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REGENERATIVE MEDICINE

Concise Review: Human Cell Engineering: Cellular Reprogramming and Genome Editing

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ABSTRACT

Cell engineering is defined here as the collective ability to both reset and edit the genome of a mammalian cell. Until recently, this had been extremely challenging to achieve as nontransformed human cells are significantly refractory to both these processes. The recent success in reprogramming somatic cells into induced pluripotent stem cells that are self-renewable in culture, coupled with our increasing ability to effect precise and predesigned genomic editing, now readily permits cellular changes at both the genetic and epigenetic levels. These dual capabilities also make possible the generation of genetically matched, disease-free stem cells from patients for regenerative medicine. The objective of this review is to summarize the key enabling developments on these two rapidly evolving research fronts in human cell engineering, highlight unresolved issues, and outline potential future research directions. STEM CELLS *2012;30:75–81*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The overarching goal of regenerative medicine is to develop processes for creating functional tissues to enable the repair or replacement of damaged and diseased tissues. The realization of this goal is typically envisioned through a two-step process: the first being to derive disease-free starting cells, ideally stem cells that are genetically matched to the recipient; and the second being to modulate these through appropriate differentiation and assembly to achieve a transplantable tissue form. However, in practice, efficacious fulfillment of each of these steps presents serious obstacles and is thus the subject of active research by biomedical scientists. Specifically, in the quest for a suitable source of cells, pluripotent stem cells, such as embryonic stem (ES) cells derived from early embryos (Fig. 1A), offer a particularly attractive avenue to explore. This is because they possess two key features: one, an indefinite self-renewal capability in culture and, two, a very broad differentiation potential to generate all cell types [1]. Hence, in theory, if one can efficaciously derive such cells and then efficiently do gene therapy in them to correct all underlying disease causing mutations, then the resulting cells can serve as the desired inexhaustible source of healthy stem cells. These can subsequently be directed to differentiate into any desired cell type of choice, which can ultimately serve to repair the damaged or diseased tissue of interest. This review aims to provide an overview of the first step in the above cascade, specifically approaches toward engineering disease-free human stem cells that can serve as a viable source of cells for cell-based therapies.

REJUVENATING CELLS FOR REGENERATIVE MEDICINE

During the course of embryonic and subsequent development, cells starting from a pluripotent state differentiate into various cell types with progressively narrower developmental potential. Their cellular and epigenetic programs gradually become less flexible and more defined, resulting in the acquisition of a stable phenotype [2, 3]. Drawing an analogy using Waddington's epigenetic landscape of mammalian development [3], akin to marbles that lose potential energy on going downhill, cells too during the course of development (starting from the unicellular zygote stage) progressively lose their degree of multipotentiality. The marbles eventually settle into valleys that correspond to local minima's and thus represent cell types with stable phenotypes that will be normally found during homeostasis. Occasionally cells in response to external stimuli may crossover to local minima's in their immediate vicinity, if an intervening barrier is not too high. However, to affect a movement uphill toward the top or into a distant valley, two distinct processes that are termed, respectively, as dedifferentiation or transdifferentiation (and referred collectively as cellular reprogramming), a sustained stimulus or driving force is needed.

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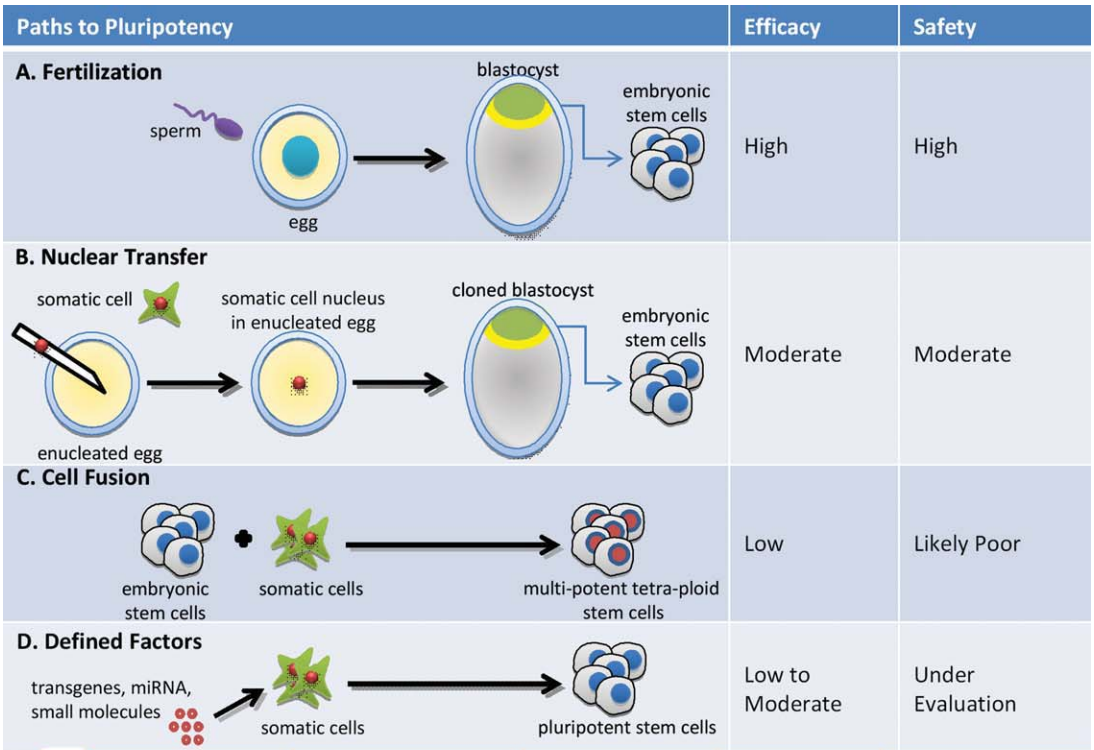


