Scientific and Clinical Advances Advisory Committee Paper

Paper title	**Update on alternative methods to derive ES and ES-like cells**
Paper number	SCAAC(06/15)04
Meeting date	10 June 2015
Agenda item	04
Author	Sarah Testori (Scientific and Clinical Policy Manager)
Information/decision	Decision
Resource implications	None
Implementation	None
Communication	Information updates summarised in this paper and SCAAC's view will be used to update the paper 'Alternative methods to derive stem cells' used by the HFEA Licence Committee when considering research licence applications which involve the use of viable embryos for research purposes.
Organisational risk	Low
Committee recommendation	Members are now asked to: • consider the progress of research (since June 2014) into alternative methods to derive embryonic or embryonic-like stem cells; • advise the Executive if they are aware of any other recent developments; and • reflect on whether their views have changed in the light of recent research.
Evaluation	None
Annexes	None

HUMAN FERTILISATION & EMBRYOLOGY AUTHORITY

1. Introduction

1.1. Human embryonic stem cells (hES cells) have the potential to form every other type of cell in the body. They are important for research into cell biology, drug testing and disease modelling, and could potentially be used in therapies for patients.

1.2. hES cells are derived from the cells of human embryos. Currently the only way to derive hES cells involves using viable embryos but researchers are investigating alternative methods of deriving hES cells, or hES-like cells, without destroying viable embryos.

1.3. Section 3A(1)(c) of Schedule 2 of HFE Act 1990 (as amended) requires embryo research to be "necessary or desirable" for defined purposes. If alternative methods of deriving ES or ES-like cells are developed, it may not be necessary for research groups to destroy viable embryos. It is, therefore, important for the Authority to keep up to date with developments regarding these alternative methods so that the HFEA Licence Committee can bear them in mind when considering research licence applications.

1.4. In February 2015, SCAAC advised the HFEA that alternative methods to derive hES cells should remain a high priority for the Committee and the Authority during 2015/16. The Committee also asked to be periodically updated with relevant research developments, and last considered research in June 2014. This paper summarises key research since June 2014 and is therefore an update to SCAAC paper SCAAC(06/14)01, however, for brevity; in some cases only a citation has been given.

2. Induced pluripotent stem cells (iPSCs)

2.1. Induced pluripotent stem cells are adult somatic cells which have been reprogrammed to an embryonic stem cell-like state. There are many factors which have been implicated in the control of this process, in particular the expression of transcription factors and epigenetic changes are thought to be key mediators.

2.1.1. The following are key papers which elucidate the basic mechanisms or pluripotency:

- Morgani SM, Brickman JM. (2014). The molecular underpinnings of totipotency. Philos Trans R Soc Lond B Biol Sci. 369. Review.
- Boroviak T, Loos R, Bertone P, Smith A, Nichols J. (2014). The ability of inner-cell-mass cells to self-renew as embryonic stem cells is acquired following epiblast specification. Nat Cell Biol.16, 516-28.
- Wu J, Izpisua Belmonte JC. (2014). Stem cells: A designer's guide to pluripotency. Nature. 516, 172-3.

2.1.2. The following papers highlight aberrations that occur in IPSCs as a consequence of reprogramming:

- Martinez-Fernandez A, Nelson TJ, Reyes S, Alekseev AE, Secreto F, Perez-Terzic C, Beraldi R, Sung HK, Nagy A, Terzic A. (2014). iPS cell-derived cardiogenicity is hindered by sustained integration of reprogramming transgenes. Circ Cardiovasc Genet. 7, 667-76.

- Ben-David U, Arad G, Weissbein U, Mandefro B, Maimon A, Golan-Lev T, Narwani K, Clark AT, Andrews PW, Benvenisty N, Carlos Biancotti J. (2014). Aneuploidy induces profound changes in gene expression, proliferation and tumorigenicity of human pluripotent stem cells. Nat Commun. 8;5:4825.

