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Final Paper
16 March 2012

Stem Cell Gene Therapy as a Treatment for Diverse Genetic Disorders

Mendelian genetic disorders are diverse in their pathophysiology, symptoms, and prognosis, yet have in common the underlying cause of a DNA lesion. Can this shared source of causation translate into a shared approach for treatment of these disorders, or perhaps even a shared cure? Gene therapy, in which diseases are treated by introduction of exogenous, normal DNA to supplement or replace a defective gene or genomic region, could in principle represent such a "silver bullet" for genetic disease. Yet progress in the field of gene therapy has been slow. *In vivo* approaches, in which a gene (usually borne in a viral vector) is administered directly to the patient, have generally failed to confer appreciable expression or improve symptoms, and, in some cases, have proven intolerable due to inflammatory response (Selkirk 2004).

The alternative approach of *ex vivo* gene therapy, in which autologous cells are harvested from the patient, modified, and then subsequently reintroduced, has shown greater promise and is of particular interest given accelerating progress in the stem cell field. For some genetic disorders, particularly those affecting hematopoietic-derived cell lineages, allogeneic transplantation of stem cells from an HLA-matched donor is considered the best curative modality (e.g., for Wiskott-Aldrich syndrome; Boztug et al. 2010). A number of clinical trials are investigating use of genetically modified, autologous stem cells in this context (ClinicalTrials.gov). At present, such *ex vivo* correction of autologous stem cells seems to represent the most realistic and feasible form of gene therapy for genetic disease. Here, I will explore whether this approach might realistically provide an approach to treating diverse types of genetic disease, considering a) technical challenges in generation of safe, clinically suitable modified stem cells, and b) how the properties of a given genetic disease may affect its suitability for stem cell gene therapy.

A major and general concern in stem cell gene therapy is whether patient-derived stem cells can be genetically modified in a way that avoids (or adequately minimizes) the risk of genotoxicity, clonal expansion, and the development of malignancies in the modified cells. To date, clinical trials involving

genetic correction of stem cells have typically employed integrating viral vectors to deliver a complementing transgene (ClinicalTrials.gov; rev. in Riviere et al. 2012). As such vectors insert randomly or near-randomly in the genome, transduction of stem cells represents a form of "obligatory insertional mutagenesis" and can result in disruption or altered expression of endogenous genes (Riviere et al. 2012). In particular, disruption of a repressive regulatory element or ectopic activation due to sequences within the transgene (such as the LTR sequences of γ-retroviruses) can result in overexpression of proto-oncogenes, causing clonal expansion and ultimate malignant transformation of the genetically modified stem cells. Severe adverse events of this type have been observed in two separate clinical trials, with several patients who received stem cell therapy for X-linked severe combined immunodeficiency (X-SCID) or Wiskott-Aldrich syndrome developing subsequent leukemias associated with the transgene insertion (rev. in Krause 2011).

As discussed for hematopoetic stem cells in an excellent review by Riviere et al. (2012), there are a number of alterations to the gene modification process, including changes to viral vectors, genetic transfer methods, and cell types used, that might reduce the chance of genotoxic events in transduced stem cells. Foremost among these is the use of alternative viral vectors that differ from first-generation γ-retroviral vectors in their regulatory elements and genomic integration patterns. Recently developed vectors lack the transcriptional-activatory LTRs present in the original γ-retroviral vectors (known as "self-inactivating vectors"), and many are also based on HIV-1 derived lentiviruses, which have the advantage of integrating nearly completely randomly in the genome (in contrast to γ-retroviruses, which favor promoter regions and may thus be inclined to perturb gene expression; rev. in Riviere et al. 2012). Additional design factors that might enhance safety of integrating viral vectors are the use of weaker promoters to drive transgene expression, the inclusion of "insulator" sequences to buffer nearby genes from enhancers within the transgene, and, when feasible, the use of promoters limited in their expression to a defined cell type, reducing the likelihood of aberrant activation of neighboring genes in other cell types (Riviere et al. 2012). Unfortunately, even a perfectly insulated transgene cannot fully eliminate the risk of transgene-associated oncogenesis (for instance, by insertional disruption of a negative regulatory

element upstream of a proto-oncogene, or by alteration of chromatin structure in the neighborhood of an oncogene). Nonetheless, these vector modifications might, in combination, reduce the frequency of malignant mutagenic events to a low, clinically acceptable level.

