

HUMAN FERTILISATION AND EMBRYOLOGY AUTHORITY

LICENSED EMBRYO RESEARCH CENTRE

INITIAL APPLICATION FOR A RESEARCH LICENCE

The completed form and all supporting documents should be returned together with the appropriate fee (£200) to:

The Human Fertilisation and Embryology Authority
21 Bloomsbury Street
London
WC1B 3HF

Guidance notes for completing this application can be found at the end of this form

Form revised June, 2001

Details of the centre undertaking research

Name of centre/institution Roslin Institute
(including department) Division of Gene Function and Development

Address: Address for correspondence
(if different):

Division of Gene Function and Development
Roslin Institute
Roslin, Midlothian
EH25 9PS

Tel No: 0131 527-4219 (I. Wilmut) Tel No: 0131-527-4267 (P. De Sousa)

Fax No: 0131-527-4493

Fax No:

e.mail address (if applicable): Ian.Wilmut@bbsrc.ac.uk
Paul.DeSousa@bbsrc.ac.uk

Are the premises used for the provision of licensed treatment? (Please tick appropriate box below.)

Yes ☐ No ☒

If "yes" please give centre number:

Corporate information

2.1 Is the centre a NHS facility or a private operation? (Please tick appropriate box.)

NHS ☐ Private ☒

2.2 If private please give the following information:

- i. **Limited Company**
Company Name: Roslin Institute
Registration No: 157100/ Charity No. SC023592
Registered Offices: Roslin Biocentre, Roslin, Midlothian

- ii. **Partnership**

Name of Partners: The Institute is sponsored by the Biotechnology and Biological Science Research Council (BBSRC), which is a non-departmental public body of the Department of Trade and Industry.

iii. Sole Trader
Name of Owner:

Person Responsible

Name: Ian Wilmut

Position: Head of Division of Gene Function and Development

Nominal Licensee

Name: Paul De Sousa

Position: Group Leader

Research History

Please briefly summarise the history of any HFEA licensed research carried out at your centre, highlighting any previous projects in the same area of research proposed in this application:

(Please include the research licence numbers. You may continue on a separate sheet if you wish.)

Our centre (0202) first acquired an HFEA licence (R0136-1-a) on July 1st, 2003, entitled *Platform Technologies Underpinning hES cell Derivation*. The aims of this program have been to:

- I. *Derive hES cells from fresh or frozen embryos that are surplus to IVF and preimplantation genetic diagnosis treatment, or parthenogenetic embryos originating from in vitro matured eggs.*

And

- II. *Derive and maintain hES cells in a culture environment free of animal cell products.*

On February 26th, 2004, a variation to this licence was granted to permit embryo donors to consent to the possible future therapeutic use of newly derived stem cells

(R0136-1-b). An application to renew this licence is currently pending, as is an application to the Medical Healthcare-Products Regulatory Agency (MHRA) to accredit our facility as a Tissue Bank.

In support of the aims of our current licence we have to date:

- a. Used an *in vitro* bovine egg and embryo culture model system to optimise procedures to mature and parthenogenetically activate eggs.
- b. Matured human cumulus-oocyte complexes *in vitro* in medium that is completely defined or supplemented with fractionated serum and made parthenogenetic blastocysts.
- c. Evaluated human dermal fibroblasts for their capacity to support the culture of 2 existing hES cell lines.
- d. Isolated 3 new presumptive hES cell lines by outgrowth of whole human blastocysts on human dermal fibroblast feeders in media with or without supplementation with fractionated serum.

Optimisation of oocyte maturation in vitro and parthenogenesis

We have completed research in a bovine model system demonstrating that a member of the neurotrophin Growth Factor (GF) family, Brain Derived Neurotrophic Factor (BDNF), can substitute for fractionated serum during oocyte *in vitro* maturation, and benefit the development of parthenogenetic embryos. In the same model system we have arrived at an optimised parthenogenetic activation protocol requiring calcium ionophore, a serine threonine kinase inhibitor (DMAP) and cycloheximide. We have also evaluated these protocols on human oocytes, and found that both defined and serum-mediated maturation can support first polar body emission, and embryo development to the blastocyst stage following parthenogenetic activation. These accomplishments represent critical milestones with which to further develop cell nuclear replacement as proposed in the current licence application. A completely defined culture system for oocyte maturation also improves the reproducibility and clinical safety of such a technique for purposes of assisted conception by *in vitro* fertilisation.

Optimisation of hES cell culture and isolation

We have completed studies confirming that human dermal fibroblasts can serve as a source of conditioned medium to maintain the pluripotency of two existing hES cell lines (H1 and H9, originally derived by Dr. Thomson's laboratory in Wisconsin). This was assessed using a standard battery of assays evaluating surface and genetic markers of undifferentiated cell status by flow cytometry and Taqman RT-PCR, as well as differentiation of cell types representative of all three embryonic germ lineages (ectoderm, endoderm, & mesoderm). We have also demonstrated that human dermal fibroblasts on purified human laminin can support the outgrowth of small clusters of hES cells or embryoid bodies grown at a low density, intended to model presumptive ES cell isolation. We have also transitioned an existing cell line into a

completely defined feeder and serum-free culture system, wherein hES cells are grown on purified human laminin.

To date we have received 23 blastocysts at day 6 post fertilisation from which we have 3 presumptive new hES cell lines, exhibiting the expected growth and morphological attributes of existing hES cell lines. All of these lines have been isolated on mitotically inactivated human dermal fibroblasts plated on purified human laminin, although in one case this has been accomplished in a completely defined serum-free medium. The humanised and serum-free culture conditions with which these cell lines have been derived coupled with our efforts to isolate them in a quality assured manner, may well make them the most therapeutically suitable cell lines isolated in the world to date.

Title of research project

6.1 Please give the full title of the project

Derivation of human embryo stem cells by cell nuclear replacement for technology development and the study of Motor Neuron Disease

6.2 Please indicate which purpose of research (as defined in the HFE&E Act 1990) the project falls under. You may tick more than one box.

- | | |
|---|-------------------------------------|
| a. to promote advances in the treatment of infertility | <input type="checkbox"/> |
| b. to increase knowledge about the causes of congenital disease | <input checked="" type="checkbox"/> |
| c. to increase knowledge about the causes of miscarriage | <input type="checkbox"/> |
| d. to develop more effective techniques of contraception | <input type="checkbox"/> |
| e. to develop methods for detecting the presence of gene or chromosome abnormalities in embryos before implantation | <input type="checkbox"/> |
| f. i. to increase knowledge about the development of embryos | <input type="checkbox"/> |
| ii to increase knowledge about serious disease | <input checked="" type="checkbox"/> |
| iii to enable any such knowledge to be applied in developing treatments for serious disease | <input checked="" type="checkbox"/> |

Lay Summary

7.1 The Authority requires a brief summary of the project in lay terms, which will be disclosed to the public upon request. Please provide a short paragraph that we may use for this purpose. N.B., all other information provided in the application remains confidential.

We propose to develop methods for the derivation of stem cells from embryos produced by cell nuclear replacement. These methods will provide new opportunities for the study of inherited diseases in which the genetic cause has not yet been identified.

We propose to study Motor Neuron Disease. The project will make embryo stem cells from cells donated by patients with Motor Neuron Disease whose condition cannot be linked to known genetic errors that cause the disease. This would provide the first opportunity to study in cells from such patients the development of the disease, and ways to treat it. This research is not possible by any other means and would be a unique contribution to an international collaboration with experts in Motor Neuron Disease. These cells would not be used to correct the disease.

