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(54) Title: LOW OXYGEN CULTURING OF CELLS

(57) Abstract

The present invention relates to the growth of cells in culture under conditions that promote cell survival, proliferation, and/or cellular differentiation. The present inventors have found that proliferation was promoted and apoptosis reduced when cells were grown in lowered oxygen as compared to environmental oxygen conditions traditionally employed in cell culture techniques. Further, the inventors found that differentiation of precursor cells to specific fates also was enhanced in lowered oxygen where a much greater number and fraction of dopaminergic neurons were obtained when mesencephalic precursors were expanded and differentiated in lowered oxygen conditions. Thus at more physiological oxygen levels the proliferation and differentiation of CNS precursors is enhanced, and lowered oxygen is a useful adjunct for *ex vivo* generation of specific neuron types. Methods and compositions exploiting these findings are described.

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LOW OXYGEN CULTURING OF CELLS

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

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FIELD OF THE INVENTION

The present invention relates to the growth of cells in culture. More particularly, the present invention provides methods and compositions for increasing cell survival, cell proliferation and/or cell differentiation along specific pathways by growing the cells in low ambient oxygen conditions.

BACKGROUND OF THE INVENTION

In a time of critical shortages of donor organs, efforts to bring cellular transplantation into the clinical arena are urgently needed (Neelakanta & Csete, 1996). Indeed, cellular and tissue transplantation is now well recognized as a desirable technique for the therapeutic intervention of a variety of disorders including cystic fibrosis (lungs), kidney failure, degenerative heart diseases and neurodegenerative disease. However, although this may be a desirable and much needed intervention, a major impediment to this type of therapeutic intervention is the lack of an available supply of viable, differentiated cells. Generally differentiated cells cannot be readily expanded in culture. Thus, methods of increasing the number and/or availability of differentiated, viable cells are needed.

The central nervous system (CNS) (brain and spinal cord) has poor regenerative capacity which is exemplified in a number of neurodegenerative disorders, such as Parkinson's Disease. Although such diseases can be somewhat controlled using pharmacological intervention (L-dopa in the case of Parkinson's Disease), the neuropathological damage and the debilitating progression is not reversed. Cell transplantation offers a potential alternative for reversing neuropathological damage as opposed to merely treating the consequences of such damage.

Cultured CNS stem cells can self-renew, and after mitogen withdrawal, have an intrinsic capacity to generate oligodendrocytes, astrocytes, and neurons in predictable proportions (Johe *et al.*, 1996). Manipulation of this intrinsic differentiation capacity in culture has been used to define a complex array of factors that maintain, amplify, or diminish a particular differentiated phenotype. Most such studies emphasize a primary role for transcription factors in defining CNS lineage identity, as well as growth and trophic factors acting locally and over long distances (Johe *et al.*, 1996, Panchinsion *et al.*, 1998). Dopaminergic neurons and their progenitors from these cultures are of special interest as potential sources of replacement cellular therapies for Parkinson's Disease patients (reviewed in Olanow *et al.*, 1996).

The sympathoadrenal (SA) lineage of the neural crest gives rise to all of the catecholaminergic derivatives of the peripheral nervous system (PNS). These include sympathetic neurons, adrenal chromaffin cells, carotid body cells, and Small Intensely Fluorescent (SIF) cells (Doupe, *et al.*, 1985, at p. 2119 and p. 2143). The terminal differentiation and plasticity of SA derivatives have been intensively studied for over a decade (reviewed in Anderson, *et al.*, 1993). Although multipotent neural crest progenitors have been observed to give rise to SA derivatives in vivo (Fraser, 1991), relatively little is known about how neural crest stem cells (NCSCs) become committed to this sublineage. Bone Morphogenetic Proteins (BMPs) -2, -4 and -7 have been identified as inducers of SA marker expression in mass cultures of avian and mammalian neural crest cells (Varley, *et al.*, 1995; Varley, *et al.*, 1996; Reissman, et al, 1996; and Lo, *et al.*, 1999). Such BMPs can also induce autonomic neuron differentiation in clonal cultures of mammalian NCSCs (Stemple, 1992), but they do not induce SA lineage markers (Shah, *et al.*, 1996). The inability to achieve SA lineage differentiation in clonal cultures of NCSCs has severely hampered further study of the mechanisms regulating this important lineage restriction process.

The sympathetic potential of NCSCs is of clinical as well as basic interest because sympathetic lineage cells synthesize dopamine and dopaminergic cells are used to alleviate Parkinson's disease (reviewed in Gage, *et al.*, 1989). Parkinson's disease is a relatively common neurodegenerative disorder caused by the loss of dopaminergic neurons in the substantia nigra. The transplantation of a wide variety of

dopaminergic cell types, including fetal mesencephalon (Freed, *et al.*, 1992) and neural crest-derived adrenal chromaffin cells (Date, 1996) or carotid body cells (Luquin, 1999) into the substantia nigra can ameliorate the symptoms of Parkinson's disease; however, the supply of fetal tissue is very limited and even autologous transplants of dopaminergic cells may sometimes be unsuitable (Date, 1996; Stoddard, *et al.*, 1989). These constraints have prompted extensive efforts to identify alternate sources of dopaminergic neurons (Zawada, 1998) and to expand dopaminergic cells in culture (Studer, 1998).

Ideally, *ex vivo* culture conditions should reproduce the *in vivo* cellular environment with perfect fidelity. This ideal is especially pertinent when explants are used to study development, because conditions may be defined for cell fate choice and differentiation. For CNS stem cell cultures, in particular, maximizing survival, proliferation, and cell fate choice leading to dopaminergic neurons is essential for future cellular transplant therapies. Thus, understanding and control of the differentiation of such cells is crucial for providing a viable, useful product that can be used in transplantation or for studying the behavior of CNS cells, *in vitro*, in response to various conditions.

In embryogenesis, each tissue and organ develops by an exquisitely organized progression in which relatively unspecialized or "undifferentiated" progenitor or stem cells give rise to progeny that ultimately assume distinctive, differentiated identities and functions. Mature tissues and organs are composed of many types of differentiated cells, with each cell type expressing a particular subset of genes that in turn specifies that cell's distinctive structure, specialized function, and capacity to interact with and respond to environmental signals and nutrients. These molecular, structural and functional capacities and properties comprise the cell phenotype. Similarly, coupled cell proliferation and/or differentiation occurs, in the presence of changing local O_2 supply, when an injured or degenerating adult tissue undergoes repair and regeneration. The level of oxygen is especially pertinent in many regeneration paradigms in which normal blood supply is reduced or even transiently stopped by trauma or embolic events (myocardial infarction, stroke and the like).

Therefore, in clinical settings, gases are appreciated as a primary variable in organ survival, with oxygen as the critical gas parameter. Virtually all modern cell

culture is conducted at 37°C in a gas atmosphere of 5% CO_2 and 95% air. These conditions match core human body temperature and approximate quite well physiologic CO_2 concentrations. For example, mean brain tissue CO_2 is 60 mm Hg or about 7% (Hoffman *et al.,* 1998). However, in striking contract, oxygen in standard tissue culture does not reflect physiologic tissue levels and is, in fact, distinctly hyperoxic.

At sea level, (unhumidified) room air contains 21% O_2 which translates into an oxygen partial pressure of 160 mm Hg [0.21(760 mm Hg)]. However, the body mean tissue oxygen levels are much lower than this level. Alveolar air contains 14% oxygen, arterial oxygen concentration is 12%, venous oxygen levels are 5.3%, and mean tissue intracellular oxygen concentration is only 3% (Guyton, and Hall, 1996). Furthermore, direct microelectrode measurements of tissue O_2 reveal that parts of the brain normally experience O_2 levels considerably lower than total body mean tissue oxygen levels, reflecting the high oxygen utilization in brain. These studies also highlight considerable regional variation in average brain oxygen levels (Table 1) that have been attributed to local differences in capillary density. Mean brain tissue oxygen concentration in adult rates is 1.5% (Silver and Erecinska, 1988), and mean fetal sheep brain oxygen tension has also been estimated at 1.6% (Koos and Power, 1987).

Table 1

Regional rat brain tissue partial pressures of oxygen measured by microelectrode

Brain area	**%O_2**
Cortex (gray)	2.5-5.3
Cortex (white)	0.8-2.1
Hypothalamus	1.4-2.1
Hippocampus	2.6-3.9
Pons, fornix	0.1-0.4

Adapted from Silver, L, Erecinska, M. Oxygen and ion concentrations in normoxic and hypoxic brain cells. In *Oxygen Transport to Tissue XX*, 7-15, edited by Hudetz and Bruley, Plenum Press, New York (1988).

Thus, from the discussion above it is clear that under standard culture conditions, the ambient oxygen levels are distinctly hyperoxic, and not at all within physiologic ranges. These conditions of cell growth are have been historically inadequate for generating cells and tissues for transplantation into the brain or other area of the body or for providing an accurate *in vitro* model of what is occurring *in vivo*. Thus, there remains a need for methods to produce differentiated cells which can be used for therapeutic and research purposes. The present invention is directed to providing such methods.

BRIEF SUMMARY OF THE INVENTION

The present invention is directed to growing cells in low ambient oxygen conditions in order to mimic the physiological oxygen conditions with greater fidelity. The growth of these cells in such conditions provides certain surprising and unexpected results. These results are exploited and described in further detail herein. More particularly, the present invention describes methods that may independently be useful in increasing cell survival, cell proliferation and/or cell differentiation along specific pathways.

In specific embodiments, the present invention describes a method of increasing cell differentiation comprising culturing undifferentiated central nervous system (CNS) cells in low ambient oxygen conditions, wherein the low ambient oxygen conditions promotes the cellular differentiation of the neuronal cells. The definitions of low ambient oxygen conditions are described in depth elsewhere in the specification. However, it is contemplated that in specific embodiments the low ambient oxygen conditions comprise an ambient oxygen condition of between about 0.25% to about 18% oxygen. In other embodiments, the ambient oxygen conditions comprise an ambient oxygen condition of between about 0.5% to about 15% oxygen. In still other embodiments, the low ambient oxygen conditions comprise an ambient oxygen condition of between about 1% to about 10% oxygen. In further embodiments, the low ambient oxygen conditions comprise an ambient oxygen condition of between about 1.5% to about 6% oxygen. Of course, these are exemplary ranges of ambient oxygen conditions to be used in culture and it should be understood that those of skill in the art will be able to employ oxygen conditions

falling in any of these ranges generally or an oxygen conditions between any of these ranges that mimics physiological oxygen conditions for CNS cells. Thus, one of skill in the art could set the oxygen culture conditions at 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, 5.5%, 6%, 6.5%, 7%, 7.5%, 8%, 8.5%, 9%, 9.5%, 10%, 10.5%, 11%, 11.5%, 12%, 12.5%, 13%, 13.5%, 14%, 14.5%, 15%, 15.5%, 16%, 16.5%, 17%, 17.5%, 18%, 18.5%, or any other oxygen condition between any of these figures.

The cells employed in the method described may be any cells that are routinely used for CNS studies. As such, the cells may be primary tissue culture cells or derived from a cell line. The cells may be fetal cells or adult cells. In specific embodiments, it is contemplated that the cells may be selected from the group consisting of central nervous system stem cells, spinal cord-derived progenitor cells, glial cells, astrocytes, neuronal stem cells, central nervous system neural crest-derived cells, neuronal precursor cells, neuronal cells, hepatocytes, and bone marrow derived cells. In preferred embodiments, it is contemplated that the cells may be mecencephalic progenitor cells, lateral ganglionic precursor cells, cortical precursor cells, astrocytes or neuroblasts.

The method may comprise determining the amount, level or degree of differentiation. Those of skill in the art are familiar with technologies employed to determine cellular differentiation. The differentiation may determined by monitoring a differentiation specific phenotype in the cells. For example, the differentiation specific phenotype determined may be by monitoring message level, protein level, subcellular localization, functional assays or morphological changes.

There are various techniques that may be employed for determining message level including but not limited to PCR™, *in situ* hybridization, RNAse protection assay, or single cell PCR™. In specific embodiments, the present invention may monitor the message level for nestin, tyrosine hydroxylase, GAPDH; BDNF; GDNF; FGFR3; En1; FGF8; SHH; Ptx3; Nurr1; VEGF; EPO; HIF1α or VHL. Of course these are exemplary differentiation markers for CNS cells or markers of cellular responses to oxygen and it is contemplated that those of skill in the art will be able to substitute additional similar markers for the markers specifically described herein without undue experimentation. Other embodiments monitor protein level by, for example, using antibody staining, HPLC, western blotting or immunoprecipitation. In

more particular embodiments, the protein level monitored is the level of nestin, tyrosine hydroxylase, dopamine β-hydroxylase or dopamine transporter. The functional assay typically will be one that monitors a particular function of the selected CNS cells. A particularly useful functional assay may be one which monitors the rate of dopamine production.

A preferred feature of the present invention is that the low oxygen conditions produce a cell population that is enriched in dopaminergic neurons as compared to a similar cell population that is grown in 20% oxygen incubator conditions. Another preferred embodiment is that the low oxygen conditions produce a cell population that is enriched in serotoninergic neurons as compared to a similar cell population that is grown in 20% oxygen incubator conditions. In still additional embodiments, the low oxygen conditions produce a cell population that is depleted in GABAnergic neurons as compared to a similar cell population that is grown in 20% oxygen incubator conditions. Further, certain methods of the present invention will provide low oxygen conditions to produce a cell population that is depleted in glutaminergic neurons as compared to a similar cell population that is grown in 20% oxygen incubator conditions.

In preferred embodiments, the method may further comprise growing the cells in the presence of a neuronal growth stimulant, mitogen, cytokine, neuroprotective factor or an anti-apoptotic agent. The inventors have found that there was a significant increase in EPO expression as a result of lowered oxygen versus 20% O_2. In particular embodiments, the differentiated phenotype is retained after transfer of the cells from the low ambient oxygen conditions to 20% oxygen culture conditions. In specific embodiments, it is contemplated that the cells may be grown in low ambient oxygen conditions for multiple generations prior to transfer to 20% oxygen culture conditions. In other embodiments, the cells may be continuously maintained in low ambient oxygen conditions.

Another aspect of the present invention provides a method of inhibiting apoptosis of a CNS cell in culture comprising growing the cell in low ambient oxygen conditions.

Yet another embodiment provides a method of increasing the expansion of a CNS cell in culture comprising growing the cell in low ambient oxygen, wherein the

cells exhibit increased expansion in the low ambient oxygen as compared to growing the cell in 20% oxygen incubator conditions.

In an additional embodiment, the present invention further contemplates a method of increasing cell proliferation in culture comprising growing CNS cells in low ambient oxygen, wherein the growth in low ambient oxygen increases cell proliferation compared to growing the cells in 20% oxygen incubator conditions.

Also provided is a method of preparing a cell for use against a neurodegenerative disorder comprising obtaining a population of CNS cells and growing the cells in low ambient oxygen conditions wherein the low ambient oxygen conditions increases the expression of a gene involved in the neurodegenerative disease. In specific embodiments, the neurodegenerative disease is Parkinson's Disease and the gene is tyrosine hydroxylase (TH).

The method further may comprise contacting the cell(s) with a first polynucleotide encoding a dopamine biosynthetic protein under conditions suitable for the expression of the protein wherein the polynucleotide is under the transcriptional control of a promoter active in the cells. In addition, the method further may comprise contacting the cell with a first polynucleotide encoding a dopamine releasing protein under conditions suitable for the expression of the protein wherein the polynucleotide is under the transcriptional control of a promoter active in the cells. Also contemplated is a method further comprising contacting the cell with a second polynucleotide encoding a dopamine releasing protein under conditions suitable for the expression of the protein wherein the polynucleotide is under the transcriptional control of a promoter active in the cells. Other embodiments involve contacting the cell with a second polynucleotide encoding a dopamine biosynthetic protein under conditions suitable for the expression of the protein wherein the polynucleotide is under the transcriptional control of a promoter active in the cells.

In more particular embodiments, the dopamine biosynthesis protein may be TH; L-amino acid decarboxylase (AADC), erythropoietin or any other protein directly or indirectly involved in dopamine synthesis. The dopamine releasing protein is a vesicular monoamine transporter (VMAT), which may be VMAT1 or VMAT2. In specific embodiments, the first and second polynucleotides are under control of different promoters. The promoter may be any promoter known to those of skill in the

art that will be operative in the cells being used. For example, it is contemplated that the promoter may be CMV IE, SV40 IE, β-actin, TH promoter, AADC promoter, and nestin promoter. It is contemplated that the first and second polynucleotides each may be covalently linked to a polyadenylation signal.

Also encompassed by the present invention is a cell produced according to the method comprising obtaining a starting CNS cell and growing the cell in low ambient oxygen conditions wherein the conditions produce a differentiated neuronal cell. In specific embodiments, the starting cell is a nestin-positive cell. More particularly, the low ambient conditions produce a nestin-negative differentiated cell more rapidly and in greater numbers than traditional cell culture conditions. In specific embodiments, the low ambient conditions produce a TH positive cell. In other embodiments, the cell further comprises an expression vector comprising a polynucleotide encoding an exogenous gene wherein the polynucleotide is operatively linked to a promoter.

Another aspect of the present invention provides a method of treating Parkinson's disease in a subject comprised of obtaining cells suitable for transplanting in the subject; growing the cells in low ambient oxygen conditions; and implanting the cells grown in the low ambient oxygen conditions into the subject; wherein the implanted cells have an increased capacity to produce dopamine in the subject as compared to similar cells grown in 20% oxygen incubator conditions. In specific embodiments, the cells are from the subject and have been transduced with a polynucleotide that expresses a protein that increases dopamine production and are treated or expanded in lowered oxygen conditions. In other preferred embodiments, the cells are CNS cells from a source other than the subject. In preferred embodiments, the cells are transduced with a polynucleotide that expresses a protein that increases dopamine production and are treated or expanded in lowered oxygen conditions.

In a particular embodiment, a method of culturing at least one neural crest stem cell (NCSC) to enhance survival, proliferation or differentiation of the NCSC is provided. The method comprises culturing the NCSC(s) in low ambient oxygen conditions, wherein said survival, proliferation or differentiation is enhanced compared to a control NCSC cultured in ambient oxygen conditions.

In one aspect, a method is provided for enhancing differentiation of the NCSC wherein differentiation is to a neuron. In a preferred embodiment, differentiation is to a cell of the sympathoadrenal lineage or the cholinergic lineage. In another embodiment, differentiation is to a sensory neuron. In another aspect, differentiation is to a phenotype which produces dopamine and norepinephrine. In yet another aspect, differentiation is to adrenal chromaffin cells, small intensely fluorescent cells or sympathetic neurons.

The neural crest stem cell can be a single isolated cell. Moreover, the neural crest stem cell can be from a primary source or culture. The methods provided can be applied in a number of ways including storing and forming cells which can be used in transplantation into subjects. Subjects can be individuals suffering from neurodegenerative diseases or disorders or animal models of the same.

In a further aspect, a method of culturing at least one undifferentiated cell to form a cell which produces dopamine and norepinephrine is provided. In one embodiment, the method comprises culturing said cell(s) in low ambient oxygen conditions to form a cell which produces dopamine and norepinephrine.

In other preferred embodiments, the present invention provides a method for isolating, maintaining, propagating or enriching progenitor or stem cells, and/or for influencing the differentiation outcome or differentiation kinetics of such cells into particular tissue types, comprising obtaining cells derived from mammalian tissue containing at least one progenitor cell or stem cell capable of producing progeny that can assume or produce cells with one or more differentiated phenotypes, and culturing the cells derived from such mammalian tissue (various organ and tissue types) in suitable medium under empirically determined subatmospheric oxygen conditions for a time sufficient to promote the survival, proliferation, or enrichment of the stem or progenitor population, or to cause or influence entry into one or more differentiation pathways.

In certain aspects, the present invention provides a method for manipulating the yield of progenitor/stem cell cultures. This method has several embodiments, including: subatmospheric/physiologic oxygen conditions used to culture or enrich stem cell and/or progenitor cells; subatmospheric/physiologic oxygen conditions used to isolate/identify stem or progenitor cells that are not yet

identified; subatmospheric/physiologic oxygen conditions used to alter the kinetics of developmental progression or accelerate differentiation from stem cell and/or progenitor populations; subatmospheric/physiologic oxygen conditions used to enhance regeneration and subatmospheric/physiologic oxygen conditions used to select, promote or reinforce or promote a specific cell fate or fates.

The invention contemplates a method of enriching progenitor or stem cells in a population of cells comprising progenitors or stems, progeny thereof, and other "contaminating" cells, comprising obtaining a population of mammalian cells (adult or embryonic; from one or more solid tissue type) containing at least one stem cell or progenitor cell capable of producing differentiated progeny, and culturing the cells derived from mammalian tissue in suitable medium under oxygen levels reflecting physiologic oxygenation for the tissue from which they were derived or under hypoxic conditions (below normal physiologic oxygenation) for a time sufficient to enrich the population of progenitor/stem cells in said culture relative to one or more other cell types in the population.