More sophisticated analysis of transgene insertions or retrovirus-free modification techniques could, in principle, further reduce the risk of mutagenesis associated with transgene insertion (Riviere et al. 2012). For example, individual, clonal lines of transduced stem cells could be established, the insertion sites in each line analyzed, and only those lines with insertions far from proto-oncogenes used therapeutically (Riviere et al. 2012). The complication of this approach is that adult hematopoetic stem cells and, presumably, other adult stem cell types, are difficult to manipulate in culture and cannot be appreciably expanded or subcloned. Thus, for clinical applications, patient stem cells must be transduced and infused in bulk, such that a number of distinct insertion events are represented in the reintroduced cells and insertion sites cannot be characterized prior to infusion (Riviere et al. 2012). Alternatively, the genetic modification technique itself could be altered to avoid insertional mutagenesis, for example, by utilizing homologous recombination rather than viral vector-mediated insertion to introduce a normal copy of the disease gene. A major limitation of homologous recombination is its low efficiency, with modification frequencies of around 0.5% to 1.0% in hematopoetic stem cells (as compared to 25-50% for γ-retroviral vectors; values from Riviere et al. 2012 and Kohn et al. 2003, respectively). The frequency of homologous recombination can be increased dramatically by the induction of double-stranded DNA breaks using zinc finger nucleases, which may be engineered to recognize and cleave at the site of the target mutation (Yusa et al. 2011; Riviere et al. 2012). While this approach shows promise, is relatively immature, and its efficiency in primary stem cells, as well as the possibility of off-target cleavage (resulting in possible deletions or translocations), needs to be evaluated (Riviere et al. 2012).

An alternative approach that circumvents the intractability of adult stem cell types in culture is the use of patient-derived induced pluripotent stem cells (iPSCs), which can be generated from readily accessible patient cell types, reprogrammed to pluripotency, and, in principle, induced to acquire the multipotent stem cell, progenitor cell, or terminal fate of choice (Riviere et al. 2012; Stadtfeldt and

Hochedlinger 2010). Because iPSCs can be expanded in culture, clonal lines can be established following genetic modification (transduction with viral vectors, zinc finger nuclease-assisted homologous recombination, etc.), and these lines can be extensively analyzed for insertion site location, chromosomal abnormalities, copy number variants, and even point mutations acquired during culture (via genomic or transcriptomic sequencing; Yusa et al. 2011, Riviere et al. 2012). In an elegant study providing proof of concept for the use of genetically modified autologous iPSCs to treat genetic disease, iPSCs derived from tail fibroblasts of a sickle cell anemia mouse were corrected *ex vivo* via homologous recombination, induced to differentiate as hematopoetic progenitors via HoxB4 infection, and re-introduced into conditioned sickle cell animals (Hanna et al. 2007). The genetically modified cells engrafted successfully, such that modified cells were detected stably in the bloodstream for three months, and treated mice showed significant cellular and functional improvement, including an increase in red blood cell numbers, increased hemoglobin levels, better kidney function and urine concentration, and improved overall condition (increased body weight and less frequent breathing).

Despite these encouraging preliminary results, there is consensus in the research community that iPSCs are not yet ready for transplantation in a clinical context. Of particular concern are their potential for uncontrolled proliferation (especially when reprogramming factors are introduced via integrating vectors), their incompletely characterized epigenetic differences from *bona fide* embryonic stem cells, and their tendency to accumulate genomic abnormalities in culture (Stadtfeldt and Hochedlinger 2010; Riviere et al. 2012). The last point is particularly worrisome: in a recent study, two out of three iPSC lines established from fibroblasts displayed chromosomal abnormalities (duplications or deletions ranging from 20 kb to 1.3 mb; Yusa et al. 2011). Further, iPSCs genetically modified by zinc finger nuclease-assisted homologous recombination were found to bear 29 single-nucleotide exome mutations relative to the parental fibroblasts, of which 24 had occurred during the derivation of the iPSC (rather than during the modification process; Yusa et al. 2011). This observation is consistent with the hypothesis that reprogramming of cells to an iPS state may require accumulation of rare, permissive genetic or epigenetic

changes, perhaps including mutations that bias cells to proliferation and transformation (rev. in Riviere et al. 2012).