2.1.3. The following papers detail improvements to iPSCs protocols:

- Wei X, Chen Y, Xu Y, Zhan Y, Zhang R, Wang M, et al. (2014) Small molecule compound induces chromatin de-condensation and facilitates induced pluripotent stem cell generation. J. Mol. Cell Biol. 6, 409–420.
- Ji J, Sharma V, Qi S, Guarch ME, Zhao P, Luo Z, et al. (2014) Antioxidant supplementation reduces genomic aberrations in human induced pluripotent stem cells. Stem Cell Reports 2, 44–51.
- Luo L, Kawakatsu M, Guo CW, Urata Y, Huang WJ, Ali H, et al. (2014) Effects of antioxidants on the quality and genomic stability of induced pluripotent stem cells. Sci. Rep. 4, 3779.
- Kishino Y, Seki T, Fujita J, Yuasa S, Tohyama S, Kunitomi A, et al. (2014) Derivation of transgene-free human induced pluripotent stem cells from human peripheral T cells in defined culture conditions. PLoS One 9
- Mormone E, D'Sousa S, Alexeeva V, Bederson MM, Germano IM (2014) "Footprint-free" human induced Pluripotent Stem Cell-derived astrocytes for in vivo cell-based therapy. Stem Cells Dev. 23, 2626–2636.
- Hansson ML, Albert S, González Somermeyer L, Peco R, Mejía-Ramírez E, Montserrat N, Izpisua Belmonte JC. (2015). Efficient delivery and functional expression of transfected modified mRNA in human embryonic stem cell-derived retinal pigmented epithelial cells. J Biol Chem. 290, 5661-72.

2.2. Transcription factors

2.2.1. Cellular reprogramming from somatic cells to induced pluripotent stem cells (iPSCs) can be achieved through forced expression of the 'Yamanaka' transcription factors, Oct4, Klf4, Sox2 and c-Myc (OKSM). These factors, in combination with environmental cues, induce a stable intrinsic pluripotency network that confers indefinite self-renewal capacity on iPSCs.

2.2.2. Although iPSCs are pluripotent, they frequently exhibit high variation in terms of quality. Reliably high-quality iPSCs will be needed for future therapeutic applications. In their recent paper, Buganim et al. (2014) show that the interplay between reprogramming factors is an important determinant of iPSC quality and that high expression of Sall4, Nanog, Esrrb, and Lin28 (SNEL) generated high-quality iPSCs more efficiently than other combinations of factors including OSKM in mouse embryonic fibroblasts (MEFs). The authors also show that high-quality SNEL-iPSCs are correlated with faithful H2A.X deposition patterns (Buganim et al. 2015).

- Benevento M, Tonge PD, Puri MC, Hussein SM, Cloonan N, Wood DL, Grimmond SM, Nagy A, Munoz J, Heck AJ. (2014). Proteome adaptation in cell reprogramming proceeds via distinct transcriptional networks. Nat Commun. 5:5613.

2.3. Epigenetics

2.3.1. The field of epigenetics is concerned with study of cellular and physiological trait variations that are not caused by changes in the DNA sequence. The inheritance of epigenetic marks, such as DNA methylation and histone modifications, provides a molecular memory that ensures faithful commitment to gene expression programs during cellular differentiation. Upon fertilization reprogramming of the parental genomes occurs, which allows proper embryonic development and a state of naive pluripotency to be achieved (from cells that where once differentiated: the egg and sperm). Epigenetic reprogramming is also key in the production of iPSCs (H. J. Lee et al. 2014).

2.3.2. In a recent paper, Lee et al. (2014) use in number of experimental techniques to characterize the epigenomic roadmap that leads to the reprogramming of somatic cells to induced pluripotency. The authors observe that genes with CpG-rich promoters demonstrate stable low methylation and strong engagement of histone marks, whereas genes with CpG-poor promoters are safeguarded by methylation, and as such initial methylation status may act as a modulator of the dynamic changes to histone modification during reprogramming and reveals the crucial role that DNA methylation plays as an epigenetic switch driving somatic cells to pluripotency (D.-S. Lee et al. 2014).

2.4. Ground state stem cells

2.4.1. As previously mentioned, cells that acquire pluripotency have the capacity to give rise to all the somatic lineages of the embryo and to the germline. In vivo, the pluripotent state emerges during development the blastocyst, during which an initial two lineages are formed: the inner cell mass (ICM), which is the pluripotent founder population, and the trophectoderm (TE), which forms an extraembryonic epithelial layer that envelopes and supports the ICM. The ICM goes on to form two further lineages: the epiblast lineage and primitive endoderm. At this point the epiblast cells enter the developmental “ground state,” the origin of all future embryonic lineages. However, until recently the human naïve ES cell state has eluded derivation. A number of recent papers have begun to define the conditions required to generate naive ground state pluripotency in human cells.

2.4.2. In their 2013 paper Jacob Hanna and colleagues established defined culture conditions that allow human embryonic stem cells and induced pluripotent stem cells to acquire a pluripotent state that retains growth characteristics, molecular circuits, chromatin landscape and signalling pathway dependence that are very similar to mouse naive embryonic stem cells. (Gafni et al. 2013). The authors have made additional data available in a corrigendum to this paper (Gafni et al. 2015).

