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Genetic Modification of Stem Cells for Transplantation

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Abstract

Gene modification of cells for prior to their transplantation, especially stem cells, enhances their survival and increases their function in cell therapy. Like the Trojan horse, the gene modified cell has to gain entrance inside the host's walls and survive and deliver its transgene products. Using cellular, molecular and gene manipulation techniques the transplanted cell can be protected in a hostile environment from immune rejection, inflammation, hypoxia and apoptosis. Genetic engineering to modify cells involves constructing modules of functional gene sequences. They can be simple reporter genes or complex cassettes with gene switches, cell specific promoters and multiple transgenes. We discuss methods to deliver and construct gene cassettes with viral and non viral delivery, siRNA, and conditional Cre/Lox P. We review the current uses of gene modified stem cells in cardiovascular disease, diabetes, neurological diseases, (including Parkinson's, Alzheimer's and spinal cord injury repair), bone defects, hemophilia, and cancer.

Keywords

vigilant vector; stem cells; microRNA; Cre/LoxP; heart failure; cancer; diabetes; neurological diseases

1. Introduction

Gene modification of stem cells is the Trojan horse approach to making stem cells more effective. The idea of entering a cell with the help of the cell and then attacking it from the inside is even more ancient than this story. It is the process used by viruses, plasmids, bacteria and parasites. By using viruses and plasmids as the Trojan horse, we can modify genes or introduce new ones to make the cell die or survive longer, secrete proteins or switch off genes, differentiate or not differentiate.

Stem cells replicate throughout life so long as a few of them do not differentiate. The daughter cells that differentiate go on to become adult cells with specific functions in the body. Adult stem cells generally are multipotent and can transform into tissues that are produced within the organ or tissue in which they are found. Stem cells in bone marrow can become osteocytes, blood cells and lymph cells [1]. Cardiac derived stem cells can become any of the cells that are part of a functioning heart including cardiomyocytes, neurons, endothelial cells [2,3,4] It is debatable whether bone marrow cells can turn into heart cells or any other cell which is not related to blood, bone or lymph [1,5].

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2. Genetic Engineering

Genetic engineering of stem cells can be useful for increasing cell survival when transplanted, particularly into a hostile environment. They can be modified to deliver proteins to neighboring cells, kill cancer cells or reduce graft-host rejection. Obviously both embryonic and adult stem cells have great potential for treatments involving cellular repair, replacement and regeneration. One of the limitations of cell replacement therapy is that a majority of grafted cells do not survive when grafted. Even if they are autologous or from a syngenic population, cell transplantation usually results in a loss of cells. Genetic engineering can increase survival of engrafted stem cells when transgenes are inserted into the cell to prevent or reduce apoptosis and inflammatory injury. In genetic engineering a gene cassette is constructed and loaded into a vector for entry into the cell. Once inside the cell, the gene construct can express or over express specific genes. The transgene expression can be constant leading to constitutive synthesis of specific proteins or can be controlled by a gene switch. Constitutive activation of genes is unphysiological leading to overproduction of proteins which down regulates receptors and renders the gene expression ineffective. A gene switch essentially makes the cell “intelligent” because the cell will then respond to a physiological stimulus, e.g. low oxygen, high glucose levels, hormone concentrations, or to drugs or chemical agents.

A key principle to genetic engineering for cells is to mix and match modules of functional domains that are used in Nature. Thus we can take a gene module used by yeast and a human virus module to create a chimeric regulator. Wang et al first described a gene regulatory system for gene transfer by building a gene switch that responds to increases in Mifepristone, a progesterone antagonist [6]. They fused a ligand-binding domain of a mutated human progesterone receptor to the yeast transcriptional activator GAL4 DNA binding domain, and the herpes simplex virus protein VP16 activated domain. They demonstrated that this system could be activated by the exogenous administration of Mifepristone(RU 486) at low doses to activate transcription of target genes. As described below we developed a Vigilant Vector [7, 8] with a gene switch similar to this concept, but built it to automatically respond to hypoxia, so that no exogenous drug was required to turn the system on or off.

2.1 Transgenics

A very well established gene modification of embryonic stem (ES) cells is in the production of transgenic animals. Transgenic mice with genes knocked out, or genes “knocked in” (where the number of copies of genes is increased), [9] are ubiquitous gene studies in living animals. They have been very useful for studying the role of specific genes and practical for producing specific human proteins. The method involves harvesting ES cells from the inner cell mass of the blastocyst. Using recombinant DNA (rDNA) a desired gene is inserted in a vector together with promoter sequences to regulate the gene expression. To replace a normal gene by knocking one out, two drug resistant genes are added to the cassette. A neo^r gene, which is resistant to lethal effects of neomycin, and a thymidine kinase gene (tk) which phosphorylates gangcyclovir. The majority of cells fail to take the vector inside their walls. These cells can be killed by neomycin or its analogs. A few of the remaining cells allow the vector in, but the gene is inserted randomly. To avoid this, these cells are killed by gangcyclovir. That leaves only those cells in which homologous recombination has occurred. The normal gene has been knocked out and a new, specified gene knocked in. These cells are then injected into a blastocyst, which is implanted in the uterus to produce offspring that can be bred. If the new gene is nonfunctional, (i.e. a null allele), the function of the former gene may be revealed through breeding the mice with the knockout gene to homozygosity.

Ideally the function of the missing gene will be as obvious as if a limb had been cut off. In actuality several things can happen. The knocked out gene may prevent the embryo from developing (it is embryonically lethal), or the missing gene is fully compensated by other genes,

or subtle changes occur in development or in different organs so that the effect is not obvious. Nevertheless the technique has had a huge influence on revealing functional effects of proteins especially where antibodies have not been developed. The opposite of knocking in copies of a gene has been used to reveal mechanisms of diseases caused by overexpression of a protein [9]. The transgenic animal approach requires going through embryonic development. This limits the technique when a knocked out gene is embryonically lethal. However a method first used by Gu et al 1994 [10] the Cre/Lox P System is able to induce the same mutation and avoid lethality.