Beyond safety considerations, there are also significant practical obstacles to the use of iPSCs in stem cell transplantation, notably a lack of knowledge about the factors needed to induce specific multipotent stem cell identities in iPSC derivatives. For instance, for both hematopoetic and mesenchymal lineages, the multipotent stem cells derived from iPSCs via current methods differ from adult stem cells and have more “embryonic” properties (including, in the case of hematopoetic stem cells, production of derivatives that express fetal hemoglobin; Giuliani et al. 2011; rev. in Jung et al. 2011; rev. in Riviere et al. 2012). While these distinct properties may be beneficial in certain therapeutic contexts, use of iPSC-derived stem cells to replace adult stem cells in established therapies (e.g., hematopoetic stem cell transplant) would require protocols that induce iPSC differentiation into more mature stem cell types.

The questions discussed thus far concerning stem cell gene therapy have been essentially technical, concerning how a stem cell may be modified with minimal genotoxicity. Yet even if an optimal technique were available, and genetically modified stem cells of a desired type could be readily and safely generated, it remains unclear whether stem cell gene therapy would constitute a suitable treatment for all, or even many, genetic disorders. The fundamental question is simply whether most genetic diseases are, by the nature of their underlying defects and the cells they affect, amenable to the type of stem cell-based replacement therapies effectively used to treat certain hematopoetic disorders. Indeed, at present, the most successful “case studies” of stem cell gene therapy involve disorders primarily affecting hematopoetic-derived cells, such as SCID (affects T, NK, and B cells), Wiskott-Aldrich syndrome (affects platelets and immune cells, and adrenoleukodystrophy (ALD, affects hematopoetic-derived microglia; Aiuti et al. 2009; Boztug et al. 2010; Cartier et al. 2009; rev. in Thrasher and Candotti 2005).

The basic procedure used for treatment of such disorders via autologous transplant involves extraction of the relevant type of patient stem cells (hematopoietic stem cells), modification of stem cells to complement the genetic defect, partial or complete elimination of the defective cellular system within the patient’s body (via chemotherapy or irradiation), and re-introduction of the corrected stem cells to

repopulate the system. For a disease such as Wiskott-Aldrich syndrome, this process can be highly effective because the symptoms and defects caused by the disorder are reversible, because all of the affected cells are derived from a single, well-defined adult stem cell type, because gene-corrected cells have a selective advantage *in vivo*, and because the steps described above (stem cell isolation, systemic ablation, and repopulation with corrected stem cells) can be carried out successfully in the hematopoietic system (Marangoni et al. 2009; rev. in Thrasher and Candotti 2005).

It is less clear whether a similar procedure would be as effective for disorders that differ from Wiskott-Aldrich syndrome with regards to these parameters. Even among hematopoetic disorders for which established bone marrow transplantation protocols can be used, there is considerable latitude in the success of stem cell gene therapy that correlates with disease properties, notably the interactions of modified stem cells with their endogenous counterparts. In the ideal case, genetically corrected stem cells or their progeny have a selective advantage over uncorrected cells, e.g., because the disease defect reduces cell fitness. This situation facilitates engraftment of the genetically modified cells and, correspondingly, means that engraftment can be achieved with milder conditioning regimens than would otherwise be necessary (Thrasher and Candotti 2005). The importance of selective advantage for engraftment of corrected cells is illustrated by the case of adenosine deaminase deficiency-induced SCID (ADA-D SCID). When stem cell gene therapy was initially used to treat this disorder in combination with enzyme replacement therapy, disappointing results were obtained. Several lines of evidence suggest that stem cell engraftment failed because enzyme administration compromised the selective advantage of the modified cells or their progeny, and subsequent trials in which enzyme therapy was eliminated have yielded marked improvements in patient condition (Thrasher and Candotti 2005).