Also contemplated by the present invention is the use of subatmospheric/physiologic oxygen conditions to isolate/identify stem or progenitor cells that are not yet identified. In this embodiment, a culture derived from an organ or tissue system is established, consisting of a variety of cell types, both stem cell and/or progenitor and differentiated cells. Oxygen dose-response experiments are performed, in which parallel cultures are exposed to progressively decreasing levels of oxygen, starting with 12% (approximately adult arterial levels). The goal being to identify the oxygen level at which stem cell and/or progenitor cells increase in absolute number, or survive in preference to other cells in the culture, or selectively proliferate, or a combination of these effects. In any of these cases, subatmospheric oxygen is used to facilitate identification and characterization of the usually rare stem cell and/or progenitor cell population.

Another embodiment of the present invention involves shifting the kinetics or the yield of differentiation of progenitor cells and/or stem cells into various cell types by exposing them to physiologic/subatmospheric oxygen levels. In this embodiment, subatmospheric oxygen is used to alter the kinetics of progression from an undifferentiated stem cell and/or progenitor state to cells with a differentiated

phenotype. This change in kinetics, depending on the cell type and culture conditions may be an acceleration or deceleration. Moreover, the use subatmospheric oxygen may increase or decrease the fraction of stem cells or progenitor cells that differentiate. Depending on the application, such increase or decrease might be desirable.

In another embodiment, subatmospheric oxygen is used to first expand the stem cell and/or progenitor pool, from which regeneration is then allowed to proceed. Since the initial pool from which tissue/organ can be regenerated is increased in number, the ultimate amount of regeneration into differentiated cells is also increased.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF SEVERAL VIEWS OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Effect of lowered oxygen on precursor yield *in vitro* at varying plating densities. Striatal cultures were expanded with bFGF in lowered or ambient oxygen, and total cell numbers assessed after 5 days of proliferation when over 95% of cells are nestin+ precursors. Significantly increased cell numbers were detected at all densities in lowered O_2 compared to ambient oxygen.

FIG. 2. Lowered oxygen culturing leads to increased proliferation of CNS precursors. FIG. 2A. Mesencephalic precursors were pulsed with 10 μM BrdU for 60 minutes immediately before fixation, then stained for BrdU uptake. More BrdU+ cells were seen in lowered oxygen cultures during both proliferation and differentiation. Scale bar = 20 μm. FIG. 2B. Mesencephalic precursors in lowered O_2

yielded an increased percentage of BrdU+ cells and a greater absolute number of BrdU+ cells than cultures maintained at 20% O_2. Data are given as mean +/- SEM, n=40. Differences between lowered and 20% O_2 were statistically significant at all time points and for all parameters ($n=8$, $p < 0.05$) except percentage of BrdU+ cells at day 4 of expansion ($n=8$, $p = 0.10$).

FIG. 3. CNS precursors cultured in lowered (vs. 20%) O_2 have reduced rates of apoptosis. FIG. 3A. Apoptosis was assayed by TUNEL labeling of mesencephalic precursors cultured in parallel at either lowered or 20% O_2. Representative figures of the expansion phase (2 and 6 days of culture) and the differentiation phase (4 days after bFGF withdrawal) are shown. Scale bar = 20 μm. FIG. 3B. Precursors grown at lowered O_2 showed a significant decrease in the percentage of apoptotic cells ($n=8$, $p < 0.05$) compared to traditional cultures.

FIG. 4. Basic differentiation patterns of CNS stems in lowered and 20% O_2 cultures. FIG. 4A. Striatal cultures in lowered or 20% O_2 were assessed for the relative percentages of precursor-derived neurons (by TUJ1 stain), astrocytes (GFAP) and oligodendrocytes (Gal-C) after 5 days of bFGF proliferation followed by four days of cell differentiation (for quantification see text). FIG. 4B. Passaged mesencephalic precursors were proliferated for 6 days and differentiated for 5 days in lowered or 20% O_2 and analyzed for O4, a marker of oligodendrocyte precursors. O4+ cells could be detected only in lowered oxygen cultures. FIG. 4C. Nestin+ clones were derived from single passaged mesencephalic precursor cells after 20 days of bFGF proliferation (left panel). Clones in lowered oxygen differentiated into TUJ1+ neurons upon bFGF withdrawal (right panel). FIG. 4D. Lowered O_2 promotes clone formation efficiency. The yield of clones derived from single precursors was 3-fold higher in lowered O_2 compared to 20% O_2 cultures (left panel). The majority of clones derived from precursors in O_2 oxygen cultures contained 50 – 500 cells whereas clone size in 20 % O_2 cultures was generally 5 – 50 cells (right panel). Scale bar = 20 μm in all panels.

FIG. 5. Lowered O_2 culturing improves the yield of functional precursor-derived dopaminergic neurons. FIG. 5A and FIG. 5B. Precursors from E12 mesencephalon were proliferated with bFGF for 5 days followed by 5 days of

differentiation, then stained for the neuronal marker TUJ1 and for TH. A large increase in total number (and percentage) of TH+ neurons was detected ($p < 0.001$) in lowered O_2 compared to 20% O_2 cultures. Scale bar = 20 μm. FIG. 5C. Quantification of TH protein level by Western blot analysis revealed significantly more TH in samples from lowered (vs. 20%) O_2 cultures. Each lane was loaded with 2.5m μg total protein. FIG. 5D. rp-HPLC with electrochemical detection was used to quantify dopamine levels in conditioned medium (24 hrs), in HBSS after 15 minutes of conditioning (basal release), and in HBSS+56 mM KCl after 15 minutes (evoked release). Significantly more dopamine was detected in cultures maintained at lowered O_2 compared to those grown at 20% O_2 under all these conditions (conditioned medium $p < 0.01$; basal and evoked release $p < 0.05$). Inset shows typical chromatogram for dopamine detection in lowered and 20% O_2 cultures.

FIG. 6. Neuronal subtype differentiation from mesencephalic precursors in lowered vs. 20% O_2. Double immunocytochemical labeling revealed that lowered O_2 culturing markedly increased the representation of dopaminergic and serotonergic neuronal (Tuj1+) subtypes, but decreased the representation of GABA+ and Glutamate+ neurons. Colony depicted in GABA stain at 20% O_2 is an unusual example of very high GABA expression under these conditions. TH and GABA were not co-expressed as seen in some developing neurons *in vivo*. Floor plate cells (FP4+) were more numerous in lowered O_2 cultures as was the percentage of neurons expressing the midbrain transcription factor En1. Precursor markers nestin and PSA-NCAM were both reduced in lowered O_2 cultures after differentiation compared to 20% O_2 conditions (lower right panels). Scale bars = 20 μm.

FIG. 7. Differential gene expression in mesencephalic precursors at lowered and 20% O_2 assessed by RT-PCR. FIG. 7A. Expression of genes involved in the physiological response to changes in oxygen levels. The expression of HIF1α, VHL, EPO and VEGF was assessed after 2 or 6 days of expansion and after differentiation (day 4 of differentiation = day 10 of culture) in lowered and 20% O_2. Data are normalized to GAPDH expression. A significant increase in EPO expression was detected in lowered oxygen versus 20% O_2 mostly during cell differentiation, whereas VEGF was upregulated during both expansion and differentiation. Surprisingly, no

major oxygen-dependent regulation of HIF1α or VHL was observed. FIG. 7B. Candidate genes involved in midbrain development were also tested for O_2-dependent differential expression. Increased expression of TH and Ptx-3 during cell differentiation confirmed the larger number of functional dopaminergic neurons in lowered oxygen cultures (compare FIGs. 5). Significant lowered O_2–mediated changes in expression levels of FGF8 and En1 were also detected.

FIG. 8. EPO mimics the lowered oxygen effect on dopaminergic differentiation. Saturating concentrations of EPO or EPO neutralizing antibody were added to E12 mesencephalic precursor cultures during both proliferation and differentiation phase (5 days each) in lowered or 20% O_2. EPO supplementation significantly increased TH+ cell numbers in 20% O_2 cultures ($n=6$, $p < 0.05$). EPO neutralizing antibody decreased TH+ cell numbers in both lowered oxygen ($n=6$, $p < 0.01$) and 20% O_2 cultures ($n=6$, $p < 0.05$). Scale bar = 20μm.

FIG. 9. Cells indicating low oxygen promotes sympathoadrenal lineage differentiation. Sciatic nerve $p75^+P_0^-$ cells (Morrison, *et al.*, 1999) were cultured in low oxygen or in ambient oxygen. Cultures were grown for a total of 12 days, either under standard conditions or with 5 μM forskolin and 1 ng/mL BMP2 added after 6 days of standard culture. Cultures were fixed and stained with antibodies against tyrosine hydroxylase (TH) (rate limiting enzyme for dopamine production), dopamine-ß-hydroxylase (DBH), and the sympathoadrenal lineage marker SA-1. The bars show the percentage of stem cell colonies that contained cells expressing the given markers. Error bars show standard errors. Sympathetic markers were expressed at a significantly higher frequency at low oxygen and in the presence of forskolin and BMP2 ($p<0.05$).

DETAILED DESCRIPTION OF THE INVENTION

In order for cell transplantation therapies to become widely and universally used there is a need for availability of appropriately differentiated, viable cells. Preferably, these cells need to be resilient enough that they can be cryopreserved without loss of phenotypic integrity. The high incubator O_2 levels in which the cells are grown at ambient air O_2 levels (referred to herein as traditional O_2 conditions; or 20% O_2 culture conditions) do not facilitate the production of such cells. These cells

often do not survive, proliferate or differentiate in sufficient numbers to be useful. As such expansion of these cells in traditional culture yields a cell that is at best inadequate for use in *in vitro* model assay studies let alone for use in transplantation.

The present invention is directed towards providing methods and compositions for producing cells that are differentiated, viable, amenable to cryopreservation and provide an accurate indication of how such cells behave biochemically in an *in vivo* setting. As such, these methods will provide cells that can be used *in vitro* to perform characterization studies or *in vivo* as replacement therapies for cells that have been damaged by disease, injury resulting from trauma, ischemia, or a drug-induced injury. Further, it should be noted that the method leads to increased survival of undifferentiated precursors that could also be used for transplantation, which when placed in the appropriate environmental conditions will differentiate down the appropriate pathway.

The present invention particularly contemplates the use of culture conditions using subatmospheric/physiological oxygen to culture or enrich a population of neuronal cells with cells that are expanded and/or differentiated to express a particular neuronal phenotype. The increase in cell differentiation may be such that the process of a cell being converted from a primitive undifferentiated state to one in which a particular cellular phenotype (dopaminergic phenotype; GABAergic phenotype; serotoninergic phenotype or the like) is expressed. Specifically, it appears that growth in low O_2 conditions results in an enrichment in dopaminergic and serotinergic neuronal populations, whereas GABAergic and glutaminergic neurons are relatively decreased. These enriched populations may be subject to further enrichment through such methods such as cell sorting.

Alternatively, it may be that the increase in differentiation produced by this method is such that the relative percentage of cells that go on to differentiate (as opposed to remaining in an undifferentiated state) is increased in low oxygen. However, incubation of a pluripotent cell line under low O_2 incubation conditions *in vitro*, will allow the manipulation or skewing of the direction of differentiation of the cell population. Thus, the oxygen is used to control the number and percentage of one type of cell in the population increased or decreased because the differentiation pathway changes under influence of the gas. Thus, enrichment of the CNS cells by

physiologic or low levels of oxygen may be the result of one or more mechanisms that include (1) increase in the absolute number of CNS cells, (2) enrichment by selective survival of CNS cells, (3) enrichment of CNS by their selective proliferation or (4) enrichment of specific differentiation pathways. Similarly, these methods are sued for the enrichment of stems/progenitors cells. The low oxygen or physiologic oxygen conditions are part of the enrichment process from either the time of isolation or some time after (such as after attachment of cells to tissue culture plates) or throughout the process, depending on the specific cell type to be enriched and depending on the particular starting cell population and its physiologic status.

Any increase in the number of CNS, stem or progenitor cells is significant in that more cells are then available to regenerate a greater volume of new tissue. An enrichment, even without increase in number, is important in applications where limitations on total cell number are pertinent or when the effects of the non-CNS progenitor cell contaminants are negative for the desired outcome or for defining the material adequately. Any enhancement of survival of the CNS, stem or progenitor cell, even without increase in cell number or any enrichment of cell types is valuable in settings where culture is required (*i.e.*, to handle tissue before administration of cell therapy, or to permit any other procedure during which the cells must survive such as transfection of genes, drug treatment, or enrichment by cell sorting or other additional procedures).

A particular embodiment of the present invention demonstrates that growth of CNS cells, or indeed any pluripotent stem cell in subatmospheric culture conditions reduces the level of apoptotic and non-apoptotic cell death. It is likely that the increased survival of the cells may be due to both an inhibition of apoptosis and non-apoptotic death. Apoptosis or programmed cell death is a well known phenomenon and can be measured by techniques well know to those of skill in the art.

In one aspect of the invention, a method of culturing at least one neural crest stem cell (NCSC) to enhance survival, proliferation or differentiation of the NCSC is provided. In a preferred embodiment, the method comprises culturing at least one NCSC in low ambient oxygen conditions, wherein the survival, proliferation or differentiation is enhanced compared to a control NCSC cultured in ambient oxygen conditions.

The low ambient oxygen conditions thus will be used to promote differentiation of CNS cells, inhibit apoptosis of cells in culture, increase expansion of cells, and otherwise make such cells amenable for use in transplantation. Such methods and compositions are outlined in further detail below.

Definitions

The present section provides definitions of the terms used in the present invention in order to facilitate a better understanding of the invention.

A "stem cell" is a relatively undifferentiated cell that can be induced to proliferate and that can produce progeny that subsequently differentiate into one or more mature cell types, while also retaining one or more cells with parental developmental potential. In many biological instances, stem cells are also "multipotent" because they can produce progeny of more than one distinct cell type, but this is not required for "stem-ness." Self-renewal is the other classical part of the stem cell definition, and it is essential as used in this document. In theory, self-renewal can occur by either of two major mechanisms. Stem cells may divide asymmetrically, with one daughter retaining the stem state and the other daughter expressing some distinct other specific function and phenotype. Alternatively, some of the stem cells in a population can divide symmetrically into two stems, thus maintaining some stem cells in the population as a whole, while other cells in the population give rise to differentiated progeny only. Formally, it is possible that cells that begin as stem cells might proceed toward a differentiated phenotype, but then "reverse" and re-express the stem cell phenotype. In one aspect, wherein a particular phenotype is formed, a variety of cells may be used. In a preferred embodiment, the differentiated cell produces both dopamine and norepinephrine. In one embodiment, a progenitor or a stem cell is utilized.

As used herein, the term "neural crest stem cell" means a cell derived from the neural crest which is characterized by having the properties (1) of self-renewal and (2) asymmetrical division; that is, one cell divides to produce two different daughter cells with one being self (renewal) and the other being a cell having a more restricted developmental potential, as compared to the parental neural crest stem cell. The foregoing, however, is not to be construed to mean that each cell division of a neural

crest stem cell gives rise to an asymmetrical division. It is possible that a division of a neural crest stem cell can result only in self-renewal, in the production of more developmentally restricted progeny only, or in the production of a self-renewed stem cell and a cell having restricted developmental potential. The neural crest gives rise to the peripheral nervous system (PNS).

"Progenitor cells" have a cellular phenotype that is more primitive (*i.e.*, is at an earlier step along a developmental pathway or progression than is a fully differentiated cell). Often, progenitor cells also have significant or very high proliferative potential. Progenitor cells may give rise to multiple distinct differentiated cell types or to a single differentiated cell type, depending on the developmental pathway and on the environment in which the cells develop and differentiate. Like stem cells, it is possible that cells that begin as progenitor cells might proceed toward a differentiated phenotype, but then "reverse" and re-express the progenitor cell phenotype.

"Differentiation" refers to the developmental process whereby cells assume a specialized phenotype, *i.e.*, acquire one or more characteristics or functions distinct from other cell types. In most uses, the differentiated phenotype refers to a cell phenotype that is at the mature endpoint in some developmental pathway. In many but not all tissues, the process of differentiation is coupled with exit from the cell cycle; in these cases, the cells lose or greatly restrict their capacity to proliferate when they differentiate. Thus, in one embodiment, low oxygen conditions promote a higher proportion of colonies that contain only neurons and a lower proportion that contain no neurons compared to ambient oxygen conditions. In a preferred embodiment, if one third of colonies in ambient oxygen conditions have all neurons, two thirds of colonies in low ambient oxygen conditions have all neurons. In other embodiments, the proportion of colonies having all neurons is increased 25% to 50%, more preferably, 50% to 100%, more preferably, 150% to 200% and more preferably, 300% or 400%.

In one aspect of the invention, differentiation is to a particular phenotype. Preferred phenotypes include cells that express one or more of tyrosine hydroxylase, dopamine beta hydroxlase and the sympathoadrenal lineage specific antigen SA-1. In another embodiment, the cells are differentiated to become PNS sensory cells,

sympathoadrenal cells or cholinergic cells. In preferred embodiments, the method can be used to produce dopaminergic or noradrenergic cells.

In one aspect, survival is enhanced. "Survival" as used herein may include a delay or decreased rate in either apoptotic and non-apoptotic cell death. Any enhancement of survival of a cell provides a number of uses. For example, survival provides facilitation to handle tissue before administration of cell therapy or any other procedure during which the cells must survive such as transfection of genes, drug treatment, or enrichment by cell sorting or other additional procedures. In a preferred embodiment, survival is increased at least 10%, more preferably at least 20% and most preferably at least 30%. For example, in a preferred embodiment, if about 35% of cells added to culture form colonies in ambient oxygen, then about 48% of cells form colonies in low ambient conditions. Other preferred embodiments include enhancement of at least 50% or 75% to 100% or 2x or 3x the survival rate.

In another embodiment herein, neural crest stem cells are more likely to form multilineage colonies in low oxygen. Preferably, the low oxygen conditions produce at least a 10% or 20% increase, more preferably, a 30% to 40% increase, and more preferably, at least a 50% to 60% increase. For example, if in ambient oxygen only 48% of colonies are multilineage (containing neurons, Schwann cells, and myofibroblasts), with the balance being Schwann cells, then low oxygen conditions will produce colonies which are at least 80% multipotent.

In yet another aspect herein, proliferation is enhanced. "Proliferation" refers to an increase in the number of cells in a population (growth) by means of cell division. Cell proliferation is generally understood to result from the coordinated activation of multiple signal transduction pathways in response to the environment, including growth factors and other mitogens. Cell proliferation may also be promoted by release from the actions of intra- or extracellular signals and mechanisms that block or negatively affect cell proliferation. In a preferred embodiment, proliferation is increased at least 10% to 50%, more preferably at least 50% to 100% and more preferably, proliferation is 2 to 3x, or 4x to 5x. Most preferably, cells in low (also called decreased) oxygen form colonies containing about twice as many cells as those grown in ambient conditions.

The phrase "low ambient oxygen conditions" as used herein refers to any culturing conditions below atmospheric oxygen. Moreover, the phrase is sometimes used interchangeably herein with "subatmospheric" or "low oxygen" conditions. Low ambient oxygen conditions generally means any oxygen concentration below about 20%, preferably below about 15%, more preferably below about 10%, at sea level. By the term "low ambient oxygen conditions", the present invention refers to any culturing conditions below atmospheric oxygen. Thus in particular embodiments, low ambient O_2 conditions are defined as between about 0.5% and about 18%. Ideally, the culture oxygen conditions are kept as close as possible to the normal physiological oxygen conditions in which a particular cell would be found in *in vivo* the better. Clearly, this will mean that those conditions employed for cells will depend on the regional origin of a particular cell. For example, cells from an alveolar origin may prefer growth at about 14% O_2; cells from an arterial source will prefer an oxygen concentration of about 12%; whereas those from certain regions of the brain may prefer oxygen conditions as low as about 1.5%. Preferably, the culture oxygen conditions are kept as close as possible to the normal physiological oxygen conditions in which a particular cell would be found *in vivo*. Thus, in some embodiments, the conditions employed for cells will depend on the regional origin of a particular cell; such conditions are known to the skilled artisan. "Physiologic" oxygen levels are the range of oxygen levels normally found in healthy tissues and organs.

"Subatmospheric" conditions mean any oxygen concentration below about 20%, preferably below about 15%, more preferably below about 10%, at sea level. The term subatmoshpheric may be used herein interchangeably with "low oxygen conditions" defined above.

"Atmospheric O_2 conditions" are those conditions found in the air, *i.e.*, 20-21% O_2. As used herein this term is used interchangeably with the term "traditional" O_2 conditions as traditional tissue culture incubators are kept at atmospheric O_2 conditions.

"Physiologic" oxygen levels are the range of oxygen levels normally found in healthy tissues and organs. These levels vary depending on tissue type (Table 1). However, it is of note that this rate is below 15% in all tissues and below 8% in most

tissues. Thus the physiological oxygen levels can range from about 15% to about 1.5% depending upon the region of the body being measured.

"Hypoxia" occurs when the normal physiologic levels of oxygen are not supplied to a cell or tissue. It should be noted that the low ambient oxygen conditions are not to be considered the same as "hypoxic" conditions. The low ambient oxygen conditions are intended to mimic physiological conditions. As defined herein "hypoxic conditions" are those in which the oxygen level is less than 0.1% O_2 (Gross *et al.*, 1999).