2.2 Cre/Lox P System

To knockout a target gene in specific cell groups or tissue, in adult animals, the *Cre/lox P* system is a suitable technique. It is based on the viral bacteria phage P1, which produces *Cre*, a recombinase enzyme. *Cre* cuts its viral DNA into packages. *Cre* cuts all the DNA out between two separate *lox P* sites. The DNA ends, which each have a half *lox P* site are then ligated by the recombinase. Gu et al [10] used this principle with a strategy of a conventional transgenic mice, in which the *Cre* transgene plus a promoter, was inserted by homologous recombination, in a cell specific type. This mouse was crossed with a second mouse strain that had a target gene flanked by two *lox P* sites. In the offspring the target gene was only deleted in those specific cells that contained *Cre* and the *lox P* “floxed” sequences. The target gene remained functional in all the other cells and the animals survived development, so the function of the targeted gene in specific cells could be studied.

More recent developments have made the technique less laborious to use [11,12]. An example is a study by Sanniyha et al [12] who made transgenic mice with *lox P* insertions flanking the gene for angiotensinogen. Angiotensinogen is a substrate for the enzyme renin and is one of the critical components for the synthesis of the peptide angiotensin. Instead of making a separate strain of *Cre* mice and proceeding with breeding, they simply injected *Cre* into the floxed mice. This had the advantage of not only being time saving, but also of opening up a new way to study genes with site directed, conditional, gene ablation in specific cells. As they were working on the brain they were able to pinpoint anatomically a very small brain structure, the subfornical organ. By injecting *Cre* into the structure, they showed that angiotensin synthesis could be blocked and proved it is synthesized in the brain. [13,14]

To inhibit synthesis of proteins by inhibiting gene translation there are two methods; antisense and RNA interference.

2.3 Antisense inhibition

Antisense is based on the fact that mRNA is in the “sense” direction from 5’ to 3’. Antisense is a limited sequence of DNA in the antisense direction 3’ to 5’ designed from knowing the sequence of a target gene. Antisense oligonucleotides (AS-ODN) are usually built around the initiation codon of a gene (the AUG start site) and are shorter than the full length gene. This is because the AS-ODN binds to part of the appropriate mRNA sequences and prevents the mRNA from translating the protein it would otherwise produce.

For gene modification with antisense within a cell, a viral vector can be fitted with DNA in the antisense direction. We have designed these in the adeno-associated virus and shown them to have long lasting inhibitory effects on designated cell protein synthesis. [18] Antisense inhibition although widely used in research and approved for clinical treatment [19] is not perfect. When antisense is put into a cell it is competing with the cells’ own mRNA copying machinery. The presence of AS-ODN may actually increase the number of cell produced mRNA copies, thereby overcoming the endogenously administered AS-ODN. Because of this antisense as a treatment has not proven to be the killer of cells and therefore not a revolutionary

anti-cancer agent, as it was originally hoped. However it has played a pivotal role in leading to the next advance in cellular gene inhibition – RNA interference.

2.4 siRNA gene silencing

Fire and Mello [20] were using antisense to study behavioral effects on the primitive worm, *C. elegans*. They tested sense RNA and antisense RNA on the worms but there was no effect of either. However when they tested a combination of sense and antisense RNA, the worms started to twitch spontaneously. The gene that was holding back the twitching had been silenced. Fire and Mello had discovered gene silencing by double stranded (ds) RNA which acted as small interfering RNA (siRNA). RNA interference has become widely recognized as a biological mechanism for the regulation of gene expression and used for intracellular inhibition. Double stranded RNA is produced in the nucleus. In the cytoplasm it binds to an enzyme, Dicer, literally dices up the double stranded RNA into short strands (15–20 nucleotides).

One of the strands is loaded into a protein complex, RNA induced silencing complex (RISC). The RISC complex now has the single strand of short RNA as a binding site to bind to a complementary sequence on the cell's mRNA. This binding leads to cleavage of mRNA degrading the message and stopping it from translating a specific protein. And hence it is silenced.

RNAi is a fundamental cellular process of gene regulation in the cells of animals and plants. Since both animals and plants are subject to diseases induced by viruses, RNAi may have evolved to protect cells from invasion by viruses. The genome of a retrovirus is in double strands of RNA. A retrovirus, lacking cellular mechanisms and DNA, injects its genomic dsRNA into a cell to reproduce itself using the DNA of the invaded cell. RNAi protects the cell by destroying the viral RNA through the RISC complex mechanism.

SiRNA is more powerful than antisense in silencing genes, but it has its difficulties. It is not long lasting, it may silence off-target sites, and it has been not been easy to inject systemically as a therapy. We have directly compared siRNA to antisense to inhibit the Beta-1 adrenergic receptor gene [21]. The effect was measured on blood pressure in hypertensive rats and on measures of heart performance, because beta blockers have long been used for hypertension and heart failure treatments. The siRNA and AS-ODN were injected systemically in a Lipofectamine vehicle. The result was a significantly better effect on lowering blood pressure and improving heart performance with the siRNA compared to the AS-ODN. Both approaches lasted about 1 week with a single injection [21].

2.5 MicroRNA

MicroRNA offer completely new possibilities for gene modification, cell therapy and drug development. They are involved in almost every biological process regulated by genes and their absence or mutations could be the cause of many disease states from birth defects to cancer.

