NEURAL STEM CELLS

The present invention relates to neural stem cells and to culture conditions and methods of culturing neural stem cells (NS cells or NSCs) in order to promote symmetrical division and self renewal of the stem cells. Compositions, cell populations, cell lines and single neural stem cells are also provided.

While neural stem cells have been purported to be isolated *in vitro* from a variety of sources it has not, to date, been possible to expand the cells in large-scale culture in a symmetrically-dividing, undifferentiated state. The long-term expansion of the cells in this state would be highly desirable, both from an experimental and therapeutic point of view. Having pure neural stem cell populations would enable directed differentiation into the three cell types of neurons, astrocytes and oligodendrocytes.

One known method of culturing neural stem cells, albeit as a minor component in a heterogeneous population of cells, is the neurosphere system. This system involves the serial passaging of aggregates of heterogeneous cells, of which only a tiny fraction are true neural stem cells. The majority of cells in neurospheres are committed progenitors.

There are many reports of the heterogeneity and instability of neurospheres, and their limited capacity to generate neurons (e.g. Morshead et al., 2002). *Rappa et al.* Neuroscience 2003 report a method for transfecting neurosphere cells by plating them on fibronectin for 1-7 days then reforming neurospheres but provides no significant characterization of the stem cells, if any, within neurospheres.

Suslov et al 2002. provide clear data that the neurosphere system is too heterogeneous to enable meaningful characterization of any stem cells within. The major conclusions are that not only is there heterogeneity within neurospheres – "that exhibit intra-clonal neural cell-lineage diversity i.e. they contain, in addition to NSCs, neuronal and glial progenitors in different states of differentiation" - but also

that large differences exist in gene expression profiles between different clonal neurosphere lines. Suslov's gene expression profiling does not constitute a definition of stem cells within neurospheres as explicitly confirmed in the paper:– "the molecular phenotypes that were obtained indicate that clonogenic NSCs in our system are heterogeneous, with subsets reflecting distinct neural developmental commitments".

Much literature in this field describes the properties of glial cells. *Skogh et al.* MCN 2001 describe cultures of glial cells that express GFAP and stain variably for RC2 and nestin – note that mouse GFAP is not expressed in radial glia in vivo (Rakic, 2003). They report that their cells do not express Pax6, the hallmark of neurogenic radial glia (Malatesta et al., 2001, 2003), and they examine no other radial glia markers. They had heterogeneous cultures and carried out no clonal analyses.

Other accounts of radial glia *in vitro* consider them as differentiation intermediates or terminal products. Thus Bibel et al (*Nat Neurosci*, 2004) report the generation of neurons from ES cells via a transient radial glia-like cell, and Gregg and Weiss (*J Neurosci*, 23: 11587-601, 2003; US patent publication 2003/0032181) describe "differentiation" of neurosphere cells into radial glia. Hartfuss et al (*Dev Biol,* 2001) show that radial glia are present in neurospheres.

Gregg and Weiss found that radial glia are differentiation products of neurospheres, concluding that "These results suggest that neural stem cells can give rise to RGCs [radial glia cells] and that RGC-guided migration can be recapitulated in the adult CNS". The prevailing view is that radial glia are one of the differentiated cell types arising from neurospheres.

Liour and Yu (*Glia*, 2003) show that radial glia cell differentiation can be obtained from ES cells, but as with other reports in this field do not report expansion of neurogenic radial glia.

Gobbel *et al.* Brain Res 2003 report propagation of multipotent rat cells "as spherical

clusters of cells that grew loosely attached to the substrate", i.e. not as uniform monolayers. Further, the abstract of this paper concludes "these cells produce post-mitotic cells in approximately two of three divisions, thus making expansion difficult". There is extensive gliogenic differentiation in their cultures, but the authors find "relatively few" (<3%) neurons. They provide no molecular phenotype of their cells.

WO 01/30981 describes cultures of cells that can differentiate into neurons. But these cells are positive for GFAP and nestin, are described as astroglial cells and are a relatively impure, mixed population.

The concept that stem cells require specific cellular microenvironments, or niches, is an orthodoxy in stem cell biology. It is known, though not always acknowledged, that in neurospheres stem cells constitute only a small fraction of the total cells amongst progenitors, blast cells and immature differentiated progeny. This aggregation of mixed cell types may constitute a niche for the small proportion of cells within that may be stem cells.

There are no reports in the literature of deriving homogenous populations of neural stem cells from ES cells, only of deriving neuroepithelial precursor cells that may be expanded transiently before becoming glial-restricted (e.g. Brustle et al., Science 1999).

Hence a number of problems exist in the art.

All reports acknowledge that their cultures are heterogeneous. It has not been possible to use the neurosphere system to maintain large scale cultures of neural stem cells in a symmetrically-dividing and undifferentiated state. Other attempts to culture large numbers of neural stem cells have not succeeded beyond 5-20 passages, and have also been hindered by a high tendency of the cells to differentiate. An exception is work on adult rat hippocampal stem cells, but these are karyotypically abnormal and form neurons at low efficiency.

Whilst transient neural developmental precursors are known, permanent or near-permanent self-renewing stem cells have not been isolated and purified.

It is desired to derive neural stem cells from ES cells, and then maintain these neural stem cells in pure cultures, but this is hitherto not possible. It is also desired to derive neural stem cells from ES cells for transplantation but the persistence of ES and other non-neural cells in known ES-derived cell populations gives rise to tumours in recipient animals. It is further desired to obtain pure neural stem populations from foetal and post-natal CNS.

A complete understanding of the molecular and cellular events controlling the behaviour of neural stem cells is essential, not only as a route for understanding embryogenesis, but also as a framework upon which neural stem cells can be isolated, expanded and controlled for future therapeutic applications. The culturing methods for neural stem cells known in the art are, for reasons described above, unsuitable for use in such investigations and therapeutic applications. It is therefore desirable to develop methods and conditions for culturing large quantities of neural stem cells that allow the cells to be maintained in a symmetrically-dividing, self-renewing state. It is particularly desirable to have defined culture media, which meet the above requirements as the use of defined media is highly desirable in a clinical setting.

The present invention solves one or more of the above-mentioned problems.

In more detail, the present invention provides a method of promoting the symmetrical division of neural stem (NS) cells, comprising culturing said cells in a medium containing:-

(a) an activator of a signalling pathway downstream from a receptor of the EGF family; and

(b) an activator of a signalling pathway downstream from an FGF receptor.

In preferred embodiments, cells are cultured in the absence of serum, e.g. in medium that is free of serum and free of serum extract. It is further preferred that cells are cultured attached to a substrate, otherwise referred to as in an adherent culture. It is also preferred that culture medium contains insulin or another agonist of an insulin receptor on the cells.

In the context of the invention, it will be appreciated that the term "promoting" includes the maintenance of the neural stem cells in a symmetrically-dividing state.

According to another aspect of the present invention there is provided culture media that supports and preferably promotes the self-renewal and symmetrical division of neural stem cells in an undifferentiated state for many passages. The media substantially prevents the asymmetrical division and differentiation of neural stem cells.

The invention further provides methods of obtaining neural stem cells, and specifically as set out in the protocols herein, and additionally provides cells obtained by such methods.

The invention also provides neural cell populations, compositions, cell lines, clonogenic cell lines, and single neural stem cells, which comprise self-renewing, symmetrically-dividing neural stem cells.

Further aspects of the invention provide methods for promoting the differentiation of ES cells to neural stem cells, and methods for maintaining the neural stem cells obtained in a self-renewing, symmetrically-dividing, substantially undifferentiated state.

Definition Section

"Neural stem cells"

The term "neural stem cell", as used in the present specification, describes a cell that is capable of undergoing greater than 20-30 cell divisions whilst maintaining the potency to generate both neurons and glia. Preferably, said cells are capable of undergoing greater than 40, more preferably greater than 50, most preferably unlimited such cell divisions.

Neural stem cells are capable of dividing either symmetrically, or asymmetrically. When dividing symmetrically, the neural stem cell divides to form two daughter neural stem cells or two committed progenitors, though unless otherwise specified symmetrical division refers herein to symmetrical self renewal; when dividing asymmetrically, the neural stem cell divides to form one daughter neural stem cell, and one committed progenitor (e.g. either a neuron or a glial progenitor).

Neural stem cells of the present invention can be described as radial glia, and have been shown to express at least one (and preferably all) of the radial glia markers RC2, 3CB2, GLAST, BLBP and Pax6. Preferably, the neural stem cells of the invention express RC2, 3CB2 and GLAST. More preferably, the cells express RC2, 3CB2, GLAST, and at least one of BLBP or Pax-6. Neural stem cells of the invention can also be characterized in that they are positive for the expression of at least one (preferably all) of the neural precursor markers nestin or vimentin, the LewisX antigen, Musashi-1 or prominin, and negative for the expression of at least one (preferably both) of Oct-4 or Nanog (see also Example 1-3).

The neural stem cells of the invention are by definition multipotent, i.e. they are capable of differentiating into a number of neural cell types (e.g. neurons/glia). Examples below confirms that neural stem cells cultured according to the methods of the invention retain their potency and are able to differentiate into all expected cell types.

"Sources of neural stem cells"

It is possible to derive neural stem cells of the invention from a wide variety of sources. For example, neural stem cells can be derived directly from embryos, from adult tissue, from foetal tissue, or from embryonic stem (ES) cells (either wild-type or genetically modified ES cells). Preferably, the neural stem cells of the invention are derived from mouse or human ES cells, or are derived from mouse or human foetal cells.

Neural stem cells of the invention can be derived from, *inter alia*, humans, primates, rodents, and birds. Preferably, the neural stem cells are derived from mammals, especially mice, rats and humans.

"EGF Receptor family"

The term "EGF receptor family", as used in the present specification, describes a family of receptors (usually homodimeric or heterodimeric receptors) which can be activated by a family of EGF signalling factors. The receptors are made up of a family of four highly homologous transmembrane glycoproteins: ErbB-1 (also known as EGF-R), ErbB-2, ErbB-3 and ErbB-4.

Reference to an "EGF receptor" includes any monomeric or dimeric receptor complex of the EGF receptor family.

Each receptor has an extracellular ligand binding domain, a single hydrophobic membrane-spanning domain and a cytoplasmic tyrosine kinase domain that is responsible for signal transduction through Type-1 receptor tyrosine kinase activity. Ligand binding to any of the receptors results in receptor dimerisation and autophosphorylation, followed by phosphorylation of a number of cellular substrates leading to an array of biological effects. The receptor dimerisation may be triggered by various stimuli, including receptor ligands and toxic environmental stimuli such as UV radiation. Each dimeric receptor initiates a distinct signalling pathway by recruiting different SH2-containing effector proteins. For example, the EGF-R dimer can complex with the adaptor protein Grb, coupled to a guanine nucleotide releasing

factor, SOS. The Grb-SOS complex can either bind directly to phosphotyrosine sites in the receptor, or indirectly through Shc. These protein interactions bring SOS in close proximity to Ras, allowing for Ras activation. This subsequently activates the ERK and JNK signalling pathway that in turn activate transcription factors, such as c-fos, AP-1 and Elk-1, that regulate gene expression. The EGF receptors can also activate the PLCγ signalling pathway.

"FGF receptor"

The term "FGF receptor", as used in the present specification, describes any member of the family of transmembrane FGF receptor tyrosine kinases. There are four main isotypes of these receptors, FGFR1, 2, 3 and 4, and they are known to act in close association with the heparin and heparan sulphate (HS) systems. Reference to an FGF receptor includes any monomeric or dimeric (homo or heterodimer) complex of the FGF receptor family.

Cellular signalling pathways associated with FGF receptors include the MAP kinase pathway, and the PLCγ pathway.

" Culture Media"

Culture media used in the present invention preferably comprise a basal medium, optionally supplemented with additional components.

Basal medium is a medium that supplies essential sources of carbon and/or vitamins and/or minerals for the neural stem cells. The basal medium is generally free of protein and incapable on its own of supporting self-renewal/symmetrical division of neural stem cells.

Preferably, culture media used in the invention do not contain any components which are undefined (e.g. serum and/or feeder cells), that is to say components whose content is unknown or which may contain undefined or varying factors that are

unspecified. An advantage of using fully defined media, free of serum and free of serum extracts, is that efficient and consistent protocols for culture and subsequent manipulation of neural stem cells can be derived.

"Culture Surfaces"

Typical surfaces for culture of the neural stem cells in all aspects of the invention are culture surfaces recognized in this field as useful for cell culture, and these include surfaces of plastics, metal, composites, though commonly a surface such as a plastic tissue culture plate, widely commercially available, is used. Such plates are often a few centimetres in diameter. For scale up, this type of plate can be used at much larger diameters and many repeat plate units used.

The culture surface may further comprise a cell adhesion protein, usually coated onto the surface. Receptors or other molecules present on the stem cells bind to the protein or other cell culture substrate and this promotes adhesion to the surface and promotes growth. Gelatin coated plates are particularly preferred.

The present invention is based on the observation that culturing neural stem cells attached to substrates in media comprising agonists of the EGF receptor, or agonists of both the EGF and FGF-2 receptors, promotes the unlimited, symmetrical division of the stem cells, and substantially prevents their differentiation into neurons/glia.

The various aspects of the invention are now discussed in detail.

A first aspect of the invention provides a method of promoting the symmetrical division of neural stem (NS) cells, comprising culturing said cells in a medium containing:-

(a) an activator of a signalling pathway downstream from a receptor of the EGF family; and

(b) an activator of a signalling pathway downstream from an FGF receptor.

