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DATE : 25 November 2010

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Attn: Ms. Huang Qinyi

Dear Colleagues,

Post Grant Amendment of Singapore Patent No. 76376 [WO 99/53022]
(Singapore Patent Application No. 200005749-7)
Invention: Lineage Specific Cells And Progenitor Cells
Applicant: University of Edinburgh

We refer to your letter of 8 November 2010.

We submit herewith PF 99 and a copy of the new specification as amended for the present application.

We hope that the above is in order and look forward to receiving a Notification on this matter.

Yours faithfully,

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★ FICPI

LINEAGE SPECIFIC CELLS AND PROGENITOR CELLS

This invention relates to lineage specific cells and progenitor cells, methods of obtaining them and their uses. Pluripotent embryonic stem (ES) cells can be induced to differentiate in vitro into a mixture of cell types, comprising extraembryonic yolk sac and derivatives of all three embryonic germ layers. However, the disorganised and heterogeneous nature of the differentiation impedes manipulation and analysis of individual lineages. In particular, the invention provides a lineage-specific genetic selection technique to establish purified populations of neural precursors from differentiating ES cells.

Embryonic stem (ES) cells are non-transformed cell lines derived directly from the pluripotent founder tissue in the mouse or human embryo, the epiblast (Evans and Kaufman, 1981; Martin, 1981; Brook and Gardner, 1997; Thomson et al, 1998; Shamblott et al, 1998). ES cells can be propagated and extensively genetically manipulated whilst retaining the capacity for multilineage differentiation, both in vivo and in vitro (Robertson, 1987). The differentiation of ES cells in culture closely reflects differentiative events in the embryo. In principal, therefore, ES cells provide in vitro access to the instructive and selective processes by which cellular diversification is generated in the mammalian embryo (Smith, 1992).

Multilineage differentiation of ES cells can be initiated by simple aggregation (Martin and Evans, 1975; Doetschman et al., 1985). The aggregates form structures known as embryoid bodies, differentiation of which mirrors aspects of peri- and early post-implantation mouse embryogenesis (Martin et al., 1977; Doetschman et al., 1985). A diverse array of cell types are subsequently found in outgrowths from embryoid bodies (Weiss and Orkin, 1996). Although representation of particular lineages can be diminished or enhanced by treatment with dimethyl sulphoxide or retinoic acid (RA), the differentiated products always consist of a mixture of cell types. This intrinsic disorganisation and complexity have limited the exploitation of in vitro ES cell differentiation for assignment of gene functions in developmental

pathways.

Several reports have documented the presence of neuronal cells and glia (Bain, 1995; Fraichard et al., 1995; Strubing et al., 1995; Okabe et al., 1996) in embryoid body outgrowths. This could be exploited to detect, characterise and manipulate factors that regulate neurogenesis and neuronal and glial differentiation. ES cells could also be used as a source of normal or genetically manipulated neural cells for biochemical and functional studies or for transplantation. The problem is that these objectives, however, are severely compromised by the abundance of non-neural cells in the cultures and because it is not currently possible to establish or maintain a culture predominantly containing neural cell progenitors.

The art thus fails to provide a reliable method of obtaining a substantially pure population of a cell of any selected lineage, and progenitor cells of a selected lineage in particular. The art also fails to provide assays for developmental and other effects of factors on progenitor cells or differentiated cells of a selected lineage.

The present invention aims to provide progenitor cells and/or differentiated cells of a selected lineage, assays for the effect of factors on such cells and uses of these cells, such as in transplantation. The present invention further aims to provide a genetic selection technique to eliminate non-neural cells from cultures and to enable purification from embryoid bodies of differentiation competent neural precursors.

In one embodiment, there may be provided a method for obtaining a culture of cells of a selected lineage having a combination of two steps. One step is to select for cells that express a gene characteristic of cells of that lineage. The other is to so culture cells that they tend to differentiate into or proliferate as cells of that lineage. The effect is to yield a more highly purified culture of the selected-lineage cells than is otherwise possible.

By way of example, one step of the combination is to select for cells that express

a gene known to be expressed uniquely in haematopoietic progenitor cells, and the other is to culture cells in medium containing a nutrient known to promote differentiation of cells into haematopoietic progenitors.

Accordingly, the invention provides a method for generating a culture that is purified or enriched in respect of cells of a selected lineage, comprising:

(i) introducing into a multipotential cell a selectable marker that is differentially expressed in cells of the selected lineage compared with its expression in other cells, wherein cells of the selected linage constitute a sub-set of the cells obtainable from the multipotential cell;

(ii) culturing the multipotential cell *in* vitro to induce differentiation of the multipotential cell into a cell of the selected lineage or into a mixture of cells including cells of the selected lineage; and

(iii) selecting for cells of the selected lineage according to differential expression of the selectable marker introduced step (i)

wherein said multipotential cell is not of human embryonic origin.

The method is suitable for obtaining cells, optionally progenitors, of the selected lineage at high purity. Whilst following the art method for inducing differentiation of ES cells into a culture that includes neural cells can provide a maximum of about 50% neural progenitors, using the technique of the invention a purity in excess of 70% can be obtained, and in a specific embodiment described below the purity is substantially 100%.

Step (ii) results in a pure population of cells or a mixed culture at least slightly purified or enriched in respect of desired cells and can suitably be carried out by culturing the multipotential cell in the presence of a factor that induces differentiation of the cell into a progenitor cell of the selected lineage. By way of example, a mitogen specific for neural progenitors is fibroblast growth factor. Reference herein to the term"factor"is not intended to be limited to protein or polypeptide factors but is intended to encompass any biologically active molecule

or potentially biologically active molecule.

The multipotential cell may be selected from embryonic stem (ES) cells, embryonic germ (EG) cells, embryonal carcinoma (EC) cells, a primary culture of foetal cells, a primary culture of post-natal cells and a primary culture of adult cells. The method may further comprise genetically modifying cells to delete, mutate, substitute or add genes in order to assay gene function in progenitor cells of the selected lineage or adapt a cell phenotype to render it more suitable for transplantation. The cells may thus be obtained by introducing a selectable marker into a multipotential cell line or a primary culture containing the cells of interest and then selecting out the cells of interest. The selectable marker is optionally introduced by transfection or viral infection via a transgenic animal from which the primary cultures are then established, suitably using the methods described in WO-A-94/24274.

The invention thus advantageously enables a highly enriched population of cells and in particular all lineages of progenitors of a chosen lineage to be obtained. In an example below the population obtained is substantially 100% pure making it possible to isolate a single cell of known progenitor lineage. The invention is of application to all lineages of cells, particularly progenitor cells. A selectable marker expressed in cells that express a Sox gene leads to neural progenitor cells; the CD34, CD44 and SCL genes are suitable for obtaining haematopoietic progenitors, and the Nkx 2.5 or GATA-4 gene for cardiac progenitors. For generating myogenic progenitors, Myo0 or myE5 are suitable. Using retinoic acid induces differentiation of ES cells into neural cells, DMSO induces differentiation into haematopoietic cells and absence of retinoic acid induces a population enriched in cardiac progenitors.

The method optionally further comprises:-

(iv) introducing into the multipotential cell a second selectable marker that is differentially expressed in progenitor cells or other cells of a selected sub-lineage compared with its expression in other cells, wherein cells of the selected sub-lineage are formed by differentiation of cells of the selected

progenitor lineage; and

(v) when a culture of progenitor cells of the selected lineage has been obtained, allowing or inducing differentiation of the cells and selecting for cells that express the second selectable marker.

This aspect of the invention further enhances the purity of the obtained culture of cells, and is of advantage in cases that cells of the selected lineage differentially express a gene but only at a level slightly different from non-desired cells.

In a preferred embodiment of the invention, described in more detail below, the selectable marker is a gene that codes for antibiotic resistance and selecting for those cells that express the selectable marker comprises introducing antibiotic into the culture. In use, application of the antibiotic selectively kills or ablates cells that do not express the marker, leaving behind a population of cells purified or enriched in respect of those expressing the antibiotic resistance, i.e. viable cells of selected lineage. At least two ways of introducing the selectable marker are known and suitable for the invention. The selectable marker may be introduced into the multipotential cell by targeted integration or gene trapping into a gene that is differentially expressed in progenitor cells of the selected lineage. Expression of the selectable marker is thereby operatively linked to a gene differentially expressed in a desired pattern. The selectable marker may also be introduced into the multipotential cell via random integration as a transgene wherein it is expressed under control of the regulatory elements of a gene that is differentially expressed in progenitor cells of the selected lineage.

The selectable marker may more generally be a marker that when expressed results in preferential survival of cells expressing the marker, with antibiotic resistance being one such example. In this instance the marker is expressed in cells of the selected lineage. The selectable marker may also be a marker whose expression results in preferential killing of cells expressing the marker. In this instance the marker is expressed in cells other than those of the selected lineage.

In a specific embodiment of the invention described in an example below, the multipotential cell is an ES, EC or EG cell and the method comprises inducing differentiation of the multipotential cell - one way is to form an embryoid body from the cell - and dissociating the cells. Trypsin is used in one example. It is an advantage that individual cells are thereby obtained. These are all exposed to the culture medium and not being attached to neighbouring cells are free of cell-to-cell influences that might affect the pattern of growth and / or differentiation of the cells, and hinder formation of progenitor cells according to the invention.

A culture that is purified or enriched in respect of ventral progenitor cells is obtainable according to the invention, wherein the selectable marker is differentially expressed in neural progenitor cells and the second selectable marker is differentially expressed in ventral progenitor cells. The second selectable marker for this is suitably differentially expressed in cells that express Pax 6.

A culture that is purified on enriched in respect of dorsal progenitor cells is obtainable according to the invention, wherein the selectable marker is differentially expressed in the neural progenitor cells and the second selectable marker is differentially expressed in dorsal progenitor cells. The second selectable marker for this is suitably differentially expressed in cells that express Pax 3.

In another embodiment, there may be provided a cell, preferably a progenitor, of a selected lineage, obtainable according to the method of the invention. Hitherto, preparations of progenitors were too impure for certainty as to whether any chosen cell was a progenitor cell. With culture according to the invention that can give rise to substantially 100% pure preparations of progenitors, isolation of a single progenitor is achieved.

In a further embodiment, there may be provided a composition comprising a plurality of cells, wherein a majority of the cells are progenitor cells of a selected lineage. Preferable, at least 60% of the cells are progenitor cells of the selected lineage. More preferably, at least 60% of the cells are neural progenitor cells. In addition, there may be provided

an isolated neural progenitor cell.