2.4.3. In their recent paper Takashima, et al. (2014) reports that short-term expression of two components, NANOG and KLF2, is sufficient to ignite other elements of the network and reset the human pluripotent state. These reset cells self-renew continuously, are phenotypically stable, and karyotypically intact. They differentiate in vitro and form teratomas in vivo. Metabolism is reprogrammed with activation of mitochondrial respiration as in ESC and DNA methylation is dramatically reduced and transcriptome state is globally realigned across multiple cell lines. These findings demonstrate feasibility of installing and propagating functional control

circuitry for ground-state pluripotency in human cells (Takashima et al. 2014).

2.4.4. Theunissen et al. (2014) took a systematic approach to identifying small molecules that support self-renewal of naive human ESCs. The authors identified a combination of five kinase inhibitors that induces and maintain a molecular signature of ground state pluripotency, when applied directly to conventional human ESCs. These inhibitors generate human pluripotent cells in which transcription factors associated with the ground state of pluripotency are highly upregulated. Comparison with previously reported naive human ESCs indicates that these conditions capture a distinct pluripotent state in humans that closely resembles that of mouse ESCs (Theunissen et al. 2014).

2.4.5. Ware et al. (2014) describe two sets of conditions capable of generating non-transgenic naïve human ES cells (hESCs). The authors show that human naïve cells meet mouse criteria for the naïve state by growth characteristics, antibody labelling profile, gene expression, X-inactivation profile, mitochondrial morphology, microRNA profile and development in the context of teratomas, demonstrating that hESCs can exist in a naïve state without the need for transgenes (Ware et al. 2014).

- Onishi K, Tonge PD, Nagy A, Zandstra PW. (2014). Local BMP-SMAD1 signaling increases LIF receptor-dependent STAT3 responsiveness and primed-to-naive mouse pluripotent stem cell conversion frequency. Stem Cell Reports. 3, 156-68.

2.5. Alternative stem cell states

2.5.1. Various states of pluripotency have been described: those achieved through archetypal derivation of embryonic stem cells, via somatic cell reprogramming, and through the forced expression of key transcription factors in the case iPSCs. However, it is not known whether there are additional classes of pluripotent cells, or the full spectrum of reprogrammed phenotypes. This has been explored in a number of recent publications (Wu et al. 2015; Tonge et al. 2014; Clancy et al. 2014; Hussein et al. 2014; Sancho-Martinez et al. 2014).

2.5.2. In their paper, Wu el al. (2014) show that by modulating culture parameters, a stem-cell type with unique spatial characteristics and distinct molecular and functional features, designated as region-selective pluripotent stem cells (rsPSCs), can be efficiently obtained from mouse embryos and primate pluripotent stem cells, including humans. The authors comment that the ease of culturing and editing the genome of human rsPSCs offers advantages for regenerative medicine applications.

2.5.3. Tonge et al. explore alternative outcomes of somatic reprogramming by fully characterizing reprogrammed cells independent of preconceived definitions of iPSC states. They demonstrate that by maintaining elevated reprogramming factor expression levels, mouse embryonic fibroblasts go through unique epigenetic modifications to arrive at a stable, Nanog-positive, alternative pluripotent state, demonstrating that the pluripotent spectrum can encompass multiple, unique cell states .

2.5.4. The expression of multiple miRNAs play a critical role in the reprogramming process. Indeed, transcription factor-driven reprogramming can be enhanced or replaced by the expression of specific subsets of miRNAs known to be highly expressed in iPSCs. Clancy et al. (2014) use next generation sequencing (NGS) to analyse small RNA expression as MEFs transition towards distinct pluripotent states. The authors observe widespread changes, often characteristic of either the ESC-like or an alternate stable pluripotent state represented by a class of fuzzy colony forming cell lines (F-class).

2.5.5. Two additional papers examining alternative states of pluripotency:

- Hussein SM, Puri MC, Tonge PD, Benevento M, Corso AJ, Clancy JL, Mosbergen R, Li M, Lee DS, Cloonan N, Wood DL, Munoz J, Middleton R, Korn O, Patel HR, White CA, Shin JY, Gauthier ME, Lê Cao KA, Kim JI, Mar JC, Shakiba N, Ritchie W, Rasko JE, Grimmond SM, Zandstra PW, Wells CA, Preiss T, Seo JS, Heck AJ, Rogers IM, Nagy A. (2014). Genome-wide characterization of the routes to pluripotency. Nature. 516, 198-206.
- Sancho-Martinez I, Ocampo A, Izpisua Belmonte JC. (2014). Reprogramming by lineage specifiers: blurring the lines between pluripotency and differentiation. Curr Opin Genet Dev. 28:57-63.

