REVIEW

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Safeguarding clinical translation of pluripotent stem cells with suicide genes

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The generation of human induced pluripotent stem cells (hiPSCs) opens a new avenue in regenerative medicine. However, transplantation of hiPSC-derived cells carries a risk of tumor formation by residual pluripotent stem cells. Numerous adaptive strategies have been developed to prevent or minimize adverse events and control the in vivo behavior of transplanted stem cells and their progeny. Among them, the application of suicide gene modifications, which is conceptually similar to cancer gene therapy, is considered an ideal means to control wayward stem cell progeny in vivo. In this review, the choices of vectors, promoters, and genes for use in suicide gene approaches for improving the safety of hiPSCs-based cell therapy are introduced and possible new strategies for improvements are discussed. Safety-enhancing strategies that can selectively ablate undifferentiated cells without inducing virus infection or insertional mutations may greatly aid in translating human pluripotent stem cells into cell therapies in the future.

Introduction

Stem cell therapies are one of the most promising areas in medicine and hold great potential for the treatment of degenerative diseases, genetic disorders, and severe injuries that were previously considered refractory to therapeutic intervention.[1] Pluripotent stem cells (PSCs), which can undergo extensive proliferation in vitro and give rise to lineages that represent any of the three embryonic germ layers, serve as an unlimited resource for cell-replacement therapy and tissue engineering.[2] However, the use of human embryonic stem cells (ESCs), one type of PSCs, for clinical applications has been plagued by highly controversial ethical and legal questions because it requires the destruction of a human embryo.[3]

It is also possible to reprogram somatic cells to a pluripotent state through somatic cell nuclear transfer (SCNT),[4] cell fusion,[5] or gene transfer of defined transcription factors.[6] Human induced pluripotent stem cells (hiPSCs) derived from adult cells by forced expression of defined transcription factors have attracted considerable attention because their characteristics are indistinguishable from those of inner cell mass-derived hESCs and they offer relatively high reprogramming efficiency without associated ethical dilemmas. These hiPSCs offer an exciting opportunity for elucidating underlying mechanisms of pluripotency and establishing in vitro models for human disease; they also hold the potential for future clinical applications in regenerative medicine.[7,8]

Traditionally, hiPSCs have been generated from different kinds of somatic cells, including fibroblasts, hematopoietic cells, meningiocytes and keratinocytes,[9] using a variety of gene delivery methods, including retrovirus (RV) and lentivirus (LV) transduction. hiPSCs generated by these latter methods may cause permanent, and random, transgene insertion into the host genome.[6,8] More recently, various non-viral and non-integrating methods, which may enable safe, efficient derivation of hiPSCs suitable for clinical applications, have been developed. These include transient DNA transfection using transposons or minicircle plasmids, protein transduction, and RNA/miRNA (micro RNA) transfection.[10] Nevertheless, transcriptional, genetic and epigenetic abnormalities acquired from the corresponding somatic cells of origin or during reprogramming stress and culture adaptation increase the tumorigenicity of hiPSCs.[11] In a karyotype analysis of more than 1,700 human iPSC and ESC cultures collected from 97 investigators in 29 laboratories, Taapken et al. reported that trisomy 12 was the predominant abnormality in iPSCs cultures (31.9%), and trisomy 8 occurred more frequently in iPSCs (20%) than in ESCs (10%). More importantly, these authors found that the frequency and types of karyotypic abnormalities were not affected by the reprogramming method.[12] Athurva et al.[13] reported that 22 hiPSCs lines reprogrammed by different methods (RV, LV, and non-integrating methods including episomal and mRNA delivery) each contained an average of five protein-coding point mutations, and the majority of these mutations were enriched in genes that are cancer promoting or mutated in cancers. Tong et al.[14] found that mice generated from tetraploid complementation-competent iPS cells are prone to tumorigenesis. Pancreatic and bone tumors were identified among the iPS-derived mice, whereas ES-derived mice and control mice were all tumor free. Kyoko et al.[15] compared the tumorigenicity of neurospheres generated from 36 mouse induced pluripotent stem cell lines. They found that neurospheres from tail tip, fibroblast-derived miPSCs showed the highest propensity for teratoma formation owing to the persistence of undifferentiated cells. Moreover, hiPSCs need to be induced to differentiate before transplantation. To the best

