hiPSCs: Reprogramming towards cell-based therapies

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ABSTRACT

Stem cell therapies show great potential for use in regenerative medicine, though advancements in safe stem cell technology need to be realized. Human induced pluripotent stem cells (hiPSCs) hold an advantage over other stem cell types for use in cell-based therapies due to their potential as an unlimited source of rejuvenated and immunocompatible SCs which do not elicit the ethical and moral debates associated with the destruction of human embryos. Towards realization of this potential this review focuses on the recent progress in DNA- and xeno-free reprogramming methods, particularly small molecule methods, as well as addresses some of the latest insights on donor cell gene expression, telomere dynamics, and epigenetic aberrations that are a potential barrier to successful widespread clinical applications.

1. INTRODUCTION

The promise of stem cell-based rejuvenation therapy has long been heralded and has recently been previewed with interim reports of success by companies such as StemCell, Inc. In a Phase I/II trial using their proprietary human neural stem cells (HuCNS-SCs), StemCell, Inc. was able to show allogeneic SCs could improve the sensory function of chest-level complete spinal cord injuries [1]. Thus far the results have shown the treatment to be safe owing to the low immunogenicity of fetally-derived SCs, though the patients remain temporarily immunosuppressed. In another Phase I study these same allogeneic HuCNS-SCs were shown safe for injection in human brains suffering from Palizaeus-Merzbacher disease

(PMD) [2]. There is currently no cure for PMD and children normally die of this disease around ages 10 - 15 due to severe neurological dysfunction owing to defective oligodendrocytes which fail to myelinate axons (reviewed in [3]). With cautious optimism, early results show that within nine months after the procedure transplanted regions had increased myelination and neurological function.

These reports support the great expectations of animal-based SC research translated into human therapies [4]; however, fetal SCs have limited availability and the use of these and related human embryonic SCs (hESCs) still carry many ethical considerations as harvesting these viable embryos destroys potential life [5]. Moreover, though ESCs have low immunogenicity due to their immuno-suppressive capacity and do not readily provoke a T-cell response, as they differentiate and express more MHC molecules on their surface allogeneic ESCs can provoke an immune response [6] that could necessitate lifelong immuno-suppression or even make the condition worse. While an immunocompatible and potentially unlimited source of autologous hESCs for cell therapy can be derived through somatic cell nuclear transfer (SCNT), hESC generation by SCNT is technically challenging [7] and not only elicits the same ethical debate concerning the destruction of a viable embryo but also that of human cloning—globally banned by the United Nations [8,9]. Though therapeutic cloning is permitted in some countries and US states such as California and Massachusetts [10], public perception and political climates restrict funding for development of this technology. Adult mesenchymal stem cells (MSCs) do not have the ethical or moral complications associated with ESCs, are more readily available, and have already shown efficacy in cell therapy trials to treat myocardial infarction, diabetes, bone lesions, cartilage damage, and skin burns, among others [11]. However, while MSCs have been shown to have low immunogenicity due to secretion of immune modulators such as IL-10, HLA-G5, and TGFβ1, allogeneic MSCs can become immunogenic upon termi-

nal differentiation [12,13]. Moreover, while autologous MSCs are immunocompatible there are not only known differences in quality between early and late passage MSCs but also between MSCs derived from "youthful" and "aged" donors which can limit safe and efficacious use: aged and late-passage MSCs have less proliferation ability and are more prone to genetic [14], proteomic [15], and phenotypic abnormalities [16,17] and are therefore more limited in their capacity to be expanded into clinically relevant, efficacious, and safe numbers. Therefore, hiPSC technology which carries no ethical or moral debate and is capable of generating potentially unlimited numbers of immunocompatible pluripotent SCs (PSCs) with both rejuvenated bioenergetics and replicative life-spans from patients own cells has been under aggressive development.

Indeed, hiPSCs have been successfully created in many labs using a variety of reprogramming techniques; however, iPSC technology still has significant advances to be made in safety and efficiency for viable use in regulatory-compliant, clinical-scale human therapies—a focus in this review. Takahashi & Yamanaka [18] first defined the core transcription factors (TFs) for inducing pluripotency in somatic cells of mice as Oct3/4, Sox2, Klf4, and c-Myc (OSKM) using gammaretroviruses with high transduction efficiencies and then in human dermal fibroblasts [19] using lentiviruses; however, these methods resulted in more than 20 retroviral integrations per clone and oncogenic potential too high for clinical therapeutics. This is a significant hurdle to regulatory approval as the FDA code of federal regulations (21 CFR Part 1271) and compliance program (7341.002) requires that the iPSCs be xeno- and foreign DNA-free, free of growth abnormalities and mutagenesis, noncontaminated, and consistently manufactured according to cGMP before regulatory approval will be awarded [20]. Thus, advances in iPSC derivation have been made using non-integrating adenoviruses [21], lentiviral vectors [22], episomal vectors [23], minicircle vectors [24], piggyBac (PB) transposons [25,26], Sendai Virus [27], mRNA [28, 29], miRNAs [30,31], proteins [32], and small molecules [33-43]. Moreover, though hiPSC reprogramming does manifest some epigenetic aberrations [44,45] currently limiting safe clinical application, these cells appear to be rejuvenated, having both "youthful" telomere lengths [46] and mitochondria [47,48]. While there has also been great success in animal-based iPSC research, the focus of this review will be on safe and efficient hiPSC methods and genomics.

