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(54) Title: BSC CELL DIFFERENTIATION AND USE IN THERAPY

(57) Abstract: The present invention relates to the discovery of a novel multipotent progenitor cell termed pre-rosette stage ectoderm progenitor cells or BSC cells. These cells may be isolated, grown stably for a number of which are novel, self-renewing early ectodermal cell types (pre-rosette stage ectoderm progenitors) exhibiting neural progenitor-like characteristics generations and cryopreserved for stable storage. These cells may be further differentiated into neural cells such as neurons and glia cells, and other cells including neural crest derivative cells, among others, which may be used for transplantation and other therapies. Methods of producing these cells from BSC cells are also disclosed.

BSC Cell Differentiation and Use in Therapy

Field of the Invention

The present invention relates to the use of a novel pluripotent stem cell termed BSC (pre-rosette stage ectoderm progenitor cells) in the production of neural cells, in particular dopaminergic neurons, motor neurons, glial cells, neural crest cells, neural crest derivative cells and ectoderm cells, including skin cells, among others, including Schwann cells, smooth muscle cells, adipogenic cells, osteogenic cells, myogenic cells and chondrogenic cells and other cells from BSC cells. In this method, BSC cells which are produced from human Pluripotent stem cells as otherwise described herein may be further differentiated into neural cells such as neurons and glia cells, neural crest cells, neural crest derivative cells and skin cells which may be used for transplantation and other therapies.

The approaches which are presented in the present application can generate important neural, glial and skin cells for a number of therapeutic applications, including transplantation, spinal cord repair and the treatment of numerous neurodegenerative diseases such as Parkinson's disease, multiple sclerosis, Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis (ALS; Lou Gehrig's disease), primary lateral sclerosis (PLS) and progressive muscular atrophy (PMA). In addition, neural lineages have the potential to treat stroke, head trauma and spinal cord injury and multipotent precursor that has potential to form neural cells, including cranial and spinal cord neurons. Other applications include transplantation and cosmetic surgeries (with skin cells produced according to the present invention).

Related Applications and Grant Support

This application claims the benefit of priority of United States provisional application serial number US61/210,365, filed March 18, 2009, entitled "Novel Progenitors from Pluripotent Stems Cells, Methods of Using Same for Therapy", the entire contents of which application are incorporated by reference herein.

Background of the Invention

Human pluripotent stem cells such as human embryonic stem cells (hESCs;

Thomson J.A. et al., 1998, Science 282: 1145-1147) and induced pluripotent stem cells (iPSCs; Takahashi and Yamanaka, 2006, Cell 126: 663-676) have the capacity to differentiate into cells representing the three embryonic germ layers (ectoderm, definitive endoderm and mesoderm) and extra-embryonic lineages (Figure 1). Much interest has focused on the ability to differentiate hESCs into functional neural lineages and neural progenitors because of their relevance to treating central nervous system diseases such as Parkinson's disease, multiple sclerosis, Huntington's disease, amyotrophic lateral sclerosis (ALS; Lou Gehrig's disease), primary lateral sclerosis (PLS) and progressive muscular atrophy (PMA). In addition, neural lineages have the potential to treat stroke, head trauma and spinal cord injury. To generate neural lineages, hESCs transition through ectoderm progenitor states that can further differentiate to generate functional neuronal cell lineages (Figure 2).

Several studies have described a pathway for early neuro-epithelial specification from hESCs (Figure 3). In this pathway, Oct4+ Nanog+ hESCs differentiate to a primitive anterior neuroepithelial state identified by the expression of Pax6 and Otx2 and by the absence of Sox1 (Pankratz et al., 2007, Stem Cells 25: 1511-1520; Li et al., 2005, Nature Biotechnology, 23: 215-221). These very early ectoderm precursors then progress to a Pax6+ Sox1+ neuroectoderm progenitors that acquire either anterior (Pax6+ Sox1+ Otx2+) or posterior characteristics (Pax6+ Sox1+ Hoxb4+). In the context of rosette-stage neuro-epithelial cultures, which appear to reproduce many aspects of neural plate/tube formation in the embryo, these cells represent different stages of columnar cell maturation at the center of these structures. These markers are absent however, in the flat surrounding cells of rosettes (Pankratz et al., 2007, Stem Cells 25: 1511-1520; Li et al., 2005, Nature Biotechnology, 23: 215-221). The Pax6+ Sox1+ early neuroepithelial cells in rosette structures (Elkabetz et al., 2008, Genes Dev. 22: 152-165; Koch et al., 2009, Proc Natl Acad Sci USA. 106: 3225-3230) are of special significance because they appear to have a wider neural differentiation potential than the more well-characterized Sox1+ neural progenitors cells (NPCs) described by a relatively large number of groups (Ben-Hur et al 2004; Shin et al., 2006; Joannides et al 2007). Unlike Sox1+ neural progenitor cells (NPCs) and rosette progenitor cells (Elkabetz et al., 2008, Genes Dev. 22: 152-165; Koch et al., 2009, Proc Natl Acad Sci USA. 106: 3225-3230), which can be maintained over a period of several months, there have been no previous reports of a pre-rosette progenitor with neuro-epithelial potential . This cell type would be of

value because it may have a wider ectoderm differentiation potential than rosette-stage progenitor/stem cells. If achieved, this would represent a more versatile starting point from which to generate lineages of the central nervous system (CNS), including cranial and spinal cord neurons, as they have the potential to generate anterior and posterior cell lineages. Existing methods of generating anterior fated neural progenitors requires a differentiation step followed by a selection process where Sox1+ progenitors are enriched over several passages (Erceg et al., 2009, Stem Cells; 27: 78-87). The same is true for rosette stage progenitors (Elkabetz et al., 2008, Genes Dev. 22: 152-165; Koch et al., 2009, Proc Natl Acad Sci USA. 106: 3225-3230). A method whereby neural progenitors/stem cells could be generated without such cumbersome methodologies would represent a significant advance to the field.

It should be noted that several neural progenitor and stem cell populations have been described in vivo (Kelly et al., 2009, PLoS ONE 4: e4213), including those at different positions in the developing neural tube and in the adult nervous system but, the relationship between these and ESC-derived progenitors is unclear.

Brief Description of the Figures

Figure 1 shows that pluripotent hESCs or hiPSCs can self-renew as a stable population or, differentiate into extra-embryonic lineages or the three germ lineages- ectoderm, definitive endoderm or mesoderm.

Figure 2 shows that human pluripotent cells (Oct4+), such as hESCs or hiPS cells, can generate neural lineages by differentiation towards the ectoderm lineage where they first form Sox1+ multi-potent neural progenitors and/or neural stem cells before differentiating into mature neural cell types such as neurons and glia.

Figure 3 shows a proposed model describing how human pluripotent cells generate pan-neural progenitor/stem cells. These cells are proposed to be patterned by external signaling molecules which then programs them to differentiate into neural lineages. A Pax6+ Sox1+ Otx2+ progenitor associated with rosettes has been proposed to give rise to anterior and posterior progenitors which contribute to the formation of cranial neurons (dopaminergic neurons, TH+) and spinal cord (motor neurons, HB9+), respectively.

Figure 4 shows bright field images of WA09 hESCs and BSCs (passage 20) generated from WA09 hESCs. Micron bars are shown on images taken at 10x, 20x and 40x.

Figure 5 shows that Nanog and Oct4 transcripts decrease following conversion of hESCs to BSCs. Transcript profiling of pluripotency markers Nanog and Oct4 in BG02 and WA09 hESCs and BG02 and WA09-derived BSCs (passage numbers indicated) by Q-RT PCR. Transcript levels were determined after normalization to a GAPDH control. Each assay was performed in triplicate and error bars represent the standard error of the mean. Data is expressed as the fold change relative to the parent hESC line.