The methods will have other important uses. We will investigate differences between people in their response to medicine, which cause the death of large numbers of people even if the medicine is prescribed and taken appropriately. Cells produced by cell nuclear replacement may also offer advantages in the treatment of degenerative diseases as they would be genetically identical to the donor who would not require treatment to prevent rejection.

Duration of project

8.1 Please give the proposed date of commencement for the project.

January 2005

8.2 Please give the period of time for which you wish the licence to be granted.

3 years

9. Usage of material

9.1 Please indicate in the box below the estimated numbers of oocytes and embryos you expect to use during the period of the licence. If more than one year has been requested please indicate the year usage in the appropriate boxes.

Material	Year 1	Year 2	Year 3
Fresh Oocytes			
Frozen Oocytes	50	50	50
Failed to fertilise Oocytes (Frozen)	350	350	350
Fresh Embryos			
Frozen Embryos			

10. Centre(s) providing oocytes/embryos

10.1 Please give the names of the centres that will be supplying materials for this project, together with an indication of the number of embryos and or oocytes they each will be providing.

Centre	Fresh Oocytes	Failed to fertilise Oocytes (Frozen)	Frozen Embryos
Assisted conception Unit, Department of Obstetrics and Gynaecology, Forresterhill Aberdeen		400/yr	-

11. Current Research Projects

11.1 If you currently hold a research licence please give the project number(s) and title(s) of current research projects below. If you do not currently hold a research licence, please proceed to section 12.

R0136-1-b *Platform Technologies Underpinning hES cell Derivation.*

Centre 0202

Expires: June 30th, 2004 (Renewal pending)

Person Responsible: Paul A. De Sousa

Nominal Licensee: Ian Wilmut

11.2 Please indicate in the boxes below the number of oocytes and embryos received and the number that were used during the past year, in all your currently licensed research projects. This allows audit of embryos received but found to be unsuitable for research. Please give the name(s) of the centre(s) who supplied the material and give the data for each supplying centre separately.

Dates: From: July 1, 2003 To: May 18, 2004

Centre Name: UCH ACU (C044) provided to Roslin Institute (C0202) under R0136-1-b

Total number of embryos received	Total number of embryos used	Total number of oocytes received	Total number of oocytes used	Total number of embryos created for research
Fresh: <div>23</div>	Fresh: <div>23</div>	Fresh: <div>0</div>	Fresh: <div>0</div>	<div>0</div>
Frozen: <div>0</div>	Frozen: <div>0</div>	Failed to Fertilise: <div>0</div>	Failed to Fertilise: <div>0</div>	
		Frozen: <div>0</div>	Frozen: <div>0</div>	

Dates: From: July 1, 2003 To: May 18, 2004

Centre Name: New Royal Infirmary Edinburgh (Ob/Gyn Day Surgery) provided to Roslin Institute (C0202) under R0136-1-b

Total number of embryos received	Total number of embryos used	Total number of oocytes received	Total number of oocytes used	Total number of embryos created for research

Fresh: <div>0</div>	Fresh: <div>0</div>	Fresh: <div>61</div>	Fresh: <div>61</div>	<div>58</div>
Frozen: <div>0</div>	Frozen: <div>0</div>	Failed to Fertilise: <div>0</div>	Failed to Fertilise: <div>0</div>	
		Frozen: <div>0</div>	Frozen: <div>0</div>	

12. Abstract

Please give a summary of the work you propose to undertake

The objective of the project is to derive neural cells from a small number of patients with inherited forms of the neurodegenerative condition, Motor Neuron Disease, whose genetic basis is not well understood. In a small proportion of cases (2%) it is known that the disease reflects the influence of one of several mutations in a specific gene (superoxide dismutase). In a further group of patients (8%) the disease is inherited, but the molecular mechanism is not known. Neither the number nor the identities of other genes whose malfunction may cause this condition are known, although it is known that several genes are involved in this group of patients (see below).

The use of cell nuclear replacement will enable the first laboratory studies in human cells of the cellular and molecular changes that are associated with inherited cases, but in which the molecular cause of the disease is known. This research will draw upon recent progress in several different areas of research and be part of an international collaboration. The specific new opportunity is to use cell nuclear replacement to produce human embryos from which embryonic stem cells can be derived. Neural populations with the genotype of the patient will be derived from these stem cell lines. Several major steps are involved including maturation of oocytes, cell nuclear replacement, embryo development to the blastocyst stage, embryo stem cell derivation and differentiation of neural populations.

Developmentally competent human oocytes are required for embryo production by cell nuclear replacement. Under our current HFEA licence (R0136-1-b) we have determined that human oocytes can be matured *in vitro* and that a proportion retains the capacity to respond to an artificial parthenogenetic activation stimulus as is required for CNR. Our current supply of immature oocytes from fertile women undergoing laparoscopic sterilisation at the New Royal Infirmary of Edinburgh will continue to be used for research to derive parthenogenetic embryo stem cells whilst that project is funded. However, in the event that this funding is not renewed, this source of oocytes would be directed towards the current project, subject to appropriate patient consent being obtained. In addition, the program will rely on oocytes that are not suitable for purposes of conception in the course of infertility treatment. This will include those that are immature at the time scheduled for intracytoplasmic sperm injection (ICSI), and thus not injected, and those mature oocytes that fail to fertilise after ICSI or during insemination *in vitro* (IVF). These will be sourced from the Assisted Reproduction Unit, Department of Obstetrics and Gynaecology, Foresterhill, Aberdeen, where they will be cryopreserved before shipment to the Roslin Institute. All oocytes supplied by Aberdeen will undergo *in vitro* maturation if necessary using protocols optimised under our current research licence. When necessary, the enucleation procedure will be modified to include the removal of the sperm head as well as the oocyte chromosomes. Some oocytes will be enucleated before being cryopreserved in order to compare the efficiency of storage by freezing oocytes before or after enucleation. At Roslin, CNR experiments would be performed on batches of enucleated oocytes as they became available using strategies optimised in animal models.

Transfer of cell nuclei from Motor Neuron Disease patients into these enucleated, mature human oocytes will produce embryos with the specific genotype. Patients will donate skin and blood samples from which cells will be isolated and stored in liquid nitrogen for availability as nuclear donors. Skin fibroblasts are selected for the first studies because offspring of several species have been derived from such cells after nuclear transfer. Two alternative cell types will be considered as nuclear donors if fibroblasts prove unsuitable or if experiments in the mouse suggest that the alternatives are more suitable than skin derived fibroblasts. The alternatives are hair follicle stem cells and peripheral monocytes. Stem cells can be derived from human hair follicles routinely and have been shown to have considerable developmental potential (eg haematopoietic (Lako, et al., 2002)). Monocytes are selected for study, despite the fact that they have not previously been used as nuclear donors, because there is recent evidence that some peripheral monocytes have a wide developmental potential in culture (Zhao, et al., 2003). Use of adult cells with a great developmental potential would be expected to increase the efficiency of blastocyst production.

Cell nuclear replacement will be carried out according to established procedures, including the method used to derive the first embryo stem cells from an embryo produced by cell nuclear replacement in Korea. Earlier research established that cell cycle co-ordination is essential for normal development and suggested that there are advantages in using donor cells in quiescence and recipient oocytes at metaphase II of meiosis. However, species differ in the optimum time for oocyte activation in relation to nuclear transfer. The optimum procedures for embryo reconstruction and for the stimulation of the reconstructed embryos will be studied.