2. REPROGRAMMING METHODS

2.1. Adenoviral, Cre-Lox, PiggyBac

Since Takahashi & Yamanaka's pivotal research on reprogramming terminally differentiated cells to pluri-

potency, a number of vector and reprogramming factor variations and improvements have been made. In 2009, Zhou & Freed [21] showed that much more genomically stable hiPSCs could be generated using OSKM in nonintegrating, transient adenoviral transfection, for example. These reprogrammed fibroblasts were then shown to readily differentiate into neural dopaminergic cells. Regrettably, however, the method had low efficiency (~0.0002%). An improvement in efficiency of induction of pluripotency (0.005% - 0.01%) was described by Soldner et al. [22] using a self-excising Cre-recombinase method with doxycycline (DOX)-inducible lentiviral vectors containing LoxP sites in the 3' LTRs. The self-excision of oncogenic genes such as c-Myc decreased the oncogenic potential; however, though the transgenes are excised from the genome, residual LoxP sites still remain. While a great system for mouse engineering, the residual foreign DNA is a human safety issue not likely to gain regulatory acceptance in the near future. The use of DOX-inducible PB transposition of OSKM was another novel use of a vector requiring only terminal inverted repeats and transient expression of the transposase enzyme in order to catalyze insertion or excision of the transgenes [25]. Though verification of complete excision of the transgenes and vector sequences can be labor-intensive and cost-prohibitive on an industrial scale, Woltjen et al. note that the their use of established plasmid preparation techniques and commercial transfection technology under xeno-free conditions is an improvement over limited-lifetime, xeno-biotic viral methods.

2.2. Episomal, Minicircle, RNA Sendai

Further improvement in transgene and vector free hiPSC reprogramming methods were made using episomal vectors with a cis-acting oriP element and trans-acting EBNA1 gene derived from the Epstein-Barr virus [23]. This method required only a single transfection to reprogram human fibroblasts using OSKM as well as NANOG, LIN28, and an IRES2 for coexpression and did not require viral packaging. Interestingly, the addition of the SV40 large T gene (SV40LT) gene was required and was thought to have countered the toxic effects of c-Myc expression. Plasmid free clones can easily be drug-selected over time as these vectors only replicate once per cell cycle and ~5% of plasmids are lost each cycle. Efficiency, however, was very low (0.000006%), SV40 is an oncogene, the EBNA1 protein may elicit an immune response if the transgene is not completely removed, and there is still the possibility of integration when using DNA-based methods even though this DNA is extra-chromosomal. Another plasmid-transfection based but nonviral minicircle vector of supercoiled DNA using Oct4, Sox2, Lin28, and NANOG in a single, primarily eukaryotic expression cassette further improved on trans-

gene and vector free hiPSC reprogramming [24]. Transfection efficiencies (~10.8% ± 1.7%) were approx. an order of magnitude higher than with the aforementioned episomal vectors and benefitted from reduced exogenous silencing leading to longer ectopic expression; reprogramming efficiencies were (~0.005%) were also much higher than other transgene and vector-free methods. While this decreased labor associated with reprogramming, FDA-required screening for possible DNA integration can still be labor intensive. Improving safety further, a move towards DNA-free methods without the risk of integration was described by Fusaki et al. [27] using an RNA Sendai virus (SeV) which does not replicate using a DNA phase. Reprogramming efficiencies (1%) using the SeV vector carrying OSKM were much improved over retroviral methods. Additionally, the SeV genome is naturally depleted from the cytoplasm by the cell over time. However, SeV can activate innate antiviral mechanisms; anti-HN protein antibody negative selection can easily purify for viral free clones but does add extra labor.

2.3. RNAs

Truly xeno-, transgene-, vector sequence-, and DNA-free methods to reprogram human somatic cells without the risk of residual integration using mRNA were first published by Yukabov et al. [28]. Yukabov et al. showed that transfecting in-vitro produced mRNA coding Oct4, Sox2, Lin28, and NANOG could successfully induce human fibroblasts to pluripotency, albeit with low efficiency (0.0005%). While RNA reprogramming methods have been known for some time, innate immune responses such as those effected by the RNA helicase retinoic acid inducible gene I (RIG-I) which detects viral RNA [49] have generally limited success. Research by Angel & Yanik [50] first showed that knockdown of innate immune system-related genes such as Ifnb1 and Eif2ak2 using a standard siRNA cocktail could suppress this response and improve RNA delivery methods. Further advancements were made using synthetic mRNA developed by Warren et al. [29] and delivered using RNAiMAX (Invitrogen) cationic lipid delivery vehicles. Knowing that ssRNA can activate cellular anti-viral mechanisms the authors modified the mRNA by removing the 5' phosphates with phosphatases; this attenuated interferon signaling. Additionally, by mimicking normal mRNA editing through substitution of either 5-methylcytidine (5mC) for cytidine or pseudouridine (psi) for uridine, increased transcript and cell viability was observed. The addition of a type I interferon receptor decoy receptor improved viability further. This combination greatly decreased the innate antiviral responses and initial toxicity normally encountered when using unedited RNA. Under these conditions, reprogramming human

somatic cells using OSKM as well as Lin28 mRNAs resulted in much improved efficiencies of 1.4% versus 0.04% using retroviral methods; timeframes for derivation (16 days) were approximately half that using retroviral methods. However, Warren et al. noted that the mRNA reprogramming required daily (16) transfections in order to maintain high levels of ectopic expression, though some reported optimizations in reprogramming factor cocktails and the use of an Oct4-MyoD fusion protein mRNA are capable of reducing required daily transfections to approximately 1 week [51].

