemia because the vector inserted near an oncogene (a cancer-causing gene), inadvertently causing it to be inappropriately expressed in the genetically-engineered lymphocyte target cell.[3] On a more positive note, a small number of patients with adenosine deaminase-deficient SCID have been successfully treated by gene therapy without any adverse side effects.[4]

A small number of more recent gene therapy clinical trials, however, are concerned with monogenic disorders. Out of the approximately 1000 recorded clinical trials (January 2005), fewer than 10% target these diseases *(see Figure 4.1)*. The majority of current clinical trials (66% of all trials) focus on polygenic diseases, particularly cancer.

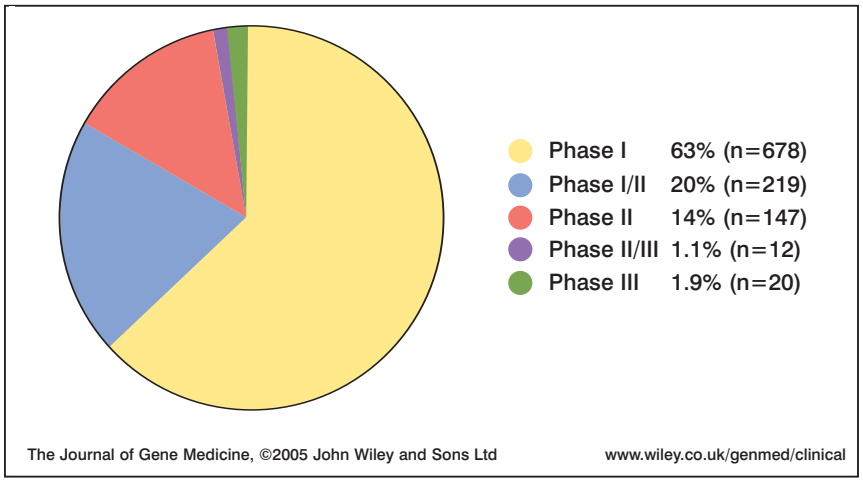

Figure 4.1. Indications Addressed by Gene Therapy Clinical Trials.

Gene therapy relies on similar principles as traditional pharmacologic therapy; specifically, regional specificity for the targeted tissue, specificity of the introduced gene function in relation to disease, and stability and controllability of expression of the introduced gene. To integrate all these aspects into a successful therapy is an exceedingly complex process that requires expertise from many disciplines, including molecular and cell biology, genetics and virology, in addition to bioprocess manufacturing capability and clinical laboratory infrastructure.

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THE TWO PATHS TO GENE THERAPY

Gene therapy can be performed either by direct transfer of genes into the patient or by using living cells as vehicles to transport the genes of interest. Both modes have certain advantages and disadvantages.

Direct gene transfer is particularly attractive because of its relative simplicity. In this scenario, genes are delivered directly into a patient's tissues or bloodstream by packaging into liposomes (spherical vessels composed of the molecules that form the membranes of cells) or other biological microparticles. Alternately, the genes are packaged into genetically-engineered viruses, such as retroviruses or adenoviruses. Because of biosafety concerns, the viruses are typically altered so that they are not toxic or infectious (that is, they are replication incompetent). These basic tools of gene therapists have been extensively optimized over the past 10 years.

However, their biggest strength — simplicity — is simultaneously their biggest weakness. In many cases, direct gene transfer does not allow very sophisticated control over the therapeutic gene. This is because the transferred gene either randomly integrates into the patient's chromosomes or persists unintegrated for a relatively short period of time in the targeted tissue. Additionally, the targeted organ or tissue is not always easily accessible for direct application of the therapeutic gene.

On the other hand, therapeutic genes can be delivered using living cells. This procedure is relatively complex in comparison to direct gene transfer, and can be divided into three major steps. In the first step, cells from the patient or other sources are isolated and propagated in the laboratory. Second, the therapeutic gene is introduced into these cells, applying methods similar to those used in direct gene transfer. Finally, the genetically-modified cells are returned to the patient. The use of cells as gene transfer vehicles has certain advantages. In the laboratory dish *(in vitro),* cells can be manipulated much more precisely than in the body *(in vivo).* Some of the cell types that continue to divide under laboratory conditions may be expanded significantly before reintroduction into the patient. Moreover, some cell types are able to localize to particular regions of the human body, such as hematopoietic (blood-forming) stem cells, which return to the bone marrow. This "homing" phenomenon may be useful for applying the therapeutic gene with regional specificity.