"Normoxia" refers to normal physiologic levels of oxygen for the particular cell type, cell state or tissue in question.

"Anoxia" is the absence of oxygen. "Hypoxic conditions" are those leading to cellular hypoxia. These conditions depend on cell type, and on the specific architecture or position of a cell within a tissue or organ, as well as the metabolic status of the cell. A critical point is that in most cell biology research of the past 25 years, ambient atmospheric oxygen levels of 20-21% are routinely called and experimentally taken to be "normoxic," but this assumption is physiologically erroneous. In this historic context, much cell culture literature refers to any condition with oxygen lower than ambient atmospheric as "hypoxic," but this usage is also physiologically incorrect.

"Acidosis" means that the pH is below normal physiologic levels.

"Enriching" of cells means that the yield (fraction) of cells of one type is increased over the fraction of cells in the starting culture or preparation.

"Proliferation" refers to an increase in the number of cells in a population (growth) by means of cell division. Cell proliferation is generally understood to result from the coordinated activation of multiple signal transduction pathways in response to the environment, including growth factors and other mitogens. Cell proliferation may also be promoted by release from the actions of intra- or extracellular signals and mechanisms that block or negatively affect cell proliferation.

"Regeneration" means re-growth of a cell population, organ or tissue after disease or trauma.

Other terms used throughout the specification will have the meaning commonly assigned by those of skill in the art unless otherwise stated.

Central Nervous System Cells

As mentioned earlier, a particular advantage of the present invention is that it can be used to generate viable cells or tissue that can be used to ameliorate neurodegenerative disorders. Such cells or tissue upon transplantation can be referred to as a graft. The cells for transplantation can include but are not limited human or animal neurons for stroke, brain and spinal cord injury, Alzheimer's Disease, Huntington's Disease and other neurodegenerative disorders; septal and GABAergic cells for epilepsy; ventral mesencephalic or other CNS dopaminergic cells for treatment of Parkinson's Disease; and trophic factor secreting cells for neurological disorders, or even certain psychiatric disorders. The cells to be used as grafts can be from primary tissue or even from certain cell lines. Further it should be understood that any of the cell types mentioned herein throughout may be adult cells or from a fetal origin.

For treatment of neurological disorders, the present invention will produce differentiated neural stem cells that proliferate and differentiate. Undifferentiated neural progenitor cells differentiate into neuroblasts and glioblasts which give rise to neurons and glial cells. During development, cells that are derived from the neural tube give rise to neurons and glia of the CNS. Certain factors present during development, such as nerve growth factor (NGF), promote the growth of neural cells. Methods of isolating and culturing neural stem cells and progenitor cells are well known to those of skill in the art (Hazel and Muller, 1997; U.S. Patent No. 5,750,376).

Regarding any methods provided herein, a single cell or cells at clonal density are preferred. As used herein, the term "clonal density" means a density sufficiently low enough to result in the isolation of single, non-impinging cells when plated in a culture dish, generally about 225 cells/100 mm culture dish. Furthermore, the cells can be primary or from cultures, adult or fetal origin. Moreover, while human cells are preferred, any mammalian cell can be used in this invention, including cells from mice, cattle, sheep, goat, pigs, dogs, rats, rabbits, and primates. Specific examples of suitable solid tissues include neurons or central nervous system supporting cells

derived from brain tissue, germ cells or embryonic stem cells. Stem cells and progenitor cells isolated from any other solid organ (liver, pancreas, spleen, kidney, thyroid, etc.) or those from marrow, spleen or blood are also amenable candidates for culturing under physiologic or hypoxic conditions. Stem cells and progenitor cells isolated from solid tissues (the exception to solid tissue is whole blood, including blood, plasma and bone marrow) which were previously unidentified in the literature are also within the scope of this invention.

In certain embodiments, if about one sixth of colonies in ambient oxygen conditions have no neurons, about a twentieth of colonies in low ambient oxygen conditions have no neurons. In other embodiments, the proportion of colonies having no neurons is increased at least 25% to 50%, more preferably, 50% to 100%, more preferably, 150% to 200%, and more preferably, 200% to 300% or 400%.

Hazel and Muller describe methods of isolating, culturing, and differentiating rat brain neuroepithelial stem cells from both fetal and adult rat brains. Briefly, neural precursors are removed from desired regions of the fetal rat central nervous system by dissection, dissociated to a single-cell suspension, and plated on tissue culture dishes in medium containing the mitogen basic fibroblast growth factor (bFGF). Initially, many of the differentiated neurons die. Proliferating cells are then harvested in a buffered solution. The passaged cells are relatively homogenous for multipotent precursors. To induce differentiation to neurons and glia, the media containing bFGF is removed and replaced with media lacking bFGF.

Subatmospheric culturing conditions can be used in such a protocol from the start of stem cell isolation, in order to enrich the stem cell pool and enhance differentiation into a greater number of cells. "Enriching" of cells means that the yield (fraction) of cells of one type is increased over the fraction of cells in the starting culture or preparation. Subatmospheric/physiologic culture conditions can also be used after initial plating and division, to up-regulate certain gene products in the more differentiated brain cells. Subatmospheric/physiologic culture conditions can also be used throughout the process to enhance the function of the entire population for transplantation.

Detection of neural stem cell derivatives can be determined by antibody staining. For example, central nervous system multipotential stems are marked by

high level expression of the intermediate filament, nestin (Hazel & Muller, 1997). The differentiated neurons are marked by the antibody TUJ1 (O'Rourke *et al.,* 1997), oligodendrocytes by GalC (Bosio *et al.,* 1996), and astrocytes by GFAP antibodies (Rutka *et al.,* 1997).

The methods of the present invention may be used to produce neural cells containing a heterologous gene. Methods of producing cells of neural origin comprising a heterologous gene and uses of such cells are described in U.S. Patent No. 5,750,376 (incorporated herein by reference).

Culture Conditions

Suitable medium and conditions for generating primary cultures are well known in the art and vary depending on cell type. For example, skeletal muscle, bone, neurons, skin, liver, and embryonic stem cells are all grown in media differing in their specific contents. Furthermore, media for one cell type may differ significantly from lab to lab and institution to institution. As a general principle, when the goal of culturing is to keep cells dividing, serum is added to the medium in relatively large quantities (10-20% by volume). Specific purified growth factors or cocktails of multiple growth factors can also be added or sometimes used in lieu of serum. As a general principle, when the goal of culturing is to reinforce differentiation, serum with its mitogens is generally limited (serum about 1-2% by volume). Specific factors or hormones that promote differentiation and/or promote cell cycle arrest can also be used.

Physiologic oxygen and subatmospheric oxygen conditions can be used at any time during the growth and differentiation of cells in culture, as a critical adjunct to selection of specific cell phenotypes, growth and proliferation of specific cell types, or differentiation of specific cell types. In general, physiologic or low oxygen-level culturing is accompanied by methods that limit acidosis of the cultures, such as addition of strong buffer to medium (such as Hepes), and frequent medium changes and changes in CO_2 concentration.

Cells can be exposed to the low oxygen conditions using a variety of means. Specialized laboratory facilities may have completely enclosed environments in which the oxygen levels are controlled throughout a dedicated, isolated room. In such

specialized areas, low oxygen levels can be maintained throughout the isolation, growth and differentiation of cells without interruption. Very few laboratories have such specialized areas. Physiologic or low oxygen culturing conditions also can be maintained by using commercially-available chambers which are flushed with a pre-determined gas mixture (*e.g.*, as available from Billups-Rothenberg, San Diego CA). As an adjunct, medium can be flushed with the same gas mixture prior to cell feeding. In general, it is not possible to maintain physiologic or low oxygen conditions during cell feeding and passaging using these smaller enclosed units, and so, the time for these manipulations should be minimized as much as possible. Any sealed unit can be used for physiologic oxygen or low oxygen level culturing provided that adequate humidification, temperature, and carbon dioxide are provided.

In addition to oxygen, the other gases for culture typically are about 5% carbon dioxide and the remainder is nitrogen, but optionally may contain varying amounts of nitric oxide (starting as low as 3 ppm), carbon monoxide and other gases, both inert and biologically active. Carbon dioxide concentrations typically range around 5% as noted above, but may vary between 2-10%. Both nitric oxide and carbon monoxide are typically administered in very small amounts (*i.e.* in the ppm range), determined empirically or from the literature.

The optimal physiologic or low oxygen level conditions for any given cell type or any particular desired outcome will vary. A skilled artisan could determine suitable subatmospheric conditions by generating an oxygen dose response curve, in which carbon dioxide is kept constant, and oxygen levels are varied (with nitrogen as the remaining gas). For example, to determine the optimal ambient oxygen culturing conditions for expansion of a CNS cell, one would establish cultures from an organ system. The initial culture is mixed, consisting of some differentiated cells, cells of other developmental lineages or pathways, as well as CNS cells. After exposure to the various oxygen levels (*e.g.* 1%, 2%, 5%, 10% and 15%), the number and function of CNS cells is assessed by methods appropriate to the system. In some cases, a constellation of molecular markers is available to rapidly identify the cell population. But in other cases, a single marker coupled with proliferation assays is appropriate, while in other cases proliferation assays alone are appropriate. In some cases all or some of the above assays are coupled with bioassays to follow the differentiation

potential of the presumed stem cells. Overall, the precise assays used to determine stem cell and/or progenitor response to oxygen levels are dependent on the nature of the system examined as well as available markers and techniques specific to that system.

The timing of physiologic or low oxygen conditions is also part of the oxygen dose response curve. Some cells may be more or less sensitive to oxygen during isolation or immediately after isolation while some cells may respond only after some time in culture. The timing of physiologic or low oxygen conditions absolutely and in relation to other manipulations of the cultures is part of assessing the optimal oxygen culturing conditions. Furthermore, the mitogenic effects of other gases may be synergistic with physiologic or low oxygen conditions. Different gene regulatory networks may be induced by low/physiologic oxygen culturing during different phases of culture. During expansion of the cells, low oxygen may induce gene expression distinct from that induced by low oxygen during differentiation.

The cells are typically exposed to low oxygen level conditions for a time sufficient to enrich the population of progenitor/stem cells compared to other cell types. Typically this is for 1 or more hours, preferably 3 or more hours, more preferably 6 or more hours, and most preferably 12 or more hours, and may be continuous. The temperature during the culture is typically reflective of core body temperature, or about 37°C, but may vary between about 32°C and about 40°C. Other important embodiments may simply achieve an increase in cell absolute number or promote the survival of cells.

Following an initial exposure to low or physiologic oxygen culturing conditions, cells can be maintained in these conditions or returned to normal laboratory oxygen conditions, depending on the desired outcome.

It is understood that the initial medium for isolating the CNS cells, the medium for proliferation of these cells, and the medium for differentiation of these cells can be the same or different. All can be used in conjunction with low or physiologic oxygen level culturing. The medium can be supplemented with a variety of growth factors, cytokines, serum, etc. Examples of suitable growth factors are basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factors (TGFα and TGFβ),

platelet derived growth factors (PDGFs), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), insulin, erythropoietin (EPO), and colony stimulating factor (CSF). Examples of suitable hormone medium additives are estrogen, progesterone, testosterone or glucocorticoids such as dexamethasone. Examples of cytokine medium additives are interferons, interleukins, or tumor necrosis factor-α (TNFα). One skilled in the art will test additives and culture components at varied oxygen levels, as the oxygen level may alter cell response to, active lifetime of additives or other features affecting their bioactivity. In addition, the surface on which the cells are grown can be plated with a variety of substrates that contribute to survival, growth and/or differentiation of the cells. These substrates include but are not limited to laminin, poly-L-lysine, poly-D-lysine, polyornithine and fibronectin.

Following culturing in physiologic or low oxygen conditions, one can determine if cells are proliferating by a variety of means including incorporation of radionucleotides (such as ^{3}H-thymidine), or by uptake of BrdU (a thymidine analog) (Assy & Minuk, 1997). Some CNS, progenitor or stem cells will be identified by their ability to differentiate into specific cell types, and this assay may be used alone or in combination with other assays as above, depending on the availability of suitable markers.

Additional Factors for Promotion of Growth and Differentiation

As described herein, the present invention provides methods of increasing the survival, differentiation and phenotypic integrity of CNS cells. This method generally involves growing these cells *in vitro* within physiological oxygen parameters. There is now a wealth of literature pointing to other factors that may increase the survival of such cells. It is contemplated that the use of some of these factors in combination with the growth conditions of the present invention will be useful.

Much of this interest has focussed on finding trophic factors. These factors are able to increase the survival of dopaminergic cells prepared for transplantation; maintain the *in situ* survival post-transplantation of embryonic neurons transplanted into the striatum; as well as increase graft volume, and thereby re-innervate a larger

part of the caudate and putamen which has been shown to have effect both *in vitro* and *in vivo*.

Trophic factors such as NGF, bFGF, EGF, IGF I and II, TGFβ1-3, PDGF, brain derived growth factor (BDNF), ganglion derived growth (GDNF), neurotrophin (NT)-3, NT-4, and ciliary neuronal trophic factor (CNTF), (Engele and Bohn, 1996; Mayer *et al.*, 1993a and 1993b; Knusel *et al.*, 1990, 1991; Poulsen *et al.*, 1994; Nikkhah *et al.*, 1993; Othberg *et al.*, 1995; Hyman *et al.*, 1991) have been investigated and shown to have pronounced effects *in vitro*.

MPTP and 6-OHDA lesions in primates are models of certain neurodegenerative disorders. It has been shown that the effects of such lesions can be reversed in primates and rats (Gash *et al.*, 1996) by the addition of NGF or bFGF to the cell suspension prior to grafting. The same factors also have been shown to increase graft survival, if added to the cell suspension prior to grafting (Chen *et al.*, 1996; Dunnett and Bjorkland., 1994). Additional studies showed an increased graft survival rate in transplanted neurons derived from a neural progenitor (CINP) cell line, that were retrovirally transduced with NGF (Martinez-Serrano *et al.*, 1995) and astrocytes transduced with BDNF (Yoshimoto *et al.*, 1995). GDNF has been shown to increase graft survival, extend fiber outgrowth and alleviate behavioral effects after 6-hydroxydopamine lesions in the striatum of rats (Sauer *et al.*, 1994; Bowenkamp *et al.*, 1995; Rosenblad *et al.*, 1996; Olson, 1996).

Thus these and other factors that may prolong the survival of the CNS cells either *in vitro* or *in vivo* are contemplated for use in the growth and maintenance conditions described in the present invention.

Induction of differentiation with EPO at atmospheric O_2

In another embodiment, EPO can be used to mimic the lowered oxygen effect on dopaminergic differentiation. That is, EPO at atmospheric oxygen levels is used to promote differentiation of stem cells, CNS progenitor cells, or progeny thereof to produce dopaminergic cells. Mesencephalic precursor cells are particularly preferred. The amount of EPO used in this embodiment is an amount sufficient to produce greater than at least about 10%, preferably 20%, more preferably 50% of dopaminergic cells. Preferably, saturating concentrations of EPO are added the

culture either during proliferation, during differentiation or during both phases. The cultures are contacted with EPO for greater than at least about 1, preferably 2, more preferably 5 days. For example, a culture of CNS progenitor cells could be cultured in an atmosphere containing 5% CO_2, 20% O_2 (balance N_2) for 5 days at saturating concentrations of EPO during the proliferative phase, and subsequently cultured for 3 days at 10% EPO during the differentiation phase. The atmosphere preferably contains 20% O_2, although lower O_2 concentrations can be used.

Transplantation Methods

The cells can be used in a variety of applications. The physiologic/ subatmospheric culturing conditions described herein can be used to differentiate specific populations of cells useful for transplantation, and to expand the number of available cells derived from a variety of culture systems.

Laboratory and clinical studies have shown the transplantation of cells into the CNS is a potentially significant alternative therapeutic modality for neurodegenerative disorders such as Parkinson's disease (Wictorin *et al.,* 1990; Lindvall *et al.,* 1990; Sanberg *et al.,* 1994; Bjorklund and Stenevi, 1985; Freeman *et al.,* 1994). In some cases, transplanted neural tissue can survive and form connections with the CNS of the recipient, *i.e.* the host (Wictorin *et al.,* 1990). When successfully accepted by the host, the transplanted cells and/or tissue have been shown to ameliorate the behavioral deficits associated with the disorder (Sanberg *et al.,* 1994). The obligatory step for the success of this kind of treatment is to have enough viable cells available for the transplant.

In addition to cell cultures described above, fetal neural tissue is another important source for neural transplantation (Lindvall *et al.,* 1990; Bjorklund, 1992; Isacson *et al.,* 1986; Sanberg *et al.,* 1994). Other viable graft sources include adrenal cells and various cell types that secrete neural growth factors and trophic factors. The field of neural tissue transplantation as a productive treatment protocol for neurodegenerative disorders has received much attention resulting in its progression to clinical trials. To date the major problem with this field has been the lack of ability to obtain enough viable cells. The present invention provides a method of

maintaining such tissue in a state that will prevent them from losing their ability to serve as an appropriate graft for neurodegenerative diseases.

Methods of grafting cells are now well known to those of skill in art (U.S Patent 5762926; U.S Patent 5650148; U.S Patent 5,082,670) . Neural transplantation or grafting involves transplantation of cells into the central nervous system or into the ventricular cavities or subdurally onto the surface of a host brain. Conditions for successful transplantation include: 1) viability of the implant; 2) retention of the graft at the site of transplantation; and 3) minimum amount of pathological reaction at the site of transplantation.

Methods for transplanting various nerve tissues, for example embryonic brain tissue, into host brains have been described in Neural Grafting in the Mammalian CNS, Bjorklund and Stenevi, eds., (1985) Das, Ch. 3 pp. 23-30; Freed, Ch. 4, pp. 31-40; Stenevi *et al.,* Ch. 5, pp. 41-50; Brundin *et al.,* Ch. 6, pp. 51-60; David *et al.,* Ch. 7, pp. 61-70; Seiger, Ch. 8, pp. 71-77 (1985), incorporated by reference herein. These procedures include intraparenchymal transplantation, *i.e.* within the host brain (as compared to outside the brain or extraparenchymal transplantation) achieved by injection or deposition of tissue within the host brain so as to be opposed to the brain parenchyma at the time of transplantation (Das, supra).

The two main procedures for intraparenchymal transplantation are: 1) injecting the donor cells within the host brain parenchyma or 2) preparing a cavity by surgical means to expose the host brain parenchyma and then depositing the graft into the cavity (Das, supra). Both methods provide parenchymal apposition between the graft and host brain tissue at the time of grafting, and both facilitate anatomical integration between the graft and host brain tissue. This is of importance if it is required that the graft become an integral part of the host brain and to survive for the life of the host.

Alternatively, the graft may be placed in a ventricle, *e.g.* a cerebral ventricle or subdurally, *i.e.* on the surface of the host brain where it is separated from the host brain parenchyma by the intervening pia mater or arachnoid and pia mater. Grafting to the ventricle may be accomplished by injection of the donor cells or by growing the cells in a substrate such as 3% collagen to form a plug of solid tissue which may then be implanted into the ventricle to prevent dislocation of the graft. For subdural

grafting, the cells may be injected around the surface of the brain after making a slit in the dura. Injections into selected regions of the host brain may be made by drilling a hole and piercing the dura to permit the needle of a microsyringe to be inserted. The microsyringe is preferably mounted in a stereotaxic frame and three dimensional stereotaxic coordinates are selected for placing the needle into the desired location of the brain or spinal cord.

The donor cells may also be introduced into the putamen, nucleus basalis, hippocampus cortex, striatum or caudate regions of the brain, as well as the spinal cord.

For grafting, the cell suspension is drawn up into the syringe and administered to anesthetized graft recipients. Multiple injections may be made using this procedure. The age of the donor tissue, *i.e.,* the developmental stage, may affect the success of cell survival after grafting.

The cellular suspension procedure thus permits grafting of donor cells to any predetermined site in the brain or spinal cord, is relatively non-traumatic, allows multiple grafting simultaneously in several different sites or the same site using the same cell suspension, and permits mixtures of cells from different anatomical regions. Multiple grafts may consist of a mixture of cell types, and/or a mixture of transgenes inserted into the cells. Preferably from approximately 10^4 to approximately 10^8 cells are introduced per graft.

For transplantation into cavities, which may be preferred for spinal cord grafting, tissue is removed from regions close to the external surface of the CNS to form a transplantation cavity, for example by removing bone overlying the brain and stopping bleeding with a material such a gelfoam (Stenevi *et al.,* Brain Res. 114:1-20 (1976)). Suction may be used to create the cavity. The graft is then placed in the cavity. More than one transplant may be placed in the same cavity using injection of cells or solid tissue implants.

Grafting of donor cells into a traumatized brain will require different procedures, for example, the site of injury must be cleaned and bleeding stopped before attempting to graft. In addition, the donor cells should possess sufficient growth potential to fill any lesion or cavity in the host brain to prevent isolation of the graft in the pathological environment of the traumatized brain.