Although microRNAs (miR) were discovered over 20 years ago in *C. elegans* [22] and later found in mammals, we are still in an early stage of discovering how many there are, what they do and how they do it. Over 500 miRs have been found in the human genome. A recent review in Nature Reviews suggests that miRs regulate one third of human genes [23]. Micro RNAs have become recognized as a new class of gene regulators and therefore important for gene modification of cells. miRNA are small non coding RNA's that modify gene expression by post transcriptional inhibition of targeted mRNA. In the nucleus miRNA is formed from introns and exons as "primary" or "pri-miRNA". But it is not a messenger RNA – it does not specify

or generate a protein. The pri-miRNA, a folded back, structure of 60–70 nucleotides, is processed in the nucleus by the enzymes Drosha and Pasha. Drosha cuts out the stem-loop structure which is the “pre-miRNA”. The pre-miRNA is exported out of the nucleus by exportin and into the cytoplasm where it is diced up by the enzyme Dicer RNase III, mentioned above in the siRNA process. The same effect occurs. Dicer cuts the stem-loop into short length (19–25 nucleotides) inverted “mature miRNA”. As with siRNA, one strand of the mature miRNA becomes part of the RISC complex and targets mRNA by binding to antisense complementary regions and cleaving or degrading the targeted mRNA. Multiple roles for miRNAs in gene regulation have been revealed by gene expression analysis PCR, and by transgenic mice with knockouts of specific miRNA. Expression arrays are revealing specific miRNAs in different tissues and cells from invertebrates to humans. Many miRNAs, (miR-1, miR-34, miR-60, miR-87, miR-124a) are highly conserved between vertebrates and invertebrates [24] including the small temporal (st) RNAs discovered in *C. elegans* (eg let-7 RNA, lin-4) that are similar to miRNAs in humans. As these stRNAs are critical for cell differentiation and timing of neural connections, the conservation may indicate functional evolution.

Although the mechanism of miRNA action is principally inhibitory on targeted mRNA, which is essential for normal growth and differentiation in cell and tissue development, miRNAs can be involved in cancer. They can be depleted or suppressed allowing oncogenes to be overproduced. Kumar et al [25] recently showed that global suppression of miRNAs in various cancer cell lines increased cancer cell transformation and enhanced tumorigenesis in mice. To suppress miRNA they targeted Drosha and Dicer with siRNA. Non cancerous cells did not become cancerous, but did not grow. This suggests that increasing miRNAs could be a new approach to treating cancer by either suppressing oncogenes or by increasing differentiation.

2.6 Reporter genes

Manipulation of genes in cells, such as stem cells, before transplantation can be done at several different levels of sophistication. If one simply wants to label cells with an internal marker so that the cells can be identified after transplantation then a reporter gene such as a fluorescent gene for example green fluorescent protein (gfp) or Luciferase (Luc) or beta galactosidase (Lac Z) gene sequence can be inserted by a vector. Each cell marker has its own advantage or disadvantage. Fluorescent labels are not easily quantified but visible with highly sensitive fluoroscope that the cells can be located, even under the skin in tissues and tumors. Luciferase has the advantage that it is quantifiable using luminometers, dual luciferase assays or relative luciferase gene expression.

2.7 Cell Specific Promoters

At the next level of sophistication, a cell or tissue specific promoter is spliced with the selected cell marker transgene so that the transgene can be observed to be expressed in one type of cell. Selecting the promoter raises some problems. A powerful promoter like cytomegalovirus (CMV) drives a gene but is non selective for tissue type. A more cell specific promoter is likely to have a weaker power and therefore there will be less gene marker expressed. Improving promoter power without losing cell specificity is a challenge. Fitting a promoter into a cassette for a vector of small loading capacity such as AAV, may require cutting the promoter into fragments and testing driving force versus specificity. For example we used the myosin light chain-2v promoter (MLC-2v) in the heart [8], which is 1700bp long. In order to fit this promoter into the AAV, we reduced the MLC-2v to a 250bp fragment that contained the heart specific cis regulatory elements [26]. To further increase power, a promoter enhancer can be added to the effective promoter fragment. SV40 and Chick beta actin or globin [27] have been tried and increase expression by several fold. A feed-forward system can be introduced by the making the product of cassette transgenes – the fusion proteins, feedback on an activating sequence to drive the promoter.

High level expression powered by CMV or even the lower level of gene expression driven by a cell specific promoter is constant. This constitutive gene expression could lead to a build up of protein and unwanted side effects. For a therapeutic approach the gene modification needs to have a gene switch in the design

2. 8 Gene switches

Several different types of gene switches have been developed. Some require application of exogenous drugs to induce expression. These include the “Tet-on Tet-off “ system using tetracycline as the switch inducer [28]. Ecdysone [29], rapamycin [30] and mifepristone [6] have also been used.

To make a transgene turn on and off to physiological stimulus, requires genetic engineering of the cassette to include naturally occurring cellular regulatory elements.. The cassette is constructed from modules which can be spliced together in a specific order. To illustrate, we have developed a “Vigilant Vector™ that is switched on by hypoxia in heart cells (Figure 1) [31]. To develop the hypoxia switch there were several possibilities. The natural oxygen sensitive elements of a cell had been worked out and sequenced [32]. The hypoxia regulatory element (HRE) contains inducible factors (HIF-1alpha and HIF -1beta). When oxygen is low the HIF-1alpha combines with the HIF-1beta and the fusion product acts a transcription factor in the nucleus to generate proteins in response to low oxygen, such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO). By extracting the oxygen sensor in HIF-1a, oxygen dependent domain (ODD), and installing it as the oxygen sensor of a chimeric gene, we could control the genetic response to hypoxia and avoid the production of these and other proteins. The ODD module was spliced in an activator system. The DNA binding domain is the yeast GAL4 and the activating domain is the human p65 derived from human nuclear kappa B protein. Under normal oxygen levels, the fusion protein of p65/ODD/Gal4 is ubiquitinated and the ubiquitin tail is the signal for transport to, and destruction in, proteosomes. But as oxygen decreases, a threshold is reached where the fusion protein is not ubiquitinated or destroyed and the GAL4 component of the protein binds to an inserted upstream activating sequence in front of the TATA box that activates gene expression. The lower the oxygen concentration, the greater number of fusion proteins are generated, exponentially increasing gene activity. Combined with a heart specific promoter (MLC-2v) the whole system acts as a site specific gene switch for hypoxia. Further the system allows for amplification of gene expression. In practical terms when the transgene was heme-oxygenase-1, an antioxidant with anti-apoptotic and anti-inflammatory effects it protected ischemic (mouse) hearts from heart failure [33].

