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Authors

Wolf, Don P
Morey, Robert
Kang, Eunju
et al.

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Concise Review: Embryonic Stem Cells Derived by Somatic Cell Nuclear Transfer: A Horse in the Race?

DON P. WOLF,[a,b] ROBERT MOREY,[c] EUNJU KANG,[a,b] HONG MA,[a,b] TOMONARI HAYAMA,[a,b] LOUISE C. LAURENT,[c] SHOUKHRAT MITALIPOV[a,b,d,e]

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[a]Center for Embryonic Cell and Gene Therapy, Oregon Health & Science University, [b]Division of Reproductive & Developmental Sciences, Oregon National Primate Research Center, Oregon Health & Science University, Beaverton, Oregon, USA; [c]Department of Reproductive Medicine, Sanford Consortium for Regenerative Medicine, University of California, San Diego, La Jolla, California, USA; [d]Knight Cardiovascular Institute, [e]Departments of Obstetrics and Gynecology, Molecular and Medical Genetics, and Biomedical Engineering, Oregon Health & Science University, Portland, Oregon, USA

Correspondence: Shoukhrat Mitalipov, Ph.D., Center for Embryonic Cell and Gene Therapy, Oregon Health & Science University, 3303 SW Bond Avenue, Portland, Oregon 97239, USA. Telephone: 503-614-3709; Fax: 503-533-2494; e-mail: mitalipo@ohsu.edu

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ABSTRACT

Embryonic stem cells (ESC) hold promise for the treatment of human medical conditions but are allogeneic. Here, we consider the differences between autologous pluripotent stem cells produced by nuclear transfer (NT-ESCs) and transcription factor-mediated, induced pluripotent stem cells (iPSCs) that impact the desirability of each of these cell types for clinical use. The derivation of NT-ESCs is more cumbersome and requires donor oocytes; however, the use of oocyte cytoplasm as the source of reprogramming factors is linked to a key advantage of NT-ESCs—the ability to replace mutant mitochondrial DNA in a patient cell (due to either age or inherited disease) with healthy donor mitochondria from an oocyte. Moreover, in epigenomic and transcriptomic comparisons between isogenic iPSCs and NT-ESCs, the latter produced cells that more closely resemble bona fide ESCs derived from fertilized embryos. Thus, although NT-ESCs are more difficult to generate than iPSCs, the ability of somatic cell nuclear transfer to replace aged or diseased mitochondria and the closer epigenomic and transcriptomic similarity between NT-ESCs and bona fide ESCs may make NT-ESCs superior for future applications in regenerative medicine. STEM CELLS *2017;35:26–34*

SIGNIFICANCE STATEMENT

This review describes recently developed human ESCs from somatic cell nuclear transfer blastocysts and discusses their advantages and limitations compared to other types of human pluripotent stem cells.

INTRODUCTION

While a general appreciation of the existence of pluripotent cells in early embryos has existed for decades, they were first experimentally isolated in mammals as mouse embryonic stem cells (ESCs) in 1981 [1, 2]. The defining features of these cells included unlimited proliferation in vitro without differentiation; a normal diploid karyotype; in vivo differentiation into teratomas containing cells from all three primary germ layers and, in vitro, the ability to differentiate into a wide variety of cell types. Like inner cell mass (ICM) cells in preimplantation blastocysts, mouse ESCs expressed pluripotency markers and could contribute to both somatic tissues and the germline of chimeric animals. The first primate ESCs were successfully isolated from the rhesus monkey [3]. Three years later, the same team reported ESC derivation from human embryos that had been generated by in vitro fertilization (IVF) for reproductive purposes, and which had been stored frozen and donated by IVF patients after completion of their families and after Institutional Review Board-approval [4]. The immediate reaction expressed in the popular press predicted that such pluripotent cells could be transplanted to cure a multitude of human diseases, and the new field of regenerative medicine slowly emerged from the shadows. However, while human ESCs from in vitro fertilized embryos may represent a "gold standard," they are allogeneic to potential recipients and their derivation and use carry substantial ethical and technological limitations and significant hurdles. Further slowing progress in this area has been the fact that federal research funding in the United States has been restricted or inconsistent over the past 15 years and available human ESC lines are limited in number and, for the most part, not developed or approved to be products suitable for clinical use (see review by Simonson et al., 2015 [5]). Fortunately, there are now alternatives to ESCs that may provide

histocompatible cells for regenerative medicine: ESCs derived by somatic cell nuclear transfer (NT-ESC), induced pluripotent stem cells (iPSCs), and ESCs derived by parthenogenesis. In this review, we will focus on NT-ESCs, as the Progenitor Cell Biology Consortium focused on comparative studies of iPSCs generated using a variety of reprogramming methods [6], and the potential utility of parthenogenetic ESCs has been reviewed recently [7]. The aim of this review is to consider and contrast NT-ESCs and genetically matched transcription factor-mediated-iPSCs, and since the rhesus monkey has long been a useful primate model, to reference its role in pluripotent stem cell protocol development.

SOMATIC CELL NUCLEAR TRANSFER AND NT-ESCS

Factors present in mature, metaphase II (MII)-arrested oocyte cytoplasm are uniquely capable of reprogramming the identity of transplanted somatic cell nuclei to the oocyte-like state. Since the initial discovery in amphibians [8], somatic cell nuclear transfer (SCNT) success has been achieved in a range of mammalian species, including agriculturally important species, pets, horses and rare/endangered animals, suggesting that such reprogramming activity in enucleated or spindle-free oocytes (cytoplasts) is universal [9–12].