Measurement of Phenotype of Cells

In specific embodiments, it may be necessary to monitor the phenotype of the cell that has been grown in the subatmospheric oxygen conditions so as to determine whether differentiation or other modification of the cell has occurred. Various methods may be used to achieve this, including monitoring message level, protein level, subcellular localization, functional assays or morphological changes. The methods for monitoring message level include PCR™ (U.S. Patent 5,364,790; U.S. Patent 4,800,159; U.S. Patent 4,683,195), In situ hybridization (U.S. Patent 4,888,278; U.S. Patent 4,886,741; U.S. Patent 5,506,098; U.S. Patent 5,225,326; U.S. Patent 5,521,061; U.S. Patent 5,538,869; U.S. Patent 5,665,540), RNAse protection assay, and single cell PCR™. The methods for monitoring protein level may use antibody staining, HPLC, western blotting or immunoprecipitation. These techniques are all well known to those of skill in the art.

The ability to detect genes that are differentially expressed in two cell types or populations combined with advances of rapid gene detection and sequencing technologies may be used to compare gene expression in cells cultured under varying oxygen concentrations.

Methods of differential display have been used to elucidate the genes responsible for a difference in phenotypes between two relatively similar cell types or during sequential changes of a cell from one state to another. For example, using the differential display technique, Kocher *et al.* (1995) selected for genes that were up-regulated in renal cell carcinoma compared with normal renal parenchyma. Through this method, Kocher *et al.* (1995) were able to isolate a gene (DD96) that was rarely expressed in normal epithelial cell populations, expressed diffusely in malignant epithelial cells of the wide majority of carcinomas, and markedly overexpressed in carcinomas originating from the colon, breast, and lung. A similar technique can be used to compare gene expression in cells incubated under traditional versus low oxygen level conditions. Genes up-regulated in one population over the other then may be used as a probe to screen for expression of that gene in other cell populations or the same cell population under different culturing conditions (*i.e.*, in the presence of compounds or environmental stimuli that may affect the expression of the gene).

Kang *et al.* (1998) have developed a reciprocal subtraction differential RNA display (RSDD) method that permits the rapid and efficient identification and cloning of both abundant and rare differentially expressed genes. The technology was used to analyze gene expression alterations resulting during cancer progression as adenovirus-transformed rodent cells developed an aggressive transformed state (Kang *et al.*, 1998). The approach resulted in the identification and cloning of known and unknown sequences that displayed expression as a function of progression and suppressed expression as a function of progression (Kang *et al.*, 1998). The RSDD technique may be used to compare gene expression between cells during maintenance, proliferation and/or differentiation of the cells from progenitor or stem cells to fully differentiated cells in room air versus subatmospheric conditions.

The methods of differential display may be used in conjunction with rapid DNA sequencing and detection methods, allowing for the ability to screen for or sequence a large number of genes in a relatively short amount of time. U.S. Patent No. 5,800,992 provides methods for detecting the differential expression of a plurality of genes between two cell types using complimentary polynucleotides in an array. Such technology is commonly referred to as "DNA chip" technology because the polynucleotides are deposited on a substrate that resemble computer microprocessor chips. Also described are methods of sequencing genes using DNA chips.

Additionally, similar techniques are described in U.S. Patent No. 5,834,181 which utilizes similar technology to detect minor alterations in genes such as single nucleotide substitution, allowing detection of mutations in genes that lead to a change in the phenotype of a cell.

Single-cell reverse transcriptase-polymerase chain reaction (RT-PCR) technique, also will be useful in monitoring the phenotype of the cells grown in the present invention. Such a technique is described by, for example, Cornelison and Wold (1997).

The single cell RT-PCR technique of Cornelison and Wold allows determination of expression of a number of genes at one time and may be used to identify skeletal muscle satellite cells and determine their activation state when incubated in the low/physiological oxygen conditions of the present invention.

Another detection method commonly used is an RNAse protection assay in which a radiolabeled RNA probe is mixed with a test RNA population, such as total cellular RNA from an individual, under conditions where complementary segments of the RNA probe and the test RNA will hybridize. RNAse is then added to the mixture to destroy unprotected (unhybridized), single-stranded probe and test RNA. When all single-stranded RNA has been destroyed, only short fragments of protected RNA remains that can be analyzed electrophoretically to diagnose the particular RNA composition of the test RNA. The protected double-stranded RNA fragments are denatured before analysis, to make available the detectable, labeled single stranded RNA probe fragment.

Disease Models

Once a particular set of cells have been generated it will of course be necessary to test that these cells would apply to a disease model. Animal models of Parkinson's Disease and other neurodegenerative diseases are now well known to those of skill in the art.

For example, a rat model of Parkinson's disease can be created by giving a unilateral injection of saline-ascorbate 6-hydroxy-dopamine (6-OHDA) into the medial forebrain bundle. This produces a lesion that ultimately mimics Parkinsonian behavior. Completeness of the lesion produced can by monitoring either apomorphine or amphetamine induced rotational behavior (Ungerstedt and Arbuthnott, 1970). Animals turning at a rate of more than 7 turns per minute (Schmidt *et al.,* 1982) can be inferred to have the appropriate lesion (at least 7 contralateral rotations/min following apomorphine administration and at least 7 ipsilateral rotations/min towards the side of the lesion following amphetamine administration).

Using such a model a baseline rotation behavior can be established. After that, the cells grown in the present invention can then be transplanted into the rat model as described herein above. Any decrease in the rotational behavior would be indicative of the cellular transplant having an appropriate therapeutic value.

Gene Replacement/Augmentation Applications

Optionally, the CNS cells obtained using the method of the present invention can be manipulated to express desired gene products. Gene therapy can be used to either modify a cell to replace a gene product, to facilitate regeneration of tissue, to treat disease, or to improve survival of the cells following implantation into a patient (*i.e.* prevent rejection).

In this embodiment, the CNS cells are transfected prior to expansion and differentiation. Techniques for transfecting cells are known in the art.

A skilled artisan could envision a multitude of genes which would convey beneficial properties to the transfected cell or, more indirectly, to the recipient patient/animal. The added gene may ultimately remain in the recipient cell and all its progeny, or may only remain transiently, depending on the embodiment. For example, genes encoding tyrosine hydroxylase, or a monoamine transporter such as VMAT 1 or VMAT 2 could be transfected into certain CNS cells to provide an appropriate therapeutic cell suitable for grafting into a subject with Parkinson's disease. Other genes that could be used include GABA-decarboxylase, enkephalin, dopa decarboxylase (AADC), ciliary neuronal trophic factor (CNTF), brain derived neurotrophic factor (BDNF), neurotrophin (NT)-3, NT-4, and basic fibroblast growth factor (bFGF).

The added gene may ultimately remain in the recipient cell and all its progeny, or may only remain transiently, depending on the embodiment. For example, genes encoding angiogenic factors could be transfected into progenitor cells isolated from smooth muscle. Such genes would be useful for inducing collateral blood vessel formation as the smooth muscle tissue is regenerated. It some situations, it may be desirable to transfect the cell with more than one gene. In some situations, it may be desirable to transfect the cell with more than one gene. Of course the above therapeutic genes are only exemplary and those of skill in the art will understand that any neurodegenerative disorder that results from an aberration in gene expression and/or function can be treated by gene replacement and/or augmentation. Such disorders and their related genes are well known to those of skill in the art.

In some instances, it is desirable to have the gene product secreted. In such cases, the gene product preferably contains a secretory signal sequence that facilitates secretion of the protein.

The viral vectors used herein may be adenoviral (U.S. Patent No. 5,824,544; U.S. Patent No. 5,707,618; U.S. Patent No. 5,693,509; U.S. Patent No. 5,670,488; U.S. Patent No. 5,585,362; each incorporated herein by reference), retroviral (U.S. Patent No. 5,888,502; U.S. Patent No. 5,830,725; U.S. Patent No. 5,770,414; U.S. Patent No. 5,686,278; U.S. Patent No. 4,861,719 each incorporated herein by reference), an adeno-associated viral (U.S. Patent No. 5,474,935; U.S. Patent No. 5,139,941; U.S. Patent No. 5,622,856; U.S. Patent No. 5,658,776; U.S. Patent No. 5,773,289; U.S. Patent No. 5,789,390; U.S. Patent No. 5,834,441; U.S. Patent No. 5,863,541; U.S. Patent No. 5,851,521; U.S. Patent No. 5,252,479; each incorporated herein by reference), an adenoviral-adenoassociated viral hybrid (U.S. Patent No. 5,856,152 incorporated herein by reference), a lentiviral vector, a vaccinia viral or a herpesviral (U.S. Patent No. 5,879,934; U.S. Patent No. 5,849,571; U.S. Patent No. 5,830,727; U.S. Patent No. 5,661,033; U.S. Patent No. 5,328,688; each incorporated herein by reference) vector.

Delivery of the expression constructs through non-viral vectors also is contemplated. Such delivery may employ microinjection (U.S. Patent No. 5,612,205), electroporation (U.S. Patent No. 5,507,724; U.S. Patent No. 5,869,326; U.S. Patent No. 5,824,547; U.S. Patent No. 5,789,213; U.S. Patent No. 5,749,847; U.S. Patent No. 5,019,034; Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), calcium phosphate coprecipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990), DEAE dextran introduction (Gopal, 1985), receptor mediated introduction (Wu and Wu, 1987; Wu and Wu, 1988), liposome mediated introduction (U.S. Patent No. 5,631,018; U.S. Patent No. 5,620,689; U.S. Patent No. 5,861,314; U.S. Patent No. 5,855,910; U.S. Patent No. 5,851,818; U.S. Patent No. 5,827,703, U.S. Patent No. 5,785,987; Nicolau and Sene, 1982; Fraley *et al.*, 1979), dendrimer technology (U.S. Patent 5,795,581; U.S. Patent 5,714,166; U.S. Patent 5,661,025), naked DNA injection (Harland and Weintraub, 1985) and particle bombardment (U.S. Patent No. 5,836,905; U.S. Patent No. 5,120,657; Yang *et al.*, 1990).

The desired gene is usually operably linked to its own promoter or to a foreign promoter which, in either case, mediates transcription of the gene product. Promoters are chosen based on their ability to drive expression in restricted or in general tissue types, or on the level of expression they promote, or how they respond to added chemicals, drugs or hormones. Particularly contemplated promoters include but are not limited to CMV IE, SV40 IE, β-actin, collagen promoter, TH promoter, AADC promoter and the nestin promoter.

Other genetic regulatory sequences that alter expression of a gene may be co-transfected. In some embodiments, the host cell DNA may provide the promoter and/or additional regulatory sequences.

Other elements that can enhance expression can also be included such as an enhancer or a system that results in high levels of expression.

Methods of targeting genes in mammalian cells are well known to those of skill in the art (U.S. Patent Nos. 5,830,698; 5,789,215; 5,721,367 and 5,612,205). By "targeting genes" it is meant that the entire or a portion of a gene residing in the chromosome of a cell is replaced by a heterologous nucleotide fragment. The fragment may contain primary the targeted gene sequence with specific mutations to the gene or may contain a second gene. The second gene may be operably linked to a promoter or may be dependent for transcription on a promoter contained within the genome of the cell. In a preferred embodiment, the second gene confers resistance to a compound that is toxic to cells lacking the gene. Such genes are typically referred to as antibiotic-resistance genes. Cells containing the gene may then be selected for by culturing the cells in the presence of the toxic compound.

Application to Other Cell Types

Although the majority of the discussion above is focussed on the growth and culturing of CNS cells, it should be appreciated that techniques of the present invention also will be useful for growth of other types of cells. As such it is contemplated that the techniques provided herein will be useful for growing any cells that are routinely used in transplant therapies. For example, such cells may be islets cells for diabetes; myoblasts for muscular dystrophy; hepatocytes for liver disease;

skin grafts for wound healing and/or burns, and bone marrow or stem cells for hematopoietic and genetic disorders. In addition, the disclosure of U.S. Application number 09/195,569 filed November 18, 1998, (specifically incorporated herein by reference) will provide additional examples that may be useful in conjunction with the present invention.

Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1

Material and Methods

Culture of CNS Stem Cells. Animals were housed and treated following NIH guidelines. Cells dissected from rat embryonic lateral ganglionic eminence (E14) or mesencephalon (E12) were mechanically dissociated, plated on plastic 24-well plates (Costar) or 12 mm glass cover slips (Carolina Biologicals) precoated with polyornithine/fibronectin, and grown in defined medium with bFGF (Studer *et al.*, 1998; Johe *et al.*, 1996). In general, bFGF was withdrawn from the medium after 4 - 6 days of culture. Clonal assays were carried out in plastic 48-well plates (Costar). In some studies, recombinant human (rh)EPO, rhVEGF$_{165}$ or recombinant mouse (rm)FGF8b, or their neutralizing antibodies (all from R&D Systems) were added to cultures at the following concentrations: EPO 0.5 U/ml, EPO neutralizing antibody 10 μg/ml, FGF8 250 ng/ml, FGF8b neutralizing antibody 5 μg/ml, VEGF 50 ng/ml, VEGF neutralizing antibody 0.5 μg/ml. Dose response for EPO was carried out at

0.05 U/ml, 0.5 U/ml, 5 U/ml and 15 U/ml; for anti-EPO at 10 μg/ml and 100 μg/ml. Results of all experiments were confirmed by at least 2 independent culture series.

Culture of Sciatic Nerve Progenitor Cells. Sciatic nerve progenitors were cultured at clonal density as described previously (Morrison *et al.*, 1999). Briefly, plates were coated with poly-d-lysine (Biomedical Technologies, Stoughton MA) and human fibronectin (Biomedical Technologies). The culture medium contained DMEM-low glucose (Gibco) with 15% chick embryo extract (Stemple, *et al.*, 1992), 20 ng/ml recombinant human bFGF (R&D Systems, Minneapolis), N2 supplement (Gibco), B27 supplement (Gibco), 50 μM 2-mercaptoethanol, 35mg/ml retinoic acid (Sigma), and penicillin/streptomycin (BioWhittaker). This composition is described throughout as "standard medium". Under standard conditions, cells were cultured for 6 days in standard medium, then switched to a similar medium (with 1% CEE and 10ng/ml bFGF) that favors differentiation for another 8 days before immunohistochemical analysis of colony composition. To promote sympathetic differentiation, 5μM forskolin and 1ng/ml BMP2 (Genetics Institute) were sometimes added after the first 6 days of standard culture, and the cultures were allowed to develop for another 6 days. To further promote sympathetic neuron differentiation the cultures were switched to standard medium containing 50 ng/ml NGF and 50 ng/ml NT3 for a final 6 days.

Normal humidified tissue culture incubators with around 6% CO_2 were used for 20% oxygen cultures. For decreased oxygen cultures, plates were inserted into gas-tight modular incubator chambers (Billups-Rothenberg, Del Mar, CA) that were flushed with a custom gas mixture containing 1% O_2/6% CO_2/balance N_2. The incubator chambers were flushed for one and a half to two minutes daily at a rate of 15 liters per minute, then inserted into normal tissue culture incubators. This achieved an actual concentration inside the chamber of around 5% oxygen, based on direct measurement with a microelectrode (Animus Corp., Malvern PA). Although 5% oxygen is commonly described as hypoxic relative to atmospheric oxygen, it actually generates a tissue culture environment closer to physiological levels (Guyton, *et al.*, 1996).

Low oxygen culture. Cultures were placed in humidified portable isolation chambers (Billups-Rothenberg, Del Mar CA), flushed daily with a gas mixture 1%

O_2, 5% CO_2 + 94% N_2. Precise O_2 levels in the surrounding atmosphere depended on the length of chamber flush (90 sec at 15 L/min achieved 6% O_2, 6 minutes of flush achieved 1.5% O_2), which was not standardized until availability of an O_2-sensitive electrode system (OS2000, Animus Corp., Frazer PA). Thus "lowered O_2" conditions represent a range of ambient O_2 of 3±2%, which approximates normal brain tissue levels (Table 1). The entire chamber was housed in an incubator to maintain temperature at 37°C.

BrdU uptake and TUNEL analysis. Bromodeoxyuridine (10 μM) was added to cultures for exactly 60 minutes, just prior to fixation. Anti-BrdU staining was performed according to the manufacturer's protocol (Amersham Life Sciences). The TUNEL reaction (Boehringer-Mannheim) was also performed according to manufacturer's protocol. TUNEL+ cells were visualized by metal-enhanced DAB reaction (Pierce) after peroxidase conversion of the FITC label.

Immunohistochemistry. Cells were fixed in 4% paraformaldehyde + 0.15% picric acid/PBS. Standard immunohistochemical protocols were followed. The following antibodies were used: Stem cell/progenitor characterization: Nestin polyclonal #130 1:500 (Martha Marvin & Ron McKay), PSA-NCAM, En1 and FP4 (all monoclonal 1:2, Developmental Studies Hybridoma Bank, provided by Tom Jessel). Stem cell differentiation: β-tubulin type III (Tuj1) monoclonal 1:500 and polyclonal 1:500 (both BabCO), O4 monoclonal 1:5 (Boehringer-Mannheim), galactocerebroside (GalC) monoclonal 1:50 (Boehringer-Mannheim), glial fibrillary acidic protein (GFAP) 1:100 (ICN Biochemicals). Neuronal subtype differentiation: Tyrosine hydroxylase (TH) polyclonal 1:200-1:500 (PelFreeze, Rogers AK) or monoclonal 1:2000 (Sigma), GABA polyclonal 1:500 (Sigma), glutamate 1:500 (Sigma), dopamine-β-hydroxylase (DBH) 1:100 (Protos Biotech Corp). Appropriate fluorescence-tagged (Jackson Immunoresearch) or biotinylated (Vector Laboratories) secondary antibodies followed by metal-enhanced DAB reaction (Pierce) were used for visualization.

For routine analysis of neural crest cell culture compositions, cultures were fixed in acid ethanol and stained with antibodies against peripherin (Chemicon AB1530, Temecula CA), smooth muscle actin (Sigma A-2547), and GFAP (Sigma G-

3893) as described previously (Morrison, *et al.*, 1999). In order to stain for sympathetic markers, cultures were fixed in 4% paraformaldehyde, blocked in PBS with 4% goat serum plus 0.2% BSA plus 0.1% NP-40, then stained with antibodies against tyrosine hydroxylase (Boehringer Mannheim, Indianapolis, IN), and/or dopamine-beta-hydroxylase (Pharmingen, San Diego CA), or SA-1 (a gift of Paul Patterson). VAChT staining was performed on 4% paraformaldehyde fixed cultures, blocked in horse serum, and stained for an hour at room temperature in goat polyclonal antibody (Chemicon AB1578), followed by immunoperoxidase detection (nickel/diaminobenzidine precipitation).

Cell Counts and Statistical Procedures. Uniform random sampling procedures were used for cell counts and quantified using the fractionator technique (Gundersen, *et al.*, 1988). Statistical comparisons were made by ANOVA with posthoc Dunnett test when more than 2 groups were involved. If data were not normally distributed, a non-parametric test (Mann-Whitney U) was used to compare lowered vs. 20% O_2 results. Data are expressed as mean $\pm$SEM.

Reverse-phase HPLC determinations of dopamine content. Culture supernatants of medium, HBSS, and HBSS+56 mM KCl were stabilized with orthophosphoric acid and metabisulfite, and stored at –80°C until analysis. Stabilization, aluminum adsorption, equipment, and elution of dopamine have been previously described (Studer *et al.*, 1998; Studer *et al.*, 1996). Results were normalized against dopamine standards at varying flow rates and sensitivities.

A Shimadzu solvent delivery system was used with the following mobile phase: 92% 75 mM NaH_2PO_4, 1.7 mM octanesulfonic acid, 0.05 mM EDTA, pH 3.1; 8% acetonitrile, at a flow rate of 0.8 ml/min. An Alltech Absorbosphere HS C18 reverse phase column (10 x 4.6 mm, 3 μm) was connected to an electrochemical detector (ESA: Coulochem II) set at an applied potential of +0.02V at detector 1 and + 0.40V at detector 2. Detector response was linear for 0.05 - 10 ng.($r = 0.99$ for linear regression calculations of all compounds assayed; within-assay variance was less than 5%). Peak areas were quantitated with a Rainin MACintegrator system. No DA or NE were detected in blank samples prepared from solvent, or from a control colony cultured under standard conditions in 20% oxygen.

Western blots. Cell pellets were stored at –80°C. Pellet was lysed in 20 mM Hepes, pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton-X-100, with protease inhibitors (Complete, Boehringer-Mannheim), homogenized, and incubated on ice for 1 hr. After centrifugation, supernatant protein concentration was assayed by BCA (Pierce). For western, block was 5% milk in TBST, primary TH antibody (Pel-Freeze, Rogers, AK) was used at 1:500, and secondary was HRP-conjugated goat anti-rabbit (Pierce) at 1:5000. Signal was detected with SuperSignal (Pierce).