Another method of the invention, for obtaining neural stem cells comprises:-
(1) obtaining a mixed population of cells containing a neural stem cell;
(2) replating the cells in medium comprising (a) an activator of a signalling pathway downstream from a receptor of the EGF family; and (b) an activator of a signalling pathway downstream from an FGF receptor;
(3) culturing the cells;
(4) harvesting aggregates of cells;
(5) replating the cells in medium comprising (a) an activator of a signalling pathway downstream from a receptor of the EGF family; and (b) an activator of a signalling pathway downstream from an FGF receptor;
(6) culturing the cells;
(7) replating cells as single cells in medium containing (a) an activator of a signalling pathway downstream from a receptor of the EGF family; and (b) an activator of a signalling pathway downstream from an FGF receptor.

The method can be supplemented by selecting for cells that express a neural stem cell specific marker, for example by linking expression of such a marker to a promoter preferentially active in neural stem cells compared to its expression in differentiated progeny thereof. Preferably the method comprises passaging the cells at or below 65% confluence, more preferably at or below 55% confluence, especially below 50% confluence.

Other preferred features of methods of the invention are as set out in the protocols of the invention in Example 9.

The cultures of the invention are preferably adherent cultures, i.e. the cells are attached to a substrate.

The substrate is typically a surface in a culture vessel or another physical support,

e.g. a culture dish, a flask, a bead or other carrier. Preferably, the substrate is coated to improve adhesion of the cells and suitable coatings include laminin, poly-lysine, poly-ornithine and gelatin. It is also preferred that the cells are grown in a monolayer culture and not in suspension and not as balls or clusters of cells. At higher densities, cells may begin to pile up on each other, but the cultures are essentially monolayers or begin as monolayers, attached to the substrate.

One or more signaling pathways downstream from a receptor of the EGF family can preferably be activated using an agonist of a receptor of the EGF family. An agonist of a receptor of the EGF family is suitably a member of the EGF family of signaling factors, and preferably binds to the extra cellular domain of the EGF receptor. The term "agonist" also embraces mimetics, fusion proteins, antibodies to or chimeras of the EGF family of signalling factors, and fragments, variants and derivatives thereof, capable of activating receptors of the EGF family.

The molecules that make up the EGF family of signalling factors can be characterized in that they contain at least one EGF-like domain. This domain can be defined by 6 cysteine residues that generate three peptide loops through the formation of disulphide bonds.

Specific agonists capable of acting through receptors of the EGF receptor family, and thus of activating pathways downstream of these receptors, include EGF, TGF-α, amphiregulin, heparin binding-EGF, epiregulin, betacellulin, neuregulins 1-4, and Cripto-1. Preferably, the agonist is EGF itself.

One or more signalling pathways downstream from a receptor of FGF can preferably be activated using an agonist of a receptor of FGF. An agonist of a receptor of FGF is suitably a member of the FGF family of signalling factors. The term "agonist" is also intended to embrace mimetics, fusion proteins, antibodies to or chimeras of the FGF family of signalling factors, and fragments, variants and derivatives thereof, capable of activating receptors of FGF.

Preferably, the agonist of the FGF receptor is FGF-2, or laminin-FGF.

It will be appreciated that activation of a signalling pathway downstream of a receptor of either the EGF receptor family or of an FGF receptor can also be effected by constitutively active receptors, or by downstream effectors (e.g. MEK or Bcl2) of the respective signal transduction pathways. In a particularly preferred embodiment of the invention, the signalling pathways are activated by cell-permeable small molecules, which can bypass the respective receptors and activate the signalling pathways directly. Thus, in the present invention, the term "activator" embraces all molecules capable of activating a signalling pathway downstream of receptors of the EGF family of receptors, or of an FGF receptor.

The effectiveness of the activators in the maintenance of neural stem cell cultures of the present invention is demonstrated in Examples 1-1 and 1-2 below. Here, bulk and clonal populations of neural stem cells are maintained in media comprising EGF and FGF-2 and may be passaged many times. Moreover, there is negligible/no differentiation of the neural stem cells, as is shown by the ubiquitous presence of the neural stem cell markers in the cell populations tested (see Example 1-3), and they retain their potency and are able to differentiate into all expected cell types (see Example 1-4).

Thus, the invention provides an efficient method of maintaining large populations of neural stem cells, in a self-renewing, symmetrically-dividing, undifferentiated state. Compositions of the invention include compositions comprising neural stem cells, wherein the neural stem cells are in an adherent culture and at least 50%, 70% or 80% of the cells in the composition are neural stem cells. The proportion of neural stem cells is further preferably at least 90%, more preferably at least 95%, very preferably at least 97%. Further, neural stem cells of the invention can be passaged extensively. It is preferred that the neural stem cells have been passaged at least 30 times, more preferably at least 60 times, very preferably at least 90 times. Still further, in a population of neural cells of the invention at least 80%, preferably at least 90%, more preferably at least 95%, of said cells are symmetrically-dividing

neural stem cells. The cells in this compostion can be further characterized by any and all features of the invention, alone or in combination

Also provided are compositions, which comprise:-

neural stem cells;

an activator of a signalling pathway downstream from a receptor of the EGF family; and

an activator of a signalling pathway downstream from an FGF receptor.

The compositions preferably comprise a basal medium. It is also preferable that at least 80%, preferably 90%, more preferably 95% of the neural stem cells in the composition are symmetrically-dividing neural stem cells.

In both the neural cell populations and compositions described above, the neural stem cells are preferably free from exogenous genetic material encoding an oncogene, i.e. that they have not been subjected to immortalisation strategies. In specific embodiments, the neural stem cells of the populations/compositions are characterized in that they are positive for the expression of at least one of:-

the neural precursor markers nestin or vimentin;

Sox-2;

the radial glia cell specific markers RC2, 3CB2, GLAST, BLBP or Pax-6;

the LewisX antigen;

Musashi-1; and

Prominin;

and are negative for the expression of at least one of:-

Oct4, and

Nanog.

Preferably, the cells are positive for the expression of RC2, 3CB2 and GLAST. In other embodiments, (optionally in addition to the preceding marker profile) the cells are positive for the expression of at least one of BLBP, Pax-6, the neural precursor markers nestin or vimentin, the LewisX antigen, Musashi-1 or prominin. In particularly preferred embodiments (and optionally in addition to the preceding marker profiles) the cells are negative for the expression of at least one of Oct4 or Nanog.

In further embodiments (optionally in addition to the above marker profiles), the cells are positive for the expression of Sox-2, and negative for the expression of Sox-1. In certain compositions/populations comprising mouse-derived NS cells, no more than 1% of the cells are positive for the expression of GFAP or βIII tubulin, thereby confirming that there is negligible differentiation of the cells to astrocytes or neurons. In other compositions/populations, no more than 1% of the cells are positive for the expression of markers for mature astrocytes, neurons or oligodendrocytes.

The invention has also been carried out with rat cells. Thus, we have taken cells from the rat CNS and using the methods of the invention obtained a culture of rat neural stem cells having the properties of (i) high purity, typically in excess of 80% or 90% rat neural stem cells, and (ii) being cells a high proportion (a minimum of 50%) of which after many doublings, in excess of 50, in excess of 100, even in excess of 200 doublings, retained the ability to form neurons and glia. In the art cells from the rat were clearly highly heterogenous and only from the adult hippocampus. We have not obtained cells from this source, but instead from rat CNS. It is notable in this respect

that the method of the invention has worked across all three species – rat, mouse, human. It is generally seen that if a single cell will form a neural stem cell colony following the methods of the invention then neurons can be obtained from cells in that colony. This is measured for example by staining separately for (i) nuclei (i.e. staining all cells) and (ii) neurons (i.e. only neurons). By comparing the relative numbers of stained cells the proportion of cells that form neurons can be calculated.

A second aspect of the invention provides a method for the preparation of a neural stem cell, comprising (i) culturing neural stem cells according to the methods described above, thereby obtaining a culture of neural stem cells, and (ii) isolating a neural stem cell from said culture. Preferably, the isolated neural stem cell is one that has been conditioned to divide in a symmetrical manner.

A number of sources of cells can be used from which to derive neural stem cells. In one method, cells are obtained from nervous tissue of an animal and cultured according to the invention, to obtain a culture of symmetrically dividing neural stem cells. The nervous tissue can be from adult or foetal tissue. The nervous tissue can be from CNS, and symmetrically dividing populations of neural stem cells can be obtained from extracts taken from both adult and foetal CNS, from both human and mouse. The nervous tissue preferably comprises cells identified as having a radial glial phenotype, for example cells having this phenotype have been identified in extracts from the cerebellum and retina, both of which represent suitable further sources of cells. Neural stem cells can be obtained from diseased nervous tissue, useful for modeling disease, e.g. cells can be obtained from brain tumours for this purpose.

An option is to use the invention to derive neural stem cells from a diseased individual then (i) use those cells for assays, or (ii) carry out genetic modification prior to transplantation of cells back into that individual. Cells may thus be obtained from an individual with a neurodegenerative disorder, examples including Alzheimer's and Parkinson's diseases; or with a brain tumour.

Another method of obtaining a neural stem cell comprises:
(i) obtaining a multipotent or pluripotent stem cell capable of differentiating into a neural stem cell,
(ii) culturing the cell of (i) in medium which is non-permissive for pluripotent cells and in the presence of (a) an agonist of a signalling pathway downstream of a receptor of the EGF family and (b) an agonist of a signalling pathway downstream of a receptor of the FGF family.

In use, potentially contaminating ES cells are removed by this method, or at least substantially reduced in number, giving a purer culture and one less likely to give rise on transplantation to teratomas.

The stem cell is suitably a pluripotent stem cell, especially an ES or EG cell. The method preferably comprises culturing the cell in the presence of an agonist of the EGF receptor and an agonist of the FGF receptor.

Using NS cells of the invention, we have obtained astrocytes by removing EGF and FGF and adding serum or BMP4, we have obtained neurons by taking away EGF, then taking away FGF after an interval of about a week and plating the cells on laminin and we have obtained oligodendrocytes by taking away both EGF and FGF. Generally, all methods of the invention used to generate a neural stem cell optionally comprise a further step of differentiating the neural stem cell into a neuron, an astrocyte or an oligodendrocyte, and then further optionally using the neuron, astrocyte or oligodendrocyte e.g. in an assay, for transplantation or otherwise.

Once a neural stem cell culture has been isolated, it can then be used to establish a neural stem cell line. The establishment of such a cell line preferably includes the steps of:-

(a) obtaining a single neural stem cell,

(b) culturing the neural stem cell according to any of the methods

described previously,

and thereby obtaining a clonal population of neural stem cells.

In specific embodiments, the single neural stem cell used to establish the cell line is a symmetrically-dividing neural stem cell, or a neural stem cell that has been conditioned to divide in a symmetrical manner. Preferably, the single neural stem cell is obtained according to the method described above for preparing a neural stem cell.

In preferred embodiments, the neural stem cell line obtained by the above method is a clonal population of neural stem cells, i.e. wherein all the cells are progeny of a single neural stem cell.

Single neural stem cells and cell lines (optionally obtainable by the methods described above) also form part of the invention. In a first embodiment, cell lines are provided wherein the cells are maintained in the presence of an activator of a signalling pathway downstream of a receptor of the EGF receptor family, and an activator of a signalling pathway downstream of an FGF receptor. In other embodiments, the single neural stem cells and the cells of the cell lines are characterized in that they are positive for the expression of at least one of:-

the neural precursor markers nestin or vimentin;

Sox-2;

the radial cell specific markers RC2, 3CB2, GLAST, BLBP or Pax-6;

the LewisX antigen;

Musashi-1; and

Prominin;

and they are negative for the expression of at least one of:-

Oct4, and

Nanog.

Preferably, the cells are positive for the expression of RC2, 3CB2 and GLAST. In other embodiments, (optionally in addition to the preceding marker profile) the cells are positive for the expression of at least one of BLBP, Pax-6, the neural precursor markers nestin or vimentin, the LewisX antigen, Musashi-1 or prominin. In particularly preferred embodiments, (and optionally in addition to the preceding marker profiles) the cells are negative for the expression of at least one of Oct4 or Nanog.

In other specific embodiments (optionally in addition to the above marker profiles), the cells are positive for the expression of Sox-2, and negative for the expression of Sox-1. In certain mouse-derived cell lines, no more than 1% of the cells are positive for the expression of GFAP or β III tubulin. In other (e.g. human) cell lines, no more than 1% of the cells are positive for the expression of markers for mature astrocytes, neurons or oligodendrocytes.

It is preferable that both the single neural stem cells, and the cells of the cell lines, are free from exogenous genetic material encoding an oncogene, i.e. that they have not been subjected to immortalisation strategies. It is also preferable that the cell lines described are neural stem cell lines.

In a third aspect, the invention provides a method of obtaining and maintaining a transfected population of symmetrically-dividing neural stem cells, comprising:-

(a) transfecting ES cells with a construct encoding a selectable marker A, wherein in use said selectable marker is expressed under the control of a

neural precursor cell-specific promoter;

(b) promoting the differentiation of the ES cells into neural precursor cells;

(c) selecting for neural precursor cells that express the selectable marker A; and

(d) culturing the selected cells according to any of the methods previously described.

The selectable marker A may encode antibiotic resistance, a cell surface marker or another selectable marker as described e.g. in EP-A-0695351. The neural precursor cell-specific promoter may be selected from the group consisting of Sox-1, Sox-2, Sox-3, BLBP and nestin neural enhancer. Further details of this selection strategy are provided in Example 1-1.

The neural stem cells of the invention (i.e. single neural stem cells, and neural stem cells present in the compositions, cell lines and populations of the invention) can be genetically modified . There is therefore provided a method of transfecting the neural stem cells of the invention comprising:-

(a) transfecting the neural stem cells with a construct encoding a selectable marker B and a polypeptide; and

(b) selecting for neural stem cells that express the selectable marker B.

The selectable marker B may encode antibiotic resistance or a cell surface marker, and may be the same as or different from selectable marker A. Suitable transfection methods are known ones, including electroporation, lipofection, necleofection and retro-and lenti-viral transfection.