2.6. Development of iPSC-derived retinal pigment epithelium cell for clinical use

2.6.1. When an update on alternative methods to derive ES and ES-like cells was last presented to SCAAC in June 2014, it was concluded that, "while iPSCs are extremely useful for studying diseases, their variability and genetic instability (increased chance of carrying mutations and uncertain epigenetic status) is higher due to the way they are derived. This may make them unsuitable for clinical use." However, since then, research by Masayo Takahashi 's group has shown that techniques for generating iPSCs are moving ever closer to a point where they can be used clinically.

2.6.2. In a recent study, Assawachananont et al. (2014) demonstrated that a protocol for 3D differentiation of mouse iPSC-derived retinal tissue resulted in the formation of aggregates, which as they differentiated evaginated to form optic-vesicle-like structures. These 3D-differentiated iPSC-derived retinal sheets were transplanted into the subretinal space of 6 to 8 week old rd1 mice[1]. The cells were capable of developing a structured outer nuclear layer (ONL) with complete inner and outer segments even in an advanced rd1 mice that lacked ONL. The authors observed host-graft synaptic connections by immunohistochemistry, providing a ‘‘proof of concept’’ for retinal sheet transplantation therapy for advanced retinal degenerative diseases (Assawachananont et al. 2014).

2.6.3. In their paper, Kamao, et al. (2014) characterized human iPSC-derived retinal pigment epithelium cell sheets. The authors generated hiPSC-derived RPE (hiPSC-RPE) cell sheets optimized to meet clinical use requirements,

[1] Rd1 mice are a model of rapid progressive retinitis pigmentosa, with end-stage retinal degeneration. In this model, most rod photoreceptor cells are lost by 3 weeks of age

including quality, quantity, consistency, and safety. The cell sheets were generated as a monolayer of cells without any artificial scaffolds, and expressed typical RPE markers, formed tight junctions that exhibited polarized secretion of growth factors, and showed phagocytotic ability and gene-expression patterns similar to those of native RPE. The authors also generated iPSC-RPE from cynomolgus monkey (*Macaca fascicularis*) somatic cells (miPSC-RPE) and transplanted them into the eyes of recipient monkeys to examine their immunogenicity. These cell sheets showed no immune rejection or tumor formation, and the authors concluded that their results that autologous hiPSC-RPE cell sheets may serve as a useful form of graft for use in tissue replacement therapy for age-related macular degeneration (AMD) (Kamao et al. 2014).

2.6.4. In a culmination to this work, researchers at the RIKEN Centre for Developmental Biology, Japan have now begun the world's first iPSC clinical trial using sheets of retinal pigment epithelium cells, derived from iPSCs, to try to halt the progression of AMD. In September 2014, Nature reported that a Japanese woman in her 70s, suffering from AMD became the first person to undergo the procedure. Some cells from the patient's skin were reprogrammed to produce a sheet of retinal pigment epithelium iPSCs, a 1.3 by 3.0 millimetre piece of which was implanted into the patient's eye. The procedure is unlikely to restore his patient's vision, however, it will allow researchers to see whether the cells are able to halt further destruction of the retina while avoiding potential side effects, such as bringing about an immune reaction or inducing cancerous growth (http://www.nature.com/news/japanese-woman-is-first-recipient-of-next-generation-stem-cells-1.15915).

2.6.5. Other papers demonstrating the therapeutic potential of iPSCs:

- Little MH, Takasato M. (2015). Generating a self-organizing kidney from pluripotent cells. Curr Opin Organ Transplant. 20, 178-86. [PMID: 25856180]
- Xia Y, Sancho-Martinez I, Nivet E, Rodriguez Esteban C, Campistol JM, Izpisua Belmonte JC. (2014). The generation of kidney organoids by differentiation of human pluripotent cells to ureteric bud progenitor-like cells. Nat Protoc.9, 2693-704. [PMID: 25340442]
- Salewski RP, Mitchell RA, Li L, Shen C, Milekovskaia M, Nagy A, Fehlings MG. (2015). Transplantation of Induced Pluripotent Stem Cell-Derived Neural Stem Cells Mediate Functional Recovery Following Thoracic Spinal Cord Injury Through Remyelination of Axons. Stem Cells Transl Med. May 15.
- Hanna CB, Hennebold JD. (2014). Ovarian germline stem cells: an unlimited source of oocytes? Fertil Steril. 101, 20-30.

