# CELL CULTURE METHOD

The present invention relates to cell culture methods, and in particular to cell culture methods for the production of an essentially homogeneous population of cells (for example neuronal cells) in vitro. The invention also relates to neural cells (e.g. human neural cells) having introduced therein a selectable marker (e.g. a positive and/or negative selectable marker).

The central nervous sytem is presently the subject of intense research, but its enormous complexity at the cellular level has militated against a full understanding of its function. While increasingly selective methods of labelling specific sub-populations of neural cells ex vivo (such as immunostaining, in situ hybridization histochemistry etc.) have been developed, the separation and purification of such sub-populations as living cells presents severe difficulties.

Various approahes have been adopted to address this problem. For example, techniques such as differential centrifugation have been used to enrich for specific neural cell phenotypes. Other methods have been based on the use of fluorescent cell type-specific substrates to label sub-

populations of cells,or the use of fluorescent tracer dyes injected into a specific termination area of a given set of neurones (Schaffner et al. (1987) J. Neuroscience 7:3088). In each case, separation of labelled from unlabelled cells is by fluorescence-activated cell sorting (FACS).

Another method is based upon the identification of extracellular membrane-bound markers specific for a given cell type (Urakami & Chiu (1990) J. Neuroscience 10:620). Here, separation in this example is achieved by panning the mixed cell population on an adherent antibody layer to produce cell-antibody complexes from which the cells of interest can later be dissociated for further study.

According to another known method, progenitor cells which give rise to specific cell populations are immortalized by oncogene transduction or by the sub-culturing of spontaneous cell outgrowths.

With the exception, perhaps, of the precursor cell immortalization technique, most of these methods have not acquired widespread application. This is partly due to the enormous heterogeneity of phenotypes within the central nervous system, such that the markers and the separation techniques utilised have to be extremely specific to avoid

cross-reacting with unwanted cell types.

Secondly, the degree of purification required to obtain homogeneity is often two or more orders of magnitude: such an enrichment is too great for FACS or panning, the result being a degree of contamination.

Thirdly, the availability of sufficiently abundant, yet adequately specific, extracellular markers which are also common to the better characterized neural cell groups (e.g. dopaminergic cells, or glutamatergic cells) is limited.

Moreover, most of these known enrichment techniques require relatively immature neural cells, to prevent their destruction during the procedure.

Finally, these considerations are even more restrictive for human neural cells because of the difficulties in obtaining suitable amounts of fresh human brain tissue.

The present invention provides a method for selectively culturing a pre-selected sub-population of cells from a heterogenous population in vitro, comprising the steps of: (a) introducing a selectable marker (e.g. a positive and/or negative selectable marker) into the heterogenous cell

population, which marker is subject to differential expression/activity in the pre-selected sub-population; and (b) selectively culturing the pre-selected sub-population on the basis of the differential expression/activity therein of the selectable marker.

The pre-selected sub-population of cells may be an essentially homogeneous population of cells of a particular cell type or cell class. For example, the pre-selected sub-population may be a particular class of neural cells. In the case of neural cells, the pre-selected population may be selected on the basis of transmitter characteristics, e.g. dopamine- or acetylcholine-containing neurones may be selectively cultured according to the method of the invention.

The selectable marker need not be introduced into every cell making up the heterogenous cell population: for most purposes it is sufficient if a significant proportion of the cells receive the selectable marker.

Preferably, however, the selectable marker(s) are introduced into a large proportion (for example essentially all) of the heterogenous cell population.

As explained herein, the method of the invention finds particular application in the selective culture of particular classes of essentially normal neural cells. However, the method of the present invention is of general application and may be used to selectively culture other sub-populations of cells.

It is known in the art that mammalian neural cells can be transduced with heterologous genetic material. Many methods exist for transducing eukaryotic and other cells, but the characteristics of neural cells are such that natural methods of transfection are presently the most useful (Miller (1992) Nature 357:455). Thus, transduction with virally-packaged genetic material, for example, is not only more efficient, but also results in a lower neural cell mortality during the actual process than does, for example, calcium phosphate precipitation, electroporation, microprojectile bombardment or microinjection. Transduction of mammalian neural cells from the central nervous system both in vivo (Culver et al. (1992) Science 256:1550) and in vitro (Stringer & Foster (1994) Brain Res 79:267) has been described.

The genetic material that can be introduced into living cells may include both positive and negative selectable

markers. A positive selectable marker is one that permits survival of the transduced cell under conditions which would kill cells not expressing the selectable phenotype. A negative selectable marker confers sensitivity on the cells which express it, such that they are destroyed under conditions which are relatively innocuous to other cells.