Indeed, moderation of innate immune activities may benefit the use of directly transfected mature double-stranded miRNAs such as mir-200 c, mir-302 s, and mir-369 which induce reprogramming by global demethylation, indirectly promoting expression of pluripotency TFs Oct4 and Sox2 [30,31], for example. miRNAs have shown feasibility in reprogramming human cells but efficiency has suffered, presumably due to detection by RIG-I. While these methods present a much more viable method capable of meeting the strict requirements for safety and regulatory approval while being commercially feasible, the industrial production of modified RNAs could increase costs considerably.

2.4. Directed Delivery of Proteins

Employing bioprocesses already in place for the production of recombinant proteins may improve the commercialization of hiPSC technology. Such methods would also bypass the inherent risks of DNA-based genome manipulation and the added complexity of RNA-based methods. It has already been shown feasible that the direct reprogramming of human somatic cells with OSKM proteins is possible, for example, as performed by Kim et al. [32], but with low efficiency (0.001%). A major hurdle to improving protein-based methods is the ability to deliver them across the cell membranes; Kim et al. have made progress in overcoming this hurdle by taking advantage of the viral HIV transactivator of transcription (TAT) protein containing a high proportion of basic amino acids known as cell penetrating peptides (CPPs). These CPPs are capable of efficiently entering the cell and nucleus—a quality Kim et al. exploited by anchoring them to OSKM proteins. Further advances in protein-based methods have aimed at stabilizing the protein in culture and improving endosomal release upon uptake. In an optimization of direct protein delivery methods, Their et al. [52] employed media containing KnockOut D-MEM medium supplemented with 2% fetal calf serum, 7.5% serum replacement, and 2.5% lipid rich Albumax to confer enhanced protein stability and transduction efficiencies. This media supported recombinant Oct4-TAT reprogramming efficiencies only slightly reduced and on the same order of magnitude as viral meth-

ods using transduction of the Oct4 gene. Using similar media, Their *et al.* [53] have shown recombinant Sox2-TAT is reprogramming competent, though getting the proteins delivered to the right parts of the cell for efficient expression needs improvement.

2.5. Small Molecules

The use of small molecules represents an advantageous approach to consistent and safe derivation of hiPSCs: using small molecules not only circumvents the need for laborious assays proving the absence of adventitious agents and/or foreign genetic elements required for FDA approval, particularly if the molecules are already part of an FDA-approved drug library, but also takes advantage of existing industrial drug development infrastructure. Moreover, small molecule platforms are amenable to clinical- and industrial-scale high-throughput (HT) platforms that not only include automated cell culture systems such as the CompacT SelecT (TAP Biosystems) used by StemCell, Inc. to manufacture their line of HuCNS-SCs for human cell therapy [54] but also HT, label-free microfluidic platforms capable of separating hiPSCs based on their unique adhesion signature [55]—greatly reducing labor while increasing reliability and regulatory standardization. Many compounds which increase hiPSC reprogramming efficiency such as through reduced extrinsic and intrinsic apoptosis and modulation of master TFs involved in pluripotency have already been identified, for example.

Inhibition of Caspase3-mediated Rho kinases (ROCKs) which mediate caspase cascades, cell detachment, membrane blebbing, and nuclear fragmentation have shown beneficial to cell survival [56]. Thiazovivin, a ROCK inhibitor, is just one example of a small molecule which increases hiPSC survival and colony derivation after cell-cell and cell-substratum detachment during splitting [39]. Vitamin C (Vc) is a cheap, readily available, and FDA approved molecule which has also been shown to increase survival of viral OSKM reprogrammed somatic (fibroblast) cells [33], for example. Here, Estaben *et al.* showed Vc could decrease senescence of hiPSC through suppression of p53, research that is supported by [57] who have shown that suppression of the p53-p21 pathway increases virally-based generation of hiPSCs. The exact mechanisms of Vc in cell reprogramming were further elucidated by Wang *et al.* [41] who showed that Vc promotes the histone demethylase (HDM) activities of Jhdm1a/1b in Oct4 transduced mouse fibroblasts; Jhdm1a/1b (kdm2a/b) not only demethylated H3K36-me2/3 marks in the Ink4/Arf locus—repressing it and senescence while promoting cell cycle progression—but also activated miRs 302/367 in conjunction with Oct4. Estaben *et al*'s hypothesis that Vc can modulate dioxygenase hypoxia inducible factor (HIF) target genes as a

cofactor for HIF reactions is supported in research by Yoshida *et al.* [58] whom had first shown that hypoxic (5% O_2) culture conditions could improve hiPSC reprogramming efficiency; HIF-2α is known to directly bind hypoxia response elements (HREs) located in Oct4 promoter but also the conserved regions 3 & 4 of the Oct4 distal enhancers (DE) known to drive expression in the ICM and in ES cells [59]. Building on this discovery, a small molecule activator of 3'-phosphoinositide-dependent kinase-1 (PDK1) discovered by Zhu *et al.* [60], PS48, has been shown to activate Akt and stimulate a transition from aerobic to glycolytic metabolism. This transition to anaerobic metabolism is also hypothesized to improve reprogramming through a reduction in mitochondrial oxidation-associated ROS. Supporting research on this change in bioenergetics, mt morphology and energetics analysis [47,48] have shown that the cristae and energetic capacity of hiPSCs mts undergo a transition and are rejuvenated to a youthful ESC-like state during reprogramming. The addition of the glycolytic intermediate L-lactate can improve this mt metabolic shift and reprogramming efficiency [36].

