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HUMAN FERTILISATION AND EMBRYOLOGY AUTHORITY

Application for a Research Licence

Centre: Newcastle Fertility Centre at Life and the Institute of Human Genetics, University of Newcastle

Centre No: 0017

Research Project No: R0152

Person Responsible: Professor Alison Murdoch

Feedback from Peer Reviewers

Does proposed work justify need for the creation of embryos specifically for research? Could researchers feasibly utilise other avenues in order to conduct the proposed work?

Referee I: Unfortunately, the authors fail to comment on the practicality of alternate methods that might be used to achieve a similar result. The authors mention the usefulness of homologous recombination in altering CNR produced human ES cell lines. Others have suggested that this procedure could potentially be used to eliminate histocompatibility issues and create disease models using existing stem cells lines. Additionally, it has been suggested that co-transplantation of differentiated ES cell derived cells with haematopoetic cells derived from the same ES cell lines could reduce or eliminate immune rejection in recipients. It is important for the authors to acknowledge this previous work, and state why they believe that therapeutic cloning would be a preferred method.

Our comments: We are very thankful to the referee I for the comments and very helpful suggestions which definitely improve the quality of our application. We completely agree that there is a huge possibility to use homologous recombination to eliminate histocompatibility or create disease models using existing hES cell lines as previously demonstrated in mouse ES cells (Hamilton et al., 1997; Sotilo et al., 2001; Oishi et al., 2002; Brook et al., 2003; Westervelt et al., 2003). However, to our knowledge there are not many studies which describe the successful elimination of histocompatibility or the creation of disease models using existing hES cell lines (Zwaka and Thomson, 2003). In addition, using this procedure in hES cells, we can foresee several different problems. In contrast to mouse ES cells, hES cells cannot be cloned efficiently from single cells (Amit et al., 2000) making it difficult to screen for recombinant events (Zwaka and Thomson, 2003). Furthermore, some hES cell

lines grow in the presence of feeder cells which prevent their efficient genetic manipulation. However, homologous recombination in hES cells will be useful in the future not only to generate knock-in cell lines with a tissue specific expression pattern or to purify a specific hES cell-derived cell type from a mixed population but also for combining therapeutic cloning with gene therapy (Rideout et al., 2002; Zwaka and Thomson, 2003).

We believe that therapeutic cloning is preferred method to eliminate immune rejection in recipients for several reasons. The successful application of differentiated hES cells in cell therapy will require the elimination of histo-incompatibility. Possible options are considered below.

1. Cell line matching: Because MHC alloantigens are the primary target for destruction by host T lymphocytes, many efforts are made to match HLA isotypes before transplantation. Theoretically the same rationale can be used to match the isotypes of the patient and the hES cells. However, a very large bank of immunotyped hES cell lines would be required to match the majority of the population.
2. Transplantation into immune-privileged sites: In various animals, the immune response to grafts placed in the immunologically privileged sites is considerably restricted. These sites are anterior part of the eye, brain or testis and therefore the broader application of hES cells in cell therapy using this method is limited.
3. Genetic modification: It is theoretically possible to use this procedure to reduce the expression of MHC molecules, for instance by knockout of the beta2-microglobulin gene, which is essential for MHC-I presentation. Because hES cells do not express MHC-II, unless they are differentiated hematopoietically, such a cell line would be almost totally deprived of MHC expression and therefore might have reduced immunogenicity to alloreactive T cells. However, it is important to note that transplantation of tissues from MHC-I and MHC-II knockout mice to an allogeneic host still results in rejection, probably through T cell-dependant mechanisms (Grusby et al., 1993; Dierich et al., 1993). Moreover, lowering MHC expression can also induce killing by natural killer cells (Bix et al., 1991). Alternatively, one can create a universal hES cell line by using the natural mechanisms of immune-privileged sites. FasL expression, one of the hallmarks of immune-privileged sites, is known to induce apoptosis of T-cells that express its receptor (Fas; Griffith et al., 1995). However, transgenic FasL expression in cardiac and pancreatic transplants resulted in tissue damage or accelerated rejection (Green and Ferguson, 2001).
4. Haematopoietic chimerism: This process is accomplished by injection of donor-derived bone marrow cells to the patient, which leads to hematopoietic coexistence (Shizuru et al., 2000; Beilhack et al., 2003). Using this procedure it would be possible to generate both hematopoietic cells and other cell types and the tolerance could be achieved allowing cellular transplantation of various differentiated cells. But the broad use of this method requires myeloablation (elimination of haematopoiesis) by

irradiation or cytotoxic drugs (van den Brink and Burakoff, 2002) so induction of hematopoietic chimerism will mainly suit those patients undergoing myeloablative treatments. In addition, it was demonstrated that rat embryonic stem cell-like cells differentiated into monocytes and B cells allowed acceptance of a cardiac graft only from the same donor rat strain but not from a different strain (Fändrich et al., 2002) and to our knowledge this procedure has not been tried in humans with hES cells. Thus the potential in this regard is still not clear (Drukker and Benvenistry, 2004).