A significant application of the invention is in the field of assaying factors for the effects they may have on a selected progenitor. Accordingly, the invention still further provides an assay of the effect of a factor on a culture of progenitor cells of a selected lineage, comprising:

A (i) introducing into a multipotential cell a selectable marker that is differentially expressed in progenitor cells of the selected lineage compared with its expression in other cells, wherein the selectable marker is operatively coupled to the promoter of a gene that is differently expresses in cells of the selected lineage, and wherein progenitor cells of the selected lineage constitute a sub-set of the cells obtainable from the multipotential cell;

(ii) culturing the multipotential cell *in vitro* to induce differentiation of the multipotential cell into a cell of the selected lineage or into mixture of cells including cells of the selected lineage or to induce preferential survival, in a mixed culture of cells of the selected lineage;

(iii) selecting for progenitor cells of the selected lineage according to differential expression of the selectable marker introduced in step (i), and

B culturing the thereby obtained cells of selected lineage in the presence of the factor.

The assay method is preferably for assay of the effect of a factor on a culture of progenitor cells of selected lineage, wherein the selectable marker is differentially expressed in those progenitor cells. Reference to a"factor"in the assay, is intended to be a reference to any actual or potential biologically active molecule that may be introduced into culture of cells of the selected lineage, and is thus not intended to be limited to protein and polypeptide factors. The term is also intended to encompass a gene introduced or modified in a multipotential cell from which cells of the selected lineage are obtained. The assay can thus conveniently be used to assay for the effect

of a particular gene, as well as for the effect of polypeptide and/or protein factors or small molecules of interest.

The method can be used to assay whether the factor has a proliferative, maturation, toxic or protective effect on progenitor cells of the selected lineage, or whether a factor has a proliferative, maturation, cytotoxic or glial protective effect on neural progenitor cells or on other differentiated cells obtained following withdrawal of selection and differentiation of the progenitors.

It is an advantage of the assay method that it enables a population of terminally differentiated cells of selected lineage to be obtained and used for such assays as gene and drug discovery screens.

In yet a further embodiment, there may be provided a neural progenitor cell, or a culture comprising a majority of neural progenitor cells, for transplantation. In an example below, a neural progenitor cell of the invention has been successfully isolated and transplanted into the brain of a rat. The neural cell may optionally be a neuronal cell or a glial cell.

This transplantation can also be carried out using neuronal cells obtained from neural progenitors of the invention. A suitable method of generating purified neurons comprises obtaining a culture purified in respect of neural progenitors, using the method of the invention wherein the selectable marker is differentially expressed in cells that express a Sox gene, and culturing the progenitors obtained in the presence of medium suitable for differentiation of the progenitors into neurons. A suitable method of generating a purified glial cells comprises obtaining a culture purified in respect of neural progenitors, using the method of the invention wherein the selectable marker is differentially expressed in cells that express a Sox gene, and culturing the progenitors obtained in the presences of medium suitable for differentiation of the progenitors into glial cells.

In still a further embodiment, there may be provided a neural progenitor cell for transplantation to treat neurodegenerative disease or neuronal/brain injury, a neural progenitor cells for transplantation, obtainable from a cell selected from an ES cell, an EC cell, an EG cell a primary culture of foetal cells and a primary culture of post-natal cells, for

transplantation to treat neurodegenerative diseases or neuronal/brain injuries, a method of treatment of neurodegenerative disease or neuronal/brain injury comprising transplantation of a neural progenitor cell, and a method of preparing a neural progenitor cell or a differentiated progeny thereof for storage, comprising obtaining the cell in a method according to the invention and freezing the cell in the presence of a cryoprotectant, such as 10% dimethyl sulfoxide.

In an embodiment of the invention, a selection/reporter gene, βgeo, was integrated by homologous recombination into the sox2 gene, which is expressed uniformly in precursor cells in the neural plate and neural tube. Application of G418 to differentiating cultures of sox2-targeted cells resulted in the efficient isolation of viable βgeo-positive cells. These cells expressed markers of neuroepithelial precursor cells. They differentiated efficiently into networks of neuron-like cells that expressed a variety of neuronal markers. Thus, an in vitro system for genetic and molecular dissection of mammalian neural differentiation is provided by the invention, and also a route for the production of pure populations of normal or genetically modified neural precursors and neurons for functional studies including transplantation. Furthermore, the lineage selection approach of the invention is applicable to the isolation of other precursor populations from differentiating ES cell cultures.

In still yet a further embodiment, there is provided a method of amplifying a purified population of progenitor cells of a selected lineage, comprising

maintaining the cells in culture in the presence of

a mitogen; and

a growth factor.

The progenitor cells preferably comprise a gene coding for a selectable marker, which gene is differentially expressed in the progenitor cells compared with its expression in other cells, and the method further comprises selecting for cells that express the selectable marker. It is an option to maintain the culture over a plurality of generations and periodically select for cells that express the selectable marker.

Another option is to maintain the culture over a plurality of generations and continuously select for cells that express the selectable marker. Where the selectable marker is antibiotic resistance the method may thus comprise continuous culture in the presence of antibiotic.

The invention is now described with reference to the accompanying drawings in which:-

fig. 1 shows morphology and characterization of neurons and glia in day 4 cultures;

fig. 2 shows phase contrast picture of day 0 cells (2a - 4 hours following plating), and Sox1 antibody staining (2b) - scale bar is 50 microns;

fig. 3 shows a schematic diagram of lineage selection according to the invention;

fig. 4 shows culture enrichment for Sox2-positive cells by G418 selection;

fig. 5 shows expression of region-specific neural precursor markers;

fig. 6 shows Sox2 positive cells proliferate in vitro in response to FGF-2;

fig. 7 shows ES cell-derived neurons differentiated following Sox2-selection;

fig. 8 shows results of assay of effect of sonic hedgehog on neural progenitors; and

fig. 9 shows an ES cell-derived neuron after transplantation *in utero*.

In more detail, fig 1 shows morphology and characterization of neurons and glia in day 4 cultures. E14TG2a ES cells were induced to differentiate in aggregates for

8 days, plated on poly-D-lysine/laminin substrate in DMEM/F12 supplemented with N2. Cells were photographed alive with phase-contrast optics after 4 days in culture. The majority of the cells are neuronal-like, their neuritic processes connected into a cellular network. (a), (b) and (c) show cultures that were stained with anti-NFL, anti-MAP2, and anti-GFAP, respectively.

In fig 4, ES cells with a targeted insertion of βgeo into the Sox2 gene were induced to differentiate by culture as aggregates ("embryoid bodies"). After 4 days they were exposed to 10^{-6} M retinoic acid for a further 4 days. The aggregates were then dissociated and cells plated on poly-D-lysine and laminin-coated dishes in serum-free medium with N2 supplement. Cultures were unselected or exposed to G418 either for the final 48 hours of aggregate culture (b, f, h, i) or for 24 hours after plating (d):

a. Sox2-linked β-galactosidase expression in unselected culture 3 hours after plating.

b. Sox2-linked β-galactosidase expression in G418-selected culture 3 hours after plating.

c. Sox2-linked β-galactosidase expression in unselected culture 24 hours after plating.

d. Sox2-linked β-galactosidase expression in G418-selected culture 24 hours after plating.

e. Immunostaining with anti-Sox2 of unselected culture 3 hours after plating.

f. Immunostaining with anti-Sox2 of G418-selected culture 3 hours after plating.

g. DAPI counterstaining of panel e.

h. DAPI counterstaining of panel f.

i. Double-labelling with anti-nestin (green) and anti-Sox2 (red/orange) of G418-selected culture 3 hours after plating.

The scale bar indicates 100 μm for (a,b,c,d), 66.7 μm for (e,f, g, h) and 50μm for (i).

For fig 5, Sox2 expressing cells were selected with G418 for 2 days in EB cultures, cells were dissociated and allowed to attach to culture dishes for 3 hours before fixation. The expression for Pax3 (a), delta-1 (b), Mash-1 (c) and Math-4a (d) were detected by in situ hybridisation with specific anti-sense riboprobes; the presence of Pax6-expressing cells were detected by staining the culture with an anti-Pax6 antibody (e), culture e was double labelled with DAPI to localize nuclei of all cells (f). Scale bar, 100 μm.

For fig 6, following 8 days induction of neural differentiation, cells were dissociated by trypsinization, plated at low density, and cultured in DMEM/F12 supplemented with N2 medium in the presence of 10 ng/ml of bFGF and 200 μg/ml of G418. (a) overnight culture; (b), 4 day culture. Cell numbers increased around 10 fold over the culture period. Cells expanded in the presence of bFGF maintained Sox2 expression as shown via X-gal staining. Scale bar: 50 μm.

For fig 7, Sox2-expressing cells selected by 48 hours exposure to G418 during embryoid body culture were plated and grown in the absence of G418 in N2 medium for 48 hours (a and b) or 96 hours (c-e), or for 72 hours followed by a further 72 hours in Neurobasal medium plus B27 supplement and 2% horse serum (f-i) (Scale bar, 100 μm):

a,b. Double immunolabelling showing down-regulation of Sox-2 (a) in

newly differentiating cells expressing the neuronal marker β-tubulin 3 (b).

c. Immunostaining for neuronal marker β3-tubulin with propidium iodide counterstaining showing neuronal differentiation of >90% of cells.

d,e. Double immunolabelling for neuronal markers synapsin-I (d) and MAP2/Tau (e).

f. Immunostaining for GABA

g. Phase contrast image of field in f.

h. Immunostaining for glutamate.

i. Phase contrast image of field in h.

In fig. 8, CCE-sox2 ES embryoid bodies undergoing g418 selection were exposed to the indicated concentrations of recombinant sonic hedgehog protein. Cells were fixed and immunostained for the motorneuron marker islet 1/2 48 hours after dissociation.

In fig. 9, sox-2 expressing cells selected by exposure to g418 during embryoid body culture were dissociated, labelled with PKH26-GL (Sigma) and injected into the forebrain vesicles of E16 rat foetuses *in utero*. Pregnancy was then allowed to go to term. Pups were sacrificed on P2. Vibratome sections were prepared and examined by fluorescence microscopy. The figure shows a representative PKH26-GL labelled cell within the cortex exhibiting typical immature neuronal morphology. This study demonstrates that Sox2 selected cells can integrate and differentiate in the brain.

Materials and Methods

ES cell culture

ES cell lines used in this study were: E14TG2a (Hooper et al., 1987), CGR8 (Mountford et al., 1994) and CCE-Sox2, a derivative of CCE (Bradley et al., 1984) in which one copy of the Sox2 gene has been disrupted by homologous recombination. All ES cell lines were maintained in gelatin-coated tissue culture plastic in Glasgow modified Eagle's medium (GMEM) supplemented with 10^{-4} 2-mercaptoethanol, 10% fetal calf serum (FCS) and 100 U/ml LIF (Smith, 1991).

Induction of differentiation.