The genetically modified neural stem cells also form part of the invention, and the

invention therefore provides a single neural stem cell, or neural stem cells as present in the compositions, populations or cell lines of the invention, further comprising the construct. It will be appreciated that such neural stem cells may already comprise selectable marker A, in addition to selectable marker B.

As mentioned previously, the methods of the invention are suitable for use with neural stem cells derived from any source. In one particular embodiment, the invention provides a method for obtaining neural stem cells from a source of ES cells. According to the method, differentiation of ES cells into neural stem cells is promoted by converting ES cells into neural progenitors, e.g. by culture in a monolayer or embryoid body differentiation, and then culture of neural progenitors in NSA medium. In addition, it is contemplated that the NSA medium may comprise supplemental components, such as supplemental glucose and HEPES. As an alternative, media (preferably basal media) supplemented with glucose and HEPES can also be used to promote this differentiation.

In the presence of NSA and/or glucose and HEPES, the conditions are such that the propagation of neural stem cells is favoured, with the added advantage that any non-neural cells present in the culture preferentially die. This results in a substantially pure culture of neural stem cells (e.g. at least 80%, preferably 90%, more preferably 95% of all cells present). Example 1-5 provides further details of this method.

In a preferred embodiment, the method of Example 1-5 may be used to prepare a population of symmetrically-dividing neural stem cells, which are subsequently maintained using any of the culturing methods of the invention described above.

The method of Example 1-5 can also be used to assay for the effect of factors on the differentiation of ES cells into neural stem cells. In a preferred assay embodiment, ES cells are cultured using the method described in Example 1-5, in the presence of the factor to be tested. The effect of the factor can be assessed by, for example, determining the marker profile of the resultant cells, i.e. to show whether the cells have a similar marker profile to the cells of the invention, or whether the ES marker

profile is maintained. The factors tested may be either inductive or blocking factors.

There is much interest in the use of neural stem cells in the treatment of neurological and neurodegenerative diseases, and brain injuries; in particular in the treatment of diseases such as Parkinson's and Alzheimer's disease, multiple sclerosis and amyotrophic lateral sclerosis. The methods, compositions, cell populations, cell lines and single cells of the present invention are all capable of being used in such treatment, as well as in the manufacture of preparations for such treatment. Particular neurological/neurodegenerative diseases that can be treated using the invention include:- Parkinson's disease, motor neuron disease, stroke, multiple sclerosis, and Huntington's disease.

In a fifth aspect, therefore, the invention provides for the use of the cell lines, neural cell populations, single neural cells and compositions described above for cell therapy and for the manufacture of a preparation for the treatment of neurodegenerative diseases and brain injuries. Such preparations may be formulated in phosphate buffered saline (PBS).

Methods of treatment of the diseases listed above can comprise the transplantation of single cells, cell lines, compositions, or cell populations of the invention into a patient. Preferably, the patient is mammalian, more preferably the patient is human. Such transplantation has been shown to be successful in both embryonic and adult CNS and is described in more detail in Example 1-6.

The cells of the invention, and in particular the cell lines, can be used to assay the effect of inductive or blocking factors on the differentiation of neural stem cells. Such an assay may comprise contacting a neural stem cell of the invention (i.e. as present in the compositions, cell lines, and populations, or a single neural stem cell) with the factor to be tested. The effect of the factor on the differentiation of the neural stem cell can be suitably assessed by determining the marker profile of the resultant cells, i.e. to show whether the cells have a similar marker profile to the cells of the invention, or whether these markers have been lost. The cells of the invention are

also suitable for assaying pharmaceuticals.

To assess the proportion of cells that will form neurons or glia, cells are propagated clonally; cells are plated individually and not all cells form clonal colonies but of those which do generally in excess of 50% will make neurons (under the appropriate protocol) or glia (again, under the appropriate protocol). The cells are distinguished from the art as while the art alleges identification of and even propagation of cells which have neural stem cell properties the populations were highly heterogenous. This is an important distinction as impure populations include cells which provide signalling to remaining neural stem cells which stimulates differentiation and further reduction of the proportion of neural stem cells. While the prior art methods can generate neurospheres from individual cells the invention enables generation of substantially pure populations from individual cells.

In particular embodiments of the invention, described in more detail in an example below, all NS cell colonies produce 15% or greater, preferably at least 20%, more preferably about 20 to 30% TuJ positive neurons. In higher density cultures we have counted TuJ positive cells. 35% of LC1 NS cells acquired neuronal morphology and expressed beta III-tubulin after 115 passages, longer than one year of continuous culture. These cells retained a diploid karyotype at this stage. LC1 is an uncloned population. Therefore the data indicate that there was minimal selective pressure for glial-restricted precursors or for genetic transformation.

Cells of the invention can be grown without co-culture with heterologous cells and without undefined conditioned media, extracellular matrix fractions or serum, a feature not previously shown for any stem cell type other than ES cells. Hence following the invention, neural stem cells are preferably grown in homogenous culture in defined conditions. Even a single NSC isolated in a microwell can expand, indicating minimal dependence on extracellular signals other than EGF and FGF and that essential extrinsic self-renewal signals for the NSCs can be reduced to EGF and FGF in vitro.

In further embodiments illustrated in an example in more detail, substantially every colony, that is at least 90%, preferably at least 95%, more preferably at least 97% of colonies that develop from plating single cells (i) show identical homogenous expression of neural precursor markers and absence of differentiation markers in FGF plus EGF, and (ii) generate neurons on growth factor withdrawal. In a specific example, every colony had these properties. This is evidence for symmetrical self-renewal. Furthermore, data from colony assays on non-cloned NS cell cultures were comparable with data from the clonal NS5 line. Serial formation of expandable undifferentiated colonies from NS5 shows that the clonogenic cells are stem cells and that their numbers expand in proportion with the NS population. Formally an increase in stem cell numbers can occur via two mechanisms: de novo generation or symmetrical self-renewal divisions. The former requires a pre-stem cell source i.e. a pluripotent cell or a foetal anlage, neither of which are present in the NS cell cultures herein, so symmetrical self-renewal must be ongoing in the NS cell cultures.

Specific NS cells, obtained in an example below, express Sox2, Pax6, Emx2, Olig1, Olig2, Nestin, BLBP, GLAST, Vimentin, and are immunoreactive for RC2, 3CB2, SSEA-1, and Prominin. Preferably they do not express Sox1 and are negative for GFAP and neuronal antigens. These NS cells lack pluripotency markers and markers of other germ layers. We contend that neural stem cell character is masked during CNS development and only truly demonstrable *ex vivo*, as is the case with ES cells. The finding that Sox1 expression was not maintained in the NS cells is new, unpredicted, and indicates that continuous lineage selection using Sox1 would not be productive.

In further embodiments of the invention NS cells, exemplified by ES cell-derived uncloned (CGR8-NS) and cloned (NS5) NS lines and foetal cortex-derived uncloned (Cor1) and cloned (Cor1-3) NS lines, show expression of radial glia markers 3CB2, BLBP, GLAST, Nestin, RC2 and Vimentin in culture. Expression of GFAP is preferably in fewer than 10%, more preferably fewer than 5% especially in fewer than 2% of cells in any of these cultures. In a specific example GFAP expression was seen in fewer than 1% of cells. We have also shown uniform expression of nestin

and RC2 with absence of GFAP and βIII-tubulin in ES cell derived NS cells after 115 passages indicating that the NS cell phenotype is stable. Preferred cells of the invention are differentiated from ES cells and (1) continue to express nestin and RC2 and (2) continue not to express GFAP and βIII-tubulin after 30 passages, preferably after 60 passages, more preferably after 100 passages. Non-expression of GFAP and beta III-tubulin means expression is seen in fewer than 5% of cells, preferably fewer than 2% of cells.

We have carried out analysis via RT-PCR which confirmed absence of pluripotent, mesodermal, or endodermal specific transcription factors in specific NS cells. We have also carried out Affymetrix (RTM) expression profiling that confirms the absence of lineage inappropriate transcripts and demonstrates consistent expression of neural and radial glia markers in three different NS cell cultures (ES derived, foetal cortex-derived, and clonal foetal cortex-derived).

Obtaining NS cells by differentiation from ES cells preferably comprises maintaining the cells in continuous adherent culture, and more preferably omits a step of forming a neurosphere. However, obtaining NS cells by differentiation of cells from primary cell isolates from foetal or adult brain optionally comprises (i) first forming a suspension aggregate or a neurosphere, and (ii) subsequently maintaining the cells in adherent culture. We have found in examples that after a few days the aggregates can be attached to gelatin-coated plastic and NS cells will then grow out.

We determined the proportion of neurons and astrocytes generated at early and late passages of cells of the invention, specifically for the LC1 NS cells at passages 8 and 115. The latter culture represents 12 months of expansion with a doubling period of 24 hours. There is a modest decline in the number of neurons obtained at late passage, but this still totals >35%. The NS5 clonal line, independently derived from 46C ES cells, shows a similar level of neuronal differentiation efficiency at passage 30. Generation of GFAP immunoreactive astrocytes approached 100% at both time points for the LC1 NS cells and also for NS5 cells. Furthermore, the *in vivo* data, which show extensive differentiation of both neurons and astrocytes, were obtained

using LC1 NS cells grown for multiple passages, then transduced with GFP lentivirus and expanded further before transplantation. In combination with the uniformity of radial glia marker expression, the data on stable differentiation potential for the uncloned LC1 NS cells indicate that adherent culture in FGF and EGF favours stem cell self-renewal and suppresses commitment to either glial or neuronal fates.

We found NS cells, obtained by differentiation from ES cells, could be transplanted without formation of tumours over a period of 5 weeks, thus being distinct from ES cells which would give rise to macroscopic teratomas within 4 weeks in the mouse brain.

The present invention also relates to nuclear reprogramming methods and to cells and animals obtained via those methods.

Nuclear reprogramming is a technique by which the nucleus of a somatic cell, optionally a stem cell or a terminally differentiated cell, is reprogrammed so as to behave as a nucleus of a cell of relatively higher potency; ultimately a completely reprogrammed nucleus acts as the nucleus of a pluripotent cell and reprogramming is often taken to mean reprogramming all the way to pluripotency. Methods of reprogramming by nuclear transfer are well established in the art and became well publicized after the invention described in WO 96/07732, sometime referred to as the "Dolly the sheep" invention. Nuclear transfer can thus be used to clone non-human animals.

Nuclear transfer methods have a number of problems. Primarily they remain of low efficiency, in that the cells obtained are rarely reprogrammed so as to be truly pluripotent. It is also desirable to be able to carry out genetic manipulation on the nucleus as part of the reprogramming. But, at present this manipulation is not possible or is unreliable due to the difficulty in obtaining clonal populations of donor nuclei. Even with clonal nuclei the reprogramming methods are complex procedures that require many individual steps. Lastly, ES cells from some species remain difficult to isolate. Using reprogramming techniques, if such were available and reliable, may

offer an alternative route to pluripotent cells in these species.

Further aspects of the present invention have as an object to provide alternative approaches to the above problems and to provide solutions thereto.

Accordingly, the invention provides a method of nuclear reprogramming, wherein the donor nucleus is obtained from a neural stem cell of the invention.

Neural stem cells of the invention have been found to be reprogrammable at high efficiency, and hence the invention provides an efficient reprogramming method and also facilitates production of genetically modified reprogrammed cells, especially pluripotent stem cell, of many species. The ability, via the invention, to propagate individual neural stem cells clonally means that after genetic manipulation clonal populations of cells can be obtained, all with the same genetic modification, and used in reprogramming methods.

It is also possible to identify neural stem cells according to the presence of specific cell surface markers and/or the absence of others – this has the advantage that the step of culturing according to aspects of the invention described herein can be omitted, as the neural stem cell can be picked directly from a mixed population, e.g. a brain homogenate.

A method of nuclear reprogramming is hence provided herein, wherein the donor nucleus is from a neural stem cell obtained according to any embodiment or aspect of the invention.

A particular method of nuclear reprogramming, comprises:-

- obtaining a donor cell;
- obtaining a recipient cell;
- transferring the nucleus of the donor cell into the recipient cell, wherein the donor cell is (i) a neural stem cell, or (ii) a cell obtained according to a method of the invention; and

- culturing the cell so as to reprogram the donor cell nucleus, thereby obtaining a reprogrammed cell.

The nucleus of the recipient cell will generally be removed at one stage in the process so the resulting cell is diploid, this is done optionally prior to transfer of the donor cell nucleus and optionally after transfer of the donor cell nucleus.

One of the interesting possibilities offered by NS cells of the invention is to introduce genetic lesions e.g. that may induce malignancy. A further option is thus to genetically manipulate the donor cell nucleus. This can be used to introduce mutations or genes of interest, e.g. to generate cells or animals for assays or other test purposes wherein a plurality of cells are obtained all with the same manipulation. The range of manipulation is wide. One example is to introduce a disease-causing genetic sequence or a putative disease-causing genetic sequence into the donor cell nucleus, useful for drug screening. A further option is to obtain an animal comprising tissue derived from the reprogrammed cell and carry out an assay using the tissue. It is preferred that the cell is reprogrammed back to pluripotency, e.g. by nuclear transfer into an oocyte.

In particular embodiments of the invention, taking advantage of the knowledge from the invention of cell surface makers in the desired neural stem cells allows omission of cultures steps otherwise needed to isolate a neural stem cell from a mixed population. One such reprogramming method comprises providing a mixed population of cells, isolating a neural stem cell from the mixed population based upon its cell surface marker profile, and transferring the nucleus of the isolated cell to a recipient cell.

As with the other methods, the isolated cell can be genetically manipulated prior to transferring its nucleus to the recipient cell. Also, the isolated cell can be cultured to obtain a clonal population of cells prior to transfer of the nucleus of one cell in the population to the recipient cell.

