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(54) Title: METHODS OF PRODUCING AND PROPAGATING NEURAL PRECURSOR CELLS

(57) Abstract: This invention relates to the production and propagation of neural precursor cells (NPCs) by a combination of automated mechanical passaging and culture in a fully humanised and defined culture human neuralising medium (NNM). The methods described herein are amenable to automation and expansion to bulk culture and may be useful in the generation of clinical grade NPCs for therapeutic applications, as well as a source of human neural cells and glia for high-throughput drug screening.



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Methods of Producing and Propagating Neural Precursor Cells

This invention relates to the production and propagation of mammalian cells, in particular neural precursor cells (NPCs).

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Realising the promise of stem cells as a source of defined cells for cell based therapies or drug screening for neurological disorders requires totally defined and controlled differentiation conditions. In addition, the ability to control the conditions of neuralisation will enable examination of the molecular mechanisms underlying human neural development. Although recent advances have been made in the derivation and propagation of undifferentiated hESC lines (Klimanskaya, I. et al. Lancet 365, 1636-1641 (2005), Ludwig, T.E. et al. Nat. Biotechnol. 24, 185-187 (2006), Lu, J. et al Proc. Natl. Acad. Sci. USA 103, 5688-5693 (2006), Yao, S. et al. Proc. Natl. Acad. Sci. USA 103, 6907-6912 (2006)), definitive and scaleable methods of neural differentiation that do not depend on exposure to non-human or proprietary products have not been described; and yet this is an essential prerequisite if hESCs are to be used to treat diseases characterised by neuron loss or damage, such as Parkinson's disease and multiple sclerosis. The most widely applied stem cell neuralising systems employ spontaneous differentiation, addition of retinoic acid to stem cell aggregates or co-culture with stromal cells or conditioned media (Hoffman, L.M. & Carpenter, M.K. Nat. Biotechnol. 23, 699-708 (2005)).

Lineage selection using cell surface epitopes or genetically modified reporter lines as a method of neural enrichment have thus far been restricted to mouse ESC studies (Li, M. et al Curr. Biol. 8, 971-974 (1998)). Studies reporting derivation of human neural precursors under serum-free defined conditions (Zhang, S.C. et al Nat. Biotechnol. 19, 1129-1133 (2001), Itsykson, P. et al. Mol. Cell Neurosci. 30, 24-36 (2005)) have relied on the use of commercial, proprietary formulations such as "knock-out" serum replacement and B27 supplement, which are not fully humanised. In addition, the

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presence of retinoids in B27 has limited studies addressing physiological mechanisms of neural induction and patterning.

The present inventors have developed simple and fully defined methods of producing and propagating neural precursor cells using fully humanised and generic culture conditions. These methods are amenable to automation and expansion to bulk culture and may be useful in the generation of clinical grade neural precursors for therapeutic applications, as well as a source of human neural cells and glia for high-throughput drug screening.

An aspect of the invention provides a method of production of a population of neural precursor cells (NPCs) comprising:

- i) slicing a colony of stem cells using an automated tissue sectioning device to produce colony fragments, and;
- ii) culturing one or more of said colony fragments in human neutralising medium (HNM) to produce said NPCs.

A neural precursor cell (NPC) is a daughter or descendant of a undifferentiated stem cell, with a more committed phenotype and/or a more reduced differentiation potential compared to the original stem cell (i.e. multipotent but not totipotent). NPCs are committed to a neural lineage and are capable of giving rise to a plurality of distinct neuronal and glial phenotypes i.e. an NPC is able to differentiate under appropriate conditions into all cell types in the nervous system, including neurons with different neurotransmitter subtypes, astrocytes, oligodendrocytes. An NPC may express one or more NPC associated markers, such as Nestin, Musashi1, Pax6 and Sox1.

A stem cell is a pluripotent cell which is undifferentiated and is not committed to a neural phenotype. A stem cell may express one or more of the following stem-cell associated markers: Oct-4; Nanog; Sox2; stage specific embryonic antigens (SSEA-3 and 4), and the tumor rejection antigens TRA-1-60 and -1-81. A stem cell may lack

markers associated with specific neuronal fates, such as tyrosine hydroxylase and markers associated with NPCs, such as Nestin, Musashil, Pax6 and Sox1.

5 Suitable stem cells may be derived from any source and may include embryonic, fetal and adult stem cells, e.g. skin derived stem cells. In some embodiments, suitable stem cells may be obtained by reprogramming adult somatic cells (Cowan et al, Science 2005 Aug 26;309(5739):1369-73). For example, a stem cell may be obtained from
10 an individual in need of therapy, for example for the treatment of a medical condition associated with degeneration, damage to, the loss of, or a disorder in nervous tissue. The stem cell may be obtained directly from the individual or may be obtained by reprogramming a somatic cell obtained from the individual. A population of cells
15 produced from the stem cell as described herein may be administered to the individual to treat the condition.

Preferably, stem cells are human stem cells.

20 Stem cells include embryonic stem cells. An embryonic stem cell (ESC) is a cell derived from an early stage embryo, which exhibits an undifferentiated phenotype and is potentially totipotent i.e. it is capable of giving rise to any cell type in an individual. An ESC may be a cell obtained from an embryo or may be a descendent of such
25 a cell.

In some embodiments, the stem cells are human ESCs (hESCs). Suitable stem cells may be obtained from a cultured hESC cell line, such as H9 or HUES9. Further examples of suitable human embryonic stem cells
30 are described in (Thomson JA et al Science 282: 1145-1147 (1998); Reubinoff et al. Nat Biotechnol 18:399-404 (2000); Cowan, C.A. et al. N. Engl. J. Med. 350, 1353-1356(2004), Gage, F.H., et al. Ann. Rev. Neurosci. 18 159-192 (1995); and Gotlieb (2002) Annu. Rev. Neurosci 25 381-407); Carpenter et al. Stem Cells. 5(1): 79-88
35 (2003); see also: the NIH stem cell registry

<http://stemcells.nih.gov/research/registry/>. Potentially clinical grade hESCs are described in Klimanskaya, I. et al. Lancet 365, 1636-1641 (2005), Ludwig, T.E. et al. Nat. Biotechnol. 24, 185-187 (2006). In other embodiments, the stem cells are not hESCs.

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A colony of stem cells, for example hESCs, may be obtained by culturing cells from an cell line using conventional techniques (Vallier, L. et al Dev. Biol. 275, 403-421 (2004), Cowan, C.A. et al. N. Engl. J. Med. 350, 1353-1356 (2004), Joannides, A. et al. Stem Cells 24, 230-235 (2006) Klimanskaya, I. et al. Lancet 365, 1636-1641 (2005), Ludwig, T.E. et al. Nat. Biotechnol. 24, 185-187 (2006)) For example, hESCs may be cultured in a culture dish on a layer of feeder cells, such as irradiated mouse embryonic fibroblasts (MEF), at an appropriate density (e.g. 10^5 to 10^6 cells/60mm dish), or on an appropriate substrate with feeder conditioned or defined medium. hESC colonies may be passaged by enzymatic or mechanical means. Suitable culture media for hESCs include Knockout Dulbecco's Modified Eagle's Medium (KO-DMEM) supplemented with 20% Serum Replacement, 1% Non-Essential Amino Acids, 1mM L-Glutamine, 0.1mM β -mercaptoethanol and 4ng/ml to 10ng/ml human bFGF.

If a human embryo is used as a source of hESCs, the human embryo is one that would otherwise be destroyed without use, or stored indefinitely, especially a human embryo created for the purpose of IVF treatment for a couple having difficulty conceiving. IVF generally involves creation of human embryos in a number greater than the number used for implantation and ultimately pregnancy. Such spare embryos may commonly be destroyed. With appropriate consent from the people concerned, in particular the relevant egg donor and/or sperm donor, an embryo that would otherwise be destroyed can be used in an ethically positive way to the benefit of sufferers of severe neurodegenerative disorders such as Parkinson's disease. Furthermore, ES cells may be derived from one embryo cell without destroying the whole embryo (Chung Y et al, Nature

2006;439(7073):216-9.)). The present invention itself does not concern the use of a human embryo in any stage of its development. As noted, the present invention minimizes the possible need to employ a material derived directly from a human embryo, whilst
5 allowing for development of valuable therapies for common and devastating diseases. Any therapeutic interventions based on the present invention must also be performed according to the relevant national laws and ethical guidelines.

10 A stem cell may be genetically manipulated, for example to reduce or silence expression of one or more genes or to express a heterologous polypeptide.

A stem cell may have the genotype of a genetic disease or disorder.
15 For example, the stem cell may be derived from an individual having a genetic disease.

A stem cell for use in the present methods may be substantially free from one or more other cell types. Stem cells may, for example, be
20 separated from other cell types, using any technique known to those skilled in the art, including those based on the recognition of extracellular epitopes by antibodies and magnetic beads or fluorescence activated cell sorting (FACS) including the use of antibodies against extracellular regions of molecules found on
25 embryonic stem cells such as SSEA4.

Prior to sectioning, a stem cell colony is preferably detached from feeder cells using standard techniques, for example collagenase treatment, as described herein.

30 A stem cell colony may be sliced or sectioned by making a series of incisions through the colony which divide the colony into a series of separate pieces or fragments. Preferably, the separate pieces or fragments are uniform in size.

Preferably, colonies are sliced in two different (i.e. non-parallel) directions by making a first series of parallel incisions through the colony in a first direction, and then making a second series of parallel incisions through the colony in a second direction. The
5 first and second series of incisions intersect to divide the colony into separate pieces or fragments. The first and second directions may, for example, be perpendicular to produce substantially square fragments. A colony may be conveniently sliced in two directions by rotating the colony relative to the cutter of the device, for
10 example by mounting the culture dish containing the colony on a rotatable platform or table.

Preferably, the incisions are separated such that each colony fragment which is defined by the incisions contains about 40 to 150
15 cells, preferably about 70 cells.

The incisions in a series of parallel incisions may be at intervals of 50µm to 250µm, preferably 100µm to 200µm, and typically 150µm.

20 Any suitable automated sectioning device may be used to section the colony. The device may conveniently comprise a blade or cutter mounted on a chopping arm which is positioned to section a subject located on a platform. The platform may be rotatable to allow the cutter to section the subject in different directions. The device
25 may be adapted to section colonies in a culture dish for the passaging of hESCs. For example, the chopping arm may comprise an indentation which is positioned to receive the side of the dish during sectioning, to prevent contact between the dish and the chopping arm. Such adaptation may be unnecessary for passaging cells
30 other than ESCs, including neuralisation fragments, since intact colonies of such cells may be detached from the culture dish, and placed on a suitable stage, and sectioned to the desired fragment size.

Suitable automated sectioning devices include automated tissue sectioning devices such as the McIlwain Tissue Chopper (TC752: Campden Instruments Ltd, Loughborough, LE12 7XT England)

- 5 Following sectioning, the colony fragments may be transferred to one or more secondary culture dishes. Preferably, the cells are transferred at a cell density which is equivalent to 0.5 to 1.5 x 10⁶ cells per 10 cm dish.
- 10 In the one or more secondary culture dishes, the fragments of the stem cell colony are cultured in human neutralising medium (HNM). The secondary culture dishes are preferably non-adherent, such that colony fragments form small spheres in the HNM medium and the cells therein differentiate into neural precursor cells (NPCs).
- 15 Differentiation is characterised by a loss of expression of stem cell markers such as Oct4 and SSEA4 and a gain of expression of neural progenitor markers, such as nestin, Pax6 and mushash1.

HNM is a defined medium which is devoid of serum, exogenous
20 mitogens, retinoids, and neural inducers. HNM comprises a basal cell culture medium, such as DMEM, with the addition of human serum albumin (1.80E-02 to 1.20E-01 mM), human transferrin (30% saturation: 3.80E-05 to 2.85E-03 mM), glutathione (reduced) (up to 1.00E-02mM), recombinant human insulin (3.51E-04 to 5.26E-03 mM),
25 superoxide dismutase (up to 1.50E-04mM), L-ascorbic acid (5.00E-02 to 2.00E+00 mM) and N-acetyl-L-cysteine (up to 7.50E+01 mM).

As described below, recombinant human EGF (up to 1.29E-05 mM) and recombinant human FGF2 (up to 5.77E-06 mM) may also be included in
30 some contexts EGF to maintain long-term cell survival and proliferation.

A suitable HNM medium may comprise the ingredients set out in table 1, each ingredient being independently at a concentration between
35 the maximum and minimum concentrations set out for that ingredient.

Where no maximum and minimum concentrations are provided, the ingredient may be omitted or may be present at a concentration which is within the range of +/-50% of the stated preferred concentration.