5. Therapeutic cloning: Therapeutic cloning offers the only real possibility of rejection of differentiated hES cells. In combination with other techniques, therapeutic cloning generates genetically matched pluripotent hES cells for in vitro differentiation into the desired cell type with several potential benefits:
    a) no requirement of long-term administration of immunosuppressive drugs to prevent rejection of the transplanted cells,
    b) the opportunity to repair genetic defects within NT-hES cells to treat or cure inherited diseases, and
    c) the possibility to repeatedly expand and differentiate the NT-hES cells into the desired cell type for continued therapy as needed.

Lanza et al. (2002) recently showed that in cattle differentiated NT-hES didn't lead to graft rejection. Furthermore, transplantation of mouse NT-ES cell-derived dopaminergic neurons corrected the phenotype of a mouse model of Parkinson disease, demonstrating an in vivo application of therapeutic cloning in neural disease (Barberi et al., 2003). Again, the availability of NT-hES cell lines would opens the prospect for repairing a gene defects by homologous recombination as demonstrated in mice (Rideout et al., 2002). Therefore, therapeutic cloning will not only allow us to derive new hES cell lines which contain virtually identical genetic information to that contained in the patient; therapeutic cloning will help us to learn more about the pathogenesis of many debilitating human diseases for which mouse models have proven inadequate (for instance Hprt-1 deficiency and Lesch-Nyhan syndrome).

Do you consider that the objectives are clearly defined and the methods proposed are likely to yield relevant and clear results? If not, what are the problems?

Referee I: The methods for creating CNR embryos are outlined in the grant application. The source of the oocytes and somatic cells is clear and detailed, and consent forms appear to be complete and exhaustive. There is little information detailing the handling of oocytes once collected. The authors indicate that they expect many of the gametes will be immature at acquisition, but provide no details regarding if or how these oocytes will be matured. Furthermore, there are no nuclear transfer or embryo culture protocols included. Additionally, while the authors indicate that "different chemical, mechanical and/or electrical stimuli" will

be used to activate the nuclear transfer oocytes, no other detail is provided. While we concede that there is little human data on which to draw at this point, there is sufficient animal data to provide at least some more specific potential activation protocols. Overall, more detail would be useful.

Our comments: We would like to remind the referee I that we submitted our application before scientists from South Korea (Hwang et al., 2004) announced that they derived first nuclear transfer (NT) hES cell line. This confirms our statement that we believe that derivation of hES cells using NT technique is possible. The same group announced that the first NT-hES cell line was derived using slightly modified protocol for bovine NT which is available in numerous studies including those of Dr. Stojkovic. In addition, if our application will be granted, we are kindly invited to visit Prof. Hwang's facilities in South Korea.

However, the protocols have been now included (please see below) but we would like to underline that they might be slightly modified as new publications appear.

Source of cytoplasts/maturation of oocytes

It is known that mammalian oocytes spontaneously mature in vitro, following liberation from the follicle (reviewed in Hovatta, 2004). Human oocytes undergo normal cleavage following the addition of gonadotrophins to culture medium (Armstrong et al., 1991). The first children, triplets from oocytes matured in vitro after being taken from ovarian tissue, were born in 1991 (Cha et al., 1991). In vitro maturation of human oocytes has been further developed in the recent years and more children have been born from in vitro matured oocytes (for review see Hovatta, 2004).

Newcastle Fertility Centre at Life performs approximately 600 IVF/ICSI treatment cycles per year. About 30% of the oocytes fail to fertilise and we wish to use these oocytes. In most cases these will be identified by the absence of pronucleii on day 1 after insemination and we be allocated to research on that day. If no normal embryos have been identified in that treatment cycle, the oocytes will be retained until day 2 to allow transfer if cleavage has occurred.