Because SCNT, in the context of reproductive cloning, could reduce inter-animal genetic variability, thereby, decreasing the number of animals required to reach statistical significance in experimental endeavors, we first undertook protocol development in the rhesus macaque. The successful production of monkeys by nuclear transfer, using embryonic blastomeres as the source of donor nuclei, was described in 1997 [13]. However, despite successful application of SCNT in many species, as referenced above, reproductive cloning of monkeys from somatic cells (rather than embryonic blastomeres) has not yet been accomplished [14, 15]. The production of autologous pluripotent cells for therapeutic use in personalized drug selection or cell-based regenerative medicine (NT-ESCs) is another story. The first primate success was again in the rhesus macaque [16], in which the SCNT procedure was used to reprogram adult skin fibroblasts into SCNT oocytes that upon parthenogentic activation developed into zygotes, blastocysts and then formed NT-ESCs [16, 17]. Success was based on the concept that during the first critical step in SCNT, the transplanted somatic cell nucleus must become oocyte-like when exposed to MII-arrested oocyte cytoplasm (Fig. 1). This notion is contrary to the common misconception that SCNT immediately induces formation of an embryo-like or pluripotent entity. We presumed that somatic cell-specific transcription and epigenetic factors maintaining cellular identity must be first dissociated from the metaphase chromatin and actively replaced by oocyte-specific programs. We soon realized that suboptimal SCNT micromanipulation procedures caused premature exit of oocyte cytoplasm from the meiotic metaphase thus precluding formation of MII oocyte-like SCNT spindles. Ultimately, we employed enucleation using noninvasive imaging and hemagglutinating virus of Japan envelope (HVJ-E)-based cell fusion in the presence of caffeine, a protein phosphatase inhibitor, that protects the cytoplast from premature activation to induce efficient de novo spindle formation as the measure of reprogramming (Fig. 1) [18]. The next step in SCNT reprogramming is exit from the MII arrest and formation of the diploid interphase zygote, without sperm and without segregation of chromosomes into the second polar body (PB2). This can be achieved using artificial activation treatments involving exposure to agents inducing Ca^{2+} oscillations (ionomycin) and protein synthesis inhibitors (6-DMAP) that prevent segregation of PB2. While these standard activation procedures were sufficient to induce formation of interphase (pronuclear stage) SCNT zygotes, most embryos failed to progress beyond the 4-8 cell stage. During normal fertilization, in addition to release from meiotic arrest, sperm entry also triggers release of oocyte maternal factors responsible for reprogramming the chromatin to totipotency. To accomplish this challenging step, we used a combination of activation agents along with electroporation in the presence of the histone deacetylase inhibitor, trichostatin A (TSA) (Fig. 1). Although an electroporation stimulus and TSA are not required for cell fusion or exit from meiosis, they are supportive of proper totipotency reprogramming following SCNT [19, 20]. We initially tested and characterized several optimized SCNT protocols in the monkey model and concluded that these approaches were adequate to serve as a starting point for experimentation with human oocytes.

After a number of years of trial-and-error, the efficient formation of SCNT blastocysts and derivation of human NT-ESCs was finally achieved in 2013 [20]. A schematic presentation of the approach is included in Figure 1. As with the monkey, initial failure in human SCNT was characterized by early embryonic arrest or poor quality embryos at the blastocyst stage. However, when caffeine was incorporated to maintain meiotic arrest during spindle removal and somatic cell fusion, somatic cell nuclei efficiently formed spindle-like structures. Further, incorporation of electroporation and TSA enhanced SCNT reprogramming and blastocyst development with visible and prominent ICMs, similar to those observed for IVF-produced embryos. With one exceptional oocyte donor, eight oocytes were recovered and, following SCNT, five SCNT embryos developed into blastocysts and were utilized for ESC isolation. Four of these blastocysts formed ICM outgrowths and gave rise to ESC-like colonies. Subsequent passaging resulted in the propagation of four stable ESC lines of diploid cells with typical morphology and growth characteristics (Table 1). The apparent oocyte or nuclear donor fibroblast dependency observed in this pioneering experience was challenged when MII oocytes were collected from different egg donor volunteers and used for SCNT with fibroblasts from an infant with a mitochondrial DNA-based disease (Leigh syndrome) or a 72-year-old ALS patient [20, 23, 25]. Most oocytes survived spindle removal and successfully fused with nuclear donor cells. Following activation and culture, blastocysts were also produced from these oocyte and fibroblast donors supporting the establishment of stable NT-ESC lines—one from each oocyte cohort (Table 1). In an effort to relate oocyte donor dependency to controlled ovarian stimulation, the number and maturity of oocytes recovered as well as the use of a gonadotrophin releasing hormone antagonist versus an agonist was considered. Low oocyte numbers (less than 10) and the use of an antagonist were associated with the best outcomes. The implications of this accomplishment to the future of the assisted reproductive technologies have been discussed [26].