A wide variety of selectable markers is available. Genes that are widely applied as positive selectable markers include the bacterial neomycin phosphotransferase (neo; Colbere-Garapin et al. (1981) J. Mol. Biol. 150:1), hygromycin phosphotransferase (hph; Santerre et al. (1984) Gene 30:147) and xanthineguanine phosphoribosyl transferase (gpt; Mulligan & Berg (1981) Proc. Natl. Acad. Sci. USA 78:2072).

Also used as positive selectable markers are the herpes simplex virus type 1 thymidine kinase (HSV-1 TK; Wigler et al. (1977) Cell 11:223), adenine phosphoribosyl transferase (APRT; Wigler et al. (1979) Proc. Natl. Acad. Sci., USA 76:1373) and hypoxanthine phosphoribosyl transferase (HPRT; Jolly et al. (1983) Proc. Natl. Acad. Sci., USA 80:477). These latter markers must be used in cells having a particular mutant genotype (viz. one which leads to a deficiency in the gene product on which the selection is

based).

Preferred negative selectable markers include genes encoding products involved in programmed cell death (apoptosis), for example the gene for p53. Such negative selectable markers may be activated by inducing the expression of the gene in question (for example by use of a tetracycline-responsive promoter, as described infra). The use of genes encoding products involved in apoptosis has the advantage that transient expression (in many cases 30 minutes or less) of the gene may be sufficient to commit the cell to death, permitting reliable and very stringent negative selection.

Some of the aforementioned genes also confer negative as well as positive selectable phenotypes. They include the HSV-1, APRT, HPRT and gpt genes. These genes encode enzymes which can catalyze the conversion of certain nucleoside or purine analogues to cytotoxic intermediates. For example, the nucleoside analogue ganciclovir (GCV) is a good substrate for the HSV-1 thymidine kinase, but a poor substrate for the natural thymidine kinase found in mammalian cells. Consequently, GCV can be used for efficient negative selection against cells expressing the HSV-1 TK gene (St. Clair et al. (1987) Antimicrob. Agents

Chemotherap. 31:844).

Xanthineguanine phosphoribosyl transferase can be used for both positive and negative selection which expressed in wild type cells (Besnard et al. (1987) Mol. Cell Biol. 7:4139). Cytosine deaminase can also be used as a negative selection marker, converting the innocuous 5-fluorocytosine to the cytotoxic 5-fluorouracil (Polak & Scholer (1976) Chemotherapy (Basel) 21:113).

Selectable markers are usually used in both prokaryotic and eukaryotic genetic engineering to permit the recovery from a mixed population of cells those which have undergone a rare genetic change. For instance, they can be used in physical association with another gene which encodes a product of interest to select cells which have taken up that other gene along with the selectable marker. As an example, the neo gene has been used to monitor genetically modified cells taken from patient samples after gene therapy has taken place.

It has also been proposed to use negative selectable markers as a safety device in gene therapy (Lupton et al. (1991) Mol. Cell Biol. 11:3374). Many gene therapies involve the removal of somatic cells from the patient, the

introduction therein of a therapeutic gene (the expression of which repairs a biochemical lesion), followed by reintroduction of the cells into the patient. Since the reintroduced genetically modified cells may ultimately prove deleterious to the health of the patient (for example, if they prove to be immunologically incompatible or become malignant), a negative selectable marker may be introduced along with the therapeutic gene to permit (if necessary) subsequent selective elimination of the genetically modified cells.

A number of vectors bearing positive or negative selectable markers have been made and are readily available to those skilled in the art (for review see Miller (1992) Nature 357:455). Others may be readily assembled using standard gene cloning techniques.

The method of the invention can include the prior induction of replication in mixed populations of embryonic neural cells, using supplements to the culture medium such as epidermal growth factor or fibroblast growth factor, or by prior transfection with immortalizing oncogenes, to elicit such replication. Alternatively, non-expanding cell cultures can be used.

Preferably, at an early stage after plating, the cells are transduced with a positive selectable marker and a negative selectable marker, both linked operably to an expression element. The expression element may be specific for a given central nervous system region, a given neural cell type, or a specific sub-population of neurones. After the cultures have reached the required level of expansion, the cells may be allowed, at least partially, to differentiate. The appropriate drug can then be applied, such that non-transduced cells and those transduced cells without the active specific expression element are eliminated, while transduced cells with the active element (which leads to the expression of the downstream selectable markers) will be resistant.