In more detail, an application of the cells of the invention is in high throughput drug screening. Both neural stem cells of the invention and neurons, glia etc obtained therefrom can be used for the screening, e.g. to identify factors active on either type of cell. The cells or progeny can be used in models of brain cancer. Cells and progeny can be obtained from a tumour of the nervous system, especially the CNS and the neural cells derived therefrom and their progeny used in screens: the nature of the screen is believed to be apparent to all, but in outline comprises obtaining a cell of the invention or a differentiated progeny thereof, culturing the cell or progeny in the presence of a test factor and determining an effect of the factor on the cell or progeny. In a particular screening use, a cell is provided, which is a cell of the invention or obtained by the invention and which has been modified so as to express an EGF receptor or an additional EGF receptor.

The cells can be modified before being used in a screen. For example, the cells can be genetically modified to introduce a mutation in a gene, or to introduce a nucleic acid encoding a gene product known to be or suspected to be implicated in disease, especially a disease of neural cells including Parkinsonism and Alzheimer's. The Parkin mutation can be introduced. A gene encoding a protein, e.g. APP, implicated in Alzheimer's can be expressed or mutated or have it expression altered.

Cells of the invention are of use as a source of cells for cell therapy. They offer a source of stem cells from a patient which potentially can be a source of nuclei for nuclear transfer. They can be used for delivery of gene therapy, including neuroprotective gene therapy. In an example therapy, a cell of the invention expresses glial cell derived neurotrophic factor (GDNF). These cells can be obtained by following the invention and genetically manipulating the cells. The cells can be transplanted to restore damaged neural circuitry and/or restore brain function.

An advantage of the invention lies in the purity of the cells obtained. A pure population is easier to control in transplantation or culture, as in a heterogenous culture a reduced percentage of neurons is obtained as more cells are already committed to a glia fate. Some therapies will need both neurons and astrocytes.

Other therapies will need glial cells (e.g. oligodendrocytes for MS, astrocytes, migratory cells, for other applications).

Methods of cloning of non-human animals are provided by the invention. One method of cloning a non-human animal, comprises (i) obtaining a neural stem cell from the non-human animal, (ii) obtaining an oocyte of the same species as the non-human animal, (iii) transferring the nucleus of the neural stem cell into the oocyte, and (iv) implanting the cell obtained in (iii) into a female of the same species. The invention hence provides an efficient method of non-human animal cloning based upon isolation of neural stem cells. The cloning method is believed to be of application to substantially all non-human animals, though especially domestic animals including cows, pigs, sheep, cats, dogs, chickens and others and also laboratory animals including mice and rats.

The invention enables, for the first time, growth of a pure population of diploid, clonogenic, transfectable, tissue stem cells in the same way as ES cells. This is a significant step forward in stem cell biology that opens up a range of new experimental opportunities. For example, previous studies on profiling "neural stem cells" are seriously compromised by reliance on heterogeneous neurosphere cultures (e.g. Suslov et al.). Moreover, the radial glia characteristics of NS cells define their *in vivo* counterpart. The ability to culture and genetically manipulate pure populations of radial glia also opens up new opportunities for analysing the cell biology of these remarkable cells, that can function both as stem cells and as specialised scaffold cells. Finally, the ability to propagate NS cells in simple media without heterologous cells or cell extracts establishes that self-renewal can be driven by growth factors alone and does not require a complex microenvironmental niche, hitherto considered indispensable by stem cell biologists. The invention opens up new approaches to nuclear reprogramming and optional genetic manipulation using the cells.

The invention provides efficient generation of neurons from NS cells. In a method, set out in more detail in a protocol herein, a method of obtaining neurons comprises (a) culturing neural stem cells in the presence of an agonist of an FGF receptor and in the absence of an agonist of an EGF receptor; and (b) thereafter, culturing the

cells in the absence of an agonist of an FGF receptor and in the absence of an agonist of an EGF receptor. It is found that the period during which e.g. EGF is absent primes the cells to become neurons when e.g. FGF is subsequently withdrawn. The neural stem cells are preferably plated in monolayer culture. Typically, the NS cells are transferred to medium free of EGF but which contains FGF-2 and cultured for a period, say at least 2 days, or at least 4 days, before FGF-2 is also removed from the medium (which includes that cells are transferred to medium that does not contain it). In a specific method the cells are grown in FGF-2 but no EGF for a week and then FGF-2 is withdrawn. Withdrawal of FGF leads to some cell death in the culture, but a good percentage survive and form neurons. This method may be used as an optional addition to any of the methods described herein for derivation of NS cells.

It will be appreciated that methods/uses according to all aspects of the invention can be carried out either *in vivo* or *in vitro*.

The methods and compositions of the invention are illustrated in the accompanying drawings in which:-

Fig. 1 shows generation of neural stem (NS) cells from ES cells;
Fig. 2 shows NS cells can be derived from various ES cell line and foetal forebrain;
Fig. 3 shows NS cells incorporate and differentiate within the adult brain;
Fig. 4 shows human ES cell or foetal-derived NS cells; and
Fig. 5 shows electrical activity under voltage- and current-clamp conditions of NS cells cultured in differentiating medium.

Referring to the figures in more detail, Fig. 1 shows generation of neural stem (NS) cells from ES cells: **A.** The adherent NS cell culture (LC1) propagated in EGF and FGF-2 (**a**) shows no expression of neuronal (**b**) or astrocyte (**c**) antigens and uniform expression of the precursor marker RC2 (**d**) and nestin (not shown). LC1 cells differentiate into immunopositive astrocytes (**e**) on addition of serum and generate neurons (**f-h**) on growth factor withdrawal. The proportion of neurons obtained

remains >35% of total cells after 115 passages (**i**). **B,** Clonal NS-5 cells were generated through *Sox1* neural lineage selection. **a** and **c**, Phase image of neural precursors at passage 1 and 5 respectively. **b** and **d** corresponding *Sox1*-GFP fluorescence. **e**, Single cell 1 hour after plating in Terasaki well. **f**, Phase contrast image of clonal cell line at passage 20. **C**, RT-PCR for ES cell and neural stem cell/radial glia cell markers (46C, parent ES line; P5, 5 passages after neural differentiation; NS-5 clonal NS line; LC1, NS population (passage 17); brain, E12.5/E16.5 mouse brain). **D**, NS-5 immunoreactivity for neural stem cell/radial glia markers. **E**, NS-5 differentiation into astrocytes (a,b) and neurons (d,e) with loss of nestin immunoreactivity (c, f) **F**, Colonies of NS-5 cells (**a**) generate neurons on growth factor withdrawal (**b**) and in the presence of EGF/FGF retain homogenous expression of RC2 and BLBP with no immunoreactivity for GFAP (c,d). **G**, Metaphase spread of NS-5 (passage 31).

Fig. 2 shows NS cells can be derived from various ES cell line and foetal forebrain: NS cells were derived from independent ES cell lines (CGR8, E14Tg2a) or primary cortical (Cor-1) and striatal (Str-1) tissue. **A**, RT-PCR of stem cell/radial glia markers. **B**, RT-PCR for transcriptional regulators. **C**, The ES-derived line (CGR8-NS) and foetal-derived line (Cor-1) are indistinguishable from LC1 by morphology (**a**, **f**) and neural stem cell/radial glial marker immunoreactivity (**b**, **c**, **g**, **h**), and can each differentiate into neurons (**d**, **i**) and astrocytes (**e**, **j**).

Fig. 3 shows NS cells incorporate and differentiate within the adult brain: **a-h** Confocal images of LC1 NS cells, lentivirally transduced with eGFP, four weeks post-grafting into hippocampus (**a**, **b**) or striatum (**c-h**). **b, d**, higher magnification of the insets in panels **a** and **c**, respectively. **e, f,** Examples of eGFP grafted NS cells (green) showing co-expression (yellow) of the neuronal markers TuJ (**e**, red) or MAP-2 (**f**, red). **g**, astroglial marker GFAP (red). **h**, neural progenitor marker nestin (red). **i**, Quantitative analysis of graft-derived neuronal (MAP2), astroglial (GFAP), progenitor (Nestin), and proliferating (Ki67) cells, four weeks after transplantation into adult mouse striatum. Data are means (± standard deviation) of at least 500 eGFP+ cells from five independent animals. Scale bars: **a,c,** 100 □m; **b, d ,e**, 40 □m.; **f-h**, 20 □m.

Fig. 4 shows human ES cell or foetal-derived NS cells: **A**, Derivation from human ES cells. **a**, human ES primary culture. **b**, differentiation of human ES cells into neural-rosette structures. **c**, passage 9 in NS expansion medium. **d**, individual cells exhibit radial glial morphology. **e-h**, immunostaining for neural stem cell/radial glia markers. **B**, Derivation from human feotal forebrain. **i**, neurospheres generated from cortex. **j**, attachment and outgrowth. **k**, passage 5 in NS expansion medium. **l**, radial glia morphology. **m-p**, neural stem cell/radial glial markers. **C**, Differentiation. **q**, TuJ positive immature neuron. **r**, GFAP positive astroglia.

Fig. 5 shows: A. Superimposed inward and outward current tracings obtained at different membrane potentials (between -70 and +40 mV from a holding potential of -90 mV), from three different NS cells following incubation in differentiating medium for six (a), twenty (b) and thirty days (c). B. superimposed voltage responses obtained following injection of depolarising rectangular current pulses in the same three cells (a, b and c) as in (A) by switching from voltage- to current-clamp immediately after current recordings shown in (A) were obtained. The dashed line represents a voltage level of -60 mV. C. Average Na^+ currents elicited at -20 mV from cells cultured in differentiating medium for increasing times as indicated by labels. Bars indicate SE. D. Superimposed inward currents elicited at -40 mV and 0 mV in 10 mM Ba^{2+} and in the presence of TTX; the holding potential was -90 mV. E. Current/voltage relationship from the same cell as in (D).

The invention is demonstrated in use by the following examples.

EXAMPLES

Example 1-1.

Isolation and culture of bulk populations of NS cells

An ES cell differentiation protocol was recently established that allows efficient and consistent generation of between 50-70% Sox1 positive neural precursors in adherent monolayer culture. In order to isolate, expand, and characterise the neural

precursors (derived from mice) within these cultures, a previously generated cell line (46C cells) was used which contained a GFP-IRES-puromycin reporter cassette targeted to the Sox1 locus (Ying et al., 2003b). Puromycin was added to the differentiation cultures after 7 days, and within 3 days more than 95% of remaining cells were GFP+ neural precursors. At this point, cells were re-plated in N2B27 media supplemented with EGF/FGF-2 (without puromycin). These cells grew rapidly, within a few passages taking on a homogeneous morphology.

These 46C neural precursor cells (46C-NP) were kept continuously in culture for more than 20 passages retaining a characteristic bipolar morphology. These cultures showed very little cell death and had a high plating efficiency with doubling time of approximately 25hrs.

Example 1-2.

Culture of cell lines

To isolate clonal cell lines, single cells from passage 5 bulk cultures of 46C-NP cells were plated into separate wells of a microwell plate. Of 95 single cells scored, 15 were grown up into colonies and, of these, 4 lines were kept growing continuously through more than 10 passages. One line (NP5) showed the characteristic bipolar cells with homogenous morphology identical to that observed in the bulk population. This NP5 line has been maintained in culture indefinitely (NP5 passage 41; more than 5 months).

Example 1-3.

Characterization of NS cells

In order to characterise both the bulk cell population and clonal lines immunocytochemistry was used for a range of markers. As expected, each of the cell lines was found to be positive for the neural precursor markers, nestin or vimentin. Importantly, these cells were also positive for the radial glia specific markers RC2, 3CB2 and astrocyte-specific glutamate transporter (GLAST). Less than 1% of cells were positive for either GFAP or b III tubulin suggesting that very little spontaneous differentiation to astrocytes or neurons occurs during passage of these cells. Unlike

primate/human cells, rodent radial glia are negative for GFAP. Furthermore, these cells are immunoreactive for SSEA-1, an antibody that recognises the LewisX antigen, previously used to enrich for adult neural stem cells.

RT-PCR was performed with a range of markers to confirm their identity as radial glia. All four clonal lines as well as the bulk population were negative for Oct4 or Nanog confirming that they were not ES cells, while they did express nestin, vimentin and GLAST, consistent with the immunocytochemistry. Furthermore, each cell line showed expression of BLBP, a radial glia marker. These cells also express Musashi-1 and prominin.

Interestingly, the bulk culture of 46C-NP gradually lost expression of Sox1, as assessed by the GFP reporter, such that by passage 5 (~2 weeks) very few cells remained green. Also, none of the clonal lines showed Sox1 driven GFP expression RT-PCR confirmed that these cells did not express Sox1, but instead expressed the related SoxB1 class protein, Sox2. Taken together these results suggest that a range of Sox1 expressing neural precursors can be isolated using a puromycin selection strategy, and that Sox1-, Sox2+ cells with properties of forebrain radial glia can be isolated and clonally expanded.

Example 1-4.

NS cell line differentiation into neurons and glia

The molecular markers expressed by the NS cell lines confirm that they are a type of neural precursor cells. To confirm that these cells were true multipotent stem cells (i.e. capable of differentiation as either neurons or glia) a range of conditions designed to test the potency of the cells were tested.

Previous investigations of neural precursors induced differentiation through protocols involving withdrawal of mitogens and/or addition of serum or other cytokines. In the present case, EGF, FGF, or both, was withdrawn from proliferating NP5 and 46C-NP cells cultured on plastic. Simultaneous removal of both EGF and FGF resulted in

rapid and extensive cell death within 24hrs. Culture with FGF alone, by contrast, lead to cell death over the course of 3 days. Culture in EGF alone resulted in no cell death or differentiation, but cell proliferation was reduced. Thus, EGF clearly acts an essential cell survival signal (partially compensated by FGF signalling) for NS cells cultured on plastic.