- 5 In preferred embodiments, the HNM medium may comprise the ingredients set out in table 1, wherein one or more, two or more, three or more or preferably all of the ingredients in the group consisting of human serum albumin, human transferrin, glutathione (reduced), recombinant human insulin, superoxide dismutase, L-
10 ascorbic acid and N-acetyl-L-cysteine are at the preferred concentrations stated in table 1. Preferably, one or more, two or more, three or more or preferably all of the remaining ingredients are at the preferred concentrations stated in table 1.
- 15 Preferably, when cultured in HNM, the colony fragments are agitated to prevent re-aggregation of the fragments and maintain small particle size. Agitation may include mechanical stirring or, more preferably, orbital shaking, for example at about 40rpm. Standard mammalian cell culture conditions may be employed, for example 37°C,
20 21% Oxygen, 5% Carbon Dioxide. Media is preferably changed every two days and cells allowed to settle by gravity. Techniques for mammalian cell culture are well-known in the art (Handbook of Stem Cells (ed. R. Lanza) ISBN: 0124366430).

The cells in the stem cell colony fragment may be cultured in HNM
25 for 12 to 18 days (D12-D18) from the initial seeding of the medium (D0), preferably about 16 days (D16), to allow differentiation into NPCs.

To improve the yield of NPCs, human recombinant fibroblast growth factor 2 (FGF2) may be added to the culture medium. Preferably, FGF2
30 is added to the culture medium (HNM) 4 to 10 days (D4 to D10) after the colony fragment (D0), preferably about 8 days (D8). Human recombinant FGF2 is available from commercial suppliers (e.g. R&D, Minneapolis, MN, USA).

After differentiation, the NPCs produced as described above may be further maintained and propagated in HNM as described herein.

After the initial differentiation, the NPCs can be further purified,
5 for example by using a standard enzyme dissociation method (trypsin, accutase, EDTA) to obtain single cells and FACS sorting for one or more NPC markers, such as CD133+, syndecan-1+/ notch-1+/integrin beta-1+, integrin alpha6 or beta 1, dsd-1, nestin, musashil, Pax6 and/or Sox1 (Uchida N et al, PNAS 2000 Dec 19;97(26):14720-5;
10 Nagato M et al, J Neurosci Methods 2005 May 15;80(4):456-66; Hall PE et al, Stem cells 2006 May 11).

Methods of the invention may also be useful in the propagation of neural precursor cells (NPCs). Suitable NPCs may be produced by a
15 method described above or may be NPCs obtained from an individual, for example NPCs from the fetal or adult brain, or cells descended therefrom.

A method of propagating NPCs may comprise:

- 20 (i) slicing a colony of NPCs using an automated tissue sectioning device to produce colony fragments,
(ii) culturing said colony fragments in HNM with FGF2 and EGF, and,
(iii) repeating steps i) and ii) every 6-10 days.

25 The use of an automated tissue sectioning device to slice cell colonies is described in more detail above.

HNM is described in more detail above. Preferably the fragments are
30 agitated during culturing, for example by orbital shaking, in order to prevent re-aggregation of colony fragments.

Preferably, the cell density is reduced at least 2-fold, at least 3 fold or at least 4 fold for each repetition of steps i) and ii)

NPCs propagated using the present methods may be purified, for example by FACS sorting as described above.

5 NPCs produced as described above may be allowed to terminally differentiate into neural cells such as neurons and glia (including astrocytes and oligodendrocytes). For terminal differentiation, NPCs may be cultured in the absence of growth factors, for example in DMEM/2% B27 (Invitrogen) or HNM or other suitable media, which may also include combinations of established neurotrophic factors
10 (such as BDNF, GDNF and NT3).

The methods described herein are suitable for the generation of clinical grade neural precursors and terminally differentiated neural cells for therapeutic applications. The present invention
15 extends in various aspects not only to an NPC or terminally differentiated neural cell produced in accordance with any one of the methods disclosed herein or a population of such cells but also a pharmaceutical composition, medicament, drug or other composition comprising such a cell or population, use of such a cell or
20 population or composition in a method of medical treatment, particularly the treatment of a medical condition associated with degeneration, damage to, the loss of, or a disorder in nervous tissue, for example neuronal and/or glial cells, a method comprising administration of such a cell or population or composition to a
25 patient, e.g. for treatment (which may include preventative treatment) of a medical condition associated with degeneration, damage to, the loss of, or a disorder in nervous tissue, use of such a cell or population in the manufacture of a composition for administration, e.g. for treatment of a medical condition associated
30 with degeneration, damage to, the loss of, or a disorder in nervous tissue, and a method of making a pharmaceutical composition comprising admixing such a cell or population with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally one or more other ingredients, e.g. a neuroprotective
35 molecule, a neuroregenerative molecule, a retinoid, growth factor,

astrocyte/glial cell, anti-apoptotic factor, or factor that regulates gene expression in NPCs or neural cells or in the host brain. Such optional ingredients may render the cell independent of its environment, i.e. such that its survival is not dependent on the presence of one or more factors or conditions in its environment.

A cell or cell population which is administered to an individual may be genetically manipulated to produce a therapeutic molecule, for example a drug or growth factor (Behrstock S et al, Gene Ther 2006 Mar;13(5):379-88, Klein SM et al, Hum Gene Ther 2005 Apr;16(4):509-21)

The present invention provides a composition containing a cell or population produced in accordance with the invention, and one or more additional components. Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may comprise, in addition to the neuron or cell, a pharmaceutically acceptable excipient, carrier, buffer, preservative, stabiliser, anti-oxidant or other material well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the activity of the neuron. The precise nature of the carrier or other material will depend on the route of administration. The composition may include one or more of a neuroprotective molecule, a neuroregenerative molecule, a retinoid, growth factor, astrocyte/glial cell, or factor that regulates gene expression in stem, neural stem, precursor or progenitor cells or neuronal cells. Such substances may render the neuron independent of its environment as discussed above.

Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, tissue or cell culture media, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

The composition may be in the form of a parenterally acceptable aqueous solution, which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride, Ringer's Injection, or Lactated Ringer's Injection. A composition may be prepared using artificial cerebrospinal fluid.

Cells may be implanted into a patient by any technique known in the art (e.g. Lindvall, O., (1998) *Mov. Disord.* 13, Suppl. 1:83-7; Freed, C.R., et al., (1997) *Cell Transplant*, 6, 201-202; Kordower, et al., (1995) *New England Journal of Medicine*, 332, 1118-1124; Freed, C.R., (1992) *New England Journal of Medicine*, 327, 1549-1555, Le Blanc et al, *Lancet* 2004 May 1;363(9419):1439-41).

Administration of a composition in accordance with the present invention is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Damage to or loss of nervous tissue, for example neuronal or glial cells, may occur within the CNS. The CNS includes the brain, the spinal cord, and neurons whose cell bodies lie within, or have a primary synapse in, the brain or spinal cord. Examples of such neurons are neurons of origin of the corticospinal tract,

rubrospinal tract and retinal ganglion cells and the CNS branch of sensory axons. There are also neurons whose cell body lies within the CNS, but the axon is largely in the PNS, such as the neurons of the cranial nerves (damage to which can e.g. cause Bell's palsy) and
5 motor neurons that innervate the musculature and whose cell bodies are in the ventral horn of the spinal cord.

A condition associated with neuronal cell loss or damage may be a spinal cord injury, for example an injury caused by assault,
10 accident, tumour, intervertebral disc or bone abnormality, or surgery, e.g. surgery for spinal problems and/or surgery to remove tumours.

The neuronal cell loss or damage may be CNS damage other than spinal
15 cord injury, particularly CNS damage of the following kinds: hypoxic injury including stroke and perinatal hypoxia; brain injury, including (without limitation) injury caused by assault, accident, tumour (e.g. a brain tumour or a non-brain tumour that affects the brain, such as a bony tumour of the skull that impinges on the
20 brain) or surgery, e.g. surgery to remove tumours or to treat epilepsy; immune-mediated disease including multiple sclerosis; CNS damage following infection, neurodegenerative diseases and genetic diseases affecting the nervous system, including metabolic disorders (e.g. Gaucher's) and leukodystrophies (e.g. Krabbe's).

25 Neurodegenerative diseases may include diseases characterised by the intracellular accumulation of protein aggregates. Aggregates may accumulate, for example, in the cytoplasm of a cell. Commonly, aggregates may form in neuronal cells, such as brain cells, for
30 example in disorders such as Alzheimer's disease, Huntington's disease, Parkinson's disease, and motor neurone disease (amyotrophic lateral sclerosis).

Another aspect of the invention provides an HNM medium for use in
35 culturing mammalian cells which comprises human serum albumin, human

transferrin (30% saturation), glutathione (reduced), recombinant human insulin, superoxide dismutase, L-ascorbic acid and N-acetyl-L-cysteine.

5 Human serum albumin may be present at $1.80\text{E-}02$ to $1.20\text{E-}01$ mM), human transferrin (30% saturation) may be present at $3.80\text{E-}05$ to $2.85\text{E-}03$ mM, glutathione (reduced) may be present at up to $1.00\text{E-}02$ mM, recombinant human insulin may be present at $3.51\text{E-}04$ to $5.26\text{E-}03$ mM, superoxide dismutase may be present at up to $1.50\text{E-}04$ mM, L-
10 ascorbic acid may be present at $5.00\text{E-}02$ to $2.00\text{E+}00$ mM and N-acetyl-L-cysteine may be present at up to $7.50\text{E+}01$ mM.

As described below, recombinant human EGF (for example, up to $1.29\text{E-}05$ mM) and/or recombinant human FGF2 (for example, up to $5.77\text{E-}06$
15 mM) may also be included in some contexts EGF to maintain long-term cell survival and proliferation.

Preferably, the medium allows the neuralising of non-neural stem cells and the propagation of NPCs cultured therein.

20 A suitable HNM medium may comprise the ingredients set out in table 1, each ingredient being independently at a concentration which in the range defined by the maximum and minimum concentrations set out for that ingredient in table 1. Where no maximum and minimum
25 concentrations are provided, the ingredient may be omitted or may be present at a concentration which is within the range of $\pm 50\%$ of the stated preferred concentration.

In preferred embodiments, the HNM medium may comprise the
30 ingredients set out in table 1, wherein one or more, two or more, three or more or preferably all of the ingredients in the group consisting of human serum albumin, human transferrin, glutathione (reduced), recombinant human insulin, superoxide dismutase, L-ascorbic acid and N-acetyl-L-cysteine are at the preferred
35 concentrations stated in table 1. Preferably, one or more, two or

more, three or more or preferably all of the remaining ingredients are also at the preferred concentrations that those ingredients set out in table 1. Thus, in a preferred embodiment, an HNM medium comprises all the ingredients set out in table 1, with each
5 ingredient being at the concentration which is set out as preferred for that ingredient in table 1.

Another aspect of the invention provides the use of an HNM medium as described above in the neuralisation of stem cells or the
10 propagation of NPCs, for example in accordance with a method described herein.

Other aspects of the invention relate to methods of screening for compounds suitable for use in the treatment of medical conditions
15 associated with degeneration, damage to, the loss of, or a disorder in neuronal cells. A method of screening for a compound for use in the treatment of a medical condition associated with degeneration, damage to, the loss of, or a disorder in nervous tissue, may include;

- 20 (i) bringing a cell produced by a method described herein into contact with a test compound;
(ii) determining the growth, proliferation, function, maintenance, viability or neurotransmitter release of the neural cell in the presence of said test compound.

25 Cells produced by the present methods include NPCs and fully differentiated neural cell types, such as neurons, astrocytes and oligodendrocytes.

30 A cell may be derived from a naturally occurring stem cell disease lines, for example an hESC line with the genotype of a disease such as Down's syndrome and Huntington's disease (embryos with these genetic disorders are commonly discarded following IVF).

A cell may be derived from a genetically modified stem cell line, for example a cell line which over-expresses an aggregation-prone protein such as alpha-synuclein, which may be useful in determining neuronal/glial dysfunction and possible responses to test compounds as described below, in the study of protein aggregation disorders, such as Parkinson's disease.

The growth, proliferation, function, maintenance, viability or neurotransmitter release of the cell in the presence of test compound may be compared with the growth, function, maintenance, viability or neurotransmitter release of the cell in the absence of test compound. An increase in growth, neurotransmission, maintenance, or viability in the presence relative to the absence of test compound may be indicative that the test compound is useful in the treatment of a medical condition associated with degeneration, damage to, the loss of, or a disorder in nervous tissue.

The growth, neurotransmission, or maintenance, or viability of the cell may be determined by any method known to those skilled in the art, for example by cell counting, e.g. by the TUNEL technique or active caspase-3 immunostaining, by BrdU or ³H-Thymidine incorporation, by monitoring morphology, (e.g. sprouting, axonal elongation and/or branching), and/or by monitoring biochemistry, (e.g. TH activity, e.g. neurotransmitter uptake/release/content), and/or by gene or protein expression (e.g. immunohistochemistry, PCR, genechip, proteome) and/or by electrophysiological screening using multi-electrode arrays.

A screening method may employ any known method for analyzing a phenotypic difference between treated and untreated cells and may be at the DNA, mRNA, cDNA or polypeptide level. Differential screening and gene screening are two such techniques. A substance identified by any of the methods of screening described herein may be used as a test substance in any of the other screening methods described herein.