Embryos will be cultured to the blastocyst stage according to standard culture systems for IVF clinics. The proportion of embryos from which embryo stem cell lines can be derived may be reduced in embryos produced by cell nuclear replacement as the number of cells in the inner cell mass is sometimes reduced in these embryos. Human embryo stem cell isolation would be in dedicated state of the art facilities working to high standards of quality assurance. Isolations would follow protocols established in the lab by research carried out under our current licence (R0136-1-b). Currently this involves outgrowth of whole blastocysts on human dermal fibroblast feeders on purified human laminin in media with or without serum supplementation.

The different cell populations required for the research project will be derived according to protocols available at the time. Steps that are known to influence development of neural populations from murine embryo stem cells include formation of embryoid bodies, exposure to retinoic acid, cytokines such as FGF4 and morphogens such as sonic hedgehog. As several laboratories have independently described methods for derivation of neural populations the approaches that are being used appear to be robust. The specific derivation of motor neurons from murine embryo stem cells has been described (Wichterle, et al., 2002). Equivalent methods for differentiation of human cells are likely to be available when required for this project as intensive research is driven by the possibility of using such cells in treatment of human neurodegenerative diseases, and an MRC funded programme for the development of this protocol is presently underway at Roslin Institute. Comparison of cells known to be liable to Motor Neuron Disease that are derived by these different means will be very informative.

The proposed research is part of a wider collaboration to be able to study human cells that are liable to Motor Neuron Disease. The unique contribution of the proposed research will be to produce cells from inherited cases of the disease that are not known to be associated with mutation in Cu/Zn superoxide dismutase. These observations will complement those made in cells produced by genetic modification of existing human embryo stem cell lines. As mutant superoxide dismutase alleles have dominant effects in patients it is expected that over-expression of mutant superoxide dismutase alleles in cells by gene transfer will mimic effects in the disease. Comparisons will be made between cells that over-express either normal or mutant forms of the protein. Over a period of time, as resources allow, cell nuclear replacement will be used to produce cells from several patients with non-superoxide dismutase inherited Motor Neuron Disease cases as it is known that several genes are involved in this group of patients (other MND loci have been identified on Chromosomes 9,15, 16, 18 and 20).

Methods will be developed for inducing differentiation into astrocytes or support cells that may play a causal role in the degeneration of motor neurons. In order to assess the role of the motor neurons and their surrounding cells in susceptibility, methods will be developed for co-culture of affected and non-affected glia cells. We also aim to develop an assay with motor neurons genetically identical to those of patients with Motor Neuron Disease that can be used to screen for compounds that protect the cells from gene mutation induced death. This may require some added stress to the cells to reveal their vulnerability. Removal of serum for the final 24 hours before study has been used as a method of oxidative stress.

Please continue on a separate sheet if required

13. Objectives

Please state the aims and objectives of the project. If the project involves human embryonic stem cells, please provide justification for their use, e.g. could the same results be obtained using other material such as animal stem cells or adult stem cells?

The aims of this program of research are 1). to develop methods for the derivation of embryo stem cells from human embryos produced by cell nuclear replacement (CNR) and 2). to use those methods to gain new understanding of cellular and molecular changes associated with Motor Neuron Disease. *In vitro* differentiated neural cell populations derived from Motor Neuron Disease patient-CNR hES cells will be compared with several other populations. Introducing specific mutations into existing embryo stem cell lines will produce other cells known to be susceptible to Motor Neuron Disease. These lines will be compared both with cell lines obtained by cell nuclear replacement from patients not known to be susceptible to Motor Neuron Disease and to hES cells obtained from surplus to fertility requirement embryos where there is no reason to expect susceptibility to the disease.

Cell nuclear replacement

A recent publication has described the first derivation of human embryo stem cells from an embryo produced by cell nuclear replacement (Hwang, et al., 2004) establishing the feasibility of this objective. Our first research objective will be to optimise these procedures by experiments in both human and animal models. Factors that will be evaluated using human oocytes include the use of cryopreserved oocytes as recipient cell cytoplasts for CNR, methods of enucleation, choice of donor cell, co-ordination of the cell cycle of donor and recipient cell, method of activation of the reconstructed embryo and method of embryo culture.

Studies of Motor Neuron Disease

The aetiology of Motor Neuron Disease is largely unknown. In the past it has not been possible to follow the development of the disease in affected tissues because studies have been limited to post-mortem samples in which the motor neurons are depleted and many secondary changes may have taken place. Over a period of time the objective of a large collaborative project of which this research is part, is to obtain human neural populations from CNR derived embryo stem cells for study in the laboratory. The availability of human ES cells now makes it possible to study human neural cells associated with Motor Neuron Disease. Overall these neural cells will be of several types:

1. Those not known to be vulnerable to Motor Neuron Disease as a control
2. Those expressing a mutant form of the superoxide dismutase gene introduced by gene transfer.
3. Those from patients with inherited Motor Neuron Disease not associated with a superoxide dismutase mutation.

The aim of the research proposed in this application is to develop the methods of cell nuclear replacement necessary to obtain cells of type 3. The steps involved in this process are:

- 1) To develop methods for blastocyst production after cell nuclear replacement with nuclei from adult somatic cells.
 - a). Assess development to the blastocyst stage after transfer of nuclei.
 - b). Examine those embryos that fail to cleave in search of an understanding of the cause of developmental failure.
 - c). Examine a proportion of blastocysts to determine the number of cells and the proportion allocated to the inner cell mass.
 - d). Isolate and characterise the cells derived by CNR.
 - e). If cell lines are abnormal then examine reconstructed embryos to confirm normal re-assembly of the transferred nucleus.
- 2) To derive neural cell populations and compare them with equivalent cells derived from embryo stem cells from surplus to fertility requirements embryos.

Justification for CNR-hES cell isolation

The study of a human cellular disease *in vitro*, such as Motor Neuron Disease, requires the capacity to produce and maintain the relevant affected differentiated cell lineages *in vitro*. Ideally these cells should be produced by the pathways of differentiation that occur *in vivo*. Further, the affected cells should be produced without too intrusive an intervention for the patient and have a normal life span in culture. Most importantly they should have the same genotype as found by individuals affected with the disease. In principle, three approaches have the potential to meet these requirements. Each approach requires further technical development and has strengths and weakness. The three approaches would be to either use;

- 1) multipotent cells from adult tissue from individuals affected by the disease under study.
- 2) cell nuclear replacement to derive human embryo stem cells known to be liable to Motor Neuron Disease, from which affected cell lineages could be differentiated. Or,
- 3) genetically modify either existing adult multiprogenitor cells or hES cell lines to replicate the disease under study.

Multipotent adult cells

Some adult cell populations are apparently revealing a far wider developmental potential than was expected (see review by (Verfaillie, et al.,2003). A pluripotent cell population that has been derived from bone marrow of several species including mouse and human has demonstrated extraordinary potential (Jiang, et al.,2002). The pluripotent cell populations grow in culture for very long periods where they have been induced to differentiate into all three major lineages, including specifically, cells with many of the morphological characteristics of neurectoderm. After injection into blastocysts, mouse cells were able to contribute to all lineages including nerve tissue. However, functional assessments of neurectoderm derivatives remain to be made.

If neural cells from Motor Neuron Disease patients were obtained from adult cells they would be of the specific genotype. However, it is unlikely that differentiation would be through normal pathways. It also remains to be confirmed that the cells can be fully functional. Experience suggests that it may take some time, perhaps years, before the expertise to isolate these cells becomes routine, or if indeed it proves possible. Finally, at present these adult progenitor cells are derived from bone marrow requiring a comparatively intrusive sampling procedure. In the context of this research objective it should be noted that cells displaying some characteristics of neural cell populations have been derived from a sub-population of human peripheral blood Monocytes (Zhao, et al., 2003).