Small molecule modulation or replacement of master TFs involved in pluripotency has also been shown to benefit iPSC reprogramming efficiencies. While not shown in human iPSCs, Ichida *et al.* [34] first showed that Sox2 and C-Myc can be replaced by a small molecule inhibitor of TGF-β signaling (E-616452, RepSox) through induction of Nanog in retrovirally reprogrammed mouse embryonic fibroblasts (MEFs). Other research using commercially available small molecule inhibitors of the pan-Src family kinase (SFK) have also shown that activation of Nanog can replace Sox2 to virally reprogram MEFs [61]; SFk inhibitors have not been adequately assayed in hiPSC reprogramming but have been shown to promote epithelial differentiation in hESCs, however [62]. Moreover, it has been shown that removal of C-Myc can increase OKS lentiviral hiPSC reprogramming efficiency in combination with other small molecule inhibitors of TGF-β receptor kinase (SB 431542) as well as inhibitors of MEK signaling (PD 0325901), GSK3β signaling (CHIR 99021), and ROCK inhibition with Thiazovivin [40]. Indeed, Valamehr *et al.* showed that using Thiazovivin to decrease apoptosis in combination with small molecules that inhibit TGF-β, MEK, and GSK3β signaling increases hiPSC reprogramming efficiency over methods without Thiazovivin-mediated ROCK inhibition or just ROCK inhibition alone. Interestingly, lithium (Li) was found to be able to replace some core factors in O alone, OK, and OS transduced hiPSCs through partial inhibition of GSK3β signaling, increased transcriptional activity of Nanog, and through inhibition of the H3K4 HDM Kdm1a (LSD1) [42].

However, complete replacement of all reprogramming

factors with nongenetic, small molecule methods is desirable. Oct4 is known to be the most important factor [18] and can be used alone to reprogram cells, though with greatly reduced efficiency as compared to OSKM. Thus, selecting cells which endogenously express some of the master TFs OSKM may be a consideration. Human fetally-derived neural SC (NSC) which endogenously express Sox2 and c-Myc were reprogrammed by Kim *et al.* [63] with just retroviral transduction of Oct4, for example; however, fetally-derived NSCs are not a reliable supply of cells. In the previously mentioned research by Zhu *et al.* [60], human keratinocytes—a clinically feasible source of cells for patient-specific hiPSCs which endogenously express Klf4 and c-Myc—were successfully reprogrammed using just retroviral trans duction of Oct4 and the small molecule PS48. Small molecules which can increase the endogenous expression of Oct4 through interactions with epigenetic modifiers of pluripotency which reduce the suppressed state and increase activation have also been shown to increase hiPSC reprogramming efficiency. Valproic acid (VPA) is a histone deacteylase (HDAC) inhibitor (such as H3K9ac in mESCs [64]) which increases access of the transcriptional machinery to the Oct4 promoter [65]; sodium butyrate (NaBu) is another HDAC inhibitor that has been shown to increase OS reprogramming efficiencies in human fibroblasts [37]. Wang *et al.* [66] have further shown VPA cooperates with Klf4 to increase the activity of the histone acetyltransferase (HAT) EP300 and the H3K27me2/3 HDM Kdm6b (JMJD3) in the proximal promoter (PP) of Oct4 while the H3K4me2/3 HDM Kdm5a (Jarid1a) and H3K27 HDM Kdm6a (Utx) activity are increased at the Oct4 PP and proximal enhancers (PEs). Likewise, BIX-01294-mediated inhibition of the H3K9 histone methyltransferase (HMT) KMT1C (G9a) has been shown to increase OK reprogramming efficiency in MEFs [67] by inhibiting both G9a-mediated heterochromatinization and H3K9 trimethylation at the Oct4 promoter [68]. Benefiting safe derivation with chemical methods, UNC0638 is a small molecule inhibitor of G9a/GLP in human cells with higher potency and lower cytotoxicity than BIX-01294 [69]; Chen *et al.* [70] have shown UNC0638-mediated inhibition of G9a/GLP in human hematopoietic stem and progenitor cells (HSPCs) repressed lineage-specific genes and supported "stemness" during expansion. Shi *et al.* also showed that inhibition of the pluripotency gene silencing DNA methyl transferases (DNMTs) 3a/3b (responsible for DNA methylation during differentiation of ES cells) by 5-azacytidine and RG108 can act synergistically to enhance OK-transduced MEF reprogramming. Relevant to clinical hiPSC production, RG108 has higher potency and is less cytotoxic than 5-azacytidine in human cells [71]. Moreover, RG108 does not result in covalent trapping of DNMTs and may have another advantage over other inhibitors in that RG108 seems to support stability of satellite DNA and centromere methylation states that are commonly found perturbed in hiPSCs. Silencing lineage-specific gene expression is also critical to successful hiPSC reprogramming; inhibition of the H3K79 HMT Dot1L and somatic gene expression can be improved with the small molecule EPZ004777 [38]. While there are currently no validated small molecules which can substitute for the human Oct4 reprogramming factor, recent HT screens of heterocyclic chemical libraries have described Oct4-activating cpds (OACs) capable of promoting Oct4 expression through direct interactions with the Oct4 promoter [35]. Indeed, it is this reviews opinion that future hiPSC reprogramming research focus on identifying small molecules which activate endogenous expression of OSK pluripotency factors.