A screening method may employ a nucleic expression array, e.g. a cDNA expression array. In this approach, an array of different nucleic acid molecules is arranged on a filter, quartz or another surface, e.g. by cross-linking the nucleic acid to the filter. A test solution or extract is obtained and the nucleic acid within it is labelled, e.g. by fluorescence. The solution or extract is then applied to the filter or genechip. Hybridisation of the test nucleic acid to nucleic acid on the filter or genechip is determined and compared to the hybridisation achieved with a control solution. A difference between the hybridisation obtained with the test and control samples is indicative of different nucleic acid content. For further information on nucleic acid arrays, see Clontech website (e.g. www.clontech.com) or Affymetrix website (e.g. www.affymetrix.com), findable using any available web browser.

Those of skill in the art may vary the precise format of any of the screening methods of the present invention using routine skill and knowledge.

Test compounds may be natural or synthetic chemical compounds used in drug screening programmes and, in particular, may include established compounds used to treat unrelated conditions, whose pharmacological properties have been well-characterised. Extracts of plants which contain several characterised or uncharacterised components may also be used.

Combinatorial library technology (Schultz, JS (1996) Biotechnol. Prog. 12:729-743) provides an efficient way of testing a potentially vast number of different substances for ability to modulate activity of a polypeptide.

The amount of test substance or compound that may be added to an assay will normally be determined by trial and error depending upon the type of compound used. Typically, from about 0.1 to 100 μ M

concentrations of putative inhibitor compound may be used, for example from 1 to 10 μ M. The test substance or compound may be membrane permeable in order to access intracellular targets.

- 5 The compound may be identified as a compound useful in the treatment of a medical condition associated with degeneration, damage to, the loss of, or a disorder in nervous tissue.

10 A compound identified by any one of the methods provided by the invention may be isolated and/or purified and/or further investigated. It may be manufactured and/or used in the preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug.

- 15 Following identification of a compound as described above, a method may further comprise modifying the compound to optimise the pharmaceutical properties thereof. The modification of a 'lead' compound identified as biologically active is a known approach to the development of pharmaceuticals and may be used to avoid randomly
20 screening large number of molecules for a target property. The optimisation commonly comprises several steps. Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. These parts or residues constituting the active region of the compound are known as
25 its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled to according its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g.
30 spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the modified compound is easy to synthesise, is
5 likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the autophagy inducing activity of the lead compound. The modified compounds found by this approach can then be screened to see whether they promote or increase the growth and/or proliferation of the NPCs, or the survival of their differentiated
10 derivatives, or to what extent.

Further optimisation or modification can then be carried out to arrive at one or more final compounds for *in vivo* or clinical testing.

15 The test compound may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals, e.g. for any of the purposes discussed
20 elsewhere herein. A method may comprise formulating the test compound into a pharmaceutical composition with a pharmaceutically acceptable excipient, vehicle or carrier as discussed further above.

Other aspects of the invention relate to methods of propagating and
25 expanding hESC populations. A method of propagating or expanding a population of hESCs may comprise:

- (i) culturing said hESCs in a differentiation-inhibiting culture medium to produce an hESC colony,
- (ii) slicing said colony using an automated sectioning device
30 to produce colony fragments,
- (iii) culturing one or more of said colony fragments in differentiation-inhibiting culture medium to produce an hESC colony.

Steps (ii) and (iii) may be repeated every 6-10 days. Preferably, the cell density is reduced at least 2-fold, at least 3 fold or at least 4 fold for each repetition of steps ii) and iii).

5 Methods of culturing hESCs are well known in the art. Suitable differentiation-inhibiting culture media include Knockout Dulbecco's Modified Eagle's Medium (KO-DMEM) or DMEM/F12 supplemented with 20% Serum Replacement or fetal bovine serum, 1% Non-Essential Amino Acids, 1mM L-Glutamine, 0.1mM β -mercaptoethanol and 4ng/ml and
10 10ng/ml human bFGF. The hESCs may conveniently be cultured on a layer of feeder cells, for example, irradiated mouse embryonic fibroblasts (MEF) at a density of 10^5 - 10^6 cells/ 60mm dish. Other suitable media formulations which do not required feeder cells are well known in the art (Xu et al. Nat Biotechnol 2001 Oct;19(10):971-
15 4.; Amit et al., Biol Reprod 2004 Mar;70(3):837-45. Epub 2003 Nov 19; Stojkovic et al., Stem Cells, 2005 Aug;23(7):895-902; Ludwig et al, Nat Biotechcnol 2006 Feb;24(2):185-7).

Preferably the stem cell colonies are sliced or sectioned at 100 μ m
20 to 300 μ m intervals in two directions. The mechanical sectioning of stem cell colonies using an automatic sectioning device is described in more detail above.

Various further aspects and embodiments of the present invention
25 will be apparent to those skilled in the art in view of the present disclosure.

All documents mentioned in this specification are incorporated
herein by reference in their entirety.

30 Certain aspects and embodiments of the invention will now be illustrated by way of example and with reference to the figures described below.

Figure 1 shows a schematic of the culture system with corresponding representative phase micrographs (Scale bar, 100µm).

5 Figure 2 shows representative immune micrographs and graphs showing quantification of the temporal profile of Oct4, SSEA4, Nestin and Pax6 in H9 cultured in HNM at D0, D8 and D16 demonstrating efficient neuralisation in defined conditions (Scale bar, 100µm).

10 Figure 3 shows representative immune micrographs and graphs showing quantification of the temporal profile of Oct4, SSEA4, Nestin and Pax6 in HUES-9 cultured in HNM at D0, D8 and D16 demonstrating efficient neuralisation in defined conditions (Scale bar, 100µm).

15 Figure 4 shows the overall expansion of hESC-derived neural precursors over 100 days from one representative hESC plate.

20 Figure 5 shows FACS characterisation and quantification with corresponding immune micrographs of D50 NPCs for the neural progenitor markers Nestin and Musashi1.

25 Figure 6 shows an exemplar family of superimposed whole-cell currents from NPC-derived neurons after 30 days of terminal differentiation obtained under voltage-clamp conditions showing voltage-activated, rapidly inactivating inward currents (marked as ●) followed by sustained outward currents (marked as ■).

Figure 7 shows mean (n=4) current-density versus voltage relationships for inward (●) and outward currents (■) of NPC-derived neurons after 18 days of terminal differentiation.

30 Figure 8 shows membrane potential recording from the same cell as in figure 6 under fast current-clamp conditions, demonstrating action potential generation.

Figure 9 shows a family of superimposed current-clamp recordings of membrane potential from the same cell as in figures 6 and 8.

5 Figure 10 shows a scatter representation of hESC colony size before and after mechanical chopping (bars represent mean values).

Figure 11 shows a graph demonstrating sphere growth following addition of FGF2 at D4, D8 or D12. Addition of FGF2 at D8 is optimal for maximising cell yield without persistence of undifferentiated
10 hESCs (*p<0.05, **p<0.01, ***p<0.001).

Figure 12 shows a graph demonstrating cell yield following addition of FGF2 at D4, D8 or D12.

15 Figure 13 shows a graph showing persistence of Oct4 at D16, following addition of FGF2 at D4, D8 or D12.

Figure 14 shows a schematic of the automated mechanical passaging technique.

20

Figure 15 shows a scatter representation of total hESC number per fragment analyzed after mechanical and enzymatic methods of passaging. Bars represent median values.

25 Figure 16 shows a frequency distribution of colony fragment sizes analyzed immediately after passaging. x-axis values represent the average value for each frequency interval

Figure 17 shows a graph showing colony sizes of passaged human
30 embryonic stem cell populations at 1, 2, and 3 days after passaging.

Figure 18 shows a growth curve showing net expansion of individual colonies in culture after passaging at days 2 and 3 (expressed relative to day 1 after passaging).

35

ExamplesMethods*hESC culture*

The hESC lines H9 (WiCell Research Institute, Madison; WI, USA) and
5 HUES-9 (hES facility; Harvard University, Cambridge, MA, USA)
derived in accordance with local and national guidelines were used,
between passages 21-60. Cells were cultured as previously described
(Vallier, L. et al Dev. Biol. 275, 403-421 (2004), Cowan, C.A. et
al. N. Engl. J. Med. 350, 1353-1356 (2004)) on a layer of irradiated
10 mouse embryonic fibroblasts (MEF) at a density of 3×10^5 cells/60mm
dish for H9, and 1×10^6 cells/60mm dish for HUES-9, and passaged by
enzymatic or mechanical means (Joannides, A. et al. Stem Cells 24,
230-235 (2006)). Culture medium (KSR) consisted of Knockout
Dulbecco's Modified Eagle's Medium (KO-DMEM) supplemented with 20%
15 Serum Replacement, 1% Non-Essential Amino Acids, 1mM L-Glutamine,
0.1mM β -mercaptoethanol (all from Invitrogen; Carlsbad, CA, USA) and
4ng/ml and 10ng/ml human bFGF (R&D, Minneapolis, MN, USA) for H9 and
HUES-9 respectively.

20 *NPC generation and propagation*

Human ESCs were washed in phosphate buffered saline (PBS) and
incubated in 1mg/ml collagenase IV (Invitrogen) for approximately
90min until colonies could be easily detached from the underlying
feeder layer by gentle pipetting. Detached colonies were placed in a
25 15ml conical tube and allowed to settle by gravity for 5min, before
being transferred with a sterile Pasteur pipette onto the lid of a
60mm culture dish. Colonies were chopped at 150 μ m intervals using a
McIlwain tissue chopper (Mickle Engineering; Gomshall, Surrey, UK)
before being plated at a low density in human neuralising medium
30 (HNM) into bacteriological grade 10cm culture dishes (Sterilin;
Stone, Staffordshire, UK) on an orbital shaker to prevent sphere
aggregation. HNM consisted of a modified version of chemically
defined medium as previously described for mESC differentiation
(Johansson, B.M. & Wiles, M.V. Mol. Cell Biol. 15, 141-151 (1995),
35 Wiles, M.V. & Johansson, B.M. Exp. Cell Res. 247, 241-248) and with

all non-human components replaced with recombinant or human-derived products (Table 1). Medium was changed by harvesting the growing spheres by gravity in conical tubes. Cultures were passaged approximately every 8 days using a McIlwain tissue chopper. For
5 terminal differentiation, NPCs were plated onto poly-D-lysine/laminin coated coverslips and cultured in DMEM / 2% B27 (Invitrogen) or HNM without added growth factors for up to 35 days.

Transcriptional analysis

10 Total RNA was extracted from harvested NPCs using the RNeasy Mini Kit (Qiagen; Valencia, CA, USA) according to the manufacturer's instructions. Samples were treated with RNase-free DNase (Qiagen) to remove DNA contamination. cDNA was synthesized from 2.5µg of RNA using Moloney Murine Leukaemia Virus reverse transcriptase
15 (Invitrogen) and oligo-dT primers according to the manufacturer's instructions. PCR was carried out using Taq polymerase (Invitrogen). Sequences and PCR conditions for each primer are shown in Table 2. PCR products were separated on a 2% agarose gel and visualised with SYBR-Green (Invitrogen). The expression of the housekeeping gene
20 GAPDH was used to normalise PCR reactions.

Immunocytochemistry

Immunocytochemistry was carried out using standard procedures. hESC colonies and coverslip plate-downs were fixed with 4% fresh
25 paraformaldehyde (PFA) for 20min at room temperature. NPC spheres were fixed with 4% fresh PFA for 30min at 4°C, and cryoprotected with 30% sucrose before embedding in OCT compound (BDH; Dorset, UK). Blocks were sectioned at 12µm intervals using a Leica cryostat. Fixed cells and sections were blocked for 1h at room temperature
30 with PBS / 5% goat serum / 0.1% Triton X-100 and then incubated overnight with primary antibody at 4°C. Secondary antibody (Invitrogen, 1:1000) was applied for 1h at 37°C in PBS/Hoechst (1:5000). Primary antibodies used included Oct4 (Santa Cruz, 1:100), SSEA4 (DSHB, 1:50), Nestin (Chemicon, 1:500), Pax6 (DSHB, 1:50),
35 Musashi1 (Chemicon, 1:500), β-III tubulin (Sigma, 1:200), MAP2ab

(Sigma, 1:200), Glutamate (Sigma, 1:500), GABA (Sigma, 1:500), Tyrosine Hydroxylase (Chemicon, 1:500), Synapsin1 (Calbiochem, 1:500), GFAP (DakoCytomation, 1:200) and O4 (supernatant diluted 1:5, 6). Cells were viewed under a Leitz microscope with appropriate
5 filters for cell identification and counting.

FACS analysis

Harvested NPCs were prepared for FACS analysis as previously described⁷. NPCs were dissociated with accutase, washed in FACS
10 buffer (PBS / 2% serum / 10mM sodium azide - PFN) and fixed in ice cold 4 % PFA for 30 min. Cells were incubated with primary antibodies (at similar concentrations to immunocytochemistry) in PFN with 0.14% saponin (Sigma) for 30 min at 4°C. Secondary antibodies were applied for 30 min at 4°C. Cells were then washed in PFN and
15 analysed using a CyAn flow cytometer (Dako). Data analysis was performed using Summit v4.2 software (Dako).