2.7. Generation of clinical-grade stem cells

2.7.1. Generating clinical-grade cells from pluripotent stem cells (PSCs) for use in patients is not simply a matter of complying with current good manufacturing practices (cGMPs) and chemistry and manufacturing controls (CMCs). A range of other issues demand careful attention, including accessing tissue in an ethical manner and adhering to the varied rules and regulations of specific local and national jurisdictions. In their paper, Andrews at al. (2015) suggest that the current patchwork of practices represents a major hindrance to progress in regenerative medicine, and propose the

establishment of an international body tasked with developing, evaluating and harmonizing the technical, ethical, legal and regulatory frameworks that govern the production of therapies based on PSCs (Andrews et al. 2014).

2.7.2. In another recent article, a broad range of international authors revisit the 2009 consensus on principles of best practice for the procurement, cell banking, testing and distribution of human embryonic stem cell (hESC) lines for research purposes, which was broadly also applicable to human induced pluripotent stem cell (hiPSC) lines, published by contributors from the International Stem Cell Banking Initiative (ISCBI) and the Ethics Working Party of the International Stem Cell Forum. The paper examines in detail the issues surrounding governance and ethics, provenance and selection of donor tissue, safety and characterisation of human pluripotent stem cell (hPSC) stocks, regulation and quality assurance, and future applications of hPSCs, providing an aid to the development of clinical grade materials by providing points to consider in the preparation of seed stocks of stem cell lines for use in cell therapy (Stacey G et al. 2015).

- Heslop JA, Hammond TG, Santeramo I, Tort Piella A, Hopp I, Zhou J, Baty R, Graziano EI, Proto Marco B, Caron A, Sköld P, Andrews PW, Baxter MA, Hay DC, Hamdam J, Sharpe ME, Patel S, Jones DR, Reinhardt J, Danen EH, Ben-David U, Stacey G, Björquist P, Piner J, Mills J, Rowe C, Pellegrini G, Sethu S, Antoine DJ, Cross MJ, Murray P, Williams DP, Kitteringham NR, Goldring CE, Park BK. (2015). Concise review: workshop review: understanding and assessing the risks of stem cell-based therapies. Stem Cells Transl Med.;4, 389-400.

3. Somatic Cell Nuclear Transfer (SCNT)

3.1. The field of SCNT, which involves transferring the nucleus of a somatic cell into an enucleated oocyte, was launched in 1997 with the cloning of Dolly the sheep, and since then has been developed by the successful cloning of more than twenty different mammalian species. SCNT is a potentially valuable tool for generating genetically matched stem cells for research and therapeutic purposes. However, using human SCNT (hSCNT) to generate hESCs has proved more challenging, as early embryonic arrest of human SCNT embryos has prevented derivation of stable nuclear transfer embryonic stem cells (NT-ESCs).

3.2. Last year, for the first time, a group led by Shoukhrat Mitalipov published their success in generating hESCs using clone human embryos. In their paper, Tachibana et al. identified premature exit from meiosis in human oocytes and suboptimal activation as key factors that are responsible for this embryonic arrest, and designed optimized SCNT approaches to overcome these roadblocks. They found, that when applied to premium quality human oocytes it was possible to generate karyotypically normal NT-ESC lines with nuclear genomes inherited exclusively from parental somatic cells, from as few as two oocytes (Tachibana et al. 2013). Since then a number of papers have explored fundamental aspects of hSCNT, reasserting this technique as a powerful research and therapeutic tool.

3.3. For many cell types, reprogramming is more difficult for adult cells than for fetal/infant cells, presumably at least in part due to the fact that their epigenetic landscape is further removed from the pluripotent state, and due to age-related changes such as shortened telomeres and oxidative DNA

damage. In a recent paper, Chung et al. (2014) examined the effect of nuclear donor age on SCNT. By making modification to Tachibana et al's recently developed methodology, the authors were able to generate two karyotypically normal (46, XY) diploid ESC lines, using dermal fibroblasts (DFBs), one from the 75-year-old donor and one from the 35-year-old donor (Chung et al. 2014). This is a landmark achievement as it refutes the hypothesis that cells from older individuals are harder to dedifferentiate (Cibelli 2014).

3.4. In another recent paper Yamada et al. (2014) advanced their previous work investigating the parameters affecting efficiency of blastocyst development and stem-cell derivation from SCNT derived hESCs. The authors showed that improvements to the oocyte activation protocol, including the use of both kinase and translation inhibitors, and cell culture in the presence of histone deacetylase inhibitors, promote development to the blastocyst stage. Further to this, they showed that developmental efficiency varied between oocyte donors, and was inversely related to the number of days of hormonal stimulation required for oocyte maturation. Using a modified nuclear transfer protocol, in which diluted Sendai virus in calcium-free medium was used to prevent premature oocyte activation, the authors derived diploid pluripotent stem- cell lines from somatic cells of a newborn and, for the first time, an adult, a female with type 1 diabetes (Yamada et al. 2014).