In the following the author of this review presents a theoretical nongenetic, small molecule reprogramming recipe for hiPSCs which focuses on activating endogenous Oct4 expression. Fibroblasts are a reprogrammable and clinically viable source of donor cells which can be obtained from a patient with relatively little nuisance; thus, these will be the cell types considered in the following recipe. It is likely that the assay will require significant development as normal Oct4 expression promoting pluripotency must be maintained: repression of expression by half will result in trophoectoderm while twofold overexpression will result in endoderm and mesoderm differentiation [72]. This methodology posits that early reprogramming (**Figure 1**, (a)-(j)) steps should focus on decreasing epigenetic repression with DNMT inhibitors (e.g. RG108), HMT G9a inhibitors (e.g. UNC 0638), and HDAC inhibitors (e.g. VPA, NaBu). OACs should be added next. It is likely that an initial direct delivery of the Oct4 protein (e.g. Oct4-TAT) may substantially jumpstart Oct4 expression: it has been shown that Oct4 alone is capable of binding its own DE and maintaining an active and transcriptionally competent nucleosome-depleted region (NDR) once cytosines have been demethylated [73]. Supporting Oct4 enhancer binding stability after cytosine demethylation is research by Bhutani *et al.* [74] showing that activation-induced deaminase (AID) is only required within the first 72 hrs of lentiviral transduced OSKM MEFs. AID overexpression only leads to a 2-fold increase in efficiency, however, and small molecule activators of AID may not be as advantageous as other targets. Additionally, Sox2 upstream enhancers are known to be a downstream target of Oct4 while Sox2 reciprocally regulates transcription of Oct4 via Oct4-Sox2 elements in the DE [75]; therefore, epigenetic derepression of these TFs along with initial Oct4 proteins and TF substitutes is hypothesized to be sufficient for robust activation of Oct4 and the pluripotency

network. Simultaneously, one should begin silencing lineage-specific somatic gene expression (e.g. EPZ 004777). While Onder *et al.* [38] found that early and middle stage Dot1L inhibition could not only substitute for Klf4 and c-Myc in OS transduced human fibroblasts but could also increase expression of Nanog and Lin28, interestingly, TGF-β inhibitors did not increase efficiency with this combination and are not a part of this cocktail. Addition of the GSK3β and HDM LSD1 inhibitor—Li—should be considered. ROCK inhibitors (e.g. Thiazovivin) as well as Vc should be included in this initial cocktail and throughout reprogramming. Mid-stage reprogramming (**Figure 1**, (a)-(m)) should continue using the same cocktail but should include a transition from early stage aerobic culture conditions which promote cell cycle progression to hypoxic culture conditions (5% O_2) as well as the PDK1 activator PS48 and lactate to further promote a shift to glycolytic metabolism. Late-stage reprogramming should continue using the same cocktail, though it would be interesting to further optimize the assay for a reduction and/or complete discontinuation of HMT, HDM, DNMT, and HDAC modifiers so as to limit the potential for global epigenetic perturbance, to be discussed next.

3. hiPSC GENOMICS CONCERNS

Even with safer xeno- and DNA-free methods and efficient reprogramming of somatic cells improving, further characterization of altered epigenetic signatures and differentiation potential of hiPSCs is required. hESC pluripotency is often characterized as Oct4- and Nanog-positive, express surface antigens SSEA-3 & 4, TRA-1-60 & TRA-1-81, show *in vitro* differentiation capacity, and teratoma assays showing the ability of the cells to differentiate into ectodermal, endodermal, and mesodermal lineages [76]. hiPSCs are characterized similarly and resemble hESCs under these definitions; however, while hiPSCs display globally similar gene expression profiles they often show persistent donor cell gene expression signatures not completely silenced [77] as well as epigenetic differences [44,45].