Quantification and statistical analysis

All experiments were repeated at least 3 times unless stated
20 otherwise. Cells were viewed using a Leitz microscope at high magnification (x40) for determination of cell numbers, hESC colony fragment sizes after seeding in HNM, and NPC sphere diameter in subsequent culture using a calibrated eyepiece. A minimum of twenty random fields were counted for each experiment and hESC colony
25 fragment sizes following passaging were obtained by determining the number of cells per cluster in a total of 50 random fields for each method over five different passages. Neuralisation experiments were performed a minimum of 6 times per line, with quantitative immunocytochemical analysis undertaken twice for each line. The
30 proportion of positive cells was expressed as a mean $\pm$ s.d. A paired t-test was used to determine any significant difference in mean cell counts between lines for the markers studied. P values for statistical significance in all other two sample comparisons were calculated using a student's unpaired t-test.

Colony population growth was calculated by determining the number of cells per colony in 15 randomly selected fields at each time point for every condition. Colony growth curves were constructed by determining the size of 10 randomly selected colonies from each condition (on two separate occasions) 24 hours after passaging (day 1) and at two subsequent 24-hour intervals (days 2 and 3). Net expansion was calculated by dividing total cell number for a given colony at day 2 or 3 by the total cell number for the same colony at day 1. All figures and error bars are represented as mean $\pm$ standard error from the mean unless stated otherwise. For parametric analysis, a twotailed Student's t-test to compare population means and a twotailed F-test to compare population variances were used. A Mann-Whitney rank-sum test to compare median values was performed for nonparametric analysis.

Statistical analysis was carried out using GraphPad Prism 3.03 (GraphPad Software, San Diego, CA, <http://www.graphpad.com>).

Karyotypic analysis

Chromosome number and size was scored using G-banding by the Department of Haematology, Cambridge University Hospitals NHS Trust.

Electrophysiological characterisation

Terminally differentiated NPCs on glass coverslips (as described above) were transferred into a perfusion chamber mounted on the stage of an Olympus CK40 inverted microscope equipped with phase-contrast optics. Cells were continuously perfused at room temperature using a gravity fed system at a rate of 2-4 ml/min with a bath solution containing: 135mM NaCl, 5mM KCl, 1.2mM MgCl₂, 2.5mM CaCl₂, 5mM HEPES and 10mM glucose with the pH adjusted to 7.4 with NaOH. Some of cells which displayed a neuronal phenotype (cell bodies of 8-12 μ m with long branching projections) were voltage- or current-clamped following establishment of the whole-cell configuration of the patch-clamp technique. Whole cell pipettes had measured tip resistances of 6-8 M Ω when filled with a solution

containing: 117mM KCl, 10mM NaCl, 2mM MgSO₄, 1mM CaCl₂, 11mM EGTA, 2mM Na-ATP and 11mM HEPES with the pH adjusted to 7.2 with KOH. Whole cell capacitance was measured and compensated whilst series resistance compensation was employed at 80-85%. The standard voltage-clamp protocol consisted of holding cells at -90mV and sequentially stepping at 10mV intervals for 200ms to voltages between -120mV and +60mV using a P/N=4 on-line subtraction protocol. Inward currents were measured as the peak current between 1 and 20 ms into the voltage step whilst outward currents were measured as the mean current between 180 and 195ms into the voltage step. All currents were normalized to cell membrane area by dividing cell capacitance to yield current density (pA/pF). Further, cells were current-clamped at 0pA and two protocols employed: i) continuous recording of resting membrane potential interspersed with current injection to bring membrane potential about threshold for action potential generation (typically 50-100pA) and; ii) stepped current injection of 100ms duration from 0pA to that which elicited action potential generation and beyond (typically from between 10 to 200pA). All recordings were obtained using an Axopatch 700A amplifier (Axon Instruments; Foster City, CA, USA) and a DigiData 1320 A/D interface. Current and voltage protocols were generated by PClamp 9 software and recordings were obtained at a digitisation rate of 10kHz through a 4-pole Bessel low pass filter set at 2kHz.

Results

NPC Production

To initiate neuroectodermal differentiation, hESC colonies were selectively detached, seeded in HNM and cultured as free-floating embryoid bodies (EBs) in suspension culture. Preliminary studies showed that neural specification occurred over approximately 16 days. However, EBs were heterogeneous in nature. Analysis revealed a correlation between EB size and degree of neurogenesis; small aggregates had a uniform, compact structure and were highly neurogenic, while larger aggregates had a non-uniform internal organisation, with limited neurogenic regions dispersed as rosette

islands (Supplemen. This finding is consistent with previous mESC observations showing that low cell plating density in suspension culture is critical for efficient neurogenesis (Bouhon, I.A. et al Brain Res Bull. 68, 62-75 (2005), Watanabe, K. et al. Nat. Neurosci. 8, 288-296 (2005)). Unlike mESCs however, dissociated hESCs had very poor survival in HNM. In order to overcome this problem, we adapted our protocol of hESC automated mechanical passaging (Joannides, A. et al. Stem Cells 24, 230-235 (2006)) to chop detached hESC colonies before seeding into HNM. A mini-orbital shaker was also used to prevent re-aggregation of chopped colony fragments. This resulted in consistently small hESC aggregates of about 70 cells which now formed uniform compact spheres over 16 days (Figure 10). Serial RT-PCR analysis revealed a gradual loss of the pluripotency markers POU5F1, NANOG and TERT, with a concomitant and marked upregulation of the neuroectodermal markers NCAM, PAX6 and SOX1. SOX2, a marker of embryonic and neural stem cells, was present throughout. Importantly, negligible expression of pluripotent, endodermal (HNF-3 β) and mesodermal (T, MYOD, SMA) markers was evident by D16.

Whilst HNM efficiently supported the neuralisation of hESCs, culture in HNM alone did not support the longer-term propagation of the hESC-derived neural precursor cells (NPCs) much beyond D16. Therefore, to improve cell yield, we supplemented HNM with human recombinant FGF2, an established mitogen for human fetal-derived neural precursors¹³. Since FGF2 contributes to the maintenance of hESC pluripotency (Xu, R.H. et al. Nat. Methods 2, 185-190 (2005)), we first determined the optimal time point for adding FGF2 to HNM to support NPC proliferation. Addition of FGF2 at D0 increased total cell yield, but as expected maintained a significant persistence of Oct4 expression (Figures 11 to 13). Instead, supplementing cultures with FGF2 from D8 onwards was optimal for maximising NPC yield without contaminant undifferentiated hESCs.

Combining mechanical chopping of hESC colonies at D0, neuralisation in HNM, orbital shaking, and addition of FGF2 at D8 resulted in cell

acquisition of nestin and Pax6 protein expression by D16 with loss of Oct4 and SSEA4 expression (Figures 2 and 3). Together these findings are consistent with the generation of a highly enriched hESC-derived NPC population. There was no significant difference
5 between H9 and HUES9 in the mean proportion of cells expressing these markers at the studied time-points ($p=0.439$).

Following neuralisation, NPCs at D16 were dissociated and subsequently propagated in HNM under similar conditions to those
10 used for human fetal-derived neural precursors (Svendsen, C.N. et al. J. Neurosci. Methods 85, 141-152 (1998)); automated mechanical passaging and addition of FGF2 together with EGF resulted in long-term NPC propagation with complete loss of Oct4 / SSEA4 at all subsequent timepoints analysed. Using this system, NPCs had an
15 overall expansion in excess of 250,000-fold by D100 (Figure 4) and maintained a stable karyotype. The behaviour of hESC-derived NPCs was comparable across H9 and HUES9. The use of the orbital shaker allowed up to 3×10^7 cells to be cultured in a single 10cm plate, thus making large scale expansion practical. Propagated NPCs
20 maintained high expression of the neural progenitor markers Nestin and Musashi1 at D50 (Figure 5), and later timepoints analysed. To date, NPCs have been propagated using this method for over 180 days. NPCs could also be cryopreserved allowing efficient banking and recovery of expanded neuralised cell stocks. Critically, NPCs were
25 multipotent and could be differentiated into mature neurons (expressing β -tubulin, MAP2ab and SynapsinI) with different neurotransmitter subtypes, astrocytes and oligodendrocytes. NPC-derived neurons were electrically active, demonstrating fast, voltage-activated and rapidly inactivating inward currents followed
30 by slowly activated but sustained outward currents (Fig. 6) indicative of voltage-activated sodium currents and delayed-rectifier potassium currents (Fig. 7), and were able to generate action potentials upon current injection (Figs 8 and 9).

hESC Cell Automated Mechanical Passaging

To compare enzymatic and mechanical passaging, hESC cultures were established. The H9 line had been previously passaged using collagenase. Briefly, after incubation with collagenase, colonies were detached using a cell scraper, gently triturated, and replated in a 1:3 splitting ratio. To establish a mechanical passaging method, initial studies determined that single colonies could be successfully passaged by manual dissection using a scalpel blade, a finding consistent with previous observations (Reubinoff BE et al. Nat Biotechnol 2000; 18:399-404, Schulz TC et al. BMC Neurosci 2003; 4:27, Oh SK et al. Stem Cells 2005;23:605- 609). However, this method is extremely time-consuming and impractical for regular use or bulk passaging. To explore whether this method could be automated, a McIlwain tissue chopper was adapted. The McIlwain tissue chopper, originally developed for sectioning fresh brain tissue, has been successfully used for passaging of human fetal neural precursor cultures (Svendsen CN et al. J Neurosci Methods 1998;85:141-152). The tissue chopper was modified by removing a small square on the chopper arm to avoid contact with the side of the culture dish. Colony plates were fixed on the chopper stage and automatically sectioned. The McIlwain tissue chopper allows the sectioning interval to be calibrated between 50 and 950 μm . Pilot studies identified 200- μm intervals as optimal for AMP, dividing colonies into approximately 15 fragments, depending on the starting colony size.

AMP is rapid, with a single 6-cm dish being passaged in less than 60 seconds. The chopped fragments were readily detached from the culture dish and replated at a ratio of 1:3. A striking observation after AMP was the consistency of fragment size after chopping in contrast to the variability of size produced by conventional collagenase passaging.

Quantitative analysis of this phenomenon was next undertaken by counting the total number of cells in randomly selected

fragments (immediately after passage) at different passage numbers. A narrower size range was evident in AMP (40 to 250 cells/fragment) compared with enzymatic passaging (10 to > 500 cells/fragment). Initial analysis revealed similar mean values that were not significantly different (138 ± 8 [AMP], 133 ± 30 [collagenase]; $p > .10$), an observation consistent with comparable starting cell numbers. However, a large variance was apparent in collagenase-treated cultures, which was significantly different from mechanically passaged cultures ($p > .0001$). The scatter representation and frequency distribution confirmed large numbers of very small or large fragment sizes in collagenase-passaged cultures in contrast to the narrower distribution after AMP (Figs. 15 and 16). This difference was statistically significant (median values: 148 [AMP], 31 [collagenase]; $p > .0001$).

We next examined growth of colonies derived by enzymatic and mechanical passaging. Population analysis at days 1 to 3 after passaging revealed comparable mean cell number per colony between the two methods ($p > .10$ for all time points). However, a significantly larger variability of colony cell number persisted in collagenase-passaged cultures compared with AMP after seeding (Fig. 17; $p > .02$ for all time points).

Serial examination of individual colonies at days 1 through 3 after passaging showed no significant difference in net expansion between the two methods (D2: 2.21 ± 0.14 [AMP], 1.96 ± 0.15 [collagenase]; D3: 4.61 ± 0.27 [AMP], 4.01 ± 0.37 [collagenase]; values relative to day 1; $p > .10$ for both time points; Fig. 18).

Cultures were passaged approximately every 3 to 4 days and, to date, have been expanded exclusively by AMP for over 100 days. AMP-passaged cells could also be successfully freeze-thawed. Cytogenetic analysis showed maintenance of a stable karyotype by AMP (data not shown). In addition to the H9 line, HuES9 was also successfully propagated with AMP for multiple passages.

Spontaneous differentiation was occasionally observed in hESC cultures. However, substantially less differentiation was found in cultures maintained by AMP. This may reflect the uniformity of size achieved by the technique, given that differentiation was predominantly evident within the centre of large colonies. Furthermore, expression analysis revealed maintenance of pluripotency of hESC cultures with AMP.

Semi-quantitative reverse transcription (RT)-PCR did not reveal any significant differences in the expression of POU5F1, NANOG, SOX2, or TERT between the two methods. Immunocytochemistry confirmed that mechanically passaged colonies remained positive for Oct-4 and SSEA-4.

The methods described herein therefore provide a method for bulk expansion of hESCs by automated mechanical chopping that is both rapid and maintains greater uniformity of colony size than conventional methods.