The basic protocol is based on that described (Bain, 1995). ES cells were lightly trypsinized into small clumps and allowed to aggregate in suspension culture in the absence of LIF. After 4 days culture, all trans-retinoic acid (RA) was added at a concentration of 10^{-6}M. After a further 4 days aggregates were dissociated by incubation with trypsin and trituration. Cells were seeded at 3×10^5 cells/well into 4-well dishes (Nunc) coated with poly-D-lysine and laminin. Culture medium was serum-free DMEM/F12 (50/50) supplemented with N2 and, where specified, B27 (Gibco-BRL) plus 2% horse serum.

Substrate preparation.

Tissue culture plates were precoated with poly-D-lysine (PDL, 30-70 kDa, Sigma) for 20 min at a concentration of 10 μg/ml in PBS. Excess PDL was withdrawn and the plates were rinsed with PBS three times before coating with laminin (Sigma) at a concentration of 2-10 ?g/ml in PBS overnight at 4 °C.

Immunocytochemistry

Staining for Sox1 and Sox2 was carried out on cultures fixed in MEMFA (4% formaldehyde, 100 mM MOPS pH 7.4, 20 mM EGTA, 1 mM $MgSO_4$) for 1 hour. To stain cells with antibodies against GABA and glutamate, cells were fixed with 1% glutaraldehyde in PBS (Turner, Neurochemistry). For detection of other intracellular antigens, cultures were fixed in 4% paraformaldehyde in PBS for 15 min. Fixed cells were rinsed with PBS, incubated with blocking buffer (PBS, 1 mg/ml BSA, 0.1% Triton X-100, and 1% goat serum) for 30 min followed by

incubation with primary antibodies in blocking buffer overnight at 4 °C. Cells were then rinsed with PBS, and incubated with a species specific secondary antibody in blocking buffer for 1 hr. Cultures were washed with three changes of PBS, mounted with Vectashield mounting media (Vector) and examined under a fluorescent microscope.

Double labelling experiments were performed by simultaneously incubating cells in appropriate combinations of primary antibodies, followed by incubation with non-cross-reactive secondary antibodies. In some experiments, cultures were counter stained with propidium iodide or DAPI at concentrations of 1ng/ml and 5 μg/ml respectively.

The following dilutions were used for primary and secondary antibodies: rabbit anti-Sox1 and anti-Sox2 (1:500), rabbit anti-GABA (1:2000, Sigma), rabbit anti-glutamate (1:4000, Sigma), mouse anti-β-tubulin 3 (1:200, Sigma); mouse anti-NF68 (1:400, Sigma); rabbit anti-MAPs, which reacts with MAP2 and Tau (1:400, Sigma); mouse anti-GFAP (1:400, Sigma), mouse anti-synapsin-I (1:50, Chemicon), mouse anti-nestin (1:50, DSHB), mouse anti-Pax6 (1:10, ---). Cy3-conjugated goat anti-mouse IgG (1/50, Jackson ImmunoResearch), FITC-conjugated goat anti-mouse IgG (1:100, Sigma), FITC-conjugated goat anti-rabbit Ig (1:100, Sigma), TRITC-conjugated goat anti-rabbit Ig (1:50, Sigma).

In situ hybridization of cultured cells.

The protocol is based on that of (Rosen and Beddington, 1993) adapted for cultured cells. Briefly, cultures were fixed in 4% paraformaldehyde and permeabilized at -20°C in 100% methanol. The cells were then rehydrated through a series of methanol and finally put into PBS with 0.1% Tween-20. Prehybridization was performed for 1-4 hr in hybridization buffer (50% ultrapure formamide, 5 x SSC, pH 4.5, 50 mg/ml heparin, 100 μg/ml herring sperm DNA and tRNA, 0.1% Tween-20), the digoxigenin (DIG)-labeled probes were added at 1 μg/ml overnight at 70 °C. Washes the following day were three times for 30 min at 65oC in wash buffer (50% formamide, 2 x SSC, 0.1% Tween-20), three 5 min washes in TBST

(137 mM NaCl, 25 mM Tris-HCl, pH 7.6, 3 mM KCl, 0.1% Tween-20) and 1 hr block in 10% serum/TBST. Cells were then incubated overnight at 4°C with alkaline phosphatase-coupled anti-DIG antibody (1:2000, Boehringer-Mannheim). The following day cells were washed three times 1 hr with TBST and three times for 10 min with alkaline phosphatase buffer (APB; 100 mM NaCl, 100 mM Tris pH 9.5, 50 mM $MgCl_2$, 0.1% Tween-20). The alkaline phosphatase staining reaction was allowed to proceed for 3 hr to overnight with 4.5 µl/ml NBT an 3.5 µl/ml BCIP in APB (Boehringer).

DIG-labeled RNA probes

The murine cDNAs used as templates for riboprobes were a 519 bp Pax3 fragment (provided by Dr Rosa Beddington), a 700 bp Delta-1 clone (provided by Dr Domingos Henrique), a 670 bp Mash-1 fragment and a 1.5 kb Math-4A cDNA (both were provided by Dr Francois Guillemot). DNAs were linearized and RNA synthesis was directed using T7, T3 or SP6 RNA polymerase, including DIG-labeled nucleotide mix as recommended by the suppliers (Boehringer). Products were analyzed on a 0.8% agarose gel and approximately 1 µg/ml of the DIG-labeled antisense RNA was used for hybridization of cells.

Detection of β-galactosidase

Cells were fixed in fix buffer (0.2% glutaraldehyde, 0.1 M phosphate buffer pH 7.2, 2 mM MgCl2, 5 mM EGTA) for 10 min at 4°C and washed three times with wash buffer (0.1 M phosphate buffer pH 7.2, 2 mM $MgCl_2$). The cells were then incubated at 37°C overnight with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), 4 mM potassium fericyanide, 4 mM potassium ferrocyanide in wash buffer (Beddington et al., 1989) .

Results

Efficient generation of neurons and glia from ES cells

Induction of neural differentiation was based on published methods (Bain, 1995; Fraichard et al., 1995) in which ES cells are aggregated in suspension to form embryoid bodies, exposed to retinoic acid, and then allowed to reattach and

outgrow on a substrate. Under these conditions neuronal like cells become evident in the outgrowths after several days accompanied by a variety of other cell types. We introduced two variations into the protocol which enhanced the final representation of neuronal cells. First, the embryoid bodies were dissociated before plating. This results in a homogeneous dispersion and immediately terminates inductive and selective effects within the embryoid bodies. Second, cells were plated in a defined neuronal culture medium (DMEM/F12 plus N2) on substrates coated with poly-D-lysine and laminin which support attachment and outgrowth of neuronal cells. Each of these procedures had an additive effect on the proportion of neural cells in the cultures. When combined, more than 50% of viable cells 4 days after plating had extended processes and where immunoreactive for neuronal markers neurofilament light and heavy chains, MAP2/tau, or β tubulin III (Figure 1 and data not shown). A smaller proportion of cells, around 20%, expressed the astrocyte marker GFAP (not shown). Significantly this observation is not cell line specific as comparable results were obtained with three independent ES cell lines and several subclones.

Non-neural cell types remained in these cultures, however, often identifiable as large, flat non-refractile cells. If, at any point, DMEM/F12 plus N2 was supplemented with serum or mitogens (FGF-2 or B27 supplement), these non-neural cells expanded rapidly and progressively became the predominant cell type.

Detection of Sox1/Sox2 expressing neural progenitors

The generation of a large proportion of differentiated neurons and glia suggested that neural precursor cells might be detectable at an earlier time point in the differentiation protocol. Figure 2a shows a representative culture 4 hours following plating. At this stage, cells appear to be morphologically undifferentiated. The soma of the majority of cells are small, elongated or oval shaped. Some cells have short processes similar in length to their cell bodies. These morphological features are similar to primary neural precursors reported previously (Kalyani et al., 1997). These cells do not express detectable NF-L or GFAP 3 hours after plating. After overnight culture, less than 1% of the population were positive for either of these

two markers.

The cultures were examined for presence of the intermediate filament, nestin (Lendahl et al., 1990), which is expressed in neuroepithelial cells. Almost all cells were nestin-positive, however, presumably because the expression of nestin is not strictly restricted to neuroepithelium [Hockfield, 1985 #868]. We therefore sought a more specific marker. The SRY-related transcription factor Sox1 is confined to the neuroepithelium of the neural plate and dividing neural progenitors in the early mouse embryo (Pevny, unpublished data). The related Sox2 gene is expressed in an overlapping pattern that also encompasses floor plate and early neural crest cells. Immunostaining of freshly plated cells with antibodies raised against Sox1 and Sox2 revealed that 40-50% of the cells were positive (Fig. 2b and Fig. 3e). These cells likely correspond to neural progenitors.

Selection and purification of Sox2-expressing neural progenitor cells.

To isolate the neural progenitor pool we used ES cells in which the bifunctional selection marker/reporter gene βgeo has been integrated into the Sox2 gene by homologous recombination (ref). When induced to differentiate as described above, approximately 50% of these cells stained for β-galactosidase activity (Figure 2 and Table 1), consistent with the proportion of cells that express Sox2 protein. We applied G418 to the differentiating cultures to eliminate Sox2-negative non-neural cells (Figure 3).

G418 (200μg/ml) was added after retinoic acid induction, either during embryoid body culture or upon plating. In both conditions appreciable cell killing was evident. Crucially, however, large numbers of cells survived that exhibited typical neuroepithelial morphology. Over 90% of these cells gave prominent β-galactosidase staining (Figure 4). Concordance with Sox2 protein expression was confirmed by immunostaining (Figure 4, Table 1). The related HMG-box factor Sox1, an exclusive marker of pluripotential neural plate stage cells and of CNS-restricted precursors in the neural tube, was detectable in the great majority of cells. Almost all viable cells expressed nestin.

Sox2-selected cells express markers of neural progenitor specification.

The developmental organisation and subdivision of the central nervous system is underpinned by the temporal and spatial patterning of gene expression (Tanabe and Jessell, 1996). In order to gauge the diversity of neural differentiation that could be achievable from ES cells in the absence of embryonic axial organisation, we have begun to examine expression of key determinants, Pax genes and neurogenic bHLH transcription factors.

The paired box transcription factors Pax3 and Pax6 are found in dividing neural precursors throughout the length of the embryonic neural tube. Pax3 is initially expressed in the neural plate and subsequently becomes confined to the dorsal half of the neural tube (Liem et al., 1995). Pax6 is expressed predominantly in the ventral region of the neural tube (Walther and Gruss, 1991; Tanabe and Jessell, 1996). Widespread expression of both pax genes was found in sox-2 selected cells analysed on the day of plating (Figure 5 and Table 2). This suggests that ES cell-derived neural precursors can acquire both dorsal and ventral identities.