RTPCR™. Cultures were washed once in PBS before solubilization in 2 ml (per 6 cm dish) Trizol (Life Technologies) then stored at –80°C. RNA extraction was carried out according to manufacturer's recommendations (Gibco Life Technologies). The Superscript kit (Gibco Life Technologies) was used for reverse transcription of 10 μg RNA per condition. PCR conditions were optimized by varying MgCl concentration and cycle number to determine linear amplification range. Amplification products were identified by size and confirmed by DNA sequencing. TH was kindly provided by Vera Vikodem, NIDDK, 30 cyc., 56°C, 300bp. For the other products the primer sequences, cycle numbers, and annealing temperatures were as shown in Table 2.

Table 2: primer sequences, cycle numbers, and annealing temperatures for PCR

Identity	Forward primer	Reverse primer	Conditions
GAPDH	CTCGTCTCATAGACAAGATGGTGAAG (SEQ ID NO:1)	AGACTCCACGACATACTCAGCACC (SEQ ID NO:2)	28 cyc., 59°C, 305bp
VHL	CCTCTCAGGTCATCTTCTGCAACC (SEQ ID NO:3)	AGGGATGGCACAAACAGTTCC (SEQ ID NO:4)	35 cyc., 60°C, 208bp
HIF1α	GCAGCACGATCTCGGCGAAGCAAA (SEQ ID NO:5)	GCACCATAACAAAGCCATCCAGGG (SEQ ID NO:6)	30 cyc., 59°C, 235bp
EPO	CGCTCCCCCACGCCTCATTTG (SEQ ID NO:7)	AGCGGCTTGGGTGGCGTCTGGA (SEQ ID NO:8)	30cyc., 60°C, 385 bp
VEGF	GTGCACTGGACCCTGGCTTTACT (SEQ ID NO:9)	CGCCTTGCAACGCGAGTCTGTGTT (SEQ ID NO:10)	30 cycles, 60°C, 474bp (detects VEGF-1, VEGF-2 AND VEGF-3)
Nurr1	TGAAGAGAGCGGAGAAGGAGATC (SEQ ID NO:11)	TCTGGAGTTAAGAAATCGGAGCTG (SEQ ID NO:12)	30 cyc., 55°C, 255 bp
Ptx3	CGTGCGTGGTTGGTTCAAGAAC (SEQ ID NO:13)	GCGGTGAGAATACAGGTTGTGAAG (SEQ ID NO:14)	35 cyc., 60°C, 257 Bp
SHH	GGAAGATCACAAGAAACTCCGAAC (SEQ ID NO:15)	GGATGCGAGCTTTGGATTCATAG (SEQ ID NO:16)	30 cyc., 59°C, 354bp
FGF8	CATGTGAGGGACCAGAGCC (SEQ ID NO:17)	GTAGTTGTTCTCCAGCAGGATC (SEQ ID NO:18)	35 cyc., 60°C, 312bp
En1	TCAAGACTGACTACAGCAACCCC (SEQ ID NO:19)	CTTTGTCCTGAACCGTGGTGGTAG (SEQ ID NO:20)	30 cyc., 60°C, 381bp
FGFR3	ATCCTCGGGAGATGACGAAGAC (SEQ ID NO:21)	GGATGCTGCCAAACTTGTTCTC (SEQ ID NO:22)	30 cyc., 55°C, 326 bp
GDNF	according to Moreau *et al.*, 1998		
BDNF	GTGACAGTATTAGCGAGTGGG (SEQ ID NO:23)	GGGTAGTTCGGCATTGC (SEQ ID NO:24)	35 cycles, 56°C, 213 bp

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Isolation of sciatic nerve $p75^{+}P_{0}$-. Pregnant Sprague-Dawley rats were obtained from Simonsen (Gilroy, CA). For timed pregnancies, animals were put together in the afternoon and the morning on which the plug was observed was designated E0.5. Neural crest stem cells were isolated from E14.5 sciatic nerves as described previously (Morrison, *et al.*, 1999). Briefly, nerves were dissected and dissociated by incubating in trypsin plus collagenase for 4 minutes at 37° C followed by mechanical trituration. The cells were stained with monoclonal antibodies against p75 (192Ig), the low-affinity neurotrophin receptor, and P_0 (P07), a PNS myelin component. All sorts and analyses were performed on a FACSVantage flow-cytometer (Becton-Dickinson, San Jose). NCSCs were sorted as live $p75^{+}P_{0}$- cells.

Example 2

Results: Lowered O_2 augments expansion of striatal and mesencephalic precursors.

E14 rat striatum, widely used for derivation of CNS precursors, cultured in lowered O_2 yielded an average 2- to 3-fold more cells than 20% O_2 cultures over a wide range of plating densities in the presence of bFGF. (Basic FGF acts as mitogen for stem cells obtained from many regions of the developing brain. The withdrawal of bFGF initiates differentiation to neurons, astrocytes and oligodendrocytes). (FIG. 1). Identical results were obtained with E12 mesencephalic precursors. For all results, data were verified by at least two independent culture series.

Effects on cell proliferation and cell death. To test whether increased cell yield in lowered O_2 is due to increased proliferation, reduced cell death, or both, precursors were pulsed with bromodeoxyuridine (BrdU) for 1 hr immediately before fixation at multiple time points while precursor cells were proliferating or differentiating. Both mesencephalic and striatal precursors showed increased BrdU labeling indices when grown in lowered O_2 as compared to traditional cultures. Lowered O_2 increased the BrdU labeling index in the presence of bFGF and during cell differentiation following mitogen withdrawal from mesencephalic precursors (FIG. 2). BrdU incorporation rates for striatal precursors showed similar patterns

{*Day 2 of expansion* : 18 ± 6% in lowered O_2 vs. 11 ± 6% in 20% O_2 (n=24, $p < 0.05$). *Day 6 of expansion* : 30 ± 8% in lowered O_2 vs. 22 ± 5% in 20% O_2 (n=24, $p < 0.05$). *Day 4 of differentiation (10 days in vitro)* : 12 ± 5% in lowered O_2 vs. 3 ± 3% in 20% O_2 (n=24, $p < 0.05$)}.

In addition to this apparent increase in cell proliferation in lowered O_2 cultures, precursor cells were also less likely to undergo apoptosis than CNS precursors grown in 20% O_2. Both mesencephalic and striatal precursors revealed significantly reduced percentages of TUNEL-positive cells both during expansion and after bFGF withdrawal. TUNEL data for mesencephalic precursors are summarized in FIG. 3. Thus, both reduced apoptosis and increased cell proliferation contribute to elevated yield of cells at the end of the expansion phase. Cell death is reduced but not entirely eliminated during the differentiation phase by lowering the O_2 levels.

Cell lineage and clonal growth. A series of molecular markers were used, together with morphologic assessment in order to characterize how lowered O_2 culturing affects the choice of differentiation pathways and the kinetics of differentiation. Immunoreactivity for the intermediate filament nestin was used to discriminate CNS stem and progenitor cells from more differentiated progeny (Lendahl *et al.*, 1990). Six days after bFGF withdrawal the percentage of nestin-positive cells derived from expanded precursors was grossly reduced in lowered O_2 cultures compared with 20% O_2 cultures, suggesting that differentiation might have been accelerated in lowered O_2 (FIG. 4A and FIG. 6). The sialic acid substituted form of NCAM (PSA-NCAM), a proposed marker for committed neuronal progenitors (Mayer *et al.*, 1997), was conversely reduced in differentiated lowered O_2 cultures (FIG. 6). The idea of accelerated progression to a more differentiated phenotype was supported by the earlier appearance of neuronal and glial markers in lowered O_2. Neurons were assessed by β-tubulin III (TUJ1) staining, astrocytes by glial fibrillary acidic protein (GFAP), oligodendrocyte precursors by O4, and oligodendrocytes by GalC galactocerebroside staining (FIG. 4). Five days after bFGF withdrawal striatal cultures held at low O_2 contained 46% Tuj1-positive cells vs. 34% in 20% O_2 (n=12, $p < 0.05$); Six percent were GFAP+ vs. 2% in 20% O_2 (n=12, $p < 0.05$); and 4% were Gal-C+ vs. 5% in 20% O_2 (p=n.s.). In mesencephalic cultures

held at lowered oxygen, 73% were Tuj1+ vs. 63% in 20% O_2 (n=12, p = 0.06); no GFAP+ cells were detected in either oxygen conditions; 1% were O4+ versus 0% in 20% O_2 (n=12, p < 0.01). (FIG. 4B).

To investigate O_2 effects at clonal densities, mesencephalic precursors were first expanded in bFGF for 6 days in 20% O_2, replated at a density of 1-5 cells/well, then maintained at either lowered or 20% O_2. After 20 days, 20 ng/mL bFGF was withdrawn. Clonal cultures with typical multi-lineage differentiation responses were observed in both lowered and 20% O_2 conditions. FIG. 4C illustrates a typical nestin+ clone (left panel) and clonally derived cells undergoing neuronal differentiation 4 days after mitogen withdrawal (right panel). As expected of stem cells, all three lineages were represented in the clones grown in low oxygen conditions. However, the efficiency of clone formation was 3 times higher in lowered O_2 and the average clone size also increased from <50 cells in 20% O_2 to 50-500 cells in lowered O_2 (FIG. 4D, FIG. 4E).

Example 3

Results: Neuronal subtype differentiation

The results above establish that lowered oxygen conditions support stem cell proliferation and differentiation to neurons and glia. The inventors' previous work has shown that nestin-positive mesencephalic precursors differentiate into functional dopaminergic neurons (Studer *et al.*, 1998). Next it was determined whether this specific neuronal fate was influenced by lowered O_2. Mesencephalic precursors in lowered oxygen displayed a striking increase in both the absolute number and fraction of neurons expressing TH (FIG. 5A, FIG. 5B). In lowered O_2, large neuronal clusters were seen in which virtually all neurons were TH+. On average, 56% of neurons (marked by Tuj1 staining) generated in lowered O_2 were TH+ vs. 18% in traditional cultures (n=12, p <0.001). Increased TH-immunoreactivity in lowered O_2 cultures correlated with increased TH protein content in Western blots (FIG. 5C). The functional dopaminergic capacity of the TH-positive neurons was further assessed by reverse phase HPLC, which showed significantly increased levels of dopamine in lowered vs. 20% O_2 cultures (FIG. 5D): Conditioned medium (24 hours) showed a 5-

fold increase in dopamine (n=5, $p < 0.01$). Basal release in HBSS revealed a 2- to 3-fold increase (n=5, $p < 0.05$) and evoked release was 3-fold increased (n=5, $p < 0.05$). These results confirm that lowered oxygen favors the differentiation of functional dopaminergic neurons.

Mesencephalic precursors give rise to neurons with several distinct neurotransmitter phenotypes in addition to dopaminergic fate (Studer *et al.,* 1998). Interestingly, the percentage of serotonergic neurons was also increased in lowered O_2, 3.2±1.2% vs. 1.2±0.3% in 20% O_2 (n=12, $p < 0.05$, FIG. 6). On the other hand GABA+ and Glutamate+ neurons were less likely to be generated in lowered O_2 (FIG. 6: GABA+ cells 6.6±1.8% in lowered O_2 vs. 10.4±1.5% n=12, $p < 0.05$; Glutamate+ cells 12.8%±3.8% in lowered O_2 cultures vs. 23.6±4.0% in 20% O_2 (n=12, $p < 0.01$). No double labeling of TH with GABA was detected indicating that TH immunoreactivity corresponded to differentiated dopaminergic neurons and was not a transient developmental phenomenon seen in developing GABAergic neurons (Max *et al.,* 1996). Furthermore, the TH-positive neurons were not fated to a noradrenergic phenotype, since no dopamine-β-hydroxylase staining could be demonstrated.

Since lowered O_2 promoted differentiation of dopaminergic and serotonergic neurons, both ventral neuronal phenotypes (Yamada *et al.,* 1991; Hynes *et al.*, 1995; Ye *et al.,* 1998), it was determined whether these changes were associated with an increase in floor plate cells. Immunohistochemistry revealed expanded zones of FP4+ cells in lowered O_2 (FIG. 6). A more striking feature was the increased occurrence of neurons expressing the transcription factor engrailed-1 (En1) in lowered O_2. (FIG. 6). Engrailed-1 is critical for normal midbrain development (Joyner, 1996; Danelian and McMahon 1996; Wurst *et al.,* 1994) and has been implicated in control of dopaminergic neuronal fate (Simone *et al.,* 1998).

It is important to establish whether the low oxygen condition enhanced dopaminergic differentiation by acting during the proliferation or differentiation phases of the culture system. Mesencephalic precursors were expanded for 5 days in either lowered or 20% O_2. Each group was then subdivided for differentiation in either lowered or 20% O_2. Precursors expanded in lowered O_2 but differentiated in 20% O_2 yielded 38±6% TH+ neurons, similar to those maintained in lowered O_2

throughout (41±7%, n=12, p = n.s.) but significantly higher than those maintained in 20% O_2 throughout (17±4%, n=12, $p < 0.01$). Exposure to lowered O_2 limited to the differentiation phase did not significantly increase the yield of dopaminergic neurons (21±2%, n=12, p=n.s.) compared to cultures maintained in 20% O_2 throughout. From these data, it is shown that the major effect of low O_2 is during the expansion phase.

Semi-quantitative RT-PCR was used to assay RNA from cultures at various time points for differential expression of candidate genes involved in dopaminergic neuron development (FIG. 7). A small increase in TH message was detected from lowered O_2 cultures after differentiation, compared to 20% O_2. The Ptx3 homeobox gene has also been implicated in dopamine neuron development (Smidt *et al.*, 1997) and was also expressed at increased levels in lowered O_2 suggesting that these conditions promoted the dopaminergic phenotype, not simply upregulation of TH gene expression. Strong evidence links sonic hedgehog (Echelard *et al.*, 1993); and Nurr1 (Saucedo-Cardenas *et al.*, 1998) genes to the differentiation of midbrain dopaminergic neurons but no O_2-dependent changes in expression were detected. However, engrailed-1 was upregulated in lowered O_2, paralleling the immunohistochemical results (FIG. 6). Fibroblast growth factor 8b (FGF8b) message was dramatically upregulated in lowered O_2, by the end of the expansion phase. Messages for other regulators of dopaminergic differentiation did not differ significantly between O_2 conditions.

Example 4

Discussion: Lowered oxygen cultures favor proliferation and survival of CNS stem cells.

Standard conditions for the culture of mammalian cells are 37°C in a gas atmosphere of 5% CO_2 and 95% air. Thus ambient temperature is adjusted to reflect core mammalian body temperature and CO_2 is adjusted to reflect approximate venous concentrations, while in striking contrast, O_2 levels in culture are not adjusted to reflect physiologic levels. At sea level, unhumidified room air contains 21% O_2, and a 95% air/5% CO_2 mixture contains 20% O_2. Alveolar air contains 14% O_2, arterial O_2 concentration is 12%, venous O_2 levels are 5.3%, and mean tissue intracellular O_2

concentration is 3% (Guyton, and Hall, 1996). Directly relating to this study, mean brain O_2 in the adult rat and in fetal sheep have both been measured at 1.6% (Silver and Erecinska, 1988; Koos and Power, 1987). Physiological tissue O_2 levels in some brain regions are even lower (Table 1). In this work the impact of lowered, more physiologic oxygen levels on CNS stem cell culture was analyzed and showed four major effects: 1) increased proliferation of progenitors; 2) reduced apoptosis; 3) accelerated progression to differentiated states; and 4) elevated absolute number and proportion of TH+- neurons.

Lowered O_2 culturing consistently enhanced proliferation of CNS stem cells. A 2- to 4-fold increase in cell number was observed during the proliferation phase when most of the cells are nestin+ precursors. This increase in cell number was also maintained after mitogen withdrawal when proliferation was vastly reduced and differentiation takes place. Although more cells were present in differentiated cultures in lowered O_2, the proportions of neurons and glia were similar in the two culture conditions. In neural tissue, there is one supporting, though specialized, precedent for mitogenic activity of lowered O_2 in neural crest-derived carotid body chromaffin cells (Nurse and Vollmer, 1997). These dopaminergic glomus cells are functionally specialized O_2-sensitive chemoreceptors, and so would be expected to be specifically responsive to changes in O_2 levels in the artery. The present results show that lowered oxygen enhances the proliferation and survival of CNS stem cells.

Two specific signaling pathways, FGF8 and EPO, were identified as candidates for significant roles in the lowered O_2 response and showed that each can recapitulate part of the lowered O_2 phenotype at 20% O_2. Lowered O_2 culturing led to relative increases in RNAs encoding erythropoietin and FGF8. In early midbrain development, FGF8 functions as a mitogen (Danelian and McMahon 1996), but significant mitogenic or trophic effects of FGF8 on CNS stem cell cultures have not been reported. Here, the increased cell yield from mesencephalic precursors maintained in 20% O_2 and exposed to 250 ng/ml FGF8 partly recapitulated the proliferation/trophic effects of lowered O_2, with a 30% increase in total number compared to a 200-400% increase in lowered O_2. In addition to increased proliferation, less apoptosis occurs in CNS stem cells cultured in lowered O_2. There is

a potential toxic role for reactive oxygen intermediates (ROI) produced in room air cultures. However, it cannot simply be assumed that 20% O_2 cultures generate more oxidative stress than lowered O_2 cultures, since free radicals are generated in ischemic conditions (Perez Velazquez *et al.*, 1997).

In contrast to the increased cell number seen in lowered O_2, only minor effects were detected on the final ratio of neurons to astrocytes to oligodendrocytes that were derived from expanded striatal or mesencephalic precursors. This result together with clonal analysis confirms that the nestin+ precursors expanded in lowered oxygen have stem cell properties.

Example 5

Discussion: Dopaminergic commitment and differentiation.

There is a great deal of evidence that CNS stem cells can give rise to multiple neuron types (Johe *et al.*, 1996; Gritti *et al.*, 1996; Kalyani *et al.*, 1998). For several years the midbrain has been studied as model for neuron subtype specification (Hynes *et al.*, 1995;Ye *et al.*, 1998; Wang *et al.*, 1995; Ericson *et al.*, 1995; (reviewed in Hynes and Rosenthal, 1999). Recently, conditions have been established that allow midbrain precursor cells to proliferate and differentiate to dopaminergic neurons *in vitro* (Studer *et al.*, 1998). In contrast to primary rat fetal mesencephalic cultures where only 5% of the neurons are immunoreactive for TH, this number was increased to 24% of neurons in dissociated precursor cultures from E12 mesencephalon. Here it is shown that 56% of neurons generated from mesencephalic precursors are TH+, and this finding is associated with increased dopamine production by HPLC. Serotonergic neurons, another ventral neuron type found in this region of the brain, were also generated in increased numbers in lowered O_2. In contrast the number of GABAergic and Glutamatergic neurons were reduced. The lowered oxygen conditions were most effective in generating dopaminergic neurons during the phase of precursor cell expansion. These results suggest that lowered oxygen conditions enhance the production of ventral fates by a mechanism that acts prior to differentiation.

Transcript levels of FGF8 and En1, accepted mediators of midbrain dopaminergic neuron development (Ye *et al.*, 1998; Simone *et al.*, 1998; Shamim *et al.*, 1999), were upregulated in lowered vs. 20% O_2 cultures. FGF8 has also been

implicated in the commitment of serotonergic neurons (Ye *et al.*, 1998). These findings are consistent with a role for FGF8 in the expansion of dopaminergic and serotonergic neuronal subtypes seen in lowered O_2 cultures. However, addition of FGF8 to 20% O_2 cultures or neutralization of FGF8 in lowered O_2 cultures did not reproduce the O_2-dependent neuronal subtype differentiation patterns. The secreted morphogen sonic hedgehog (SHH) has been shown to induce dopaminergic neuron differentiation in explants of the early neural plate (Hynes *et al.*, 1995; Ye *et al.*, 1998; Wang *et al.*, 1995). Purified sonic hedgehog had no effect on expanded mesencephalic precursors under both oxygen conditions.

Engrailed-1 mRNA and protein levels were increased in lowered oxygen. Engrailed-1 is thought to act in a pathway with pax2, wnt-1 and FGF8 to regulate the fate of midbrain neurons (Joyner, 1996; Danelian and McMahon, 1996; Wurst *et al.*, 1994; Simone *et al.*, 1998). The FGF8 gene contains a binding site for engrailed (Gemel *et al.*, 1999). In addition it was found that the FGF8 5'-UTR sequence (accession #AF065607) contains a 9 base sequence (CCTCCCTCA) that is also known to control oxygen responsiveness in VEGF and EPO regulatory elements (Scandurro, and Beckman, 1998). The inventors have not yet determined if En1 acts as a direct upstream regulator of FGF8 in lowered O_2 cultures, or whether they act independently. Nonetheless, the prominent expression of En1 in young neurons (FIG. 6) suggests it may be a good candidate for regulating neuronal subtype differentiation.