Cell nuclear replacement to derive human embryo stem cell lines of specific genotype

As they theoretically have the ability to form all of the tissues of an adult, the availability of human embryo stem cells in the laboratory has made it possible to begin to define the mechanisms that regulate cellular differentiation during normal development. Human embryo stem cell lines are developmentally very similar to the early human embryos from which they were derived (Henderson, et al., 2002) and show appropriate developmental changes during early differentiation (Kaufman, et al., 2002). Culture conditions have been established that encourage the development of neural cells (see (Stavridis, et al., 2003) allowing studies of the cellular and molecular changes in normal and disease conditions.

Genotypes associated with Motor Neuron Disease could be replicated by use of cell nuclear replacement. An embryo stem cell line would be derived from an embryo produced by transfer of a nucleus from a patient with Motor Neuron Disease. In this case the genotype is known to be associated with the condition.

Once established, embryo stem cell lines are expected to be available for study over very long periods and the pathway of differentiation is expected to be normal. The major biological limitation to this approach is that cell nuclear replacement may introduce into the cells lines epigenetic changes that perturb gene expression. There is direct evidence of such variation in gene expression in tissues from animals produced by cell nuclear replacement (Humphreys, et al., 2002). However, there is contrasting evidence that murine embryo stem cells derived from embryos produced by cell nuclear replacement are able to contribute to chimeras as efficiently as other lines derived from embryos produced by fertilisation (e.g. Wakayama et al, 2001). The technical challenge to be addressed in this approach is in applying nuclear transfer with limited supplies of oocytes and then being able to derive human embryo stem cells. In these circumstances, we do not propose to produce control cells by transfer of nuclei from patients not known to be susceptible to Motor Neuron Disease. Rather we will collaborate with other groups carrying out cell nuclear replacement to obtain such cells, subject to regulatory approval. We will compare all of these populations with cells derived from donated embryos.

Two groups of patients would be involved. Patients with Motor Neuron Disease would donate skin and blood, both less intrusive procedures than aspiration of bone marrow. Oocytes unsuitable for clinical use would be obtained from patients undergoing IVF or ICSI treatment and in these circumstances no additional procedures would be required.

Genetic modification in existing cell lines as a means for studying Motor Neuron Disease

Mutations known to be associated with Motor Neuron Disease, such as those in superoxide dismutase, could be introduced into embryo stem cells by homologous recombination (Zwaka, et al., 2003) or gene transfer. This would allow the analysis of the effect of specific mutations on differentiation and cell function.

This approach would provide long-lived cell cultures that could be used over many years. The pathway of differentiation is expected to be normal. However, there is a significant technical challenge in achieving this objective, as the aim may be to change a single nucleotide in the coding sequence, rather than to disrupt the expression of the gene. It is also very likely that other, as yet unknown, genes modify the effect of superoxide dismutase mutations so that the precise phenotype might vary from one genetic background to another. Nevertheless this approach offers the only means of analysing the effect of several specific genetic changes in one genetic background. There are no implications for donors as the cell lines are already available.

Conclusion

There are several exciting new potential approaches to the study of human genetic diseases such as Motor Neuron Disease *in vitro*. The greatest potential benefit would arise from being able to compare directly a number of different cell lines whose vulnerability to Motor Neuron Disease reflected the influence of several genes.

All of the approaches require technical development before cells for research could become available and each has advantages and limitations. Human cells required for this research project might one day be produced from multipotent cells from adult human tissues. However, it is not certain that their differentiation would mimic that during normal development and this prospect may be several years into the future, as the different necessary procedures have not yet been described for any species. By contrast, far more is known of embryo stem cells. The individual steps proposed for this project have all been completed in the mouse and rapid progress is being made in developing most of the procedures in human, aided in part by the experience in the mouse.

Proposed research strategy

The objective of the collaborating group is to derive neural cell populations from patients with inherited forms of Motor Neuron Disease and to compare them with cells not known to be vulnerable to the disease. There are several means of obtaining cells vulnerable to Motor Neuron Disease.

Genetic changes known to be associated with Motor Neuron Disease will be introduced into existing human embryo stem cell lines by gene transfer and gene modification. These cells will be compared with the original cells without the genetic modification. The particular value of cell nuclear replacement is to produce cell lines known to be vulnerable to Motor Neuron Disease without needing to know the genes

responsible for the disease. A comparison between cells vulnerable to Motor Neuron Disease derived by the different routes would be very informative. These cells would all be compared with two groups of control cells, not known to be susceptible to Motor Neuron Disease. These would be derived from donated embryos and from embryos obtained by transfer of nuclei from donors not known to be vulnerable to Motor Neuron Disease.

Please continue on a separate sheet if required

14. Background

Please state how the project fits in to the current state of knowledge on this subject (maximum of 1500 words).

Derivation of cells from embryos produced by cell nuclear replacement

Important new opportunities in research and therapy will be created by the production of cells from human embryos produced by cell nuclear replacement. The potential applications are broadly of two types being either for laboratory research or for therapy. Representative potential uses in the laboratory will be considered first as it seems probable that they will be realised more quickly. For the purpose of this analysis it is assumed that it will become possible to produce human embryos after transfer of somatic cell nuclei, to derive human embryo stem cells from them and then to obtain cells of specific phenotypes by controlled differentiation.

Studies of human genetic diseases

As has been discussed above, the only means of obtaining cells from patients with inherited disease in which the causative genetic difference has not yet been identified is by derivation of cells from embryos produced by cell nuclear replacement. Motor Neuron Disease is the example on which we propose to concentrate.

Pharmacogenetics

An extraordinary number of people are killed each year by taking medicine. In a survey in 1994 an adverse response to medicine was the fifth most common cause of death in the USA (Phillips, et al., 2001). Many millions of other patients had to receive treatment from general practitioners or hospitals. This variation in response to medicines that are being prescribed and taken appropriately reflects genetic differences in either clearance or response to the medicine. While several organs are known to be involved, differences in clearance by the liver are responsible for a significant proportion of the cases. This presents a second, similar opportunity to use cells from embryos produced by cell nuclear replacement for research and to complement this approach by the use of gene targeting in established embryo stem cells lines to introduce alleles that are known to cause susceptibility to medicine.

Using donor cells from patients in nuclear transfer would produce embryos and embryo stem cell lines of appropriate genotype. In turn, cells of the relevant type would be derived from such cells for research in the laboratory. Hepatocytes of different genotype would be used in analyses of genetic effects on drug clearance. At the present time hepatocytes are only available from casualties if the liver is not suitable for transplantation and in order to obtain a sufficient number of cells it is sometimes necessary to pool cells from more than one person. Inevitably the genotype of the cells is not known in advance and it may not be relevant for many studies. There would be considerable benefit in having hepatocytes from the same population available over a period of years. Ideally, several different genotypes would be represented. Cells derived from embryo stem cells lines would offer those opportunities. Cells with many of the characteristics of hepatocytes have been derived from human embryo stem cells (Rambhatla, et al., 2003). If derived from a donated

embryo they may not be of a susceptible genotype, but nuclear transfer could be used to provide cells of those genotypes.

The benefits to us all of research with such cells would be considerable. In time doctors prescribing medicine will be able to know the genotype of the patient and select the most appropriate medicine and dose for that patient. At present, much the same dose is offered to us all. In these circumstances the selected dose will be too low for some people, but unfortunately fatal for others. Given the more accurate information that will become available it will be possible not only to reduce the risk of serious ill effects, but also to increase the dose to those patients who clear the drug quickly. This case has been made for hepatocytes, but similar arguments also apply for other organs.