The effect of serum on proliferating cells after withdrawal of cytokines was also tested. Cells treated in this way survived, even in the absence of EGF, and rapidly differentiated in a synchronous manner such that 100% of cells acquired a large flat astrocyte that stained positive for GFAP and negative for nestin. Therefore, all NS cells within a proliferating population are capable of differentiation as astrocytes. This effect can be mimicked by addition of BMP4 in absence of serum following EGF/FGF withdrawal on plastic. CNTF and TGF-b behaved similarly to BMP4, although the astrocyte morphology was different and there was more cell death initially. BMP, CNTF and LIF have each been shown to induce a astrocyte fate from primary cortical progenitors (Gross et al., 1996; Lillien and Raff, 1990; Nakashima et al., 1999). Consistent with these studies, a rapid induction of Id genes (>30 fold) with BMP-4 or serum treatment was found (data not shown).

In an attempt to induce neuronal differentiation, the withdrawal of EGF on laminin-treated dishes was tested. To avoid cell death seen with culture of cells on plastic, the cells were plated on a laminin substrate in the presence of FGF, but no EGF. The PCD seen in these conditions on plastic or gelatin did not occur and cells survived. There was a change in morphology of the cells to more extended bipolar processes, with characteristic endfeet of radial glia. These cells showed a drastic reduction in proliferation (through BrdU incorporation) but maintained radial glia markers (RC2, vimentin, nestin) and did not differentiate, as only a small proportion of neurons or astrocytes was seen in regions of high density. To induce neuronal differentiation, after 6 days the media was changed from NSA/N2 +FGF2 to NSA/B27 without FGF2. This promoted around 40-60% neuronal differentiation of cells as judged by MAP2 and bIII tubulin antibody staining. These neurons were GABAergic as assessed by GABA immunoreactivity, while there were few astrocytes

(GFAP). These results are consistent with the previously described role of FGF in preventing neuronal differentiation.

It was also found that each cell line could be frozen and thawed with similar success to ES cells.

In further tests, the differentiations were attempted starting from single cells. This clonal expansion and differentiation showed all the cells to have ability to form neurons.

Example 1-5.

Isolation of NS cells from any ES cell line without use of genetic selection strategies

The N2B27 media used to isolate NP5 was originally optimised for conversion of ES cells to neural precursors and hence allowed good survival of both ES cells and neural progenitors. Thus, in the absence of puromycin selection, it was not possible to efficiently expand 46C-NP cells with EGF and FGF-2 due to carry-over of ES cells and non-neural cell type (data not shown). To overcome this and allow the generation of radial glia cells from other non-targeted ES lines, other basal media combinations were tested that allowed survival and expansion of neural precursors but not other cell types.

Using a commercially available media, NS-A media (Euroclone), it was found that re-plating of a 46C ES cell monolayer differentiation at day 7 resulted in formation of clumps/spheres of cells, as well as a cell death of non-neural cells. Subsequent attachment of these cell clumps occurred and a homogeneous population of cells were outgrown. Further characterization of this passagable population (>75 passages) of cells revealed an identical profile of expressed markers seen previously using the puromycin selection strategy. Thus, the cells were positive for neural precursor markers (nestin, vimentin) and also radial glia markers (RC2, GLAST, BLBP and Pax6). 46C-NP cells behaved similarly in either N2B27 or NSA media

once established with only small differences in morphology, and no differences in marker genes.

Using this protocol, cell lines from six other ES lines were isolated: CGR8, E14T. Oct4-GIP, S11, R1 and V6.5. Each of these cell lines has a similar morphology and expression profile and can be passaged extensively.

Example 1-6.

Transplantation of NS cells

To test the potential of the NS cells in vivo, they were transplanted into both embryonic, and adult CNS, as well as in kidney capsule graft.

Electroporation was tested on NP5. They were found to be electroporated efficiently using a square wave elecroporator. The cells are also transfectable using lipofectamine plus reagent. This is a major advantage as all genetic manipulations used in mouse now become available.

To allow rapid evaluation of the grafted cells, 46C-NP were transduced with lentiviral particles carrying the eGFP marker gene. The infection was highly efficient and almost 95% of the cells were successfully marked, the eGFP signal being strong and stable with passages. The use of the lentiviral infection allowed the transgene to be stably integrated and the signal to remain stable for long term analyses of the grafted cells.

The behaviour of the cells following transplantation in the embryonic brain (an environment that contains all the molecules and factors able to sustain and direct differentiation of immature neural cells) was evaluated. 100,000 cells were resuspended in a final volume of 2 microlitres following the procedure previously described by Magrassi and colleague (Magrassi et al., Development 1999) using E14.5 mouse embryos as recipients. The cells survived well after transplantation (an approximative evaluation of around 20000 cells in the graft was made), the eGFP

signal was easily detectable and they displayed migrational activity already at early time points after the grafting. The fates that the grafted cells acquired at different time points (4 days, 7days, 2 weeks and 1 month) after the grafting were analysed. At the 4 day and 1 week time points, the majority of the cells were still immature as indicated by the nestin immunoreactivity, while 23 % expressed the neuronal marker Tuj-1 and 16.3% acquired the glial marker GFAP. These data indicate that the transplanted NS are able to generate both neurons and glia in vivo as expected from multipotent NS cells.

The NS cells were also transplanted into the adult striatum. In these transplants, the cells survived well (the survival is anyway lower than in the embryonic grafts) and differentiated toward both neuronal and glial fates. Quantitative analyses performed at 2 weeks after transplantation indicated that 43.3% of the cells expressed the neuronal-specific marker Tuj-1, while 26.6% displayed immunoreactivity for the glial marker GFAP. A small fraction of the cells (11.1%) retained an immature phenotype as indicated by the expression of nestin.

NP5-GFP cells into kidney capsule did not give rise to teratomas (n=4, data not shown).

EXAMPLE 2

Example 2-1

Methods

Mouse cell culture and differentiation

ES cells and neural differentiation are detailed by Ying & Smith, 2003. NS cells termed LC1 and other ES cell-derived NS cells were routinely generated by re-plating day 7 neural differentiation monolayer cultures on uncoated plastic in NS-A medium (Euroclone) supplemented with N2 and 10ng/ml of both EGF and FGF-2 (NS expansion medium). Cells formed aggregates which subsequently attached and outgrew NS cells. Cells termed 46C-NS cells were generated after addition of

0.5μg/ml of puromycin to differentiating adherent cultures at day 7. Cells were re-plated 3 days later into an uncoated T75 flask in N2B27 media with 10ng/ml of both EGF and FGF-2 (Peprotech) in the absence of puromycin. A clonal line, NS-5, was generated by plating single cells into 96-well microwell plates (Nunc) by limiting dilution (single cells scored one hour after plating). Primary cultures were generated using standard protocols from cortex/striatum of E16.5 mouse embryos and allowed to attach on flasks treated with 0.1% gelatin. Cor-1 and Str-1 cells were then expanded on gelatin using NS expansion medium. For astrocyte differentiations, NS cells were replated onto 4-well plates at 1 x 10^5 cells/well in NS-A medium supplemented with 1% fetal calf serum or 10ng/ml BMP4 (R&D System). For neuronal differentiation 5 x 10^4 NS cells were plated into poly-ornithine/laminin treated wells in NS-A supplemented with FGF-2 alone. After 7 days the media was switched to NS-A supplemented with B27 (Gibco) without growth factor. For clonal differentiation, 1000 cells from NS-5 or Cor-1, cultures were plated in 10cm plates pre-treated with laminin, expanded for 12 days in EGF/FGF-2, and differentiated in situ as above.

Characterization of NS cells

Immunocytochemistry was performed using appropriate TRITC or FITC secondary conjugates and nuclear counterstaining with DAPI. Primary antibodies were used at the following dilutions: Nestin (1:10), Vimentin (1:50), Pax6 (1:5), 3CB2 (1:20), RC2 (1:50) (DSHB); TuJ (1:200) (Covance); GFAP (1:300) (poly and mono, Sigma), MAP2 (1:200) (Chemicon and Becton Dickinson); NeuN (1:200), GABA (1:200), Gad65/67 (1:200) (Chemicon); Synaptophysin (1:200) (Sigma); Olig2 (1:5000) (H. Takebayashi); Emx2 (1:2000) (A. Corte); BLBP (1:500) (N. Heintz); prominin/mAb13A4 (1:200) (W. Huttner). Negative controls were ES cells, differentiated NS cells or secondary alone. For RT-PCR, total RNA was extracted using RNeasy kit (Qiagen), and cDNA generated using Superscript II (Invitrogen). PCR was performed for 30 cycles for all markers except β-actin (25 cycles). For metaphase spreads cells were treated with 5mls of 0.56% KCl for 20mins, fixed in methanol:acetic acid (3:1) on ice for 15mins, spread onto glass slides and stained

with TOPRO-3 (Molecular probes).

Human embryo and foetal cultures

Research on human tissue with informed consent was approved by the Research Ethics Committee of Lothian Health Board. Frozen supernumerary human embryos were donated for research under licence R0132 issued by the Human Fertilisation and Embryology Authority. Human cortex was dissected from a Carnegie stage 19/20 foetus following elective termination with consent for research under the Polkinghorne guidelines.

Transplants

Foetal surgery was performed as described by Magrassi et al, 1998. Using a glass capillary, $5x10^4$ cells in a volume of 1μl of HBSS were injected into the telencephalic vesicles of E14.5 Sprague Dawley rat foetuses exposed under transillumination. Injected foetuses were replaced into the abdominal cavity for development to term. After delivery animals were sacrificed at seven days (Postnatal day (P) 1, n=16) and five weeks (P30, n=8) post transplantation. For adult transplantations, 129 or CD1 mice were placed in a Kopf stereotaxic frame and received an injection of $2x10^5$ NS cells suspended in 5μl of HBSS into the striatum (n=22) or hippocampus (n=21). Transplanted mice were sacrificed after two (n=16) and four weeks (n=10) and perfused transcardially with 4% paraformaldehyde. Cryosections (16μm) were stained with the following antibodies: (mouse): NeuN (1:100) and Ki67 (1:10) (Chemicon), MAP2 (1:200; Becton Dickinson), Nestin (1:5; Ron McKay); (rabbit): βIII tubulin (1:500; Covance); GFAP (1:200; Dako); secondary antibodies, Texas Red (Vector) (Jackson ImmunoResearch) and AlexaFluor 488 (Molecular Probes). Sections were preserved in antifading solution and analysed on Nikon TE2000-S ECLIPSE and Biorad Radiance 2100 confocal microscopes.

Example 2-2

We induced monolayer differentiation of ES cells for 7 days then replated in basal medium (NS-A plus N2) supplemented with EGF and FGF-2. Cells surviving under

these minimal conditions (non-permissive for ES cell survival) predominantly associate into floating clusters. After 3-5 days these aggregates were harvested and replated in fresh medium. They attached within 2-3 days and outgrew a morphologically homogeneous population of bipolar cells, named LC1. Upon passaging LC1 cells continued to grow as adherent cultures, often forming lattice networks. They can be continuously and rapidly propagated with a doubling time of approximately 24 hours. LC1 cells display the neural precursor markers nestin and RC2 but expression of the astrocyte differentiation marker GFAP or of neuronal antigens is negligible (Fig 1A). On exposure to serum or BMP, LC1 cells adopt typical astrocyte morphology within 48 hours and subsequently uniformly express GFAP (Fig 1A,e). In contrast, cells with neuronal processes appear after replating on laminin without EGF for 5-7 days and then withdrawing FGF-2. These cells express neuronal markers type III β-tubulin, MAP2 (Fig 1A, f,g) and neuN (not shown). Most neurons stain for GAD67 (Fig 1A,g) and GABA (not shown) and by 7 days a sub-population show expression of the mature marker synaptophysin (Fig 1A, h). High numbers of neurons (>35%) are generated even after 115 passages (Fig 1A,i). Together with the observation that LC1 cells retain a diploid chromosome content at late passages (not shown), this confirms the presence of self-renewing neural stem (NS) cells.

Example 2-3

To determine whether cell aggregation is essential for generation of NS cells, we maintained cell attachment throughout the derivation process. For this we exploited lineage selection (Li et al, 1998) using 46C ES cells in which the GFPires*pac* reporter/selection cassette is integrated into the *Sox1* gene, a specific marker of neural specification (Aubert et al, 2003). Transient puromycin selection after differentiation induction yielded a purified population of neural precursors without residual ES cells (Stavridis et al, 2003) (Fig 1B a,b). FGF-2 and EGF were then applied to the Sox1 expressing neural precursors in enriched medium. Cells remained adherent in this condition. Cellular heterogeneity reduced over 3-4 passages as bipolar cells progressively increased in number and began to form

extensive lattices. Intriguingly, expression of Sox1 was lost at this stage (Fig 1B c,d) but the cells remained positive for Sox2 and nestin. To establish the presence of neural stem (NS) cells, single cells were isolated in Terasaki wells and expanded as adherent cultures (Fig 1B, e,f). Initially 5 clonal lines were derived of similar morphology and growth characteristics to the bulk LC1 population. These cells lacked detectable expression of the pluripotency factors Oct4 and Nanog, and also of the early neural marker Sox1, but retained the pan-neuroepithelial markers Sox2 and nestin (Fig 1C). NS cells were thus generated through a *Sox1* positive early neuroectodermal precursor via continuous adherent culture.