Figure 6: Nanog and Oct4 protein levels decrease following conversion of WA09 hESCs to BSCs. Immunofluorescent staining of fixed WA09 hESCs and BSCs (p15) probed with antibodies raised against Oct4 and Nanog. Images taken at 10x and 20x magnification. DAPI staining indicates DNA (nucleus).

Figure 7 shows that Nanog and Oct4 protein levels decrease following conversion of BG02 hESCs to BSCs. Immunofluorescent staining of fixed BG02 hESCs and BSCs (p9) probed with antibodies raised against Oct4 and Nanog. Images taken at 10x and 20x magnification. DAPI staining indicates DNA (nucleus).

Figure 8A-C shows the transcript profiling (Q-RT PCR) of BG02 hESCs and BSCs (p9, p11, p12) derived from BG02 hESCs. Transcript levels are normalized against a GAPDH control and expressed relative to normalized transcript levels in hESCs. All assays were performed in triplicate. Error bars represent the standard error of the mean.

Figure 9: A-C shows the transcript profiling (Q-RT PCR) of WA09 hESCs and BSCs (p9, p22) derived from WA09 hESCs. As depicted, transcript levels are normalized against a GAPDH control and expressed relative to normalized transcript levels in hESCs. All assays were performed in triplicate. Error bars represent the standard error of the mean.

Figure 10A shows that BSCs express Pax6. BG02 and WA09 hESCs and BSCs derived from them, p9 and p15 respectively, were fixed and stained by probing with antibodies raised

against Pax6. DNA (nuclei) was visualized by staining with DAPI. **B.** BG02 hESCs and BG02-derived BSCs (p11) were fixed and probed with antibodies raised against Occludin (ZO1), N-cad, Sox1, Nestin, Sox2 and Pax6. Nuclear DNA was detected by staining with DAPI. **C.** BSCs can give rise to rosette structures. WA09 hESCs and WA09-derived BSCs (p23) were fixed and probed with antibodies raised against Occludin (ZO1), N-cad, Sox1, Nestin, Sox2 and Pax6. Nuclear DNA was detected by staining with DAPI. In latter passages BSCs derived from WA09 cells, contaminating rosettes were often observed in BSC cultures. Rosettes contain Sox1+ cells, in contrast to BSCs which are Sox1-. Occludin and N-cad accumulate in cells at the center of rosettes whereas Occludin and N-cad localization is punctate in BSCs and does not coincide with any regional structure such as the core of a rosette.

Figure 11 shows the flow cytometry analysis of BG02 hESCs and BSCs derived from BG02 hESCs. Antibodies for CXCR4, CD90, CD117, CD31, CD105, CD133 , CD166, CD56, CD44 were used to probe unfixed cells as indicated.

Figure 12 shows the flow cytometry analysis of WA01 hESCs and WA01-derived BSCs. Antibodies for CXCR4, CD44, CD117, CD90, CD166, CD105, CD133, CD31 and CD56 were used to probe unfixed cells as indicated.

Figure 13 shows the flow cytometry analysis of WA07 hESCs and WA07-derived BSCs. Antibodies for CXCR4, CD44, CD117, CD90, CD166, CD105, CD133, CD31 and CD56 were used to probe unfixed cells as indicated

Figure 14 shows the flow cytometry analysis of hiPSC-derived BSCs. Antibodies for CXCR4, CD44, CD117, CD90, CD166, CD105, CD133, CD31 and CD56 were used to probe unfixed cells as indicated

Figure 15 shows the flow cytometry analysis of BSCs derived from WA09 hESCs (p9, p24). Antibodies for CXCR4, CD31, CD166, CD90, CD44, CD117, CD133, CD56, CD105 were used to probe unfixed cells as indicated.

Figure 16 shows WA09 and WA07 hESCs and BSCs derived from these lines were analyzed by flow cytometry using an antibody raised against Trk-B

Figure 17 shows a scheme for differentiation of BSCs into dopaminergic neurons and motor neurons.

Figure 18 shows that BSCs (derived from WA09 hESCs) do not express tyrosine hydroxylase (TH). Some cells in these cultures were positive when probed with anti-dopamine decarboxylase (AADC) antibodies.

Figure 19 shows the immunostaining of WA09-derived BSCs differentiated along stage 1-3 of the DA pathway (see Figure 11) or, stage 2 and 4 of the MN pathway. Left-hand panels: DA differentiation of BSCs to stage 3 (day 10). Cells were stained for AADC, TH and DAPI. Bright field images are shown at the bottom of each column. Right-hand panels: MN differentiation of BSCs to stage 2 (day 8) and stage 4 (day 30). Cells were stained for AADC, TH and DAPI. Bright field images are shown at the bottom of each column.

Figure 20 shows the immunostaining of WA09-derived BSCs differentiated along stage 1-3 of the DA pathway (see Figure 11) or, stage 2 and 4 of the MN pathway. Cells were stained for HB9 and DAPI. Bright field images are shown at the right.

Figure 21 shows the immunostaining of WA09 hESCs, p13 BSCs, and differentiating cells along motor neuron and dopaminergic neuron pathways. Cells were probed with antibodies for TH and dopamine decarboxylase (AADC). DNA was detected by staining with DAPI.

Figure 22: Immunostaining for AADC and β-tubulin in WA09 hESCs, BSCs and DA, MN differentiated cells.

Figure 23 shows a flow cytometry analysis of different hESC lines and BSCs generated from these lines. Cells were probed with antibodies for SSEA3 and SSEA4. Flow profiles show hESCs are +ve for these markers but BSCs are negative.

Figure 24, Table 1 shows a summary of upregulated genes following conversion of hESCs to BSCs (WA09, BG02). Analysis was performed in biological triplicate. BG02-derived BSCs were analyzed at passages 9,11 and 12. WA09-derived BSCs were analyzed at p9,11

and 22.

Figure 25, Table 2 shows a summary of flow cytometry analysis defining cell surface markers for BScs. Scoring (designated by '+') represents the proportion of cells expressing the marker, i.e., '++++' is generally >90%; '+' represents ~ ~25% positive. NA- not applicable/not determined. 'hi' and 'low' indicate the presence of multiple populations (usually 2) where the 'high' population is more positive in intensity than the 'low' population.

Brief Description of the Invention

In a first aspect, the present invention relates to a method for producing BSC cells (pre-rosette stage ectodermal progenitors) which are novel, self-renewing early ectodermal cell types exhibiting neural progenitor-like characteristics. These cells may be produced from human embryonic stem cells (hESCs), human induced pluripotent stem cells (hiPSCs) or human umbilical cord stem cells (hUCSCs) or placental blood stem cells (hPBSCs) by exposing these pluripotent precursor cells in a differentiation medium to a combination of effective amounts of a GSK inhibitor (e.g. BIO), a TGFβ/Activin A signaling inhibitor (.e.g., SB431542, among others as described herein) and a AMPK signaling inhibitor (e.g., Compound C) for a period effective to differentiate the pluripotent precursor cells to BSC (multipotent) cells. Modifications of the present invention are described in detail or may be readily inferred from the description of the invention which follows. In an alternative aspect, BSC cells according to the present invention are cryopreserved using standard methods in the art and stored.

The present invention is also directed to an isolated population of early ectodermal type BSC cells (aka, pre-rosette stage ectoderm progenitors) derived from human pluripotent cells as otherwise described herein. These cells are capable of being regenerated (renewal), being stably cryopreserved and may be further differentiated into mature neural cells, including neural and glia cells, neural crest cells, neural crest derivative cells and ectoderm cells, including skin cells.