A major disadvantage, however, is the additional biological complexity brought into systems by living cells. Isolation of a specific cell type requires not only extensive knowledge of biological markers, but also insight into the requirements for that cell type to stay alive *in vitro* and continue to divide. Unfortunately, specific biological markers are not known for many cell types, and the majority of normal human cells cannot be maintained for long periods of time *in vitro* without acquiring deleterious mutations.

STEM CELLS AS VEHICLES FOR GENE THERAPY

Stem cells can be classified as embryonic or adult, depending on their tissue of origin. The role of adult stem cells is to sustain an established repertoire of mature cell types in essentially steady-state numbers over the lifetime of the organism. Although adult tissues with a high turnover rate, such as blood, skin, and intestinal epithelium, are maintained by tissue-specific stem cells, the stem cells themselves rarely divide. However, in certain situations, such as during tissue repair after injury or following transplantation, stem cell divisions may become more frequent. The prototypic example of adult stem cells, the hematopoietic stem cell, has already been demonstrated to be of utility in gene therapy.[4,5] Although they are relatively rare in the human body, these cells can be readily isolated from bone marrow or after mobilization into peripheral blood. Specific surface markers allow the identification and enrichment of hematopoietic stem cells from a mixed population of bone marrow or peripheral blood cells.

After *in vitro* manipulation, these cells may be re-transplanted into patients by injection into the bloodstream, where they travel automatically to the place in the bone marrow in which they are functionally active. Hematopoietic stem cells that have been explanted, *in vitro* manipulated, and re-transplanted into the same patient (autologous transplantation) or a different patient (allogeneic transplantation) retain the ability to contribute to all mature blood cell types of the recipient for an extended period of time (when patients' cells are temporarily grown "outside the body" before being returned to them, the *in vitro* process is typically referred to as an *"ex vivo"* approach).

Another adult bone marrow-derived stem cell type with potential use as a vehicle for gene transfer is the mesenchymal stem cell, which has the ability to form cartilage, bone, adipose (fat) tissue, and marrow stroma (the bone marrow microenvironment).[6] Recently, a related stem cell type, the multipotent adult progenitor cell, has been isolated from bone marrow that can differentiate into multiple lineages, including neurons, hepatocytes (liver cells), endothelial cells (such as the cells that form the lining of blood vessels), and other cell types.[7] Other adult stem cells have been identified, such as those in the central nervous system and heart, but these are less well characterized and not as easily accessible.[8]

The traditional method to introduce a therapeutic gene into hematopoietic stem cells from bone marrow or peripheral blood involves the use of a vector derived from a certain class of virus, called a retrovirus. One type of retroviral vector was initially employed to show proof-of-principle that a foreign gene (in that instance the gene was not therapeutic, but was used as a molecular tag to genetically mark the cells) introduced into bone marrow cells may be stably maintained for several months.[9] However, these particular retroviral vectors were only capable of transferring the therapeutic gene into actively dividing cells. Since most adult stem cells divide at a relatively slow rate, efficiency was rather low. Vectors derived from other types of retroviruses (lentiviruses) and adenoviruses have the potential to overcome this limitation, since they also target non-dividing cells.

The major drawback of these methods is that the therapeutic gene frequently integrates more or less randomly into the chromosomes of the target cell. In principle, this is dangerous, because the gene therapy vector can potentially modify the activity of neighboring genes (positively or negatively) in close proximity to the insertion site or even inactivate host genes by integrating into them. These phenomena are referred to as "insertional mutagenesis." In extreme cases, such as in the X-linked SCID gene therapy trials, these mutations contribute to the malignant transformation of the targeted cells, ultimately resulting in cancer.