By way of example only, the expression elements for use in the invention may be selected from: promoters and/or enhancers which are specifically active in: (i) dopaminergic, serotoninergic, GABAergic, cholinergic or peptidergic neurones, or sub-populations thereof; (ii) Schwann cells, oligodendrocytes, astroycytes, microglia and sub-populations thereof; (iii) particular stages of embryogenesis and (iv) other specific non-neural tissues. Particularly preferred for use in the present invention are the tetracycline-responsive promoters described in e.g.

Furth et al: (1994) PNAS USA (Vol. 91, pp. 9302-9306). Such promoters can be used as an element in a tetracyclin-responsive regulatory system to effect temporal and/or spatial control of gene expression in vivo.

Alternatively, they may be selected from promoters and/or enhancers which direct the transcription of genes for: (i) neurotransmitter-specific receptors; (ii) ion channels; (iii) receptors involved in ion-channel gating, (iv) cytokines, growth factors and hormones and (v) any substance that is specifically produced and secreted in a paracrine, autocrine or endocrine fashion. For examples, see Table 1.

The invention provides a method of culturing human and other mammalian cells (e.g. neural cells) and, by selecting for a sub-population of cells on the basis of the genetic material contained within them, producing homogeneous cultures of a single cell type.

Such cultures can be put to a variety of uses including basic electrophysiological, neurochemical and developmental experimentation. In addition, the purified neural cell populations will be useful in more clinically applied studies, such as assessment of the feasibility of

transplantation to alleviate the symptoms of central nervous sytem degenerative disease, and find application in various forms of therapy, prophylaxis and diagnosis.

Such diseases include: (i) Parkinson's disease or parkinsonism, the pre-selected sub-population of cells being dopaminergic neurones of the substantia nigra; (ii) Huntington's disease, the pre-selected sub-population of cells being neural cells of the striatum; (iii) Alzheimer's disease, the pre-selected sub-populations of cells being acetylcholine-, serotonin-, and/or noradrenaline-containing neurones associated with the neo- and palæcortex; or (iv) multiple sclerosis, the pre-selected sub-population of cells being brain oligodendrocytes.

TABLE 1

| Promoter | Tissue/cell type | Application | Reference |
|---|---|---|---|
| Tyrosine hydroxylase | Catecholaminergic neurones | Alzheimer's Parkinson's | Stachowick et al. (1994) Mol Brain Res 22,309-19 |
| TSH receptor | Thyroid cells | Hypothyroidism | Ikuyama & Nakata (1994) Jap J Clin Med 52,962-8 |
| BSF1 | GABAergic neurones | Epilepsy | Motejlek et al. (1994) J Biol Chem 269,15265-73 |
| Human dopamine β-hydroxylase | Noradrenaline neurones | Alzheimer's | Hoyle et al. (1994) J Neurosci 14,2455-63 |
| Thyroglobulin | Thyroid cells | Hypothyroidism | Pichon et al. (1994) Biochem J 298,537-41 |
| Serotonin 2-receptor | Glial cells in seroton-inergic projection areas | Neurodegenerative diseases | Ding et al. (1994) Mol Brain Res 20,181-91 |
| CD4 receptor | CD4 expressing T-lymphocytes | AIDS | Nakayama et al. (1993) Int Immunol 5,817-24 |
| Human choline acetyl transferase | Acetylcholine neurones | Alzheimer's Motoneurone disease | Li et al. (1993) Neuro-chem Res 18,271-5 |

Example 1: Preparation of a homogeneous culture of human dopaminergic neurones

The Herpes simplex virus (HSV) thymidine kinase gene (tk) and the neo gene are operably linked to a promoter which is active only in dopamine-containing neurones, e.g. that controlling expression of tyrosine hydroxylase (see e.g. that described by Harrington et al. (1987) Nucl. Acids Res. 15:2363). The construct is then cloned into the appropriate cloning site of a retroviral vector, and used to transfect an amphotropic retroviral packaging cell line (e.g. ψ-crip (for review see Molecular Virology: A Practical Approach (Eds. AJ Davison & RM Elliott) IRL Press, 1993).

Tissue is dissected from embryonic (approximately 5-8 weeks of gestation) human ventral mesencephalon and grown in dissociated culture. The dopaminergic precursor cells are induced to replicate, by application of fibroblast growth factor (Mayer et al. (1993) Neuroscience 56:389), epidermal growth factor (Reynolds & Weiss (1992) Science 255:1707) or by oncogene transduction (Stringer et al. (1994) Brain Res. 79:267). Shortly after plating, the cultured cells are transduced with the retrovirally-packaged selectable

markers, and the cultures allowed to expand. When sufficient numbers of cells are produced, the cultures are incubated under conditions leading to cessation of neuronal division. The cultures are then treated with geneticin to eliminate non-transduced cells as well as transduced cells not expressing tyrosine hydroxylase, but leaving transduced, tyrosine hydroxylase-containing neurones.