In addition, some oocytes are retrieved during the follicle reduction procedure after superovulation which has produced too many follicles. Oocytes from both these sources would normally be discarded if not used for research. During routine hysterectomy +/- oophorectomy procedures, we have access to normal ovarian tissue. For several years now we have recovered oocytes from these ovaries as part of other approved research. Most of these are immature gametes. Therefore, isolated immature human oocytes will be evaluated/classified and in vitro matured as follows. Cumulus-oocytes-complexes (COCs) will be placed in maturation medium (Tissue Culture Medium 199 supplemented with 10% heat-inactivated serum or 2 mg/ml human serum albumin, 0.075 IU/l rFSH, 0.5 IU/l rLH, 0.29 mmol/l pyruvate) for 36-48h. Lyophilized rFSH (Gonal-F; Serono, Geneva, Switzerland) and rLH (Serono, Geneva, Switzerland) will be reconstituted in sterile water and added to the maturation medium prior to COCs culture. Human follicular cells have been

shown to be highly responsive to rFSH and rLH (Bergh *et al.*, 1997) and human embryonic development was improved by the in vitro maturation of oocytes with rFSH and rLH (Anderiesz et al., 2000).

The matured MII oocytes will be used for enuclation and further steps of NT-technique.

Source of karyoplast /nuclei

For karyoplast we will use different somatic cell types.

The nuclei to be used for transfer will be from three sources.

- Stem cell lines
  We will use nuclei from cells from our existing derived ES cell line (hES-NCL1, Stojkovic et al., Stem Cells, 2004, and for more details please see http://www.isscr.org/science/sclines.htm). The donors of the embryo from which the line was derived consented for further studies using these cells despite the fact that they would not be individually informed of the details of the study.
- A woman undergoing a gynaecological procedure.
  A 1cm skin biopsy will be taken from a woman undergoing a routine gynaecological operation. It is anticipated that sufficient cells will be obtained from one biopsy only.
- A patient with Type 1 diabetes.
  A 1cm skin biopsy will be taken from one patient who has Type 1 diabetes. This will allow us to develop a stem cell line from a patient with a specific disease.

Preparation and culture of donor cells

Human embryonic stem cells (our hES-NCL1 cells) will be cultured as already described (Stojkovic et al., 2004). Briefly, hES cell colony will be mechanically dispersed into several small clumps and cultured on Matrigel precoated dishes (feeder-free culture of hES cells). As culture medium we will use mouse or human embryonic feeder cell medium conditioned by Knockout-DMEM supplemented with 100 μM β-mercaptoethanol, 1 mM L-glutamine, 100 mM non-essential amino acids, 10% serum replacement, 1% penicillin-streptomycin and 8 ng/ml bFGF.

Primary Culture of Skin Fibroblasts

Small pieces of biopsies from a healthy person and person with diabetes type 1 will be separately washed three times in Dulbecco's phosphate buffered saline (PBS) and will be digested in 0.25% (v/v) trypsin–EDTA solution at 37°C. Trypsinised cells will be washed once in Ca^{2+} and Mg^{2+} free PBS by centrifugation at 200*g* for 3 min, and subsequently seeded onto 100 mm plastic culture dishes. Seeded cells will be subsequently cultured for 6–8 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%(v/v) FCS, 1 mM glutamine, 25 mM NaHCO3, and 1% (v/v) MEM amino acid solution.

Enucleation

After maturation cumulus cells of cumulus oocytes complexes (COCs) will be removed by repeated pipetting in 0.1% (v/v) hyaluronidase in HEPES-buffered Ca^{2+}-free medium supplemented with amino acids. Oocytes will be then enucleated with a micromanipulator (Leitz and Eppendorf) in manipulation medium supplemented with 10% (v/v) FCS and 5 μg/ml cytochalasin B. Each oocyte will be held with a holding micropipette and the zonae pellucida will be either partially dissected with a fine glass needle to create a slit or directly punctured by injection pipette. The first polar body and adjacent cytoplasm presumably containing the metaphase II chromosomes will be extruded either by squeezing with the needle or by the injection pipette. The removal of DNA will be confirmed by staining with Hoechst 33342 and visualisation under UV light. Alternatively, Hoechst staining will be performed prior to enucleation so that the nuclear material will be visible and therefore simpler to remove. Oocytes still containing DNA-material will be excluded. The enucleated oocytes will be placed in TCM-199 medium supplemented with 10% (v/v) FCS and used for NT.