The present invention relates to the unexpected discovery that fresh or reconstituted (from cryopreserved cells) pre-rosette stage ectoderm progenitor cells (BSC cells) are a novel source of cells which can be differentiated into mature cells including neural cells (neurons

and glia cells) which can be used for neuronal transplantation (autografting as well as allografting), thus obviating the need to use pooled neuronal fetal tissue or bone marrow tissue, which is often hard to obtain. In addition, BSC cells may be differentiated into ectoderm progenitors, which can be further differentiated into skin cells, and neural crest cells and neural crest derivative cells, which may be further differentiated into peripheral neurons and Schwann cells, as well as cells which fall within mesenchymal lineages, such as smooth muscle cells, adiogenic (fat) cells, osteogenic (bone) cells, myogenic cells and chondrogenic (cartilage) cells. Thus, the present invention may be used for producing numerous mature cell lines of varying utility by differentiating BSC cells and autografting (cells from an individual are used in that same individual), allografting (cells from one person are used in another person) and xenografting (transplantation from one species to another) those cells in order to treat numerous disease states and conditions.

In another aspect of the present invention, it has unexpectedly been discovered that BSC cells may be induced to become neurons (including central nervous system neurons and peripheral neurons, such as motor neurons), glial cells, Schwann cells, smooth muscle cells, adipogenic cells, osteogenic cells and chondrogenic cells. These cells may be used in autologous or allogeneic transplantation (grafting) procedures to effect transplantation and repair of neural tissue, in particular, tissue of the brain and spinal cord and to treat neurodegenerative diseases of the brain and spinal cord, as well as repair or introduce smooth muscle, adipogenic cells, osteogenic cells and chondrogenic cells to a patient in need of therapy. In addition, the present method may also result in the production of ectoderm cells, in particular, skin cells, for transplantation in therapeutic and cosmetic applications.

In one aspect according to the present invention, BSC cells or BSC derived neural cells are suitable for grafting into a patient's brain or spinal cord. These cells may be used without further purification, purified and/or incubated with a differentiation agent by any one or more of the methods otherwise described in the present specification and administered to a patient in need of therapy with the cells obtained.

In another aspect of the present invention, there is presented a method for obtaining one or more of neural cells, including neuronal and glial cells, neural crest cells, neural crest derivative cells, ectoderm cells, including skin cells, Schwann cells, smooth muscle cells, adipogenic cells, osteogenic cells, myogenic cells and chondrogenic cells and other cells from

BSC cells, the method comprising the steps of providing BSC cells, and incubating the BSC cells with at least one differentiation agent in a differentiation medium to change the phenotype of the cells to produce a population of neural cells, including neuron and glial cells, neural crest cells, neural crest derivative cells, ectoderm cells, including skin cells, Schwann cells, smooth muscle cells, adipogenic cells, osteogenic cells and chondrogenic cells which are capable of being transplanted. The steps of the method may also be changed such that all of the cells are incubated with a differentiation agent prior to separation of the mature phenotypically distinguishable (from BSC) cells.

The method of the present invention may include the step of separating and expanding the mature cells obtained from the differentiation of BSC cells prior to transplantation in a patient. Alternatively, an enriched cell population of mature cells may be obtained from a population of BSC cells without further separation, purification or expansion. In addition, BSC cells may be used directly in transplantation methods according to the present invention, neat or in combination with a medium which contains at least one differentiation agent. . The BSC cells may be used directly and transplanted into a patient, grown in a cell medium containing a differentiation agent as generally described above in order to change the phenotype of the BSC cells to change the phenotype of the cells to cells selected from the group consisting of neural cells, including neuron and glial cells, neural crest cells, neural crest derivative cells, ectoderm cells, including skin cells, Schwann cells, smooth muscle cells, adipogenic cells, osteogenic cells and chondrogenic cells and other cells which cells may be used in transplantation procedures directly without further purification.

The BSC may be used in administration to a patient in need thereof (e.g., transplantation) directly or after a separation/purification step. These cells have the ability to migrate and localize to specific anatomical regions where they differentiate into one or more of neural cells, including neuron and glial cells, neural crest cells, neural crest derivative cells, ectoderm cells, including skin cells, Schwann cells, smooth muscle cells, adipogenic cells, osteogenic cells and chondrogenic cells and other cells at the cite of transplantation and integrate into the tissue in a characteristic tissue pattern.

The present invention also is directed to a kit for transplantation comprising a flask with dehydrated culture medium and a population of BSC cells or alternatively, neural cells, including neuron and glial cells, neural crest cells, neural crest derivative cells, ectoderm

cells, including skin cells, Schwann cells, smooth muscle cells, adipogenic cells, osteogenic cells and chondrogenic cells and other cells which can be used to effect a transplantation or therapeutic outcome.

The present invention is also directed to a method for treating a neurodegenerative disorder comprising administering to (preferably, transplanting in) a patient suffering from a neurodegenerative disorder an effective amount of a BSC cell or a neural cell according to the present invention. Neurodegenerative disorders and other conditions and/or injuries which can be treated using the method according to the present invention include, for example, Parkinson's disease, Alzheimer's disease, ischemia, spinal cord damage, head trauma, stroke, ataxia, Parkinson's disease, multiple sclerosis, Huntington's disease, amyotrophic lateral sclerosis (ALS; Lou Gehrig's disease), primary lateral sclerosis (PLS) and progressive muscular atrophy (PMA) and alcoholism, among others, including a number which are otherwise described herein.

The present invention is also directed to a method of treating neurological damage in the brain or spinal cord which occurs as a consequence of damage from a stroke, heart attack or cardiovascular disease in a patient, the method comprising administering, preferably transplanting, an effective number or amount of neural cells which are derived from BSC cells into a patient's brain or spinal cord. Administering cells according to the present invention to a patient and allowing the cells to migrate to the appropriate cite within the central nervous system is another aspect of the present invention.

A method of obtaining BSC, neural cells, including neuron and glial cells, neural crest cells, neural crest derivative cells, ectoderm cells, including skin cells, Schwann cells, smooth muscle cells, adipogenic cells, osteogenic cells and chondrogenic cells and other cells cells for autologous transplantation from an individual's own induced human Pluripotent Stem Cells (ihPSCs) comprises the steps of 1) preparing BSC cells from a patient's ihPSCs 2) exposing said BSC cells prepared in step 1 to a differentiation medium comprising at least one differentiation agent to produce autologous neural cells, including neuron and glial cells, neural crest cells, neural crest derivative cells, ectoderm cells, including skin cells, Schwann cells, smooth muscle cells, adipogenic cells, osteogenic cells and chondrogenic cells and other cells; and 3) administering said cells obtained from step 2 to a patient in need of therapy.

Detailed Description of the Invention

The following terms shall be used to describe the present invention. Unless otherwise noted, the terms used herein are to be understood according to conventional usage by those of ordinary skill in the relevant art. In addition to the definitions of terms provided below, definitions of common terms in molecular biology may also be found for example, in Rieger *et al.*, 1991 Glossary of genetics: classical and molecular, 5th Ed., Berlin: Springer-Verlag; and in Current Protocols in Molecular Biology, F.M. Ausubel *et al.*, Eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1998 Supplement). It is to be understood that as used in the specification and in the claims, "a" or "an" can mean one or more, depending upon the context in which it is used. Thus, for example, reference to "a cell" can mean that at least one cell can be utilized.

The present invention may be understood more readily by reference to the following detailed description of the preferred embodiments of the invention and the Examples which are included herein. However, before the present compositions and methods are disclosed and described, it is to be understood that this invention is not limited to specific conditions, or specific methods, or specific characteristics, etc., as such may, of course, vary, and the numerous modifications and variations therein will be apparent to those skilled in the art.