Cells for therapy

Cells derived from embryo stem cells will offer new treatments for some very unpleasant degenerative diseases including spinal cord injury, Parkinson's Disease, diabetes and liver damage. All of these diseases reflect the death or loss of function of specific cells that are neither repaired nor replaced. There is not a fully effective treatment for these conditions at present and in some cases there is no treatment at all. It may become possible to treat Motor Neuron Disease by the transfer of cells, but it is not our objective to produce cells that might be used for that purpose. Rather the aim of this research proposal is to provide the understanding that is necessary for the development and assessment of such a therapy.

Ideally, any such cell treatment should meet several requirements. Either the cells must be immunologically matched or a treatment must be available to prevent rejection of incompatible cells. The significance of this requirement in different diseases is not known as some groups argue that immune response may be limited in some locations, such as the central nervous system. It would be essential that transferred cells survived for the life-time of the patient who might in some cases be treated in childhood and function normally throughout a period of several decades. Finally, there must be no risk of tumours arising from the cells or alternatively procedures must be in place to destroy cells within the patient if necessary.

Several different approaches are being considered for treatment of these degenerative diseases. Embryo stem cells offer a potential source of cells for therapy and methods for the derivation of specific cells types are being established, although in most cases it remains to be confirmed that the cells function normally after transfer. In addition a great deal remains to be learned about the most effective means of introducing the cells into patients.

The avoidance of immune rejection presents a significant challenge. If derived from donated embryos the cells would almost certainly be immunologically different from the patient and require a means of preventing rejection. Present methods of immunosuppression carry significant disadvantages. Nuclear transfer could offer histocompatible cells either by deriving cells from the patient or by producing cells from donors that are homozygous at the major antigens. As such donors would be extremely rare there is a very low probability of such an embryo being donated. Derivation of cells from such donors by cell nuclear replacement would provide an

effective means of meeting the requirement of a significant proportion of the population, although not the entire population (Bradley, 2004). This is a potential alternative to the proposal to derive cells for each individual patient by use of cell nuclear replacement.

Immunologically matched cells would clearly offer a great advantage in cases in which rejection is expected, but the disease is not autoimmune, such as in repair of liver or heart tissues. It has been suggested that there is no immune response within the central nervous system, but this is the subject of further research. Immunologically match cells would also offer an advantage in treatment of diseases of the central nervous system if rejection occurs.

Treatment of genetic diseases

Cells from cloned embryos will also offer an opportunity to treat some human genetic diseases. A proof of principle experiment in mice corrected a mutation that results in immune deficiency in mice and human patients (Rideout, et al., 2002). In this case cells from the animal were used as nuclear donors to produce cloned embryos and stem cell lines. The mutation was corrected before the cells were differentiated down haematopoietic lineages and returned to the mice.

This approach would offer opportunities in the following combination of circumstances. The genetic cause must have been identified, it must be possible to derive cells of the affected type and to return them if not to their usual location to another in which they can function effectively.

Conclusions

This analysis has identified a number of circumstances in which derivation of cells by cell nuclear replacement would create opportunities in research and therapy that are not available in any other way. In these circumstances we will miss opportunities to understand and treat some very unpleasant human diseases if we fail to address the challenge of cell nuclear replacement in humans.

This proposed research application

Degeneration of motor neurons is the common cause of the fatal condition variously known as Motor Neuron Disease, Amyotrophic Lateral Sclerosis and Lou Gehrig's disease. There are approximately 5,000 patients in the United Kingdom. Typically the disease is fatal within 3-5 years of first diagnosis usually because of respiratory failure. The causes of the degeneration are not understood, although a number of candidate mechanisms are under investigation. It seems likely that several genetic and environmental factors contribute to the pathogenesis of Motor Neuron Disease.

The objective of this proposal is to create a new opportunity to study the aetiology of the disease by creating in the laboratory neural populations that are genetically identical to those of patients. This will be achieved by transfer of nuclei from somatic cells provided by patients to enucleated oocytes. By first deriving embryo stem cells and then inducing these to differentiate into the required phenotypes, we would obtain neural and other populations. The specific proposal in this project is to develop the necessary methods for cell nuclear replacement.

The nature of Motor Neuron Disease

Studies of human patients and their families have revealed the mechanisms of inheritance of Motor Neuron Disease and animal models have provided the present understanding of the molecular and cellular mechanisms that lead to Motor Neuron Disease (reviewed (Dib,2003)).

The great majority of cases of Motor Neuron Disease are sporadic, but between 5 and 10% are inherited. Among these inherited cases mutations in the gene that encodes superoxide dismutase account for approximately 20% of cases and linkage analysis suggest that at least four other genes remain to be identified. The cause of Motor Neuron Disease was at first assumed to be reduced function of the gene, but this seems not to be the case. Mice in which the endogenous superoxide dismutase gene has been deleted do not develop Motor Neuron Disease, whereas those that express mutant forms of the human gene develop paralysis (reviewed by (Cluskey, et al.,2001)). As the transgenic mice carrying the human gene also had their own two copies of the gene this observation suggests that the effect of the mutation is through a cytotoxic effect of the abnormal protein, rather than a loss of function.

Although a number of encouraging avenues of research have been identified there is at present no clear explanation as to how this protein causes neurodegeneration. Progress of research is very seriously limited by the absence of human cells affected by the disease. This is because of the impossibility of obtaining neural tissue from patients in early stages of the disease. Evidence has been gained from studies in animals and immortalised mouse cells in culture. These cells were obtained by fusion of mouse motor neuron and neuroblastoma cells. Together these studies provide evidence of oxidative stress, glutamatergic excitotoxicity, mitochondrial dysfunction, disrupted axonal transport, aberrant cell signalling and protein aggregation leading to apoptosis (reviewed by (Cleveland, et al.,2001); (Shaw, et al.,2001); (Robertson, et al.,2002); (Friedlander,2003); (Allen, et al.,2003)). These hypotheses are not mutually exclusive, but the lack of consensus about the primary disease mechanisms after a decade of research underlines the limitations of existing models. The fact that differences in cell and molecular function are apparent in the cell cultures suggests that human motor neurons known to be liable to Motor Neuron Disease will provide new understanding of the mechanisms that lead to the disease, despite the fact that in many patients symptoms of the disease are not apparent until middle age.

It is in these circumstances that we propose to derive human motor neurons known to be liable to Motor Neuron Disease to provide a more informative cellular model of the disease. The availability of these cells will allow detailed studies of the disease pathogenesis and assessment of drugs and cell therapy as novel means of treatment of the disease.

The proposal to study human neural populations

The entirely new and important opportunities to study the development of the disease and assess new therapies if human neural populations from Motor Neuron Disease patients could be produced in the laboratory are described later in this section. Meeting this objective would depend upon bringing together three new techniques;

the creation of blastocyst stage embryos by cell nuclear replacement, human embryo stem cell derivation, and *in vitro* differentiation of human embryo stem cells into neural populations. The resulting cells would have the same genotype as the patient.

As discussed earlier, a major advantage of this approach is that the causative genes need not be known. Once established, embryo stem cell lines are expected to be available for study over very long periods. The major biological limitation to this approach is that present methods of cell nuclear replacement may introduce into the cells epigenetic changes that perturb gene expression. There is direct evidence of such variation in gene expression in tissues from cloned animals (Humphreys, et al., 2002). However, there is contrasting evidence that embryo stem cell lines derived from embryos produced by transfer of nuclei of adult skin fibroblasts were able to contribute to chimeras as efficiently as other lines derived from embryos produced by fertilisation (e.g. (Wakayama, et al., 2001).