3. The application of genetic modification of stem cells

3. 1 Cardiology

3.1.1 Increase graft cell survival—Adult stem cells have been proposed as a promising source for the heart repair, however, cell-based therapy is confronted with the problem of poor survival in host myocardium [34]. Graft cell survival is limited by various pathological processes such as the inflammatory response, rejection, and ischemia-reperfusion. The survival of engrafted stem cells requires adaptation to adverse environment in ischemic myocardium. Different strategies have been developed to increase cell survival after grafting. Pharmacologic preconditioning has been tested successfully in skeletal myoblasts and shows cytoprotective effects both in vitro and in vivo [35]. Suzuki et al. [36] reported that heat-shock treatment could improve cell tolerance to hypoxia-reoxygen insult in vitro and enhance survival when grafted into the heart. Exploiting cell growth and apoptotic regulatory factors to enhance the proliferation of viable stem cells or confer apoptosis resistance to donor cells, by gene modification, is a potential way to improve cell transplant efficiency. Akt is a powerful survival

signal in many systems [37]. Akt gene modification of stem cells have been reported by Mangi et al [38]. Their work demonstrated that a direct intramuscular injection of 5×10^6 Akt-engineered MSCs improved the function of infarct rat hearts. However, the overall application of constitutively active Akt gene may increase the risk of tumorigenesis [39]. HO-1 is the rate-limiting enzyme in the catabolism of heme, followed by production of biliverdin, free iron and carbon monoxide (CO). All three byproducts exert beneficial actions that protect the cells from oxidative damage and death [40]. Hypoxia-inducible HO-1 plasmid modification of graft mesenchymal stem cells can protect cells from subsequent hypoxia injury in vitro, and improve graft cell survival in ischemic myocardium in vivo via anti-inflammatory and anti-apoptosis [41]. These findings underscore the role of HO-1 for protecting grafted cells from ischemia/inflammation induced death.

3.1.2. Gene-modified stem cells to increase angiogenesis in ischemic heart disease—Myocardial ischemia associated with coronary artery disease is a leading cause of morbidity and mortality in the United States [42]. Although percutaneous transluminal angioplasty (PTCA) and operative coronary revascularization (CABG) procedures are effective for revascularization, there are increasing numbers of patients with extensive atherosclerotic coronary artery disease not amenable to traditional methods of revascularization. Several growth factors have appeared recently as adjuncts to regular revascularization, including vascular endothelial growth factor (VEGF) [43]. Although viruses carrying VEGF gene can maintain a therapeutic angiogenesis, VEGF expression is not under tight control and thus might cause unwanted side effects, such as angioma formation. To develop an approach for safe and long-lasting angiogenesis, we investigated neovascularization in ischemic myocardium via autologous mesenchymal stromal cells (MSCs) transplantation. Our finding suggested that bone marrow derived MSCs play a crucial role in improving regional blood flow in ischemic myocardium, and provides an optimal strategy for therapeutic angiogenesis by secreting a broad spectrum of angiogenic cytokines, including VEGF[44], HGF[45], bFGF[46], and SDF-1 α [46]. Increased blood supply from neovascularization would inhibit apoptosis and necrosis of hibernating and stunned myocardium in border zone. Moreover, autologous MSC have high proliferative and self-renewal capability, which is critical for maintaining lasting effects fit for clinic treatment of patients with extensive atherosclerotic coronary disease [44]. Although autologous MSC transplantation can be administrated as “sole therapy” for neovascularization, many laboratories have developed strategies to use MSCs as vehicles for angiogenic gene therapy to enhance the benefits of neovascularization Table 1 lists genetically modulated cells carrying exogenous genes encoding for angiogenic factors and the MSCs, which have an inherent ability to secrete multiple paracrine factors to achieve superior revascularization. Lei et al [47] have reviewed improvements in angiogenic outcome via deliver of multiple growth factors with synergic effects.

3.2 Gene-modified stem cells to form surrogate β cells for treating diabetes

A leading cause of type 1 diabetes is the failure of pancreatic islet β cells to survive and produce insulin. Current cell therapy mainly relies on replacing of functional insulin-producing pancreatic β cells via pancreatic islet transplantation. However, the shortage of donor cells, and the number of donors required (3:1 recipient) limits the application of this treatment. The use of stem cells (SC), a potential renewable source of pancreatic β -like cells, is currently being investigated as an alternative cell source to isolated pancreatic islet transplantation for the treatment of type 1 diabetes mellitus. Stem cell-derived insulin producing cells could be a renewable source of insulin-producing cells for cell transplantation. To enhance the maturation process of human embryonic stem cells (ESCs)-derived insulin-producing cells, recent studies used genetic manipulation methodologies to deliver specific pancreatic transcription factors or developmental control genes to hESCs. Lavon et al [54] made hESCs over-express two

different transcription factors, Foxa2 and pancreatic duodenum homeobox protein-1 (Pdx1). Foxa2 is found in the early endoderm layer [55], and expressed at a very early stage in pancreas development [56]. Pdx1 is a pancreatic specific transcription factor expressed downstream of Foxa2, and specifically involved in stem cell differentiation into β -cell progenitors [57]. Pdx1 binds and activates insulin promoter in β cells [57,58]. Their study demonstrated that the constitutive expression of Pdx1 enhances the differentiation of hESCs toward pancreatic endocrine and exocrine cell types. The expression of Pdx1 also increased the expression of several transcription factors that are downstream of Pdx1, such as Ngn3, PAX4, NKX2.2, and ISL1. However, this group also found that the expression of the insulin gene could be demonstrated only when the cells differentiated in vivo into teratomas, therefore, additional work is necessary to induce insulin expression by hESCs without forming teratomas.