Standard techniques for growing cells, separating cells, and where relevant, cloning, DNA isolation, amplification and purification, for enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like, and various separation techniques are those known and commonly employed by those skilled in the art. A number of standard techniques are described in Sambrook *et al.*, 1989 Molecular Cloning, Second Edition, Cold Spring Harbor Laboratory, Plainview, New York; Maniatis *et al.*, 1982 Molecular Cloning, Cold Spring Harbor Laboratory, Plainview, New York; Wu (Ed.) 1993 Meth. Enzymol. 218, Part I; Wu (Ed.) 1979 Meth. Enzymol. 68; Wu et al., (Eds.) 1983 Meth. Enzymol. 100 and 101; Grossman and Moldave (Eds.) 1980 Meth. Enzymol. 65; Miller (ed.) 1972 Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Old and Primrose, 1981 Principles of Gene Manipulation, University of California Press, Berkeley; Schleif and Wensink, 1982 Practical Methods in Molecular Biology; Glover (Ed.) 1985 DNA Cloning Vol. I and II, IRL Press, Oxford, UK; Hames and Higgins (Eds.) 1985 Nucleic Acid Hybridization, IRL Press, Oxford, UK; and Setlow and Hollaender 1979

Genetic Engineering: Principles and Methods, Vols. 1-4, Plenum Press, New York. Abbreviations and nomenclature, where employed, are deemed standard in the field and commonly used in professional journals such as those cited herein.

The term "human Pluripotent Stem cells" (hPSCs) of which "human Embryonic Stem Cells" (hESCs), human induced Pluripotent Stem Cells (hiPSC) and umbilical cord/placental tissue or blood stem cells refer to pluripotent stem cells which are used in the present invention. hESCs are pre-embryonic, embryonic or fetal tissue cells at any time after fertilization, and have the characteristic of being capable under appropriate conditions of producing progeny of several different cell types that are derivatives of all of the three germinal layers (endoderm, mesoderm and ectoderm), according to a standard art-accepted test, such as the ability to form teratomas in 8-12 week old SCID mice. The term includes both established lines of stem cells of various kinds, and cells obtained from primary tissue that are pluripotent in the manner described.

Included in the definition of human pluripotent stem cells or hPS cells (hPSCs) useful in the present invention are human embryonic cells of various types, especially including human embryonic stem cells (hESCs), described by Thomson et al. (Science 282: 1145, 1998). Other types of pluripotent cells are also included in the term. Human Pluripotent Stem Cells includes stem cells which may be obtained from human umbilical cord or placental blood as well as human placental tissue or umbilical cord tissue. Any cells of human origin that are capable of producing progeny that are derivatives of all three germinal layers are included, regardless of whether they were derived from embryonic tissue, fetal, or other sources, including induced Pluripotent Stem Cells. The pPS cells are preferably not derived from a malignant source. It is desirable (but not always necessary) that the cells be karyotypically normal.

pPS cell cultures are described as "undifferentiated" when a substantial proportion of stem cells and their derivatives in the population display morphological characteristics of undifferentiated cells, clearly distinguishing them from differentiated cells of embryo or adult origin. Undifferentiated pPS cells are easily recognized by those skilled in the art, and typically appear in the two dimensions of a microscopic view in colonies of cells with high nuclear/cytoplasmic ratios and prominent nucleoli. It is understood that colonies of

undifferentiated cells in the population will often be surrounded by neighboring cells that are differentiated.

Pluripotent stem cells for use in the present invention may express one or more of the stage-specific embryonic antigens (SSEA) 3 and 4, and markers detectable using antibodies designated Tra-1-60 and Tra-1-81 (Thomson et al., Science 282:1145, 1998). Differentiation of pluripotent stem cells in vitro results in the loss of SSEA-4, Tra-1-60, and Tra-1-81 expression (if present) and increased expression of SSEA-1. Undifferentiated pluripotent stem cells typically have alkaline phosphatase activity, which can be detected by fixing the cells with 4% paraformaldehyde, and then developing with Vector Red as a substrate, as described by the manufacturer (Vector Laboratories, Burlingame Calif.) Undifferentiated pluripotent stem cells also typically express Oct-4 and TERT, as detected by RT-PCR.

Another desirable phenotype of propagated pluripotent stem cells is a potential to differentiate into cells of all three germinal layers: endoderm, mesoderm, and ectoderm tissues. Pluripotency of pluripotent stem cells can be confirmed, for example, by injecting cells into severe combined immunodeficient (SCID) mice, fixing the teratomas that form using 4% paraformaldehyde, and then examining them histologically for evidence of cell types from the three germ layers. Alternatively, pluripotency may be determined by the creation of embryoid bodies and assessing the embryoid bodies for the presence of markers associated with the three germinal layers.

Propagated pluripotent stem cell lines may be karyotyped using a standard G-banding technique and compared to published human. It is desirable to obtain cells that have a "normal karyotype," which means that the cells are euploid, wherein all human chromosomes are present and not noticeably altered.

The types of pluripotent stem cells that may be used include established lines of pluripotent cells derived from tissue formed after gestation, including pre-embryonic tissue (such as, for example, a blastocyst), embryonic tissue, or fetal tissue taken any time during gestation, typically but not necessarily before approximately 10-12 weeks gestation. Non-limiting examples are established lines of human embryonic stem cells or human embryonic germ cells, such as, for example the human embryonic stem cell lines WA01, WA07, and WA09 (WiCell). Also contemplated is use of the compositions of this disclosure during the

initial establishment or stabilization of such cells, in which case the source cells would be primary pluripotent cells taken directly from the source tissues. Also suitable are cells taken from a pluripotent stem cell population already cultured in the absence of feeder cells. Also suitable are mutant human embryonic stem cell lines, such as, for example, BG01v (BresaGen, Athens, Ga.), as well as normal human embryonic stem cell lines such as WA01, WA07, WA09 (WiCell) and BG01, BG02 (Novocell, Athens, Ga.).

Epiblast stem cells (EpiSCs) and induced pluripotent stem cells (iPS) fall within the broad definition of pluripotent cells hereunder and in concept, the technology described in the present application could apply to these and other pluripotent cell types as set forth above. EpiScs are isolated from early post-implantation stage embryos. They express Oct4 and are pluripotent. See, Tesar et al, *Nature,* Vol 448, p.196 12 July 2007. iPS cells are made by dedifferentiating adult somatic cells back to a pluripotent state by retroviral transduction of four genes (c-myc, Klf4, Sox2, Oct4). See, Takahashi and Yamanaka, Cell 126, 663–676, August 25, 2006.

Human embryonic stem cells may be prepared by methods which are described in the present invention as well as in the art as described for example, by Thomson et al. (U.S. Pat. No. 5,843,780; *Science* 282:1145, 1998; *Curr. Top. Dev. Biol.* 38:133 ff., 1998; *Proc. Natl. Acad. Sci. U.S.A.* 92:7844, 1995).

The term "human embryonic stem cell" refers to pluripotent cells, of humans, which are isolated from the blastocyst stage of a human embryo. Human embryonic stem cell refers to a stem cell from a human and are preferably used in aspects of the present invention which relate to human therapy or diagnosis. The following phenotypic markers are expressed by human embryonic stem cells:

SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, CD9, alkaline phosphatase, Oct 4, Nanog, Rex 1, Sox2 and TERT. See Ginis, et al., *Dev. Biol,* 269(2), 360-380 (2004); Draper, et al., *J. Anat.*, 200(Pt. 3), 249-258, (2002); Carpenter, et al., *Cloning Stem Cells,* 5(1), 79-88 (2003); Cooper, et al., *J. Anat.*, 200(Pt.3), 259-265 (2002); Oka, et al., *Mol. Biol. Cell*, 13(4), 1274-81 (2002); and Carpenter, et al., *Dev. Dyn.,* 229(2), 243-258 (2004). While any primate pluripotent stem cells (pPSCs), including especially human embryonic stem cells can be used in the present methods to produce mesendoderm cells, mesoderm Isl1+ cells and multipotent migratory cells (MMCs)

according to the present invention, preferred pPSCs for use in the present invention include human embryonic stem cells, including those from the cell lines BG01 and BG02, as well as numerous other available stem cell lines.