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Table 1. Vector targeting strategies

Vector	Integrate	Transduction efficiency for PSCs	Advantages	Obstacles
Adenovirus	no	low	Transduce dividing and non-diving cells	Transient gene expression; post-transduction silencing
Retrovirus	yes	high	Stable transduction of dividing cells	Post-transduction silencing; random chromosome integration/oncogene activation
Lentivirus	yes	high	Transduce dividing and non-diving cells; stable transgene expression	Random chromosome integration/oncogene activation
Adeno-associated virus	yes	low	Stable transduction, integrate in specific site	Accommodates a small amount of foreign DNA
Epstein Barr virus	no	high	Large cloning capacity (≤ 330 kb)	Transient gene expression
Herpes simplex virus	no	high	Large cloning capacity (≤ 20 kb); neuron specificity	Transient gene expression
Baculovirus	no	high	Large cloning capacity (≤ 30 kb)	Transient gene expression
Non-viral plasmid	yes	low	Stable transfection	Random chromosome integration; membrane damage

of our knowledge, all methods previously used to trigger in vitro differentiation of ES/iPS cells have yielded diverse cell mixtures. These may include undifferentiated or partially differentiated cells that proliferate inappropriately. Cell transplants may also de-differentiate or become transformed to produce tumors, particularly in an in vivo microenvironment.[16] Accordingly, it is crucial that these methodological hurdles be overcome before hiPSCs can be translated into the clinic.

A number of strategies, including the use of monoclonal antibodies, recombinant proteins and pharmaceuticals, have been developed to eliminate transferred cells that have gone awry and thereby prevent or minimize the aforementioned adverse events. However, the application of such approaches to date has been limited because they have a finite half-life and/or are only active in dividing cells.[16] Suicide genes that can be stably expressed in both quiescent and replicating cells can lead to selective ablation of gene-modified cells without the likelihood of causing collateral damage to contiguous cells and/or tissues. Therefore, suicide gene applications are considered among the most attractive approaches for controlling wayward stem cell progeny in vivo.

Here, we focus on the key strategies that will be used for suicide gene applications in hiPSCs-based therapy, including the choice of vectors, promoters selection markers, and suicide genes, then subsequently provide an update on recent advances of suicide gene modifications of PSCs in order to control the behavior of their wayward progenies in vitro and in animal models.

Vector Targeting Strategies

Vectors or vehicles are required in suicide gene therapy for efficient and selective delivery of function genes to hiPSCs. A wide variety of vectors for use as gene transfer delivery vehicles have been developed to date for cancer gene therapy, including viral vectors[17] and non-viral plasmids[18] (**Table 1**). RV, LV, adenovirus (AV), and adeno-associated virus (AAV) are widely used in cancer gene therapy studies, whereas Epstein Barr virus (EBV), herpes simplex virus (HSV), and baculovirus (BV) are occasionally used. Of these, AV[19] and RV[20] have been used in clinical trials. Each of these viral vectors has certain drawbacks that restrict their applications in hiPSCs-based cell therapies. Although AVs have wide cellular tropism, they do not integrate into the host genome and cannot be expressed in progenies of transplanted cells, resulting in limited duration of gene expression. AAV,[21] a small virus that does not cause disease in humans, integrates at a specific site on chromosome 19 (AAVS1) in the human genome; however, it has a lower transduction efficiency in embryonic stem cells and cannot accommodate a large amount of foreign DNA (≤ 4.7 kb). RVs have relatively high transduction efficiency, but are readily silenced. And when randomly incorporated into the host genome, they may cause insertional mutations. Although LVs[22] show high transduction efficiency and are seldom inactivated, they too pose a risk of insertional mutagenesis. EBV,[23] HSV,[24] and BV[25] are all non-integrating viruses and mediate transient transgene expression in human cells, although they can transduce both dividing and non-dividing cells. Non-viral plasmids usually inefficiently transfect target cells and may cause insertional mutagenesis when transferred by liposome or electroporation.