Example 2: Preparation of a homogeneous culture of human oligodendrocytes

The Herpes simplex virus (HSV) thymidine kinase gene (tk) and the neo gene are operably linked to a promoter which is active only in oligodendrocytes, that controlling expression of the oligodendrocyte-specific enzyme galactocerebrosidase. The construct is virally packaged as in Example 1. A virus such as adenovirus could alternatively be used. Tiossue from embryonic or adult brain is dissected and grown in dissociated culture. If necessary, cell replication is induced (for example by using cells from HS2ts6 mice (Noble et al. (1991) WO 91/13150), and shortly after plating, the cells are transduced with the genes coding for the positive selectable marker linked to, for example, the galactocerebrosidase promoter, and for the negative

selectable marker linked to a constitutively active promoter, such as cytomegalovirus. Cell selection is obtained as in Example 1, yielding pure populations of oligodendrocytes.

Example 3: Preparation of a homogeneous culture of essentially normal human dorsal root ganglion cells expressing calcitonin gene related peptide

Neurones from dorsal root ganglia (DRG) can be grown in vivo using either embryonic, neonatal or adult tissue as a source material. The mixed cell population will be grown on, for example, a background layer of e.g. previously prepared neomycin resistant non-neuronal cells to provide trophic support (Brenneman et al. (1987) J. Cell Biol. 104:1603). The DRG cells are transfected using adenoviral technology with a neo gene linked operably to the promoter for calcitonin gene related peptide (CGRP) expression. After several days to allow activation of the promoter and thereby induction of neo expression, DRG cells not actively expressing neo will be destroyed by application of neomycin. The result will be a pure population of differentiated CGRP-positive neurones from the human DRG, for use in vitro.

Example 4: Transduction of neural cells

Primary cells from the foetal (at week 8 of gestation) human cortex, striatum, hypothalamus, mesencephalon, raphe complex, medulla oblongata and ventral horn of the spinal cord were separately plated onto gelatin/poly-L-lysine coated flasks. The medium was a mixture of DMEM/Ham's F12 (1:1) containing penicillin/streptomycin, L-glutamine (2mM) and a modified stock solution (Bottenstein and Sato, PNAS 76(1979)514-517; Romijn et al., J. Neurophysiol. 40(1981)1132-1150) containing basic fibroblast growth factor (5ng/ml).

Once the cells had adhered, retroviral particles comprising a construct (tsA58) incorporating a resistance marker to geneticin ($G418^r$) linked to an SV4OT promoter were added to the medium together wtih 0.8 μg/ml polybrene. After 1h the culture medium was replaced with fresh medium. After 5 days, geneticin was added to the culture medium (0.4 mg/ml) for a further 10 days to eradicate cells which had not incorporated the retroviral vector.

After 15 to 20 days, clusters of human neural precursor cells could be found from all of the areas listed above which were able to replicate in the FGF-containing medium

and which were also geneticin resistant. All exhibited a neuronal phenotype (they were for example neurone specific enolase positive).

Thus, it is possible to transduce stably human neural cells from a variety of areas of the central nervous system with retrovirally packaged gene constructs. Secondly, the construct can include a selection marker such as geneticin resistance.

CLAIMS

1. A method for selectively culturing a pre-selected sub-population of cells from a heterogeneous cell population in vitro, comprising the steps of:

(a) introducing a selectable marker (e.g. a positive and/or negative selectable marker) into the heterogeneous cell population, which marker is subject to differential expression/activity in the pre-selected sub-population; and

(b) selectively culturing the pre-selected sub-population on the basis of the differential expression/activity therein of the selectable marker.

2. The method of claim 1 wherein the selectable marker comprises a negative selectable marker not subject to differential expression/activity in the pre-selected sub-population (e.g. a constitutively-expressed negative selectable marker).

3. The method of claim 1 or claim 2 wherein the cells are neural cells, for example neurones.

This Sheet Has Been Replaced

4. The method according to any one of the preceding claims wherein the heterogeneous cell population is a primary cell culture, for example a human (e.g. foetal) neural primary cell culture, for example comprising neural stem/precursor cells.