The term "differentiation" is used to describe a process wherein an unspecialized ("uncommitted") or less specialized cell acquires the features of a more specialized cell such as, for example, a BSC cell, a neural cell or a glial cell. The term "differentiated" includes the process wherein a multipotent stem cell, including a hESC, becomes a more specialized intermediate cell such as a BSC progenitor cell, where the more specialized intermediate cell (BSC cell) becomes an even more specialized cell. A differentiated or differentiation-induced cell is one that has taken on a more specialized ("committed") position within the lineage of a cell. The term "committed", when applied to the process of differentiation, refers to a cell that has proceeded in the differentiation pathway to a point where, under normal circumstances, it will continue to differentiate into a specific cell type or subset of cell types, and cannot, under normal circumstances, differentiate into a different cell type or revert to a less differentiated cell type. "De-differentiation" refers to the process by which a cell reverts to a less specialized (or committed) position within the lineage of a cell. As used herein, the lineage of a cell defines the heredity of the cell, i.e., which cells it came from and what cells it can give rise to. The lineage of a cell places the cell within a hereditary scheme of development and differentiation. A lineage-specific marker refers to a characteristic specifically associated with the phenotype of cells of a lineage of interest and can be used to assess the differentiation of an uncommitted cell to the lineage of interest.

The terms "BSC cells", "pre-rosette stage ectodermal progenitors" or "BSCs" are used interchangeably to refer to a cell or cells produced according to the present invention. BSC cells are early ectodermal like cells which may be readily differentiated into neural and glial (neuroglia) cells, such as mature neuron cells of the central nervous system, including cranial neurons, such as dopaminergic neurons, and spinal cord neurons, such as motor neurons, and neuroglia cells such as dendrocytes, oligodendroglia cells and microglia cells. BSC cells exhibit up-regulation (positive) relative to the parental pluripotent cell from which they were derived of the following genes as described:

Positive up-regulated (about 75%+ in most, but not all BSC cell types) for CD56 and CD90;

Expressed in >75% in most , but not all, BSC cell types: Sox2, Pax6, nestin in these other lines Sox2 and Pax6 can be expressed in 20-75% of the population

Negative for: SSEA3, SSEA4, Nanog, Oct4, Sox1.
Most BSC cultures consist of cells that are predominantly negative for Otx2.

The following markers are less important, but identifiable in BSC cells according to the present invention:

Expressed (positive up-regulation) in >50% of BSC cells (>50% of cells are positive in a sample): CXCR4, Trk-B, CD133, CD105 and CD166. These markers are commonly found in the majority of BSCs prepared from different hESCs, hiPSC lines, but in certain lines, they are expressed in a minor % of the population, or can be absent.

The following markers are expessed in some BSC lines. These markers are infrequently expressed in different BSC lines: Sox1, CD105.

BSC cells according to the present invention preferably do not substantially express (are negative for) the following markers: SSEA3, SSEA4, Nanog and Oct4; do not substantially express the following markers for mesoderm cells: FOXF1 and MEOX1, and do not substantially express the following markers for definitive or extra-embryonic endoderm: SOX17, THBD, AFP, GSC, FOXA2.

BSC cells according to the present invention may be readily differentiated into neurons, including dopaminergic neurons, motor neurons and glial cells using techniques which are readily available in the art or otherwise as described herein.

In addition, at least two, and preferably all of the following characteristics are found in BSC cells according to the present invention:

BSCs do not form neural rosettes under standard BSC conditions;

BSCs can differentiate into cranial neurons (dopaminergic neurons, for example) and motor neurons (based on data presented); and

BSCs may differentiate into neural crest progenitors and neural crest derivatives;

In addition, two or more of the following characteristics preferably are found in BSCs:

BSCs can differentiate into glial cells;

BSCs can differentiate into a wide range of other ectoderm lineages (skin etc);

BSCs can be frozen (cryopreserved), and thawed without loss of potency and at high efficiency; and

BSCs can be maintained as a stable population over ~25 or more passages. They are self-renewing (self-renew).

BSCs express low levels of Occludin (ZO1) and do not form rosette structures, unlike the situation in rosettes where Occludin is present at the centers of neuro-epithelialized rosettes (Elkabetz et al., 2008 Genes Dev. 22: 152-165). Occludin is expressed in a distinctly punctate manner in BSCs (see Figure 10B), showing no obvious organization within the core of rosettes. N-cadherin displays a similar expression pattern. The staining pattern for Occludin and N-cadherin is therefore different than that for hESCs or neural rosettes.

BSCs according to the present invention are clearly distinguishable from other ectoderm progenitors such as neural stems cells or neural rosette progenitors as follows:

Neural stem/progenitor cells- are a later stage of neural specification and have a more limited differentiation potential. Typically they are mechanically isolated from neural rosettes and after sequential passage they lose some differentiation potency.

Neural rosette progenitors (Elkabetz et al., 2008, *Genes Dev.* 22: 152-165; Koch et al., 2009 Mar 3;106(9):3225-30, e-pub. Feb 13 *Proc Natl Acad Sci* USA): these are clusters of cells comprising polarized neuroepithelia. These cells arrange into a rosette structure and have the ability to generate dopaminergic and motor neurons- they therefore, have a wider neural differentiation potential than NPCs.

BSCs exhibit one or more of the following advantages over existing neural ectoderm stem/progenitor cells:

- BSCs can be made directly from human pluripotent cells without the need to go through a rosette stage;

- BSCs do not require mechanical isolation to generate the cells;
- BSCs can be differentiated to neural and other ectoderm lineages without going through a rosette stage;
- BSCs have a wider range ectoderm differentiation potential than rosette progenitors or NPCs;
- BSCs can be more easily amplified and characterized due to their uniform epithelial properties; and
- BSCs are uniform in composition at the molecular and cellular level.

As used herein, the terms "differentiation medium", "cell differentiation medium", "culture media", "basal cell medium", "basal cell media" or "basal media" or "stabilizing medium" are used synonymously to describe a cellular growth medium in which (depending upon the additional components used) the hESCs, iPSCs, BSCs are produced, grown/cultured or alternatively, differentiated into more mature cells, including neural and glial cells. Differentiation media are well known in the art and comprise at least a minimum essential medium plus one or more optional components such as growth factors, including fibroblast growth factor (FGF), including fibroblast growth factor 2 (Fgf2), insulin-like growth factor LR-IGF, ascorbic acid, glucose, non-essential amino acids, salts (including trace elements), L-glutamine, insulin (where indicated and not excluded), Activin A, transferrin, beta mercaptoethanol, Heregulin, and other agents well known in the art and as otherwise described herein. Preferred media includes basal cell media which contains between 1% and 20% (preferably, about 2-10%) fetal calf serum, or for defined medium (preferred) an absence of fetal calf serum and KSR, but including bovine serum albumin or other albumin such as probumin(about 1-5%, preferably about 2%). Preferred differentiation medium is defined and is serum free. In certain embodiments wherein BSCs are produced, Activin A is eliminated and/or Activin A inhibitor is used, the medium preferably may eliminate or substantially reduce the amount of Activin A.