Methods of Reprogramming

Mode of Delivery	Duration of Expression	Genomic Integration	Repeat Infections	Observed Efficiency*
Retro/Lenti-Viruses	Permanent	Yes	No	High
Transposons	Permanent	Yes, but excisable	No	Moderate
Adenoviruses	Short but starting with high level of DNA transfer	No, but could occur	No/Yes	Low to Moderate
EBNA1/OriP Plasmids	Prolonged	No, but could occur	No/Yes	Low to Moderate
Mini-circle Plasmids	Extended short term	No, but could occur	No/Yes	Low to Moderate
Conventional Plasmids	Short Term	No, but could occur	Yes	Low
Proteins	Transient	No	Yes	Low
mRNA	Transient	No	No/Yes	High

* Observed efficiency is also a function of choice of starting cell type: fetal versus adult, somatic versus stem, underlying epigenetic state, and innate proliferation potential.

Stimulatory Factors

Target Function	Target (Reagents)
Modulate immortalization, proliferation and survival potential	p53 inhibition (SV40 large T, p53 shRNA), TERT over-expression, ROCK inhibition (Y27632, Thiazovivin), Anti-oxidants and other small molecules
Modulate underlying epigenetic state	HDAC inhibition (Butyrate, VPA), H3K4 methylation agonists (trancyclopromine hydrochloride), H3K9 methylation antagoinists (BIX01294), CpG DNA methylation inhibition (5-Aza-Deoxycytidine, RG108).
Modulate signal transduction pathways	TGF-beta inhibition (A83-01, SB431542), MEK inhibition (PD0325901), Wnt agonists (Wnt3A, CHIR99021), L-type calcium channel agonist (BayK8644).
Modulate metabolic pathways	Glycolytic Metabolism (PS48), Pleiotropic effects (Myc, Hypoxia, Butyrate)
Modulate miRNA pathways	miR302a, miR302b, miR302c, miR302d, miR367, and miR372

Figure 1. Paths to pluripotency. **(A):** Fertilization of an egg by a sperm results in a totipotent cell that gives rise to the entire embryo proper and to the extraembryonic tissues. This is the process that nature takes and is associated with near perfect efficiency of reprogramming. **(B):** Alternatively one can introduce nuclei of somatic cells into oocytes, however, only a few percent of embryos develop to term. Furthermore, both these processes are also saddled by serious ethical and technical concerns. **(C):** One can also achieve reprogramming by simple cell fusion of somatic cells to embryonic stem cells, but the resultant cells, although multipotent, have tetraploid nuclei and hence are of little clinical relevance. **(D):** Most recently, in vitro reprogramming of somatic cells into pluripotent stem cells was achieved in pioneering experiments by Yamanaka and coworkers, which relied on just the forced expression of four transcription factors: *Oct4*, *Sox2*, *c-Myc*, and *Klf4*. This defined recipe remarkably suffices to restart the cells endogenous pluripotency network. This approach has since been refined and several techniques to achieve reprogramming have now been developed. The appended table summarizes these. In vitro reprogramming back to pluripotency is thus now feasible by various means, but it is also important to note that each of these reprogramming approaches can still have limitations (such as process associated introduction of genetic mutations, incomplete epigenetic reprogramming, etc.) and these need to be fully deciphered and resolved before any attempt at clinical translation. Abbreviations: EBNA, Epstein-Barr nuclear antigen; HDAC, histone deacetylase; miRNA, microRNA; shRNA, small hairpin RNA; TERT, telomerase reverse transcriptase; VPA, valproic acid.