Example 2-4

Clone NS-5 was examined in further detail and found to express Pax6, Glast, and BLBP mRNAs, and to be immunopositive for RC2, vimentin, 3CB2, SSEA1/Lex1 and prominin (Fig 1D). This set of markers is considered diagnostic for neurogenic radial glia, the precursors of both neurons and astrocytes during development of the nervous system (Campbell et al, 2002; Hartfuss et al, 2001). As with uncloned LC1 cells, NS-5 cells were competent for astrocyte and neuronal differentiation (Fig 1E). NS-5 cells plated at clonal density in EGF plus FGF-2 gave rise to colonies of bipolar cells. Every colony showed expression of RC2 and BLBP in virtually all cells and absence of GFAP (Fig 1F, c,d). These subclones could be picked and continuously expanded. To determine the frequency of cells within NS cultures that are capable of neuronal differentiation we again plated NS-5 cells at clonal density, expanded for 12 days in EGF/FGF-2 followed by FGF-2 alone for 5 days, then a further 7 days without growth factor. Every colony (126/126) produced TuJ positive cells (Fig 1F, b). These data indicated that all colony forming cells in NS cultures were competent for neuronal differentiation. Finally, like LC1, NS-5 cells maintained a diploid chromosome complement (Fig 1G) and represented a non-transformed clonal neural stem (NS) cell line that self-renewed without a requirement for a complex cellular microenvironment.

Example 2-5

To assess whether the serum-free monolayer induction protocol is a prerequisite for NS cell generation, ES cells were induced to differentiate by embryoid body formation and exposure to retinoic acid in serum-containing medium (Bain et al, 1995). Aggregates were subjected to Sox2 lineage selection (Li et al, 1998; Billon *et* al, 2002) with G418 for 48 hours then replated in the presence of FGF-2 and EGF without serum. Following attachment, Sox2 positive, nestin positive, cells proliferated that displayed the bipolar morphology and lattice growth typical of NS cells plus capacity for astrocyte and neuronal differentiation after multiple passages (data not shown). Thus NS cells were derived from embryoid bodies.

Example 2-6

NS cells were derived using monolayer induction without lineage selection as described for LC1 from three independent ES cell isolates, E14TG2a, CGR8 and R1. All NS lines examined expressed nestin and RC2 in at least 95% of cells. More detailed inspection of CGR8 and E14TG2a derived NS cells showed the full panel of radial glia markers (Fig 2A), the neural precursor markers Sox2 and Sox3, plus the bHLH transcription factors Olig2 and Mash1 (Fig. 2B). Down-regulation of Sox1 but maintenance of Sox2 was a striking feature of these NS cells, in view of the postulated determinative function of these transcription factors (Pevny et al, 1998). Thus whilst Sox1 marks all neuroectodermal precursors, it was not retained in stem cells where Sox2 is likely to play a key role. The NS cells also expressed Emx2 which is implicated in expansion of neural precursor cells (Hein et al, 2001; Galli et al, 2002). All NS cultures tested underwent astrocyte and neuronal differentiation assessed by both morphology and immunostaining (Fig 2C).

Example 2-7

In light of their apparent relationship to radial glia, we examined whether NS cells resulted from the pre-adaptation of ES cells to *ex vivo* propagation or could be derived from foetal neural tissue. Primary foetal CNS cells from E16.5 foetal brain adhered poorly to plastic in basal medium plus growth factors and spontaneously

formed aggregates. After 6-7 days these aggregates settled onto gelatin-coated plastic. Fourteen days later, outgrowths were trypsinised and plated onto gelatin-coated plastic. In three separate experiments cells morphologically identifiable as NS cells proliferated and were subsequently expanded into continuous cell lines. These foetal brain derivatives expressed the same radial glia and neurogenic markers as the ES cell derived NS cells (Fig 2A,B) and showed consistent mRNA profiles. They were likewise competent for astrocyte and neuronal differentiation (Fig. 2C). Cortex-derived Cor-1 cells were plated as single cells then colonies subjected to sequential growth factor withdrawal as described for NS-5. Every colony produced TuJ positive neurons. This indicated that all clonogenic cells in the Cor1 culture were neurogenic. Cor-1 cells were also readily sub-cloned and continuously expanded from individual cells, indicative of self-renewal. Thus NS cells were derived from foetal brain and shared the key properties of ES cell derived NS cells.

Most NS cells had the elongated bipolar morphology, lamellate extensions, end-feet and oval nuclei anticipated for radial glia (Rakic, 2003). Flattened and compact cells with short extensions were also present. Immunostaining for the metaphase marker phosphorylated histone H3 indicated that the compact cells are mitotic. Time lapse videomicroscopy confirmed the dynamic change in morphology prior to cell division In addition, the time lapse revealed that NS cell undergo interkinetic nuclear migration, a well-characterized feature of neuroepithelial and radial glia cells *in vivo*.

The NS cells were hence continuously expandable *in vitro* analogues of neurogenic radial glia.

Example 2-8

Frozen/thawed passage 40 mouse neurospheres were allowed to attach to gelatin-coated plastic in NS expansion medium. Bipolar cells outgrew that are indistinguishable from NS cells. These cells can be serially propagated as uniformly RC2 positive, GFAP negative, populations and then induced to differentiate into astrocytes or neurons.

Example 2-9

We investigated the behavior of NS cells upon transplantation into mouse brain. ES cell derived LC1 cells transduced with a lentiviral eGFP expression vector were introduced into the developing brain by intra-uterine injection at E14.5 (Magrassi et al, 1998). Animals were sacrificed after birth and the presence of eGFP positive cells examined in brain sections. NS cell progeny had migrated into various brain regions. Immunohistochemical analyses revealed co-expression of eGFP with the precursor marker nestin, neuronal markers TuJ, NeuN and MAP2, and in lesser numbers with GFAP. NS cells were also injected into the adult mouse striatum. In this case GFP-positive cells remained localised to the vicinity of the injection site. Four weeks after grafting, 44.4±5.7% of GFP expressing cells had neuronal morphology and were immunopositive for MAP2, 37.4±6.1% expressed GFAP, and 4.2±1.9% retained expression of nestin (Fig 3). The proliferative marker Ki67 was detected in only 1.0±0.6% of GFP positive cells indicating that NS cells withdraw from the cell cycle *in vivo.* Consistent with this we observed no histological evidence of un-regulated proliferation or tumour formation in a total of 35 brains examined one month after transplantation. Furthermore, NS cells grafted to mouse kidney capsules did not proliferate or give rise to teratomas. These data indicated that NS cells can survive and differentiate in both foetal and adult brain environments and unlike ES cells (Brustle et al, 1997), they do not give rise to teratomas. Moreover, the relatively high frequency of neuronal differentiation is in marked contrast to grafts of passaged neurospheres (Rossi et al, 2002).

Example 2-10

Finally we investigated whether similar NS cells could be isolated from human sources. In the process of deriving human ES cells from donated supernumerary embryos, we observed after 5-6 weeks of culture extensive differentiation into rosette structures typical of neuroepithelial cells. These cells were transferred into NS expansion medium. After a further 3-4 weeks bipolar cells similar to NS cells emerged from these cultures (Fig. 4A) and have been continuously cultured for 5

months. We also sourced Carnegie stage 19-20 human foetal cortex tissue from an elective termination. Dissociated cells initially formed floating aggregates that after 7 days were replated and allowed to attach to gelatin-coated plastic as for derivation of NS cells from mouse foetal brain. A proliferating culture was established (Fig 4B). Human NS cultures from both ES cells and fetal tissue were characterized by the presence of flattened cells associated with the bipolar cells. However, all cells expressed immature precursor markers nestin, vimentin and 3CB2 (Fig 4B). Time lapse monitoring confirmed that the two cell morphologies are plastic and interconvertible. These human cells also exhibited low levels of GFAP consistent with the activity of the human GFAP promoter in radial glia (Rakic, 2003; Malatesta et al, 2000). They proliferated more slowly than the mouse cells, with doubling times of several days. After sequential withdrawal of EGF and FGF-2 they appeared to generate immature neuronal cells (Fig 4C). Cells of typical astrocyte morphology with intense GFAP immunoreactivity were produced after passaging in serum (Fig. 4C).

Example 3

The electrophysiological properties of NS cells in culture were investigated during *in vitro* differentiation by employing the whole-cell variant of the patch-clamp technique, in order to know whether, following specific treatments, these cells could be efficiently transformed into mature and functional neurons from an electrophysiological point of view.

Solutions for electrophysiological recording

Seals between electrodes and cells were established in a bath solution consisting of (in mmoles/l):155 NaCl, 1.0 $CaCl_2$, 1 $MgCl_2$, 3.0 KCl, 10 glucose, 10 HEPES/NaOH (pH 7.4). After establishing the whole-cell configuration, for current-clamp recording and for total current recording in voltage-clamp, the pipette filling solution contained (in mmoles/l): 128 KCl, 10 NaCl, 11 EGTA, 4 Mg-ATP, 10 HEPES/KOH (pH 7.4). For the study of voltage-gated Na^+ channels under voltage clamp conditions, the patch pipette was filled with (in mmoles/l): 130 CsCl, 10 NaCl, 20 TEA-Cl, 10 EGTA, 2 $MgCl_2$, 4 Mg-

ATP, 10 HEPES/CsOH (pH 7.4) and the extracellular solution contained (in mmoles/l): 130 NaCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 5 tetrethylammonium-Cl, $CdCl_2$ 0.2, 10 HEPES/NaOH (pH 7.4). For the study of voltage-gated Ca^{2+} channels, the patch pipette was filled with (in mmoles/l): 120 CsCl, , 20 TEA-Cl, 10 EGTA, 2 $MgCl_2$, 4 Mg-ATP, 10 HEPES/CsOH (pH 7.4) and the extracellular solution contained (in mmoles/l): 130 NaCl, 10 $BaCl_2$, 10 glucose, 5 tetrethylammonium-Cl, 10 4-AP 1, TTX 10^{-3}, HEPES/NaOH (pH 7.4).

Patch Clamp Recording

Ionic currents were recorded under voltage-clamp conditions using the patch-clamp whole-cell configuration (19) at room temperature (20–24°C) with an Axopatch 200B patch-clamp amplifier (Axon Instruments Inc., Burlingame, CA) and digitized at sampling intervals of 26-100 µsec using a Digidata 1322A A/D converter (Axon Instruments Inc., Burlingame, CA) interfaced with an IBM-compatible PC. Stimulation, acquisition, and data analysis were carried out using the following software pachages: pClamp 9 (Axon Instruments Inc., Burlingame, CA) and ORIGIN 6 (Microcal Software Inc., Northampton, MA). For voltage-clamp experiments linear components of leak and capacitive currents were first reduced by analogue circuitry and then almost completely canceled with the P/N method. Patch pipettes were made from borosilicate glass tubing and fire polished. Pipettes had a final resistance of 3-4 MΩ when filled with internal solution. Currents were filtered at 5 KHz.

Fig. 5A shows current recordings obtained during whole-cell voltage-clamp steps to depolarising test potentials in three NS cells at different stages of *in vitro* differentiation (cultured respectively for 6, 20 and 30 days in a differentiating medium), using bath and pipette filling solutions suitable for the isolation of both inward (Na^+) and outward (K^+) voltage-gated ionic currents. From a simple inspection of the current tracings it is evident that a sizeable outward voltage-gated current is already present at early stages of *in vitro* differentiation (6 days, trace a). This current, blocked by application of an extracellular saline containing 5 mM tetraethylammonium-Cl, has the features of a delayed-rectifier K^+ current. Its

amplitude increased only slightly at later stages of differentiation (20-30 days, traces b and c respectively). By contrast, the amplitude of the inward current, negligible after six days, increases dramatically by increasing the time of exposure to the differentiating medium. Fig 5B shows the voltage responses elicited following intracellular injection of rectangular depolarizing current stimuli between 70 and 300 pA from a holding potential of about –80 mV, after switching from voltage-clamp to current-clamp mode in the same cells shown in fig. 5A. Indeed, the increase in amplitude of the voltage-gated inward current reflects the capacity of the differentiating cells to elicit action potentials. In fact, the cell exposed for only six days to the differentiating agents and showing a negligible inward current was not able to elicit a regenerative voltage response (trace labelled with (a) in Fig. 5B). By contrast, an overshooting action potential with a relatively fast depolarisation rate could be elicited in the cell cultured in differentiating medium for thirty days (trace labelled with (c) in Fig. 5B) and displaying a large inward current (trace c in fig. A). An intermediate situation, with an abortive action potential, was found in the cell treated for twenty days with the differentiating agents (trace labelled with (b) in Fig. 5B) and displaying a moderate inward current (trace b in fig. A). From this preliminary analysis it turns out that the excitability properties of these cells are strictly correlated to the magnitude of the inward voltage-gated conductance. For a quantitative analysis of this conductance as a function of the time of exposure to the differentiating medium, the inward currents were elicited using cells at different stages of differentiation and applying intra- and extracellular salines specific for the study of the activity of voltage-gated Na^+ channels (see methods). At any time of cell differentiation the fast inactivating inward current was completely blocked by the selective Na channel blocker tetrodotoxin (1 □M) and peaked at a test potential between –20 and –10 mV, showing the typical features of voltage-gated Na^+ currents in neurons (data not shown). In fig 5C the development of the Na^+ current amplitude at –20 mV as a function of the time of exposure to the differentiating medium is shown. Interestingly, the increase of the Na^+ conductance well correlates with the increasing cell exposure to the differentiating medium. Indeed, on average the Na^+ current amplitude increases of about a factor ten within 30 days of treatment (from -

55 ± 14 pA (n=17) during the first 5 days to -434 ± 135 pA (n=13) for more than 25 days). In the same way, the regenerative potential (the □V measured between the threshold and the peak) elicited by the Na^+ current under current clamp conditions ranged between 0 and +20 mV during the first 15 days of in vitro differentiation (n=6), whereas following more than 25 days of treatment it reached values between +30 and +70 mV (n=6). On the whole, the excitability properties and those of the underlying voltage-gated Na^+ conductance found in NS cells treated longer than 25 days with the differentiating medium are typical of neurons developing toward their adult phenotype. Another feature corroborating the previous conclusions is the presence of voltage-gated Ca^{2+} channel conductances in most of the tested cells following exposure to the differentiating medium for at least seven days or longer. Fig. 5D shows sample tracings obtained by membrane depolarization to the indicated test potentials from the same cell bathed in 10 mM Ba^{2+}. The fast activating and relatively fast inactivating ($□_h$ = 21 ms) current component elicited at -40 mV is reminiscent of the neuronal LVA Ca^{2+} channel current (Carbone and Lux, 1987). By contrast, the Ba^{2+} current elicited at 0 mV, displaying a slow ($□_h$ = 73 ms) and incomplete inactivation, has the typical features of the neuronal HVA Ca^{2+} channel current. The presence in this cell of two distinct, LVA and HVA, Ca^{2+} channel conductances is confirmed by the current-voltage relationship of Fig 5E. On average the LVA current peaked at -40 mV, while the I/V relationship for the HVA current peaked at 0 mV. A HVA Ba^{2+} current was detectable in 19 out of 27 cells, while a LVA current component was measured in 60% of the cells already expressing a HVA Ca^{2+} current (n=13).