Promoter and Selection Strategies

The promoters for controlling suicide gene expression can mediate control of cell transplants in vivo and a number of promoters has already been investigated with positive results in cancer gene therapy.[16] The promoter chosen to control wayward pluripotent

stem cells and their progenies in vivo for clinical applications of hiPSCs should be capable of mediating stable, highly active suicide gene expression in human PSCs. Three kinds of promoters are available for driving suicide gene expression: constitutive, cell specific and inducible.[16]

Constitutive promoters, such as the cytomegalovirus (CMV) promoter, phosphoglycerate kinase (PGK) promoter and ubiquitin C promoter, are commonly used to drive ectopic gene expression in various gene transfer applications in vitro and in vivo. They usually achieve high levels of gene expression in most cell types.[26] However, these promoters may be transcriptional silenced due to extensive methylation. Previous studies have also shown that elongation factor 1α (EF1α) and constitutive cytomegalovirus (CMV) enhancer/chicken β-actin promoter (CAGG promoters are consistently strong in all mammalian cell types tested, whereas the CMV promoter is the most variable, being very strong in some cell types and rather weak in others.[27] Norrman et al. found that ACTB (human β-actin promoter), EF1α and PGK promoters showed stable activities during long-term culture of undifferentiated hESCs.[26] Therefore, a promoter that can mediate stable, highly active suicide gene expression in human PSCs should be chosen for the clinical translation of hiPSCs in order to control wayward pluripotent stem cells and their progenies in vivo.

Cell-specific promoters can be used for applications in which the suicide gene is active in certain cell types in order to restrict transgene expression to targeted tissues, thereby reducing side effects while increasing therapeutic efficacy. Wu et al.[28] developed a viral vector platform combining glial fibrillary acidic protein (GFAP) promoter-based transcriptional targeting with miRNA regulation to control the expression of a gene for glioma suicide gene therapy in the mouse brain. They found that these vectors enabled effective elimination of human glioma xenografts while producing negligible toxic effects on normal astrocytes. Niess et al.[29] used bone marrow mesenchymal stem cells (MSCs) that were modified by a suicide gene under the control of the Chemokine (C-C motif) ligand 5 (CCL5) promoter for gene therapy of hepatocellular carcinoma. They found that transplanted MSCs were recruited to the tumor site, where they differentiated and participated in tumor angiogenesis following tumor-specific activation of CCL5 promoters. CCL5/HSV-TK-transfected MSCs in combination with ganciclovir (GCV) supplementation significantly reduced tumor growth by 56.4% compared with the control group. As for the removal of PSCs in vitro and in vivo, control of suicide gene expression using a pluripotent cell-specific promoter (e.g., OCT4, Nanog) is a better choice and helps to specifically eliminate residual PSCs or progeny cells that have reverted to a pluripotent state.

An ideal promoter for hiPSC-based cell replacement therapies should allow a suicide gene to be regulated quickly and effectively in vivo by an exogenous signal that activates/inactivates the control cassette. For example, the expression of a suicide gene might be under the control of a regulatable promoter so as to turn on only when the stem cell or its progeny form tumors or overproliferate following addition of an exogenous signal. Inducible promoters regulated by tetracycline (Tet), cAMP, rapamycin, or steroid hormones have been constructed and successfully utilized in cell lines and animal models.[16] The construction of TetON/TetOFF regulatable systems has been reported for all AV, LV, RV, HSV, and AAV vectors. These vectors can tightly regulate transgene expression downstream of a TRE promoter in the presence/absence of tetracycline in vitro and in vivo.[30]

It is necessary that the genetic modification of all hiPSCs-derived cells should be confirmed before cell replacement therapy. Thus, an efficient selection method is essential for obtaining a pure population of transduced cells. Insertion of an antibiotic coding sequence into vectors is an effective strategy that allows single colonies to be selected for further expansion after antibiotic selection.