For somatic cells, the ascent (dedifferentiation) has classically been achieved by one of two means: either nuclear transfer into oocytes [4] (Fig. 1B), or fusion with ES cells [5] (Fig. 1C). Transplantation of a somatic cell nucleus into an enucleated oocyte can initiate a striking conversion to an embryonic phenotype. Akin to the use of blastocysts for human ES cell derivation, this process is, however, also saddled by serious ethical and technical concerns [6]. However, it must be noted that not only is cloning inefficient because most cloned embryos die shortly after implantation but also the few that survive to birth frequently have developmental abnormalities and usually a short lifespan. This implies that compared with a fertilized egg from natural mating, the reprogramming of the transplanted nucleus is relatively incomplete. A similar result is also achieved by fusion of somatic cells to ES cells (Fig. 1C); however, the resulting multipotent cells have tetraploid nuclei and thus possess only limited developmental and clinical potential. It has also been found that exposure of somatic cells or nuclei to cell extracts from ES cells or embryonal carcinoma cell lines (roughly a tumor-version of ES cells) can lead to reprogramming to a ES cell-like or more undifferentiated state [7, 8]. Together, these experiments demonstrate that nuclear reprogramming is indeed possible through several means. However, the precise identity and nature of the underlying players for somatic cell reprogramming using these techniques is not easily elucidated.

Merely 5 years ago, a fourth method was developed by Takahashi and Yamanaka[9], first with the murine system in 2006 (Fig. 1D). This approach relied simply on the forced gene expression of four transcription factors: *Oct4*, *Sox2*, *Klf4*, and *Myc* (OSKM) to restart the pluripotency network (Fig. 1D). The resulting cells called induced pluripotent stem (iPS) cells are phenotypically and functionally very similar to ES cells in that they can self renew indefinitely and are pluripotent. This pioneering work and subsequent early publications [10–12] presented the first successful approach to reprogram a mammalian genome to a pluripotent state using defined factors.

Overcoming Hurdles Toward Human Cell Engineering

The publication of this seminal work by the Yamanaka laboratory for mouse cells sparked a frenzy of activity to extend it to the human system. However, it quickly became evident that a direct translation of this approach to human cells was plagued by multiple roadblocks. First, the reprogramming efficiencies for human cells were found to be significantly lower (typically one colony per 10^4 input cells or even lower) [13–16]. Second, the derivation of iPS cells from these also took a significantly longer duration of time, typically 4 weeks or even more (as opposed to just 2 weeks in mouse cells). Both may reflect the fact that normal human cells proliferate much slower than murine cells in culture. Together, these aspects made derivation from human cells a technically challenging process in early days. Furthermore, adult cells were observed to be typically significantly more refractory than embryonic or fetal cells to reprogramming [15]. However, it was imperative for biomedical research to be able to do this reprogramming efficiently in cells from adult or postnatal tissue sources.

Towards addressing these issues, it is instructive to first consider the following analogy for the pluripotency network: the four transcription factors OSKM can be considered as key nodes (genes) of the ES cell regulatory network graph from which it is possible to efficiently reach (activate) all other nodes, that is, kick-start the pluripotency network and effect reprogramming of a somatic cell to a pluripotent state. Now, although these four factors form a sufficient set, however, they need not represent a necessary or optimal starting set. Specifically, judicious inclusion of additional factors (nodes) could hasten this graph traversal, that is, speed up reprogramming, as also would conditions that improve overall graph connectivity, for instance, modulation of the epigenetic state of the somatic cell type. Finally, appropriate choice of a starting cell type that already has a partially activated pluripotency network or favorable epigenetic status would also make it highly amenable to this reprogramming process.

Consequently, toward the goal of improving reprogramming efficiency and efficacy approaches exploring each of these possibilities have been considered by researchers, and entail introduction of additional stimulatory factors to the basic four-factor cocktail. Per the above, these fall into four broad categories (Fig. 1D, table): first, factors that promote cell immortalization, proliferation, and improvement of survival potential of cells, such as SV40 large T antigen, telomerase reverse transcriptase, and reagents that reduce *p53* levels [15, 16]; second, modulation of the underlying epigenetic state of the cells to promote active chromatin marks, specifically, histone deacetylase inhibition (butyrate or valproic acid) [17, 18], H3K4 methylation agonists (trancyclopromine hydrochloride), H3K9 methylation antagonists (BIX01294) [19], and CpG methylation inhibitors (5-aza-deoxycytidine or RG108); third, modulation of key signal transduction and metabolic pathways known to be active in ES cells: specifically, MAPK/ERK kinase inhibition (PD0325901), Wnt agonists (*Wnt3A* or CHIR99021), L-type calcium channel agonist (BayK8644), transforming growth factor β inhibition (A83-01 or SB431542), promotion of glycolytic metabolism (PS48) [20], and factors with pleiotropic effects on metabolism as well as global gene expression (such as Myc, hypoxia, and butyrate) [21]; and finally fourth, modulation of microRNA (miRNA) pathways based on those selectively expressed in the pluripotent state [22]. In fact, in a recent publication, enforced expression of few exogenous miRNAs alone was shown to be sufficient to reprogram mouse and human somatic cells to a pluripotent state [23].