3.5. One of the ultimate aims of stem cell research is to enable the generation of autologous pluripotent stem cells for specific patient-matched therapies. The recent advances in SCNT have raised the prospect that this technique could indeed be used in such a way. However, the immunogenicity of mismatched mitochondria in NT-ESCs, may act as an obstacle for clinical application of SCNT-derived cell therapy. This is due to the fact that after transplantation of NT-ESC-derived cells or tissue back into the nucleus donor, mismatched mtDNA-coded proteins may induce alloimmunity.

3.6. In their recent study, Deuse et al. (2015) sought to assess mitochondria-specific alloantigenicity, by conducting a series of experiments transplanting SCNT-derived murine NT-ESCs across defined immunological barriers. Briefly, NT-ESCs were created with nuclear BALB/c nuclear DNA but with BDF-1 mtDNA. These cells were then injected into the thighs of various inbred strains of mice, including the BALB/c and BDF-1 donor strains. The authors used an assay quantifying the activation of T helper cells (Th), which are key in the orchestration of graft rejection. Mitochondria-mismatched transplants initiated marked Th activation and NT-ESC-directed antibody production, although the immune activation was significantly lower than MHC mismatched mice.The authors concluded that mitochondrial alloantigenicity should be considered when developing therapeutic SCNT-based strategies (Deuse et al. 2015).

4. SCNT versus iPSCs

4.1. The quest of regenerative medicine is to produce clinical grade, patient matched human pluripotent stem cells. Embryonic stem cells (ES cells) from in vitro fertilized embryos (IVF ES cells) represent the ‘gold standard’, but they are allogenic to patients. iPSCs represent one method to overcome this allogenicity, as does the newfound capacity to generate hECSs via SCNT, described above. This has ignited a debate over which technique, iPSC or

SCNT, provides a better tool for creating autologous pluripotent stem cells for specific patient-matched therapies.

4.2. In a recent paper, Ma et al. (2014) sought to examine whether the method of reprogramming affects epigenetic and transcriptional aberrations in human pluripotent stem cells. iPCSs have been shown to have high frequencies of genetic and epigenetic abnormalities, including subchromosomal duplications and deletions detected as copy number variations (CNVs), protein-coding mutations and defects in DNA methylation and gene expression at regions subject to imprinting and X-chromosome inactivation. To determine whether such abnormalities are intrinsic to somatic cell reprogramming or secondary to the reprogramming method, the authors undertook genome-wide analyses of genetically matched sets of human IVF ES cells, iPS cells and nuclear transfer ES cells (NT ES cells) derived by somatic cell nuclear transfer (SCNT). Using high-throughput SNP genotyping the authors determined that both NT ES cells and iPS cells derived from the same somatic cells contained comparable numbers of *de novo* CNVs. In contrast, examination of global DNA methylation, methylation at imprinted and XCI regions, and at autosomal non-imprinted loci, revealed that the profiles of NT ES cells corresponded closely to those of IVF ES cells, whereas iPS cells differed and retained residual DNA methylation patterns typical of parental somatic cells. Consistent results were seen when global gene expression patterns were assessed by strand-specific RNA-seq. The authors conclude that NT ES cells combine significant advantages of both IVF ES and iPSCs, combining epigenetic stability and histocompatibility (Ma et al. 2014).

4.3. In their recent paper Johannesson et al. (2014) also examined the differences between reprogrammed human pluripotent stem cells derived by SCNT and those reprogrammed by induced expression of defined factors. Similarly to Ma et al. (2014), the authors compare the genetic and epigenetic integrity of human nuclear-transfer embryonic stem cell (NT-ESC) lines and isogenic induced pluripotent stem cell (iPSC) lines, derived from the same somatic cell cultures of fetal, neonatal, and adult origin. However they find that the two cell types showed similar genome-wide gene expression and DNA methylation profiles, as well as comparable numbers of de novo coding mutations, but significantly more than parthenogenetic ESCs. In contrast to Ma et al. (2014), the authors conclude that the occurrence of these genetic and epigenetic defects in both NT-ESCs and iPSCs suggests that they are inherent to reprogramming, regardless of derivation approach (Johannesson et al. 2015).