Other agents which optionally may be added to differentiation medium according to the present invention include, for example, nicotinamide, members of TGF-β family, including TGF-β 1, 2, and 3, Activin A, nodal, serum albumin, members of the fibroblast growth factor family, especially including Fgf2 and Fgf8, platelet-derived growth factor-AA, and -BB, platelet rich plasma, insulin growth factor (IGF-I, II), growth differentiation factor

(GDF-5, -6, -8, -10, 11), glial cell line-derived neurotrophic growth factor (GDNF), glucagon like peptide-I and II (GLP-I and II), GLP-1 and GLP-2 mimetobody, Exendin-4, parathyroid hormone, insulin, progesterone, aprotinin, hydrocortisone, ethanolamine, epidermal growth factor (EGF), gastrin I and II, copper chelators such as, for example, triethylene pentamine, forskolin, Na-Butyrate, betacellulin, ITS, noggin, neurite growth factor, nodal, valporic acid, trichostatin A, sodium butyrate, cyclic AMP (cAMP), retinoic acid (RA), hepatocyte growth factor (HGF), sphingosine-1, VEGF, MG132 (EMD, CA), N2 and B27 supplements (Gibco, CA), steroid alkaloid such as, for example, cyclopamine (EMD, CA), keratinocyte growth factor (KGF), Dickkopf protein family, bovine pituitary extract, islet neogenesis-associated protein (INGAP), Indian hedgehog, sonic hedgehog, proteasome inhibitors, notch pathway inhibitors, sonic hedgehog inhibitors, heregulin, or combinations thereof, among a number of other components. Each of these components, when included, are included in effective amounts.

By way of further example, suitable media may be made from the following components, such as, for example, Dulbecco's modified Eagle's medium (DMEM), Gibco #11965-092; Knockout Dulbecco's modified Eagle's medium (KO DMEM), Gibco # 10829-018; Ham's F12/50% DMEM basal medium (DMEM/F12); 200 mM L-glutamine, Gibco #15039-027; non-essential amino acid solution, Gibco 11140-050; β-mercaptoethanol, Sigma #M7522; human recombinant basic fibroblast growth factor (bFGF), Gibco #13256-029. Preferred embodiments of media used in the present invention are as otherwise described herein, especially in the Examples, described infra. These media are suitable for culturing/renewing or differentiating human pluripotent stems cells as otherwise disclosed herein. In the case of differentiating BSC cells to neural and/or glial cells, neurobasal media (Gibco) containing N2 supplements with additional components as otherwise disclosed herein are used.

A particularly preferred differentiation medium for growing/culturing hPSCs (especially, hESCs, and ihPSCs) and for differentiating cells in the present invention is DMEM/F12 (50:50) which contains about 2% proalbumin (albumin; Millipore/Serologicals), 1x Pen/Strep, 1x NEAA, 1x Trace Elements A,B, C (Mediatech), Ascorbic Acid (10-100 ng/ml, about 25-65 ng/ml, about 50 ng/ml), about 0.1mM (0.025-0.5mM) β-Mercaptoethanol (Gibco), about 2-10 ng/ml, about 5-9 ng/ml, about 8 ng/ml bFGF (Sigma), 200 ng/ml (5-500

ng/ml) R-IGF (referred to as IGF-I; JRH Biosciences), 10 ng/ml Activin A (about 1ng/ml to no more than about 20ng/ml) and 10ng/ml (about 1-20ng/ml or more) Heregulin. It is noted that Activin A or Activin A signaling is *not* required for the production of BSC cells, but may be included in low concentrations.

Differentiation media useful in the present invention are commercially available and can be supplemented with commercially available components, available from Invitrogen Corp. (GIBCO), Cell Applications, Inc. and Biological Industries, Beth HaEmek, Israel, among numerous other commercial sources, including Calbiochem. In preferred embodiments at least one differentiation agent such as fibroblast growth factor (FGF), LR-IGF (an analogue of insulin-like growth factor) and Heregulin (preferably all three in effective amounts) is added to the cell media in which a pluripotent stem cell is cultured to produce a stable self-renewing stem cell population which may be differentiated into BSC cells.

When a human pluripotent cell is differentiated into a BSC cell pursuant to the present invention, the preferred defined media is DM-HIF, and the cells are grown on/plated onto a differentiation protein (preferably Matrigel) preferably containing effective amounts of a GSK3 inhibitor such as BIO, Calbiochem cat. #361550 (about 0.1 to about 10 μM, about 1-5 μM, about 2 μM), a TGFβ/Activin A signaling inhibitor such as SB431542, TOCRIS cat. #1614 (about 0.5 to about 50 μM, about 5.0 to about 35 μM, about 15-25 μM, about 20 μM) and a AMPK inhibitor such as Compound C, Calbiochem Cat.##171261(about 0.05 to about 20 μM, about 0.1 to about 10 μM, about 0.5 to about 5 μM, about 1 μM). The human pluripotent cells are preferably plated on Matrigel or other

In the case of differentiating BSC cells to dopaminergic neurons, a neurobasal medium supplemented with N2 (N2A) supplement and including effective amounts of L-glutamine, Fgf2 (e.g., about 1 to about 50-100 ng/ml, about 20-25 ng/ml, about 20 ng/ml), EGF (e.g., about 1 ng/ml to about 50 ng/ml, about 10-15 ng/ml, about 10ng/ml) is preferably used in a first stage (about 3-7 days, about 4-6 days, about 5 days) of differentiation; a neurobasal medium supplemented with N2 (N2A) supplement, L-glutamine, sonic hedgehog (about 10ng to about 2 mg/ml, about 50 to 500 ng/ml, about 200 ng/ml), TGFbeta 3 (1, 2 or 3, preferably 3, may be used in amounts ranging from about 0.1 ng/m to about 25 ng/ml, about 0.5 ng/ml to about 10 ng/ml, about 1-5 ng/ml, about 1 ng/ml) in a second stage (about 3-6,

about 3-6, about 4-5, about 4 days) of differentiation; and a neurobasal medium with N2 (N2A) supplement and including effective amounts of L-glutamine, sonic hedgehog (as described above), Fgf8 (e.g., about 10 to 500 ng/ml, about 25 to about 250 ng/ml, about 50 to 150 ng/ml, about 100 ng/ml), TGFbeta 3 (1, 2 or 3 as described above) and ascorbic acid (about 25 μM to about 2 mM, about 50 μM to about 1 mM, about 200μm,) in a third and final stage (about 4-8, about 5-7, about 6 days) to produce dopaminergic neurons. See, Cho, et al., *PNAS,* 105, 3392-3397- see Figure 17. Dopaminergic cells are generally produced from BSC cells during a (total) period ranging from about 13-21 days, about 14-20 days, about 15-19 days, about 15 days.

In the case of differentiating BSC cells according to the present invention to motor neurons, a neurobasal medium supplemented with N2 (N2A) supplement and including effective amounts of L-glutamine, Fgf2 (e.g., about 1 to about 50-100 ng/ml, about 20-25 ng/ml, about 20 ng/ml), EGF (e.g., about 1 ng/ml to about 50 ng/ml, about 10-15 ng/ml, about 10ng/ml) is preferably used in a first stage (about 3-7 days, about 4-6 days, about 5 days) of differentiation; a neurobasal medium supplemented with N2 (N2A) supplement, L-glutamine, cAMP (about 100nM to about 10 μM ng, about 500 nM to about 5 μM, about 1-2 μM, about 1 μM), all-trans retinoic acid (RA, in amounts ranging from about 0.01 to about 10 μM, about 0.05 to about 1 μM, about 0.1 to about 0.2 μM, about 0.1 μM in a second stage (about 5-9, about 6-8, about 7-8, about 8 days) of differentiation; a neurobasal medium with N2 (N2A) supplement and including effective amounts of L-glutamine, cAMP (as described above), all-trans retinoic acid (as described above), and sonic hedgehog (e.g., about 25 ng/ml to about 2 μg/ml, about 50 ng/ml to about 1 μg/ml, about 100 to about 500 ng/ml, about 200 ng/ml), in a third stage (about 10-20, about 11-17, about 14-15, about 14 days); and a neurobasal medium with N2 (N2A) supplement and including effective amounts of L-glutamine, cAMP (as described above), all-trans retinoic acid (as described above), and sonic hedgehog (e.g., about 5 to 500 ng/ml, about 10 to about 250 ng/ml, about 25 to 150 ng/ml, about 50 ng/ml), in a fourth and final stage (about 5-15, about 6-12, about 7-11, about 7-10 days) to produce motor neurons. See, Li, et al., *Nat. Biotech.* 23, 215-221- see Figure 17). Motor neuron cells are generally produced from BSC cells during a (total) period ranging from about 18-36 days, about 20-35 days, about 25-32 days, about 25-30, about 26-29 days.

