FOURSIGHT
ASSOCIATES PTY LTD

12th September 2006

The Premier, Hon. Steve Bracks
Government of Victoria
Parliament House
Spring Street
MELBOURNE, VIC 3000

The Minister for Innovation, Hon. John Brumby
Government of Victoria
Parliament House
Spring Street
MELBOURNE, VIC 3000

Re: **Progress in Stem Cell R&D**

Dear Premier, Minister

As departmental Chief Scientists, we herewith provide commentary on technological developments over the past few years in the field of human stem cells in regenerative medicine. Our views have been informed by a comprehensive literature review and analysis by Nick Gough and Associates Pty Ltd commissioned by the Department of Innovation, Industry and Regional Development. As indicated, it is our firm opinion that recent advances in this technically-challenging, highly-regulated field have been very substantial and are worthy of notice.

Yours sincerely,

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PROGRESS ON HUMAN ES CELLS FOR NEW REGENERATIVE MEDICINES

The field of stem cell research can only be sensibly addressed if the long time frame of medical discoveries is realized.

Human embryonic stem (ES) cell lines were first created in 1998 from very early human embryos (blastocysts), the techniques used being based on at least a decade of research on mouse ES cells. Technical developments over the ensuing 8 years have been on 4 fronts: (a) discovery of better methods for growth and maintenance of human ES cell lines in vitro, including major advances to ensure regulatory Good Manufacturing Practice (GMP) compliance and even commercial scale production; (b) advances in methods to more reliably drive ES cells along particular pathways of specialization, for example to develop into cells of muscle, brain, pancreas etc – a process known as differentiation; (c) demonstration of the (medically-relevant) capabilities of human ES cells and their differentiated progeny in at least 5 animal models of human disease; and (d) isolation of many new ES cell lines and establishment of international, collaborative cell banks and networks for sharing of lines and techniques.

In a field with such potential for new clinical treatments, it is not surprising that progress over an 8 year period has been substantial and certainly on a par with another cell-based therapy with excellent prospects, namely dendritic cell treatments for boosting immunity. Whilst the R&D has advanced in the ES cell area, so it has with tissue (so-called adult) stem cells. Having examined progress, ES cells will not be superceded anytime soon and their special, if not unique, characteristics will continue to be highlighted; certainly “breathless advocacy” for one versus the other cell type in development of new regenerative medicines (as well as a variety of cell-based assays, see below) is misplaced at the present time. In regard to adult stem cells, some studies have demonstrated greater developmental potency than previously thought but generally with more limited potential than ES cells; some are entirely tissue-specific, others more plastic but not totipotent. Growth to required quantities is a major limitation in adult stem cell R&D.

Differentiated ES cell progeny have been used for drug screening and, most recently, for toxicology testing thereby potentially reducing animal use in new drug development. As further indication of progress, the US company Geron has signaled its intention to initiate a clinical trial with human ES cell-derived cells for spinal cord injuries in 2007. However, achieving appropriate regulatory compliance still presents considerable challenges.

The field of stem cell transplantation in humans faces 3 serious technical hurdles:

(a) Transplant rejection, as happens with kidney or heart transplants. This must be countered by strong drugs, but monitoring rejection will be particularly difficult with cells injected as a single cell suspension into particular organs of the recipient. Tissue matching is one possibility but large banks of tissue-typed ES cell lines create serious practical difficulties. Nevertheless, it has been estimated that 150 carefully-selected ES cell lines could be suitable for use, and rejection managed, in up to 50% of patients. Teaching the patient to “tolerate” the graft is a second possibility, and some progress has been made in experimental systems to induce tolerance to grafted cells (actually by injecting ES cells themselves). However, general methods to achieve tolerance are not available and progress is slow in this area.

(b) Guidance of the ES cells down the correct pathways of differentiation. We still have much to learn about the mixture of specialized growth factors which will be required.
(c) Ensuring that cells of such great proliferative potential do not develop into cancers, even on rare occasions.

If transplant rejection is the biggest single concern then this is where the extraordinary, legislatively-constrained technology of SCNT – somatic cell nuclear transfer- comes into its own. Clearly, SCNT has the potential to overcome the transplantation barrier through "personalization" of the ES cells.

SCNT uses similar technology to that used in reproductive cloning. The scientific community unanimously rejects cloning of human beings. However, this should be differentiated from the use of SCNT to produce stem cells for medical research, which should only be allowed within the strict regulatory framework that is currently applied to stem cell research. The likelihood of any SCNT construct surviving if implanted in the womb is incredibly low. Nonetheless, such an occurrence would continue to be strictly prohibited.

SCNT involves replacing the nucleus of an egg cell (the ovum or oocyte) with that of a tissue (somatic) cell (e.g a patient's own skin cell) and deriving SCNT-ES cell lines from the subsequent SCNT embryo. Questions have been raised about the integrity of SCNT-ES cells but 2 publications in 2006 demonstrate that, in mice, they can be indistinguishable from ES cells derived in the usual way according to a number of criteria. It has also been demonstrated (Rideout et al., 2002) that genetically-modified SCNT-ES cells can function to partially restore a defective immune system in mice. The key biological event, and necessary outcome in SCNT, is the reprogramming of the somatic cell genome to take on embryonic characteristics such as broad differentiation capabilities.

To date, there has been no successful development of human SCNT-ES cell lines and fraudulent claims from South Korea made headlines in late 2005. Licenses to use human SCNT-ES cells for research purposes have been issued in the UK and US groups have indicated their intention to similarly advance human SCNT technology.

In regard to sources of oocytes, those derived from assisted reproductive technologies (e.g IVF) offer possibilities though they may not be entirely satisfactory. With limited availability of human oocytes (and with access suitably controlled – see Lockhart Report recommendations 31-33), other cells types (including ES cells) are being trialled to determine whether the reprogramming that is necessary in SCNT can be achieved with non-oocyte (embryonic) cell lines already available. Whether the oocyte has unique properties for reprogramming has yet to be determined.

Of course, major advances will occur in animal systems where the 2 key applications of SCNT technology will continue to be put to the test:

- Research applications where SCNT-ES cells using diseased tissue cells for their generation will lead to deeper understanding of complex multigenic diseases, dissection of genetic versus epigenetic phenomena, and the screening for new drugs, and
- Therapeutic applications to treat degenerative diseases through grafting of genetically matched (± genetically modified) SCNT-ES cells.

Concluding Comment

In the opinion of these reviewers and in the current and appropriate cautious and regulated environment, a broad SCNT approach is required for stem cell-based regenerative medicine to achieve its undoubted promise. On the specific question of whether the field has actually progressed in a technological sense, we can respond unequivocally in the affirmative. Formidable challenges confronting the field have been addressed particularly around the generation of clinically-acceptable human ES cells and production of medically-relevant tissue cells from human ES cells (tested in animal systems). SCNT appears to be the best current approach to address the fundamental issue of rejection by the recipient of transplanted cells. Finally, very obvious progress has been made in the use of ES cells and their progeny in cell-based screening for new drugs, for toxicology assays, and for the identification of molecules involved in ES cell self renewal and, conversely, differentiation into tissue cells.

We have mentioned the long time frames for product development in the medical field; 20 years from discovery to clinical practice is not unusual. In that context, the amount of progress that has been made in a scant 8 years with human ES cells is breathtaking. Australian scientists have been prominent in this global endeavor and should not be excluded from the next exciting chapter involving SCNT-ES cells. The Lockhart Report of December 2005 is a wise, considered, balanced report and its recommendations should be accepted and broadcast.