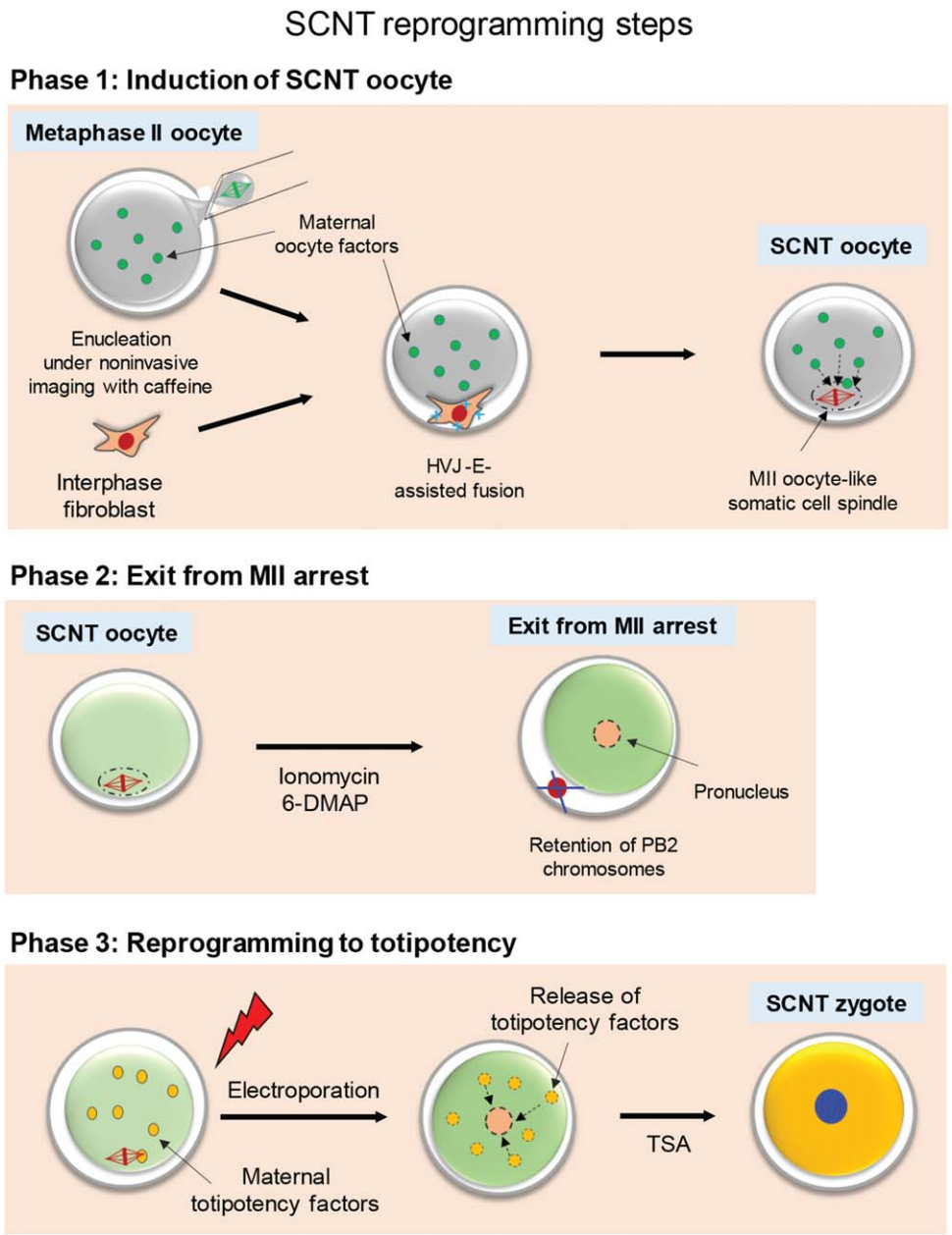

Figure 1. Details and steps of human SCNT-based reprogramming. During the first phase, SCNT into the enucleated MII-arrested oocyte should result in conversion of the somatic cell interphase chromatin into meiotic, MII-like chromosomes. This process is greatly augmented when the MII oocyte enucleation is carried out under noninvasive spindle imaging in the presence of caffeine. Somatic cell fusion is accomplished using HVJ-E that further protects the cytoplast from premature activation. After efficient induction of SCNT oocytes, a second phase is required to exit meiotic arrest and progress to mitotic interphase without chromosome loss in the PB2. This is routinely achieved using a CA^{2+} stimulus (ionomycin) combined with 6-DMAP that inhibits cytokinesis and PB2 segregation. Maternal, totipotency factors are present in the cytoplasm of unfertilized oocytes but normally remain inactive until fertilization with sperm. The signaling involved during natural, sperm-induced release of totipotency factors is poorly understood. However, the electroporation stimulus mimics this signaling and induces release of oocyte totipotency factors. This, phase 3, in reprogramming is further enhanced by exposure to the histone deacetylase inhibitor, TSA. Abbreviations: HVJ-E, hemagglutinating virus of Japan envelope; MII, metaphase II; PB2, second polar body; SCNT, somatic cell nuclear transfer; TSA, trichostatin A.