Successful reprogramming of somatic cells requires prolonged overexpression of reprogramming factors. Consequently, retroviruses/lentiviruses were the initial preferred choice of delivery vectors, because upon infecting a cell, they can efficiently integrate into the genome and thus provide the required stable and high levels of transgene expression. However, this feature results in permanent modification of the genome, and hence also significantly raises the risk of insertional mutagenesis [24–28]. Moreover, it was soon realized the expression of reprogramming genes are required only transiently; indeed, they have to be adequately silenced in successfully reprogrammed iPS cells to avoid interference of differentiation programs [11, 12, 29]. Thus, for iPS cells to be relevant in a clinical setting, it was imperative that one derives them using techniques that result in minimal genomic alterations. Consequently, there have also been significant efforts in exploring alternative reagents and approaches to enable derivation of integration-free iPS cells. Specifically, several virus-free and integration-free methods were reported, which generated mouse and human iPS cells by using purified proteins, modified mRNAs, and novel plasmid systems [29–36]. However, as expected, the reprogramming efficiencies using some of these approaches were vanishingly small, and in other cases (such as using proteins or mRNAs), needed cumbersome serial delivery (daily and up to 3 weeks) of adding multiple reprogramming molecules to reprogram fibroblasts. However, recent literature has highlighted that certain

cell types such as fetal neural stem cells [31] and postnatal blood cells (after stimulation in culture) [37, 38] are easy cell types to reprogram to the ES cell-like state and thus also amenable to facile reprogramming even using transient stimulatory techniques as above. Taken together, the above advances have enabled robust and reproducible derivation of human iPS cells from most accessible sources.

If the thus derived iPS cells are to be eventually relevant in a regenerative medicine paradigm, it is also critically important to rid them of any underlying mutations that cause diseases. As an alternative approach of correcting the mutation in iPS cells, one could do gene therapy in the starting somatic cells and subsequently reprogram them to a pluripotent state. However, the lack of self-renewal ability of most somatic cell types makes selection and expansion of rare corrected clones difficult and thus this approach is often not feasible. Thus ES/iPS cells are typically the cell type of choice for effecting genetic mutations. Precise gene targeting by homologous recombination (HR) has played a critical role in genetic studies of various systems, including the generation of knockout/knockin transgenic mouse models using mouse ES cells. However, the efficiency of HR-mediated gene targeting in human ES cells, as in nontransformed human cells, remains low even after nearly a decade since its first report [39]. Only a few studies have been published to date using methods that are commonly performed in mouse ES cells. Using standard plasmid-based systems, the current HR rates are approximately 10^{-6} and usually even lower in normal human ES cells and other nontransformed mammalian cells (Fig. 2). This is further compounded by the fact that human ES cells and iPS cells grow very poorly when plated as single cells (a practice required for selection of rare targeted clones), compared with mouse ES cells. A promising approach toward the same has been the use of zinc-finger nuclease (ZFN) generated site-specific double stranded breaks to stimulate HR efficiencies. Simply speaking, ZFNs are engineered sequence-specific nucleases comprising of two domains: a customized array of zinc-fingers (engineered to bind to a specific DNA sequence) fused to the DNA endonuclease domain from the FokI restriction enzyme [40, 41]. Each zinc-finger domain recognizes 3–4 bp of DNA and a three-finger ZFN can thus recognize approximately 9–10 bp of DNA sequences. When two ZFNs bind cognate target sequences in the proper orientation, the FokI domains can dimerize and create a nuclease that makes a DNA double-stranded break (DSB) between the two cognate sequences. The use of a ZFN pair in this manner also increases the overall sequence specificity, enabling them to precisely target a single unique locus in the genome ($\geq$18 bp by a pair of ZFNs, each with three zinc finger domains). Stimulated by ZFN-induced DSBs, endogenous loci can be targeted with high efficiency by either HR (in the presence of an exogenous donor DNA fragment serving as a repair template) or error-prone nonhomologous end-joining (especially in the absence of a DNA template). Thus, ZFNs have been used to make site-specific genomic modifications with high efficiencies in a variety of (mammalian and plant) cell lines and small organisms. Indeed, now several studies have also reported successful gene targeting by HR-mediated gene insertion at a few selective loci in normal or disease-specific human iPS cells [42–46].