4.4. Another potential obstruction to the effective use of reprogrammed pluripotent stem cells in clinical treatment is that of telomere length. Telomeres play key roles in maintaining chromosome stability and cell replicative capacity. Telomere length is maintained by telomerase, and progressive telomere shortening due to absent or insufficient telomerase activity can eventually lead to loss of telomere capping function, which plays important roles in driving degenerative pathologies in humans.

4.5. In their recent paper, Le et al. (2014) examined the capacity of iPSC and SCNT reprogramming approaches to rejuvenate telomeres using late-generation telomerase-deficient ($Terc^{-/-}$) mice that exhibit telomere dysfunction and premature aging. The authors found that SCNT derived

embryonic stem cells had greater differentiation potential and self-renewal capacity than their iPSCs counterparts. In addition, SCNT resulted in extensive telomere lengthening in cloned embryos and improved telomere capping function in the established Terc$^{-/-}$ NT-ESCs, and while mitochondrial function was severely impaired in Terc$^{-/-}$ iPSCs and their differentiated derivatives, in Terc$^{-/-}$ NT-ESCs this was significantly improved. The authors concluded that SCNT-mediated reprogramming mitigates telomere dysfunction and mitochondrial defects to a greater extent than iPSC-based reprogramming (Le et al. 2014).

5. Stem cells from 'waste products'

5.1. Many different cell types have been found within amniotic fluid, with much potential for pluripotent cells isolated from this source.

5.2. As reported in the previous update on alternative methods to derive ES and ES-like cells presented to SCAAC in June 2014, it has been suggested that amniotic epithelial (AE) cells, whaich are found within the amniotic fluid, possess ES- and iPS cell-like pluripotent differentiation characteristics (Miki 2011), with the additional advantage of being retrieved in a non-invasive way and being easy to freeze and store. In the past, studies have suggested that stem cells derived from this source have increasing potential to maintain genetic stability and possess pluripotent characteristics and therefore this source has been monitored as part of our annual review.

5.3. In a recent study, Romani et al. (2015) characterized two distinct types of human amniotic stem cells (HASCs) isolated from residual human ammonic fluid material derived from prenatal diagnostic amniocentesis. The authors observed that the two cell types differed in their morphology and growth kinetics, showing fast (fHASCs) or slow (sHASCs) population-doubling times. Both types expressed pluripotent stem-cell markers, but unlike sHASCs, clonogenic fHASCs would generate embryoid bodies and maintain their original phenotype during prolonged in vitro passaging. Differential proteomic analysis revealed thirty-six proteins that were differentially expressed by the two cell types, and bioinformatic cluster analysis revealed differential occurrence of cytoskeletal proteins (Romani et al. 2015).

5.4. In another paper, Riva et al. (2014) characterized different cell populations in the human ovary, collected during routine IVF procedures. Morphological analysis revealed a heterogeneous cell population, with cells displaying a fibroblast-like, epithelial-like and also neuron-like features. Morpho-functional characteristics of fibroblastlike cells were similar to mesenchymal stem cells, and were positive for mesenchymal stemness markers. Their work suggests that cells with mesenchymal plasticity can be easily isolated from waste follicular fluid, without the need for scraping the ovaries, and that that the cells can be cultivated in minimal conditions. The authors comment that successful growth of such progenitor cells on a three-dimensional scaffold provides the basis for future developments in tissue engineering (Riva et al. 2014).

6. Genome editing stem cells

6.1. While the intended scope of this paper concerned with techniques which could be used to replace the use of human embryos for the generation of

hESCs, the Executive feels it would be remiss not to mention the recent advances in genome editing[2] technologies and the impact these may have on efforts to use stem cells in regenerative medicine.

6.2. A recent study by Crane et al. (2014) highlights the power of these techniques. In their paper, the iPSC lines were derived and characterized from skin fibroblasts from patients diagnosed with cystic fibrosis. The authors then utilized zinc-finger nucleases (ZFNs) to correct the CFTR mutation in these cell lines. These cells, when induced to differentiate in vitro, expressed the corrected CFTR gene and resulted in restoration of expression of the mature CFTR glycoprotein and CFTR chloride channel function. This raises the possibility of using corrected CF iPSCs as a potential source of patient-specific cells capable, in vitro, of differentiation into various lung stem/progenitor cells, either for transplantation of autologous lung cells or for seeding de-vitalized lung scaffolds ex vivo to generate autologous lungs (Crane et al. 2015).