EPO levels are known to be regulated by oxygen in the erythropoietic system. EPO and its receptor are expressed in brain from early development through adulthood (Juul *et al.*, 1999), but no specific role for EPO in CNS development has been described. In the adult CNS, however, EPO has received attention as a neuroprotective agent (Sakanaka *et al.*, 1998), and EPO treatment of PC12 cells has been demonstrated to increase intracellular monoamine levels (Masuda *et al.*, 1993). Here the results show that at 20% O_2, EPO can mimic part of the lowered O_2 effect. Increases in yield of dopaminergic neurons in 20% O_2 cultures was dose-dependent, but no additional increase in yield was mediated by EPO in lowered oxygen, suggesting that the EPO levels in lowered O_2 were at maximal functional levels for this response. Though EPO supplementation of 20% O_2 cultures significantly

improves dopaminergic yield, the full effect of lowered O_2 could not be recapitulated, suggesting that additional factors are involved in promoting dopaminergic differentiation in lowered O_2. Nonetheless, the finding that EPO affects the differentiation patterns of expanded CNS precursors is novel and identifies EPO as a component of increased dopaminergic neuron yield in lowered oxygen conditions.

A recent report highlighted increased dopamine content after differentiated dopaminergic mesencephalic neurons were exposed to hypoxic conditions (O% O_2 gas mixture) (Gross *et al.*, 1999). Another study described a relative increase in TH-expressing neurons in primary neuronal cultures from E14 rats after exposure to 5% O_2 (Colton *et al.*, 1995). It is also known that hypoxic conditions favor expression of the TH gene (Czyzyk-Krzeska *et al.*, 1994; Paulding, and Czyzyk-Krzeska, 1999). However, this is the first report that lowered oxygen conditions support CNS stem cells during the expansion phase and enhance the production of ventral neuronal subtypes.

Compared to 20% O_2 the net expansion of dopaminergic neurons in lowered O_2 was at least 9-fold increased (a three-fold increase in total cell numbers, and a 3-fold increase in the percentage of TH+-neurons). HPLC shows that these neurons produce dopamine. The present results show that oxygen levels much lower than those traditionally used in culture may be useful in mimicking *in vivo* phenomena. Lowered O_2 culturing has the practical implication of contributing to a more efficient production of dopaminergic neurons for transplant therapy in Parkinson's disease. Finally, effects of lowered, more physiological O_2 on cell cultures are not limited to the CNS, and extend to the PNS and to other non-neuronal tissues.

The effect of EPO on dopaminergic differentiation was studied. Saturating concentrations of EPO or EPO neutralizing antibody were added to E12 mesencephalic precursor cultures during both proliferation and differentiation phase (5 days each) in lowered or 20% O_2 using the conditions set forth in example 3. EPO was added to medium at a dose 5 U/ml for 5 days at the time or plating (isolation) or starting at the time of bFGF withdrawal. At the end of 10 days total time in culture, cells were fixed and stained for TH to mark dopaminergic neurons. The number of positive-staining cells were counted. EPO supplementation significantly increased TH+ cell numbers in 20% O2 cultures ($n=6$, $p < 0.05$).

The experiment was repeatedly with anti-EPO in place of EPO. The anti-EPO concentration was in a saturating concentration. The timing was the same as the EPO experiments, and they were stained and counted at the same 10 day time point. EPO neutralizing antibody decreased TH+ cell numbers in both lowered oxygen ($n=6$, $p < 0.01$) and 20% O2 cultures ($n=6$, $p < 0.05$). Figure 8 shows the results when EPO was added early. Similar results were obtained with the late addition of EPO.

Example 6

Results: Decreased oxygen culture promotes the survival, proliferation, and neuronal differentiation of NCSCs

NCSCs were isolated by flow-cytometry from the sciatic nerves of E14.5 rats and sorted into culture at clonal density under standard conditions (Morrison, *et al.*, 1999). Some cultures were kept in normal incubators containing 6% CO_2 and 20% O_2 (from air), while other cultures were kept in gas-tight chambers that were flushed with 1% O_2/6% CO_2/balance N_2 to generate an actual O_2 level of 5%. After 15 days the plates were stained with antibodies against peripherin (to detect neurons), glial fibrillary acidic protein (GFAP; to detect glia), and smooth muscle actin (SMA; to detect myofibroblasts). The results are presented in Table 3. The ability of $p75^+P_0$-cells to survive and form colonies was significantly greater in decreased oxygen: 35% of cells added to culture formed colonies in 20% oxygen versus 48% of cells in 5% oxygen ($p<0.01$).

Table 3: Culture of sciatic nerve $p75^{+}P_0$- cells in decreased oxygen promotes neural crest stem cell survival and multilineage differentiation.

	Plating Efficiency	frequency of colony types (%)				
	%	N+S+M	N+S	S+M	S only	M only
20% O_2	35.0±9.1	48.0±16.4	1.0±2.1	4.8±3.4	43.7±18.5	2.5±3.0
5% O_2	48.0±5.8*	82.8±12.0*	0.4±1.0	8.1±7.4	6.2±3.6*	2.5±3.1

$p75^{+}P_0$- cells were sorted into culture at clonal density and cultured for 15 days followed by immunohistochemistry. Plating efficiency indicates the percentage of cells sorted into culture that went on to form colonies detected 15 days later. N, S, and M mean that neurons, Schwann cells, and myofibroblasts respectively were observed within colonies. For example, N+S+M colonies contained neurons, Schwann cells and myofibroblasts. Data are presented as mean±standard deviation and were derived from 6 independent experiments in which an average of more than 70 colonies were counted per treatment per experiment. Results were compared by t tests and significantly different statistics are noted by * ($P<0.05$).

In addition to improved survival, $p75^{+}P_0$- cells were more likely to form multilineage colonies in 5% oxygen. In 20% oxygen only 48% of colonies were multilineage (containing neurons, Schwann cells, and myofibroblasts; N+S+M in Table 3), with the balance being mainly Schwann-only colonies (S-only, Table 3). In contrast, in 5% oxygen cultures significantly more colonies were multipotent (82%) and significantly fewer colonies were Schwann-only (6%). One possible explanation for this difference is that more than 80% of $p75^{+}P_0$- cells are multipotent but that multipotent progenitors sometimes give rise only to Schwann cells when cultured in 20% oxygen. The other possibility is that decreased oxygen levels are toxic to glial-committed progenitors such that their frequency is underestimated. The significantly higher plating efficiency in decreased oxygen argues against the latter possibility. Nonetheless this was tested by culturing E18.5 sciatic nerve cells in standard medium in either 5% or 20% oxygen. Multipotent progenitors have not been detected in the

sciatic nerve after E17.5, so most or all neural progenitors in the E18.5 nerve appear to be glial committed (Morrison, *et al.*, 1999; Jessen, *et al.*, 1999) . The frequencies of Schwann-only or myofibroblast-only colonies did not differ between 5% and 20% oxygen cultures: 23% of E18.5 sciatic nerve cells formed Schwann-only colonies, and 22% formed myofibroblast-only colonies irrespective of oxygen level (the remaining 55% of cells died without forming colonies). Consistent with this, decreased oxygen did not affect the overall plating efficiency in mass cultures of E14.5 sciatic nerve cells, of which NCSCs constituted only 15-20% (data not shown). These data support the idea that decreased oxygen culture promotes the survival and multilineage differentiation of NCSCs, without biasing against the survival of more restricted progenitors. The $p75^{+}P_0$- population appears to contain more than 80% NCSCs.

To test whether $p75^{+}P_0$- cells self-renew in decreased oxygen culture a single cell was deposited per well of a 96 well plate then seven colonies were subcloned after 7 days. All founder clones gave rise to multipotent subclones, averaging 158±149 multipotent subclones per founder, demonstrating that individual $p75^{+}P_0$- cells self-renew in decreased oxygen as was previously documented in 20% oxygen (Morrison, *et al.*, 1999).

Decreased oxygen culture also promoted the proliferation of NCSCs. As described previously (Morrison, *et al.*, 1999), multipotent colonies can be distinguished from other colony types based on their appearance. After 6 days of culture colonies predicted to be multipotent contained 289±237 cells in 20% oxygen (n=9) or 589±156 cells in 5% oxygen (n=6). This difference in colony size (p=0.018) must have been due to a difference in proliferation since after only 6 days of culture few dead cells were observed in the colonies at either oxygen level: 7.1±5.6 cells per colony in 20% oxygen or 17.5±7.0 cells per colony in 5% oxygen appeared dead by morphology. Thus in decreased oxygen NCSC clones proliferated to a significantly greater extent forming colonies that contained around twice as many cells by the sixth day of culture.

Under standard culture conditions neurons are almost never observed in colonies that do not also contain Schwann cells and myofibroblasts (Table 3; (Morrison, *et al.*, 1999). Thus neuronal potential is associated with NCSC activity. BMP2 instructs NCSCs to differentiate into neurons (Shah, *et al.*, 1996; Morrison, *et*

al., 1999). As an independent test of neuronal potential BMP2 was added to cultures of $p75^{+}P_0$- cells in either 5% or 20% oxygen. After 4 to 6 days, the cultures were stained for peripherin to analyze the extent of neuronal differentiation. The results are shown in Table 4.

Table 4: BMP2 instructed neuronal differentiation of sciatic nerve $p75^+P_0$- cells is promoted by culture in decreased oxygen.

		Plating	types of colonies (%)		
		Efficiency (%)	all neurons	some neurons	no neurons
no add	20%	22.7±8.5	0.0±0.0	0.0±0.0	100.0±0.0
	5%	33.4±7.3*	0.0±0.0	0.4±0.9	99.6±0.9
+ BMP2	20%	26.8±8.9	31.5±17.9	52.5±18.9	15.9±5.7
	5%	43.4±11.5*	59.5±10.8*	35.0±11.1	5.5±1.4

$p75^+P_0$- cells were sorted into cultures at clonal density and cultured for 5 or 6 days followed by staining for peripherin, a marker of peripheral neurons. Plating efficiency indicates the percentage of cells initially sorted into culture that went on to form colonies. Data are presented as mean±standard deviation for 5 independent experiments. Results at different oxygen concentrations (within no add or +BMP2 treatments) were compared by t tests and significantly different statistics are noted by * ($P<0.05$). Decreased oxygen culture significantly increased plating efficiency in both treatments and neuronal differentiation in the presence of BMP2.

As seen previously, a higher proportion of $p75^+P_0$- cells survived and formed colonies in decreased oxygen, irrespective of whether BMP2 was added. Almost no colonies contained $peripherin^+$ cells in the cultures that did not receive BMP2 (peripherin expression is normally not evident for at least 12 days under standard culture conditions). However, among cultures to which BMP2 was added, decreased oxygen was associated with a significantly higher proportion of colonies that contained only neurons and a significantly lower proportion of colonies that contained no neurons. In decreased oxygen nearly 60% of colonies contained only neurons and almost 95% of colonies contained at least some neurons. Thus, neuronal differentiation was significantly promoted in decreased oxygen culture and up to 95% of $p75^+P_0$- cells may be NCSCs.

Example 7

Results: Decreased oxygen culture reveals that virtually all NCSCs isolated from sciatic nerve have SA lineage potential

$p75^+P_0$- cells were added to culture at clonal density in standard conditions in either 5% of 20% oxygen. After 6 days of culture, 5 μM forskolin and 1ng/ml BMP2 were added to some cultures. Following a total of 12 days in culture, colonies were stained for three markers of sympathetic lineage differentiation: TH, DBH, and SA-1 a marker of chromaffin cells and sympathoadrenal progenitors (Carnahan, *et al.*, 1991). In both standard cultures and cultures supplemented with forskolin and BMP2, decreased oxygen significantly increased the proportion of colonies that contained cells expressing TH, DBH, or SA-1 (FIG. 9). In decreased oxygen cultures supplemented with forskolin and BMP2 nearly all stem cell colonies contained TH^+ cells, DBH^+ cells and $SA\text{-}1^+$ cells (FIG. 9). In addition to increasing the proportion of colonies expressing sympathetic markers, decreased oxygen and/or the addition of forskolin and BMP2 also tended to increase the proportion of cells within colonies expressing the markers and the intensity of marker expression. There was an induction of TH expression by low oxygen culture in BMP2 plus forskolin as monitored by epifluorescence illumination of anti-TH antibody staining. There was no TH expression was seen in cultures grown in 20% oxygen without BMP2 or forskolin in this experiment. In general, only rare TH^+ cells were observed in ambient oxygen cultures.

In cultures grown in BMP2 plus forskolin in 5% O_2, cells often co-expressed TH and DBH, or TH and SA-1. In colonies double stained for TH and DBH, many TH^+ cells were also DBH^+ (and vice versa) though there were also subsets of TH^+DBH^- and TH^-DBH^+ cells. In colonies double labeled for TH and SA-1, all or nearly all $SA\text{-}1^+$ cells were TH^+ and many TH^+ cells were also $SA\text{-}1^+$, but some were $SA\text{-}1^-$. The cultures were grown in low oxygen plus 1 ng/ml BMP2 plus 5 μM forskolin and were double-labeled with anti-TH and anti-DBH or anti-SA1. Because the antibodies against SA-1 and DBH are of the same isotype it was not possible to double stain for those markers. These data demonstrate that virtually all NCSCs isolated from E14.5 sciatic nerve and cultured under these conditions have SA lineage potential.

Example 8

Results: TH^+ cells derived from NCSCs co-express neuronal markers and release dopamine and norepinephrine

To promote overt neuronal differentiation by these cells, they were cultured $p75^+P_0$- cells in decreased oxygen at clonal density for 6 days in standard medium, followed by 6 days supplemented with forskolin and low BMP2, followed by a final 6 days supplemented by 50ng/ml each of NGF and NT-3 (which have been shown to promote the differentiation and survival of sympathetic neuroblasts in vitro (Birren, *et al.*, 1993; Verdi, *et al.*, 1994). Under these conditions, 3.6±2.5% of cells in each colony were TH^+. These stem cell colonies contain more than 10^5 cells (Morrison, *et al.*, 1999), so individual $p75^+P_0$- cells gave rise to thousands of SA-lineage cells.

Double-labeling of cultures grown under these conditions with antibodies to TH and neurofilament middle molecular weight subunit (NFM) revealed that the colonies contained three TH^+ cell types that were distinct with respect to morphology and NFM staining. One type displayed a polygonal morphology with small or absent neurites, very strong TH staining, and low NFM staining, resembling SIF or chromaffin-like cells (Doupe, *et al.*, 1985) and references therein). There were also polygonal cells with longer varicose processes that had very strong TH staining and intermediate levels of NFM staining, appearing intermediate in phenotype between SIF cells and sympathetic neurons. Finally, other cells had round cell bodies, long neurites, a lower level of TH staining, and strong NFM staining, resembling immature sympathetic neurons. Of 29 NCSC colonies cultured under neuron-promoting conditions and then double labeled for TH and NFM, all contained cells resembling SIF cells and sympathetic neurons.

HPLC analysis was used to determine whether colonies derived from individual NCSCs that contained TH^+ and DBH^+ cells also produced both DA and NE. Single $p75^+P_0$- cells were sorted into individual wells of 96 well plates and then cultured under sympathetic neuron-promoting conditions. Some colonies were trypsinized, resuspended in 0.2M perchloric acid, and analyzed for DA and NE content by HPLC. DA and NE peaks were identified by retention times that were similar to authentic standards (<0.1 min). 3/3 colonies contained DA and 2/3 contained NE; the DA:NE molar ratio in colonies that contained both was

approximately 1:1. Other colonies were depolarized by incubation for 5 minutes in 40mM KCl in HBSS to induce transmitter release, then the supernatants and the cells were analyzed by HPLC. Five of 8 such supernatants contained DA and 6 of 8 contained NE; in three of the 5 samples containing both transmitters the ratio was close to 1:3 (DA:NE). In cells extracted after depolarization, 4/4 contained some residual DA but none contained detectable NE. These data are consistent with the fact that DA is both vesicular and cytoplasmic, whereas NE is exclusively vesicular in catecholaminergic cells.

The neurotransmitter phenotype of the neurons generated by NCSCs that did not express SA markers was of particular interest. It is possible that these cells are cholinergic, given the close developmental relationship between the sympathetic and parasympathetic (cholinergic) lineages. Most of the neurons that developed in NCSC colonies grown under standard conditions were stained by a commercial polyclonal antibody to vesicular acetylcholine transporter (VAChT). Although the staining was weak and could only be detected by the immunoperoxidase method, it had the appropriate punctate vesicular localization. Attempts to perform double-labeling of cultures containing SA lineage cells with antibodies to TH and VAChT were unsuccessful due to the low level of expression of the latter antigen, although expression of VAChT in such cultures could be detected by immunoperoxidase single labeling (not shown). Nevertheless, the fact that all colonies contained $VAChT^+$ neurons under standard conditions, while almost all colonies switched from standard conditions to BMP2 plus forskolin contained TH^+ cells (FIG. 9), indicates that isolated NCSCs have both parasympathetic and SA lineage capacities.

The finding that decreased oxygen culture can promote the production of greatly expanded numbers of dopaminergic neurons from individual, purified stem cells is an important tool to facilitate transplantation into Parkinson's patients. Under the culture conditions presented, individual NCSCs gave rise to an average of more than 3000 TH^+ cells per colony. This was without any special effort to promote stem cell expansion or to optimize sympathetic differentiation. Thus, modest efforts in those regards as known to the skilled artisan could yield additional substantial increases in the numbers of dopaminergic cells. The ability to generate thousands or

millions of dopaminergic cells from a single stem cell provides both a defined precursor cell and adequate dopaminergic cells for therapeutic transplantation.

Example 9

Low Oxygen Culturing Of Skeletal Muscle Progenitor Cells

In adult skeletal muscle, the progenitor cell is referred to as a satellite cell. Normally, satellite cells are dormant, but when muscle is traumatized, these cells divide and differentiate, and so are the source of regenerated skeletal muscle. As disclosed herein, subatmospheric oxygen culture conditions (1% oxygen) significantly increase the number of dividing satellite cells associated with myofibers over the first few days of culture.

Using BrdU labeling and morphologic criteria, the number of satellites per unit length of muscle were quantified in 12 hour intervals. (The unit length of fiber was a 20X power diameter microscope field.) At each interval for the first few days, the number of satellites dividing under hypoxic conditions was about twice that under traditional room air cultures.

Table 5. Quantification of the number of satellites per unit length of muscle in 12 hour intervals.

Hours of assay	mean # BrdU positive satellites/ unit length (mean ± S.E.)	
	21% oxygen	1% oxygen
0-12	0	rare
12-24	0.7±0.1	1.6±0.2
24-36	0.7±0.2	2.1±0.4
36-48	3.7±0.6	5.6±0.9
48-60	2.5±0.5	6.3±1.0

Methods of isolating, identifying, culturing and differentiating satellite cells are well known to those of skill in the art. For example, in U.S. Patent No. 5,328,695, Lucas *et al.* describe a myogenic protein isolate from mammalian bone that stimulates lineage commitment and differentiation of skeletal muscle stem cells. Primary cultures of muscle progenitor cells were obtained from chicken embryos, cultured and caused to differentiate *in vitro*. The inventors contemplate that the hypoxic culturing

conditions they describe herein, used in conjunction with the methods of U.S. Patent No. 5,328,695, will further increase the isolation, activation, and differentiation of such stem cells as well as satellites derived from mammalian systems.

Cornelison and Wold (1997) isolated satellite cells from adult murine skeletal muscle and characterized the expression of certain genes in quiescent and activated satellite cells. Traditionally, quiescent satellite cells have been hard to distinguish from contaminating fibroblasts because there were no known molecular markers that could be used to distinguish the two. Using a single-cell reverse transcriptase-polymerase chain reaction (RT-PCR) technique, Cornelison and Wold (1997) demonstrated that c-met is expressed in quiescent satellite cells but not in muscle-derived fibroblasts or other mononucleated cells from healthy muscle explants. Furthermore, c-met was expressed throughout activation, proliferation, and differentiation of satellite cells. Therefore, c-met may be used as a molecular marker to detect satellite cells and cells differentiated therefrom (Cornelison & Wold, 1997).

Using the single-cell RT-PCR technique, Cornelison and Wold (1997) went on to show that, upon activation, the satellite cells showed a distinct progression of MyoD family regulators of muscle determination and differentiation (mrf's) gene expression. Activated satellite cells began to express either c-met and MyoD or c-met and myf5 first among the mrf's (Cornelison & Wold, 1997). Most cells then expressed c-met and both myf5 and MyoD simultaneously (Cornelison & Wold, 1997). This state was followed by one in which the cells expressed c-met, myf5, MyoD, and myogenin (Cornelison & Wold, 1997). Although rare cells later expressed c-met and myogenin, others expressed all the MRFs of the previous state plus MRF4 (Cornelison & Wold, 1997). In the next state, myf5 and MyoD expression is turned off leaving expression of c-met, myogenin and MRF4 (Cornelison & Wold, 1997). Thus, whereas quiescent satellite cells may be determined by c-met expression, activation and differentiation of satellite cells may be determined by the expression of c-met, myf5, MyoD, myogenin, and mrf4.

The inventors showed, in particular, that expression of mrf group muscle transcriptional factors is accelerated when skeletal muscle fibers are cultured under 1% oxygen levels. For example, myogenin and mrf4 are not detected in satellites when cultured under normal laboratory oxygen (21%) conditions for 24 hours.

However, some satellites cultured with 1% oxygen express mrf4 and some express myogenin after just 24 hours of culture. In addition, a greater percentage of satellites cultured under 1% oxygen conditions express MyoD at 24 hours when compared to the normal culture conditions. This is an example of changes in the timing of differentiation genetic pathways as a consequence of low oxygen culturing conditions.