The bHLH genes, mash1 and math4A (neurogenin) show a more restricted localisation to subsets of neural progenitors. Expression of each was readily detected in sox2-selected cultures, but in significantly fewer cells than the pax gene products (Figure 5, Table 2). It is likely that the expression of mash1 and math4A specifies distinct sub-populations of progenitors, as the distribution of these two transcription factors is mutually exclusive in most CNS regions (Gradwohl et al., 1996).

Two early markers of neuronal differentiation were also examined. The notch ligand delta1, which is found in committed cells immediately prior to neuronal differentiation (ref), was expressed in only 1-2% of cells immediately after plating, indicating that the majority of the cells are not yet committed to terminal differentiation (see also Discussion). Expression of the LIM homeodomain protein islet-1/2, an early marker of differentiation of motor neurons and ventral interneurons (Ericson et al., 1992), was examined in Sox2-selected cultures 48

hours after plating. Reproducibly 1-2% of cells were immunoreactive at this stage (not shown).

Sox2-selected cells proliferate in response to FGF-2.

Several studies have presented evidence that basic fibroblast growth factor (FGF-2) can support proliferation of primary neural progenitors and immortalised progenitor cell lines(Palmer et al., 1997). Addition of FGF-2 (10ng/ml) to Sox-2 selected cultures likewise stimulated cell proliferation. Figure 6 shows a typical Xgal-stained culture following addition of FGF-2. It can be seen that all cells retain relatively undifferentiated morphology and show strong Xgal staining. Such cultures could be expanded and serially passaged for at least two weeks.

Neuronal differentiation of Sox2-selected cells.

In order to determine whether the Sox2-selected precursor cells retained the capacity for neuronal differentiation, G418 was removed from the medium. Within 48 hours the cells began to extend neuritic processes and by 96 hours a network of neuron-like cells had formed (Figure 7). β-galactosidase activity was lost from the majority of cells (not shown) consistent with the down-regulation of Sox2 in differentiating neurons (ref). Immunostaining confirmed the disappearance of Sox2 protein (Figure 7b). Pan-neuronal markers neurofilament light and heavy chain (not shown), microtubule associated proteins (MAPs/tau), β-tubulin III (Lee et al., 1990) and synapsin I were detectable from 48 hours onwards, co-incident with down-regulation of Sox2. By 96 hours over 90% of cells had long dendritic processes and expressed neuronal markers (Figure 7). Supplementation of the culture medium with B27 and horse serum allowed further maturation of the neuronal cells, evidenced both by increased sprouting of dendrites and by production of excitatory and inhibitory neurotransmitters GABA and glutamate (Figure 7)

The mammalian nervous system is derived from neuroepithelial cells of the neural tube and its derivative neural crest. During neurogenesis, these neural progenitors proliferate, progressively lose their multipotentiality and finally differentiate into various type of post mitotic neurons and glial cells [Anderson, 1993 #673, McKay,

1989 #672(Tanabe and Jessell, 1996; McKay, 1997)]. The mechanisms involved in the determination and differentiation of neural precursor cells are the subject of intense investigation. This has been hitherto hindered, however by the relative inaccessibility and tissue complexity of the mammalian embryo. An in vitro model system of the present invention in which neuroepithelial cells can be derived and undergo proliferation and differentiation provides a powerful tool to study intrinsic and extrinsic factors that determine neural specification and differentiation.

ES cells have the capacity to develop into any cell type as evidenced by their colonisation of all lineages in chimaeras (Beddington and Robertson, 1989). The prospect of using ES cells to dissect developmental pathways in vitro has been frustrated, however, by an inability to control or direct differentiation. In an embodiment of the invention described above, there is provided a strategy for selecting precursors of the lineage of interest from developing embryoid bodies. Our results demonstrate that viable neural precursors can be isolated by selection for Sox2 gene activity. These cells can be induced to proliferate or to differentiate into neurons. Therefore the survival and development of the neural lineage in vitro does not require continued interaction with other cell types. Moreover, the finding that significant cellular components can be ablated without causing disintegration of the embryoid bodies, nor apparently perturbing development of the surviving cells is both surprising and encouraging for the application of this approach to other lineages. Indeed it is possible that such ablation may favour the maintenance and expansion of specific stem cell populations by removing sources of differentiation inducing signals, which may be either cells from other lineages or more mature cells of the same lineage (Mountford and Smith, in personal communication).

The heterogenous expression of determination genes (Figure 5) within the Sox2 selected cultures appears reflective of the subspecification of progenitors within the neural tube. Requirements for induction, growth and differentiation of individual progenitor types could therefore be investigated, both by addition of extracellular regulators to the culture system and by genetic manipulation of the ES cells prior to differentiation. The latter particularly relevant to situations where homozygous

gene deletion or transgene expression in vivo cause embryonic lethality. In addition, the ability to produce genetically modified neurons is likely to find significant applications in neuronal cell biology and biochemistry.

It has recently been reported that differentiated ES cells injected into the developing (Brustle et al., 1997) or even adult (Deacon et al., 1998) rodent brain can colonise the host nervous system and give rise to mature neuronal phenotypes. Such transplants also contain non-neuronal cells, however. These foreign cells can give rise to teratomas and other benign or malignant growths. They may also interfere with trophic signalling and guidance cues from host tissue to injected neural cells. Prior isolation of the neural precursors according to the invention eliminates these problems. Furthermore, following application of lineage selection purified neural cells can be accessed at any stage of in vitro maturation and harvested for transplantation.

The invention opens the way for development of human multipotential stem cells analogous to the mouse ES cells of an example above for clinical use in transplantation. Neurodegenerative conditions such as Parkinson's and Huntington's diseases are potentially treatable by cell replacement strategies and present compelling cases for development of a renewable stem cell resource for production of transplantable cells (Svendsen and Rosser, 1995; Rosenthal, 1998). The lineage selection approach, in combination with appropriate instructive factors, is a valuable component of such a system, by enabling the generation of a defined cell population from a multipotent source.

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Table 1

Expression of neuroepithelial markers following G418 selection

Markers	- G418	+G418
	% positive staining (mean +/- SEM)	
β-galactosidase	43.8 ± 9.4	91 ± 2.6
Sox2	48.4 ± 1.1	95.5 ± 1.1
Sox1	46.6 ± 5.6	88.7 ± 5.1
Nestin*	90 ± 6.5	94 ± 3.2

G418 was applied to EB cultures from the 6th day of induction at a concentration of 200 μg/ml. Two days later aggregates were trypsinized and the cell suspension was plated on poly-D-lysine/laminin coated substrate in DMEM/F12 plus N2. Cultures were fixed 3 hours after plating followed by histochemical staining for β-galactosidase with X-gal or immunocytochemical staining for Sox1, Sox2 and nestin with specific antibodies. Positively stained cells were scored under a 40x objective, seven to ten fields were counted for each sample. The result is given as an average percentage from two independent experiments (*Nestin is expressed in somitic mesoderm in addition to neuroepithelium).

CLAIMS

1. A method for generating a culture that is purified or enriched in respect of cells of a selected lineage, comprising:-

(i) introducing into a multipotential cell a selectable marker that is differentially expressed in cells of the selected lineage compared with its expression in other cells; wherein cells of the selected linage constituted a sub-set of the cells obtainable from the multipotential cell;

(ii) culturing the multipotential cell *in vitro* to induce differentiation of the multipotential cell into a cell of the selected lineage or into a mixture of cells including cells of the selected lineage; and

(iii) selecting for cells of the selected lineage according to differential expression of the selectable marker introduced in step (i)

wherein said multipotential cell is not of human embryonic origin.

2. A method according to Claim 1 for generating a culture that is enriched or purified in respect of progenitor cells of a selected lineage.

3. A method according to Claim 1 or 2 wherein the multipotential cell is selected from embryonic stem (ES) cells, embryonic germ (EG) cells, embryonic carcinoma (EC) cells, a primary culture of foetal cells, a primary culture of post- natal cells, and a primary culture of adult cells.

4. A method according to Claim 1,2 or 3 comprising genetically modifying multipotential cells to delete, mutate, substitute or add genes in order (i) to assay gene function in progenitor cells of the selected lineage, and/or (ii) to render selected cells more suitable for transplantation.

5. A method according to any of Claims 1 to 4 further comprising:-

(iv) introducing into the multipotential cell a second selectable marker that is differentially expressed in cells of a selected sub-lineage compared with its expression in other cells, wherein cells of the selected sub-lineage are formed by differentiation of cells of the selected progenitor lineage; and

(v) when a culture of progenitor cells of the selected lineage has been obtained, allowing or inducing differentiation of the cells and selecting for cells that express the second selectable marker.

6. A method according to any of Claims 1 to 5 wherein the selectable marker is introduced into the multipotential cell by targeted integration or random gene trap integration so as to be operatively coupled to a gene that is differentially expressed in progenitor cells of the selected lineage.

7. A method according to any of Claims 1 to 5 wherein the selectable marker is introduced into the multipotential cell via random integration of a transgene in which the selectable marker is operatively coupled to a gene that is differentially expressed in progenitor cells of the selected lineage.

8. A method according to any of Claims 1 to 7 wherein the multipotential cell is an ES, EG or EC cell and the method comprises forming an embryoid body, or otherwise inducing differentiation of the cells.

9. A method according to Claim 8 wherein the differentiated cells are dissociated so as to form a culture substantially of individual cells.

10. A method according to Claim 8 or 9 wherein differentiated cells of an embryoid body are dissociated using a protease, such as trypsin.

11. A method according to any of Claims 1 to 10, for generating a culture that is purified or enriched in respect of neural progenitors, comprising introducing into the multipotential cell a selectable marker that is differentially expressed in neural progenitor cells.

12. A method according to Claim 11 wherein the selectable marker is expressed in cells that express a Sox gene.

13. A method according to Claim 12 wherein the Sox gene is selected from Sox 1, Sox 2 and Sox 3.

14. A method according to any of Claims 1 to 10 for generation of cardiac progenitor cells, wherein the selectable marker is expressed in cells that express the Nkx 2.5 or GATA-4 gene.

15. A method according to any of Claims 1 to 10 for generating a culture that is purified on enriched in respect of haematopoietic progenitors.

16. A method according to Claim 15 wherein the selectable marker is expressed in cells that express CD34, CD44 or SCL.

17. A method according to any of Claims 1 to 16 wherein the selectable marker is an antibiotic resistance gene and the method comprises culture in the presence of antibiotic.

18. A method according to Claim 5, for obtaining a culture that is purified or enriched in respect of ventral progenitor cells, wherein the selectable marker is differentially expressed in neural progenitor cells and the second selectable marker is differentially expressed in ventral progenitor cells.

19. A method according to Claim 18 wherein the second selectable marker is differentially expressed in cells that express Pax 6.