The following cells are also produced from BSC cells:

Neural crest: BSCs are cultured in N2 media supplemented with ascorbic acid (~0.2mM), sonic hedghog (200ng/ml), Fgf8 (100ng/ml), BDNF (20ng/ml). Media is changed every 2-3 days for ~21-30 days. Cultures (which may contain rosettes) are re-plated on polyornithine-laminin coated plates in N2 medium supplemented with Fgf2, ascorbic acid, BDNF and/or as determined empirically Wnt (Wnt3A or Wnt1A 4 to 400 ng/ml, preferably about 40 ng/ml), BMP4 (about 5 to 500 ng/ml, preferred about 50 ng/ml), FGF8 (about 10 ng to 1 μg/ml, preferred about 100 ng/ml), RA (about 0.05 to 5 μM, preferred about 0.5 μM), Shh (about 20ng to 2 μg/ml, preferred about 200 ng/ml), SU5402 (about 1 nM to 100 nM, preferred about 10 nM), Dkk1 (about 10 ng/ml to about 1μg/ml, preferred about 100 ng/ml) and Noggin (about 50 ng/ml to about 5μg/ml, preferred about 500ng/ml). Neural crest is heavily enriched for p75 and HNK1. Cells are typically plated on polyornithine laminin/fibronectin coated plates. Neural crest cells can then be maintained in N2 media supplemented with Fgf2 (20ng/ml), EGF (20ng/ml) and passaged every 6-7 days.

Neural crest derivatives: neural crest cells can be differentiated into peripheral neurons, Schwann cells) and mesenchymal lineages (smooth muscle, adipogenic, osteogenic and chondrogenic cells).

(i) Peripheral nerve: Neural crest are grown in N2 medium supplemented with effective amounts of BDNF, GDNF, dbcAMP (no Fgf2, EGF)

(ii) Schwann cells: Neural crest cells are grown in medium containing effective amounts of CNTF, neuregulin, Fgf2 (10ng/ml) and dbcAMP.

(iii) mesenchymal lineages: neural crest cells were cultured in αMEM, 10% FCS for 12-16 days on uncoated plastic dishes. To generate specialized lineages these cultures are then treated as indicated.

adipogenic differentiation: cells were grown to high density in dexamethasone (about 0.1 mM to about 10mM, preferred about 1mM), insulin (about 0.05 μg/ml to about 5 μg/ml, preferred about 0.5 μg/ml), isobutylxanthine (about 0.05 mM to about 5 mM, preferred about 0.5mM), FCS (about 10%) in α-MEM for about 3-5 weeks

chondrogenic differentiation: cells were treated with about 1 ng/ml to about 100 ng./ml, preferred about 10ng/ml TGFbeta-3200mM (about 300mM to about 32M) ascorbic acid in α-MEM, FCS (preferred about 10%) for about 4-6 weeks

osteogenic differentiation: cells were plated at low density about 10^2-10^4, preferred about 10^3 cells/cm^2

in the presence of 1mM to about 100mM (preferred about10mM) beta-glycerophosphate, about 0.01 to about 1 μM (preferred about 0.1μM) dexamethasone, about 20mM to about 2M, (preferred about 200mM) ascorbic acid, FCS (preferred about 10%) in αMEM for 3-4 weeks.

myogenic (cardiac myocytes) differentiation: differentiated for 2-3 weeks in αMEM, FCS (preferred about 10%). Cells are then cultured in N2 medium.

As described, one of ordinary skill in the art will be able to readily modify the cell media to produce any one or more of the target cells pursuant to the present invention. Cell differentiation medium for producing BSC cells is essentially synonymous with basal cell medium but is used within the context of a differentiation process and includes cell differentiation agents to differentiate cells into other cells. In the case of producing neuron or glial cells from BSC cells, the differentiation medium is a neurobasal medium (preferably containing N2 or N2A supplement) containing differentiation agents to differentiate the BSC cells into neuron and glial cells. Stabilizing medium is a basal cell medium (defined medium) which is used either before or after a differentiation step in order to stabilize a cell line for further use.

Pluripotent stem cells, BSC (which are multipotent) cells, and neural cells (neurons and glial cells) and other cells also may be cultured on a layer of feeder cells that support the pluripotent or multipotent cells in various ways which are described in the art. Alternatively and preferably, pluripotent stem cells and BSC cells in the present invention are cultured in the absence of feeder cells on a cellular support, for example, Matrigel, laminin or poly-L-ornithine laminin, among numerous others as described herein, coated plates or culture dishes. Alternatively, these cells may be cultured in a culture system that is essentially free of feeder cells, but nonetheless supports proliferation of these cells without undergoing substantial differentiation. The growth of pluripotent stem cells in feeder-free culture without differentiation is supported using a medium conditioned by culturing previously with another cell type or alternatively, in feeder-free culture without differentiation is supported using a chemically defined medium. These approaches are well known in the art. In preferred aspects of the present invention, the cells are grown in feeder cell free medium.

Approaches for culturing cells on a layer of feeder cells are well known in the art. For

example, Reubinoff et al. (*Nature Biotechnology* 18: 399-404 (2000)) and Thompson et al. (*Science* 6 Nov. 1998: Vol. 282. no. 5391, pp. 1145-1147) disclose the culture of pluripotent stem cell lines from human blastocysts using a mouse embryonic fibroblast feeder cell layer. Richards et al, (*Stem Cells* 21: 546-556, 2003) evaluated a panel of 11 different human adult, fetal and neonatal feeder cell layers for their ability to support human pluripotent stem cell culture. Richards et al, states: "human embryonic stem cell lines cultured on adult skin fibroblast feeders retain human embryonic stem cell morphology and remain pluripotent". US20020072117 discloses cell lines that produce media that support the growth of primate pluripotent stem cells in feeder-free culture. The cell lines employed are mesenchymal and fibroblast-like cell lines obtained from embryonic tissue or differentiated from embryonic stem cells. US20020072117 also discloses the use of the cell lines as a primary feeder cell layer. In another example, Wang et al (Stem Cells 23: 1221-1227, 2005) disclose methods for the long-term growth of human pluripotent stem cells on feeder cell layers derived from human embryonic stem cells. In another example, Stojkovic et al (Stem Cells 2005 23: 306-314, 2005) disclose a feeder cell system derived from the spontaneous differentiation of human embryonic stem cells. In a further example, Miyamoto et al (+ 22: 433-440, 2004) disclose a source of feeder cells obtained from human placenta. Amit et al *(Biol. Reprod* 68: 2150-2156, 2003) discloses a feeder cell layer derived from human foreskin. In another example, Inzunza et al (Stem Cells 23: 544-549, 2005) disclose a feeder cell layer from human postnatal foreskin fibroblasts.

Approaches for culturing pPSCs in media, especially feeder-free media, are well known in the art. U.S. Pat. No. 6,642,048 discloses media that support the growth of primate pluripotent stem (pPS) cells in feeder-free culture, and cell lines useful for production of such media. U.S. Pat. No. 6,642,048 states: "This invention includes mesenchymal and fibroblast-like cell lines obtained from embryonic tissue or differentiated from embryonic stem cells. Methods for deriving such cell lines, processing media, and growing stem cells using the conditioned media are described and illustrated in this disclosure." In another example, WO2005014799 discloses conditioned medium for the maintenance, proliferation and differentiation of mammalian cells. In still another example, Xu et al (*Stem Cells* 22: 972-980, 2004) discloses conditioned medium obtained from human embryonic stem cell derivatives that have been genetically modified to over express human telomerase reverse transcriptase. In another example, US20070010011 discloses a chemically defined culture medium for the maintenance of pluripotent stem cells.