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Key Recent Advances in Human Embryonic Stem Cell Research

A Review of Scientific Literature Commissioned by the Department of Innovation, Industry and Regional Development, Government of Victoria, Australia

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September 8 2006

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Important Notice

This Report has been prepared by Dr. Nicholas Gough FTSE at the request of The Department of Innovation, Industry and Regional Development, Government of Victoria, Australia for the purpose of providing an objective scientific summary of key recent advances in human embryonic stem cell research that are of particular relevance to the ultimate medical and clinical deployment of embryonic stem cell-derived tissues in regenerative medicine and to summarise key recent advances with respect to Somatic Cell Nuclear Transfer (SCNT).

Dr. Gough was assisted in the preparation of this document by Dr. Megan Munsie, Scientific Development Manager of Stem Cell Sciences Ltd.

Interests Statement

Dr. Gough is Chairman of the Scientific Advisory Board of the Cooperative Research Centre for Innovative Dairy Products which is engaged, inter alia, in bovine embryonic stem cell research. Nick Gough & Associates Pty Ltd (ACN 087 390 501), of which Dr. Gough is a director and shareholder, holds options to acquire ordinary shares in the Singaporean stem cell company ES Cell International Pte Ltd.

Preamble

The purpose of this paper is to summarise key recent advances in human embryonic stem cell research that are of particular relevance to the ultimate medical and clinical deployment of embryonic stem cell-derived tissues in regenerative medicine and to summarise key recent advances with respect to Somatic Cell Nuclear Transfer (SCNT).

This paper does not consider advances in research associated with stem cells derived from adult (non-embryonic) tissues, nor compare and contrast the relative merits of adult versus embryonic stem cells. Nor does this paper address issues associated with the ethics and societal acceptance of human embryonic stem cell research, the current legislative framework governing the field, nor the Australian Legislation Review of the *Prohibition of Human Cloning Act* 2002 and *Research Involving Human Embryos Act* 2002, known as the Lockhart Report.

This review is extensively referenced, principally to primary peer-reviewed research papers and to reviews in authoritative journals. Where appropriate or necessary, some references to news items and the such like are included. Most source material used in compiling this review is available on a CD accompanying this report, or in the case of on-line material, by direct link from the reference in the footnote in the text of the review.

Executive Summary

The purpose of this scientific literature review is to summarise

- key recent advances in human embryonic stem (ES) cell research, particularly those of relevance to the ultimate medical and clinical deployment of ES cell-derived tissues in regenerative medicine, and
- key recent advances with respect to Somatic Cell Nuclear Transfer (SCNT).

It is evident from a review of the relevant scientific literature that the field of human ES cell research has advanced substantially since human ES cells were first derived in 1998. Indeed for a field as new and as complex as this, the rate of progress has arguably been dramatic – certainly it compares favourably with the development times associated with other complex new biomedical and biopharmaceutical modalities.

Significant progress has been made on a number of fronts, demonstrating the growing likelihood of achieving the promise of clinically-valuable new regenerative medicines addressing major disease targets.

In particular, in recent years

- A number of major international cooperative efforts to develop standardised Stem Cell Banks and Registries to facilitate the exchange of cell lines internationally through reciprocal deposition, and to develop recommended best practice with respect to culturing techniques and media development have been initiated.
- Processes for growing human ES cells have developed significantly from relatively rudimentary laboratory-based procedures, to fully-defined culture systems potentially compliant with Good Manufacturing Practice (GMP) and more amendable to development of commercial scale propagation processes.
- A large number of new human ES lines have been isolated and in particular a number of new lines have been isolated under conditions that are potentially compliant with GMP.
- There has been a steady increase in the number of reports where human ES cells can be coaxed in vitro to produce various mature cell types, including neuronal, cardiac, erythroid, myeloid, hepatic and pancreatic.
- A number of recent studies have reported promising physiologically- or therapeutically-relevant function of human (or in one case monkey) ES cell-derived cells when transplanted into animal models of various human diseases, including
    - Dopaminergic neurones that are capable of promoting partial behavioural recovery in rat and primate models of Parkinson's disease.
    - Cardiomyocytes that can restore proper heart rate when grafted into a pig model of impaired cardiac function.
    - Oligodendrocyte progenitor cells that are capable of enhancing remyelination and promoting improvement in motor function when transplanted early after spinal cord injury in a rat model.
    - Pancreatic islet-like cells that are capable of short-term reversal of the hyperglycaemic state in diabetic mice.

  - Myogenic (muscle) precursor cells that are capable of incorporating into regenerating muscle fibres, and giving rise to myotubes, myofibres and muscle satellite cells in mice.

- Whilst the potential issue of immune rejection of transplanted tissues derived from ES cells has yet to be resolved, developments that will lead to possible resolutions to this issue include
  - Initiation of large broadly representative stem cell banks to include diverse HLA types.
  - Demonstration in a rat model of the potential for inducing immune tolerance.
- There is burgeoning interest in the use of embryonic stem cells and differentiated cell types derived from them in drug screening and in vitro toxicology testing. An important recent development in this regard is the demonstration of the capacity of ES cell-derived hepatocyte cells to be used in in vitro hepatotoxicity tests.

With respect to SCNT, a number of key developments and advances have occurred, including

- The demonstration that the molecular and developmental parameters of murine ES cells generated by SCNT are indistinguishable from ES cells derived from a normal blastocyst generated by fertilisation of an oocyte by a sperm.
- The demonstration that several different cell types derived from SCNT-stem cells engraft and are not rejected in a bovine model.
- Proof-of-concept that stem cells generated from somatic cells through the technique of SCNT could restore function to damaged tissues in animal models of Parkinson's disease and immuno-deficiency.
- Demonstration of the value of SCNT to investigate the epigenetic factors influencing malignant phenotype.
- The first reports of the derivation of human ES cell lines by SCNT by the South Korean group of Hwang et al in 2004 and 2005 were retracted in January 2006 after they were concluded to be fraudulent.
- Human SCNT-derived ES cells are yet to be generated. This is perhaps not surprising given the restrictive legislative environment governing these studies in most jurisdictions. A small number of licences to conduct such research have been recently issued.

Key Advances in Human ES Cell Research

In the eight years since the first derivation of human ES lines[1], there has been a progressive and steady increase in understanding the basic biology of these cells and in understanding and progressing their potential in regenerative medicine.

From the first sporadic reports of derivation in the late 1990s, there are now more than 75 fully characterised lines[2] and perhaps as many as 300 lines, with unstated levels of characterisation[3].

There is a major international effort, the International Stem Cell Initiative[4], whose charter is to compare cell lines isolated under different conditions and develop recommended best practice with respect to culturing techniques and media development.

National Stem Cell Banks and Registries are being developed to facilitate the wide exchange of cell lines internationally through reciprocal deposition.

In an important move, the Australian stem cell company Stem Cell Sciences, together with Melbourne IVF and the Australian Stem Cell Centre, are making all six of the intended MEL series of embryonic stem cell lines available to Australian and international researchers and companies fully unencumbered[5]. More recently the first of these lines was accepted for inclusion in the UK Stem Cell Bank.

It should also be noted that in the past four years there has also been progress and improvements in the tissue stem cell field, encompassing stem cells isolated from adult, neonate and foetal tissues. A growing list of studies has identified ES cell-like subpopulations of tissue stem cells, in particular, the multipotent adult progenitor cells (MAPC) in the bone marrow[6] and the unrestricted somatic stem cells in cord blood[7]. While both these studies have demonstrated greater developmental potency than previously thought, they also demonstrated some restriction, either in the developmental potential of the isolated stem cells or in the ability to grow the cells in culture to a required quantity necessary for any therapeutic application. Indeed, as Catherine Verfaillie, in whose laboratory the MAPCs were identified, is reported[8] "We need a whole football field to grow them."