Looking Forward to the Future

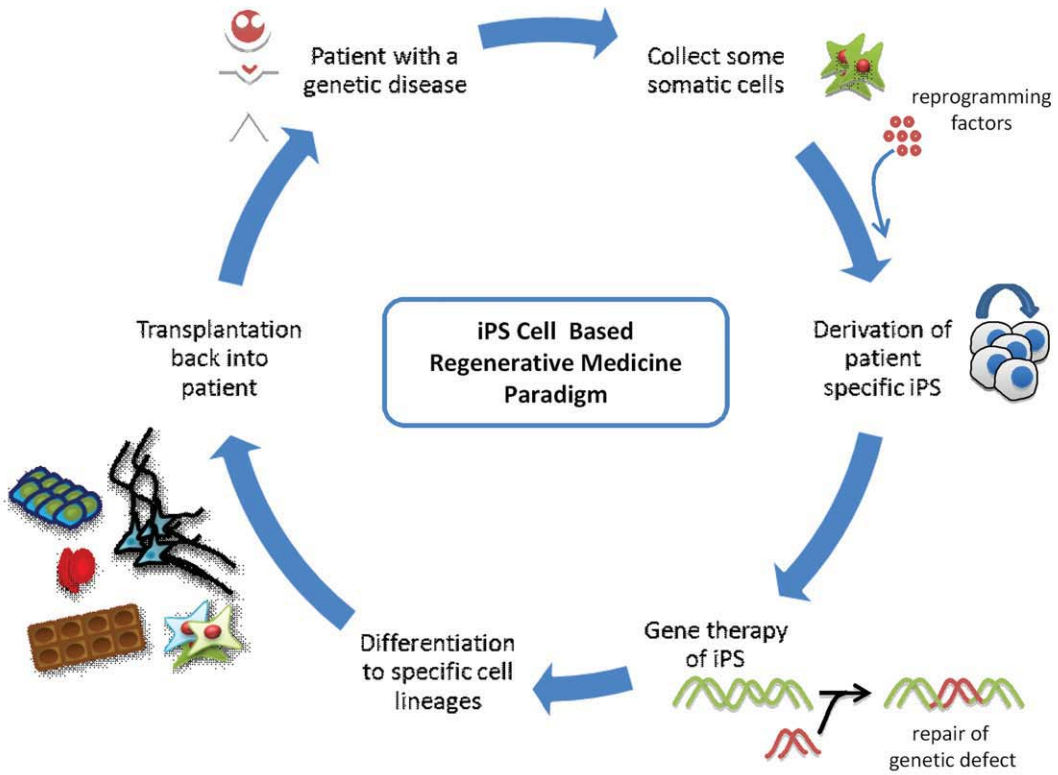
Human iPS cells that are derived from adult somatic cells hold great promise as a renewable cell source for developing patient-specific cell therapies. As we look to understand the issues that still need to be overcome before clinical translation becomes feasible, we recapitulate below the three key steps toward enabling such a regenerative medicine paradigm. These are, first, efficient and efficacious derivation of patient-specific pluripotent stem cells from accessible somatic cell types; second, subsequent facile correction of all underlying genetic mutations to obtain disease-free stem cells; and finally third, scalable differentiation to a functional tissue form suitable for transplantation. Below we analyze the first two steps (the focus of this review) in detail and identify in particular the critical aspects that still need to be addressed and also potential directions that may be explored toward these.

With regards to the first step, that is, derivation of human iPS cells, efficient reprogramming is currently no longer an impeding research issue. Recent improvements have resulted in development of protocols that enable highly robust derivation of integration-free human iPS cells from multiple postnatal human cell types in a research laboratory (Fig. 1B). The facile method by episomal vectors after one round transfection of plasmid DNA (that can be cheaply produced and are stable) for generating high-quality, integration-free iPS cell lines from blood [37, 38] is a particularly attractive approach towards development of a robust technology compliant to future clinical uses.

Recent articles have suggested that early passage iPS cells may also retain a degree of epigenetic memory of their starting somatic cell types which may influence their differentiation ability [47–49]. Preliminary studies into the potential tumorigenecity and other aberrant properties of early versions of iPS cell lines have also been initiated [50–52]. Thus, development of assays for qualifying the efficacy (especially, the safety quotient) of derived iPS *vis-à-vis* ES cells, which are the gold standards for pluripotency, will be a key next step for the reprogramming field. It is important to point out that for most applications in somatic cell regenerative medicine, iPS cells do not need to be the identical to ES cells with an epigenetic signature of an embryonic cell. For instance, the residual epigenetic memory left in derived iPS cells could also provide advantages to differentiate back to the original cell type where the iPS cell line is derived from [47–49].

With regards to the second step, that is, facile correction of genomic mutations in iPS cells, the field still has a long way to go. While the use of ZFNs can stimulate HR rates significantly, it is still not high enough that the step of gene targeting or correcting can be assumed to be either facile (especially for transcriptionally silent loci) or of short enough duration to be adaptable to a clinical setting. Thus, it is important to look beyond just ZFNs and explore other technologies. A few of note are (Fig. 2): the recently emerging TAL effector nucleases (TALENs) (which are significantly more modular than ZFNs) [53, 54], adeno-associated viruses (which are efficient at targeting several human somatic cell types) [55, 56], gutless adenoviral vectors that allow high-level gene transfer and large cargos of longer homology arms for increasing HR [57], and bacterial artificial chromosome-based plasmid vectors with extremely large homology arms [58] are all active avenues that merit further exploration. We believe that development of enabling technologies on this front will be a very intense field of research in the near future, and progress here will have far reaching impact not just in regenerative medicine but also in the general field of gene therapy and disease modeling.