Amino Acids		Min (mM)	Preferred (mM)	Max (mM)	importance
Glycine		1.00E-01	2.50E-01	5.00E-01	
L-Alanine		2.00E-02	1.91E-01	4.00E-01	
L-Alanyl-L-Glutamine		present	2.50E+00	8.00E+00	**
L-Arginine hydrochloride		5.00E-02	6.99E-01	5.00E-01	
L-Asparagine		2.00E-02	1.44E-01	5.00E-01	
L-Aspartic acid		2.00E-02	1.63E-01	5.00E-01	
L-Cystine		5.00E-02	1.12E-01	5.00E-01	
L-Cysteine hydrochloride		2.00E-02	1.03E-01	4.00E-01	
L-Glutamic acid		2.00E-02	3.05E-01	6.00E-01	
L-Histidine hydrochloride-H2O		2.00E-02	1.50E-01	5.00E-01	
L-Isoleucine		2.00E-02	4.16E-01	1.00E+00	
L-Leucine		5.00E-02	4.51E-01	1.00E+00	
L-Lysine hydrochloride		5.00E-02	4.99E-01	1.00E+00	
L-Methionine		2.00E-02	1.16E-01	3.00E-01	
L-Phenylalanine		2.00E-02	2.15E-01	5.00E-01	
L-Proline		5.00E-02	3.24E-01	5.00E-01	
L-Serine		5.00E-02	2.50E-01	6.00E-01	
L-Threonine		5.00E-02	4.49E-01	1.00E+00	
L-Tryptophan		5.00E-03	4.42E-02	8.00E-02	
L-Tyrosine disodium salt		5.00E-02	2.31E-01	6.00E-01	
L-Tyrosine		5.00E-02	1.49E-02	6.00E-01	
L-Valine		5.00E-02	4.52E-01	1.00E+00	
N-Acetyl-L-Cysteine		present	7.66E-01	7.50E+01	**
Vitamins					
L-Ascorbic acid		5.00E-02	2.27E-01	2.00E+00	**
Biotin		present	4.16E-05	1.00E-04	*
Choline chloride		1.50E-02	6.43E-02	1.50E-01	
D-Calcium pantothenate		1.00E-03	4.72E-03	1.00E-02	
Folic acid		2.00E-03	6.01E-03	1.50E-02	
i-Inositol		2.00E-02	7.00E-02	1.50E-01	
Niacinamide		3.00E-04	1.65E-02	3.00E-02	
Pyridoxine hydrochloride		5.00E-04	1.46E-04	5.00E-02	
Pyridoxal hydrochloride		5.00E-03	9.80E-03	5.00E-02	
Riboflavin		1.00E-04	5.79E-04	5.00E-03	
Thiamine hydrochloride		1.00E-04	6.40E-03	2.00E-02	*
Vitamin B12		1.00E-05	5.20E-04	2.00E-03	*

Proteins					
Human serum albumin		1.80E-02	7.52E-02	1.20E-01	**
Human transferrin (30% saturation)		3.80E-05	3.80E-04	2.85E-03	***
Recombinant human EGF *		present	3.23E-06	1.29E-05	**
Recombinant human FGF2 *		present	1.15E-06	5.77E-06	****
Recombinant human insulin		3.51E-04	1.75E-03	5.26E-03	***
Glutathione (reduced)		present	4.48E-03	1.00E-02	***
Superoxide dismutase		present	4.62E-05	1.50E-04	**
Inorganic salts					
Calcium Chloride (CaCl ₂ ·2H ₂ O)			8.95E-01		
Cupric sulfate (CuSO ₄ ·5H ₂ O)			5.00E-06		
Ferrous sulfate (FeSO ₄ ·7H ₂ O)			1.50E-03		
HEPES			1.25E+01		*
Magnesium Chloride (MgCl ₂ ·6H ₂ O)			3.01E-01		
Magnesium Sulfate (MgSO ₄ ·7H ₂ O)			4.06E-01		
Potassium Chloride (KCl)			3.69E+00		
Potassium Nitrate (KNO ₃)			3.76E-04		
Sodium Bicarbonate (NaHCO ₃)			2.50E+01		
Sodium Chloride (NaCl)			1.04E+02		
Sodium Phosphate monobasic (NaH ₂ PO ₄ ·2H ₂ O)			5.10E-01		
Sodium Phosphate dibasic (Na ₂ HPO ₄)			5.00E-01		
Sodium Selenite (Na ₂ SeO ₃ ·5H ₂ O)		present	3.23E-05	1.50E-04	**
Zinc sulfate (ZnSO ₄ ·7H ₂ O)			1.50E-03		
Lipids					
Arachidonic acid		6.57E-06	6.57E-05	9.85E-05	
Cholesterol		5.69E-04	5.69E-03	8.53E-03	
DL-alpha-tocopherol acetate		present	1.48E-03	3.00E-03	*
Linoleic acid		4.00E-05	4.00E-04	5.99E-04	
Linolenic acid		3.60E-05	3.60E-04	5.40E-04	
Lipoic acid		4.86E-05	4.86E-04	7.28E-04	
Myristic acid		4.38E-05	4.38E-04	6.57E-04	
Oleic acid		3.54E-05	3.54E-04	5.31E-04	
Palmitic acid		3.90E-05	3.90E-04	5.85E-04	
Palmitoleic acid		1.57E-05	1.57E-04	2.36E-04	
Stearic acid		2.77E-05	2.77E-04	4.16E-04	

Other components					
1-Thioglycerol			4.50E-01		*
D-Glucose			1.75E+01		*
Hypoxanthine			1.47E-02		
Phenol Red			2.15E-02		
Pluronic F68			1.19E-01		
Putrescine 2HCl		present	5.00E-04	2.50E-01	**
Sodium Pyruvate			1.00E+00		*
Thymidine			1.45E-03		
Tween 80			8.74E-02		

* added at time points indicated in the text.

Table 1

Gene	Forward	Reverse	Cycles	MgCl ₂ (mM)	DMSO (%)	Tann
<i>GAPDH</i>	AGCCACATCGCTCAGACACC	GTACTCAGCGGCCGAGCATCG	25	1.5	0	60
<i>HNF-3β</i>	GACAAGTGAGAGAGCAAGTG	ACAGTAGTGGAACCGGAG	25	1.5	0	60
<i>MyoD</i>	GGCCTTTGAGACACTCAAGC	AGGCAGTCTAGGCTCGACAC	35	1.5	5	55
<i>NANOG</i>	TTCTTGCACTCTGCTGGAGGC	AGTGTGGATCCAGCTTGTCCT	35	3	0	55
<i>NCAM</i>	AGGAGACAGAAACGAAGCCA	GGTGTGGAAATGCTCTGGT	25	1.5	0	60
<i>PAX6</i>	AACAGACACAGCCCTCACAACA	CGGGAACTTGAACTGGAAGTAC	25	1.5	0	60
<i>POU5F1</i>	CTTGCTGCAGAAGTGGGTGGAGGAA	CTGCAGTGTGGGTTTCGGGCA	25	3	0	55
<i>SMA</i>	GGAGTTATGGTGGGTATGGGTC	AGTGGTGACAAAGGAGTAGCCA	20	2	0	60
<i>SOX1</i>	TACAGCCCATCTCCAACTC	GCTCCGACTTCACCAGAGAG	30	1.5	0	65
<i>SOX2</i>	ATGCACCGCTACGACGTGA	CTTTTGACCCCTCCCATTTT	25	1.5	0	55
<i>T</i>	GTGACCAAGAACGGCAGGAGG	TGTTCCGATGAGCATAGGGGC	30	1.5	5	65
<i>TERT</i>	AGCTATGCCCGGACCTCCAT	GCCTGCAGCAGGAGGATCTT	20	1.5	0	60

Table 2

Claims:

1. A method of producing a population of neural precursor cells
5 comprising:
 - i) slicing a colony of stem cells using an automated tissue sectioning device to produce colony fragments, and;
 - ii) culturing one or more of said colony fragments in human neutralising medium (HNM) to produce said population of neural
10 precursor cells (NPCs).
2. A method according to claim 1 wherein the colony is sliced at intervals of 50µm to 250µm
- 15 3. A method according to claim 1 or claim 2 wherein the colony is sectioned by the automated tissue sectioning device in two different directions to produce the colony fragments.
4. A method according to any one of claims 1 to 3 wherein each
20 colony fragment contains about 40 to 150 cells.
5. A method according to any one of claims 1 to 4 wherein the HNM is a mammalian cell culture medium comprising human serum albumin, human transferrin, glutathione (reduced), recombinant human insulin,
25 superoxide dismutase, L-ascorbic acid and N-acetyl-L-cysteine.
6. A method according to claim 5 wherein the HNM comprises the ingredients set out in table 1, each ingredient being independently at a concentration between the maximum and minimum concentrations
30 set out for that ingredient.
7. A method according to claim 6 wherein the ingredients of the HNM are at the preferred concentrations set out in table 1.

8. A method according to any one of claims 1 to 7 wherein the one or more colony fragments are agitated in the HNM to prevent re-aggregation of the fragments.

5 9. A method according to any one of claims 1 to 8 wherein the one or more colony fragments are cultured in HNM for 12 to 18 days to produce the NPCs.

10 10. A method according to claim 9 wherein human recombinant fibroblast growth factor 2 (FGF2) is added to the one or more colony fragments after 4 to 10 days.

15 11. A method according to any one of the preceding claims wherein the stem cells express one or more markers selected from the group consisting of Oct-4; Nanog; Sox2; stage specific embryonic antigens (SSEA-3 and 4), and the tumor rejection antigens TRA-1-60 and -1-81.

20 12. A method according to any one of the preceding claims wherein the NPCs express one or more markers selected from the group consisting of Nestin, Musashil, Pax6 and Sox1.

13. A method of propagating neural precursor cells (NPCs) comprising:

- 25 (i) slicing a colony of NPCs using an automated tissue sectioning device to produce colony fragments,
(ii) culturing said colony fragments in HNM supplemented with FGF2 and EGF and,
(iii) repeating the steps (i) and (ii) above every 6-10 days.

30 14. A method according to claim 13 wherein the cell density is reduced by at least 2-fold density each time steps (i) and (ii) are repeated.

35 15. A method according to claim 13 or claim 14 wherein the colony is sliced at intervals of 50µm to 250µm.

16. A method according to any one of claims 13 to 15 wherein said NPCs are produced by a method according to any one of claims 1 to 12.

5 17. A method according to any one of claims 1 to 16 comprising purifying said NPCs.

18. A method according to claim 17 said NPCs are purified by FACS sorting.

10

19. A method according to any one of claims 1 to 16 comprising causing or allowing the NPCs to terminally differentiate to produce a population of differentiated neural cells.

15 20. A population of cells produced or propagated in accordance with a method according to any one of claims 1 to 19.

21. A population of cells according to claim 20 for use in a method of treatment of the human or animal body.

20

22. A population of cells according to claim 21 for use in the treatment of a medical condition associated with degeneration, damage to, the loss of, or a disorder in nervous tissue.

25 23. Use of a population according to claim 22 in the manufacture of a composition for use in the treatment of a medical condition associated with degeneration, damage to, the loss of, or a disorder in nervous tissue.

30 24. A method of screening for an agent for use in the treatment of a medical condition associated with degeneration, damage to, the loss of, or a disorder in nervous tissue, comprising;
(i) contacting a cells produced by a method according to any one of claims 1 to 19 into contact with a test compound;

(ii) determining the growth, proliferation, neurotransmission, maintenance, viability or neurotransmitter release of the cell in the presence of said test compound.

5 25. A method according to claim 24 wherein an increase in growth, proliferation, neurotransmission, maintenance, or viability in the presence relative to the absence of test compound is indicative that the test compound is useful in the treatment of a medical condition associated with degeneration, damage to, the loss
10 of, or a disorder in nervous tissue.

26. A method according to claim 24 or claim 25 comprising identifying the compound as a compound useful in the treatment of a medical condition associated with degeneration, damage to, the loss
15 of, or a disorder in nervous tissue.

27. A mammalian cell culture medium comprising human serum albumin, human transferrin, glutathione (reduced), recombinant human insulin, superoxide dismutase, L-ascorbic acid and N-acetyl-L-
20 cysteine.

28. A method according to claim 27 wherein the HNM comprises the ingredients set out in table 1, each ingredient being independently at a concentration between the maximum and minimum concentrations
25 set out for that ingredient.

29. A method according to claim 28 wherein said ingredients at the preferred concentrations set out in table 1.

30 30. Use of a mammalian cell culture medium according to any one of claims 27 to 29 in a method of neuralising stem cells.

31. Use of a mammalian cell culture medium according to any one of claims 27 to 29 in a method of propagating NPCs.

32. A method of propagating or expanding a population of hESCs comprising:

(i) culturing said hESCs in a differentiation-inhibiting culture medium to produce an hESC colony,

5 (ii) slicing said colony using an automated sectioning device to produce colony fragments,

(iii) culturing one or more of said colony fragments in differentiation-inhibiting culture medium to produce an hESC colony.

10 33. A method according to claim 32 wherein the colony is sliced at intervals of 50µm to 300µm

34. A method according to claim 32 or claim 33 wherein the colony is sectioned by the automated tissue sectioning device in two
15 different directions to produce the colony fragments.

35. A method according to any one of claims 33 to 34 wherein each colony fragment contains 40 to 250 cells.

20 36. A method according to any one of claims 32 to 35 wherein steps (ii) and (iii) are repeated every 6-10 days.