Suicide Gene Strategies

It is of great importance to choose an appropriate functional gene to eliminate tumor-initiating cells before and/or after cell implantation. Suicide genes, including toxin, apoptotic, tagging and drug-conversion genes, are widely used in cancer gene therapy.[16] Genes for toxins (e.g., Shiga toxin A1, Pseudomonas exotoxin A) induce cell death by inhibiting protein synthesis,[31,32] whereas apoptotic genes (caspase-3, caspase-8, et al.) eliminate cancer cells by inducing apoptosis.[33,34] Both kinds of genes are active in dividing and non-dividing cells. For such genes, control by inducible promoters is much preferred to ensure that their expression is totally shut off and is activated only when appropriate signals are administered. A tagging gene expressed in the plasma membrane can be transferred into cells before implantation.[18] An anti-tag monoclonal antibody carrying a therapeutic agent is then able to subsequently destroy the tagged cells. This tagging strategy also risks bystander toxicity because of the specificity and affinity of the anti-tag antibody and potential immunogenicity to our body.

Gene-directed enzyme prodrug therapy (GDEPT) is the most widely used strategy in cancer gene therapy.[35] This type of suicide gene converts a nontoxic drug to a toxic drug in gene-modified cells. Examples of genes used in GDEPT include herpes simplex virus thymidine kinase (HSV-TK), cytosine deaminase (CD), varicella-zoster thymidine kinase (VZV-TK), and nitroreductase (**Table 2**). HSV-TK phosphorylates nucleoside analogs, including acyclovir and ganciclovir (GCV, a prodrug approved by the US Food and Drug Administration). The resulting acyclovir triphosphate or GCV triphosphate incorporate into DNA via the action of DNA polymerase, leading to chain termination and cell death.[36] As such, they are highly toxic toward dividing cells. The CD gene encodes cytosine deaminase, which converts 5-fluorocytosine (5FC) into the cytotoxic 5-fluorouracil (5-FU), which eradicates cells.[37] The VZV-TK enzyme monophosphorylates 6-methoxypurine arabinonucleoside (ara-M) to ara-MMP. Then ara-MMP is metabolized to the highly toxic form, ara-AMP, which can significantly inhibit cell growth.[38] Nitroreductase (NR) converts CB1954 to its 4-hydroxylamino derivative and then to acetylate, becoming a powerful bifunctional alkylating agent that causes the formation of poorly repaired DNA cross-links.[39] One other promising suicide gene is carboxypeptidase

Table 2. Prodrug-activating system

Gene	Prodrug	Function	Bystander effect
HSV-TK	Ganciclovir	Metabolizes to a triphosphate nucleotide that competes with dGTP for DNA polymerases	weak
CD	5-fluorocytosine	Inhibits thymidylate synthetase and DNA synthesis	strong
VZV-TK	ara-M	Competes with dATP for incorporation into DNA, leading to termination of DNA synthesis	weak
NR	CB1954	DNA interstrand crosslinking	strong
CPG2	Nitrogen mustard L-glutamates	DNA interstrand crosslinking	strong