Ultimately, targeted differentiation and assembly into a transplantable tissue form of the disease-free iPS cells will be critical to achieve the goal of cell-based patient-specific therapies. Although there are still several hurdles to surpass and not



Techniques for Gene tic Modifications

Technique	Observed Efficiency*	Notes
Homologous recombination (HR) using a repair donor template	~ 10^{-6}	Typical donor templates bear two large homology arms (>4-10-Kb).
HR using repair donor templates delivered by BACs or gutless adenoviral vectors	~ 10^{-6}	Efficient delivery of larger homology arms is enabled using these vectors, and can improve efficiencies by several fold.
HR stimulated by using a target site-specific nuclease (ZFN or TALEN) in conjunction with a repair donor template	~10^{-1}-10^{-6}	Efficiencies upon induction of a double-strand-break (DSB) at the target site are stimulated by several orders of magnitude, esp. when the donor template homology arms are small. However, off-target DSB induction can cause cytotoxicity, and NHEJ can also create mutations in the genome.
HR using adeno associated virus (AAV)	~10^{-2}-10^{-6}	Is a moderately efficient tool for targeting some somatic cells and certain expressed genes in ES/iPS cells.
Therapeutic transgene insertion into a random locus using retro/lenti-viruses, transposons	Very high	Can be used to deliver genes to most cell types, but often associated with genome alterations by vector integration and potential oncogenic transformation
Therapeutic transgene insertion into a defined locus (e.g., the AAVS1 safe harbor) via HR	~10^{-1}-10^{-6}	Transgene is inserted into a characterized and safe locus using (nuclease stimulated) HR.

•Observed efficiency is also often a function of the target genomic locus, in particular, if it is an expressed or silent region.

Figure 2. A human induced pluripotent stem (iPS) cell-based regenerative medicine paradigm. This approach proceeds in three steps and entails, first, collecting a patients somatic cells (such as their skin or blood cells); next, directly converting them into pluripotent stem cells (that is into cells which now have the ability to differentiate into all three germ layers); and then correcting their endogenous disease causing mutations to obtain immune-matched disease-free stem cells suitable for potential cell-based therapies. The appended table lists the various techniques for performing genetic modifications. Finally, the thus derived healthy stem cells are modulated through appropriate differentiation and assembly into a transplantable tissue form. Note that, the derived iPS cells can also serve as a valuable tool for basic science research, enabling disease modeling, and potential drug screening and toxicological studies of human cells that are otherwise not directly feasible in human subjects. Abbreviations: AAV, adeno-associated virus; BAC, bacterial artificial chromosome; DSB, double-stranded break; ES cell, embryonic stem cell; HR, homologous recombination; iPS cell, induced pluripotent stem cell; TALEN, TAL effector nuclease; ZFN, zinc-finger nuclease.

all differentiation paradigms are equally mature, nonetheless, examples of attainment of functional differentiated tissues are regularly emerging (such as in instances of neural, gut, and retinal differentiation, to name but a few) [59, 60]. Clearly, advances in the years to come will lead to further refinement of these technologies making them more efficacious and also eventually scalable to enable ready clinical use.

Of particular relevance has also been the recent growing interest in the field of transdifferentiation or lineage conversion, that is, the process of converting one somatic cell type to another. Demonstrations of successful reprogramming on this front have been rapidly increasing in recent literature. While the earliest among these were the conversion of fibroblasts into muscle cells decades ago [61], lately conversion of B lymphocytes into macrophages [62] and more recently of fibroblasts into neurons [63] and blood progenitors [64] has also been successfully effected. This was achieved following the forced expression of a few transcription factors that provide the necessary transformative force to the target tissue type of interest. While maturity of the derived tissues as well as overall scalability of these processes still needs to be demonstrated, these studies offer us an unprecedented insight into the key players that govern tissue specification. More importantly, we believe these studies will eventually guide us to development of techniques for both harvesting and subsequent faithful and sustained in vitro culturing of adult human tissues or tissue progenitors. This could potentially obviate the very need to ever reprogram cells! As in such a scenario, barring the step of correcting underlying mutations, one would simply need to transiently culture such tissues to the desired scale of expansion before their eventual transplantation back into the patient to enable repair or replacement directed cell-based therapies.