Another major limitation of using adult stem cells is that it is relatively difficult to maintain the stem cell state during *ex vivo* manipulations. Under current suboptimal conditions, adult stem cells tend to lose their stem cell properties and become more specialized, giving rise to mature cell types through a process termed "differentiation." Recent advances in supportive culture conditions for mouse hematopoietic stem cells may ultimately facilitate more effective use of human hematopoietic stem cells in gene therapy applications.[10,11]

EMBRYONIC STEM CELL: "THE ULTIMATE STEM CELL"

Embryonic stem cells are capable of unlimited self-renewal while maintaining the potential to differentiate into derivatives of all three germ layers. Even after months and years of growth in the laboratory, they retain the ability to form any cell type in the body. These properties reflect their origin from cells of the early embryo at a stage during which the cellular machinery is geared toward the rapid expansion and diversification of cell types.

Murine (mouse) embryonic stem cells were isolated over 20 years ago,[12,13] and paved the way for the isolation of nonhuman primate, and finally human embryonic stem cells.[14] Much of the anticipated potential surrounding human embryonic stem cells is an extrapolation from pioneering experiments in the mouse system. Experiments performed with human embryonic stem cells in the last couple of years indicate that these cells have the potential to make an important impact on medical science, at least in certain fields. In particular, this impact includes: a) differentiation of human embryonic stem cells into various cell types, such as neurons, cardiac, vascular, hematopoietic, pancreatic, hepatic, and placental cells, b) the derivation of new cell lines under alternative conditions, c) and the establishment of protocols that allow the genetic modification of these cells.

THE POTENTIAL OF HUMAN EMBRYONIC STEM CELLS FOR GENE THERAPY

Following derivation, human embryonic stem cells are easily accessible for controlled and specific genetic manipulation. When this facility is combined with their rapid growth, remarkable stability, and ability to mature *in vitro* into multiple cell types of the body, human embryonic stem cells are attractive potential tools for gene therapy. Two possible scenarios whereby human embryonic stem cells may benefit the gene therapy field are discussed below.

First, human embryonic stem cells could be genetically manipulated to introduce the therapeutic gene. This

gene may either be active or awaiting later activation, once the modified embryonic stem cell has differentiated into the desired cell type. Recently published reports establish the feasibility of such an approach.[15] Skin cells from an immunodeficient mouse were used to generate cellular therapy that partially restored immune function in the mouse. In these experiments, embryonic stem cells were generated from an immunodeficient mouse by nuclear transfer technology. The nucleus of an egg cell was replaced with that from a skin cell of an adult mouse with the genetic immunodeficiency. The egg was developed to the blastula stage at which embryonic stem cells were derived. The genetic defect was corrected by a genetic modification strategy designated "gene targeting." These "cured" embryonic stem cells were differentiated into hematopoietic "stem" cells and transplanted into immunodeficient mice. Interestingly, the immune function in these animals was partially restored. In principle, this approach may be employed for treating human patients with immunodeficiency or other diseases that may be corrected by cell transplantation.

However, significant advances must first be made. The levels of immune system reconstitution observed in the mice were quite modest (<1% of normal), while the methodology employed to achieve hematopoietic engraftment is not clinically feasible. This methodology involved using a more severely immunodeficient mouse as a recipient (which also had the murine equivalent of the human X-linked SCID mutation) and genetically engineering the hematopoietic engrafting cells with a potential oncogene prior to transplantation.

Embryonic stem cells may additionally be indirectly beneficial for cellular gene therapy. Since these cells can be differentiated *in vitro* into many cell types, including presumably tissue-specific stem cells, they may provide a constant *in vitro* source of cellular material. Such "adult" stem cells derived from embryonic stem cells may thus be utilized to optimize protocols for propagation and genetic manipulation techniques.[16] To acquire optimal cellular material from clinical samples in larger quantities for experimental and optimization purposes is usually rather difficult since access to these samples is limited.