Mammalian cell nuclear replacement

The recent derivation of the first stem cell line from a human embryo obtained by cell nuclear replacement provides an important proof of principle. However, a great deal remains to be achieved in the optimisation and application of the procedure.

Factors that are believed to have been important in the development of methods able to obtain development to term after transfer of nuclei from differentiated cells include the use of nuclear donor cells that had been induced to exit the cell cycle and become relatively inactive, "quiescent" and the recognition of the importance of controlling the time of oocyte activation in relation to the time of nuclear replacement (Campbell, et al., 1996). Since this initial success, offspring have been obtained after transfer of nuclei from somatic cells of nine species; namely sheep, cow, goat, pig, mouse, rabbit, cat, mule and horse (see (Wilmut, et al., 2002); (Galli, et al., 2003) (Woods, et al., 2002); (Zhou, et al., 2003). However, offspring have not been produced in two other species despite extensive efforts by experienced teams. These are rhesus monkey and dog.

The failure to obtain rhesus monkey offspring is of particular importance to proposals to derive embryo stem cells from cloned human embryos. Although a significant proportion of rhesus cell nuclear replacement embryos developed to the blastocyst stage, none were able to develop to term. Inspection revealed that a high proportion of the cells in the blastocysts were apoptotic, that there was a very high incidence of chromosomal anomalies and associated this outcome with the removal of essential activities at the time of enucleation of the oocyte (Simerly, et al., 2003). Recent observations during rat cell nuclear replacement also revealed abnormal assembly of the spindle after cell nuclear replacement in that species (Hirabayashi, et al., 2003). In both species preliminary observations suggest that delaying enucleation until after transfer of the donor nucleus may provide an effective alternative approach. Development to term of sheep cell nuclear replacement embryos has also been shown after such delayed enucleation (Peura, 2003).

Together these recent observations emphasise the importance of observing events during early development of cell nuclear replacement embryos to confirm normal nuclear assembly and ploidy while systematically varying the timing of each step in

cell nuclear replacement. In the recent research with human embryos only one embryo stem cell line was obtained from 30 human blastocysts obtained by cell nuclear replacement (Hwang, et al., 2004). Only the karyotype of the cell line was defined. It will be important early in this project to confirm that the proportion of cells in blastocysts that are undergoing apoptosis is not increased in those obtained by cell nuclear replacement.

Several factors may influence the proportion of embryos produced by cell nuclear replacement that develop to the blastocyst stage including the choice of donor cell population. In a recent publication describing production of over 1,000 cattle embryos with three different adult skin fibroblast lines 52, 47 and 27% developed to compact morula or blastocyst stages from which derivation of embryo stem cell lines might be attempted (Wells, et al., 2003). Offspring were obtained from lines 1 and 3, but not from line 2, although after transfer of only 12 embryos. Embryo stem cell lines have been derived from murine cell nuclear replacement embryos and these have been shown to contribute to chimeras with the same efficiency as that of lines derived from fertilised embryos (Wakayama, et al., 2001).

CNR is of course also critically dependent on the availability of developmentally competent oocytes. Ideally the source of these oocytes should not compete with any primary interest to conceive on the part of women and couples which donate them. Thus, oocytes should be from either donors that are fertile but not wishing to conceive. In vitro maturation of oocytes obtained from this type of donor is one avenue for developmentally competent oocytes currently being investigated under our existing HFEA research licence (R0136-1-b). Although this approach has yielded viable offspring when used clinically, the success rate of this procedure is significantly lower than that achieved by conventional IVF (reviewed in (Trounson, et al., 2001)).

Alternatively, in the course of IVF treatment approximately 20-40% of oocytes are deemed unsuitable for clinical use due to being either immature at the time scheduled for insemination by intracytoplasmic injection (ICSI), or failed to fertilise after ICSI or conventional insemination in vitro (IVF). Human oocytes that are immature at the time of aspiration can be matured *in vitro* to the metaphase II stage (Roberts, et al., 2002). Such oocytes and those that have failed to fertilise after conventional IVF or ICSI remain fertilisable or capable of having their development activated parthenogenetically even after 48 h in culture (Levron, et al., 1995); (Park, et al., 2000). Not surprisingly, the developmental potential of oocytes under these circumstances is poorer than normal and diminishes with time *in vitro*. However, re-insemination of failed to fertilise human oocytes by ICSI 24 h or 48 h post first insemination has been reported to result in normal offspring (Yuzpe, et al., 2000); (Morton, et al., 1997). In addition, parthenogenetic activation of human oocytes that had not been fertilised had been reported to enable 10 % development to the blastocyst stage, with 70 % of resulting blastocysts being euploid (Zhang, et al., 1999). Both electrical pulses and protein kinase inhibitors have been used to activate human and non-human primate oocytes (Levron, et al., 1995) (Zhang, et al., 1999); (Mitalipov, et al., 2001). The former has contributed to the derivation of parthenogenetic non-human primate ES cells, which *in vitro* or in mice can differentiate into cardiomyocytes, smooth muscle, neurons, adipocytes, and ciliated epithelium (Cibelli, et al., 2002).

Oocyte cryopreservation

The efficiency with which it will be possible to use donated oocytes would be greatly increased by establishing routines for the storage of oocytes by freezing and thawing. Development of embryos produced by transfer of nuclei to frozen and thawed bovine oocytes has been established (Kubota, et al.,1998). In particular development with oocytes stored after vitrification was equivalent to that obtained with fresh bovine oocytes (Dinnyes, et al.,2000).

Derivation of human embryo stem cells

Since the original observation by Thomson in Wisconsin (Thomson, et al.,1995) human embryo stem cell lines have been derived in several other laboratories and it is likely that methods of derivation will be improved before the time when the proposed research is being conducted. The accuracy of estimates of the proportion of embryos from which lines can be derived is limited by unknown variation in the quality of the embryos that were being used and probably by failure to report some negative results. Overall the efficiency is likely to be lower with embryos produced by cell nuclear replacement because typically such embryos are less well organised and many have fewer cells in the inner cell mass from which embryo stem cells are derived.

Differentiation of neural populations

Most published research on the control of differentiation of embryo stem cells was carried out with murine cells. In this species considerable progress has been made in defining culture conditions and procedures that direct differentiation of a large proportion of the embryo stem cells along neural lineages (see (Stavridis, et al.,2003) for a review). Research to produce specific cell types draws upon earlier experiments in which use has been made of reporter genes and the technique of gene deletion in mice in order to study the genetic control of differentiation. In turn the tissue culture studies provide an opportunity to test hypotheses on the role of specific genes.

The ability has been developed to direct cell fate through the use of extra-cellular factors introduced in several steps, each with a different purpose. Specific cytokines and morphogens are used at specific stages in the process to promote multiplication of progenitor cells before final differentiation. In particular, derivation of motor neurons by a recently published procedure involved formation of embryoid bodies, exposure to retinoic acid and exposure to a small molecule agonist of the morphogen sonic hedgehog. By varying the precise protocol, different neural populations were obtained. The cells survived in culture for considerable periods and extended long processes as is expected of motor neurones (Wichterle, et al.,2002).

Completion of the proposed research depends upon establishment of comparable protocols for derivation of human neural populations. This is a reasonable expectation in view of the great medical value of such procedures and the time required for the derivation of the human embryo stem cell lines.