37. A method according to claim 36 wherein the cell density is reduced by at least 2-fold density on each repetition of said steps.

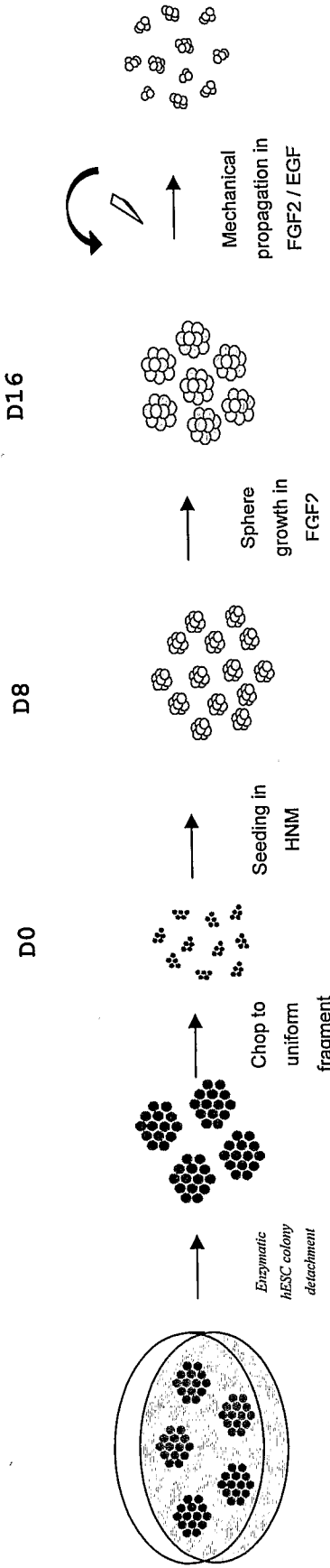
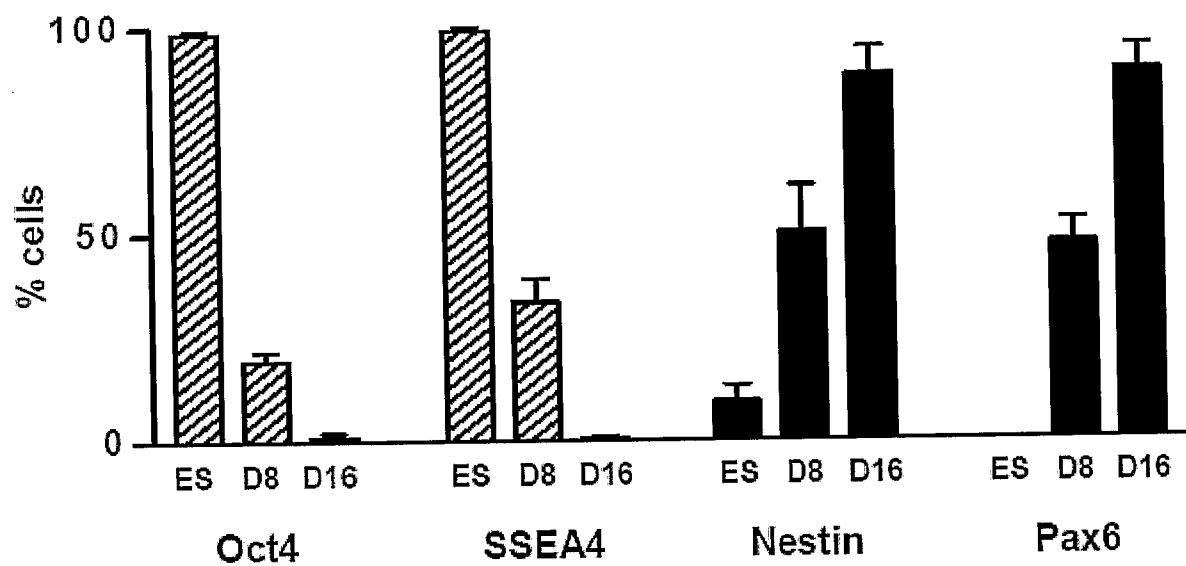


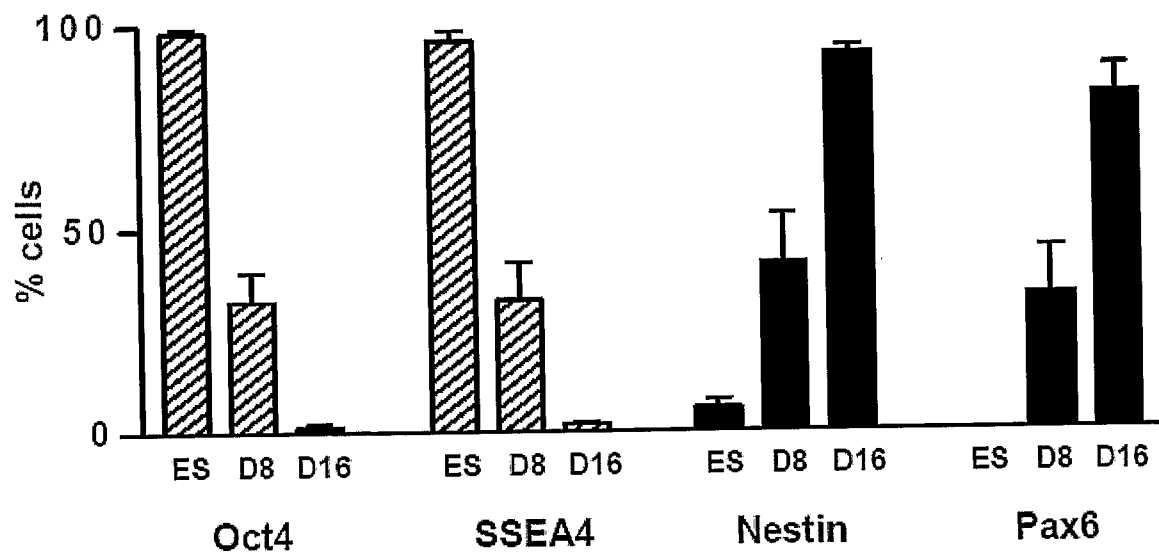
Figure 1

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H9

Figure 2



HUES9

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Figure 3

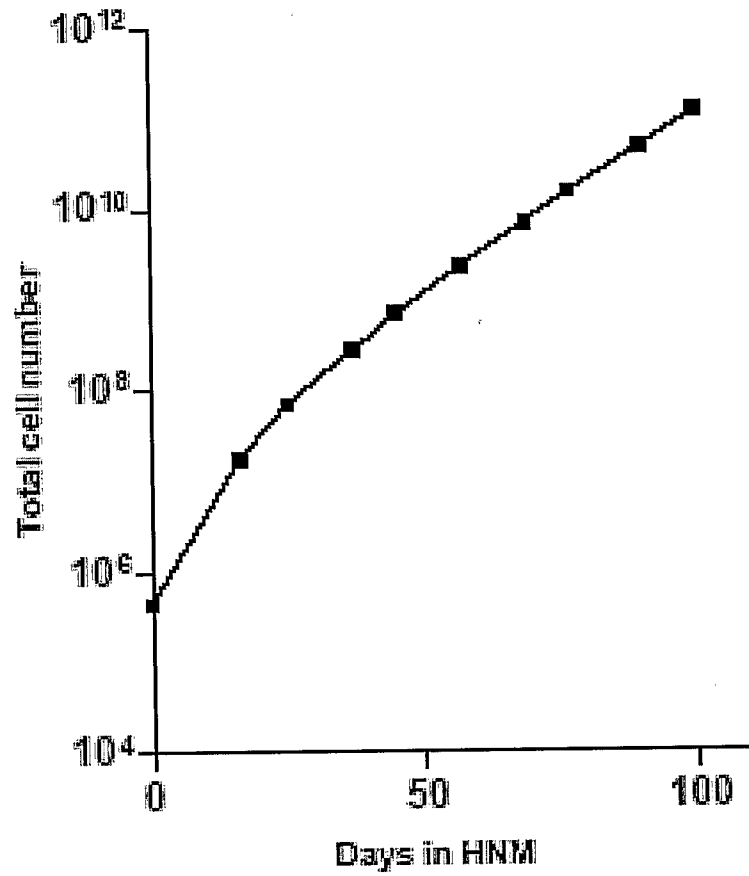


Figure 4

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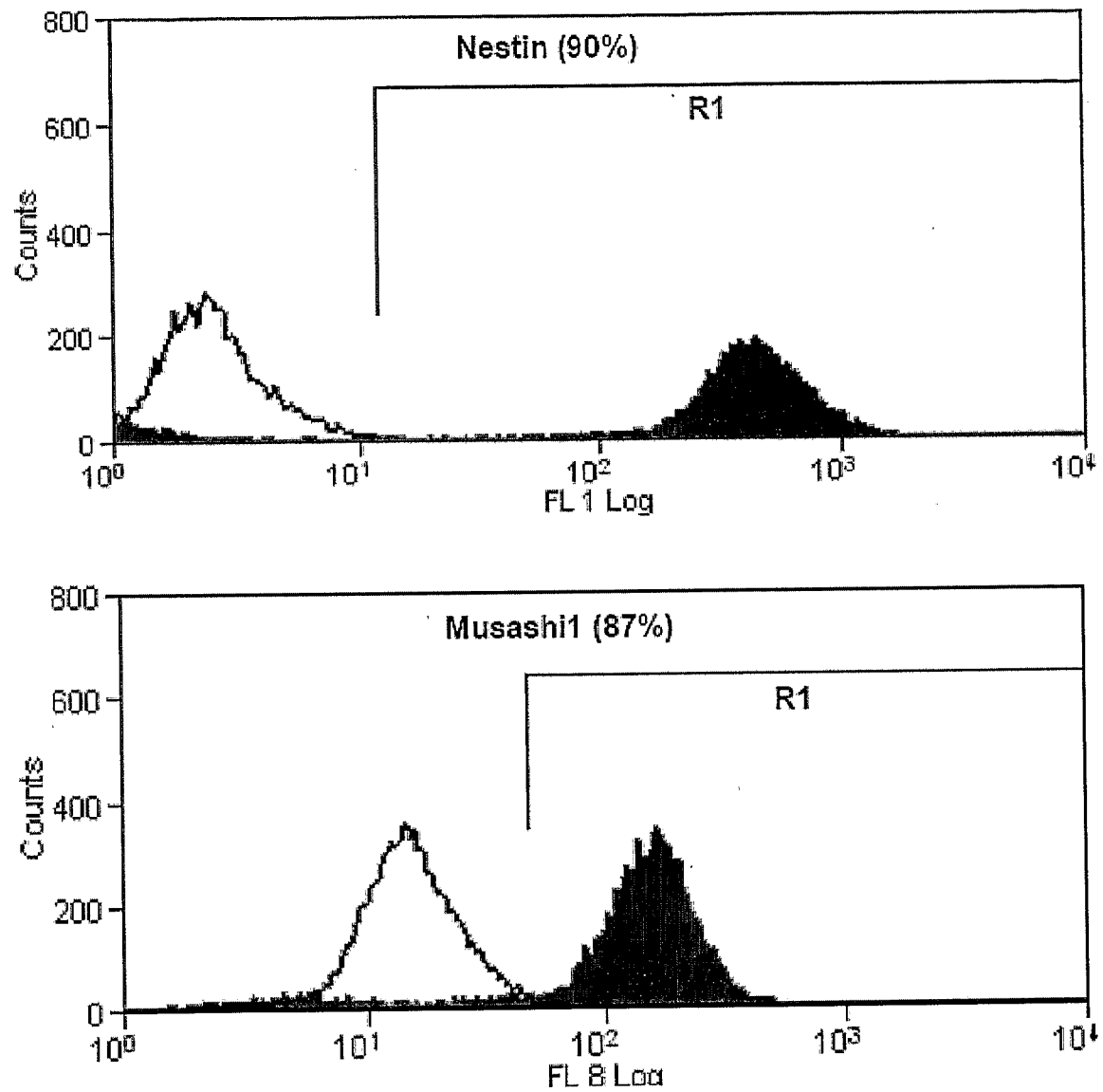


Figure 5

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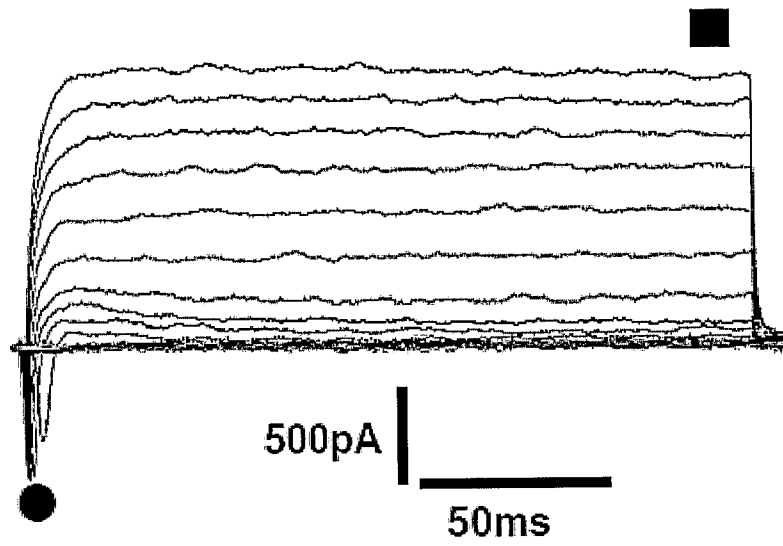