GENETIC MANIPULATION OF STEM CELLS

The therapeutic gene needs to be introduced into the cell type used for therapy. Genes may be introduced into cells by transfection or transduction. Transfection utilizes chemical or physical methods to introduce new genes into cells. Usually, small molecules, such as liposomes, as well as other cationic-lipid based particles are employed to facilitate the entry of DNA encoding the gene of interest into the cells. Brief electric shocks are additionally used to facilitate DNA entry into living cells. All of these techniques have been applied to various stem cells, including human embryonic stem cells. However, the destiny of the introduced DNA is relatively poorly controlled using these procedures. In most cells, the DNA disappears after days or weeks, and in rare cases, integrates randomly into host chromosomal DNA. *In vitro* drug selection strategies allow the isolation and expansion of cells that are stably transfected, as long as they significantly express the newly introduced gene.

Transduction utilizes viral vectors for DNA transfer. Viruses, by nature, introduce DNA or RNA into cells very efficiently. Engineered viruses can be used to introduce almost any genetic information into cells. However, there are usually limitations in the size of the introduced gene. Additionally, some viruses (particularly retroviruses) only infect dividing cells effectively, whereas others (lentiviruses) do not require actively dividing cells. In most cases, the genetic information carried by the viral vector is stably integrated into the host cell genome (the total complement of chromosomes in the cell).

An important parameter that must be carefully monitored is the random integration into the host genome, since this process can induce mutations that lead to malignant transformation or serious gene dysfunction. However, several copies of the therapeutic gene may also be integrated into the genome, helping to bypass positional effects and gene silencing. Positional effects are caused by certain areas within the genome and directly influence the activity of the introduced gene. Gene silencing refers to the phenomenon whereby over time, most artificially introduced active genes are turned off by the host cell, a mechanism that is not currently well understood. In these cases, integration of several copies may help to achieve stable gene expression, since a subset of the introduced genes may integrate into favorable sites. In the past, gene silencing and positional effects were a particular problem in mouse hematopoietic stem cells.[17] These problems led to the optimization of retroviral and lentiviral vector systems by the addition of

genetic control elements (referred to as chromatin domain insulators and scaffold/matrix attachment regions) into the vectors, resulting in more robust expression in differentiating cell systems, including human embryonic stem cells.[18]

In some gene transfer systems, the foreign transgene does not integrate at a high rate and remains separate from the host genomic DNA, a status denoted "episomal". Specific proteins stabilizing these episomal DNA molecules have been identified as well as viruses (adenovirus) that persist stably for some time in an episomal condition. Recently, episomal systems have been applied to embryonic stem cells.[19]

An elegant way to circumvent positional effects and gene silencing is to introduce the gene of interest specifically into a defined region of the genome by the gene targeting technique referred to previously.[20] The gene targeting technique takes advantage of a cellular DNA repair process known as homologous recombination.[21] Homologous recombination provides a precise mechanism for defined modifications of genomes in living cells, and has been used extensively with mouse embryonic stem cells to investigate gene function and create mouse models of human diseases. Recombinant DNA is altered in vitro, and the therapeutic gene is introduced into a copy of the genomic DNA that is targeted during this process. Next, recombinant DNA is introduced by transfection into the cell, where it recombines with the homologous part of the cell genome. This in turn results in the replacement of normal genomic DNA with recombinant DNA containing genetic modifications.

Homologous recombination is a very rare event in cells, and thus a powerful selection strategy is necessary to identify the cells in which it occurs. Usually, the introduced construct has an additional gene coding for antibiotic resistance (referred to as a selectable marker), allowing cells that have incorporated the recombinant DNA to be positively selected in culture. However, antibiotic resistance only reveals that the cells have taken up recombinant DNA and incorporated it somewhere in the genome. To select for cells in which homologous recombination has occurred, the end of the recombination construct often includes the thymidine kinase gene from the herpes simplex virus. Cells that randomly incorporate recombinant DNA usually retain the entire DNA construct, including the herpes virus thymidine kinase gene. In cells that display homologous recombination between the recombinant construct and cellular DNA, an exchange of homologous DNA sequences is involved, and the non-homologous thymidine kinase gene at the end of the construct is eliminated. Cells expressing the thymidine kinase gene are killed by the antiviral drug ganciclovir in a process known as negative selection. Therefore, those cells undergoing homologous recombination are unique in that they are resistant to both the antibiotic and ganciclovir, allowing effective selection with these drugs *(see Figure 4.2).*