One major problem with using hESCs for β -cell transplantation for therapy in diabetes is the immunological incompatibility between the cell donors and the recipients. The levels of MHC-I expression in hESCs increase after in vitro differentiation [59]. Therefore, the host immune system will recognize and attack foreign hESCs, leading to rejection of transplanted hESCs. To eliminate the problem of immuno-incompatibility and the requirement for the classic immunosuppressive therapy employed for organ transplantation, multipotential stem cells in adult tissues may offer an alternative source as functional insulin-producing cells. Tang et al [60] tested the possibility of reprogramming rat hepatic stem cell-like WB cells into functional insulin-producing cells by overexpression of Pdx1 delivered with a lentivirus. Their findings demonstrate long-term, persistent expression of either Pdx1 is effective in converting hepatic stem cells into pancreatic endocrine precursor cells that, upon transplantation into diabetic mice, become functional insulin-producing cells and restore euglycemia. Apart from liver adult stem cell, human bone marrow-derived mesenchymal stem cells (hMSCs) may be a source to produce insulin-producing cells. They are autologous, have rapid renewal capability, and low risk of graft-versus-host disease. The recent study from Li et al [61] proved that hMSCs can be induced to differentiate into functional insulin-producing cells when Pdx1 is introduced via recombinant adenoviral vector. Pdx1 gene modified hMSCs can express multiple islet-cell genes including neurogenin3 (Ngn3), insulin, GK, Glut2, and glucagon, produced and released insulin/C-peptide in a weak glucose-regulated manner. Furthermore, Pdx1 modified hMSCs seemed to contribute to the regeneration of pancreatic islets after cell transplantation in STZ-induced diabetic mice. Euglycemia can be obtained within 2 weeks and maintained for at least 42 days after the Pdx1 modified hMSCs. Transplanted cells were found in the kidney capsule of the recipient and expressed insulin at 2 weeks after cell transplantation. Therefore, gene modified hMSCs are a potential cell source for cell replacement therapy in diabetes.

Ductal progenitor cells in the pancreas were also used for beta-cell replacement because they are abundant in the pancreas of these patients. Noguchi H et al [62] used adenovirus to mediate Pdx-1, Neurogenin3 (Ngn3), NeuroD, or Pax4 expression in adult mouse and human duct cells, and found NeuroD was the most effective inducer of insulin expression in primary duct cells. Their work suggested that the overexpression of transcription factors, especially NeuroD, facilitates pancreatic stem/progenitor cell differentiation into insulin-producing cells.

3.3 Neurological Diseases

3.3.1 Gene-modified stem cells to treat spinal cord injury—Stem cell transplantation has been proposed as a strategy for CNS and spinal cord injury repair. Neural stem cells (NSCs) are capable of differentiating into neurons in the brain [63] and spinal cord [64]. Recently, three groups [65–67] demonstrated that genetically engineering NSCs with axonal growth gene or neuroprotective factor genes, such as neurotrophin-3 (NT-3), NGF and BDNF could exhibit spinal cord repair. They isolated and cultured the neural stem cells, and then modified these cells with lentivirus mediated neurotrophin-3 (NT-3). Their studies demonstrated that the NT-3

modified, grafted cells could survive for long time in vivo and migrate for long distances. Moreover, NT-3 genetically engineered NSC obviously led to a recovery of the hindlimb function of the injured rats. These experiments provide a clear indication that modifying NSC with NT-3 can make NSC act as a source of neurotrophic factors, and improve functional outcome in spinal cord injury via neuroregeneration.

3.3.2 Gene modified stem cells for stroke—About 700,000 Americans each year suffer a new or recurrent stroke. Stroke kills more than 150,000 people a year. That's about 1 of every 16 deaths. It's the No. 3 cause of death behind diseases of the heart and cancer. Americans will pay about \$62.7 billion in 2007 for stroke-related medical costs and disability. Bone marrow stem cells have been demonstrated to cross the blood–brain barrier [68], and can differentiate into neurons and glia [69]. Transplantation of bone marrow stem cells in animal models of cerebral ischemia either by intracerebral or i.v. route has demonstrated therapeutic efficacy in reducing lesion size and improving functional outcome [70–73]. Although bone marrow stem cells have potential to self-renewal, these cells had reduced replicative capacity after about 5 cell doublings over the course of about 6 weeks in culture [74]. The limitation in life span of these cells is directly correlated with telomere shortening because of the lack of telomerase activity that is necessary for maintenance of telomere [75], and may limit clinical application of bone marrow stem cells. Overexpression of hTERT(telomerase reverse transcriptase) has been demonstrated to increase or stabilize telomere length, and immortalize human cells [76–77]. The technology of hTERT-immortalization could be used to improve stem cell expansion for subsequent therapeutic cell transplantation, especially important for aging patients with stroke. Recently, hTERT-immortalized human mesenchymal stem cells have been used in rat cerebral ischemia model for brain functional repair [78]. In the experiment, human mesenchymal stem cells were isolated from healthy adult volunteers, and the primary MSCs were immortalized with hTERT-expressing retrovirus. The cell population was expanded in culture within 40 population doublings, and intravenously delivered into rats 12 h after induction of transient middle cerebral artery occlusion (MCAO), to study their potential therapeutic benefit. They found that intravenous infusion of immortalized human mesenchymal stem cells 12 h after transient MCAO in the rat results in reduction in infarction volume by histological assay and magnetic resonance spectroscopy, more importantly, behavioral performance was improved in hTERT-MSC treated group by Treadmill test and Morris water maze test. Therefore, hTERT modification of mesenchymal stem cells appears benefit to ameliorate functional deficits after stroke, and enhance the efficacy of cell transplants.