G2 (CPG2), which catalyzes the hydrolysis of nitrogen mustard prodrugs, then releases glutamic acid and the cognate drug. It has no mammalian equivalent, and no additional activating steps are required to produce the active DNA crosslinking molecule.[19,40] All GDEPT systems described above produce a toxic agent that can remove cancer cells efficiently, except for VZV-TK/ara-M, which generates weakly toxic metabolites; thus, other prodrugs need to be developed to augment the efficacy of the VZV-TK suicide gene system against cancer cells. CD, NR, and CPG2 usually convert prodrugs to diffusible toxic drugs; this offers an advantage for in vivo cancer gene therapy because it affects cells in the local milieu besides the tumor (bystander effect). However, HSV-TK and VZV-TK metabolites are phosphorylated and cannot cross the cell membrane, resulting in a lesser bystander effect. GDEPT is preferable in cancer gene therapy because the toxic drug product produces a bystander effect, acting on both the cell expressing the enzyme and cells in the local milieu. However, application of GDEPT to regenerative medicine can problematic because the bystander effect may cause undesirable damage to adjacent normal tissue.

Suicide Gene Applications for Pluripotent Stem Cells

The pluripotency of PSCs and the malignant transformation and/or reversion to pluripotency of differentiated cells pose the risks of tumor formation posttransplantation. Genetic strategies of suicide gene system can provide effective and safe control to prevent adverse events. Suicide gene applications for improving the safety of embryonic stem cells have been reported by several different groups (**Table 3**). Most have utilized lentivectors containing the HSV-TK gene under the control of a constitutive promoter (CMV, ubiquitin C, PGK).[41-43] Both prevention and elimination of teratomas has been demonstrated using these GDEPT strategies. More recently, Tang et al.[44] found that an antibody against SSEA-5 glycan on human embryonic stem cells enabled the removal of teratoma-forming cells in vitro. However, this method does not function well if a progeny cell reverts to pluripotency in vivo; moreover, it is not known whether normal somatic cells express SSEA-5. Wang et al.[45] demonstrated that addition of mifepristone-inducible caspase-1 to mouse ES cells eliminated tumor formation but spared differentiated dopamine neurons, both in vitro and in vivo. However, whether other lineages formed from differentiated caspase-1 cells can survive mifepristone is not known.

Selective ablation of undifferentiated ES cells has been achieved through expression of the HSV-TK gene under the control of the Oct4 promoter, followed by GCV treatment.[46,47] Oct4 is essential for the maintenance of the pluripotency and self-renewal capacities of ES/iPS cells. However, it has been reported that constitutive expression of Oct4 from an exogenous promoter is not sufficient to prevent ES cell differentiation,[48] and it was found that Oct4 is broadly expressed in different cell types.[49] Accordingly, Oct4-controlled suicide genes may cause undesirable damage to differentiated cell populations. Furthermore, Naujok et al. reported that Oct4-TK ES cells treated with 1 μM (255 μg/mL) GCV during in vitro differentiation over a relatively long period (14 d) prior to implantation still developed into tumors, albeit significantly smaller tumors than those formed from untreated cells.[47] Rong et al.[50] reported a scalable approach for preventing teratoma formation by human embryonic stem cells. They introduced a hyperactive variant of the HSV-TK gene into the 3' untranslated region of the endogenous Nanog gene of hESCs through homologous recombination. Using this approach, they were able to demonstrate elimination of teratomas generated by hESCs without apparent negative impacts on the differentiated cell types derived from them.

Only a few groups have reported the use of suicide genes to improve the safety of induced pluripotent stem cells. CD/5FC and iCaspase/AP20187 (caspase 9) systems have been used to safeguard nonhuman primate iPS cells.[51] However, in vitro killing of iCaspase cells by AP20187 was relatively slow compared with that observed in CodA/5-FC cells, and the in vivo function of AP20187 has not yet been determined. In our research,[52,53] we modified mouse/human ES/iPS cells to contain the suicide gene deltaTK or CodA under the transcriptional control of the EF1α or Nanog promoter. The suicide gene was introduced via lentivirus transduction without interfering with the self-renewal or pluripotency characteristics of ES/iPS cells. We found that EF1α promoter-controlled deltaTK/CodA expression efficiently eliminated pluripotent stem cells and their derivatives, both in vitro and in vivo. When the suicide gene was under the control of the Nanog promoter, tumor-initiating, undifferentiated pluripotent stem cells were selectively ablated in vitro after prodrug treatment. deltaTK was chosen because this truncated form of HSV-TK enhances the specificity of cell ablation and because deltaTK transgenic male mice are fertile, in contrast to animals expressing intact TK.[54] Chambers et al. found that transgenic expression of Nanog was sufficient for clonal expansion of ES