Figure 6

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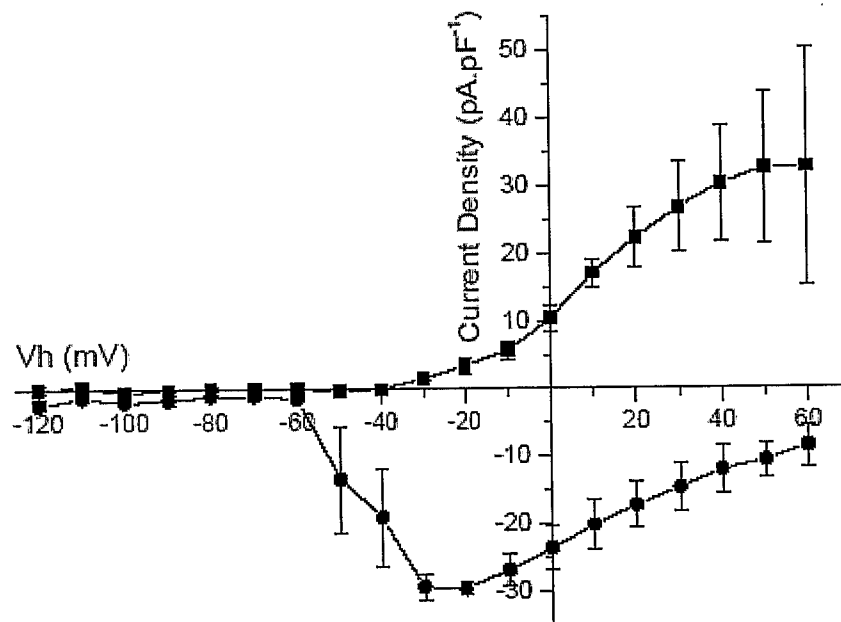


Figure 7

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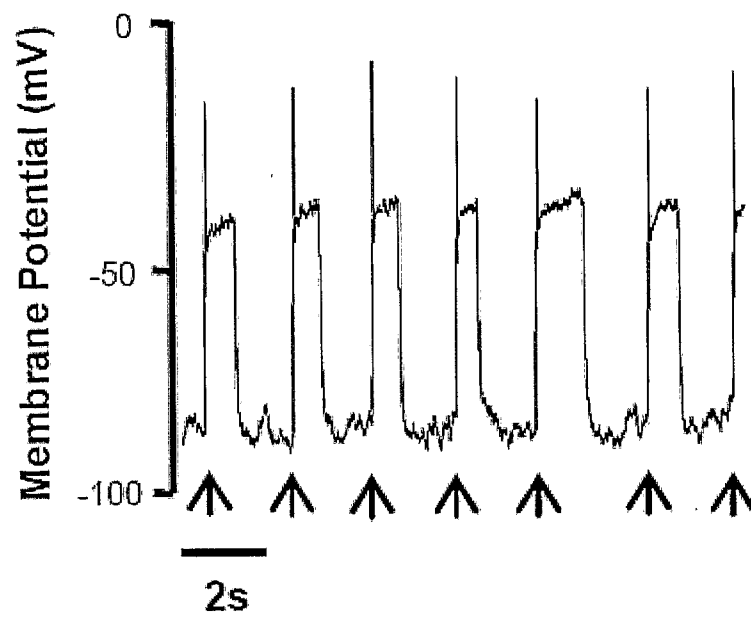


Figure 8

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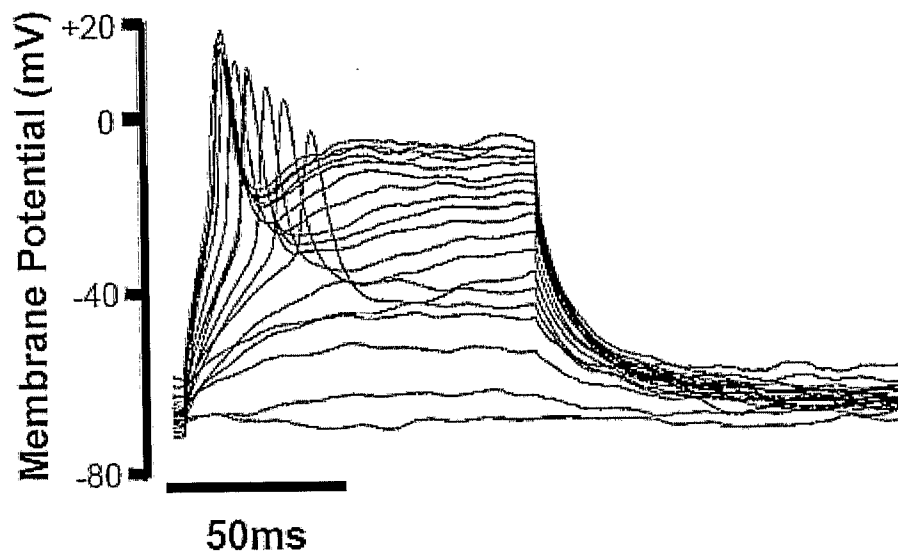


Figure 9

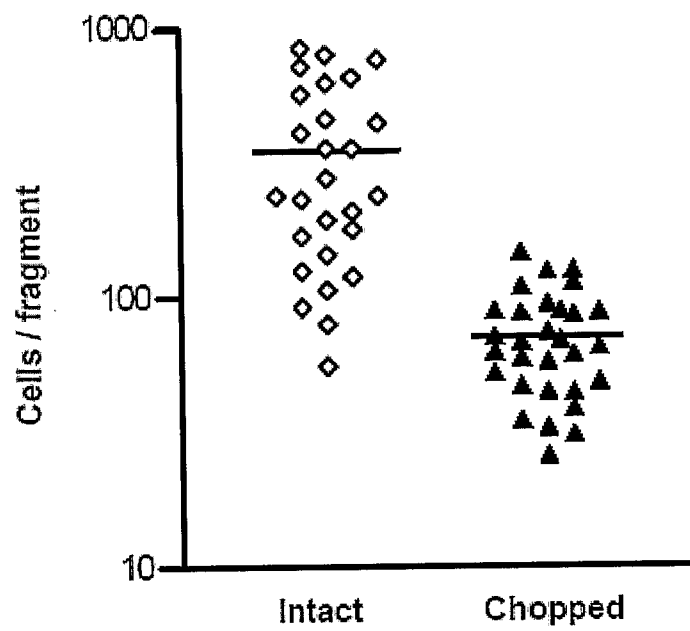


Figure 10

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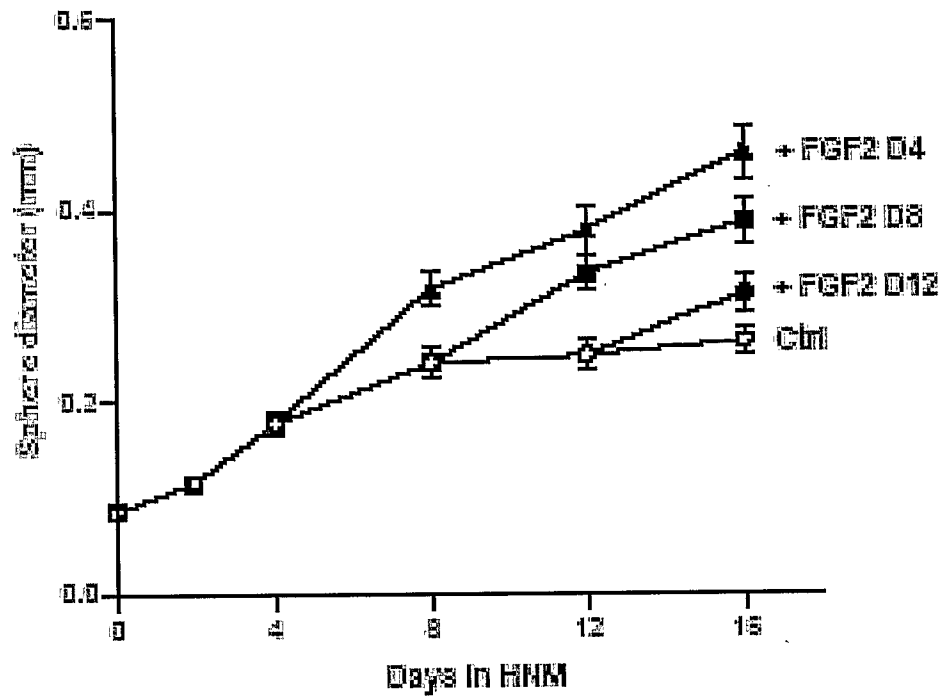


Figure 11

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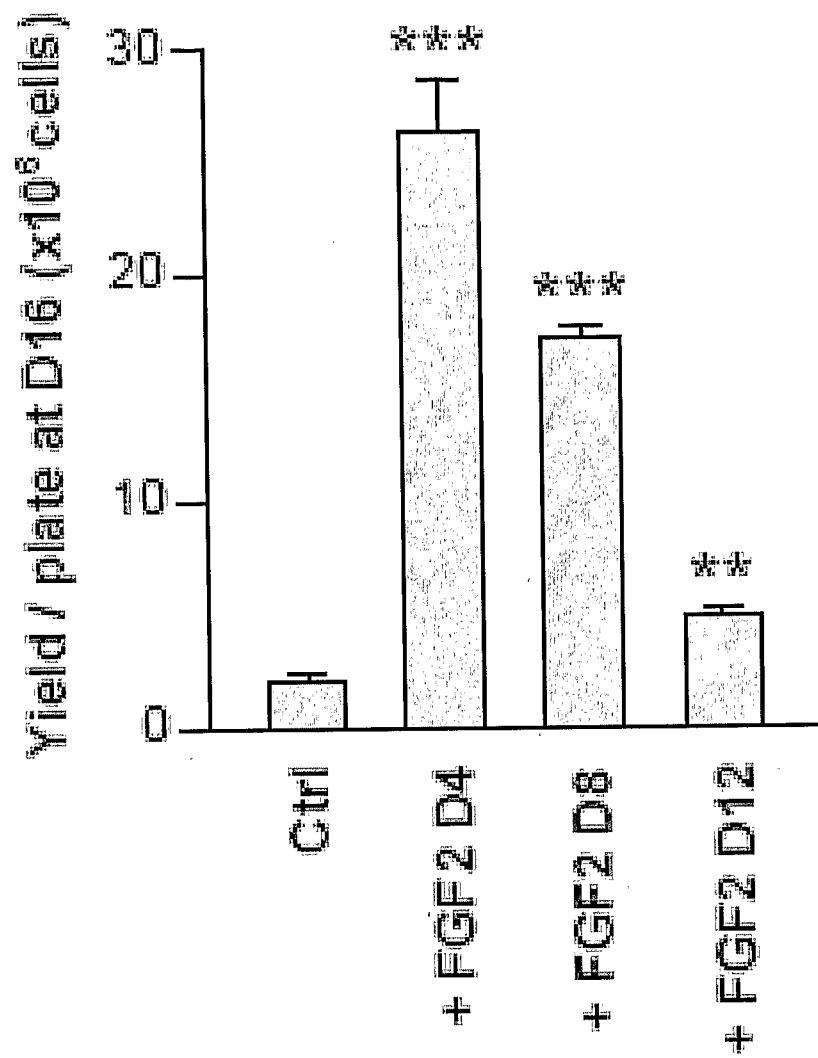


Figure 12

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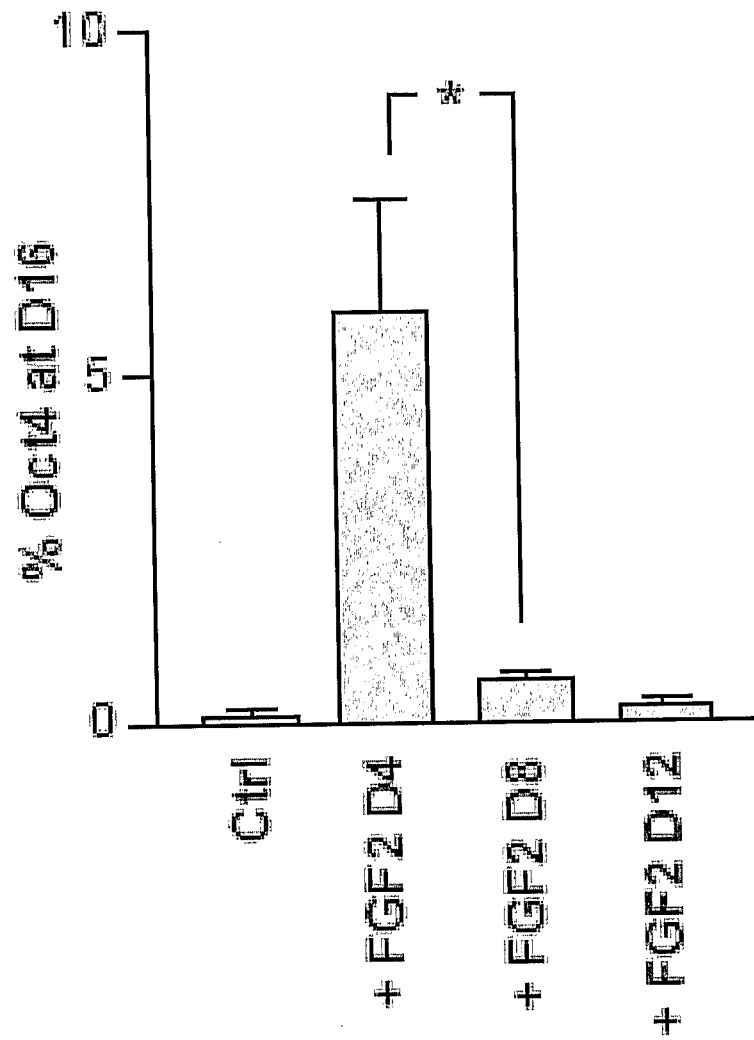