Following this successful isolation of NT-ESCs, confirmation by other groups promptly appeared (Table 1) [21, 22, 24]. Protocol alterations in one report [22] were designed to further minimize premature oocyte activation through the use of diluted HVJ-E in Ca^{2+}-free medium. Successful ESC derivation from NT embryos was also found to be dependent on the presence of a specific fetal bovine serum (FBS) batch in the embryo culture medium. No NT-ESC lines were generated without FBS, while in the presence of FBS, development to blastocysts with subsequent NT-ESC line derivation occurred at a high frequency. We note that this particular FBS batch was screened early by us (Tachibana et al. [20]) and provided to Dieter Egli's team [22]. The somatic cell donors included foreskin fibroblasts from a newborn male and skin fibroblasts

Table 1. Frequency of human SCNT blastocyst and NT-ESC development as a function of somatic nuclear donor cell age and health status

Age of nuclear donor fibroblasts	Disease	No. of MII oocytes	No. of blastocysts (%)	No. of NT-ESC lines (%)	References
N/A	Control IVF	27	16 (59.3)	9 (56.3)	Tachibana et al. (2013) [20]
Fetal	Healthy	8	5 (62.5)	4 (80.0)	Tachibana et al. (2013) [20]
8 mo. old	Leigh syndrome	20	7 (35.0)	2 (28.6)	
35 yr old	Healthy	29	2 (6.9)	1 (50.0)	Chung et al. (2014) [21]
75 yr old	Healthy	48	3 (6.3)	1 (33.3)	
Newborn	Healthy	64	10 (15.6)	3 (30.0)	Yamada et al. (2014) [22]
32 yr old	Type I diabetes	90	11 (12.2)	1 (9.1)	
72 yr old	Amyloid lateral sclerosis	25	5 (20.0)	2 (40.0)	Kang et al. (2016) [23]
42 yr old	Age-related macular	10	4 (40.0)	2 (50.0)	Chung et al. (2015) [24]
52 yr old	degeneration	36	5 (13.9)	0 (0)	
59 yr old		17	6 (35.3)	2 (33.3)	
Total SCNT	N/A	347	58 (16.7)	18 (31.0)	

All studies followed the human SCNT protocol developed by Tachibana et al., 2013 that includes enucleation under noninvasive imaging in the presence of caffeine, HVJ-E-assisted fusion, and activation with electroporation and incubation with TSA to aid reprogramming. See text for additional protocol modifications.

The percentage of blastocysts was calculated based on the total number of MII oocytes used for SCNT and the percentage of NT-ESC lines was based on the number of blastocysts used for ESC line derivation.

Abbreviations: ESC, embryonic stem cells; IVF, in vitro fertilization; MII, metaphase II; N/A, not applicable; NT-ESC, ESCs derived by somatic cell nuclear transfer; SCNT, somatic cell nuclear transfer.

from an adult female with Type 1 diabetes. Genetic characterization of the resulting NT- ESC lines was performed to verify authenticity (e.g., the origin of the nuclear DNA from the somatic cell donor, and the origin of the mitochondria from the oocyte), and an oocyte donor dependency was again implied. In a second report [21], the hypothesis that the age of the donor somatic cell nuclei could impact the efficiency of the SCNT was addressed by attempting SCNT using skin fibroblasts obtained from 35- and 75-year-old males. The waiting time, between 30 and 120 minutes from fusion to activation, was also measured and related to reprogramming success. As with the other two studies, the success of the experiments appeared to be heavily dependent on the egg donor. Although SCNT blastocysts were produced after different time periods between fusion and activation, only those in the 120-minute group hatched and supported NT-ESC line derivation. NT-ESC lines were isolated for both the 35- and 75-year-old donors (Table 1). More recently, [24, 27] a protocol modification was tested that may minimize oocyte donor variability, if it does exist. mRNA encoding a histone demethylase (KDM4A) was injected into oocytes, after somatic cell fusion, in a second batch of oocytes from donors whose first batch had failed to support development of expanded SCNT blastocysts. Reconstructed SCNT oocytes supplemented with *KDM4A* mRNA were compared to a noninjected control group. A significant beneficial effect became clear in the injected group by the morula stage and by day 6, where 26.8% of KDM4A-injected embryos developed to the blastocyst stage, as compared to only 4.2% in the controls. Some of the KDM4A-injected embryos (14.3%) developed to the expanded blastocyst stage, while none of the control embryos did, and the beneficial effect of KDM4A was observed in oocytes from all four donors. A total of eight expanded blastocysts supported the isolation of four stable NT-ESC lines and their pluripotency was established. The authors provide evidence that the KDM4A effect involves a reprogramming resistant region (RRR) found to be enriched for the histone H3 lysine 9 trimethylation (H3K9me3) mark. Hence a demethylase that removes the H3K9me3 mark from RRRs facilitates reprogramming.

As noted here and by Cibelli [28], available results now dispel the notion that SCNT is confined to a particular somatic cell type, to donor gender or age, or to the health status of the donor (Table 1). Together these reports allow the conclusion that the conditions supporting human SCNT reprogramming involve: oocyte enucleation using noninvasive imaging and the use of HVJ-E for somatic cell fusion, perhaps without extracellular calcium in the medium, but in the presence of caffeine, to minimize a major risk of premature cytoplast activation; the use of electroporation and histone deacetylase inhibitors or direct injection of demethylase mRNA to augment reprogramming. Based on the results summarized in Table 1, approximately 15%-20% of SCNT embryos should develop to normal blastocysts with a NT-ESC line derivation rate from expanded blastocysts of approximately 30%-50%. Whether or not oocyte quality varies significantly with the donor is still an open question because of the low sample numbers available. However, these results, in the context of very high blastulation rates seen in control IVF-produced embryos or in manipulated embryos, suggest that the limiting challenge in SCNT is development of blastocysts competent to produce NT-ESCs. SCNT may represent a very useful approach for further defining cytoplasmic reprogramming mechanisms.