MSCs were reported to promote neuronal cell survival and neurogenesis via secreting a variety of neuro-regulatory molecules, such as BDNF [79]. To further enhance this paracrine effects, Kurozumi K et al [80,81] transfected telomerized human MSC with the BDNF gene via a fiber-mutant F/RGD adenovirus vector and investigated whether these cells contributed to improved functional recovery in a rat transient middle cerebral artery occlusion (MCAO) model. They found that BDNF production by MSC-BDNF cells was 23-fold greater than that seen in uninfected MSC. Rats that received MSC-BDNF showed significantly more functional recovery than did control rats following MCAO. Moreover, MRI analysis revealed that the rats in the MSC-BDNF group exhibited more significant recovery from ischemia after 7 and 14 days. The apoptotic cells in the ischemic boundary zone was significantly reduced in animals treated with MSC-BDNF compared to animals in the control group. Their findings suggested that BDNF gene modification of MSC may be used as a novel strategy for the treatment of stroke by promoting functional recovery and reducing infarct size in the cerebral ischemia.

3.3.3 Gene-modified stem cells for Parkinson's disease—Bone marrow mesenchymal stem cells can be used as an alternative source of cells for neural regeneration. MSCs can be genetically modified to provide sustained production of therapeutic proteins to treat neurodegenerative disorder. Parkinson's disease (PD) is a neurological disease suited for

gene modified stem cell therapy because the mechanism of substantia nigra cell degeneration is well characterized. Early study by Schwarz EJ et al [82] tested the efficacy of genetically modified MSCs in a rat model of Parkinson disease. Rat MSCs were genetically engineered by transduction with retroviruses encoding tyrosine hydroxylase (TH) to convert tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), and GTP cyclohydrolase I, the enzyme necessary for production of the tetrahydrobiopterin cofactor for TH (BH₄). Transduced cells synthesized L-DOPA in vitro and maintained their multipotentiality after retroviral transduction. In the in vivo experiment, they injected gene modified MSCs into the striatum of 6-hydroxydopamine-lesioned rats. Their results demonstrated that L-DOPA and its metabolites can be detected in the denervated striatum of rats that received gene modified MSCs. Most importantly, they observed a significant reduction in apomorphine-induced rotation when compared with controls. Also, they reported that the engrafted cells can survive at least 87 days, whereas, the transgene expression only last about 9 days. Recently in same laboratory, they have focused on the development of a new vector system to genetically engineered autologous MSC, which is a self-inactivating retrovirus (pSIR), and contains the genes for human TH and rat GTP cyclohydrolase I separated by an internal ribosome entry site (IRES) [83]. They found that transduced rMSCs can synthesize and secrete L-DOPA (89.0--283 pmols/10(6) cells/h).

Recent studies by Shen Y et al [84] demonstrated that coexpression of TH and aromatic-L-amino-acid decarboxylase (AADC) which can convert L-DOPA to dopamine, and GTP cyclohydrolase I (GCH1) using triple transduction with adeno-associated virus (AAV)-TH, AAV-AADC, and AAV-GCH resulted in greater dopamine production than double transduction, and single transduction in denervated striatum of parkinsonian rats and improved the rotational behavior of the rats more efficiently. In a related study, Sun M et al [85] demonstrated that coexpression of four dopamine biosynthetic and transporter genes, including TH, GTPCH1, AADC, and vesicular monoamine transporter (VMAT-2, which can transport dopamine into synaptic vesicles in striatal neurons) supports efficient production of dopamine and regulated vesicular release of dopamine. The 4-gene-vector improved correction of apomorphine-induced rotational behavior better than the 3-gene-vector for 6 months. More importantly, only the 4-gene-vector supported significant K(+) dependent release of dopamine. Therefore, these investigations suggest that MSCs genetically modified with multiple dopamine biosynthetic and transporter genes may be the most suitable for cell therapy in patients with Parkinson disease.

3.3. 4. Gene-modified stem cells to treat Alzheimer' disease—Alzheimer's disease (AD) is a debilitating disorder of the central nervous system which may affect up to 50% of the population over the age of 85 years. The etiology of AD is unknown, however, cholinergic neuron loss is one of the central features of Alzheimer disease. Due to loss of cholinergic neurotransmitter systems in patients with Alzheimer's disease, early studies have focused on the development of genetically engineered cells to produce neurotrophic factors and neurotransmitters. Fisher et al [86] developed a primary fibroblast cell line that was genetically modified to express choline acetyltransferase (ChAT). They demonstrated that in vitro these cells produced and released acetylcholine at levels that varied with the amount of choline in the culture media. In their in vivo study, they found that the ChAT-expressing fibroblasts continued to produce and release acetylcholine after transplantation into the hippocampus of rats, and the levels of acetylcholine synthesized by the cells could be regulated by the localized infusion of choline in the vicinity of the grafts. Although the transplantation of genetically modified cells will not cure AD, this strategy may ameliorate the progression of cognitive impairments. Nerve growth factor (NGF) can enhance cholinergic function of neurons via cell surface receptors, such as TrkA and p75 (NTR)[98]. Recently published phase 1 clinical trial [88] demonstrated that implanting autologous fibroblasts genetically modified to express human NGF into the forebrain in eight individuals with mild Alzheimer disease showed a slower rate of cognitive decline. Also, Serial PET scans showed significant ($P < 0.05$) increases

in cortical 18-fluorodeoxyglucose after treatment. Since both NSCs and MSCs can integrate into brain and differentiate into neurons after transplantation, transplantation of gene-modified stem cells is a promising strategy to treat the Alzheimer disease by enhancing the NGF secretion and replacing degenerated neuron cells, if the appropriate neural connections are made.