3.1. Potential Aberrant Gene Expression

In transcriptome analysis Ghosh *et al.* [77] discovered significant residual fibroblastic gene expression signatures such as those involved in remodeling the extracellular matrix (ECM) (PLAT and PLAU) as well as those in cell migration (CXCL1) in fibroblast-derived hiPSCs, adipose-specific gene expression such as PALLD and COL1A1 in adipose-derived hiPSCs, and keratinocyte-specific protein expression such as keratins and proteolytic enzymes in keratinocyte-derived hiPSCs. Furthermore, many genes such as LEFTY1 and others involved in maintaining hESC pluripotency and an undifferentiated state were found to be downregulated in hiPSCs. These donor cell expression signatures can not only be found in hiPSCs reprogrammed by integrating retroviral transduction but also in cells reprogrammed with nonintegrating episomal vectors and by the directed delivery of defined reprogramming proteins. Additionally, culture conditions can play a role in genomic heterogeneity: it has been found that the use of feeder-layer
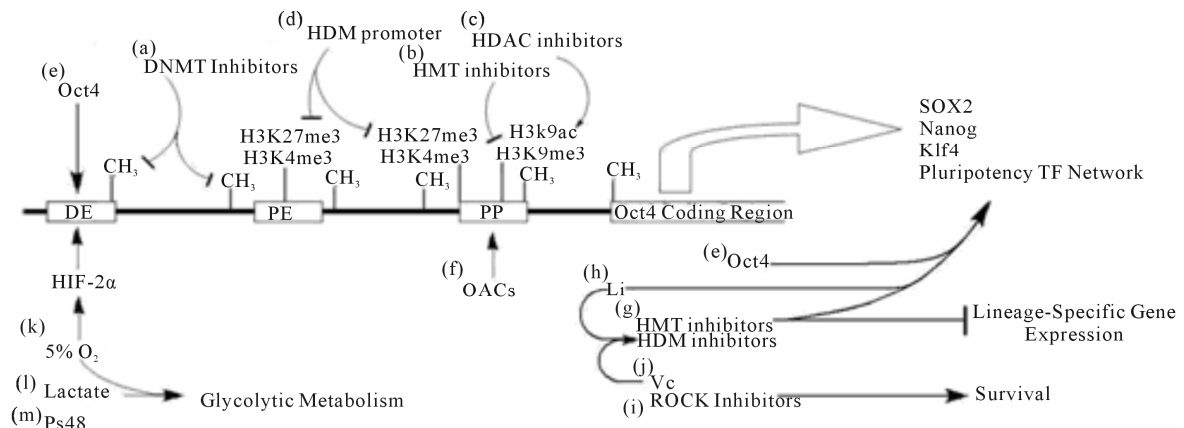
Figure 1. Stimulating Oct4 and the pluripotency network. (a) DNMT inhibitors (e.g. RG108), (b) H3K9 HMT G9a inhibitors (e.g. UNC0638), (c) H3K9ac HDAC inhibitors (e.g. VPA, NaBu), (d) promoters of H3K27me3 and H3K4me3 HDMs JMJD3, Jarid1a, Utx (e.g. VPA), and (e) Oct4 protein (e.g. Oct4-TAT) should be included first in order to render the Oct4 gene transcriptionally competent and kickstart the pluripotency network; (f) OACs should be added throughout early, middle & late reprogramming; (g) H3K79 HMT Dot1L inhibitor (e.g. EPZ004777) helps silence lineage-specific gene expression during early and middle stage reprogramming; (h) H3K4me3 HDM LSD1, GSKβ inhibitor (e.g. Li) and the cell survival promoting (i) ROCK inhibitors (e.g. thiazovivin) and (j) Vc (also a H3K36m2/3 HDM promoter) should be added during early, middle, and late reprogramming; Stimulators of glycolytic metabolism (k) 5% O_2, (l) Lactate, and (m) PS48 should be included through mid and late reprogramming.

culture of hiPSC contributes to DNA replication and cell-cycle variances in hiPSCs [78]; however, in support of xeno-free generation of hiPSCs it was found that defined, feeder-free culturing of hiPSCs on Matrigel (BD Biosciences) and mTeSR1 (StemCell Technologies) more closely resembled hESCs than those cultured with feeders. TeSR2 (StemCell Technologies) is closely related to mTeSR1 but contains no animal proteins and has been shown capable of maintaining hESCs in clinical-grade conditions [79].

It is long known that prolonged cell passaging can lead to abnormalities. In hESCs this manifests as karyotypic aberrations, most commonly in chromosomes 12, 17, and X [80]. These abnormal cells often show an increased ability to proliferate and mirror malignant transformations *in vivo*. Indeed, a rather large meta-analysis of 66 hiPSC lines from 38 independent studies conducted by Mayshar *et al.* [81] revealed that 20% of these lines contained chromosomal aberrations after prolonged culture, particularly trisomy of 12p—a region which includes the pluripotency genes NANOG and GDF3—as also found in hESCs, and general functional enrichment in cell cycle genes. In contrast to long-term passaging, mutations arising during early passaging and isolation were mostly limited to subchromosomal duplications or deletions. Mutations associated with the derivation method included trisomies of chromosome 1 and 9 in lines reprogrammed with both retroviral transduction and the directed delivery of factor proteins. Such high mutation rates regardless of integrating or nonintegrating (episomal and mRNA) methods were also found in the exomes of 22 hiPSC lines analyzed by Gore *et al.* [82]. Harboring about 6 mutations per iPSC line, Gore *et al.* found that most (83/124) were miss-sense with 53 predicted to alter protein function while 50 were known cancer-related, such as ATM, NTRK1, and NTRK3. Copy number variations (CNV) were also found to occur in common fragile sites and sub-telomeric regions at twice the rate in early-passage hiPSC lines derived by retroviral transduction and by PB transposons than in hESCs or the original fibroblasts; however, both the number and size decreased with passaging as these mutations are negatively selected for [83]. In ensuring the safe application of hiPSC-based therapies, HT qPCR assays of pluripotency gene expression will need to not only include Oct4 and other master TFs but also assays of donor cell gene expression, trisomies, and other common cancer-related mutations, including those that may have been acquired over the lifetime of the donor.

