

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 (2nd Reviewer)

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?

Objectives are clear. Methodology is vague. This reviewer would have liked to see more information on specific experimental protocols to be used. Given the experience of the PR one can assume that these 'unspecified' protocols will be used. However they are not stated in the proposal.

Our comments:

We are very thankful for the helpful comments and we hope that we are now able to improve our application and answer all questions.

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 which 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 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) with the note that they might be slightly modified as new publications appear.

Source of cytoplasts/oocytes

It is known that mammalian oocytes spontaneously mature in vitro, following liberation from the follicle (reviewed in Hovatta, 2004) and 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 been further developed in the last 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 pronuclei 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. 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 oocyte 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 enucleation 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 more details on <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 embryonic feeder cell medium conditioned by Knockout-DMEM supplemented with 100 μ M 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 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 200g 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 NaHCO_3 , 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) in manipulation medium supplemented with 10% (v/v) FCS and 5 μ g/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 somatic cell nuclear transfer (SCNT).

Activation and culture of human SCNT embryos

A single donor cell will be deposited into the perivitelline space of an enucleated oocyte treated with 100 mg/ml 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 2 h (reprogramming time, Hwang et al., 2004) chemical activation will be induced by incubating embryos in G1 medium containing 10 μ M 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 μ l microdroplets of G1 medium under mineral oil for 3 days at 37°C in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂. On Day 3, embryos will be removed into fresh droplets of culture medium (human modified Synthetic Oviduct Fluid medium supplemented with amino acids or G2 medium) and cultured for another 2-4 days.

Parthenogenetic 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 μ M A23187 for 5 min, followed by incubation with 2.0 mM 6-DMAP for 4 hours and culture activated oocytes as described above.

Derivation of hES cell from NT-blastocysts and parthenotes

Derivation of hES cells will be done as previously described by Stojkovic et al. (Stem Cells, 2004) using IVF blastocysts. 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 MEF monolayer (75,000 cell/cm²) in DMEM supplemented with 10% (v/v) Hyclone FCS. 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 μ M β -mercaptoethanol, 1 mM L-glutamine, 100 mM non-essential amino acids, 10% serum replacement (SR), 1% penicillin-streptomycin and 8 ng/ml bFGF.

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.

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?

Number of necessary gametes are given however no statistical analysis have been proposed.

Statistical analysis has been now included: Statistical analysis will be done using ANOVA and SPSS or SAS statistical analysis packages. The model will include effects of age of donor/oocyte, oocyte source/quality, maturation time, activation, culture protocols and the interaction between these factors. Comparisons between groups would be done using appropriate test (for instance Last Significant Differences test, Scheffé's test or Student's *t*-test with Levene's test for equality of variances. In addition, early cleavage and blastocyst rates could be compared by Chi-square test with Yates' continuity correction. A value of $p < 0.05$ will be considered as significant. Dr. Stojkovic has excellent background in statistical analysis (for instance see Stojkovic et al., 1995; and Stojkovic et al., 1999) and at Institute of Human Genetics we have specialised statistic team.

References:

Anderiesz C, Ferraretti A, Magli C, Fiorentino A, Fortini D, Gianaroli L, Jones GM, Trounson AO. Effect of recombinant human gonadotrophins on human, bovine and murine oocyte meiosis, fertilization and embryonic development in vitro. *Hum Reprod* 2000 15:1140-1148.

Armstrong DT, Zhang X, Vanderhyden BC, Khamsi F. Hormonal actions during oocyte maturation influence fertilization and early embryonic development. *Ann N Y Acad Sci* 1991 626:137-158.

Bergh C, Howles CM, Borg K, Hamberger L, Josefsson B, Nilsson L, Wikland M. Recombinant human follicle stimulating hormone (r-hFSH; Gonal-F) versus highly purified urinary FSH (Metrodin HP): results of a randomized comparative study in women undergoing assisted reproductive techniques. *Hum Reprod* 1997 12:2133-2139.

Cha KY, Koo JJ, Ko JJ, Choi DH, Han SY, Yoon TK. Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycles, their culture in vitro and their transfer in a donor oocyte program. *Fertil Steril* 1991 55:109-113.

Hwang WS, Ryu YJ, Park JH, Park ES, Lee EG, Koo JM, Jeon HY, Lee BC, Kang SK, Kim SJ, Ahn C, Hwang JH, Park KY, Cibelli JB, Moon SY. Evidence of

a pluripotent human embryonic stem cell line derived from a cloned blastocyst. *Science* 2004 303:1669-1674.

Hovatta O. Cryopreservation and culture of human ovarian cortical tissue containing early follicles. *Eur J Obstet Gynecol Reprod Biol.* 2004 5:50-54.

Stojkovic M, Wolf E, Büttner M, Berg U, Charpigny G, Schmitt A, Brem, G (1995). Secretion of biologically active interferon tau by in vitro-derived bovine trophoblastic tissue. *Biol Reprod* 53:1500-1507.

Stojkovic M, Westesen K, Zakhartchenko V, Boxhammer K, Wolf E (1999). Coenzyme Q₁₀ in submicron-size dispersion improves development, cell proliferation, and hatching of in vitro produced bovine embryos. *Biol Reprod* 61:541-547.

Stojkovic M, Lako M, Stojkovic P, Stewart R, Przyborski S, Armstrong L, Evans J, Herbert M, Hyslop L, Ahmad S, Murdoch A, Strachan T. Derivation of human embryonic stem cells from Day 8 blastocysts recovered after three-steps in vitro culture. (*Stem Cells*, in press).