It is unclear which type of stem cell will be best for each specific therapeutic application. In some conditions, such as repair of a cardiac infarct, it was initially thought that stem cells derived from cord blood or bone marrow may be of most

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[1] Thomson et al (1998). Embryonic stem cell lines derived from human blastocysts. Science 282: 1145 – 1147.

[2] Andrews et al (2005). The International Stem Cell Initiative: toward benchmarks for human embryonic stem cell research. Nature Biotech 23: 795 - 797.

[3] Abbott et al (2006). The lure of stem-cell lines. Nature 442: 336 – 337.

[4] Andrews et al (2005). The International Stem Cell Initiative: toward benchmarks for human embryonic stem cell research. Nature Biotech 23: 795 - 797.

[5] Stem Cell Sciences Press Release (June 6 2004). Australian company to provide human stem cell lines for world's researchers. http://www.stemcellsciences.com/press_releases/bio2004.pdf.

[6] Jiang et al (2002). Pluripotency of mesenchymal stem cells derived from adult bone marrow. Nature 418: 41 – 49.

[7] Kögler et al (2004). A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med 200: 123 - 135.

[8] Finkel (2005). Stem Cells: Controversy at the frontiers of science. (ABC Books) p. 85.

benefit, however this has recently been challenged[9, 10]. What is required, in the view of this author, to maximise the potential of regenerative medicine in its totality, is appropriate, non-polarised research across the spectrum of stem cell types.

From a technological perspective, in order for cells and/or tissues derived from human ES cells (or indeed stem cells derived from any sources) ultimately to be deployed in a clinical setting and to represent commercially-viable, clinically-valuable and routinely-applicable therapeutic modalities, a number of key issues must be addressed and satisfactory solutions obtained.

A number of key advances in human ES cell research with respect to these key issues and that are of particular relevance to the potential utility of human ES cells in human regenerative medicine, are summarised below, along with a summary of recent developments in the area of SCNT.

To date, most human ES cell lines have been propagated using cell culture systems that are laborious, manually-intensive, inefficient and involve complex components in the tissue culture environment, typically involving co-culture with other support or "feeder" cells. A typical research laboratory may have systems that enable production in the range 10^8 – 10^9 cells per week – many orders of magnitude less than will be required to underpin clinical trials and commercial production. Taken together, the traditional requirements for human ES cell culture are unacceptable for future scale-up and automation.

Systems that are ultimately to be used clinically and commercially would need to be simple, reproducible, scaleable, automatable, cost-effective and GMP-compliant. Significant progress towards these objectives are now being made on several fronts with numerous reports now suggesting that human ES cells may be grown in feeder-free, serum-free, scaleable culture systems.

Significant progress has been made in identifying various growth factors and other molecular components that promote variously survival, self-renewal, proliferation and maintenance of undifferentiated, pluripotential human ES cells in culture[11, 12, 13, 14, 15, 16, 17]. Concomitantly, culture systems omitting various poorly defined, complex and/or non-human components (such as feeder cells, condition medium, foetal calf serum

[9] Laflamme and Murry (2005). Regenerating the heart. Nature Biotech 23: 845 - 856.

[10] Passier and Mummery (2005). Cardiomyocyte differentiation from embryonic and adult stem cells. Curr Opin Biotechnol 16:1 - 5.

[11] Sato et al (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signalling by pharmacological GSK-3-specific inhibitors. Nature Med 10: 55 - 63.

[12] Valier et al (2004). Nodal inhibits differentiation of human embryonic stem cells along neuroectodermal default pathway. Dev Biol 275: 403 – 421.

[13] Beattie et al (2005). Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers. Stem Cells 23: 489 – 495.

[14] Levenstein et al (2006). Basic fibroblast growth factor support of human embryonic stem cell self-renewal. Stem Cells 24: 568 – 574.

[15] Ludwig T et al (2006). Derivation of human embryonic stem cells in defined conditions. Nature Biotechnol. 24: 185 – 187.

[16] Liu Y et al (2006). A novel chemical-defined medium with bFGF and N2B27 supplements supports undifferentiated growth in human embryonic stem cells. Biochem. Biophys. Res. Comm. 346: 131 – 139.

[17] Pyle, et al (2006). Neurotrophins mediate human embryonic stem cell survival. Nature Biotechnol. 24: 344 – 350.

etc.) have also been progressively trialled[18, 19, 20], leading to a number of recent reports of long-term propagation of human ES cells in feeder-free, conditioned medium-free, chemically-defined systems[21, 22, 23, 24, 25, 26].

In addition, there are now numerous reports of human ES culture systems in which mechanical disaggregation of human ES colonies is being replaced enzymatic passaging, which will be more amenable to scale-up[27, 28, 29].

In other developments, there are now reports of propagation of human ES cells in more genuinely scaleable culture systems, including perfusion cultures[30] and cell factories[31].

There is a major international effort, the International Stem Cell Initiative, whose charter is to undertake a detailed comparison of some 75 different human ES cell lines, to understand differences that may pertain to different lines and to develop recommended best practice with respect to culturing techniques and media development[32].

A number of studies examining the genomic stability of human ES lines have been undertaken using traditional karyotypic analyses, and have led to disparate results. One group reported[33] recurrent gains of chromosomes 17q and 12, whereas others have reported stable karyotypes for up to 2 years[34, 35, 36]. In a more recent study,

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[18] Mallon et al (2006). Toward xeno-free culture of human embryonic stem cells. Int. J. Biochem. Cell Biol. 38: 1063 – 1075.

[19] Sjögren-Jansson et al (2005). Large-scale propagation of four undifferentiated human embryonic stem cell lines in a feeder-free culture system. Developmental Dynamics 233: 1304 – 1314.

[20] Levenstein et al (2006). Basic fibroblast growth factor support of human embryonic stem cell self-renewal. Stem Cells 24: 568 – 574.

[21] Lu et al (2006). Defined culture conditions of human embryonic stem cells. Proc. Natl. Acad. Sci. USA 103: 5688 – 5693.

[22] Liu Y et al (2006). A novel chemical-defined medium with bFGF and N2B27 supplements supports undifferentiated growth in human embryonic stem cells. Biochem. Biophys. Res. Comm. 346: 131 – 139.

[23] Yao et al (2006). Long-term self-renewal and directed differentiation of human embryonic stem cells ion chemically defined conditions. Proc. Natl. Acad. Sci. USA 103: 6907 – 6912.

[24] Ludwig T et al (2006). Derivation of human embryonic stem cells in defined conditions. Nature Biotechnol. 24: 185 – 187.

[25] Li et al (2005). Expansion of human embryonic stem cells in defined serum-free medium devoid of animal derived products. Biotechnology and Bioengineering 91: 688 – 698.

[26] Ellerström et al (2006). Derivation of xeno-free human ES cell line. Stem Cells (published on-line June 1 2006).

[27] Sjögren-Jansson et al (2005). Large-scale propagation of four undifferentiated human embryonic stem cell lines in a feeder-free culture system. Developmental Dynamics 233: 1304 – 1314.

[28] Suemori et al (2006). Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. Biochem. Biophys. Res. Comm. 345: 926 – 932.

[29] Li et al (2005). Expansion of human embryonic stem cells in defined serum-free medium devoid of animal derived products. Biotechnology and Bioengineering 91: 688 – 698.

[30] Fong et al (2005). Perfusion cultures of human embryonic stem cells. Bioprocess. Biosyst. Eng. 27: 381 – 387.