In assessing the feasibility of stem cell gene therapy as general treatment for diverse types of genetic disorders, however, it is necessary to move beyond the hematopoetic system and consider the applicability of similar stem cell-based gene replacement techniques in other systems. The hematopoetic system is somewhat unique, because of both the long history of bone marrow transplantation and the extensive characterization of hematopoetic stem cells (Biochem 258 lecture, 23-Feb. Clinical trials

involving transplantation of other stem cell types (including mesenchymal stem cells, neural stem cells, and endothelial progenitor cells) are underway, and some have reported modest improvements in patients with genetic disorders (e.g., mesenchymal stem cell transplant for osteogenesis imperfecta, Hurler syndrome, and metachromatic leukodystrophy; rev. in Kassem et al. 2004; ClinicalTrials.gov). Although occasional trials involve gene therapeutic correction of autologous stem cells (e.g., in epidermal stem cell therapy for Netherton syndrome; trial NCT01545323, ClinicalTrials.gov), most involve allogeneic transplantation and are unlikely to incorporate gene therapy until a transplantation benefit has been demonstrated. It is thus difficult to assess the feasibility of stem cell therapeutic approaches for treatment of diverse genetic disorders based on clinical results, and may instead be useful to consider, from a more abstract perspective, what properties would tend to make a genetic disorder a good candidate for such an approach (Table 1).

Some properties that influence the suitability of a genetic disorder for stem cell gene therapy relate to the genetic lesion and the pathology it causes (Table 1, rows 1 and 2), while others relate to the cellular system(s) affected by the disorder (Table 1, rows 3-5). In terms of specific disease properties, one important consideration is the plasticity of disease phenotype, that is, the reversibility of disease effects. In an ideal disorder, such as Wiskott-Aldrich syndrome, disease phenotypes are largely reversible, and successful replacement of defective cell types can provide an essentially complete cure. In other cases, such as that of adrenoleukodystrophy, stem cell gene therapy may arrest symptom progression but often fails to reverse existing damage (focal demyelination; Cartier et al. 2009). Patient age may play a role in the reversibility of symptoms, and the limited success of stem cell gene therapy trials for chronic granulomatous deficiency has been attributed in part to the use of adult participants (rev. in Riviere et al. 2012). Finally, early developmental abnormalities caused by a syndrome, such as Chiari malformation of the brain and spinal cord in Ehlers-Danlos syndrome, are unlikely to be modified by stem cell therapy (Medscape). Thus, patients with disorders in which the primary defects are developmental, or early-onset and irreversible, might benefit less from stem cell gene therapy than patients with symptomatically reversible disorders.

The specific nature of the causal DNA lesion in a given genetic disorder may also influence how amenable the disorder is to stem cell gene therapy. For instance, patients with mutations affecting a single gene would tend to be good candidates for gene therapy, as provision of the normal copy of that gene could complement the defect. At the opposite end of the spectrum, patients with major chromosomal abnormalities (e.g., trisomy 21) would tend to be poorer candidates for gene therapy, as many genes would be affected and provision of large chromosomal regions (e.g., on artificial chromosomes) is not feasible given current technology. Similarly, due to the limited insert size capacity of retroviral vectors, genetic diseases caused by mutations in relatively small genes would tend to be better candidates than those caused by mutations in very large genes (e.g., Duchenne multiple dystrophy, caused by mutations in the dystrophin gene, which has a ~14 kb cDNA; Selkirk 2004). Finally, the loss-of-function or gain-of-function nature of a mutation, as considered at the level of the cell, has important implications not only for the feasibility of gene therapy but also for the type of conditioning required for symptom resolution. If a disease-causing mutation results in a simple loss of cellular function (the cell acts as an ineffectual "null"), it may be possible to treat the disease using a very mild conditioning regimen, as it is not necessary to destroy all diseased cells but rather to introduce a sufficient number of modified cells to provide the needed function. It is also possible that the modified cells have a selective advantage in this scenario, as described above for hematopoetic disorders (Thrasher and Candotti 2005). If, on the contrary, a disease mutation causes cells to acquire new, aberrant or interfering activities, it may be necessary to use a strong conditioning regiment to fully ablate endogenous cells before introducing the modified stem cells.

Additional properties that influence a genetic disorder's suitability for stem cell gene therapy relate to the cellular systems it affects. For instance, the smaller the number of cell types affected by the disorder and the more closely related these cell types are (the more recent their common progenitor), the more likely that stem cell therapy will be able to resolve a substantial fraction of the patient's symptoms. Of particular importance is the question of whether all or most involved cell types are derived from a single adult stem cell type, in which case genetic modification of that stem cell type could potentially

provide a complete cure (as in the case of hematopoetic stem cells for SCID and Wiskott-Aldrich syndrome). Involvement of cells from diverse systems would not necessarily rule out gene therapy as a treatment option, but would likely mean an incomplete resolution of symptoms, with symptoms related to the transplanted stem cell's system showing the greatest improvement.