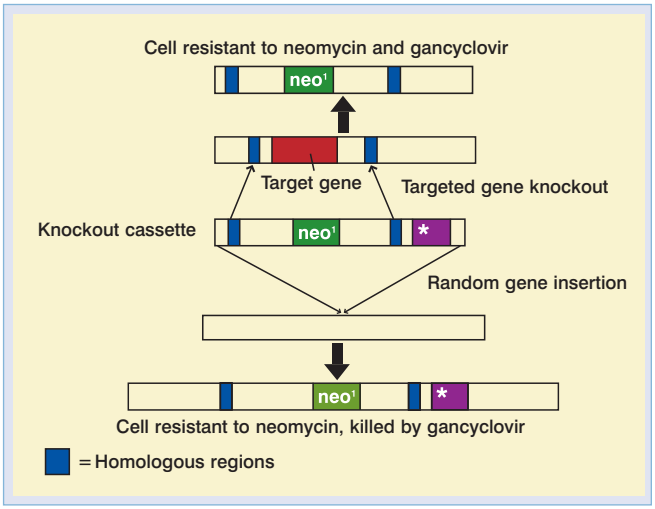

Figure 4.2. Gene targeting by homologous recombination.

Gene targeting by homologous recombination has recently been applied to human embryonic stem cells.[22] This is important for studying gene functions *in vitro* for lineage selection and marking. For therapeutic applications in transplantation medicine, the controlled modification of specific genes should be useful for purifying specific embryonic stem cell-derived, differentiated cell types from a mixed population, altering the antigenicity of embryonic stem cell derivatives, and adding defined markers that allow the identification of transplanted cells. Additionally, since the therapeutic gene can now be introduced into defined regions of the human genome, better controlled expression of the therapeutic gene should be possible. This also significantly reduces the risk of insertional mutagenesis.

FUTURE CHALLENGES FOR STEM CELL-BASED GENE THERAPY

Despite promising scientific results with genetically modified stem cells, some major problems remain to be overcome. The more specific and extensive the

genetic modification, the longer the stem cells have to remain *in vitro*. Although human embryonic stem cells in the culture dish remain remarkably stable, the cells may accumulate genetic and epigenetic changes that might harm the patient (epigenetic changes regulate gene activity without altering the genetic blueprint of the cell). Indeed, sporadic chromosomal abnormalities in human embryonic stem cell culture have been reported, and these may occur more frequently when the cells are passaged as bulk populations. This observation reinforces the necessity to optimize culture conditions further, to explore new human embryonic stem cell lines, and to monitor the existing cell lines.[23,24] Additionally undifferentiated embryonic stem cells have the potential to form a type of cancer called a teratocarcinoma. Safety precautions are therefore necessary, and currently, protocols are being developed to allow the complete depletion of any remaining undifferentiated embryonic stem cells.[25] This may be achieved by rigorous purification of embryonic stem cell derivatives or introducing suicide genes that can be externally controlled.

Another issue is the patient's immune system response. Transgenic genes, as well as vectors introducing these genes (such as those derived from viruses), potentially trigger immune system responses. If stem cells are not autologous, they eventually cause immuno-rejection of the transplanted cell type. Strategies to circumvent these problems, such as the expression of immune system-modulating genes by stem cells, creation of chimeric, immunotolerable bone marrow or suppression