[31] Choo et al (2005). Immortalized feeders for the scale-up of human embryonic stem cells in feeder and feeder-free conditions. J. Biotechnol. 122: 130 – 141.

[32] Andrews et al (2005). The International Stem Cell Initiative: toward benchmarks for human embryonic stem cell research. Nature Biotech 23:795-97.

[33] Draper et al (2004). Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nature Biotech 22: 53 – 54.

[34] Buzzard et al (2004). Karyotype of human ES cells during extended culture. Nature Biotech 22: 381 – 382.

Imreh et al have reported[37] chromosome 7 and 12 abnormalities accumulating in a high passage hES line on adaptation to growth free of extracellular matrix (ECM).

In a more detailed analysis Maitra et al[38] noted that in human ES lines stratified by culture conditions and passaging technique, there was a bias for occurrence of copy number abnormalities in late passages that were maintained in feeder-free conditioned medium and passaged by enzymatic methods. They noted however, that these observations could bear further analysis in a larger series of human ES lines.

Given that genomic alterations tend to accrue over time in tissue culture, they do not detract from the therapeutic potential of early passage lines, which seem to be largely free of such changes. These observations underscore however, the need for carefully controlled analysis of culture conditions and also careful management of ES lines – as would of course be required under conditions of Good Manufacturing Practice.

To date, most human ES cell lines have been derived and propagated in culture systems that would potentially limit their acceptability for clinical use, including the use of non-human (murine) support or "feeder" cells, non-GMP-compliant animal-derived products such as foetal calf serum and other reagents, and non-GMP-compliant procedures for obtaining embryos, deriving cell lines and establishing master cell banks.

The US FDA announced in 2000 that cell therapies involving stem cells from embryos or adults would be regulated as drugs, not as surgical techniques, therefore requiring exacting standards of purity and potency to be met[39]. With respect to the specific issue of non-human feeders, the FDA has indicated that past exposure to animal cells per se does not necessarily disqualify ES cell lines from clinical use – so long as appropriate safety issues are addressed[40].

Recent European Union directives (2003/94/EC and 2004/24/EC) require that human ES cells for transplantation must be cultured using conditions of GMP in order to guarantee the safety and quality of the cells[41, 42].

One approach to address this issue is to seek to rederive pre-existing human ES lines and adapt them to more appropriate propagation conditions, and indeed recently there has been a progressive evolution towards propagating and adapting human ES cells

[35] Brimble et al (2004). Karyotypic stability, genotyping, differentiation, feeder-free maintenance, and gene expression sampling in three human embryonic stem cell lines derived prior to August 9, 2001. Stem Cells and Dev 13: 585 – 596.

[36] Rosler et al (2004). Long-term culture of human embryonic stem cells in feeder-free conditions. Developmental Dynamics 229: 259 - 274.

[37] Imreh et al (2006). In vitro culture conditions favouring selection of chromosomal abnormalities in human ES cells. J Cell Biochem 99: 508 516.

[38] Maitra et al (2005). Genomic alterations in cultured human embryonic stem cells. Nature Genet 37: 1099 – 1103.

[39] Vogel (2005). Ready or not? Human ES cells head towards the clinic. Science 308: 1534 – 1538.

[40] Battey (2004). Letter to Suzanne Kadereit, Editor ISSCR. January 15, 2004.

[41] Rodríguez C et al (2006). Derivation of clinical-grade human embryonic stem cells. Reproductive BioMedicine Online 12: 112-118.

[42] Hovatta (2004). Derivation of human embryonic stem cell lines – towards GMP. Pasteur Institute EuroConference on Stem Cells (December 2004).

- under conditions using human rather than murine feeder cells[43, 44],
- under conditions free of feeder cells[45, 46, 47, 48] and
- under conditions free of animal components[49 50, 51].

A more robust approach (and probably in order to meet conditions of GMP in fact required[52, 53]), would be to derive and propagate new human ES cells ab initio under clinically-compliant GMP conditions.

In order to achieve this, a number of specific issues associated with human ES cell derivation and propagation processes have needed to be addressed, including development of

- GMP-compliant, preferably human, feeder cells, GMP-compliant growth media for feeder cell propagation and GMP-compliant enzymes for feeder cell passaging, or a human ES derivation and propagation system that does not require additional feeder cells.
- GMP-compliant cell growth substrates, or cell growth systems that do not require substrates.
- GMP-compliant human ES growth media and GMP-compliant human ES propagation processes.
- GMP-compliant cryopreservation protocols.
- a GMP-compliant (presumably single batch) master cell bank.

Moreover, the embryos from which the stem cells are derived would themselves also need to be obtained under GMP-compliant protocols.

Significant progress has been made over the past 6 to 12 months in this regard, with a number of reports of the derivation of new human ES lines under conditions that either partially or potentially completely, address these requirements

- Earlier this year Ludwig et al (2006)[54] derived a new human ES line under cell culture conditions free of feeder cells and from which all animal-derived

[43] Hovatta et al (2003). A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. Hum Reprod 18: 1404 - 1409.

[44] Richards et al (2003). Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem. Stem Cells 21: 546 - 556.

[45] Heins et al (2004). Derivation, characterization, and differentiation of human embryonic stem cells. Stem Cells 22: 367 - 376.

[46] Xu et al (2001). Feeder-free growth of undifferentiated human embryonic stem cells. Nature Biotech 19: 971 - 974.

[47] Amit et al (2004). Feeder layer- and serum-free culture of human embryonic stem cells. Biol Reprod 70: 837 - 845.

[48] Carpenter et al (2004). Properties of four human embryonic stem cell lines maintained in a feeder-free culture system. Dev Dyn 229: 243 - 258.

[49] Rosler et al (2004). Long-term culture of human embryonic stem cells in feeder-free conditions. Developmental Dynamics 229: 259 - 274.

[50] Simon et al (2005). First derivation in Spain of human embryonic stem cell lines: use of long-term cryopreserved embryos and animal-free conditions. Fertil Steril 83: 246 - 249.

[51] Stojkovic et al (2005). Human-serum matrix supports undifferentiated growth of human embryonic stem cells. Stem Cells 23: 895 - 902.

[52] Rodríguez C et al (2006). Derivation of clinical-grade human embryonic stem cells. Reproductive BioMedicine Online 12: 112-118.

[53] Hovatta (2004). Derivation of human embryonic stem cell lines – towards GMP. Pasteur Institute EuroConference on Stem Cells (December 2004).

[54] Ludwig T et al (2006). Derivation of human embryonic stem cells in defined conditions. Nature Biotechnol. 24: 185 – 187.

products had been removed and replaced with either recombinant or purified human-derived components. It should be noted however, that this derivation still used immunosurgery for isolation of ICM cells and hence animal components were not entirely removed from the system[55].

- Ellerström et al (2006)[56] have recently reported the isolation of a new human ES cell line under conditions from which all non-human components have been removed and which use purpose-derived human feeder cells, derived ab initio without exposure to animal products. This line has remained pluripotent and karyotypically normal for over 20 passages.
- It was recently (July – August 2006) widely reported[57, 58, 59] (plus numerous additional news reports) that the Singaporean stem cell company ES Cell International in collaboration with Sydney IVF have derived a number of new human ES cell lines using FDA approved human feeders and under conditions of GMP. Although the full details are yet to be published, the implications of the press statements are that these lines may meet all requirements for GMP.

Collectively these represent significant advances which, if substantiated, would potentially remove one of the biggest hurdles to ultimate clinical utility of human ES cells.

Current advances in the ability to produce specific, medically-relevant, mature cell types from human ES cells are well-grounded in over 20 years of work on mouse ES cells and an increasing knowledge of cellular differentiation processes in both human and other animal systems. Although advancing well, it should be recognised that cellular differentiation mechanisms are yet to be fully understood and therefore the ability to produce a range of different mature cells types is still at a relatively early stage.