Activation and culture of human NT-embryos

A single donor cell will be deposited into the perivitelline space of an enucleated oocyte treated with phytohemagglutinin in manipulation medium to improve the incorporation of donor somatic cell with recipient cytoplast. The couplets will be subsequently placed in a fusion medium comprising 0.26 M mannitol, 0.1 mM MgSO4, 0.5 mM HEPES, and 0.05% (w/v) BSA, and transferred into a cell fusion chamber with a stainless steel wire electrode after equilibration for 3 min. Fusion will be induced by two DC pulses of 1.75–1.85 kV/cm using a electro-cell manipulator. The fusion of the donor cell and the ooplast will be observed under a stereomicroscope and only fused embryos will be selected. After 2h (reprogramming time, Hwang et al., 2004) chemical activation will be induced by incubating embryos in G1 medium containing 10 μM calcium ionophore A23187 for 5 min at 37°C. Reconstructed embryos will be then washed thoroughly in G1 medium and further incubated for 4 h in G1 medium supplemented with 2.0 mM of 6-dimethylaminopurine (6-DMAP). At the end of reconstruction, embryos will be cultured in 25 μl microdroplets of G1 medium under mineral oil for 3 days at 37°C in a humidified atmosphere of 5% CO2, 5%O_2, and 90%N_2. On Day 3, embryos will be removed into fresh droplets of culture medium (G2.3 medium or human modified Synthetic Oviduct Fluid medium supplemented with amino acids) and cultured for another 2-4 days.

Parthenogetic activation of human oocytes

Oocyte activation using the calcium ionophore A23187 (calcimycin) or ionomycin and the protein synthesis inhibitor puromycin induces parthenogenetic development of human oocytes at different efficiencies. We will incubate oocytes in 10 μM A23187 for 5 min, followed by incubation with 2.0 mM 6-DMAP for 4 hours and culture activated oocytes as described above.

Are the numbers of gametes/embryos to be used realistic and are the statistical methods to be used appropriate to give meaningful results? If not, can you suggest alternatives?

Referee I: Clearly, it is difficult to determine how many oocytes would be required to achieve CNR, or how many embryos needed to ensure success isolating ES cell lines. However, the researchers have not clearly indicated what they would consider to be a success, how many stem cell lines they aim to produce if successful, or at what point the experiments would be terminated. The focus of this grant seems to be only the derivation of CNR human embryos, as there is no information in the methods section detailing the isolation of stem cell lines from these embryos. Furthermore, there is no indication in the grant application of tests that would be performed on resulting stem cell lines to determine their normality or usefulness. It would be critical that a specific test was included to ensure that resulting lines are of CNR and not parthenogenic origin.

Our comments: Derivation of one hES cell line using NT-technique could be definitely defined as success since we are aware how difficult this will be. Furthermore, complete characterisation (including SCID mice/teratoma experiment) of hES cells is time consuming job, sometimes up to six months. To make a statement how many stem cells line we aim to derive would be to speculative. Theoretically, we can produce in one week may be 2, 5 or 10 blastocysts and then able to derive 1, 3, 10 hES cell lines, or none.

Derivation of hES cells from NT-blastocysts has been now included:

Derivation of NT-hES cells will be done as previously described by Stojkovic et al. (Stem Cells, 2004) where we describe successful derivation of hES cells using consented IVF embryos. Briefly, ICMs will be isolated using the immunosurgery procedure as described by Reubinoff et al. (2000). Initially, isolated ICMs will be cultured on a γ-irradiated mouse or human feeder (75.000 cell/cm^2) in DMEM supplemented with 10% (v/v) Hyclone FCS or serum replacement. The primary hES cell colony will be mechanically dispersed into several small clumps and further cultured on a fresh MEF layer with ES medium containing Knockout-DMEM, 100 μM β-mercaptoethanol, 1 mM L-glutamine, 100 mM non-essential amino acids, 10% serum replacement (SR), 1% penicillin-streptomycin and 8 ng/ml bFGF.

If we will succeed in derivation of hES cells using NT- technique, the hES cells will be characterized, tested on their normality, usefulness and that they are not of parthenogenetic origin using the following protocols:

Characterization of hES cells. The derived hES cells will be live stained by GCTM-2, TG343, TRA1-60, TRA1-81, SSEA-3 and 4. For OCT-4 immunostaining hES cells will be fixed in 3.7% formaldehyde for 20 minutes at room temperature followed by incubation in 3% hydrogen peroxide for 10 minutes. The hES cells will be permeabilised with 0.2 % Triton x 100 diluted in 4% sheep serum for 30 minutes at 37°C. The hES colonies will be incubated with the OCT-4 primary for 30 minutes at room temperature. The hES colonies will be washed twice with PBS for 5 minutes and then incubated with 1:100 dilution of

the secondary antibody for 30 minutes at room temperature. After that, hES cells will be washed again with PBS, incubated with ABC/HRP solution for 25 minutes at room temperature and washed again with PBS. The detection will be carried out by incubation with DAB solution at room temperature for 1 minute. Final washes will be done with distilled water. The primary antibody will be omitted for the negative control. The alkaline phosphatase (AP) staining will be carried out using the Alkaline Phosphatase Detection Kit following manufacturer's instructions (Chemicon). Briefly, cells will be fixed in 90% methanol and 10% formamide for 2 minutes and then washed with rinse buffer (20 mM Tris-HCl pH 7.4, 0.05% Tween -20) once. Staining solution (Naphthol/Fast Red Violet) will be added to the wells and plates will be incubated in the dark for 15 minutes. The bright field images will be obtained using a Zeiss microscope and AxioVision software.

Reverse Transcription (RT)-PCR analysis of undifferentiated hES cells. The reverse transcription will be carried out using the cells to cDNA II kit according to manufacturer's instructions (Ambion, Huntingdon, UK). In brief, hES cells will be submerged in 100 µl of ice-cold cell lysis buffer and lysed by incubation at 75°C for 10 minutes. Genomic DNA will be degraded by incubation with DNAse I for 15 minutes at 37°C. RNA will be reverse transcribed using M-MLV reverse transcriptase and random hexamers following manufacturer's instructions. PCR reactions will be carried out using the following primers:

OCT-4F: 5'-GAAGCTGGAGAAGGAGAAGCTG-3';
OCT-4R: 5'-CAAGGGCCGCAGCTTACACATGTTC-3';
REX-1F: 5'-GCGTACGCAAATTAAAGTCCAGA-3';
REX-1R: 5'-CAGCATCCTAAACAGCTCGCAGAAT-3';
NANOGF:5'-GATCGGGCCCGCCACCATGAGTGTGGATCCAGCTTG-3';
NANOGR: 5'-GATCGAGCTCCATCTTCACACGTCTTCAGGTTG-3';
TERTF: 5'-CGGAAGAGTGTCTGGAGCAAGT-3';
TERTR: 5'-GAACAGTGCCTTCACCCTCGA -3';
GAPDHF: 5'-GTCAGTGGTGGACCTGACCT-3';
GAPDHR: 5'-CACCACCCTGTTGCTGTAGC-3'.

PCR products will be run on 2% agarose gels and stained with ethidium bromide. Reverse transcriptase negative controls will be included to monitor genomic contamination.

Karyotype analysis of hES cells. The karyotypes of hES cells will be determined by standard G-banding procedure.

Tumor formation in severe combined immunodeficient (SCID) mice after injection of undifferentiated hES cells. All procedures involving mice will be carried out in accordance with institution guidelines and institution permission. Approximately 3000 hES cells will be injected beneath the capsule of the kidney or the testis of adult male SCID mice. After 21-90 days, mice will be sacrificed, tissues will be dissected, fixed in Bouins overnight, processed and sectioned according to standard procedures and counterstained with either Haematoxylin and Eosin or Weigerts stain. Sections will be examined using bright field light microscopy and photographed as appropriate.

Ability of hES cells to differentiate in vitro. Colonies of hES cells will be grown in feeder-free conditions in ES medium and recorded on spontaneous differentiation. Differentiated cells will be fixed in 4% paraformaldehyde in PBS (Sigma) for 30 minutes and then permeabilised for additional 10 minutes with 0.1% Triton X. The blocking step will be 30 minutes with 2% FCS in PBS. Cells will be incubated with different antibody against ectoderm, mesoderm and endoderm derivatives.

DNA fingerprinting analysis and expression of imprinted genes. The DNA fingerprinting analysis with human short tandem repeat (STR) markers will indicate whether the hES cell line originated from the NT-blastocysts reconstructed from the donor cells, not from parthenogenetic activation. Total genomic DNA will be extracted from both samples and amplified with 11 microsatellite markers: D3S1358, vWA, D16S539, D2S1338, Amelogenin, D8S1179, D21S11, D18S51, D19S433, TH01, and FGA and analysed on an ABI 377 sequence detector using Genotype software (Applied Biosystems, Foster City, CA). The specific primers for paternally-expressed and *ARH1* and maternally-expressed *H19* and *UBE3A* and housekeeping gene *GAPDH* will be designed and RT-PCR amplification will be performed.

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