3.2. Potential Aberrant Epigenetics

Perturbations associated with epigenetic memory are potentially tumourigenic [84,85] and are therefore safety

issues limiting regulatory approval. While genome-scale single base resolution has shown hiPSC epigenetic signatures are similar to ESCs, differentially methylated regions (DMRs) do occur [44,45]. Assaying cell lines of varying somatic type from multiple labs reprogrammed with both integrating and nonintegrating methods and using HT methylC-Seq and ChIP-Seq methods, Lister *et al.* discovered 1175 CG dinucleotide DMRs (CG-DMRs) totaling 1.68 Mb that were either a failure to reprogram somatic memory (44% - 49%) or (51% - 56%) were particular to the hiPSC reprogramming process (iDMRs), 80% were in CG islands (CGI-DMRs) and near or within genes (62%), and 92% were hypomethylated in iPSCs and indicated insufficient methylation during reprogramming. Moreover, these CG-DMRs and iDMRs were transmitted with high frequency during differentiation and were not removed by passaging as can occur with CNVs. Common motifs included DMRs for Klf4, a reprogramming factor (that may be substituted) which may be contributing to aberrant methylation, and FOXL1 and could be useful biomarkers for complete reprogramming, for example. Lister *et al.* also discovered 29 large nonCG-DMRs comprising 32.4 Mb, 22 of which were associated with hypomethylation and H3K9me3 enrichment proximal to centromeres and telomeres. These nonCG-DMRs were associated with transcriptional disruption with 33 of 50 downregulated genes found perturbed by more than a 2 fold lower transcript abundance, 64% of which also harbored CG dinucleotides hypermethylated at the TSS. This transcriptional downregulation was associated with aberrant loss of H3K27me3. While not only potentially tumourigenic, these transcriptional aberrations in comparison to ESCs can lead to functional heterogeneity [45] and reduced efficacy in cell-based therapies.

The polycomb group proteins 1 and 2 (PRC1 and 2) are evolutionary conserved epigenetic regulators whose perturbations are known to reduce or prevent reprogramming (reviewed by Watanabe, Yamada, & Yamanaka [86]). PRC1 is largely responsible for maintaining the repressed transcriptional state that PRC2 initiates through H3K27 trimethylation, for example. It is known that both Oct4 and Nanog regulate and increase expression of DNMT1 through direct binding to DNMT1's promoter [87] while Sox2 has been shown to regulate miR-29b-catalyzed repression of DNMT3A/3B during reprogramming [88]. However, increased understanding of DNA methylation interactions suggests histone modifications regulate DNMT activity (reviewed in [89]): DNMT3A/3B-catalyzed DNA methylation of pericentric satellite repeats is dependent on HMT Suv39h-mediated H3K9 methylation [90], the PRC2 protein HMT EZH2 (Kmt6a) which catalyzes H3K27 trimethylation can physically direct DNMTs and CpG methylation [91], and

histone tails lacking H3K4 methylation have been shown to allosterically activate de novo DNA methylation by DNMT3A [92], for example. Thus, opportunity for understanding aberrant epigenetics should focus on the histone code hierarchy. Indeed, another required epigenetic regulator of reprogramming, Utx, has recently been identified [93]. In mediating demethylation of repressive H3K27me2/3 chromatin marks Utx globally regulates approximately 500 genes, is required to sufficiently activate many ES-associated genes, and physically interacts with OSK in mediating reprogramming to a state of pluripotency. In coordinating gene repression and activation Utx also forms a protein complex with the Trithorax group (TrxG) HMT MLL2/3 (MLL2/3 normally adds the activating tri-methylation mark of H3K4me3 while the HDM Jarid1a removes it), potentially functioning in bivalent domain regulation. Such suggestions are reinforced in research showing Utx complexes with MLL 2/3/4 during development in murine erythroleukemia (MEL) cells [94]. In mouse ESCs, Chaturvedi et al. have also shown crosstalk between G9a as well as Jarid1a; a significant amount of gene silencing is maintained by the repressive dimethylation of H3K9 and H3K27 mediated by G9a and the demethylation of activating H3K4me3 by Jarid1a. The coordination between these HMTs and HDMs could play a role in timely repression of lineage-specific genes and maintaining optimal stoichiometric ratios of TFs such as Oct4 during hiPSC reprogramming. It is already known that reprogramming factor stoichiometry can affect iPSC reprogramming and epigenetic states [95], for example. Moreover, perturbation of Utx has been shown to contribute to aberrant epigenetic reprogramming both *in vitro* and *in vivo*. Thus, considering Mansour *et al*'s [93] and Chaturvedi *et al*'s [94] findings, it is this reviews opinion that suboptimal correlation between levels of OSKM expression and levels of epigenetic regulators in global crosstalk and feedback mechanisms important for pluripotency and development is likely contributing to aberrant epigenetics such as the hypomethylation of H3K27 and H3K9 that Lister *et al.* observed. Further related insight is found in recent research by Parsons [96] showing that the globally acetylated state of hESCs is at least partially mediated by levels of Oct4 which influence HDAC activity; Parsons found that decreased levels of Oct4 lead to hyperacetylation and induction of differentiation. Perturbations in this crosstalk can not only lead to inefficient activation of H3K27me2—repressed pluripotency-associated genes but can also lead to H3K9me3 enrichment which prevents OSKM TF target binding [97]—another possible culprit in the low efficiency currently observed in hiPSC reprogramming. Finally, though not yet proven, perturbations in this crosstalk are also a potential culprit in H3K4me3 enrichment and the induction of and/or resid-

ual donor cell gene expression. Further elucidation of epigenetic crosstalk should benefit the precise application of reprogramming technologies which do not aberrantly perturb the delicate balance of epigenetic regulators. In light of the still enigmatic crosstalk and potential perturbations inherent to the reprogramming process, validation of hiPSCs to be used in cell therapies should include routine HT methylome analysis to ensure safe, efficacious, and nontumourigenic application of hiPSCs.