Nonetheless, there has been a steady increase in the number of reports where human ES cells can be coaxed to produce various cell types including those of the neuronal, cardiac, erythroid, myeloid, hepatic and pancreatic lineages (see Trounson 2006[60] for review and references therein).

In an important complement to the more typical potential use of ES cells to generate tissues for cell replacement therapy, Chang et al[61] recently reported correction of sickle cell anaemia in a murine model by modification of the sickle cell genetic defect

[55] Ludwig T et al (2006). Derivation of human embryonic stem cells in defined conditions. Nature Biotechnol. 24: 185 – 187: SUPPLEMENTARY TEXT.

[56] Ellerström et al (2006). Derivation of xeno-free human ES cell line. Stem Cells (published on-line June 1 2006).

[57] Abbott et al (2006). The lure of stem-cell lines. Nature 442: 336 – 337.

[58] ES Cell International Press Release (July 27 2006). ES Cell International's new clinically-compliant human embryonic stem cells will facilitate the move of hESC cell therapies from the bench to the clinic. http://www.escellinternational.com/pdfs/inthenews/ESI_27Jul06.pdf.

[59] Anon (2006). Tissue Engineering; Ortec cell line plays role in embryonic stem cell lines for human clinical trials. Stem Cell Week August 28 2006: p. 54.

[60] Trounson (2006). The production and directed differentiation of human embryonic stem cells. Endocrine Rev 27: 208 – 219.

[61] Chang et al (2006). Correction of the sickle cell mutation in embryonic stem cells. Proc Natl Acad Sci USA 103: 1036 – 1040.

in mouse ES cells followed by generation of haemoglobin-producing blood cells by differentiation of those ES cells in vitro. This approach could thus be used in human ES cells to correct haemopoietic genetic lesions such as sickle cell anaemia and thalassemia, as well as possibly other non-haemopoietic genetic lesions such as cystic fibrosis.

A number of recent studies have investigated the behaviour of human ES cell-derived cells when transplanted into animal models of human diseases

- Ben-Hur et al[62] reported promising partial behavioural recovery in a rat Parkinsonian model following transfer of neural progenitors derived from human ES cells, although Park et al[63] have reported negative findings in similar studies in which human ES-derived neural progenitor cells failed to result in improvement of behaviour deficits when injected into hemi-parkinsonian rats. Takagi et al[64] have also reported promising findings with regard to transplantation of DA neurons derived from monkey ES cells into a primate model of Parkinson's disease.
- Kehat et al[65] have shown that human ES cell-derived cardiomyocytes can restore a proper heart rate when grafted into a pig model where cardiac electromechanical function was impaired, suggesting a possible role for stem cell derived-cardiomyocytes as a biological pacemaker.
- Keirstead's group[66, 67] have demonstrated that transplantation of human ES cell-derived oligodendrocyte progenitor cells into adult rat spinal cord injuries enhances remyelination and promotes a substantial improvement in motor function when transplanted early after spinal cord injury.
- Fujikawa et al[68] have demonstrated that pancreatic islet-like cells derived from human ES cells in vitro are able when transplanted into diabetic mice to reverse the hyperglycaemic state for an interim period, but fail to fully rescue the recipient mice due to teratoma formation, underscoring the acknowledged need for addressing the teratoma risk associated with undifferentiated cells.
- Finally, Zheng et al[69] have demonstrated that myogenic precursor cells derived in vitro by differentiation from human ES cells are capable of incorporating into regenerating muscle fibres, and give rise to myotubes, myofibres and muscle satellite cells.

Underpinning these advances is a substantial increase in knowledge of the molecular biology of ES cells. Importantly, it has been demonstrated that as ES cells undergo differentiation, they recapitulate the sequential patterns of gene expression that are

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[62] Ben-Hur et al (2004). Transplantation of human embryonic stem cell-derived neural progenitors improves behavioral deficit in Parkinsonian rats. Stem Cells 22: 1246 - 1255.

[63] Park et al (2005) In vitro and in vivo analyses of human embryonic stem cell-derived dopamine neurons. J Neurochem 92:1265 - 1276.

[64] Takagi et al (2005). Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model. J Clin Invest 115: 102 – 109.

[65] Kehat et al (2004). Electromechanical integration of cardiomyocytes derived from human embryonic stem cells. Nature Biotech 22: 1282 - 1289.

[66] Faulkner and Keirstead (2005). Human embryonic stem cell-derived oligodendrocyte progenitors for the treatment of spinal cord injury. Transpl Immunol. 15: 131 - 142.

[67] Keirstead et al (2006). Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. J. Neurosci. 25 : 4694 – 4705.

[68] Fujikawa et al (2005). Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. Am J Pathol 166: 1781 - 1791.

[69] Zheng et al (2006). Skeletal myogenesis by embryonic stem cells. Cell Res 16: 713 – 722.

observed within developing mammalian embryos as their cells move from the pluripotentiality of the inner cell mass (the in vivo equivalent stage from which ES cells derive) to form cells of specific lineages. Importantly, it has been reported that this pattern of developmental stage specific gene expression is conserved between mouse and human ES cells, further attesting to their underlying similarity and boding well for the transfer of knowledge and protocols for directed differntaition between species[70].

The differentiated progeny of human ES cells will likely express human transplantation antigens (most notably ABO blood group antigens and human leucocyte antigens, HLAs) that will potentially cause rejection of transplanted cells or tissues. Thus effective strategies to overcome or avoid graft rejection, without broadly suppressing host immunity, may be needed.

The development of stem cell banks with ES cells of diverse blood group and HLA types has been advocated, enabling traditional approaches of HLA matching combined with conventional immunosuppressive therapy (as currently used for organ transplantation)[71]. Although some have expressed reservations as to the feasibility of this approach, noting the formidable challenges faced by bone marrow transplantation and suggesting that perhaps as many as a million human ES lines may be needed to ensure a high chance of finding a match, nonetheless this approach perhaps offers the most practical shorter-term strategy.

Taylor et al[72] have recently undertaken a detailed simulated analysis and have estimated that a bank of some 150 human ES lines could provide a beneficial match for 25 to 50% of potential recipients in a target population (and a 95% chance of providing a full match for at least 8% of patients). The UK launched the development of the UK Stem Cell Bank (based at the National Institute for Biological Standards and Control (NIBSC)) in 2002[73].

One strategy for avoiding graft rejection would be genomic replacement by SCNT, in which the nucleus from a somatic cell from an intended recipient is used to replace the genetic material of an oocyte, thereby generating a perfectly or near perfectly matched donor stem cell line. Whilst generation of personalised ES cells by SCNT for specific patient is a theoretical option, given the high costs and length of time involved, it is unlikely that production of personalised therapeutic tissues by genomic replacement would represent a practical strategy. SCNT may however, represent a viable approach to ensuring inclusion of rarer HLA types in a human ES cell bank.

In the longer term other strategies will doubtless develop in which immune tolerance to the donor human ES-derived tissue is induced in the recipient, using appropriate differentiated cells derived from the human ES cell line intended for the therapeutic transplant. Indeed, in a quite stunning demonstration of the potential for inducing

[70] Hirst et al (2006). Transcriptional profiling of mouse and human ES cells identifies SLAIN1, a novel stem cell gene. Dev Biol 293: 90 – 103.

[71] Taylor et al (2005). Banking on human embryonic stem cells: estimating the number of donor cell lines needed for HLA matching. Lancet 366: 2019 – 2025.