20. An assay of the effect of a factor on a culture of progenitor cells of a selected lineage, comprising :-

A (i) introducing into a multipotential cell a selectable marker that is differentially expressed in progenitor cells of the selected lineage compared with its expression in other cells, wherein the selectable marker is operatively coupled to the promoter of a gene that is differently expressed in cells of the selected lineage, and wherein progenitor cells of the selected lineage constitute a sub-set of the cells obtainable from the multipotential cell;

(ii) culturing the multipotential cell *in vitro* to induce differentiation of the multipotential cell into a cell of the selected lineage or into a mixture of cells including cells of the selected lineage, or to induce preferential survival, in a mixed culture of cells, of cells of the selected lineage; and

(iii) selecting for progenitor cells of the selected lineage according to differential expression of the selectable marker introduced in step (i), and

B culturing the thereby obtained progenitor cells of selected lineage in the presence of the factor.

21. An assay according to claim 20 characterised according to any Claims 2 to 17

22. An assay according to claim 20 or 21 to assay whether the factor has a proliferative, maturation, toxic or protective effect on progenitor cells of the selected lineage.

23. As assay according to claim 22 to assay whether a factor has a profilerative maturation, cytotoxic or glial protective effect on neural progenitor.

24. A method of preparing a neural progenitor cell or a differentiated progeny thereof for storage, comprising obtaining the cell in a method according to any of Claims 1 to 17 and freezing the cell in the presence of a cryoprotectant.

25. A method of generating purified neurons, comprising obtaining a culture purified in respect of neural progenitors, using the method of any of Claims 1 to 17 wherein the selectable marker is differentially expressed in cells that express a Sox gene, and culturing the progenitors obtained in the presence of medium suitable for differentiation of the progenitor into neurons.

FIG. 1(a)

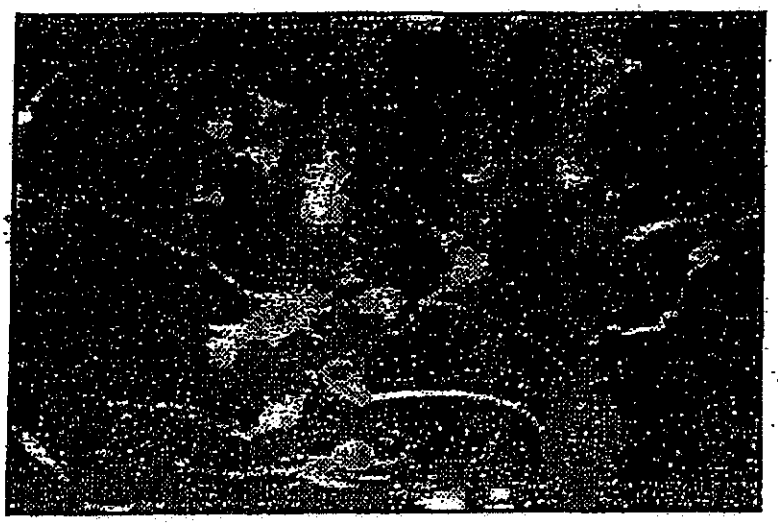


FIG. 1(b)



FIG. 1(c)

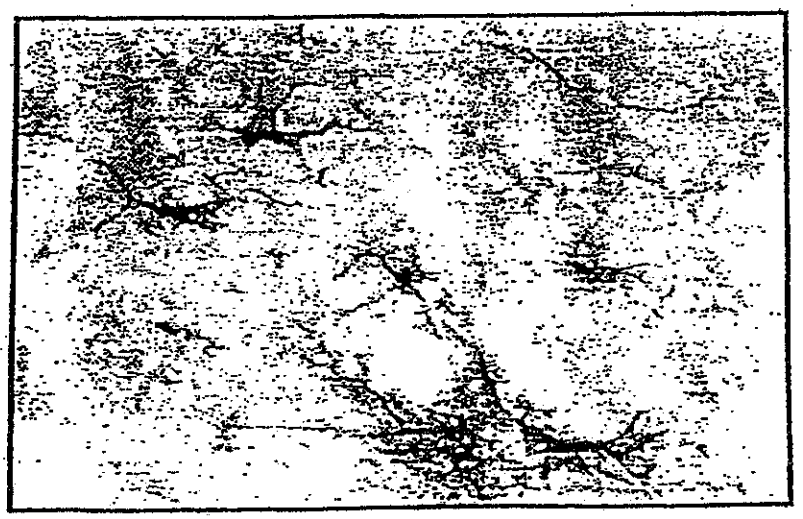


FIG. 2(a)



FIG. 2(b)

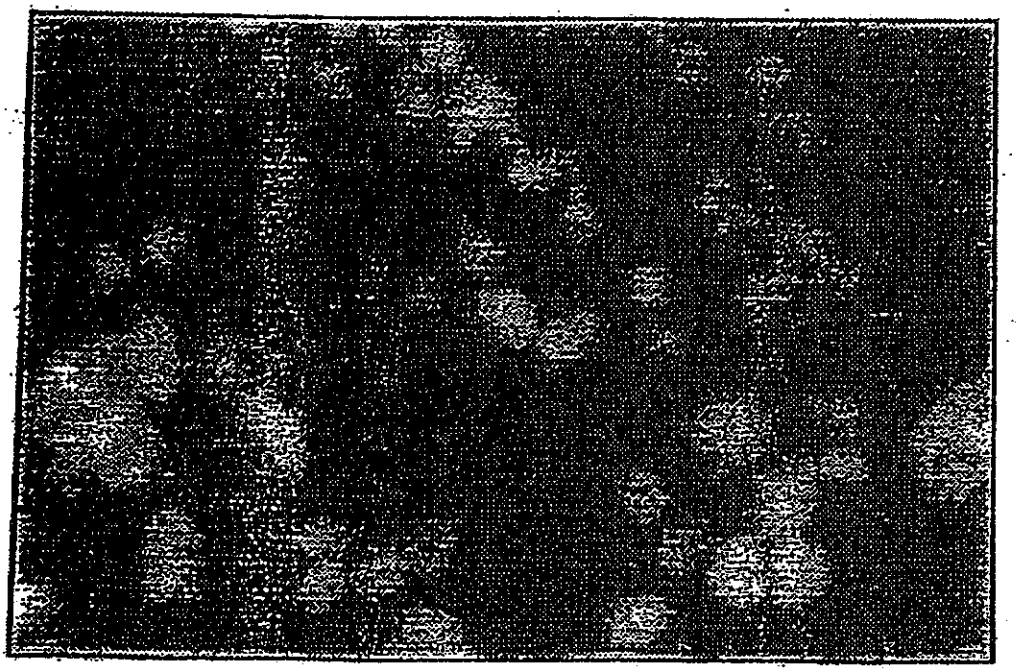
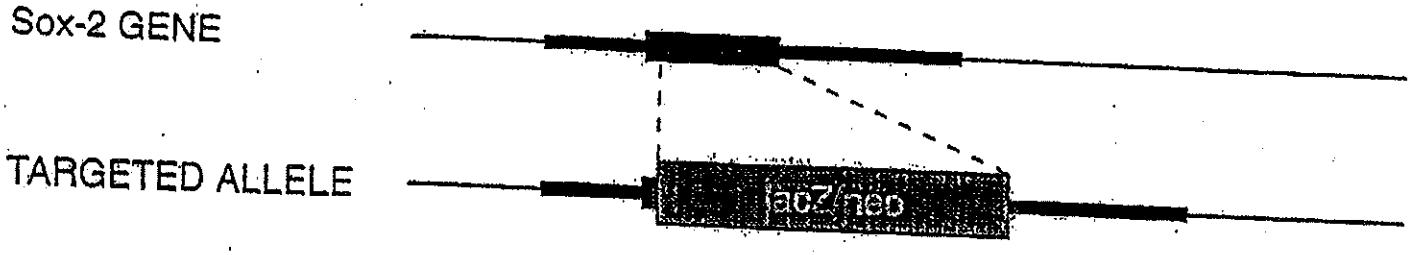


FIG. 3 NEURAL STEM CELL (NSC) SELECTION STRATEGY

I. TARGETING MARKER INTO NEURAL PRECURSOR-SPECIFIC GENE IN ES CELLS



II. ELIMINATION OF NON-NEURAL CELLS FROM DIFFERENTIATING ES CELL CULTURES

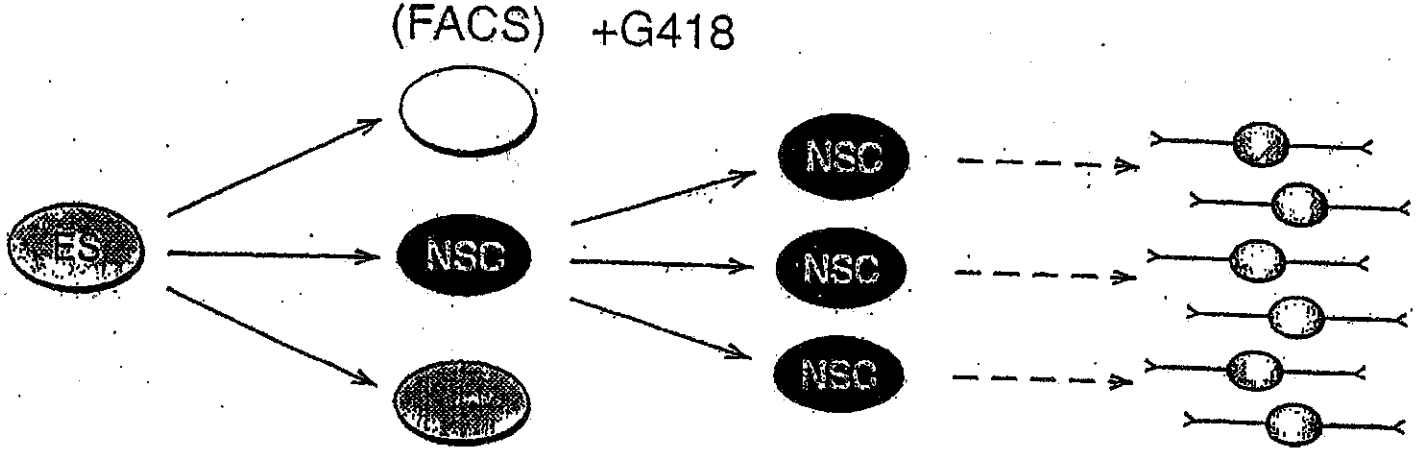


FIG. 4(a)