INDUCED PLURIPOTENT CELLS

This revolutionary approach to creating autologous pluripotent cells and cell lines involves the induction of pluripotency in somatic cells by the forced expression of several transcription factors. The pioneering efforts by Yamanaka and coworkers were published in 2006 [29] and involved the mouse as the mammalian model. Shortly thereafter, success in the human was published simultaneously by the Yamanaka and Thomson groups [30, 31] and finally in the rhesus monkey in 2008 [32]. These discoveries shifted the research focus away from ESCs and NT-ESCs, because factor-based iPSC

reprogramming is a comparatively simple process that does not require access to and use of oocytes or preimplantation embryos. However, iPSCs reportedly suffer several important limitations relating to incomplete erasure of epigenetic marks and genetic instability. There is evidence that they retain residual epigenetic memory typical of parental somatic cells [33], which may lead to bias in their propensity to differentiate to different lineages [22]. Although a variety of alternative reprogramming techniques have been developed, including use of mRNAs, miRNAs, proteins, and small molecules [34–36], only few have been reported as superior to the original integrative viral vector-based methods in terms of genetic stability [34, 36]. However, a comparison between lentiviral encoded transcription factor-mediated reprogramming of germline stem cells (GSC) to iPSCs, to culture condition-mediated reprogramming of the same GSC lines, demonstrated that the two methods were similar in both the retention of epigenetic marks characteristic of GSC and the incidence of epigenetic errors attributable to the reprogramming process. Thus, alternative reprogramming methods may not be superior to standard lentivirus-based reprogramming in terms of epigenetic memory and induction of epigenetic errors [37].

Given the existence of iPSCs and NT-ESCs, both of whom carry advantages and disadvantages, can we conclude that one is dramatically better than the other for regenerative medicine purposes? We will start with molecular epigenetic and transcriptional comparisons between isogenic iPSCs and NT-ESCs (methylation and transcriptome differences) to show that NT-ESC reprogramming produces pluripotent cells that better represent bona fide endogenous pluripotent stem cells.

Gene Expression and Epigenetic Differences Between IVF-ESCs, NT-ESCs, and iPSCs

Prior to the generation of NT-ESCs, studies were published reporting significant variability among human pluripotent stem cell lines [38], including systematic epigenetic and transcriptional differences between IVF-ESCs and iPSCs [39], some of which were attributed to “epigenetic memory” of the somatic cell source in iPSCs [40–42].

It is important to distinguish between the variability that may exist among individual pluripotent stem cell lines, which can arise from subtle experimental differences in handling of the cells (e.g., medium used, passaging method, and passage number, as well as other unrecognized factors) and differences arising from the reprogramming/derivation process. We, therefore, generated IVF-ESCs, NT-ESCs, and iPSCs that were highly matched in regard to their nuclear genomes (i.e., the NT-ESCs and iPSCs were derived using the same human fetal fibroblast [HFF] culture) and mitochondria (i.e., the oocytes used for SCNT were from the same donor who provided the fertilized blastocysts for the IVF-ESCs) [33]. Our initial gene expression microarray analysis consisted of a comparison between NT-ESCs and IVF-ESCs, and revealed few significant differences, with none of the differentially expressed transcripts coming from pluripotency-associated genes. More detailed analyses, including genome-wide DNA methylation microarray, whole genome bisulfite sequencing of the four NT-ESC lines, the four matched iPSC lines and two IVF-ESCs were subsequently performed [33]. These experiments revealed that the NT-ESCs clustered much more closely with the IVF-ESC controls (including IVF-ESC lines from another study [43]) than iPSCs (also including iPSCs from Ziller et al. [43]). Incorporating the data from the HFFs, it was apparent that the large majority of the differences between the NT-ESCs and IVF-ESCs could be accounted for by residual epigenetic and transcriptional signatures from the HFFs. The iPSCs had nearly 60-fold more differentially methylated sites than the NT-ESCs, when compared to IVF-ESCs, and about 1 out of 10 of the sites could be explained by epigenetic memory. These results suggested to us that reprogramming via SCNT better reproduced the bona fide pluripotent state, as represented by IVF-ESCs, compared to induction of pluripotency by forced expression of a small number of pluripotency-associated factors, a conclusion that is consistent with several mouse studies [44]. Two recent articles report that residual patterns in the topology of the nuclear genome from the somatic cell of origin contribute to transcriptional memory in iPSCs, accounting for some of the transcriptional memory that cannot be accounted for by changes in DNA methylation [45, 46].