[72] Taylor et al (2005). Banking on human embryonic stem cells: estimating the number of donor cell lines needed for HLA matching. Lancet 366: 2019 – 2025.

[73] UK Stem Cell Bank (2005). Development of the UK Stem Cell Bank – Phase I. Progress Report 2003 – 2005. http://www.ukstemcellbank.org.uk.

immunological tolerance with ES cells, Fändrich et al[74] were able to induce long term acceptance of allogeneic heart transplants in rats by injecting rat ES-like cells[75] (of the same type as the ultimate heart transplant) into non-immunosuppressed genetically disparate recipient rats. The injected ES-like cells persisted in the recipient and resulted in partial haempoietic chimerism, leading it would seem to immunological tolerance.

Many believe that human ES-based clinical trials are a number of years away - perhaps even 5 to 10 years[76]. One company, Geron, is however, challenging that time line with near-term plans to initiate a clinical trial with human ES cell-derived oligodendrocyte progenitor cells to treat spinal cord injuries. In 2005 it was reported that Geron would be initiating such a clinical trial in mid 2006[77] although by 2006 this is now foreshadowed to be in 2007[78, 79]. The reason for this delay is unclear, but it should be noted that many of the standards required for entering the clinic with a new human ES cell based therapy are yet to be fully detailed, and to the best of this author's knowledge it is yet to be determined whether the currently available human ES cell lines, with past exposure to non-human feeder cells, will be acceptable for clinical use. (See also Section 3. Generation of Clinically-Acceptable Human ES Cells on page 10 above).

To the best of this author's knowledge this is the only clinical trial involving human ES derived cells currently (publicly) mooted in the near term.

Need for SCNT

SCNT, whilst a single technique, has the potential to be used for two different applications in respect of ES cell research

- **Research applications** to study a number of complex disease processes and the potential identification of new drugs.
- **Therapeutic applications** to treat degenerative disease through transplantation of genetically-matched (and possibly modified) cells or tissues.

Technological advances with ES cells developed from normal blastocysts summarised in the foregoing sections, will have a profound beneficial impact on the research use and future clinical application of SCNT-derived stem cells.

At present SCNT is the only way to create "tailored" stem cell lines that would be specific for a particular patient or disease condition. The value of ES cell lines

[74] Fändrich et al (2002). Preimplantation-stage stem cells induce long-term allogenic graft acceptance without supplementary host conditioning. Nature Med 8: 171 – 178.

[75] While these cells clearly resemble murine ES cells, their phenotypic and pluripotent nature have not been fully characterised and so the authors cautiously refer to them as "ES-like" cells. See also Ruhnke et al (2003). Long-term culture and differentiation of rat embryonic stem cell-like cells into neuronal, glial, endothelial, and hepatic lineages. Stem Cells 21: 428 – 436.

[76] Vogel (2005). Ready or not? Human ES cells head towards the clinic. Science 308: 1534 – 1538.

[77] Vogel (2005). Ready or not? Human ES cells head towards the clinic. Science 308: 1534 – 1538.

[78] Geron web site: http://www.geron.com/showpage.asp?code=patiin.

[79] Coghlan (2006). First embryonic stem cell trial on the cards. NewScientist.com news service: http://www.newscientist.com/article/dn9349-first-embryonic-stem-cell-trial-on-the-cards.html

derived via SCNT is firstly that cells could be generated for patient therapy that would not be rejected by the patient and therefore avoid potentially life-long immunosuppression (as addressed in Section 5. Overcoming Transplantation Barriers on page 14 above). Secondly SCNT would also allow the generation of ES cells derived from individuals with specific genotypes for dissection of complex multigenic diseases, such as Alzheimer's disease, motor neurone disease, and others of unknown cause or multigenic origin. The ability to generate specific differentiated progeny cells that express aspects of a disease phenotype from ES cells of defined genotype will be invaluable in dissection of such diseases (see Trounson 2006 for review[80]).

Although it is possible to isolate disease-specific stem cell lines from ART embryos identified as diseased by PGD[81, 82, 83] the number of diseases that can be studied is limited to those consisting of a single gene mutation[84].

The ability to isolate embryonic stem cell lines from patients with complex, polygenic diseases associated with known or unknown genetic and epigenetic effects would be extremely valuable; such conditions include diabetes or Parkinson's diseases. Not only would this provide the opportunity to gain a fuller understanding of the disease process in vitro, but the use of embryonic stem cell lines derived from a specific patient group could assist in the identification of new drugs that may halt or even prevent onset of these debilitating conditions.

What is involved

SCNT involves the removal of maternal chromosomes and replacement with a somatic cell nucleus. The resulting reconstituted oocyte is able, following artificial activation, to undergo cell division consistent with early embryonic development. The resulting 'human nuclear transfer embryo' shares its genomic identity with the donor of original somatic cell. While there is no mixing of genomes, the bulk of the mitochondrial DNA will be derived from the oocyte cytoplasm.

Factors within the oocyte cytoplasm (unknown transcription factors and other molecules) reprogram the somatic nucleus to reset the genome to a more primitive state which allow pluripotent stem cells to be isolated and used for research. These nuclear transfer-derived stem cell lines are been shown in animal studies to be equivalent to those derived from embryos generated from fertilised oocytes - see Brambrink et al (2006)[85] and Wakayama et al (2006)[86].

While the generation of "tailored" stem cells is of great potential value to researchers, it must be acknowledged that an embryo generated by SCNT has the theoretical potential, albeit extremely poor, to implant and develop to term if transferred to the

[80] Trounson (2006). The production and directed differentiation of human embryonic stem cells. Endocrine Rev 27: 208 – 219.

[81] Verlinsky et al (2005). Human embryonic stem cell lines with genetic disorders. Reproductive BioMedicine Online 10: 105-110.

[82] Pickering et al (2003). Preimplantation genetic diagnosis as a novel source for stem cell research. Reproductive BioMedicine Online 7: 353 -364.

[83] Mateizel et al (2006). Derivation of human embryonic stem cell lines from embryos obtained after IVF and after PGD for monogenic disorders. Hum Reprod 21: 503 – 511.

[84] Sermon et al (2004). Preimplantation genetic diagnosis. The Lancet 363: 1633 – 1641.

[85] Brambrink et al (2006). ES cells derived from cloned and fertilized blastocysts are transcriptionally and functionally indistinguishable. Proc. Natl. Acad. Sci. USA 103: 933 – 938.

[86] Wakayama et al (2006). Equivalency of nuclear transfer-derived embryonic stem cells to those derived from fertilized mouse blastocysts. Stem Cells 24: 2023 – 2033.

body of a woman and therefore the use of this technology needs to be heavily regulated.

Proof of concept - Animal models

From the first proof-of-principle demonstration of SCNT in the mouse in 2000[87], there have been many subsequent stem cell lines derived from SCNT embryos in animal studies[88, 89, 90].

More recently and very importantly, it has been demonstrated that such stem cell lines are able to generate cells capable of restoring function to damaged tissue in animal models, including

- correction of the phenotype of a mouse model of Parkinson's disease by transplantation of neural progenitor cells derived in vitro from ES cells derived by SCNT (Barberi et al 2003)[91] ; and
- correction of a genetically-conferred immunological defect via repair of the gene defect in SCNT-derived "personalised" ES cells (Rideout et al 2002)[92] – thus establishing the principle use of SCNT-derived ES cells coupled with gene therapy.

It is obviously essential that cells derived via SCNT are able to function normally when transferred to a recipient patient if cell therapy using these cells is ever to be realised. Indeed, given the low frequency of normal development in animal studies[93, 94], and aberrations in gene expression patterns in cloned animals[95, 96], some have questioned the use of SCNT-derived embryonic stem cells in therapeutic applications[97, 98, 99].