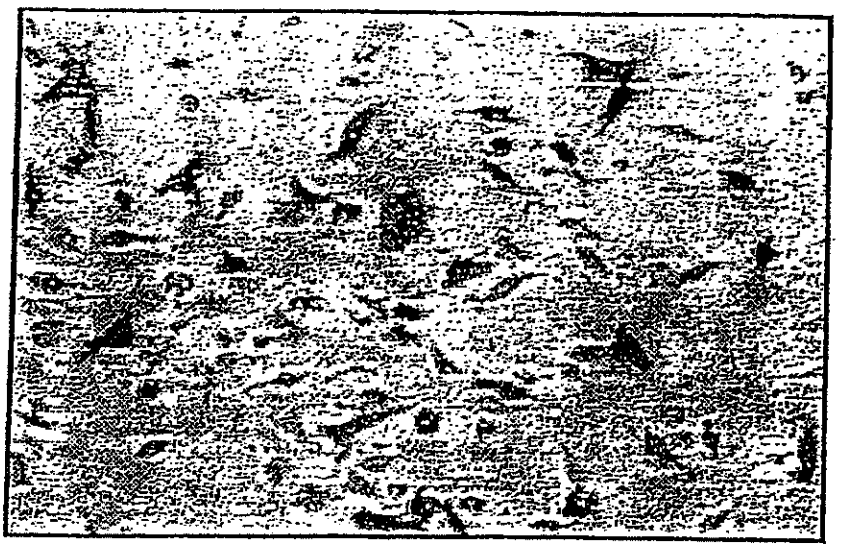


FIG. 4(b)



FIG. 4(c)

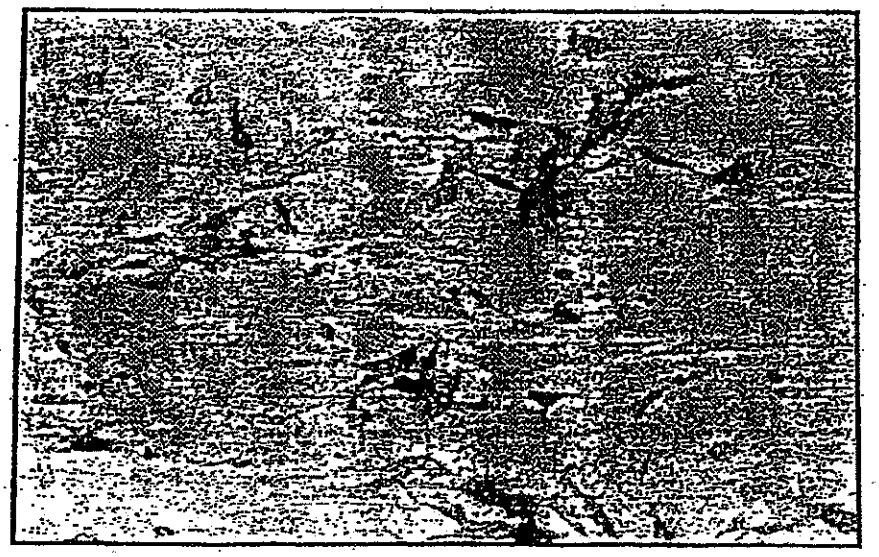


FIG. 4(d)

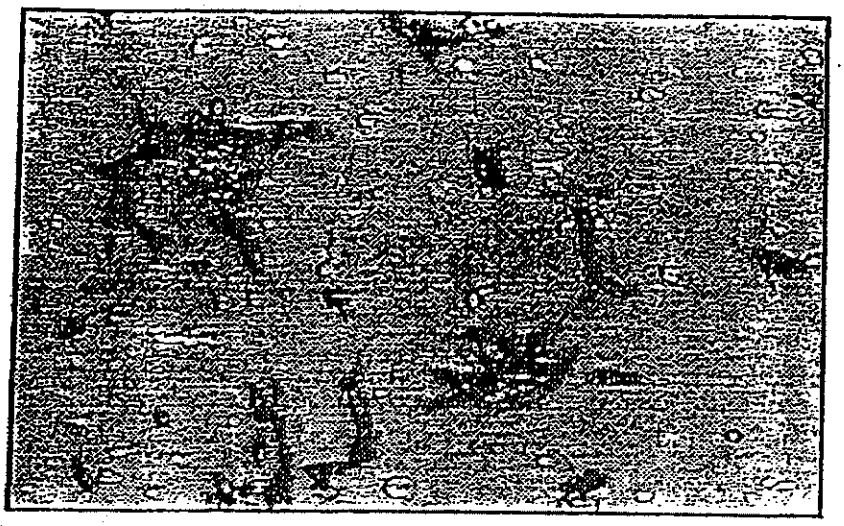


FIG. 4(e)

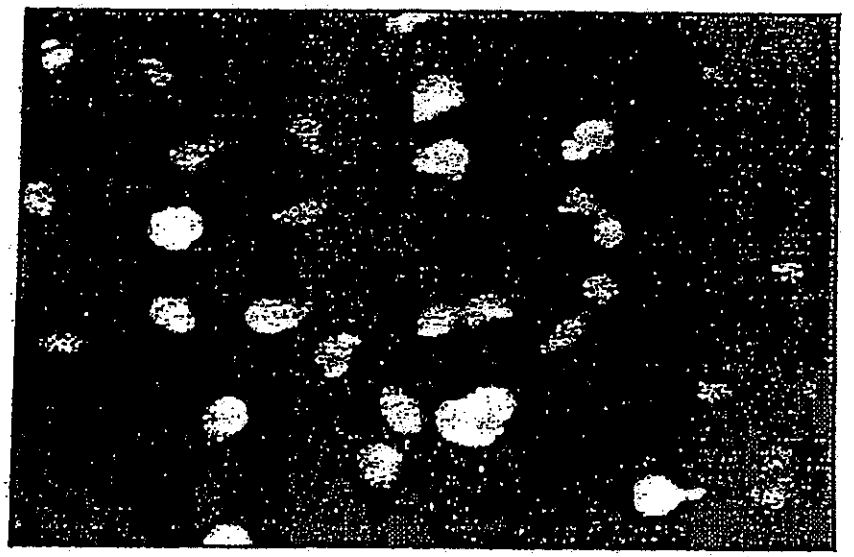


FIG. 4(f)

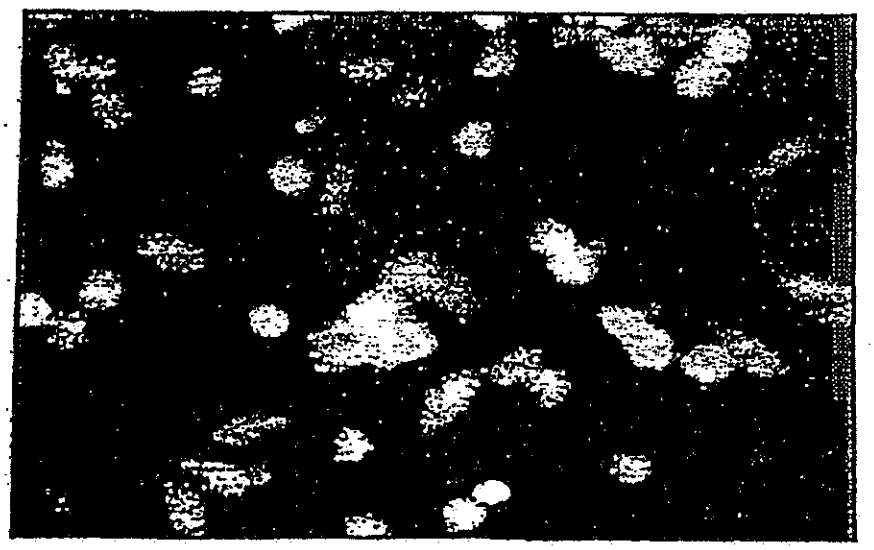


FIG. 4(g)

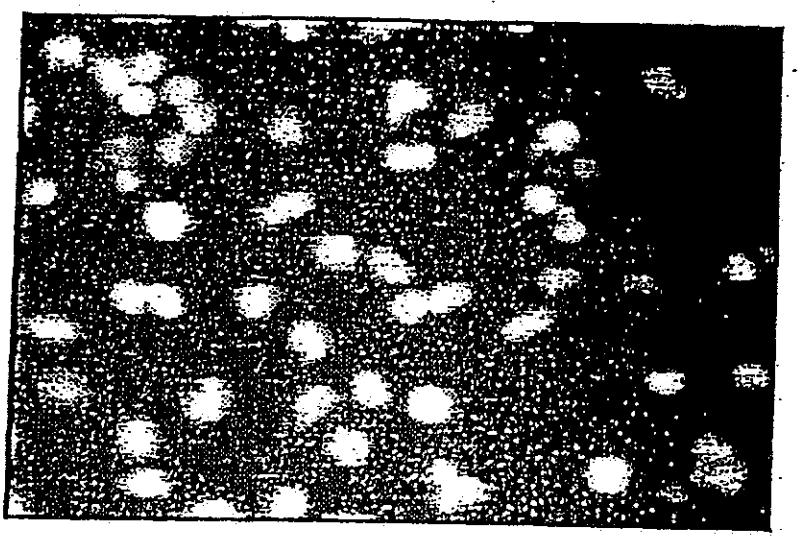


FIG. 4(h)

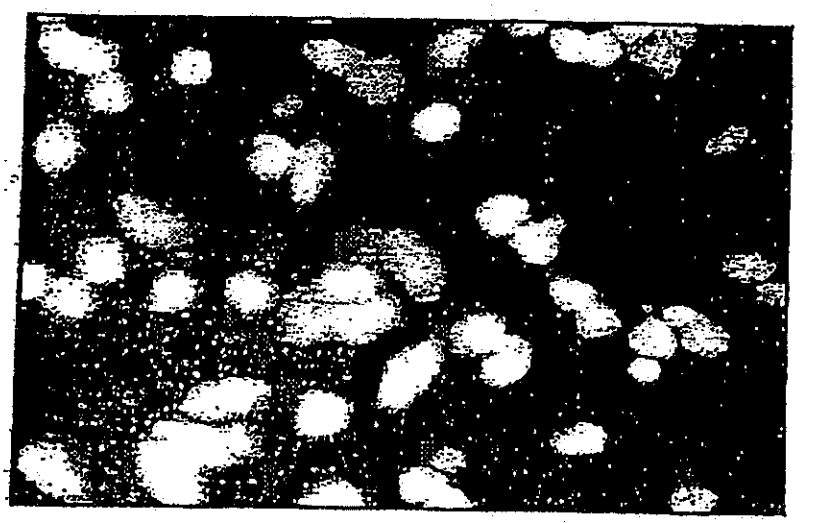


FIG. 4(i)