Subsequently, Johannesson [47] reported on DNA methylation microarray and RNAseq analysis of three NT-ESC and two iPSC lines from a neonatal fibroblast line, one NT-ESC and three iPSC lines from an adult fibroblast culture, and one unrelated IVF-ESC line. Since there was only one IVF-ESC line, statistical comparisons between the NT-ESCs and iPSCs and the IVF-ESC line could not be performed. Instead, RNAseq data from the NT-ESCs and iPSCs were compared to each other, and this analysis revealed very few (only six) differentially expressed transcripts. We speculate that variability from the use of two source fibroblast cultures (including only one NT-ESC line from the adult fibroblast culture) may have limited the ability to detect differences between the two reprogrammed cell types. Hierarchical clustering of the gene expression data revealed that the NT-ESCs from the neonatal fibroblasts clustered away from all of the other pluripotent stem cell lines, while hierarchical clustering of the DNA methylation data revealed that the NT-ESCs and IVF-ESCs clustered according to the fibroblast culture of origin, rather than the reprogramming method. We note that these results do not exclude reprogramming technique-associated differences in DNA methylation, as it is possible that both cell-of-origin and reprogramming method-associated signatures can exist in the same cell. Hierarchical clustering is not the ideal method for complex analyses, as the relationships shown reflect only the predominant source of variability in a dataset, and can be strongly influenced by the normalization and filtering methods applied to the dataset, as well as the settings used for the hierarchical clustering. We also note that in our study, we included data from 3 IVF-ESC and 3 iPSC lines from a previous study by another research group, of which all but one clustered with the corresponding 2 IVF-ESC and 4 iPSC lines from our study.

The third group that successfully generated human NT-ESCs [21] reported in 2015 [26] that the H3K9me3 mark was a barrier to successful SCNT reprogramming, and that overexpression of a H3K9me3 demethylase, KDM4A, improved SCNT. This group discovered that the overexpression of KDM4A activated the expression of many genes, and hypothesized that the failure to activate these genes might explain the low success of SCNT.

In both the Johannesson and Ma studies [33, 47], there were insufficient numbers of NT-ESC and iPSC lines examined

to adequately evaluate the differences in the frequencies of de novo genetic aberrations between these two cell types. The two studies also concurred in the observation that aberrant DNA methylation and gene expression were common at imprinted loci in both NT-ESCs and iPSCs, consistent with findings in SCNT-derived mice [48, 49]. The two studies did come to different conclusions in regard to the similarities between NT-ESCs, IVF-ESCs, and iPSCs. It is difficult to determine whether these differences are due to technical or biological factors, as the two studies differed significantly in experimental design, iPSC reprogramming techniques (retrovirus- and Sendai virus-based vectors for Ma study and mRNA transfection for the Johannesson study), and computational methods.

PROSPECTS FOR CLINICAL APPLICATION OF AUTOLOGOUS PSCS

To date, we must look to the use of adult or somatic stem cells for significant clinical applications, either ongoing, established following Food and Drug Administration approved clinical trials [50] or offered without vetting at Regenerative Medicine Centers around the world. There have been few trials using IVF-ESCs and none with NT-ESCs (see Simonson et al., review) [5]. Since IVF-ESCs are genetically divergent, their uses will likely be restricted to immune privileged areas such as the central nervous system and the eye or require immunosuppression to minimize rejection of transplanted cells [7]. An initial iPSC-based trial involved the treatment of macular degeneration [51]. However, this clinical study has been suspended by the regulatory agency in Japan due to concerns over mutations found in iPSCs. The trial could be continued with more rigorously characterized allogeneic iPSCs cells from a bank [51]. This landmark shift from the autologous to allogeneic clinical applications of iPSCs raises questions as to whether iPSCs pose other advantages if the patient-matched criteria are removed from the equation.

As discussed previously [7] the high cost of producing clinical grade iPSCs for personalized cell therapy could make it more practical to generate a bank of partially matched allogeneic stem cell lines. It is sufficient that banked human cell lines match hypothetical patients within the four critical HLA loci (HLA-A, HLA-B, HLA-C, and HLA-DRB1) to avoid transplant rejection and the need for prolonged immunosuppressive therapy. This implies that fewer iPSC, IVF-ESC or NT-ESC lines with specific HLA profiles could be banked and extensively screened for allogeneic clinical applications.

Based on the SCNT technique optimizations and outcomes described herein, the feasibility of efficient NT-ESC derivation has been established such that despite the relatively restricted availability and high cost of human oocytes, clinical applications of human NT-ESCs for allogeneic use are feasible, if not probable. Substantial numbers of immature human oocytes, either fresh or frozen, are routinely discarded from IVF programs, representing a potential alternative source for human SCNT. However, the process is resource intensive and NT-ESCs are not exact genetic copies of the somatic cell donors, given the presence of oocyte mtDNA (see section below). Interestingly, the derivation of ESCs from biopsied embryonic blastomeres has recently been reported providing yet another option [52, 53].

It is clear to us, and others [54, 55], that further studies are needed to more fully understand the epigenetic, transcriptional, and functional differences, if any, between NT-ESCs and iPSCs. Although there is a rapidly growing literature on iPSCs, ethical, regulatory, and funding considerations have been important obstacles to progress on this front for NT-ESCs.