Two important studies published earlier this year both determined that, in respect of murine ES cells, ES cells derived from fertilized blastocysts were indistinguishable from those derived by SCNT[100, 101]. These data are extremely significant in light of

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[87] Munsie et al (2000). Isolation of pluripotent embryonic stem cells from reprogrammed adult somatic cell nuclei. Curr Biol 10: 989 - 992.

[88] Kawase et al (2000). Mouse embryonic stem (ES) cell lines established from neuronal cell-derived cloned blastocysts. Genesis 28:156 - 163.

[89] Wakayama et al (2001). Differentiation of embryonic stem cells generated from adult somatic cells by nuclear transfer. Science 292: 740 - 743.

[90] Wang et al (2005). Generation and characterisation of pluripotent stem cells from cloned bovine embryos. Biol Reprod 73: 149 - 155.

[91] Barberi et al (2003). Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice. Nature Biotech 21: 1200 – 1207.

[92] Rideout et al (2002). Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. Cell 109: 17 - 27.

[93] Wilmut et al (1997). Viable offspring derived from fetal and adult mammalian cells. Nature 385: 810 - 813.

[94] Wakayama et al (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394: 369 - 374.

[95] Rhind et al (2003). Human cloning: can it be made safe? Nat Rev Genet 4: 855 – 864.

[96] Ng and Gurdon (2005). Epigenetic memory of active gene transcription is inherited through somatic cell nuclear transfer. Proc Natl Acad Sci USA 102: 1957 – 1962.

[97] Kohda et al (2005). Variation in gene expression and aberrantly regulated chromosome regions in cloned mice. Biol Reprod 73: 1302 – 1311.

[98] Fulka et al (2004). Do cloned mammals skip a reprogramming step? Nature Biotech 22: 25 - 26.

[99] Armstrong et al (2006). Epigenetic modification is central to genome reprogramming in somatic cell nuclear transfer. Stem Cells 24: 805 – 814.

[100] Brambrink et al (2006). ES cells derived from cloned and fertilized blastocysts are transcriptionally and functionally indistinguishable. Proc. Natl. Acad. Sci. USA 103: 933 – 938.

the aberrations in gene expression patterns observed in somatic tissues derived from cloned animals derived by "reproductive cloning", which have in turn raised the question of whether ES cells derived by SCNT, in contrast with fertilisation-derived ES cells, may carry epigenetic alterations causing transcriptional changes in those ES cells. Brambrink et al and Wakayama et al (vide supra) carried our extensive molecular and developmental analyses of a number of ES lines derived by SCNT with ES lines derived from fertilized blastocysts and concluded that the origins of these various lines could not be distinguished on the basis of these parameters, suggesting that any epigenetic memory of the donor nucleus is lost during the processes of selection for an in vitro proliferating cell line.

Convincingly, the study by Rideout et al diminished such concerns by showing that it was possible to generate stem cell lines from mice with defective immune systems, genetically modify those SCNT-derived embryonic stem cells and, following transplantation to the original mouse, partially restore normal function to the immune system[102].

Since that landmark study there have been two studies that have utilised SCNT to explore epigenetic factors involved in cancer[103, 104]. Blelloch et al demonstrated that developmental tumorigenic potential of murine embryonal carcinoma cells cannot be reprogrammed by SCNT suggesting that genetic rather than epigenetic restrictions are in play. Hochedlinger et al showed that while murine ES cell lines generated from melanoma nuclei could contribute to normal development, the resulting chimaeric mice were predisposed to developing malignant tumours consistent with irreversible genetic changes to the melanoma genome unaltered by SCNT.

Proof of concept – Human

The first report of the derivation of a stem cell line from a human adult cell via SCNT[105] was greeted with great interest in 2004 and was followed by a subsequent report from the same South Korean group concerning the apparent derivation of 11 further human ES cell lines by SCNT[106] with greatly improved apparent efficiency. These articles were unconditionally retracted by *Science* on 12 January 2006 after an investigation committee of Seoul National University concluded that the laboratory in question at SNU where the work was claimed to have been carried out "does not possess patient-specific stem cell lines or any scientific basis for claiming to have created one"[107, 108].

Since then there have been few reports concerning human SCNT. In 2005 production of a human SCNT blastocyst was achieved following fusion of an

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[101] Wakayama et al (2006). Equivalency of nuclear transfer-derived embryonic stem cells to those derived from fertilized mouse blastocysts. Stem Cells 24: 2023 – 2033.

[102] Rideout et al (2002). Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. Cell 109: 17 - 27.

[103] Blelloch et al (2004). Nuclear cloning of embryonal carcinoma cells. Proc Natl Acad Sci USA 39: 13985 – 13990.

[104] Hochedlinger et al (2004). Reprogramming of a melanoma genome by nuclear transplantation. Genes and Dev 18: 1875 – 1885.

[105] Hwang et al (2004). Evidence of a pluripotent human embryonic stem cell line derived from a cloned blastocyst. Science 303: 1669 - 1674.

[106] Hwang et al (2005). Patient-specific embryonic stem cells derived from human SCNT blastocysts. Science 308: 1777 - 1783.

[107] Kennedy (2006). Editorial retraction. Science 311: 335.

[108] Gerber (2006). What can we learn from the Hwang and Sudbø affairs? Med J Aust 184: 632 – 635.

undifferentiated human ES cell with an enucleate human oocyte. Unfortunately the embryo failed to result in the establishment of an ES cell line[109].

Other developments to date include the production of early stage human SCNT embryos produced from oocytes obtained that had failed to fertilise (16 – 18 hours post-insemination)[110].

Hall et al summarise the available, limited, data and conclude that the reported efficiencies for producing SCNT embryos are low and that access to human oocytes of suitable quality and quantity from women is the major limiting factor for progress in the field to date and "it is clear that further refinement of human SCNT is required to derive human ES cell lines"[111].

It should of course be noted that the major current limitations to successfully progressing human SCNT are various legislative restrictions in place in different jurisdictions[112].

Global interest in SCNT

There are several groups around the world actively pursing SCNT to generate human embryonic stem cell lines, but with licences to conduct such research few in number and only recently issued (and with such research currently prohibited in Australia) there has of course as yet been no successful development of human SCNT-derived ES lines.

In the UK, the Human Fertilisation and Embryology Authority (HEFA) have licensed two groups to undertake such work with human cells – one at the Newcastle Centre for Life in August 2004[113], to investigate embryonic development in order to develop treatments for serious diseases like diabetes[114] and the other at the University of London and Roslyn Institute in Edinburgh to study motor neuron disease[115, 116].

In the USA, Harvard has recently announced[117] a large initiative to advance SCNT for the generation of ES cells to "create a powerful new tool to explore the biology of, and hopefully find treatments for, a number of devastating diseases: juvenile diabetes, genetic blood disorders, and ALS, also known as Lou Gehrig's disease"[118], while the private biotechnology company ACT have also announced that they plan to relaunch their SCNT program and believe they are well poised to progress the field given their previous success[119].

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[109] Stojkovic et al (2005). Derivation of a human blastocyst after heterologous nuclear transfer to donated oocytes. Reproductive BioMedicine Online 11: 226 - 231.

[110] Lavoir et al (2005). Poor development of human nuclear transfer embryos using failed fertilized oocytes. Reproductive BioMedicine Online 11: 740 - 744.

[111] Hall et al (2006). Using therapeutic cloning to fight disease: a conundrum or reality? Stem Cells 24: 1628 – 1637.