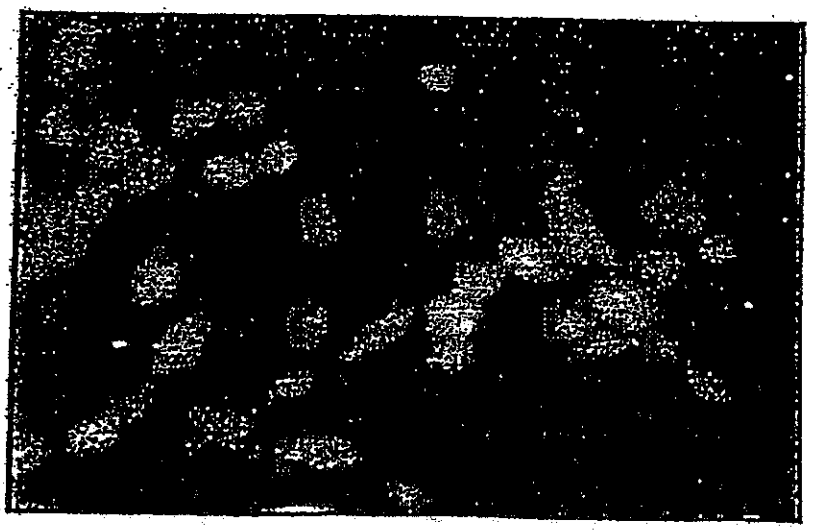


FIG. 5(a)

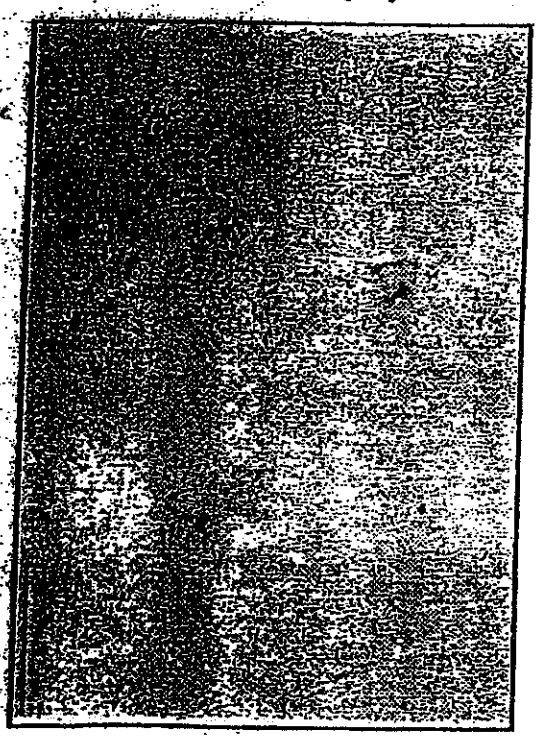


FIG. 5(b)

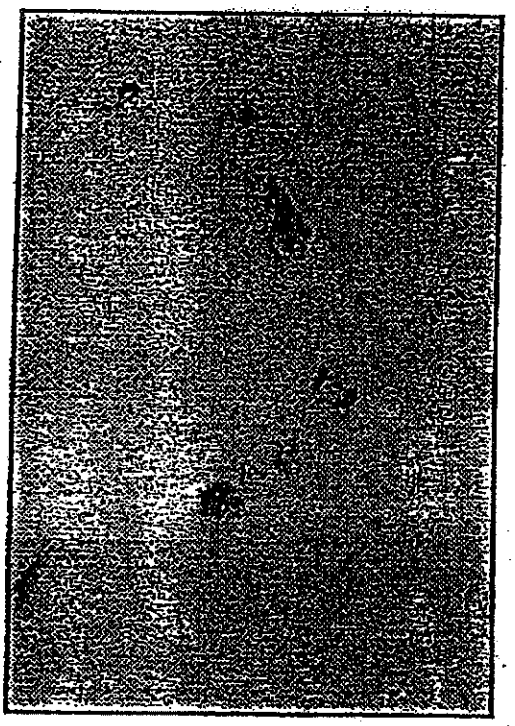


FIG. 5(c)

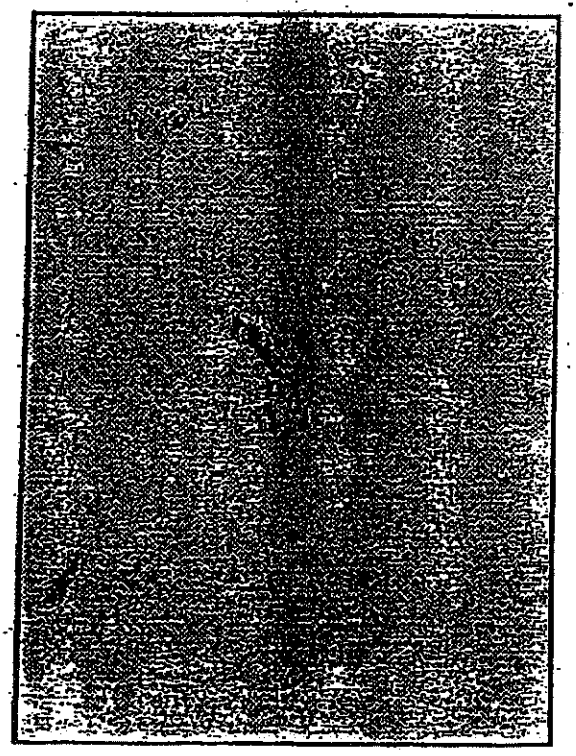


FIG. 5(d)

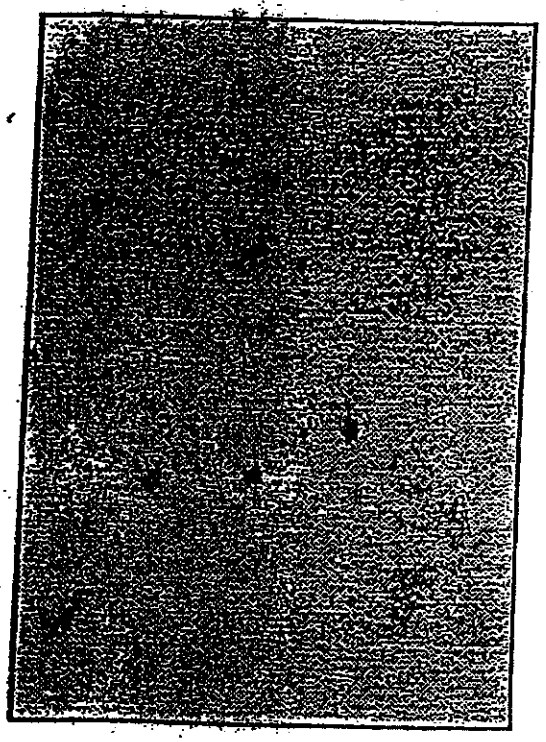


FIG. 5(e)



FIG. 5(f)

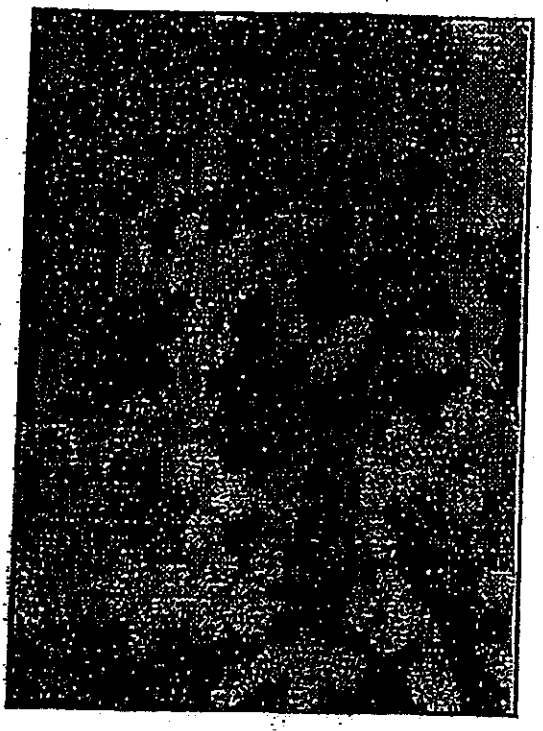


FIG. 6(a)

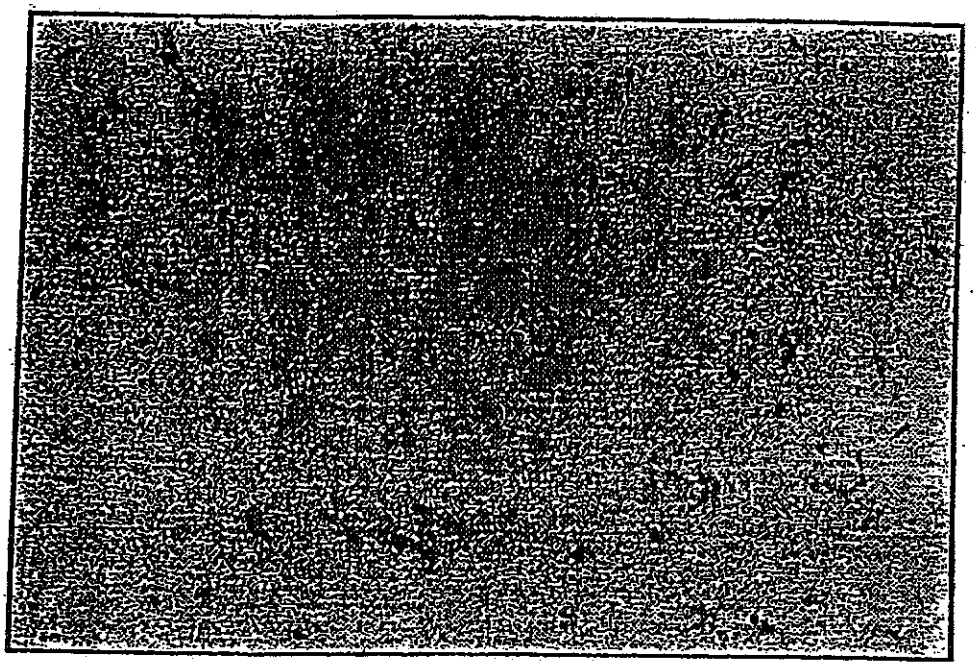


FIG. 6(b)

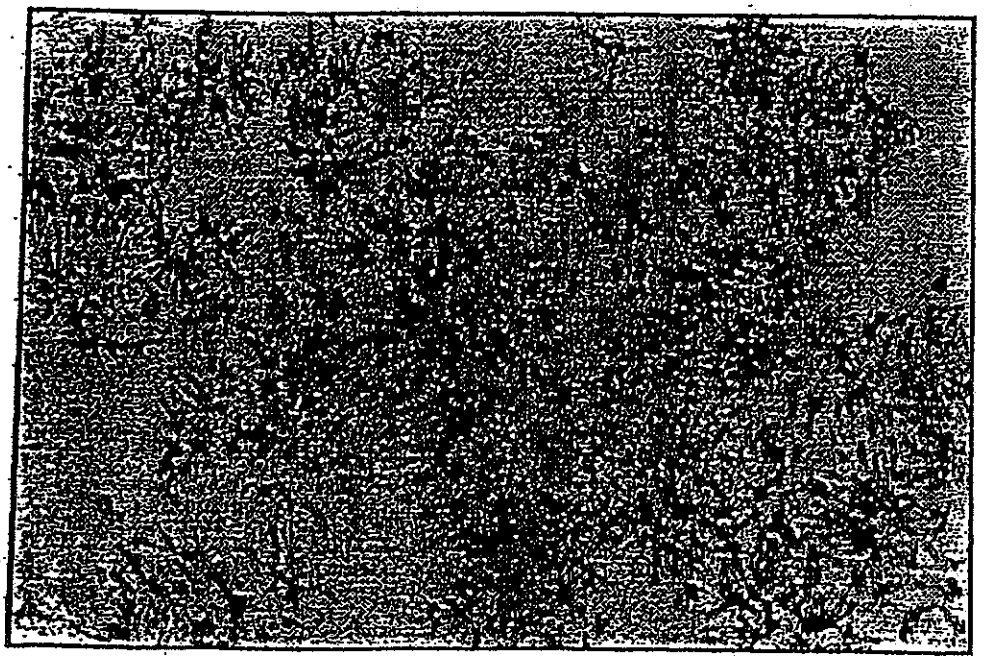


FIG. 7(a)