Figure 13

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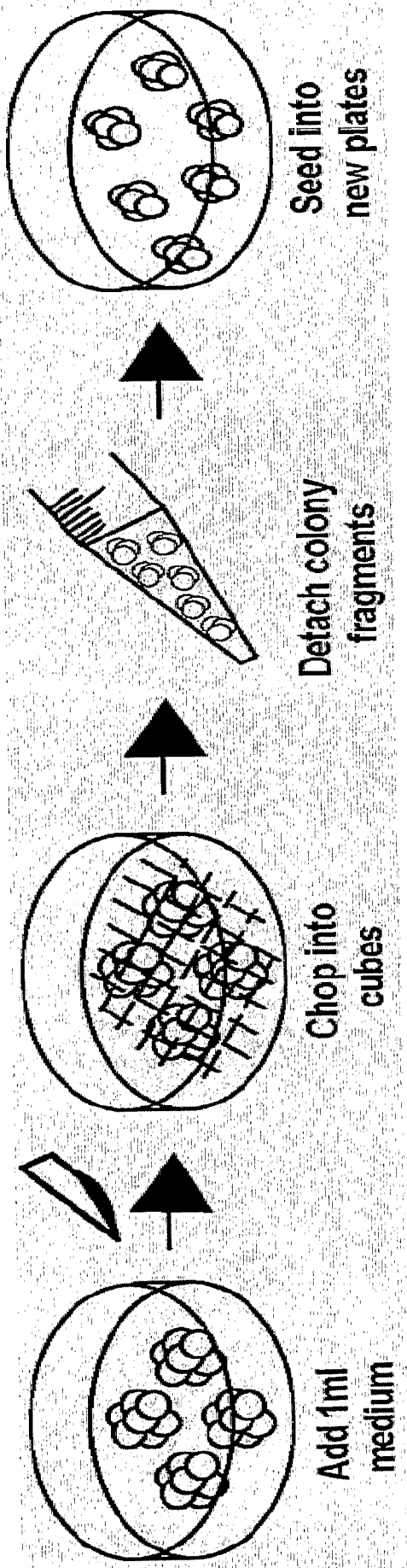


Figure 14

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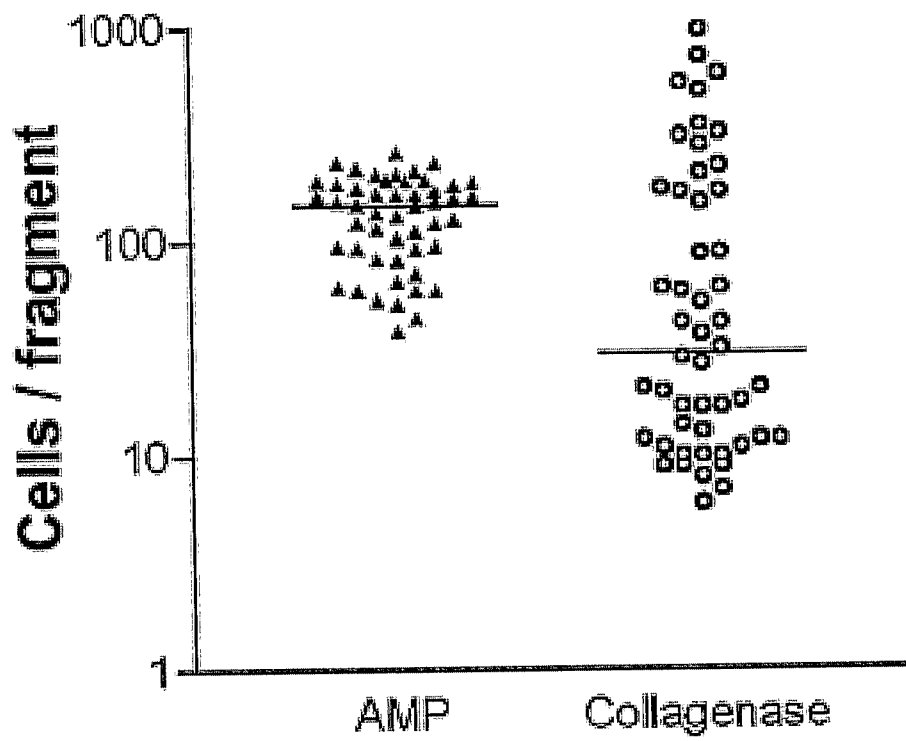


Figure 15

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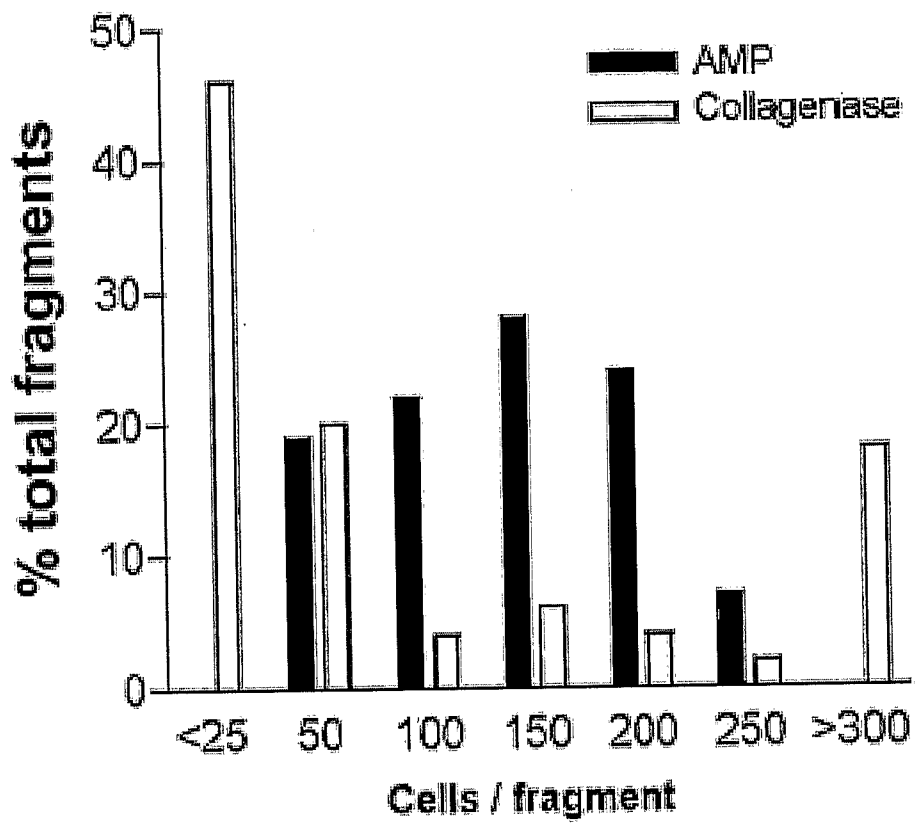


Figure 16

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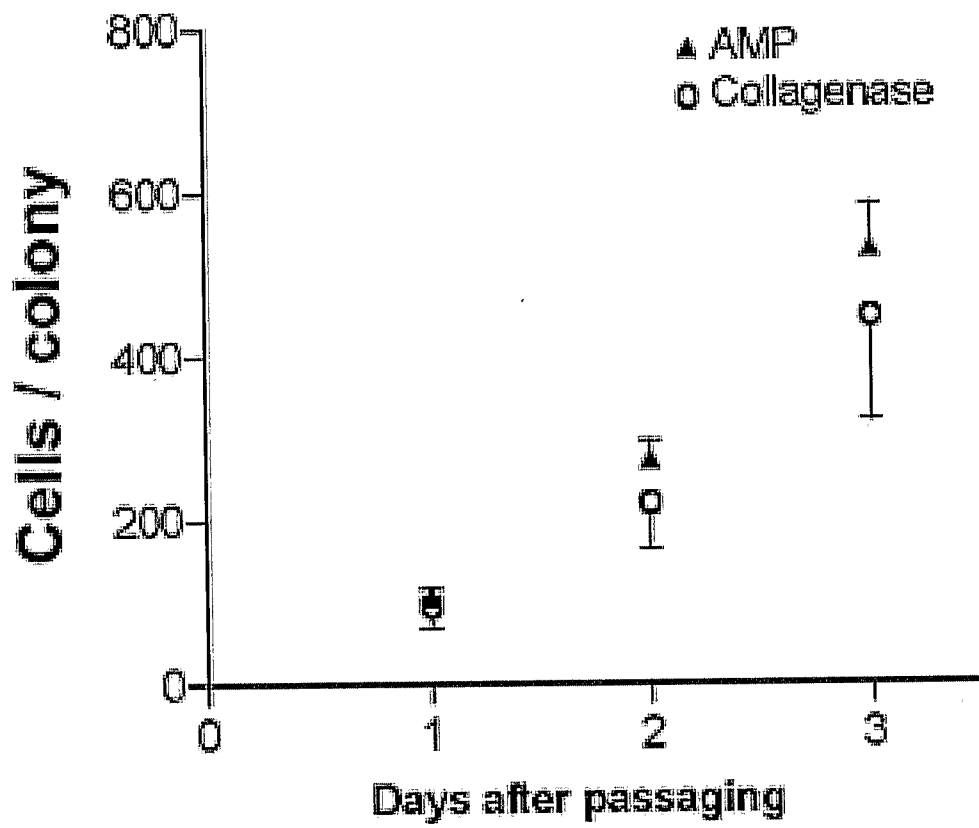


Figure 17

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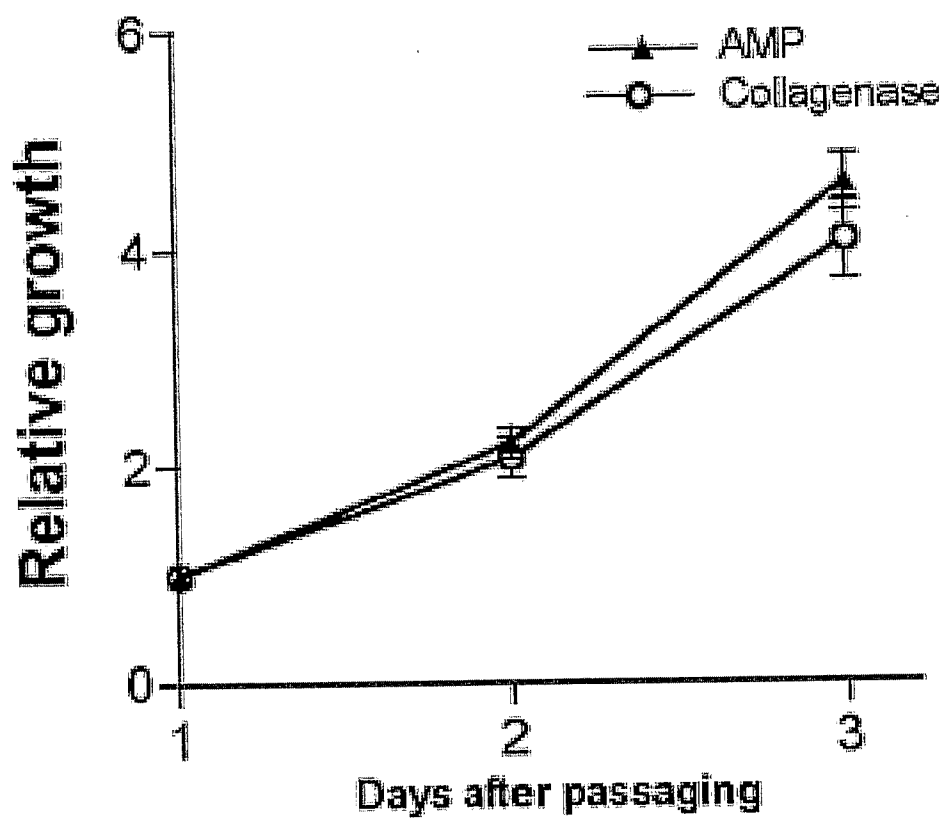


Figure 18