Experimental objectives in the longer term

Achievement of these objectives will create opportunities that are not available in any other way. The collaborative group will be able to:

1. Compare differentiation in control and disease susceptible cell populations.
2. Seek a common pathogenic mechanism by describing the aetiology of the disease in cells of several genotypes that are all liable to the disease. It may be necessary to stress the cell in culture.
3. Compare gene and protein expression in control and Motor Neuron Disease susceptible motor neurones and astroglial cells.
4. Discover whether the disease is cell autonomous by comparison of cell cultures that contain only one or several cell types.
5. Develop a high throughput assay with human Motor Neuron Disease cells that can be used to screen for compounds that protect the cells.
6. Assess the protective effects of control cells in mixed culture.

15. Methodology/experimental design/analysis of results

Please state how this work is to be carried out. Please state clearly how you intend to dispose of the embryos after the research is completed. If human embryonic stem cells are to be used, please indicate clearly the fate of the stem cells throughout the project, and how they are to be disposed of after completion of the work.

Experimental objectives

The purpose of this research is to develop methods for the derivation of human embryo stem cell lines of specific genotype and to derive cell lines from patients with Motor Neuron Disease. Neural populations derived from these lines would provide the first opportunity to study the development of the disease in human cells and to use such cells to assess new treatments with small molecule drugs or by cell therapy. Attainment of this objective depends upon successful completion of a series of procedures. Where possible established methods for these steps would be employed, but experimental comparisons would be made at specific points.

Experimental Overview

The project will initially rely on immature or failed to fertilise oocytes produced in the course of fertility treatment. At the Assisted Conception Unit (ACU), these may be cryopreserved immediately upon receipt or after culture to allow first polar body emission and development to metaphase II of meiosis. Since the number of oocytes donated at any one time is anticipated to be variable and low (<3-4), all oocytes from a donor will be randomly assigned to experimental culture/cryopreservation protocols being evaluated. This research will operate under an HFEA research licence to be sought for this purpose at the ACU. Care will be taken to ensure that where possible oocytes from each donor are allocated equally to each treatment.

Once a sufficient number of oocytes for a cell nuclear replacement trial has been collected these will be shipped to the Roslin Institute. Oocyte shipments will be in a portable liquid nitrogen dewar picked up and delivered by car either by Institute staff named in the HFEA licence, or by the same insured carrier used for transportation of embryos for stem cell derivation under licence R0136-1b (ie. World Courier). Oocytes subjected to different culture/cryopreservation protocols will be equally distributed among subsequent cell nuclear replacement protocols. Information regarding recovery of oocytes after thaw will be fed back to the ACU to inform experiments conducted under the HFEA research licence held there. Failed CNR embryos and a limited number of CNR blastocysts will be structurally characterised for cell number, nuclear morphology and apoptosis to inform development of future protocols at Roslin, after which embryo stem cells will be isolated under HFEA licence R0136-1b, using optimised protocols. Subsequent characterisation of CNR hES cell lines and their deposition in the UK stem cell bank will be under licence R0136-1b.

Oocyte donation and handling

Couples seeking fertility treatment at the ACU in Aberdeen, or additional centres as they become available, will be approached to donate oocytes unsuitable for their treatment by a member of the ACU staff not involved in the research. Only women over the age of 18, in good general health, with sufficient command of the English language who have understood the consent form will be accepted as donors. Donors will be advised that participation in the study will have no bearing on their treatment, and they will have the opportunity to withdraw consent until donated oocytes are used in experimental protocols. The medical history of patients donating oocytes will be established. Signed copies of patient information and consent forms will be kept in medical patient notes and in project records at the ACU. These will not be distributed to their General Practitioner unless specifically requested and consented by patients. Anonymous and coded patient information will be kept in project records at the Roslin Institute. This will include information on donor eligibility, medical history information collected by questionnaire, the results of any donor screening performed in support of the fertility treatment, a record of the patient information and consent forms used to obtain consent, and any variations in the terms of consent. The key relating actual and coded patient identity will be kept at the site of oocyte donation.

Oocytes will be available from two treatment procedures used in the ACU. These are 1. conventional in vitro fertilisation (IVF) in which sperm are mixed with oocytes in culture and 2. IntraCytoplasmic Sperm Injection (ICSI) when sperm are injected directly into the oocyte.

Those oocytes that are not fertilised 20-24 hours after addition of sperm to oocytes in the conventional IVF treatment will be removed from the fertility treatment at that time. Oocytes in procedures for sperm injection that are judged to be immature at the time scheduled for sperm injection will be removed from the fertility treatment before being cultured for an additional period in maturation medium to discover if they have the potential to mature. Assessment of oocyte immaturity and clinical unsuitability will be according to standard clinical criteria. Oocytes donated to the project will be cryopreserved and stored in liquid nitrogen before being shipped to Roslin Institute.

Two fundamentally different approaches to cryopreservation that have both been shown to be very effective with other cell types will be compared. Oocytes will be either cooled very rapidly in the presence of high concentrations of protective agents or cooled slowly in the presence of lower concentrations of agent. Variations to these protocols will be evaluated under an HFEA research licence to be sought by the ACU. After shipment of frozen oocytes to the Roslin Institute, groups of oocytes will be thawed and the protective agent removed. The viability of oocytes after thawing, and use in cell nuclear replacement experiments will be reported back to the ACU to inform their research program.

Cell donation by Motor Neuron Disease patients

Patients will be selected as having inherited forms of Motor Neuron Disease that are not associated with known mutations in superoxide dismutase. Fibroblasts will be derived from skin biopsies obtained under local anaesthetic. Skin samples will be cut into small pieces before dispersing the cells by exposure to trypsin. The fibroblasts will be cultured in Eagles medium supplemented with energy sources and amino acids and fetal calf serum (10%). As the cells become confluent they will be passaged up to 10 times before being frozen and stored. Samples of the cells will be examined before use as nuclear donors to confirm that chromosome number is normal and to develop effective methods for inducing quiescence.

If evidence from experiments in the mouse suggests that they may be suitable nuclear donors, monocytes will be derived from the buffy coat of peripheral blood of (500ml) Motor Neuron Disease patients. This is the volume of blood collected by the Transfusion Service. Blood will not be withdrawn from any patient for this purpose if the clinician concerned is concerned that this might cause any harm. Monocytes will be separated by centrifugation (Ficoll-Hypaque) and attachment to the dish during 8-12 hours culture in medium enriched with serum and antibiotics. Cultures may be maintained in the presence of macrophage colony stimulating factors and other cytokines. At different points in this sequence cells may be stored by freezing and thawing.

Preparation of nuclear donor cells

Nuclear donor cells will be induced to exit the cycle and enter specific stages of the cell cycle, such as G0 or G1 phases. This may be achieved by allowing the cells to reach a high level of confluence or by reducing serum concentration from 10%. Typically quiescence in other cell types is induced at 90% confluence or by 0.5% serum, but effective procedures for these cell populations will be confirmed by preliminary experiments before use as nuclear donors.

Cell Nuclear Replacement

Many factors may influence the development of cell nuclear replacement embryos. These include 1) the cell cycle stage of donor cells, 2) the cell cycle stage of the recipient oocyte, 3) time of enucleation in relation to nuclear transfer, 4) time of oocyte activation in relation to nuclear transfer and 5) donor cell type. Unless other information becomes available in the scientific literature before experiments begin, the first experiments will assess a protocol founded on the current literature and our

own best practice, which in subsequent experiments would be systematically varied. This protocol would involve the removal of the metaphase II spindle before transfer of nuclei from skin-derived fibroblasts that have been induced to become quiescent by serum deprivation. After 3 h, the oocyte would be artificially activated. (See appendix C).