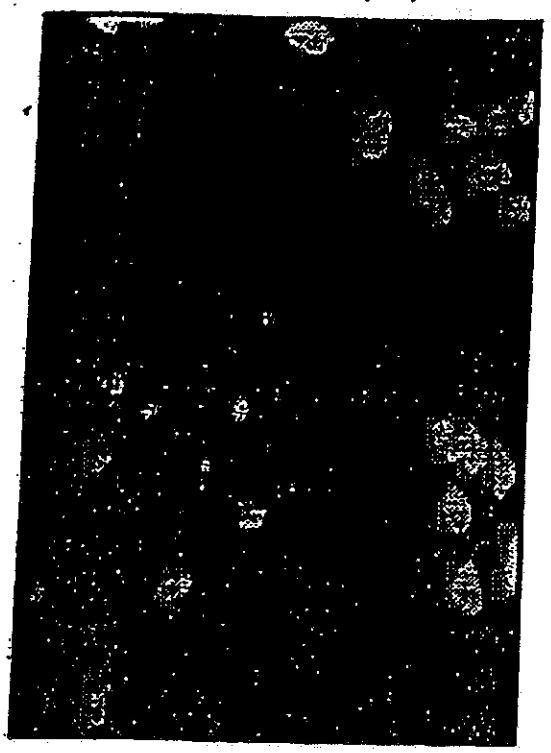


FIG. 7(b)

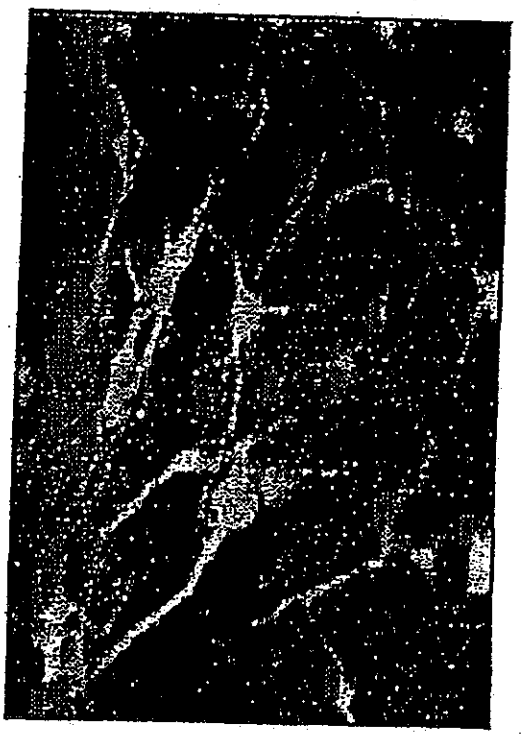


FIG. 7(c)



FIG. 7(d)



FIG. 7(e)

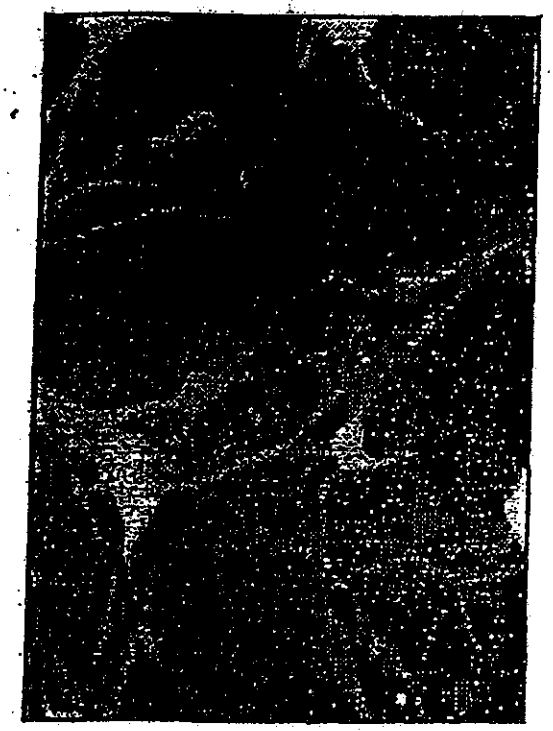


FIG. 7(f)

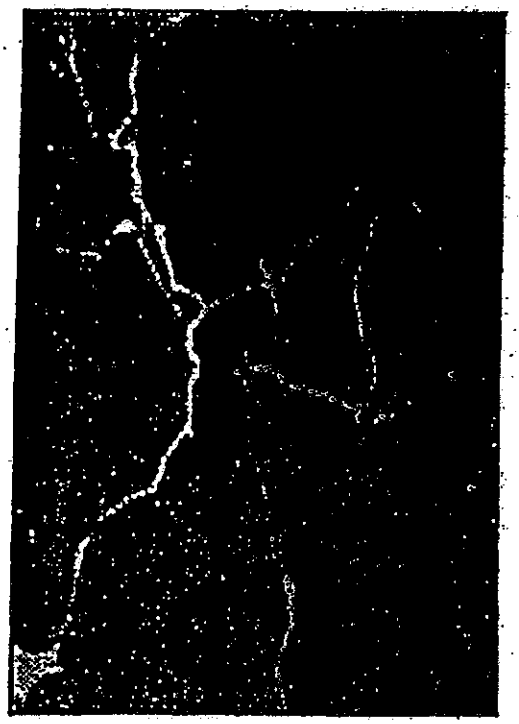


FIG. 7(g)



FIG. 7(h)



FIG. 7(i)

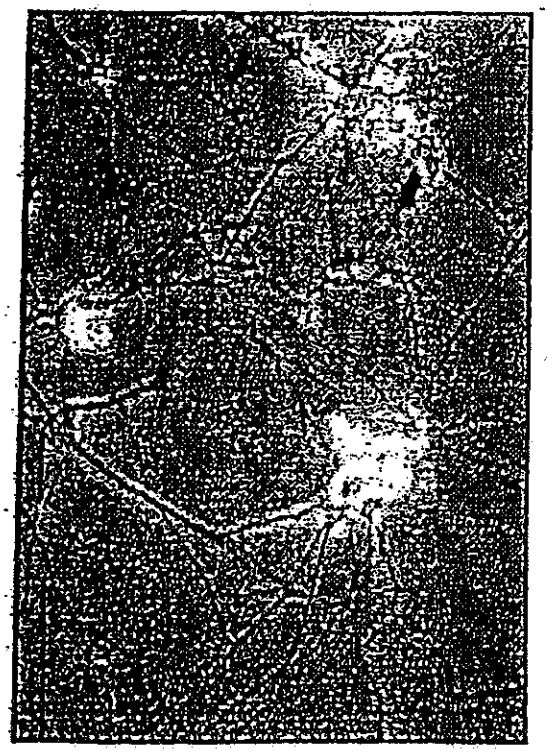


FIG. 8 INDUCTIVE EFFECT OF SONIC HEDGEHOG ON ES-DERIVED NEURAL PRECURSORS

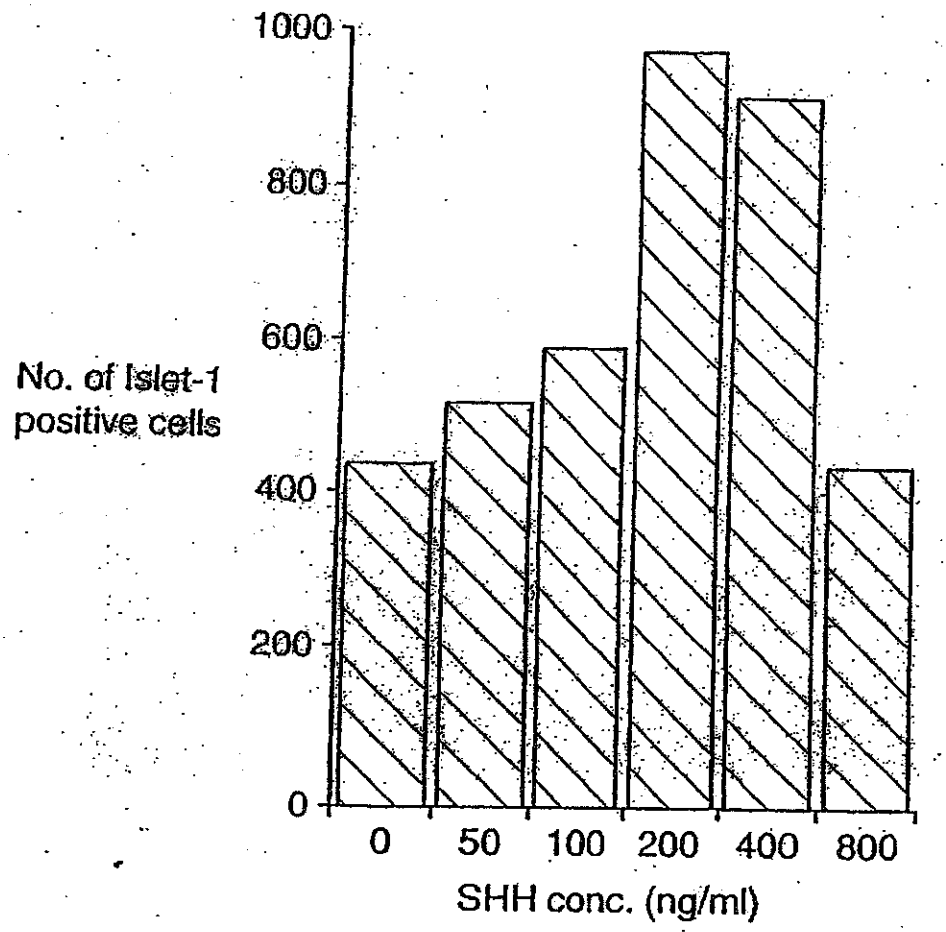


FIG. 9