Once the target stem cell and cellular system for transplantation for a given syndrome have been identified, these too can be examined to assess the likelihood of successful stem cell-based gene therapy. The ideal stem cell type for transplantation would share many properties with a hematopoetic stem cell: it would be readily isolated using minimally invasive procedures, have a well-characterized cell surface marker profile allowing purification, survive for at least a brief period *ex vivo*, and be amenable to gene integration (via retroviral insertion or homologous recombination). Additional stem cell properties that might facilitate gene therapy include the ability to grow and expand in culture and the possibility of derivation from iPSCs via expression of specific factors. Finally, the properties of the cellular system populated by the target stem cell would also influence the likelihood of successful stem cell gene therapy. In general, some degree of conditioning (ablation of the endogenous cellular system via irradiation or chemotherapy) is required for successful engraftment of transplanted cells, and in cases where modified cells do not have a selective advantage or where diseased cells exert dominant-negative effects, strong conditioning that destroys essentially all the cells of the endogenous system may be required (Thrasher and Candotti 2005). Thus, the ability to condition the target cellular system safely and specifically, with minimal effects to non-target systems, is essential for the clinical feasibility of stem cell gene therapy for a given disorder. The ease with which modified stem cells can be delivered to niche locations (or home to these locations) following gene therapy may also affect therapeutic success.

While the parameters outlined above provide a framework for assessing the utility of stem cell gene therapy for any given disorder, they do not fully answer the question of whether such therapy is in fact a "silver bullet" suitable for treatment of numerous genetic disorders. To a large extent, the answer to this question will be determined by the path of biomedical research over the next decade: not just clinical trials that investigate safety and efficacy of new methodologies, but also basic biological studies that

clarify the properties and types of adult stem cells, the safety of iPSC cells and factors required for their differentiation, and the safest techniques for generating transgenic stem cells without risk of genotoxicity. Certainly, stem cell gene therapy (as we currently conceive of it) will not provide a panacea for all genetic disorders. But with a sufficient understanding of the mechanisms underlying both genetic disease and stem cell biology, such therapy might become a realistic treatment for a substantial number of disorders, offering a potentially curative option to sufferers of otherwise intractable genetic disease.

Table 1. Properties of disease (phenotypic affects, mutation type, and cellular systems affected) that influence suitability of a genetic disease for stem cell gene therapy.

Parameter	**Positive Factors**	**Negative Factors**
Plasticity of disease phenotypes	Disease effects are reversible Disease effects are solely physiological Young patient	Disease effects are irreversible Disease causes developmental abnormalities Adult patient
Disease mutation type	Disease is caused by a single-gene lesion Disease gene is reasonably small in size (can fit in a viral vector) Mutation is loss-of-function as defined at the cellular level (diseased cells are "nulls" with reduced function/fitness)	Disease is caused by a chromosomal abnormality Disease gene is very large or requires large regions of regulatory DNA Mutation is gain-of-function as defined at the cellular level (diseased cells have new, aberrant or interfering activities)
Lineage relationships of affected cells	Fewer cell types affected Affected cell types all derived from a developmentally recent common progenitor cell Affected cell types all derived from a single adult stem cell type	More cell types affected Affected cell types distantly related, with a common progenitor only early in development Affected cell types derived from multiple adult stem cell lineages (or, cell types are not derived from known adult stem cell lineages)
Properties of relevant stem cell type	Stem cell type can be readily isolated, ideally in large numbers Stem cell type can be purified (known, unique cell surface marker profile) Media for short-term maintenance and/or culture of the stem cell type have been defined Transgenes can be readily integrated into stem cell type Stem cell type can be derived from iPSCs via expression of known factors (future direction, not yet ready for the clinic)	Stem cell type difficult to isolate, or isolation very invasive No fully unique cell surface marker profile known for stem cell type Unknown how to keep stem cells alive *ex vivo* Stem cell type not readily transduced Molecules required to derive stem cell type from iPSCs unknown and/or known to cause transformation
Properties of the relevant cell system	System (or relevant branch) can be selectively ablated without lethality Stem cell niches are accessible for re-introduction of modified cells	No ablation methods for cell system, or ablation methods have off-target effects on other systems Stem cell niches are inacessible

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Acknowledgements

Thanks to Michael Abrash for proofreading and comments on this paper.