[112] Hall et al (2006). Using therapeutic cloning to fight disease: a conundrum or reality? Stem Cells 24: 1628 – 1637.

[113] HEFA Press Release (August 11 2004). HEFA grants the first therapeutic cloning licence for research. http://www.hfea.gov.uk/cps/rde/xchg/SID-3F57D79B-101A4FE5/hfea/hs.xsl/1048.html.

[114] Coghlan (2004). UK cloners target diabetes cure. New Scientist 183: 8 – 9.

[115] HEFA Press Release (February 8 2005). HEFA grants embryonic stem cell research licence to study motor neurone disease. http://www.hfea.gov.uk/cps/rde/xchg/SID-3F57D79B-E9722E2E/hfea/hs.xsl/1061.html.

[116] Gross (2005). A new licence to clone. Current Biol 15: R143 – 144.

[117] Holden (2006). Harvard cloners get OK to proceed with caution. Science 312: 1585.

[118] http://www.boston.com/news/local/articles/2006/06/07/harvard_launches_effort_to_clone_human_stem_cells/

[119] Advanced Cell Technology web site: http://www.advancedcell.com/cellular-reprogramming/

Future possibilities

The difficulties associated with obtaining sufficient numbers of human oocytes for SCNT have led researchers to explore the option of animal oocytes as a source of reprogramming factors. Chinese researchers have reported the production of pluripotent ES cell lines derived from the fusion of enucleated rabbit oocytes with human somatic cells[120]. While other researchers are yet to reproduce this result, the use of animal oocytes may provide an opportunity for researchers to optimise the SCNT technique, identify reprogramming factors present in oocyte cytoplasm that maybe conserved between species and investigate complex human diseases. Such an approach would be for basic research and use of such cells in therapeutic applications may be questionable.

Practical and ethical limitations associated with SCNT also encourage investigation of oocyte-and-embryo-free alternatives for obtaining of adult-derived pluripotent cells[121].

An alternative approach (or alternative source of reprogramming factors) to reprogram the somatic genome involves fusion of an adult (somatic) cell with an intact pluripotent cell, or the cytoplasm derived therefrom.

Potential pluripotent fusion partners include embryonic carcinoma cells[122], embryonic germ cells[123] and embryonic stem cells[124].

Human[125] and mouse[126] somatic cells can be reprogrammed by fusion to form pluripotent hybrid cells. However, hybrid cells typically contain nuclear contribution from both the adult and pluripotent cell resulting in tetraploid cells which contain the non-autologous pluripotent nucleus as well as the adult nucleus.

Two alternative routes have been proposed to produce autologous diploid cells following fusion:

- Enucleation (removal of the nucleus) of embryonic stem cells and the fusion of such cytoplasts to somatic cell karyoblasts or intact somatic cells, and the selective elimination of the pluripotent genome following fusion to the somatic partner[127].
- Another approach that has resulted from the study of stem cell biology has explored the effect of forced expression of a small number of key genes

[120] Chen et al (2003). Embryonic stem cells generated by nuclear transfer of human somatic nuclei into rabbit oocytes. Cell Research 13: 251 – 263.

[121] McLaren (2002). Human embryonic stem cell lines: socio-legal concerns and therapeutic promise. C. R. Biologies. 325: 1009 – 1012.

[122] Miller and Ruddle (1976). Pluripotent teratocarcinoma-thymus somatic cell hybrids. Cell 9: 45 – 55. (e-copy not available).

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[124] Tada et al (2001). Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. Curr. Biol. 11: 1553 - 1558.

[125] Cowan et al (2005). Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. *Science* 309: 1369 - 1373.

[126] Tada et al (2001). Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. Curr. Biol. 11: 1553 – 1558.

[127] Pralong et al (2005). A novel method for somatic cell nuclear transfer to mouse embryonic stem cells. Cloning Stem Cells 7: 265 – 271.

(Oct3/4, Sox2, c-Myc, and Klf4) on altering pluripotentiality of murine somatic cells[128].

Various technologies such as those outlined above open new possibilities for generating autologous, diploid pluripotent cells for research and/or therapy and also provides a new dynamic model for studying factors implicated in nuclear reprogramming. Current SCNT research using oocytes will in all likelihood be transitory, but necessary, as other approaches to reprogramming somatic nuclei develop.

Whilst beyond the scope of this paper to review this area in any depth, it is noted that there is burgeoning interest and activity with regards the use of embryonic stem cells in the development of cell-based drug screening or toxicology assays - e.g. see reviews by Gorba and Allsopp (2003) [129] and Davila et al (2004)[130].

A major focus of the work in the Edinburgh laboratories of the international biotechnology company Stem Cell Sciences which has its origins in Australia, is to expand human embryonic stem cells in sufficient numbers to be able to provide cells for such assays. Stem Cell Sciences has previously participated in several R&D projects with large pharmaceutical companies to explore the use of mouse embryonic stem cells for screening. One study, conducted in collaboration with Sanofi-Aventis, provided large numbers of differentiated living nerve cells for screening of potential drug candidates for their eventual development as Alzheimer's disease therapeutics[131].

Recently Kulkarni and Khanna[132] have assessed hepatotoxicity assays developed using hepatocyte-like cells derived from mouse ES cells and demonstrated their suitability for use in in vitro hepatotoxicity assays. Such a stem cell-based system offers a promising alternative for obtaining large numbers of hepatic cells for early efficacy and toxicity screening of new drug candidates, and whilst not replacing in vivo drug screening, does have the potential to reduce the number of animals needed and offer a simpler and more robust way of elucidating the human biotransformation pathway for a new drug.

In addition potentially to using specific cell types derived from embryonic stem cells for drug identification or characterisation where appropriate cell types for those screens can only be effectively obtained from such sources, Ding and Schultz (2004)[133] make the point that the embryonic stem cells themselves are also legitimate targets for "pharmacologic" intervention in their own right, in order to identify small molecules may be used to selectively control stem cell proliferation and differentiation

[128] Takahashi and Yamanaka (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126: 663 – 676.

[129] Gorba and Allsopp (2003). Pharmacological potential of embryonic stem cells. Pharmacol Res 47: 269 - 278.

[130] Davila et al (2004). Use and application of stem cells in toxicology. Toxicol Sci 79: 214 – 223.

[131] Gorba and Allsopp (2003). Pharmacological potential of embryonic stem cells. Pharmacol Res 47: 269 - 278.

[132] Kulkarni and Khanna (2006). Functional hepatocyte-like cells derived from mouse embryonic stem cells: a novel in vitro hepatotoxicity model for drug screening. Toxicology In Vitro 20: 1014 – 1022.

[133] Ding and Schultz (2004). A role for chemistry in stem cell biology. Nature Biotech 22: 833 - 840.

– an approach vindicated by the discovery of different GSK-3β modulators that have the capacity promote neurogenesis[134] or maintain pluripotentiality[135].

[134] Ding et al (2003). Synthetic small molecules that control stem cell fate. Proc Natl Acad Sci USA 100: 7632 – 7637.

[135] Sato et al (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by pharmacological GSK-3-specific inhibitors. Nature Med 10: 55 - 63.

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9. Ben-Hur et al (2004). Transplantation of human embryonic stem cell-derived neural progenitors improves behavioral deficit in Parkinsonian rats. Stem Cells 22: 1246 - 1255.
10. Blelloch et al (2004). Nuclear cloning of embryonal carcinoma cells. Proc Natl Acad Sci USA 39: 13985 – 13990.
11. Brambrink et al (2006). ES cells derived from cloned and fertilized blastocysts are transcriptionally and functionally indistinguishable. Proc. Natl. Acad. Sci. USA 103: 933 – 938.
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