6.3. In another noteworthy, but somewhat troubling advance in this area, scientists in China have used CRISPR-Cas-9 to edit the genome of human embryos. Their research was designed to investigate the potential of CRISPR/Cas9-mediated genome editing in human cells and, in particular sought the correct mutations in the human β-globin (HBB) gene, which encodes a subunit of the adult hemoglobin and is mutated in β-thalassemia. Their results revealed that the efficiency of the technique was low, with apparent off-target cleavage, and lead to the production of mosaic embryos (Liang et al. 2015). However, it raises ethical concerns about whether the germline modification in humans is a desirable course of action to take.

- Suzuki K, Yu C, Qu J, Li M, Yao X, Yuan T, Goebl A, Tang S, Ren R, Aizawa E, Zhang F, Xu X, Soligalla RD, Chen F, Kim J, Kim NY, Liao HK, Benner C, Esteban CR, Jin Y, Liu GH, Li Y, Izpisua Belmonte JC. (2014). Targeted gene correction minimally impacts whole-genome mutational load in human-disease-specific induced pluripotent stem cell clones. Cell Stem Cell. 15, 31-6.

7. ‘Gastruloids’

7.1. Also beyond the normal scope of this paper, but worth highlighting due to the ethical concerns they raise are two recent papers which examined whether particular culture condition could induce ESCs to self-organise in a manner reminiscent of early embryogenesis.

7.2. In a recent paper, van den Brink et al. (2014) demonstrate that under certain culture conditions small aggregates of mouse ESCs, of about 300 cells, self-organise into polarised structures that display collective behaviours reminiscent of those that cells in early mouse embryos exhibit, including

[2] Genome editing refers to a range of techniques which can make double strand breaks into DNA at targeted locations and can also introduce specific DNA sequence changes at these locations. Transcription activator-like effector nucleases (TALENs) and zinc-finger nuclease (ZFNs) are powerful tools which have been recently established. However, these may be superseded by a technique known as CRISPR-Cas-9 (often referred to as CC9 or just CRISPR), which allows genome editing with unprecedented ease, speed and at low cost.

symmetry breaking, axial organisation, germ layer specification and cell behaviour, as well as axis elongation. The authors note that these movements are similar to those of cells in gastrulating embryos and for this reason term these aggregates ‘gastruloids’ (Brink et al. 2014).

7.3. In their paper Warmflash et al. (2014) showed that geometric confinement was sufficient to trigger self-organized patterning in hESCs. In response to BMP4, colonies reproducibly differentiated to an outer trophectoderm-like ring, an inner ectodermal circle and a ring of mesendoderm expressing primitive-streak markers in between. Examination of different colony sizes revealed identical dynamics of patterning as measured from the colony edge inwards, demonstrating that patterning has a fixed length scale and proceeds identically in time, regardless of the size of the colony (Warmflash et al. 2014).

8. Conclusions

8.1. When SCAAC last considered the progress of research in June 2014, the Committee was interested in new developments in iPSCs and in SCNT ES cells, and suggested that SCNT ES cells could potentially provide a more clinically relevant source of cells. The Committee noted that the quality of stem cell lines derived from IPS cells are improving but further experiments are required to fully characterise these

8.2. As in previous years, SCAAC concluded in 2014 that, despite promising developments in the iPSC creation process, there is still no viable equivalent to embryonic stem cells and therefore the creation of stem cells from embryos may still be considered “necessary or desirable” for defined purposes. The Committee noted that it has been shown that it may be possible to develop SCNT embryos for the derivation of patient-matched ES cells. The Committee agreed to continue to review research on an annual basis.

8.3. Since then further research reveals that SCNT is capable of generating hESC lines from older individuals. It has also been shown that telomere dysfunction and mitochondrial defects are mitigated to a greater extent in SCNT than iPSC-based reprogramming. However there is conflicting evidence for the transcriptional, genetic and epigenetic stability of one cell type over another. Progress also been made in our understanding of the processes that underlie pluripotency, and in particular in our understanding of naïve ground state pluripotency in humans and alternative pluripotent states. Strides have also been made in the development of iPSCs for clinical treatment, with clinical trials currently underway in Japan. In this paper the Committee is asked to reflect on these developments, and consider whether the creation of stem cells from embryos is still “necessary or desirable”.

9. Recommendations

9.1. Members are now asked to:

- consider the progress of research (since June 2014) into alternative methods to derive embryonic or embryonic-like stem cells;
- advise the Executive if they are aware of any other recent developments; and

- reflect on whether their views have changed in the light of recent research.

9.2. Information summarised in this paper and SCAAC's view will be used to update the paper 'Alternative methods to derive stem cells' used by the HFEA Licence Committee when considering research licence applications which involve the use of viable embryos for research purposes.

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