Differentiated skeletal muscle has a distinctive appearance: myotubes are large fused cells with multiple nuclei aligned coordinately. The myofiber has distinctive, patterned striations. These features are used to define the appearance of newly regenerated muscle from satellites in culture. In addition, specific proteins, such as myosin heavy chain, are expressed by the differentiated fused myotube and are detected using antibody staining.

The methods of this present invention may be utilized to produce both slow- and fast-twitch myofibers (Cornelison & Wold, 1997). Regardless of muscle fiber type, in another embodiment, the muscle produced by the methods of the present invention are used to produce muscle satellites and/or fibers for the purpose of clinical transplantation. In theory satellites and or their progeny could be transplanted to treat muscular diseases such as the muscular dystrophies, or atrophy due to trauma, nerve damage, or disuse. For muscular dystrophies, the satellites and progeny would provide missing gene product (dystrophin, for example) necessary for normal muscle strength. In other cases, missing normal muscle mass would be regenerated from transplanted satellites and progeny. Satellite cells activated under hypoxic conditions may be transplanted into a patient without further differentiation. This protocol may be particularly useful in patients who lack functional satellites to activate because they lack muscle mass. The protocol may also be useful in patients who have satellites that do not regenerate normal muscle (muscular dystrophies), if the donor cells express corrective gene product.

Obtaining a population of mammalian muscle cells. To create single muscle fiber cultures, 100-200 day old female B6D2F1 mice are euthanized (with CO_2). The back legs are skinned, amputated at the hip, and the feet removed. The legs are placed into DMEM without phenol red or sodium pyruvate, containing high glucose, L-glutamine, 25 mM Hepes buffer, with pyridoxine hydrochloride (this is a commercial preparation from GibcoBRL, catalog no. 21063-029) at room

temperature. The leg muscles are dissected out individually using a dissecting microscope, and teased into small pieces, then placed into 400 U/ml collagenase type I (Worthington Biochemical) in the DMEM formulation above, at 34°C for 45 min. After digestion, the muscle is placed into a 10-cm plastic dish, and individual live fibers are picked using a protein-coated Pasteur pipet (with a hand-polished tip) into the following medium: DMEM with phenol red + 5% chick embryo extract + 10% horse serum + antibiotics/antimycotics + L-glutamine + 25 mM Hepes, prewarmed to 34°C incubator with 5% C)2 for 15-30 min. The procedure is repeated with a finer polished tip Pasteur pipet, placing picked fibers into the same Hepes-buffered DMEM medium. A third pick of fibers is performed, this time placing the fibers into DMEM with phenol red+5% chick embryo extract + 10% horse serum + antibiotic/antimyocotic + L-glutamine, but without added Hepes (growth medium). Then the fibers are placed either in traditional incubators as above, or in a hypoxia chamber (Billups-Rothenberg, San Diego CA) flushed with the appropriate gas mixture which is then placed in the incubator. Hypoxic cultures are maintained in the chamber except during feeding, and feeding time is minimized as much as possible. The chambers are flushed daily.

The above protocol differs from that previously described (Cornelison & Wold, 1997). Significantly, (1) the fibers are dissected in a buffered medium solution rather than in PBS, (2) the temperature at which the collagenase digestion is performed was reduced, (3) Hepes buffered medium is used while the fibers are out of the incubator and cannot be buffered by CO_2.

Enriching muscle progenitor cells in population. After isolation of single skeletal muscle fibers for culture, half the fibers from one mouse are placed into a 10-cm plastic tissue culture plate containing fiber growth medium, and put into a sealed chamber flushed with 1% oxygen, 5% carbon dioxide and 94% nitrogen. The chamber, containing an open Petri dish with sterile water, is placed into an incubator maintained at 37°C. The remaining half of the fibers (for comparison purposes) are placed in the same incubator, which has carbon dioxide maintained at 5% in room air. After 12 hours, the fibers are placed in fresh, pre-warmed growth medium under a dissecting microscope, then returned immediately to their previous culture conditions.

An aliquot of fibers is removed periodically for analysis. Except during necessary cell manipulations, 1% oxygen is maintained in the low-oxygen cultures at all times.

Differentiating muscle progenitor cells. After 4-7 days in culture as above, the fibers are placed into pre-warmed medium of DMEM + 2% horse serum + penicillin/streptomycin/antimycotic + L-glutamine, then returned to the hypoxia chamber (or control cells to normal culture conditions). The medium is changed one time per week. After several days, differentiating myotubes appear both on the live and dead floating muscle fibers and attached to the bottom of the tissue culture plate. From both locations they can be picked singly using a patch clamp apparatus for analysis of messenger RNA expression patterns, or stained for specific protein products.

Example 10

Low Oxygen Culturing Of Skin Cells

In another embodiment, the hypoxic culturing methods of the present invention may be used in the culturing of keratinocytes (progenitor cells for skin). Methods of isolating, culturing, and differentiating keratinocytes are well known to those of skill in the art (Jones *et al.*, 1995; Di Cunto *et al.*, 1998). Although keratin 19 is widely considered to be the best available overall marker for keratinocytes (Michel *et al.*, 1996), in preferred embodiments the progenitor cells are distinguished by morphologic criteria because a reliable antibody is not available. Hypoxic/physiologic oxygen conditions can be used from the time of isolation of both fetal and post-natal keratinocytes, in order to facilitate isolation, expansion, and differentiation of these cells.

The skin cells produced by the methods of the present invention are used to produce skin progenitors or tissue for the purpose of clinical transplantation. Transplantation may be used to treat skin injuries such as delayed wound healing, thermal or chemical burns, or severe allergic reactions, or after massive resection of skin for malignancy.

Example 11

Low Oxygen Culturing Of Embryonic Stem Cells

Embryonic stem cells have been used widely in the generation of experimental gene knockout mice. The isolation of embryonic stem cells from human tissue has also been reported (Thomson, 1998). However, as disclosed herein, oxygen concentration has a significant effect on proliferation and differentiation of developmentally primitive cells. Therefore, variation of ambient oxygen in the culture conditions for embryonic stem cells may be an important parameter in the development of specific tissue from these lines for therapeutic purposes. Methods of varying oxygen concentration and assaying for the effect of physiologic/ subatmospheric oxygen culture conditions are disclosed herein.

Example 12

Manipulation of Pluripotent Stem Cells

When a particular stem or progenitor cell is capable of differentiating into a number of developmentally distinct cell, tissue, or organ types, the cell is said to be pluripotent. By incubating a pluripotent cell line under hypoxic conditions *in vitro*, the inventors were able to manipulate or skew the direction of differentiation of the cell population.

The result of this technique is essentially an enrichment of one or more cell type but this may also be considered a selection against other cell types. In this example, growth of the cells under hypoxic conditions enriched for muscle cells and, at the same time, selected against fat cells. The inventors contemplate that oxygen concentrations may be manipulated to direct differentiation of other pluripotent stem or progenitor cells as well.

Specifically, the mouse cell line 10T1/2 cells can be pharmacologically (5-azacytidine) induced to generate cartilage, skeletal muscle and fat (adipocytes) (Taylor & Jones, 1979). Previous work subjecting these cells to 2% oxygen suggested that the numbers of skeletal muscle cells generated was increased by low oxygen conditions (Storch TG, 1996). The present inventors have shown that culturing of 10T1/2 cells under 21% oxygen conditions in conjunction with 5-azacytidine treatment results in skeletal muscle and adipocyte differentiation within 2 weeks. Notably, adipocyte differentiation in this cell line is blocked under 1% oxygen conditions in the presence (or absence) of azacytidine. This is an example of

physiologic/subatmospheric oxygen conditions playing a role in the precise differentiation fate taken by pluripotent cells. The identification of gene networks up- or down-regulated by the change in oxygen environment could, in turn, provide pharmacologic targets for enhancement or blocking of specific differentiation pathways, in this case fat development.

* * *

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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U.S. Patent 5,328,688
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U.S. Patent 5,521,061
U.S. Patent 5,538,869
U.S. Patent 5,585,362
U.S. Patent 5,612,205
U.S. Patent 5,612,205
U.S. Patent 5,620,689
U.S. Patent 5,622,856
U.S. Patent 5,631,018
U.S Patent 5,650,148
U.S. Patent 5,658,776
U.S. Patent 5,661,025
U.S. Patent 5,661,033
U.S. Patent 5,665,540
U.S. Patent 5,670,488
U.S. Patent 5,686,278
U.S. Patent 5,693,509
U.S. Patent 5,707,618
U.S. Patent 5,714,166
U.S. Patent 5,721,367
U.S. Patent 5,749,847
U.S. Patent 5,750,376
U.S Patent 5,762,926
U.S. Patent 5,770,414
U.S. Patent 5,773,289
U.S. Patent 5,785,987

U.S. Patent 5,789,213

U.S. Patent 5,789,215

U.S. Patent 5,789,390

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U.S. Patent 5,824,547

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U.S. Patent 5,830,725

U.S. Patent 5,830,727

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CLAIMS

1. A method of culturing at least one neural crest stem cell (NCSC) to enhance survival, proliferation or differentiation of said NCSC, said method comprising culturing said NCSC in low ambient oxygen conditions, wherein said survival, proliferation or differentiation is enhanced compared to a control NCSC cultured in ambient oxygen conditions.

2. The method of claim 1, wherein survival is enhanced.

3. The method of claim 1, wherein proliferation is enhanced.

4. The method of claim 1, wherein differentiation is enhanced.

5. The method of claim 1, wherein survival, proliferation and differentiation are enhanced.

6. The method of claim 1, wherein differentiation is to a neuron.

7. The method of claim 1, wherein differentiation is to a cell of sympathoadrenal lineage.

8. The method of claim 1, wherein differentiation is to a cell of cholinergic lineage.

9. The method of claim 1, wherein differentiation is to a sensory neuron.

10. The method of claim 1, wherein differentiation is to a phenotype which produces dopamine.

11. The method of claim 1, wherein the differentiation is to a phenotype which produces norepinephrine.

12. The method of claim 1, wherein the differentiation is to a phenotype which produces dopamine and norepinephrine.

13. The method of claim 1, wherein the differentiation is to adrenal chromaffin cells.

14. The method of claim 1, wherein the differentiation is to small intensely fluorescent cells.

15. The method of claim 1, wherein the differentiation is to sympathetic neurons.

16. The method of claim 1, wherein said neural crest cell is a single isolated cell.

17. The method of claim 1, wherein said differentiation is to a phenotype which produces an neurotransmitter wherein deficiency is said neurotransmitter is associated with a neurodegenerative disease.

18. A method of preparing cells for use against a neurodegenerative disease associated with a deficiency in a neurotransmitter, said method comprising culturing at least one neural crest cell in low ambient oxygen conditions to enhance differentiation of said neural crest cell(s) to form cells which produce a neurotransmitter associated with a neurodegenerative disease.

19. The method of claim 18, wherein said neurodegenerative disease is Parkinson's disease.

20. The method of claim 19, wherein said neurodegenerative disease is Alzheimer's disease.

21. A method of culturing at least one undifferentiated cell to form a cell which produces dopamine and norepinephrine, said method comprising culturing

said cell(s) in low ambient oxygen conditions to form a cell which produces dopamine and norepinephrine.

22. A method culturing stem cells or progenitor cells, comprising:

a) obtaining a population of mammalian cells capable of forming a solid tissue containing at least one stem cell or progenitor cell;
b) culturing the population of mammalian cells in suitable medium with the oxygen level selected to maintain or enhance the survival of the stem or progenitor cell or cell type; and
c) culturing the population of stem cells or progenitor cells for a time sufficient to maintain or enhance the survival of the stem cell or progenitor cell.

23. A method culturing stem cells or progenitor cells, comprising:

a) obtaining a population of mammalian cells capable of forming a solid tissue containing at least one stem cell or progenitor cell;
b) culturing the population of mammalian cells in suitable medium with the oxygen level selected to maintain or enhance the proliferation of the stem or progenitor cell; and
c) culturing the population of stem cells or progenitor cells for a time sufficient to maintain or enhance the proliferation of the stem or progenitor cell.

24. A method of enriching stem cells or progenitor cells relative to one or more other cell types in a population of mammalian cells capable of forming a solid tissue comprising at least one stem cell or progenitor cell and one or more other cell types, comprising:

a) obtaining the population of mammalian cells containing at least one stem cell or progenitor cell and one or more other cell types; and

b) culturing the population of mammalian cells in suitable medium under subatmospheric oxygen conditions for a time sufficient to enrich stem cells or progenitor cells in the population relative to one or more other cell types in the population.

25. A method of identifying a stem or progenitor cell in a population of cells, comprising:

a) obtaining the population of mammalian cells capable for forming a solid tissue containing at least one stem cell or progenitor cell and other cell types;

b) culturing the population of mammalian cells in suitable medium under subatmospheric conditions; and

c) identifying stem cells or progenitor cells in the population.

26. A method of accelerating differentiation of a stem cell or a progenitor cell and/or the progeny of the stem or progenitor cell capable of forming a solid tissue, comprising culturing a stem or progenitor cell in suitable medium under subatmospheric oxygen conditions for a time sufficient to accelerate the developmental progression and/or differentiation of the stem cell or progenitor cell or its progeny into one or more types of cell that are more advanced in a developmental pathway or are more differentiated.

27. A method of enhancing production of desired differentiated cells or cells at a more advanced stage in a developmental progression than are the starting stem cells or progenitor cells derived from a solid tissue, comprising:

a) first culturing with subatmospheric oxygen conditions to expand the stem or progenitor cell population; and

b) then culturing the expanded population under subatmospheric oxygen conditions to encourage differentiation.

28. A method of manipulating a differentiation pathway of a stem or progenitor cell, comprising the step of:

a) culturing a stem cell or a progenitor cell in suitable medium under subatmospheric oxygen conditions selected to obtain differentiated progeny, such that the percentage of at least one type of differentiated cell produced is increased relative to the percentage of at least one other type of differentiated cell which could ultimately be produced; and

b) optionally culturing the differentiated progeny in suitable medium under subatmospheric oxygen conditions, such that the differentiation progeny of step a) are further differentiated.

29. The method of claim 21, 22, 23, 24, 25, 26, 27, or 28, wherein the stem cell or progenitor cell is derived from skeletal muscle, neural tissue, bone, skin, liver, bone marrow, smooth muscle, or embryonic stem cells.

30. The method of claim 29, wherein the stem cell or progenitor cell is derived from skeletal muscle, skin or neural tissue.

31. The method of claim 21, 22, 23, 24, 25, 26, 27, or 28, further comprising the step of transfecting the stem cell or progenitor cell with an expression vector comprising a DNA operably linked to a promoter.

32. The method of claim 21, 22, 23, 24, 25, 26, 27, or 28, further comprising the step of transfecting the stem cell or progenitor cell with an expression vector comprising a DNA or other synthetic or natural nucleic acid.

33. A method for screening for a compound which affects the survival, proliferation or differentiation and/or regeneration of a stem cell or progenitor cell, comprising:

a) contacting a population of stem cells or progenitor cells capable of forming a solid tissue with the compound under subatmospheric conditions, and

b) monitoring the various cell populations for survival, proliferation and/or differentiation.

34. The method of claim 26, which further comprises culturing the differentiated progeny in suitable medium under subatmospheric oxygen conditions, such that the differentiation progeny of step a) are further differentiated.

35. The method of claim 26, wherein the culturing is performed for at least 12 hours.

36 The method of claim 26, wherein the culturing is performed in an atmosphere containing 0-12 % oxygen.

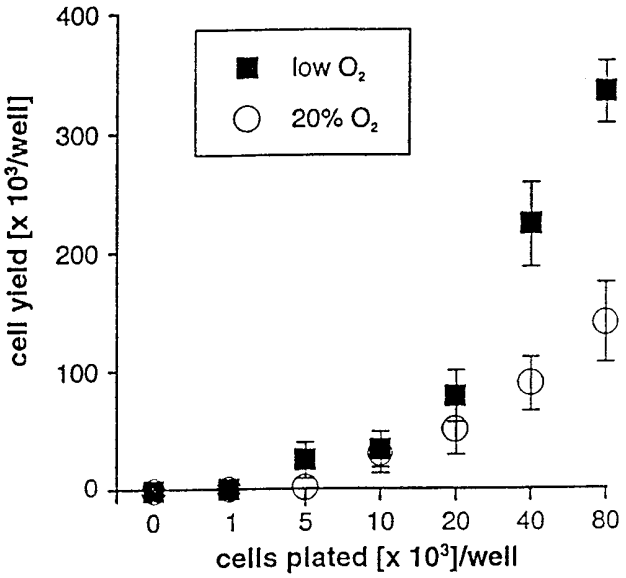
400
300
200
100
0
cell yield [x 10³/well]
low O₂
20% O₂
0
1
5
10
20
40
80
cells plated [x 10³]/well

FIGURE 1

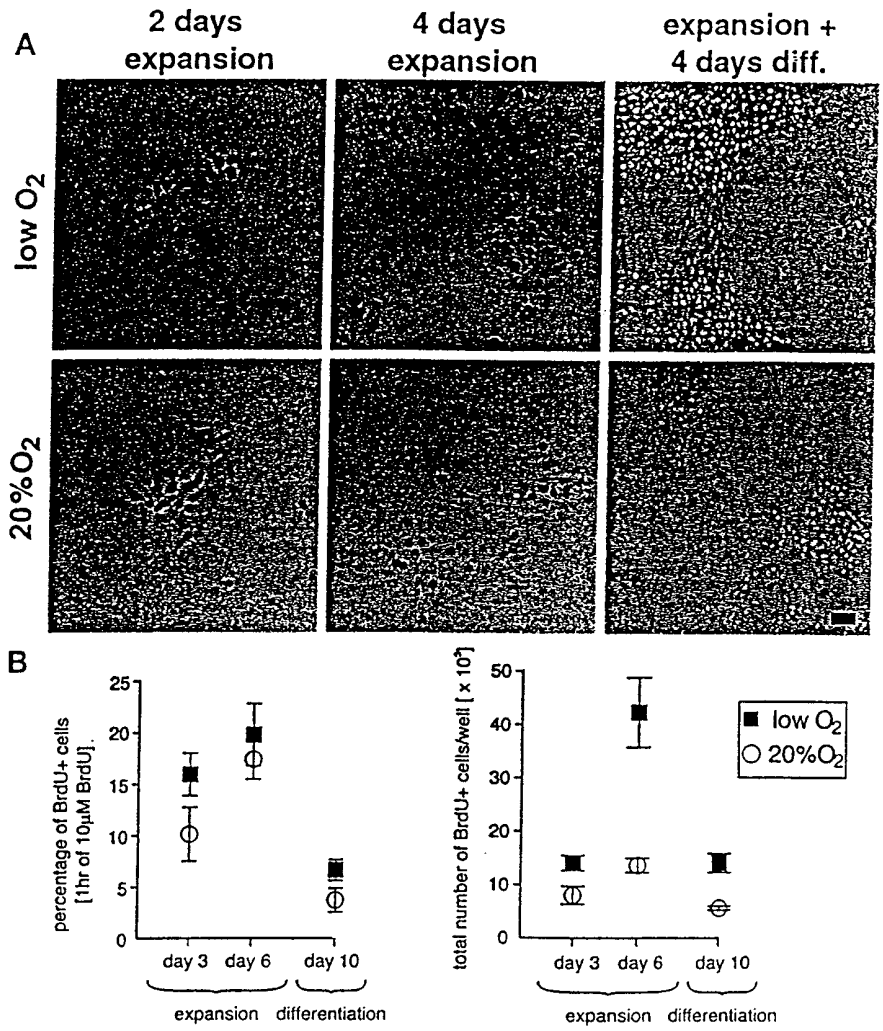
A
2 days expansion
4 days expansion
expansion + 4 days diff.
low O2
20%O2
B
percentage of BrdU+ cells [1hr of 10μM BrdU].
day 3
day 6
day 10
expansion
differentiation
total number of BrdU+ cells/well [x 10³]
low O2
20%O2