3.4 Gene-modified stem cells to treat bone defect disease

In United States, there are about 6.5 million fractures per year, and about 15% of them are hard to heal. Effective therapies are lacking for these difficult cases. Bone marrow contains a population of bone marrow derived stromal cells which are progenitor cells capable of differentiating into bone, cartilage, muscle, tendon, and other connective tissues. MSCs, can be purified and culture expanded from animals and humans [89]. Engineering pluripotent MSCs with BMPs 2 has recently been a research focus for the treatment of a variety of bone defects. Gazit et al [90] reported that MSCs, following transduction with BMP2, can spontaneously differentiated into osteogenic cells in vitro, and enhance segmental defect repair in a mouse model of radial segmental defect in vivo. In a study reported by Moutsatsos et al [91], a tetracycline regulated expression vector encoding human BMP2 was used to transduce MSCs. Their finding showed that both bone formation and bone regeneration could be controlled by doxycycline, a tetracycline analogue. Moreover, there is increased angiogenesis accompanied by the bone formation in vivo. In a related study, Hasharoni A et al [92] transduced MSCs with regulated-BMP2 expressing vector, and injected the engineered MSCs into the paraspinal muscles in mice. The nature and extent of bone formation were analysed by micro-computerized tomography scanning and histological studies. They found that newly formed bone fuses with the spine, and in 7-days highly mineralized bone formed in mice injected with regulated- BMP2-transduced cells. Injected cells induced active osteogenesis at the site of implantation for up to 4 weeks post-injection. These data suggest that BMP2 vectors provide powerful gene therapy tools for bone regeneration.

3.5 Gene-modified stem cells to treat cancer

For cancer gene therapy, high efficiency, tumor-specific targeting is crucial for success, without systemic toxicity of vector dissemination. Recent data from Hung et al [93] provided direct evidence that bone marrow MSCs possess extensive tropism for solid tumors after systemic injection and thus can be used as a delivery vehicles for cancer therapy [93]. Hung et al used micropositron emission tomography imaging with [18F]-FHBG to monitor the pTY-EFEGFP-TK vector modified human MSCs. Micropositron emission tomography imaging revealed that tracer human MSCs could migrate to the sites of microscopic tumor lesions, engraft into these microscopic tumor lesions, and contribute to the development of a significantly portion of tumor stroma. Although the mechanisms are unclear, it is believed that MSCs are likely to migrate to tumor tissues through the chemotactic effect mediated by chemokines/chemokine receptors because solid tumor tissues can express and secrete multiple chemokines such as SDF-1 α hepatocyte growth factor, vascular endothelial cell growth factors (VEGF), TGFs, FGFs, platelet-derived growth factors, monocyte chemoattractant protein-1 (MCP-1), and IL-8. Therefore, MSCs can be developed to deliver genes encoding biological agents that interfere with tumor growth. Systemic delivery of genetically modified MSCs can be used as a tumor-targeting gene therapy strategy to exert anti-tumor effects. Interferon- β (IFN- β) show capability to anti-malignant tumor through antiproliferative and proapoptotic effects in vitro [94,95], however, clinical trials of IFN- β is failed because the concentration of IFN- β to inhibit tumor via systemic administration is much higher than the maximally tolerated dose for human. To solve this problem, Studeny et al [96,97] have developed a therapeutic strategy to treat multiple tumors with IFN- β gene engineered MSCs (MSC-IFN- β cells). They treated multiple lung metastases of human tumors in SCID mice by intravenous injection of human MSCs expressing interferon- β and demonstrated that transplanted MSCs incorporated into the tumor architecture, and MSC-IFN- β suppress the growth of pulmonary metastases and

prolong the survival of mouse. Nakamizo A et al [98] extended this therapeutic strategy to the treatment of intracranial human gliomas in nude mice. They injected human MSCs expressing interferon- β into the carotid artery of mice bearing human glioma intracranial xenografts (U87), and showed that MSC-IFN- β can track human gliomas and significantly increase animal survival. More recently, the study from Xin H et al [99] demonstrated successful inhibition of the development of lung metastases and thus prolonged the survival of these tumor-bearing mice by systemic administration of CX3CL1-expressing MSCs to the mice bearing lung metastase of C26 and B16F10 cells. In the related study, Stagg et al [100] investigated whether MSCs can be exploited to deliver IL-2 and generate effective immune responses against the poorly immunogenic B16 melanoma in mice with normal immune systems. Their study showed that IL-2-producing MSCs mixed with B16 cells significantly delayed tumor growth in an IL-2 dose-dependent manner while primary MSCs mixed with B16 cells and injected subcutaneously in syngeneic recipients do not affect tumor growth. Moreover, they observed that matrix-embedded IL-2-producing MSCs injected in the vicinity of preestablished B16 tumors led to absence of tumor growth in 90% of treated mice. Their study also demonstrated that tumor-bearing mice treated with IL-2-producing MSCs developed CD8-mediated tumor-specific immunity and significantly delayed tumor growth of a B16 cell challenge.