This protocol will be used until cell nuclear replacement has been successful in producing a total of between 30 and 50 embryos. In the light of the practical experience and the results of this first study, subsequent experiments may examine the effect of using a second donor fibroblast cell line, the use of monocytes as nuclear donors, varying the time and sequence between nuclear replacement, enucleation, oocyte activation, or the method of activation. In the first instance, the CNR protocol will rely on our best practice for oocyte activation, optimised in research to generate parthenogenetic human embryos for stem cell derivation (R0136-1b). However, it is possible that this practice would have to be varied further to accommodate the requirements of CNR. Finally, donor cells in mitosis may be used.

The first embryos developing after cell nuclear replacement will be assessed for the normality of their development, as reflected by their capacity to undergo cleavage, compaction, and cavitation. In particular, blastocysts may be examined for the number of cells and the proportion allocated to the inner cell mass, the morphology of cell nuclei and for the occurrence of apoptosis. Rhesus monkey embryos produced by the original Roslin procedures for cell nuclear replacement developed to the blastocyst stage, but a large proportion of the cells were apoptotic. If research in other laboratories identifies the molecular cause of this abnormal development, experiments may be carried out on embryos at earlier stages of development to discover if similar abnormalities occur in human embryos produced by the original procedures.

Embryos will only be used for human embryo stem cell derivation when some embryos derived by cell nuclear replacement have at least half the normal number of cells.

Human embryo stem (hES) cell derivation (HFEA Licence R0136-1b)

We will attempt to isolate hES cells from CNR embryos using the best available practice established in our laboratory. Currently that involves outgrowth of whole embryos on mitotically inactivated human dermal fibroblasts plated on purified human laminin, in medium supplemented with a commercially available serum substitute (Knockout Serum Replacement-KOSR, Invitrogen). This product consisted of fractionated bovine serum, sourced from New Zealand where cattle remain free of Transmissible Spongiform Encephalopathies). To date we have isolated 2 hES cell lines under these conditions, which we have confirmed to be positive for Oct-4, SSEA-4, and Tra-1-60. We have also isolated one hES cell line as above in a completely defined medium free of serum or KOSR, which if repeated could become our best practice in the future.

HES cell culture and evaluation

Newly established CNR hES cell lines will be transitioned to culture conditions that do not require attachment to feeder cells, so that they can be subsequently

manipulated and differentiated more efficiently. A number of feeder-free culture environments are used in our laboratory. These range from reliance on fibroblast conditioned medium (ie. a Dulbecco's Minimum Essential Medium that is supplemented with KOSR and basic Fibroblast Growth Factor and conditioned by either mitotically inactivated mouse fetal fibroblasts or neonatal human dermal fibroblasts), through to a completely defined serum (and KOSR) free medium. These media are used in combination with commercially available substrates for cell attachment such as matrigel (a heterogeneous extracellular matrix sourced from mouse sarcoma tumours) or purified human laminin.

New CNR hES cell lines will also be evaluated qualitatively and quantitatively for the expression of molecular markers defining undifferentiated and differentiated cell status (see (Thomson, et al.,1995); (Thomson, et al.,1998); (Xu, et al.,2001)). This will involve an assessment of morphology, karyotype by G-banding, and expression of undifferentiated cell surface markers over prolonged passage by Fluorescence Activated Cell Sorting, in situ immunocytochemistry, and TAQ-MAN™ Reverse Transcription Polymerase Chain Reaction. FACS and immunocytochemistry markers will include SSEA 4, TRA-1-60, TRA-1-81 and alkaline phosphatase, but not SSEA1. TAQ-MAN markers will include Oct-3/4, a transcription factor expressed by undifferentiated embryo stem cells, and hTERT, the catalytic subunit of telomerase. The capacity of human embryo stem cells to differentiate into tissues representative of all 3 germ layers will be evaluated by *in vitro* differentiation assay.

Tracking and disposal of oocytes, embryos, tissues and established cell lines

At the Roslin Institute, records that accompany shipped oocytes, tissues, or cells will be filed together with forms tracking their usage. Oocyte donation and usage will also be noted in an electronic database stored on a mainframe computer backed up weekly, and stored in a separate building at the Roslin Institute. Access to written and electronic records will be restricted to Roslin Institute staff involved in the research program.

For each embryo produced by cell nuclear replacement there will be a record of the protocols used for cell culture, oocyte source, methods for cell nuclear replacement and embryo culture. Oocytes and embryos stained immunocytochemically would be stored on slides, which would be kept secured as experimental data supporting future publications of the research. Any human embryo stem cell line derived would be preserved indefinitely if possible. As such, records will be kept as to passage number, method and dates of freezing or thawing, and evaluations of karyotype, pluripotency, sterility, and infectious disease status. Human embryo stem cell lines created by the proposed research will be deposited in the national Medical Research Council stem cell bank.

Human embryo stem cell lines derived from the proposed research will be used for research and potentially non-therapeutic commercial purposes, but not transplantation into humans, as stated in patient information and consent forms.

Facilities and Staff

Dr Maureen Wood will supervise storage of oocytes by cryopreservation at the Assisted Conception Unit in Aberdeen before shipment to Roslin Institute. Dr Wood has almost 30 years experience of embryo and oocyte storage by freezing and thawing. At present she is a Research Embryologist in the Department of Obstetrics and Gynaecology, University of Aberdeen, working within the Assisted Conception Unit.

Collection of skin and blood samples from donors will be supervised by Dr C. Shaw at King's College Hospital, London before shipment to Roslin Institute. Dr Shaw is a Senior Lecturer in Neurogenetics and Honorary Consultant Neurologist, in Guy's, King's & St Thomas' School of Medicine, London. Of his 52 research publications approximately half are concerned with Motor Neuron Disease. These range from genetic analyses of the inheritance of the disease, through cell and molecular studies to clinical assessment of new treatments. At present he holds 12 grants for research into different aspects of this disease including analyses of proteins produced in motor neurons of mice carrying SOD mutations. One MRC grant is a collaborative project with Dr Jim McWhir and Professor Ian Wilmut to study Motor Neuron Disease. The specific objectives include the development of methods for the derivation of motor neurons from human embryo stem cells and the introduction of copies of SOD1 carrying mutations known to cause Motor Neuron Disease.

Dr Jim McWhir leads a laboratory at Roslin Institute whose aims are to develop human stem cell therapies. He has over 20 years experience of research on embryo stem cells of mice and more recently human. His laboratory recently developed the first *in vitro* differentiation protocol for human ES-derived osteoblasts. Dr McWhir is a member of the MRC funded collaborative group studying Motor Neuron Disease in cells derived from human embryo stem cells with the group at Guy's, King's & St Thomas' School of Medicine, London. His specific contribution is in the introduction of genetic change and differentiation of neural populations.

Oocyte cryostorage, oocyte and embryo culture, and derivation and cryostorage of human embryo stem cells will be by dedicated staff in a secured purpose built laboratory facility at the Roslin Institute, as described in R0136-1b. The atmosphere in this facility is HEPA filtered and temperature, pressure, and humidity controlled. The facility operates under evolving standards of practice that seek to comply with emerging regulatory requirements. Currently, accreditation from the Medical Healthcare-Products Regulatory Agency (MHRA) as a tissue bank is being sought. While the aim of the current project is to develop Motor Neuron Disease CNR hES cell lines for study, their derivation in a high specification quality assured facility can only maximise the likelihood of success and reproducibility.

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Research Project: Derivation of human embryonic stem cell lines by cell
nuclear transfer for technology development and the
study of motor neuron disease

List of relevant publications submitted as part of the application for a research licence

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