PLURIPOTENT STEM CELLS AND MITOCHONDRIAL DNA

As indicated above, the relative genetic stability of iPSCs and NT-ESCs could predicate the selection of one over the other of these two cell types for use in regenerative medicine. Genetic aberration could be specific or acquired during reprogramming and thus pluripotent stem cell types could be more or less affected. Other nuclear gene aberrations, like exome mutations, are probably similar since they all come from parental somatic cells. However, the most important differences between iPSCs and NT-ESCs involve mtDNA as somatic mtDNA is particularly affected by age-related, structural damage leading to a decline in OXPHOS activity [56–58]. A higher mutation rate in mtDNA in somatic cells, perhaps 10- to 20-fold, relative to that observed in the nuclear genome has been noted [59], and often both mutated and wild-type mtDNA coexist in the same cell [60]. Germline mtDNA in oocytes is more faithfully maintained and thus IVF-ESCs inherit fewer mutations [23]. It follows then that NT-ESCs clearly are at advantage over iPSCs since their mtDNA is of germline origin. Below, we describe efforts to develop pluripotent cell based approaches to circumvent first generation passage of mtDNA-based disease, to create patient-specific pluripotent cells that are mtDNA mutation-free for the carrier and for assessing age related changes in mitochondrial function and mtDNA mutation load.

Mitochondrial replacement therapy (MRT), to circumvent mother to child passage of mtDNA based disease, involves a variation of nuclear transfer, called spindle transfer, wherein an oocyte spindle-chromosomal complex from the patient is transferred into an enucleated donor oocyte containing healthy mtDNA [61–63]. More recently, in efforts to provide the mtDNA-disease patients with their own genetically corrected stem cells for therapeutic use, patient fibroblasts were reprogrammed directly into iPSCs or indirectly, by SCNT, employing donor oocytes containing healthy mitochondria, into NT-ESCs [25, 64]. While mutant-free stem cell lines could be generated by the isolation and culture of individual iPSCs recovered from patients with relatively low heteroplasmy (amount of mutated mtDNA) levels, only SCNT with subsequent derivation of NT-ESCs could be employed successfully for patients with high heteroplasmic or homoplasmic mtDNA mutations. Genetically rescued patient cell lines with normal metabolic function may eventually enable the transition from palliative care to therapeutic interventions based on regenerative medicine. Applications such as this are awaiting FDA approval.

If mtDNA based mutations are age related, it follows that iPSCs derived from older patient somatic cells should reflect the increased mutation rate. However, the extent of mtDNA defects in proliferating peripheral tissues commonly used for