Table 3. Safeguarding strategies in pluripotent stem cells

Vector	Promoter	Suicide gene	Cell types	Function	Reference
Non-viral plasmid	PGK	HSV-TK	hESCs	Ablation of teratomas	43
Lentivirus	Ubiquitin C	HSV-TK	mESCs	Prevention of teratoma formation	42
Lentivirus	CMV	HSV-TK	mESCs	Ablation of teratoma formation	41
Knock-in at the endogenous locus of OCT4	OCT4	HSV-TK	mESCs	Prevention of teratoma formation in spheroids culture	46
Non-viral plasmid	OCT4	HSV-TK	mESCs	Prevention of teratoma formation	47
SSEA5	NA	Anti-SSEA5 antibody	hESCs/hiPSCs	Prevention of teratoma formation	44
Lentivirus	SFFV	CD/iCaspase9	Macaca nemestrina iPS cells	Prevention and ablation of teratoma formation	51
Non-viral plasmid	mifepristone-inducible promoter (GAL4 UAS)	Caspase-1	mESCs	Ablation of teratoma formation	45
Knock-in at the endogenous locus of Nanog	Nanog	Enhanced mutant version of HSV-TK	hESCs	Prevention of teratoma formation	50
Lentivirus	EF1a/Nanog	DeltaTK/CD	hESCs/hiPSCs mECSs/miPSCs	Prevention and ablation of teratoma formation	52, 53

cells, independent of Stat3 (signal transducer and activator of transcription 3) activation status, indicating that Nanog is central in the transcription factor hierarchy that defines the ES cell state.[55] Our results showed that a much lower concentration of GCV (10 μg/mL vs. 255 μg/mL) and much less time (5 d vs. 14 d) was required to eradicate pluripotent stem cells using the Nanog-deltaTK system than was observed with the Oct4-TK system.

Future Directions

An optimal suicide gene strategy for the safe application of hiPSCs is one that has high transfection efficiency and does not cause insertional mutagenesis. Transgene expression should be stable and maintained in all stem cells and their progeny in vitro and in vivo. Unfortunately, the vectors described above do not yet meet all these requirements. One of the best approaches for improvement is to recombine suicide genes in "safe harbor" sites or in the intron of a specific gene (e.g., Nanog, β-actin) of the hiPSCs genome through homologous recombination using new genetic manipulation technologies with high efficiency, such as zinc finger nucleases (ZFNs)[56] and transcription activator-like effector nucleases (TALENs).[57] ZFNs combine the nonspecific cleavage domain of the FokI endonuclease with DNA-binding domains of zinc finger proteins.[59] Genomic alterations, including point mutations, deletions, insertions, inversions, duplications and translocations, can be introduced by ZFNs with high efficiency.[60] TALENs that can be very easily and rapidly designed have similar structure and function to ZFNs and have been an alternative to ZFNs for genome editing by introducing targeted double-strand breaks into specific sites of the genome with similar efficiency.[60] However, the gene-targeting technology used for controlling wayward stem cells may result in the destruction of all transplanted cells, including properly functioning cells, through constitutive expression of the suicide gene or bystander effects. And when excess proliferation, inappropriate differentiation, and/or improper localization of transplanted cells occur, pluripotent gene promoter-controlled suicide genes may not function well. Therefore, a dual system comprising a suicide gene under the control of constitutive and a cell-specific promoter, both transferred by knock-in technology, would greatly aid in controlling transplanted cells in a "failsafe" manner.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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