This data shows that neurons produced from neural stem cells of the invention can function: they can fire action potentials, the hallmark of active neurons.

Thus, according to the invention, we have derived and homogenously propagated defined NS cells as differentiated progeny of ES cells and foetal brain extracts. The NS cells proliferated clonally in simple adherent monoculture and remained diploid. After prolonged expansion, they differentiated efficiently into neurons and astrocytes

both *in vitro* and on transplantation into the adult brain. NS cells also formed oligodendrocytes *in vitro*. NS cells uniformly expressed morphological and molecular features of radial glia, foetal precursors of neurons and astrocytes. We were able to establish adherent NS cell lines from mouse and human foetal brain.

Example 6

NS cells were obtained from mouse ES cell and foetal cortex using the protocol below. The derived NS cells uniformly expressed radial glia markers. NS cells derived specifically from CGR8 ES cells or from E16 foetal cortex (internal reference: Cor-1 and clonal derivative Cor-1.3) were analysed for expression of the indicated markers by immunochemistry. Examination at high power showed that the radial glia markers were each expressed in almost all cells whilst they were uniformly negative for GFAP.

Example 7

NS cells were derived from expanded mouse foetal forebrain neurospheres using the protocol below. An NS line derived from a long-term foetal neurosphere culture (40 passages) exhibited identical morphology to ES-derived NS lines, expressed neural precursor cell/radial glial marker immunoreactivity, and could differentiate into neurons and astrocytes. This showed that the NS cells obtained from fresh foetal neurospheres were the same as those from neurospheres frozen down and then grown as neurospheres for a long time.

Example 8

LC1 mouse NS cells were transplanted into foetal rat brain. Confocal images of NS cells, lenti-virally transduced with eGFP, were taken one week after transplantation into the ventricle of E14.5 rats. Donor cells migrated from the ventricle into the parenchyma in clusters and as single cells. Grafted cells showed co-localization of eGFP and the neuronal marker MAP2, the astroglia marker GFAP or the progenitor cell marker nestin. Thus, the NS cells migrated and differentiated after transplantation in the foetal rat brain.

Example 9

Protocols For Derivation and manipulation of NS cell lines

We devised the following protocols for derivation and manipulation of NS cell lines.

Derivation of mouse NS cell lines from ES cells

ES cells can efficiently be converted to Sox1 expressing neural progenitors in adherent monolayer culture [P1]. Detailed protocols and troubleshooting and for this ES cell differentiation are described elsewhere [P2]. Briefly, ES cells are routinely cultured under feeder-free conditions in medium supplemented with 10% foetal calf serum and 100 U/ml recombinant leukaemia inhibitory factor (LIF) on gelatin-coated tissue culture plastic [P3]. Undifferentiated ES cells are expanded to ~80% confluence in a T75 flask (Iwaki), trypsinised and resuspended in N2B27 media [P2]. Cells are plated onto 9cm plates (Iwaki) that have been coated with a 0.1% gelatin solution (Sigma) for at least 10 mins then allowed to dry. As initial plating density is a crucial parameter for efficient neural induction, and can vary between ES cell lines, we routinely seed several different cell densities (0.8×10^6, 1×10^6 and 1.2×10^6) per plate. Culture medium is changed each day, in the process removing detached or dead cells. Under these conditions 50-80% of cells will undergo neural lineage specification within 4-5 days, and with overt neuronal differentiation detectable from day 5 onwards.

Conversion of heterogeneous progenitor cultures to homogeneous NS cell lines can be achieved as follows. Day 7 differentiated cultures are trypsinised and 2-3 x 10^6 cells are re-plated into an uncoated T75 flask in NS expansion media, comprising NS-A media (Euroclone) supplemented with L-glutamine, 2mM final (Gibco), modified N2 supplement (freshly prepared in house) [P2] and 10ng/ml of both mouse EGF (Peprotech) and human FGF-2 (Peprotech). Expansion media can be stored at 4°C for up to 4 weeks. Within 2-3 days the flask will contain many thousands of floating aggregates in suspension culture (absolute number varies according to efficiency of initial ES cell differentiation). Cell aggregates are harvested by mild

centrifugation or allowed to settle under gravity in a 30ml universal tube for 10mins. This step removes debris and dead cells, thus enriching for NS cell founders, and ensures complete media exchange. Cells are replated in 10ml of fresh NS expansion medium onto a gelatin coated T75 flask (Iwaki). After a further 3-7 days, cell aggregates will attach to the flask and shortly thereafter cells outgrow with characteristic bipolar NS cell morphology. Following extensive outgrowth of cells (a further 3-4 days) the entire population is trypsinised and re-plated as single cells onto a gelatin coated T75 flask in expansion medium. Cells grow very rapidly (doubling time ~25hrs) and remain adherent. Within several passages residual differentiated and blast cells are eliminated (monitored by GFAP and TuJ1 immunostaining) and cultures are uniformly positive for NS cell markers.

For 46C ES cells (*Sox1*-GFP-IRES-pac knock-in) [P4] a transient selection with puromycin (0.5□g/ml) can be used to eliminate non-neural cells and derive NS cells via continuous adherent culture. 46C ES cells (1 x 10^6) are plated in N2B27 media onto a gelatin coated 9cm dish to induce neural commitment. Six days later puromycin is added for 48hrs. The enriched Sox1 expressing cell population (~ 3-5 x 10^6) is then re-plated in N2B27 medium containing EGF (10ng/ml) and FGF-2 (10ng/ml) on gelatin coated 9cm plates. The initially morphologically heterogenous Sox1 expressing population progressively acquires a Sox1 negative character and after 3-4 passages uniform NS cell morphology and marker expression.

Derivation of mouse NS cell lines from foetal CNS and neurospheres

Cell clusters form in suspension upon dissociation of foetal E16.5 cortex and primary culture in NS cell expansion media. These primary aggregates can be readily converted into adherent NS cell lines by plating onto gelatin-coated substrate in expansion medium. In order to promote attachment, it is important that debris/dead cells are first effectively removed by sedimentation and medium is exchanged completely. Cell aggregates will attach and outgrow over 2-5 days. Outgrowing cells can subsequently be trypsinised to single cells, re-plated and propagated in NS cell expansion medium. From passaged foetal neurospheres [P5], NS cells can

conveniently be established by dissociation and plating directly on gelatin coated plastic in NS expansion medium. During the initial few passages the derived NS lines have a tendency to aggregate, detach from the flask and re-form neurospheres, particularly if the cell density becomes high. Cultures should therefore be passaged at or below 50% confluence. The tendency to spontaneously aggregate is variable, but is generally reduced upon further passaging, or through establishment of clonal cell lines.

Passage and expansion of NS cells

Once established, NS cells are propagated in NS expansion media. NS cells are grown on gelatin coated plates/flasks and are routinely split 1 in 2 to 1 in 5. NS cells have a doubling time of around 25hrs. Cells are passaged using trypsin/EDTA or through incubation with calcium magnesium-free PBS (Sigma). For establishment of clonal lines, single cells can be deposited in gelatin-coated microwells in expansion medium. Less rigorously, cells can be plated at very low density, 1000 cells per 9cm dish. Colonies appear within two weeks and can be picked and expanded.

Cryopreservation and recovery of NS cells

NS cells are readily recovered following freezing/thawing. Routinely we trypsinise a 60-90% confluent T75 flask, and resuspend the pellet into 1.5ml NS expansion media plus 10% DMSO. This is then split into 3x 1ml cryotubes (Nunc) and stored at -80°C. NS cells are recoverable following more than 6 months storage in these conditions. For long term storage frozen vials are transferred to liquid nitrogen. NS cells are thawed by rapidly bringing the vial to 37°C followed by transfer to 10ml of pre-warmed NS expansion media. Cells are pelleted and then resuspended in fresh expansion media to remove DMSO. Cell recovery following cryopreservation is >95% for NS cells.

Astrocyte and neuronal differentiation of NS cells

Rapid differentiation of NS cells to GFAP positive astrocytes occurs within 2 days of exposure of NS cells to BMP4 (10ng/ml) or 1% FCS in NS-A (with N2, withou

EGF/FGF) on gelatin coated flasks/plates. Cell density is not a crucial parameter for astrocyte differentiation.

For neuronal differentiation NS cells are harvested using Accutase (Sigma) or calcium/magnesium-free PBS to detach cells and 0.5-1.0 x 10^4 cells are re-plated into each well of a poly-ornithine/laminin coated 4-well multidish plate (Nunc) in NS-A medium supplemented with FGF-2 (5ng/ml), modified N2, and B27 (Gibco). We find that the NS-A basal medium is more permissive for neuronal differentiation, than other basal media. A half volume of medium is replaced every 2-3 days to maintain conditioning of medium. After 7 days in these conditions we exchange media to NS-A mixed with Neurobasal medium (Gibco) in a ratio of 1:1 supplemented with 0.25x N2 plus B27 and without EGF or FGF. This formulation promotes further neuronal differentiation and maturation. For longer term cultures of neurons (beyond 14 days) we exchange media to Neurobasal supplemented with B27 without N2 in the presence of BDNF (10ng/ml).

Culture and neuronal differentiation of human NS cells

Human NS cells are expanded on 0.1% gelatine (Sigma) coated flasks/plates in expansion media as for mouse NS cells additionally supplemented with 100U/ml recombinant human leukaemia inhibitory factor (LIF). Cells have a doubling time of approximately 1 week. They are passaged with trypsin once reaching ~30% confluence and split 1 in 2. Overgrowth should be avoided to maintain monolayer culture and prevent aggregation and detachment.

For neuronal differentiation, human NS cells are harvested using Accutase (Sigma) and around 1x10^4 cells re-plated into each well of poly-ornithine /laminin (Sigma) coated 12- well plate (Iwaki) in expansion media. Cells are expanded until they reach around 80% confluent. Neuronal differentiation is induced by removing EGF and LIF from expansion media. After 7 days in the absence of EGF and LIF, exchange media to NS-A mixed with Neurobasal media in a ratio of 1:1 (Gibco) supplemented with 0.5X N2, B27, FGF2 (5ng/ml), and BDNF (10ng/ml). After a further 7 days in these

conditions, media is switched to Neurobasal media supplemented with B27 and BDNF (10ng/ml) without N2 or FGF-2. Half the medium is exchanged every 2 or 3 days as for mouse NS cells throughout this protocol. After a further 10 days cells of neuronal morphology immunoreactive with TuJ1 and MAP2 represent up to 40% of total cell numbers. Significant numbers of astrocytes are also generated, in contrast to the mouse NS cell protocol for neuronal differentiation in which few GFAP positive cells emerge.

The invention thus provides methods and media for obtaining and maintaining neural stem cells of many species in a symmetrically-dividing, undifferentiated state. The invention has been carried out using cells obtained or extracted from ES cell, foetal and adult sources. In all cases the resultant cells obtained by following the methods described herein look and behave substantially the same; they all can be maintained in high purity cultures over high numbers of, e.g. hundreds of, doublings with a high proportion of the cells retaining the ability to form neurons and glia. Specifically, NS cells have been successfully obtained from human (ES, foetal CNS), mouse (ES, foetal CNS, adult CNS) and rat (foetal CNS). Mouse neural stem cells have been grown in culture in pure populations and after being transplanted grew without forming tumours but differentiated *in vivo*.

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Protocol References

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CLAIMS

1. A method of promoting the symmetrical division of neural stem (NS) cells, comprising culturing said cells attached to a substrate in a medium containing:-

   (a) an activator of a signalling pathway downstream from a receptor of the EGF family; and

   (b) an activator of a signalling pathway downstream from an FGF receptor.

2. A method according to Claim 1, wherein the activator of a signalling pathway downstream from a receptor of the EGF family is an agonist of an EGF receptor.

3. A method according to Claim 2, wherein the agonist is selected from the group consisting of EGF, TGF-α, amphiregulin, heparin binding-EGF, epiregulin, betacellulin, neuregulins 1, 2, 3 or 4, and Cripto-1.

4. A method according to any of Claims 1-3, wherein the activator of a signalling pathway downstream from an FGF receptor is an agonist of an FGF receptor.

5. A method according to Claim 4, wherein the agonist is FGF-2.

6. A composition, comprising:-

   neural stem cells;

   an activator of a signalling pathway downstream from a receptor of the EGF family; and

   an activator of a signalling pathway downstream from an FGF receptor.

7. A composition according to Claim 6, wherein at least 80% of said neural stem

cells are symmetrically-dividing neural stem cells.

8. A composition according to Claim 6 or 7, wherein the neural stem cells are characterized in that they are positive for the expression of RC2, 3CB2 , BLBP and Sox-2.

9. A composition according to Claim 8, wherein the neural stem cells are further characterized in that they are positive for the expression of at least one of:-

GLAST, Pax-6, the neural precursor markers nestin or vimentin, the LewisX antigen, Musashi-1 or prominin.