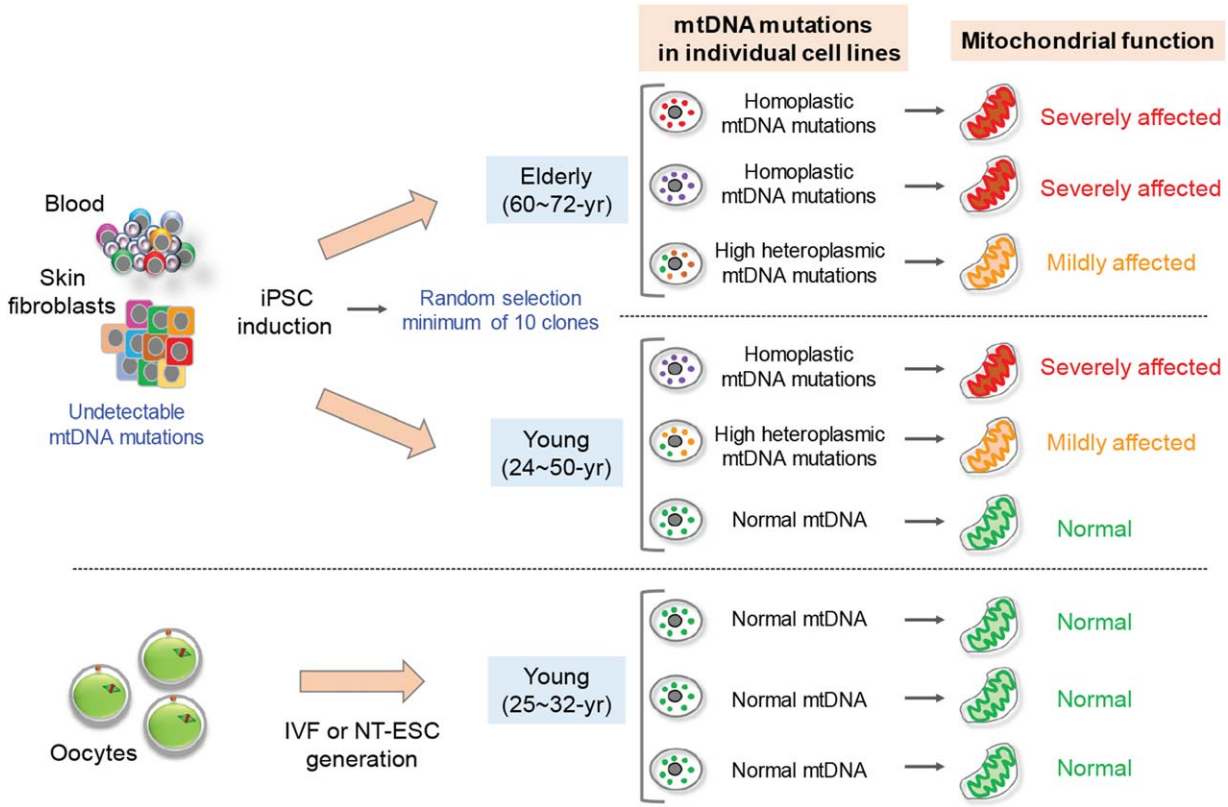

Figure 2. Prevalence of somatic mtDNA mutations in iPSCs derived from young and elderly adults. Due to the random nature of somatic mtDNA mutations, screening pooled samples containing DNA from millions of cells does not reveal individual mutations occurring in each cell. iPSC induction is based on selection and propagation of individual cell clones. Somatic mtDNA mutations, pre-existing in parental cells now become apparent in selected iPSC clones. The frequency of mtDNA mutations in iPSCs is significantly correlated with patient age. Similar to IVF-ESCs, NT-ESCs carry oocyte-derived, germline mtDNA that is greatly protected from mutagenesis compared to somatic mtDNA. Abbreviations: iPSCs, induced pluripotent stem cells; IVF-ESCs, in vitro fertilized-embryonic stem cells; mtDNA, mitochondrial DNA; NT-ESC; ESCs derived by somatic cell nuclear transfer.

iPSC induction, such as skin and blood, is reportedly low and limited to common noncoding variants [56, 65]. Moreover, accumulation of mtDNA variants in these tissues with age was not found [66, 67]. We recently suggested that somatic mtDNA mutations may be under-reported secondary to the level of sample interrogation [23]. In this study (Fig. 2), bulk mtDNA isolated from pooled skin fibroblasts and blood, derived from young and elderly subjects, showed only low heteroplasmic point mutations. However, a panel of ten individually screened iPSC lines from each tissue or clonally expanded fibroblasts carried an elevated load of high heteroplasmic or homoplasmic mutations. Many iPSC mutations were not detectable in parental blood or skin cells suggesting that somatic mutations randomly arise within individual cells. As expected, the frequency of mtDNA defects in iPSCs increased with age, and many mutations were nonsynonymous or resided in RNA genes and, thus, could lead to adverse functionality. These results carry implications not only to the role of mitochondria in aging but also to the limitations of iPSCs derived from elderly patients destined for clinical applications. In the latter case, when significant mutations are present in mtDNA, NT-ESCs are the only available alternative since mutant mtDNA is replaced with unaffected donor mtDNA from the oocyte donor, thereby mitigating the risk of carrying somatic mutations into any resultant pluripotent derivatives.

Conclusion

The critical role of the oocyte/cytoplast in SCNT is now more fully appreciated with the prevention of premature activation on the one hand, coupled with careful somatic cell fusion and ample activation to adequately reprogram somatic cell nuclei to pluripotency, on the other. The importance of oocyte quality in general has long been appreciated; human IVF programs eventually realized that fewer higher quality eggs were associated with higher pregnancy rates, and SCNT outcomes, as described herein, were often correlated with an exceptional oocyte donor both in the monkey [16] and the human [20, 22]. It is exciting to think that oocyte quality may become a less dramatic factor with the protocol modifications described in the past two years. Perhaps we are at the stage where all oocyte donors produce functional oocytes and rejected patient oocytes from IVF programs could be recovered, if not for fertility purposes, then for the production of NT-ESCs. The fact that only a quarter of SCNT embryos develop beyond the 8-cell stage suggests that either the reconstructed embryo is damaged or that the somatic cell nucleus has not been reprogrammed to the extent necessary to support development. In view of the high developmental capacity of IVF-produced and manipulated embryos coupled with the positive effects of histone modulation, incomplete or inappropriate

reprogramming is the most likely culprit. With this focus in mind, continued protocol development to improve the efficiency of reprogramming by SCNT should be pursued.

We assert that NT-ESCs, carrying mutation-free mtDNA, should be seriously considered as a potential source of cells for clinical applications in regenerative medicine. Challenges that must be addressed include: the generation of a collection of diverse and well-characterized lines, produced and handled under clinical grade protocols; the robustness of the cells to long term maintenance, expansion, and low temperature storage; and finally, but very importantly, ethical and/or legal restrictions that may impact NT-ESC use. We note that current U.S. regulations governing funding for derivation and study of NT-ESCs are equally or more restrictive than those for IVF-ESCs. Legislation pertaining to human SCNT in other countries varies, with some countries specifically allowing SCNT for research and therapeutic purposes (referred to as therapeutic cloning; U.K., Sweden, Spain, Finland, Belgium), others banning SCNT for any reason (Norway, France), or banning reproductive cloning but remaining silent on therapeutic cloning (Bulgaria, the Czech Republic, Denmark), and some lacking regulations governing SCNT (Austria, Germany).

From the perspective of addressing the relative safety and functionality of different types of PSCs nonhuman primate studies comparing iPSCs and NT-ESCs produced using the most advanced techniques will be exceedingly valuable. This system would enable rigorous evaluation the totipotency of SCNT embryos through reproductive cloning, an approach that, of course, would not be considered in humans for ethical reasons. Similarly, demonstration of the safety and efficacy of undifferentiated and differentiated PSCs in the nonhuman primate system will be a key prerequisite for clinical trials in human.

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AUTHOR CONTRIBUTIONS

D.P.W. and L.C.L.: Conception and design, manuscript writing; R.M.: Manuscript writing; E.K., H.M., and T.H.: Manuscript editing; S.M.: Conception and design, manuscript writing and editing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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