Overall, we thus look forward to an exciting future in this highly interdisciplinary field of research. Several basic science discoveries are at the cusp of being unraveled as our understanding of human development and aging rapidly expands. Most importantly, this improved understanding is directly impacting the development of clinically translatable technologies for regenerative medicine.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

References

1 Murry CE, Keller G. Differentiation of embryonic stem cells to clinically relevant populations: Lessons from embryonic development. Cell 2008;132:661–680.
2 Reik W. Stability and flexibility of epigenetic gene regulation in mammalian development. Nature 2007;447:425–432.
3 Hochedlinger K, Plath K. Epigenetic reprogramming and induced pluripotency. Development 2009;136:509–523.
4 Wilmut I, Schnieke AE, McWhir J et al. Viable offspring derived from fetal and adult mammalian cells. Cloning Stem Cells 2007;9:3–7.
5 Cowan CA, Atienza J, Melton DA et al. Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science 2005;309:1369–1373.
6 Mali P, Cheng L. Re-programming somatic cells without fusion or ethical confusion. Regen Med 2006;1:837–840.
7 Hansis C, Barreto G, Maltry N et al. Nuclear reprogramming of human somatic cells by xenopus egg extract requires BRG1. Curr Biol 2004;14:1475–1480.
8 Taranger CK, Noer A, Sorensen AL et al. Induction of dedifferentiation, genomewide transcriptional programming, and epigenetic reprogramming by extracts of carcinoma and embryonic stem cells. Mol Cell Biol 2005;16:5719.
9 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663–676.
10 Maherali N, Sridharan R, Xie W et al. Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. Cell Stem Cell 2007;1:55–70.
11 Wernig M, Meissner A, Foreman R et al. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 2007;448:318–324.
12 Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. Nature 2007;448:313–317.
13 Takahashi K, Tanabe K, Ohnuki M et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861–872.
14 Yu J, Vodyanik MA, Smuga-Otto K et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007;318:1917–1920.
15 Park IH, Zhao R, West JA et al. Reprogramming of human somatic cells to pluripotency with defined factors. Nature 2008;451:141–146.
16 Mali P, Ye Z, Hammond HH et al. Improved efficiency and pace of generating induced pluripotent stem cells from human adult and fetal fibroblasts. Stem Cells 2008;26:1998–2005.
17 Mali P, Chou BK, Yen J et al. Butyrate greatly enhances derivation of human induced pluripotent stem cells by promoting epigenetic remodeling and the expression of pluripotency-associated genes. Stem Cells 2010;28:713–720.
18 Huangfu D, Osafune K, Maehr R et al. Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. Nat Biotechnol 2008;26:1269–1275.
19 Shi Y, Desponts C, Do JT et al. Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds. Cell Stem Cell 2008;3:568–574.
20 Lin T, Ambasudhan R, Yuan X et al. A chemical platform for improved induction of human iPSCs. Nat Methods 2009;6:805–808.
21 Yoshida Y, Takahashi K, Okita K et al. Hypoxia enhances the generation of induced pluripotent stem cells. Cell Stem Cell 2009;5:237–241.
22 Subramanyam D, Lamouille S, Judson RL et al. Multiple targets of miR-302 and miR-372 promote reprogramming of human fibroblasts to induced pluripotent stem cells. Nat Biotechnol 2011;29:443–448.
23 Anokye-Danso F, Trivedi CM, Juhr D et al. Highly Efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. Cell Stem Cell 2011;8:376–388.
24 Cavazzana-Calvo M, Payen E, Negre O et al. Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. Nature 2010;467:318–322.
25 Hacein-Bey-Abina S, Von Kalle C, Schmidt M et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science 2003;302:415.
26 Howe SJ, Mansour MR, Schwarzwaelder K et al. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. J Clin Invest 2008;118:3143.
27 Ott MG, Schmidt M, Schwarzwaelder K et al. Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1–EVI1, PRDM16 Or SETBP1. Nat Med 2006;12:401–409.
28 Schröder ARW, Shinn P, Chen H et al. HIV-1 integration in the human genome favors active genes and local hotspots. Cell 2002;110:521–529.
29 Okita K, Nakagawa M, Hyenjong H et al. Generation of mouse induced pluripotent stem cells without viral vectors. Science 2008;322:949.