3.3. Telomere Rejuvenation

Despite aberrant methylation of subtelomeric regions, advocates of hiPSC research for use in regenerative medicine can remain optimistic with research showing that telomere lengths are rejuvenated in a number of cell types [46], thus ameliorating some concerns about cellular senescence when using cells from aged donors. Also, this is another advantage over using MSCs which may be prone to senescence from aged donors and/or prolonged passaging due to inactive telomerase, though telomere-induced senescence can be avoided with ectopic expression of hTERT [98]. hTERT is stably expressed during hiPSC reprogramming [99], though there is some heterogeneity in length found among hiPSC lines that could be related to suboptimal ratios of pluripotency TFs and the regulatory loops governing telomere length. It is known that Oct4 and Nanog bind the promoters of the telomerase RNA component (TERC) locus and upregulate transcription and lengthening of telomeres in dyskeratosis congenita (DC) cells [100]. Recently, Hoffemeyer *et al.* [101] have shown that the Tert promoter is a target of β-catenin and Klf4 in human carcinoma and mouse ES cells. Interestingly, Klf4 is only required for β-catenin to bind the Tert promoter: β-catenin actually drives Tert expression, possibly by recruiting HMTs. Wnt/β-catenin is also a target of Tert expression, however, and this may also form a regulatory loop governing telomere length in hiPSCs that is perturbed by suboptimal correlations of the pluripotency factors. hTERT and alternative lengthening of telomeres (ALT) is required for full telomere rejuvenation and true pluripotency [102], however. ALT lengthens telomeres in association with epigenetic modifiers such as DNMT3A/3B and HMTs Suv39h1/h2; the aberrant hyper- & hypo-methylation of subtelomeric regions that Lister *et al.* [44] identified may be associated with aberrant crosstalk between these epigenomic modifiers, possibly by the use of associated inhibitors [99]. It can be hypothesized these aberrations may even be an artifact of the previously shortened state and/or ALT. Still, Yehezkel *et al.* [99] have shown successful hiPSC reprogramming elongates telomeres on average by approximately 10 kb; hTERT and telomere elongation is then stably repressed upon differentiation, allowing

normal telomere shortening. However, a safe and effica-cious replicative lifespan for hiPSCs used in cell therapy must be shown: this review suggests routine telomere assays not only include an assay of hTERT expression but also assays of absolute telomere length such as with modified Cawthon HT qPCR [103].

4. CONCLUSION

SCs for use in cell-based therapies have recently shown interim clinical viability and hold great potential for use in regenerative medicine. There are many regula-tory and biological concerns to be resolved before com-mercialization of SCs for clinical therapy can be achie-ved, however: the use of hESCs in such therapies not only carries great ethical debate but also poses an immu-nogenicity risk; SCNT technology is technically chal-lenging and also controversial; allogeneic MSCs may still pose an immunogenicity risk while autologous MSCs may be susceptible to senescence. In contrast, autologous hiPSCs have all the potential of hESCs and are a source of potentially unlimited, immunocompatible SCs with rejuvenated bioenergetics and replicative lifespans for patient-specific cell-based therapies that pose no ethical dilemma; however, iPSC reprogramming methods still suffer from safety, efficiency, and efficacy concerns, though these obstacles are being surmounted. Since Ta-kahashi & Yamanaka first defined the core OSKM TFs required to reprogram somatic cells to iPSCs using inte-grating viral transduction methods, much progress has been made in developing safer nonintegrating-, RNA-, protein-, and small molecule-based methods. Towards the goal of clinically viable hiPSC technology, strategies exploiting modifiers of cell survival, endogenous pluri-potent TF expression, and epigenetic regulation have been developed to increase hiPSC reprogramming safety, efficiency, and efficacy. However, epigenetic memory of hiPSCs still poses a safety and efficacy concern. This review has discussed the latest discoveries benefiting an increased understanding of pluripotent TF and epigenetic regulator crosstalk perturbed during reprogramming. This knowledge is paramount to developing reprogram-ming technology which completely silences lineage-specific gene expression, maintains telomere integrity, and circumvents aberrant epigenetic methylation. Per-turbations in hiPSC methylomes may be a result of suboptimal correlations between pluripotency factors and epigenetic regulators during hiPSC reprogramming and more research is needed in this regard. The use of non-genetic small-molecule methods to very precisely restart the endogenous expression of required TFs such as OSK may ameliorate the epigenetic scars of forced OSKM TF expression characteristic of other methods; however, there is currently insufficient genomics data concerning the epigenetic landscape of hiPSCs reprogrammed using

still nascent small molecule—only methods to know at this time. Nevertheless, it is this review's final opinion that DNA- and xeno-free small molecule methods hold the most potential as a clinically viable and relatively lower-cost HT technology capable of generating hiPSCs in a safe, regulatory-compliant, and efficacious platform.

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