5. The method according to any one of the preceding claims wherein in step (b) the pre-selected sub-population is selectively cultured on the basis of:

(i) the expression/activity therein of a positive selectable marker, or

(ii) the lack of expression/ activity therein of a negative selectable marker.

6. The method of any one of the preceding claims wherein step (a) the selectable marker is introduced by retroviral transduction, for example with a recombinant amphotopically packaged retrovirus.

7. The method of any one of the preceding claims wherein the selectable marker is:

(i) a positive selectable marker selected from neomycin phosphotransferase, hygromycin phosphotransferase, xanthineguanine phosphoribosyl transferase, the Herpes simplex

This Sheet Has Been Replaced

virus type 1 thymidine kinase, adenine phosphoribosyltransferase and hypoxanthine phosphoribosyltransferase, and/or

(ii) a negative selectable marker selected from Herpes simplex virus type 1 thymidine kinase, adenine phosphoribosyltransferase, hygromycin phosphotransferase, cytosine deaminase and hypoxanthine phosphoribosyltransferase.

8. The method according to any one of the preceding claims wherein the selectable marker is operably linked to an expression element or elements, for example a regulatable expression element or elements, e.g. a tissue- or cell-specific expression element or elements.

9. The method according to claim 7 wherein the expression element is selected from:

(A) promoters and/or enhancers which are specifically active in: (i) dopaminergic, serotoninergic, GABAergic, cholinergic or peptidergic neurones and sub-populations thereof; (ii) Schwann cells, oligodendrocytes, astrocytes microglia and sub-populations thereof; (iii) particular stages of embryogenesis; (iv) the

This Sheet Has Been Replaced

endocrine glands, lungs, muscles, gonads, intestines, skeletal tissue or part or parts thereof; (v) epithelial, fibroblast, fat, mast mesenchymal or parenchymal cells, and (vi) components of the blood system (e.g. T-lymphocytes, B-lymphocytes and macrophages); or (B) promoters/enhancers which direct the transcription of genes for: (i) neurotransmitter-specific receptors; (ii) ion channels; (iii) receptors involved in ion-channel gating and (iv) cytokines, growth factors and hormones.

10. The method according to any one of the preceding claims wherein step (b) the pre-selected sub-population is selectively cultured by incubating the cells with a selective agent or drug, for example neomycin or geneticin.

11. The method according to any one of the preceding claims wherein the heterogeneous cell population is produced by the preliminary step of <u>in vitro</u> cell culture in the presence of a growth factor (for example epithelial growth factor or fibroblast growth factor) and/or cells providing trophic support.

This Sheet Has Been Replaced

12. The method according to any one of the preceding claims wherein the heterogeneous population of step (a) is expanded and/or induced to differentiate before or after the introduction of a selectable marker.

13. A sub-population of cells produced by the method of any one of the preceding claims.

14. The cells of claim 13, for use in therapy, prophylaxis or diagnosis.

15. Use of the cells of claim 13 for the manufacture of a medicament for use in therapy, prophylaxis or diagnosis.

16. The cells of claim 14 or the use of claim 15 wherein the therapy is transplantation therapy.

17. The cells or use of claim 16 wherein the transplantation therapy is for the treatment of: (i) Parkinson's disease and/or parkinsonism, the pre-selected sub-population of cells being dopaminergic neurones from the substantia nigra; (ii) Huntington's chorea, the pre-selected sub-population of cells being neual cells of the striatum; (iii) Alzheimer's

This Sheet Has Been Replaced

disease, the pre-selected sub-population of cells being acetylcholine-containing, serotonin-containing and/or noradrenaline-containing neurones associated with palaeo- and neocortex; (iv) amyotrophic lateral sclerosis, the pre-selected sub-population of cells being motoneurones from the spinal cord; or (v) multiple sclerosis, the pre-selected sub-population of cells being brain oligodendrocytes.

18. Neural cells (e.g. a human neural cells) having introduced therein one or more selectable markers (e.g. a positive and/or negative selectable marker).

19. Cells according to claim 18 wherein at least one selectable marker is subject to regulation.

20. Cells according to claim 18 or 19 wherein at least one selectable marker is a constitutively expressed negative selectable marker.

21. Cells according to any one of claims 18-20 wherein the selectable marker(s) are introduced by transfection or infection (e.g. with a retroviral vector).

22. Cells according to any one of claims 18-21 for use in

This Sheet Has Been Replaced

therapy (for example transplantation therapy, e.g. as defined in claim 17), prophylaxis or diagnosis.

23. A graft or tissue implant comprising the cells of claim 13 or any one of claims 18-22.

This Sheet Has Been Replaced