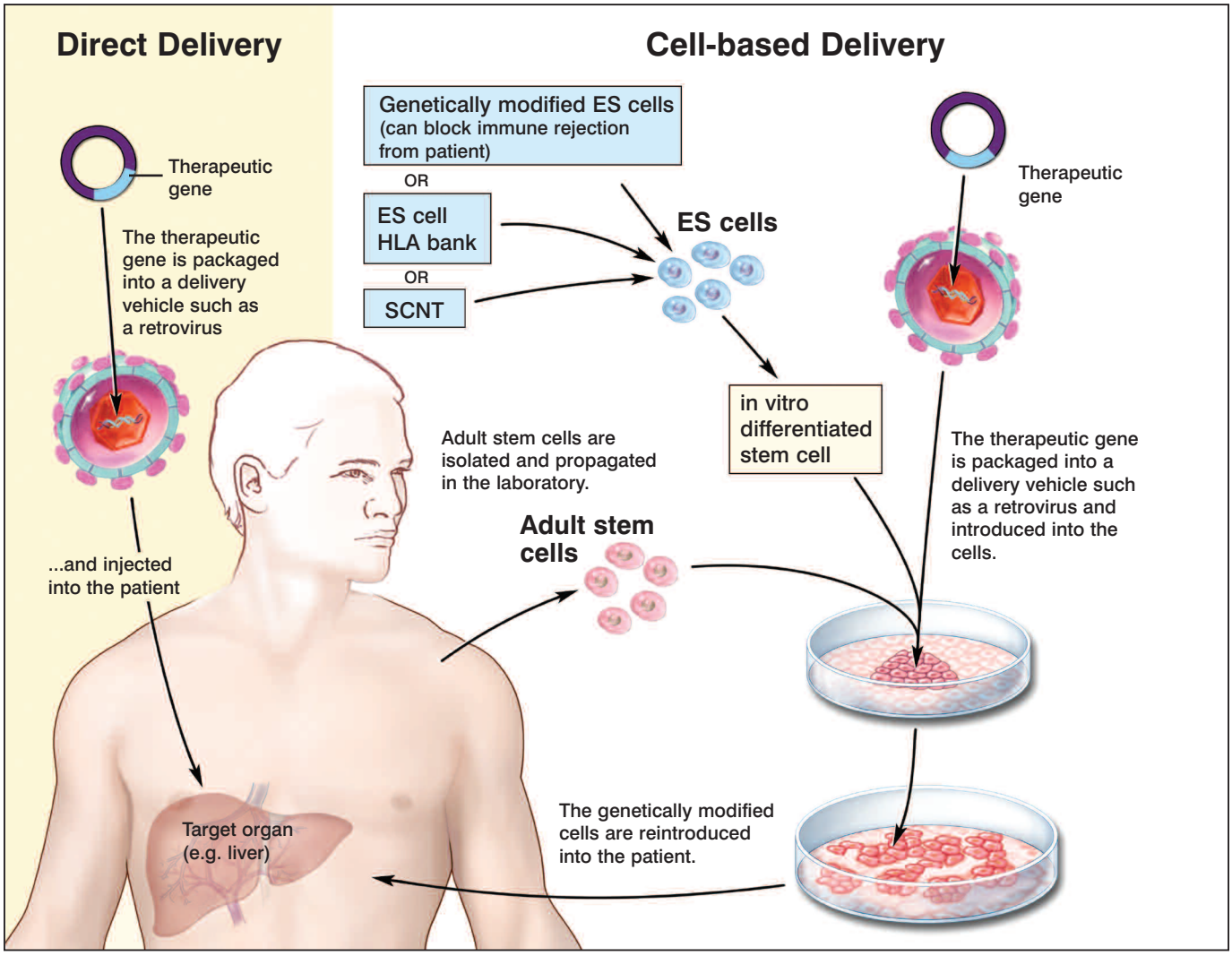

Figure 4.3. Strategies for Delivering Therapeutic Transgenes into Patients.

of HLA genes have been suggested.[25] In this context, nuclear transfer technology has been recently extended to human embryonic stem cells.[26]* Notably, immune-matched human embryonic stem cells have now been established from patients, including an individual with an immunodeficiency disease, congenital hypogammaglobulinemia.[27]* Strategies that combine gene targeting with embryonic stem cell-based therapy are thus potential novel therapeutic options.

The addition of human embryonic stem cells to the experimental gene therapy arsenal offers great promise in overcoming many of the existing problems of cellular based gene therapy that have been encountered in clinic trials *(see Figure 4.3)*. Further research is essential to determine the full potential of both adult and embryonic stem cells in this exciting new field.

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* **Editor's note:** Both papers referenced in 26 and 27 were later retracted. See Science 20 January 2006: Vol. 311. no. 5759, p. 335.

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