10. A composition according to Claim 8 or 9, wherein the neural stem cells are further characterised in that they are negative for the expression of at least one of:-

Oct4 or Nanog.

11. A composition according to any of Claims 6 to 10, wherein the neural stem cells are positive for the expression of Sox-2, and negative for the expression of Sox-1.

12. A composition according to any of Claims 6 to 11, wherein the neural stem cells are free from exogenous nucleic acid encoding an oncogene, or wherein said neural stem cells are not immortalised stem cells.

13. A composition according to any of Claims 6 to 12, wherein no more than 1% of the cells in the composition are positive for the expression of markers for mature astrocytes, neurons or oligodendrocytes.

14. A population of neural cells obtainable by the method of any of Claims 1-5,

wherein at least 80% of said cells are symmetrically-dividing neural stem cells.

15. A population of neural cells, obtainable by the method of any of Claims 1-5, wherein the neural stem cells in the population are characterised in that they are positive for the expression of markers RC2, 3CB2 and BLBP.

16. A population of neural cells according to Claim 15 wherein the neural stem cells in the population are further characterised in that they are positive for the expression of at least one of:-

GLAST, Pax-6, the neural precursor markers nestin or vimentin, the LewisX antigen, Musashi-1 or prominin.

17. A population of neural cells according to Claim 15 or 16 wherein the neural stem cells in the population are further characterised in that they are negative for the expression of at least one of:-

Oct4 or Nanog.

18. A population of neural cells according to any of Claims 14 to 17, wherein the neural stem in the population cells are positive for the expression of Sox-2, and negative for the expression of Sox-1.

19. A population of neural cells according to any of Claims 14 to 18, wherein the neural stem cells in the population are free from exogenous nucleic acid encoding an oncogene, or wherein the neural stem cells are not immortalised stem cells.

20. A population of neural cells according to any of Claims 14 to 19, wherein no more than 1% of the cells are positive for the expression markers for mature

astrocytes, neurons and oligodendrocytes.

21. A method of preparing a neural stem cell, comprising:-

culturing neural stem cells according to the method of any of Claims 1-5, thereby obtaining a culture of neural stem cells, and

isolating a neural stem cell from said culture.

22. A neural stem cell obtainable by the method of Claim 21.

23. A neural stem cell obtainable by the method of Claim 21, wherein the cell is characterised in that it is positive for the expression of the markers RC2, 3CB2 and BLBP.

24. A neural stem cell according to Claim 23, wherein the cell is further characterised in that it is positive for the expression of at least one of:-

GLAST, Pax-6, the neural precursor markers nestin or vimentin, the LewisX antigen, Musashi-1 or prominin.

25. A neural stem cell according to Claim 23 or 24, wherein the cell is further characterised in that it is negative for the expression of at least one of:-

Oct4 or Nanog.

26. A neural stem cell according to any of Claims 22 to 25, wherein the cell is positive for the expression of Sox-2, and negative for the expression of Sox-1.

27. A neural stem cell according to any of Claims 22 to 26, wherein the cell is free from exogenous nucleic acid encoding an oncogene, or wherein the cell is not

an immortalised stem cell.

28. A method of establishing a neural stem cell line, comprising:-

(a) obtaining a single neural stem cell,

(b) culturing the neural stem cell according to the method of any of Claims 1-5,

and thereby obtaining a population of neural stem cells.

29. A method according to Claim 28, wherein the population of neural stem cells is a clonal population, and all of the cells are progeny of a single neural stem cell.

30. A method according to Claim 28 or 29 , wherein the single neural stem cell is obtained according to the method of Claim 21.

31. A cell line, wherein the cells are maintained in the presence of an activator of a signalling pathway downstream of a receptor of the EGF receptor family, and an activator of a signalling pathway downstream of an FGF receptor.

32. A cell line according to Claim 31, wherein the cell line is clonogenic.

33. A cell line obtainable by the method of any of Claims 28 to 30, wherein the cells are characterised in that they are positive for the expression of the markers RC2, 3CB2, Sox-2 and BLBP.

34. A cell line according to Claim 33, wherein the cells are further characterised in that they are positive for the expression of at least one of:-

GLAST, Pax-6, the neural precursor markers nestin or vimentin, the LewisX antigen, Musashi-1 or prominin.

35. A cell line according to Claim 33 or 34, wherein the cells are further characterised in that they are negative for the expression of at least one of:-

Oct4 or Nanog.

36. A cell line, wherein the cells are characterised in that they are positive for the expression of the markers RC2, 3CB2, Sox-2 and BLBP.

37. A cell line according to Claim 36, wherein the cells are further characterised in that they are positive for the expression of at least one of:-

GLAST, Pax-6, the neural precursor markers nestin or vimentin, the LewisX antigen, Musashi-1 or prominin.

38. A cell line according to Claim 36 or 37, wherein the cells are further characterised in that they are negative for the expression of at least one of:-

Oct4 or Nanog.

39. A cell line according to any of Claims 36 to 38, wherein the cells are positive for the expression of Sox-2, and negative for the expression of Sox-1.

40. A cell line according to any of Claims 36 to 39, wherein no more than 1% of the cells are positive for the expression of the markers for mature astrocytes, neurons or oligodendrocytes.

41. A cell line according to any of Claims 36 to 40, wherein the cells are free from exogenous nucleic acid encoding an oncogene, or wherein the cells are not

immortalised cells.

42. A cell line according to any of Claims 31 to 41, wherein the cell line is a neural stem cell line.

43. Use of an activator of a signalling pathway downstream from a receptor of the EGF family for promoting the symmetrical division of neural stem cells in culture.

44. Use of a combination of:-

(a) an activator of a signalling pathway downstream from a receptor of the EGF family; and

(b) an activator of a signalling pathway downstream from an FGF receptor

for promoting the symmetrical division of neural stem cells in adherent, serum free culture.

45. A method of obtaining and maintaining a transfected population of symmetrically-dividing neural stem cells, comprising:-

(a) transfecting ES cells with a construct encoding a selectable marker A, wherein said selectable marker A is expressible under the control of a neural precursor cell-specific promoter;

(b) promoting differentiation of the ES cells to neural precursor cells;

(c) selecting for neural stem cells that express the selectable marker A; and

(d) culturing the selected cells according to the method of any of Claims 1-5.

46. A method according to Claim 45 wherein the selectable marker A encodes antibiotic resistance or a cell surface marker.

47. A method according to Claim 45 or 46, wherein the neural precursor cell-specific promoter is selected from the group consisting of Sox-1, Sox-2, Sox-3 and BLBP.

48. A method according to any of Claims 45 to 47, wherein the steps of the method are carried out in the order (a), (b), (c), (d).

49. A method of promoting the differentiation of ES cells into neural precursor cells, comprising:-

converting ES cells into neural progenitors by (1) monolayer culture or (2) embryoid body differentiation, and

culturing the neural progenitors in NSA medium.

50. A method according to claim 49 wherein the NSA medium comprises glucose and HEPES.

51. A method of preparing and maintaining a population of symmetrically-dividing neural stem cells, comprising:-

obtaining a population of neural precursor cells according to the method of Claim 49 or 50; and

culturing said population according to a method of any of Claims 1-5.

52. An *in vitro* method for assaying the effect of a factor on the differentiation of

an ES cell into a neural stem cell, comprising:-

culturing an ES cell according to the method of any of Claims 49 to 51 in the presence of said factor.

53. An *in vitro* method for assaying the effect of a factor on the differentiation of a neural stem cell, comprising:-

contacting a neural stem cell as present in the compositions of Claims 6-13, the populations of Claims 14-20, the cell lines of Claims 31-42, or a single neural stem cell of Claims 22-27, with the factor.

54. An *in vitro* method according to Claim 52 or 53, wherein the factor can be either an inductive or a blocking factor.

55. Use of neural stem cells as present in the compositions of Claims 6-13, the populations of Claims 14-20, the cell lines of Claims 31-42, or a single neural stem cell of Claims 22-27, for the manufacture of a preparation for the treatment of neurodegenerative diseases and brain injuries.

56. A method of treating neurodegenerative diseases and brain injuries comprising the transplantation of neural stem cells as present in the compositions of Claims 6-13, the populations of Claims 14-20, the cell lines of Claims 31-42, or a single neural stem cell of Claims 22-27, into a patient.

57. A method of transfecting neural stem cells as present in the compositions of Claims 6-13, the populations of Claims 14-20, the cell lines of Claims 31-42, or a single neural stem cell of Claims 22-27, comprising:-

(a) transfecting the neural stem cells with a construct encoding a selectable marker B, wherein said selectable marker B is expressible under the control

of a tissue-specific promoter; and

(b) selecting for neural stem cells that express the selectable marker B.

58. A method according to Claim 57 wherein the selectable marker B encodes antibiotic resistance or a cell surface marker.

59. A neural stem cell as present in the compositions of Claims 6-13, the populations of Claims 14-20, the cell lines of Claims 31-42, or a single neural stem cell of Claims 22-27, further comprising a selectable marker B.

60. A composition comprising neural stem cells, wherein the neural stem cells are in an adherent culture and at least 80% of the cells in the composition are neural stem cells.

61. A composition according to claim 60, wherein at least 90% of the cells in the composition are neural stem cells.

62. A composition according to claim 60 or 61, wherein the neural stem cells have been passaged at least 30 times.

63. A composition according to claim 62, wherein the neural stem cells have been passaged at least 60 times.

64. A method of obtaining a neural stem cell, comprising:
(i) obtaining a multipotent or pluripotent stem cell capable of differentiating into a neural stem cell,
(ii) culturing the cell of (i) in medium which is non-permissive for pluripotent cells and in the presence of (a) an agonist of a signalling pathway downstream of a receptor of the EGF family and (b) an agonist of a signalling pathway downstream of a receptor of the FGF family.

65. A method according to claim 64, wherein the stem cell is a pluripotent stem cell.

66. A method according to Claim 65, wherein the pluripotent stem cell is an ES cell or an EG cell.

67. A method according to any of claims 64 to 66, comprising culturing the cell in the presence of an agonist of the EGF receptor and an agonist of the FGF receptor.

68. A method according to any of claims 64 to 67, further obtaining a neuron, astrocyte or oligodendrocyte from the neural stem cell.

69. A method of nuclear reprogramming, wherein the donor nucleus is from a neural stem cell according to any of claims 22 to 27, 31 to 42, 59 and 60 to 63 or obtained according to the method of any of claims 1 to 5, 21, 28 to 30, 45 to 51 and 64 to 68.

70. A method of nuclear reprogramming, comprising:-

- obtaining a donor cell;
- obtaining a recipient cell;
- transferring the nucleus of the donor cell into the recipient cell, wherein the donor cell is (i) a neural stem cell according to any of claims 22 to 27, 31 to 42, 59 and 60 to 63, or (ii) a cell obtained according to the method of any of claims 1 to 5, 21, 28 to 30, 45 to 51 and 64 to 68; and
- culturing the cell so as to reprogram the donor cell nucleus, thereby obtaining a reprogrammed cell.

71. A method according to claim 70, comprising removing the nucleus of the recipient cell, optionally prior to transfer of the donor cell nucleus and optionally after

transfer of the donor cell nucleus.

72. A method according to claim 70 or 71, comprising genetically manipulating the donor cell nucleus.

73. A method according to claim 72, comprising introducing a disease-causing genetic sequence or a putative disease-causing genetic sequence into the donor cell nucleus.

74. A method according to claim 73, comprising:-
- culturing the reprogrammed cell so as to obtain a plurality of cells derived from the reprogrammed cell; and
- using the plurality of cells in an assay.

75. A method according to any of claims 70-74, comprising:-
- obtaining an animal comprising tissue derived from the reprogrammed cell; and
- carrying out an assay using the tissue.

76. A method according to any of claims 70 - 75, wherein the recipient cell Is an oocyte.

77. A method of reprogramming a cell, comprising:-
- providing a mixed population of cells;
- isolating from the mixed population a cell according to any of claims 22 to 27, 31 to 42, 59 and 60 to 63; and
- transferring the nucleus of the isolated cell to a recipient cell.

78. A method according to claim 77, comprising genetically manipulating the isolated cell prior to transferring its nucleus to the recipient cell.

79. A method according to claim 77 or 78, comprising:-

- culturing the isolated cell to obtain a clonal population of cells; and
- transferring the nucleus of one cell in the population to the recipient cell.

80. A method of cloning a non-human animal, comprising:-
(i) obtaining a neural stem cell from the non-human animal;
(ii) obtaining an oocyte of the same species as the non-human animal;
(iii) transferring the nucleus of the neural stem cell into the oocyte;
(iv) implanting the cell obtained in (iii) into a female of the same species.

81. A method according to claim 80, wherein the neural stem cell is (i) a neural stem cell according to any of claims 22 to 27, 31 to 42, 59 and 60 to 63, or (ii) a cell obtained according to the method of any of claims 1 to 5, 21, 28 to 30, 45 to 51 and 64 to 68.

82. A method of promoting the symmetrical division of neural stem (NS) cells substantially as hereinbefore described with reference to any of the examples.

83. A cell line substantially as hereinbefore described with reference to any of the examples.

84. A method of obtaining neurons, comprising (a) culturing neural stem cells in the presence of an agonist of an FGF receptor and in the absence of an agonist of an EGF receptor; and (b) thereafter, culturing the cells in the absence of an agonist of an FGF receptor and in the absence of an agonist of an EGF receptor.

85. A method according to claim 84, comprising plating cells in monolayer culture.

86. A method according to claim 84 or 85, comprising transferring NS cells to medium free of EGF but which contains FGF-2 and culturing them for a period of at least 2 days and them removing FGF-2 from the medium.

87. A method according to any of claims 84 to 86, wherein the neural stem cells are obtained according to the method of any of claims 1 to 5, 21, 28 to 30, 45 to 51 and 64 to 68.