Adipose tissue-derived mesenchymal stem cells (AT-MSC) have also been shown to possess the capability to migrate actively toward tumor cells, and also can be used as cellular vehicles for targeted cancer chemotherapy. Kucerova L et al [101] recently evaluated the potential of cytosine deaminase (CD)-expressing AT-MSC (CD-AT-MSC) in a human colon cancer therapy. CD is an enzyme that converts far less toxic substrate 5-fluorocytosine (5-FC) to 5-FU and the toxic metabolites production [102]. By employing retroviral vectors and G418 selection, AT-MSC transduction resulted in efficient genetic loading of AT-MSC with CD gene. CD-AT-MSC in combination with 5-fluorocytosine (5-FC) augmented the bystander effect and selective cytotoxicity on target tumor cells HT-29 in vitro. More importantly, they demonstrated that CD-AT-MSC can deliver the CD transgene to the site of tumor formation and mediate strong antitumor effect after i.v. administration of CD-AT-MSC in immunocompromised mice treated with 5-FC. Thus, AT-MSC can be used as cell vehicles to deliver prodrug converting gene for targeted cancer gene therapy.

3.6 Gene-modified stem cells to treat hemophilia

Recently gene-modified bone marrow stem cell therapy approaches have been used to target life-threatening bleeding disorder, such as haemophilia. Moayeri et al [103] used hematopoietic stem cells (HSCs) to express coagulation factor VIII (FVIII) by an oncoretroviral vector. Transduced HSCs were transplanted into immunocompetent hemophilia A mice. Therapeutic levels of FVIII were detected in the serum of transplant recipient for over 6 months. More importantly, there was only minor anti-FVIII inhibitor antibody production induced following transplantation of gene modified HSCs. In a related study, Gangadharan B et al [104] compared the therapeutic effect of achieving sustained, therapeutic levels of FVIII between gene modified MSCs and HSCs. To test this, they used retroviral mediated procine FVIII vector to genetically modified bone marrow-derived MSCs and HSCs, and transplanted cells into genetically immunocompetent hemophilia A mice. They found that the FVIII activity levels drop rapidly and returned to baseline in MSC group due to the formation of anti-procine FVIII neutralizing antibodies, however, FVIII levels stayed high in mice treated with HSCs. They found that FVIII expression was sustained beyond 10 months because of immunologic tolerance. This investigation demonstrates that HSCs, other than MSCs, offers a sufficient and durable approach for delivering curative FVIII for treating hemophilia A.

4. Problems and Perspectives

The persistence of incurable diseases and the ever present threat of new ones suddenly appearing, constantly challenges us as scientists and clinicians to find new answers. This review has mostly been about the potential of stem cells to provide solutions when they are suitably modified to express genes that help them survive transplantation longer in hostile conditions, secrete or inhibit proteins that provide benefits to patients we could not have thought possible before. Gene modification has its roots in gene therapy – an approach that still has to be proven. Perhaps cell therapy will be the successful gene therapy using modified cells as vectors. Like the Trojan horse of old, the vectors and plasmid that evolved to enter impenetrable walls of a viable cell are useful to modify genes inside cells. New weapons to inhibit genes are available – siRNA, microRNA and Cre/LoxP. New ideas for gene switches and chimeras are constructable to control genes. In the simplest form, transgenes can be markers or reporter genes to visualize, measure and follow transplanted stem cells. In more sophisticated forms, genetically modified transplanted cells can be self protected, and release key proteins and transcription factors to provide, for example dopamine to Parkinson's patients, insulin to diabetics, acetylcholine to Alzheimer's patients, angiogenesis to restore injured tissue in the heart or brain after stroke and to heal the battlefield injured. The same principles of genetic engineering and delivery of gene cassettes into stem cells may give controlled blood clotting to hemophiliacs, repair bone defects and kill cancer cells. But the challenges are formidable for actual use in cell therapy. Much of what we do today is learning in detail exactly how to modify the genes of cells, what proteins to tailor for specific diseases. The end result may be new drugs derived from the proteins produced in genetically modified cells, and eventually new drugs will play the therapeutic roles for which we modified genes in cells.

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Figure 1.

Diagram of Vigilant Vector™ designed in this version as a cardiac specific, hypoxia regulated vector system that can amplify the power of promoters. There are two components delivered together. The sensor plasmid (pS) containing the gene switch for low oxygen (see text) and the MLC-2v promoter. The effector plasmid (pE) which contains a GAL4 upstream activation sequence (USA) in front of an adenovirus E1b TATA box and the Gene/6His fused gene. ITR: inverted terminal repeats for rAAV packaging. In normal oxygen the fusion protein (GAL4ODDp65AD) is ubiquitinated and destroyed in proteosomes. Under hypoxia more and more fusion protein is made and not destroyed so that it acts as an amplifying system by binding to the UAS and activating the transgene. DBD: DNA binding domain, AD: activation domain, UAS: upstream activating sequence, ODD: oxygen-dependent degradation domain

TABLE 1
Gene modification of stem cells to improve angiogenesis in the heart

Heart disease	Cells	Gene modification	Method of transplant	Efficacy	Adverse effects	Follow-up period	Reference
Mouse MI	MSCs	Adenovirus-hVEGF ₁₆₅	MSC i.m w/cytokine mobilization	superior therapeutic angiomyogenesis and LV-function recovery	None	4 w	[59]
Rat MI	skeletal myoblasts	non-viral hSDF-1 α	Myoblast i.m	enhances angiomyogenesis	None	4 w	[60]
Rat MI	MSCs	Adenovirus-Akt + Ang-1	MSC i.m.	enhanced cell survival, improved angiomyogenesis, and restored global cardiac function	None	4 w	[61]
Rat MI	MSCs	lentivirus-hSDF-1 α	MSC i.v.	Enhanced angiogenesis effects and improve function	None	5w	[62]
Rat MI	MSCs	Adenovirus-Ang-1	MSC i.m.	improved angiogenesis and arteriogenesis effects	None	4w	[63]
Pig chronic ischemia	MSCs	Adenovirus-Ang-1	MSC i.m.	improvement of heart perfusion and function	None	4w	[64]