FIGURE 2

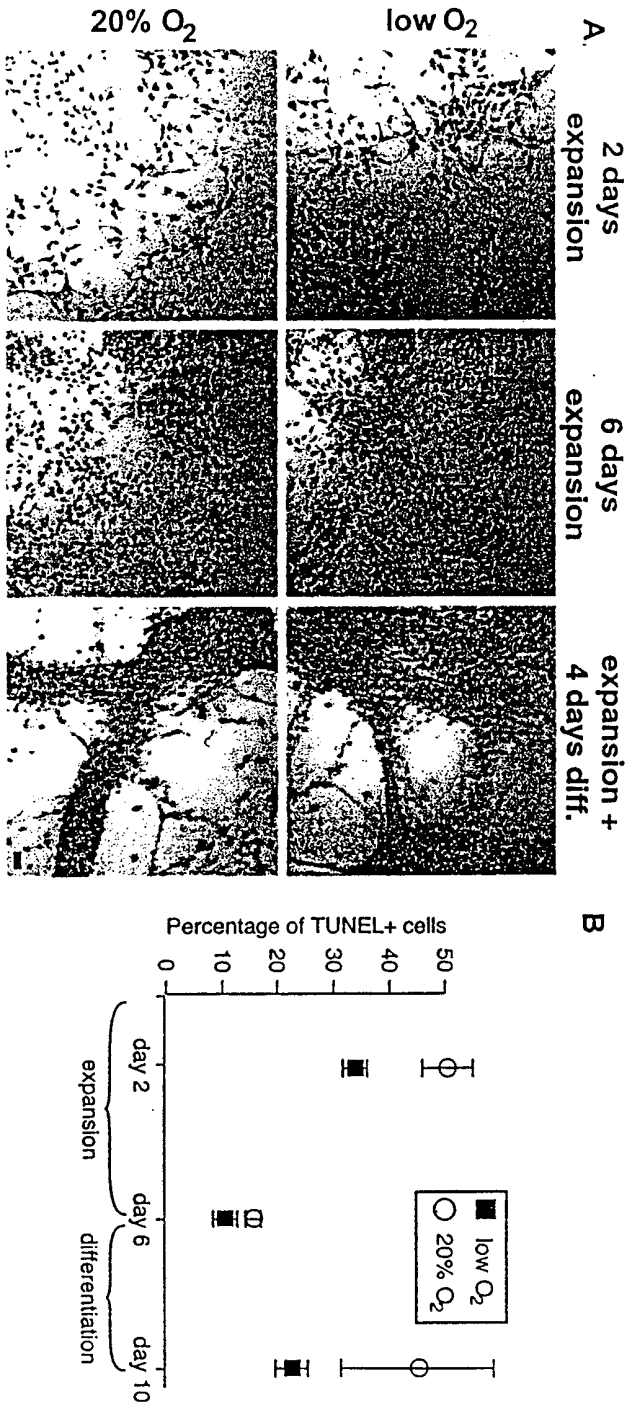
20% O2
low O2
A
2 days expansion
6 days expansion
expansion + 4 days diff.
B
Percentage of TUNEL+ cells
0
10
20
30
40
50
day 2
day 6
day 10
expansion
differentiation
low O2
20% O2

FIGURE 3

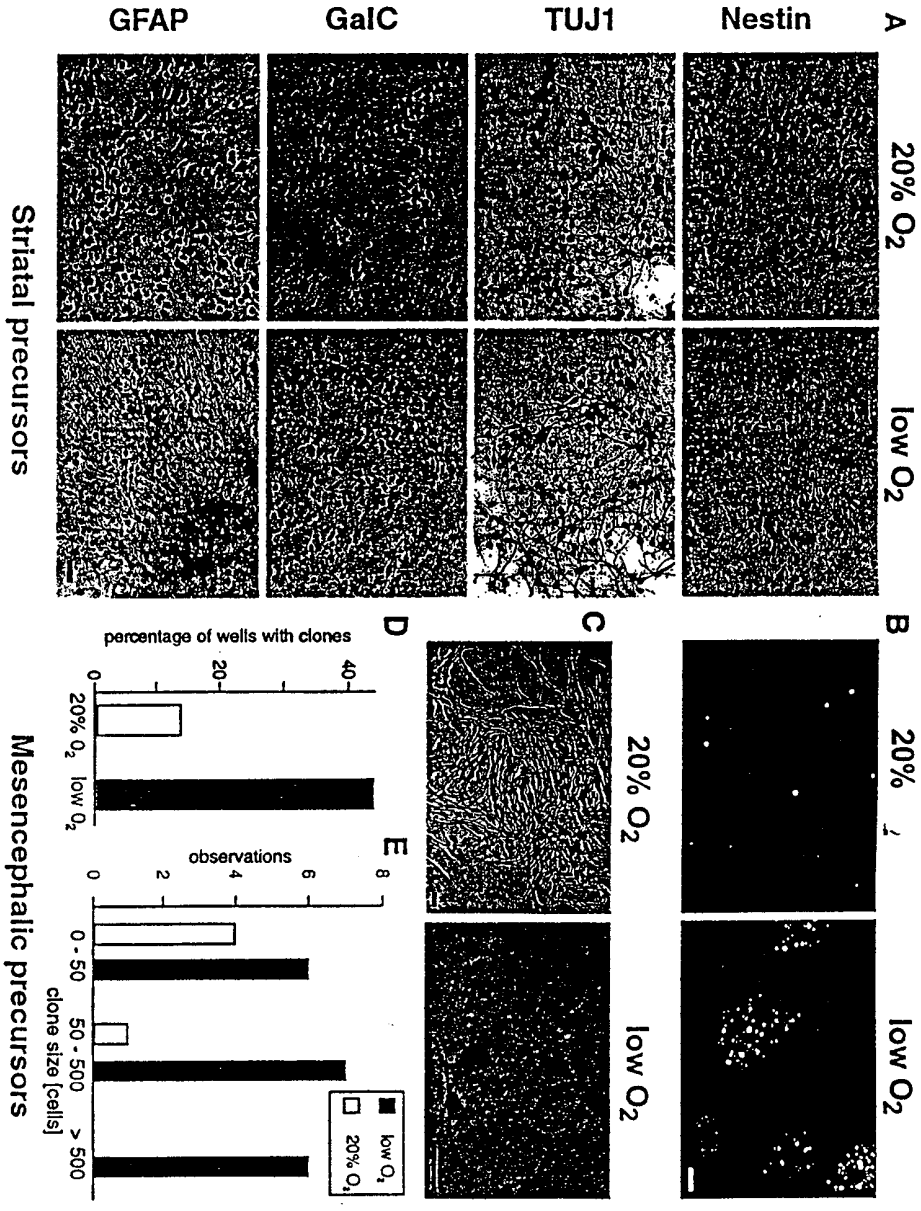
GFAP
GalC
TUJ1
Nestin
A
20% O2
low O2
Striatal precursors
Mesencephalic precursors
B
C
D
E
percentage of wells with clones
0
20
40
20% O2
low O2
observations
0
2
4
6
8
0 - 50
50 - 500
> 500
clone size [cells]
low O2
20% O2

FIGURE 4

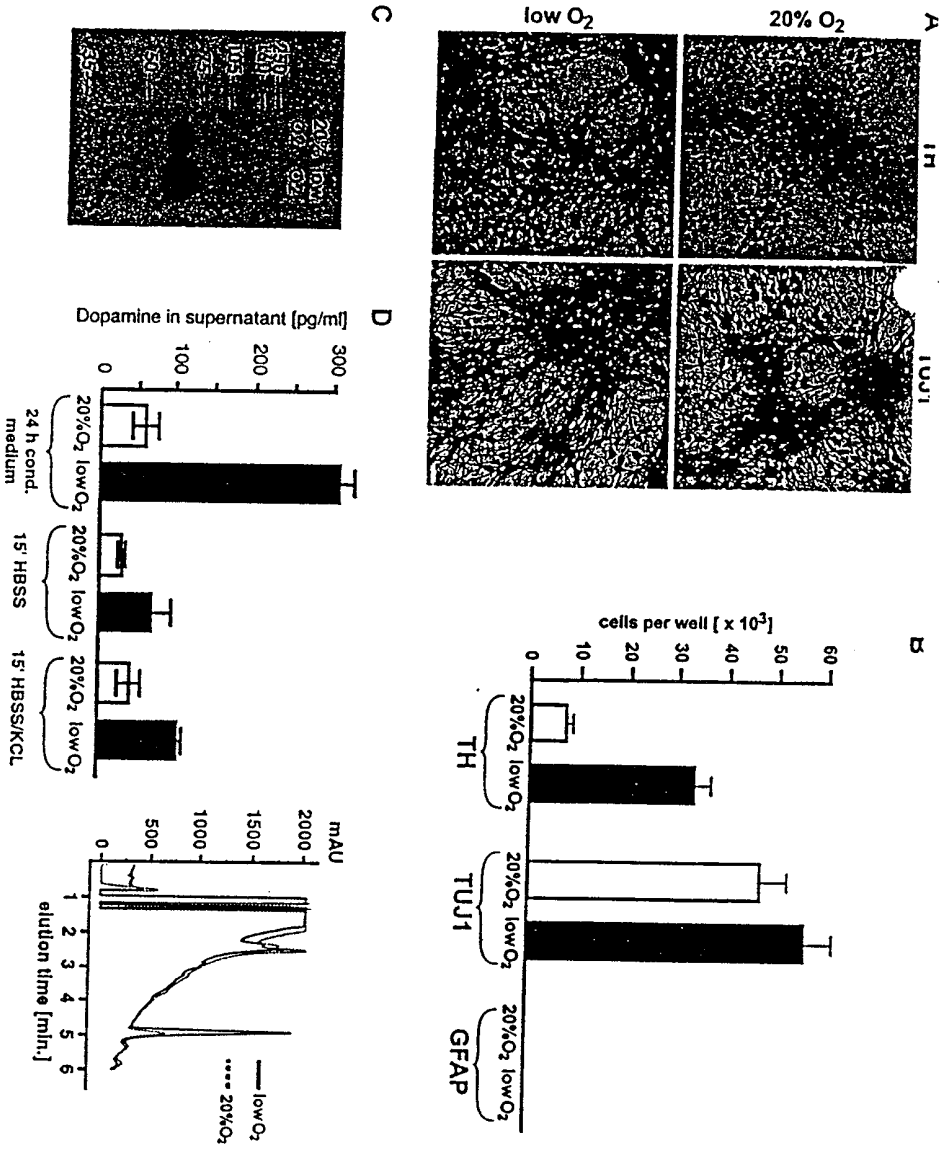
A
low O_2
20% O_2
TH
TUJ1
B
cells per well [x 10^3]
0
10
20
30
40
50
60
TH
TUJ1
GFAP
20%O_2 lowO_2
C
20% low O2
D
Dopamine in supernatant [pg/ml]
0
100
200
300
24 h cond. medium
15' HBSS
15' HBSS/KCL
mAU
0
500
1000
1500
2000
elution time [min.]
1
2
3
4
5
6
lowO_2
20%O_2

FIGURE 5

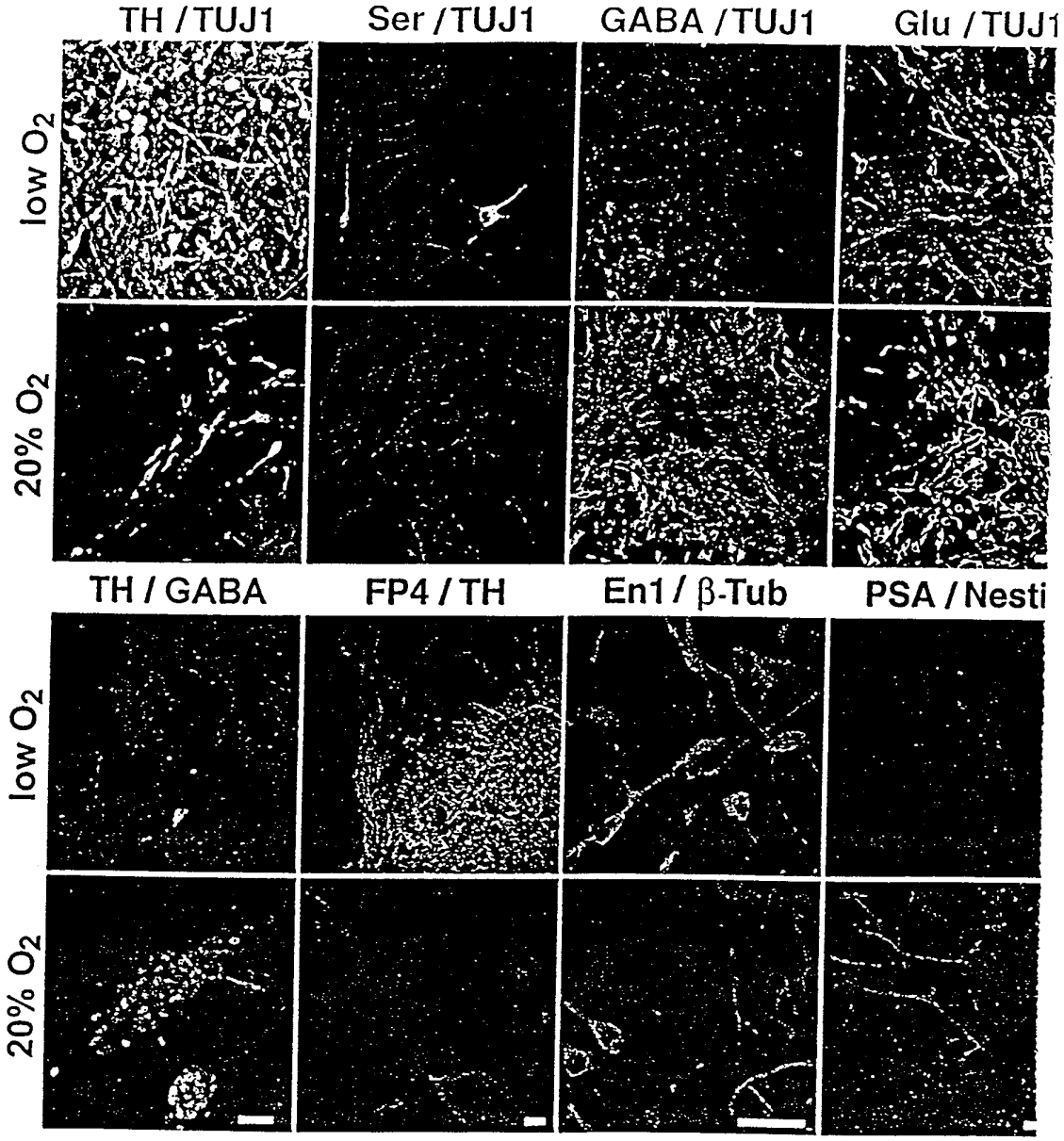
TH / TUJ1
Ser / TUJ1
GABA / TUJ1
Glu / TUJ1
low O_2
20% O_2
TH / GABA
FP4 / TH
En1 / β-Tub
PSA / Nesti
low O_2
20% O_2

FIGURE 6

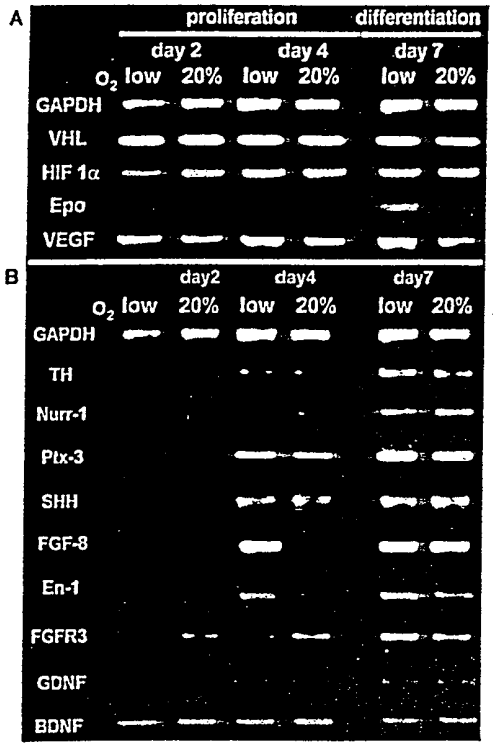
A
proliferation
differentiation
day 2
day 4
day 7
O2 low 20% low 20% low 20%
GAPDH
VHL
HIF 1α
Epo
VEGF
B
day2
day4
day7
O2 low 20% low 20% low 20%
GAPDH
TH
Nurr-1
Ptx-3
SHH
FGF-8
En-1
FGFR3
GDNF
BDNF

FIGURE 7

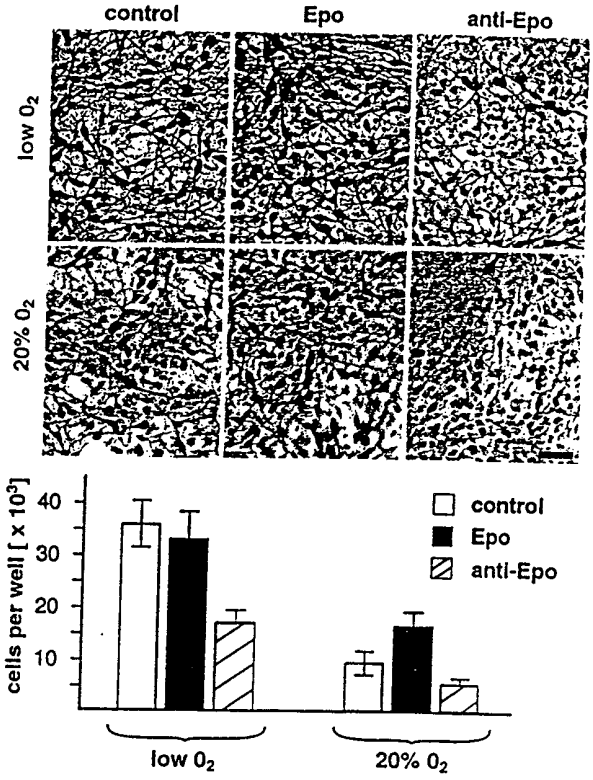
control
Epo
anti-Epo
low 0_2
20% 0_2
cells per well [x 10^3]
40
30
20
10
control
Epo
anti-Epo
low 0_2
20% 0_2

FIGURE 8

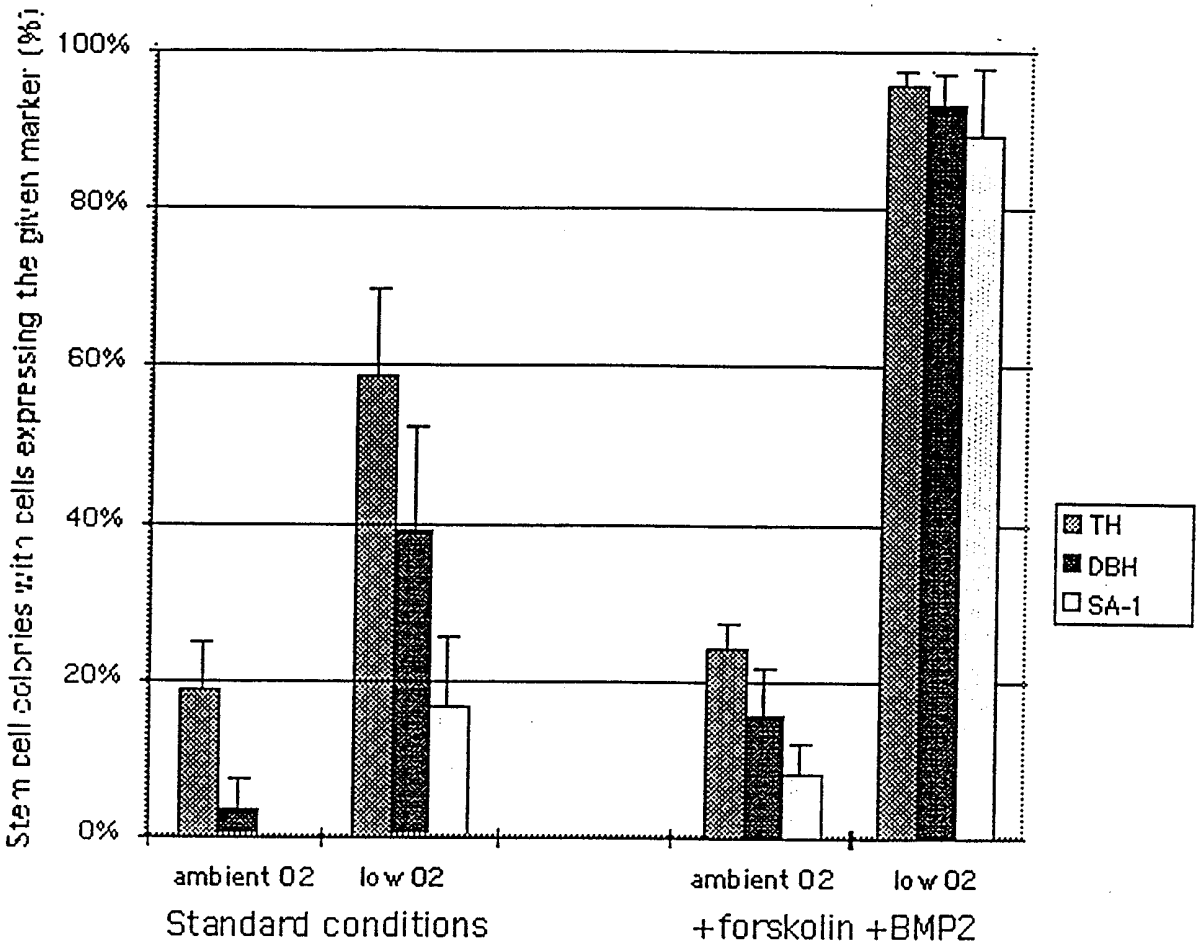
Stem cell colonies with cells expressing the given marker (%)
100%
80%
60%
40%
20%
0%
ambient 02
low 02
ambient 02
low 02
Standard conditions
+forskolin +BMP2
TH
DBH
SA-1

FIGURE 9

SEQUENCE LISTING

<110> Csete, Marie
Doyle, John
Wold, Barbara J.
Morrison, Sean J.
Anderson, David J.

<120> Low Oxygen Culturing of Cells

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