30 Zhou H, Wu S, Joo JY et al. Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell 2009;4:381–384.
31 Kim D, Kim CH, Moon JI et al. Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell Stem Cell 2009;4:472–476.
32 Yu J, Hu K, Smuga-Otto K et al. Human induced pluripotent stem cells free of vector and transgene sequences. Science 2009;324:797–801.
33 Marchetto MC, Yeo GW, Kainohana O et al. Transcriptional signature and memory retention of human-induced pluripotent stem cells. PLoS One 2009;4:e7076.
34 Fusaki N, Ban H, Nishiyama A et al. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. Proc Jpn Acad Ser B Phys Biol Sci 2009;85:348–362.
35 Jia F, Wilson KD, Sun N et al. A nonviral minicircle vector for deriving human iPS cells. Nat Methods 2010;7:197–199.
36 Warren L, Manos PD, Ahfeldt T et al. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell 2010;7:618–630.
37 Chou BK, Mali P, Huang X et al. Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. Cell Res 2011; 518–529.
38 Hu K, Yu J, Suknuntha K, Tian S et al. Efficient generation of transgene-free induced pluripotent stem cells from normal and neoplastic bone marrow and cord blood mononuclear cells. Blood 2011;117: e109–e119.
39 Thomson JA, Itskovitz-Eldor J, Shapiro SS et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145.
40 Kim YG, Cha J, Chandrasegaran S. Hybrid restriction enzymes: Zinc finger fusions to Fok I cleavage domain. Proc Natl Acad Sci USA 1996;93:1156–1160.
41 Porteus MH, Baltimore D. Chimeric nucleases stimulate gene targeting in human cells. Science 2003;300:763.
42 Zou J, Maeder ML, Mali P et al. Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. Cell Stem Cell 2009;5:97–110.
43 Hockemeyer D, Soldner F, Beard C et al. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. Nat Biotechnol 2009;27:851–857.
44 Zou J, Sweeney CL, Chou BK et al. Oxidase deficient neutrophils from X-linked chronic granulomatous disease iPS cells: Functional correction by zinc finger nuclease mediated safe harbor targeting. Blood 2011;117:5561–5572.
45 Soldner F, Laganiere J, Cheng AW et al. Generation of isogenic pluripotent stem cells differing exclusively at two early onset Parkinson point mutations. Cell. 2011;146:318–331.
46 Zou J, Mali P, Huang X et al. Site-specific gene correction of a point mutation in human iPS cells derived from sickle cell disease patient. Blood 10.1182/blood-2011-02-335554.
47 Kim K, Doi A, Wen B et al. Epigenetic memory in induced pluripotent stem cells. Nature 2010;467:285–290.
48 Polo JM, Liu S, Figueroa ME et al. Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. Nat Biotechnol 2010;28:848–855.
49 Bar-Nur O, Russ HA, Efrat S et al. Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet Beta cells. Cell Stem Cell 2011;9:17–23.
50 Ohm JE, Mali P, Van Neste L et al. Cancer-related epigenome changes associated with reprogramming to induced pluripotent stem cells. Cancer Res 2010;70:7662–7673.
51 Stadtfeld M, Apostolou E, Akutsu H et al. Aberrant silencing of imprinted genes on chromosome 12qF1 in mouse induced pluripotent stem cells. Nature 2010;465:175–181.
52 Liu L, Luo GZ, Yang W et al. Activation of the imprinted Dlk1–Dio3 region correlates with pluripotency levels of mouse stem cells. J Biol Chem 2010;285:19483–90.
53 Christian M, Cermak T, Doyle EL et al. Targeting DNA double-strand breaks with TAL effector nucleases. Genetics 2010;186:757.
54 Miller JC, Tan S, Qiao G et al. A TALE nuclease architecture for efficient genome editing. Nat Biotechnol 2011;29:143–148.
55 Mitsui K, Suzuki K, Aizawa E et al. Gene targeting in human pluripotent stem cells with adeno-associated virus vectors. Biochem Biophys Res Commun 2009;388:711–717.
56 Khan IF, Hirata RK, Wang PR et al. Engineering of human pluripotent stem cells by AAV-mediated gene targeting. Mol Ther 2010;18: 1192–1199.
57 Suzuki K, Mitsui K, Aizawa E et al. Highly efficient transient gene expression and gene targeting in primate embryonic stem cells with helper-dependent adenoviral vectors. Proc Natl Acad Sci USA. 2008; 105:13781.
58 Song H, Chung SK, Xu Y. Modeling disease in human ESCs using an efficient BAC-based homologous recombination system. Cell Stem Cell 2010;6:80–89.
59 Spence JR, Mayhew CN, Rankin SA et al. Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. Nature 2011;470:105–109.
60 Swistowski A, Peng J, Liu Q et al. Efficient generation of functional dopaminergic neurons from human induced pluripotent stem cells under defined conditions. Stem Cells 2010;28:1893–1904.
61 Davis RL, Weintraub H, Lassar AB. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 1987;51:987–1000.
62 Xie H, Ye M, Feng R et al. Stepwise reprogramming of B cells into macrophages. Cell 2004;117:663–676.
63 Vierbuchen T, Ostermeier A, Pang ZP et al. Direct conversion of fibroblasts to functional neurons by defined factors. Nature 2010;463: 1035–1041.
64 Szabo E, Rampalli S, Risueno RM et al. Direct conversion of human fibroblasts to multilineage blood progenitors. Nature 2010;468:521–526.