An alternative culture system employs serum-free medium supplemented with growth factors capable of promoting the proliferation of embryonic stem cells. For example, Cheon et al (*BioReprod DOI*:10.1095/biolreprod. 105.046870, Oct. 19, 2005) disclose a feeder-free, serum-free culture system in which embryonic stem cells are maintained in unconditioned serum replacement (SR) medium supplemented with different growth factors capable of triggering embryonic stem cell self-renewal. In another example, Levenstein et al (*Stem Cells* 24: 568-574, 2006) disclose methods for the long-term culture of human embryonic stem cells in the absence of fibroblasts or conditioned medium, using media supplemented with bFGF. In still another example, US20050148070 discloses a method of culturing human embryonic stem cells in defined media without serum and without fibroblast feeder cells, the method comprising: culturing the stem cells in a culture medium containing albumin, amino acids, vitamins, minerals, at least one transferrin or transferrin substitute, at least one insulin or insulin substitute, the culture medium essentially free of mammalian fetal serum and containing at least about 100 ng/ml of a fibroblast growth factor capable of activating a fibroblast growth factor signaling receptor, wherein the growth factor is supplied from a source other than just a fibroblast feeder layer, the medium supported the proliferation of stem cells in an undifferentiated state without feeder cells or conditioned medium.

US20050233446 discloses a defined media useful in culturing stem cells, including undifferentiated primate primordial stem cells. In solution, the media is substantially isotonic as compared to the stem cells being cultured. In a given culture, the particular medium comprises a base medium and an amount of each of bFGF, insulin, and ascorbic acid necessary to support substantially undifferentiated growth of the primordial stem cells. In a further example, WO2005065354 discloses a defined, isotonic culture medium that is essentially feeder-free and serum-free, comprising: a. a basal medium; b. an amount of bFGF sufficient to support growth of substantially undifferentiated mammalian stem cells; c. an amount of insulin sufficient to support growth of substantially undifferentiated mammalian stem cells; and d. an amount of ascorbic acid sufficient to support growth of substantially undifferentiated mammalian stem cells.

In still another example, WO2005086845 discloses a method for maintenance of an undifferentiated stem cell, said method comprising exposing a stem cell to a member of the transforming growth factor-beta (TGF.beta.) family of proteins, a member of the fibroblast growth factor (FGF) family of proteins, or nicotinamide (NIC) in an amount sufficient to

maintain the cell in an undifferentiated state for a sufficient amount of time to achieve a desired result.

The pluripotent stem cells, BSC cells, neural cells (neuron cells and glial cells) and other cells described herein which are produced according to the present invention are preferably grown on a cellular support or matrix, as adherent monolayers, rather than as embryoid bodies or in suspension. In the present invention, the use of Matrigel as a cellular support for pluripotent stem cells is preferred and poly-L-ornithine laminin support for BSC cell differentiation is preferred. Cellular supports preferably comprise at least one differentiation protein. The term "differentiation protein" or "substrate protein" is used to describe a protein which is used to grow cells and/or to promote differentiation (also preferably attachment) of an embryonic stem cell or BSC cells. Differentiation proteins which are preferably used in the present invention include, for example, an extracellular matrix protein, which is a protein found in the extracellular matrix, such as laminin, tenascin, thrombospondin, and mixtures thereof, which exhibit growth promoting and contain domains with homology to epidermal growth factor (EGF) and exhibit growth promoting and differentiation activity. Other differentiation proteins which may be used in the present invention include for example, collagen, fibronectin, vibronectin, polylysine, polyornithine and mixtures thereof. In addition, gels and other materials such as methylcellulose of other gels which contain effective concentrations of one or more of these embryonic stem cell differentiation proteins may also be used. Exemplary differentiation proteins or materials which include these differentiation proteins include, for example, BD Cell-Tak™ Cell and Tissue Adhesive, BD™ FIBROGEN Human Recombinant Collagen I, BD™ FIBROGEN Human Recombinant Collagen III, BD Matrigel™ Basement Membrane Matrix, BD Matrigel™ Basement Membrane Matrix High Concentration (HC), BD™ PuraMatrix™ Peptide Hydrogel, Collagen I, Collagen I High Concentration (HC), Collagen II (Bovine), Collagen III, Collagen IV, Collagen V, and Collagen VI, among others. The preferred material for use in the present invention includes Matrigel™ and Geltrex™.

A preferred composition/material which contains one or more differentiation or substrate proteins is BD Matrigel™ Basement Membrane Matrix. This is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in ECM proteins. Its major component is laminin, followed by collagen IV, heparan sulfate, proteoglycans, entactin and nidogen.

The pluripotent stem cells are preferably plated onto the differentiation or substrate protein. The pluripotent stem cells may be plated onto the substrate in a suitable distribution and in the presence of a medium that promotes cell survival, propagation, and retention of the desirable characteristics. All these characteristics benefit from careful attention to the seeding distribution and can readily be determined by one of skill in the art.

As used herein, the term "activate" refers to an increase or upregulation of a marker associated with a pluripotent stem cell, a BSC cell or neural cell as otherwise disclosed herein. Neural cells (neurons and glial cells as otherwise described herein) have utility in treating treating numerous diseases of the central nervous system diseases such as Parkinson's disease, multiple sclerosis, Huntington's disease, amyotrophic lateral sclerosis (ALS; Lou Gehrig's disease), primary lateral sclerosis (PLS) and progressive muscular atrophy (PMA). In addition, neural lineages have the ability totreat stroke, head trauma and spinal cord injury.

As used herein when referring to a cell, cell line, cell culture or population of cells, the term "isolated" refers to being substantially separated from the natural source of the cells such that the cell, cell line, cell culture, or population of cells are capable of being cultured *in vitro*. In addition, the term "isolating" is used to refer to the physical selection of one or more cells out of a group of two or more cells, wherein the cells are selected based on cell morphology and/or the expression of various markers.

As used herein, the term "express" refers to the transcription of a polynucleotide or translation of a polypeptide (including a marker) in a cell, such that levels of the molecule are measurably higher in or on a cell that expresses the molecule than they are in a cell that does not express the molecule. Methods to measure the expression of a molecule are well known to those of ordinary skill in the art, and include without limitation, Northern blotting, RT-PCT, *in situ* hybridization, Western blotting, and immunostaining.

As used herein, the term "Markers" describe nucleic acid or polypeptide molecules that are differentially expressed in a cell of interest. In this context, differential expression means an increased level for a positive marker and a decreased level for a negative marker. The detectable level of the marker nucleic acid or polypeptide is sufficiently higher or lower

in the cells of interest compared to other cells, such that the cell of interest can be identified and distinguished from other cells using any of a variety of methods known in the art.

As used herein, the term "contacting" (i.e., contacting a cell with a compound) is intended to include incubating the compound and the cell together *in vitro* (e.g., adding the compound to cells in culture). The term "contacting" is not intended to include the *in vivo* exposure of cells to a differentiation agent that may occur naturally in a subject (i.e., exposure that may occur as a result of a natural physiological process). The step of contacting the cell with differentiation medium and one or more growth factors (e.g. FGF, EGF, IGF, etc.)and inhibitors (inhibitors of GSK, Activin A (signaling) or AMPK (signaling)) as otherwise described herein can be conducted in any suitable manner. For example, the cells may be treated in adherent culture as an adherent layer, as embryoid bodies or in suspension culture, although the use of adherent layers are preferred because they provide an efficient differentiation process oftentimes providing differentiation to a target cell population (BSC cells, neuron or glial cells) of up to 90% or more and preferably are produced where physical separation is not required. It is understood that the cells contacted with the differentiation agent may be further treated with other cell differentiation environments to stabilize the cells, or to differentiate the cells further, for example to produce neural (neuron and glial), or skin cells.

In the case of producing BSC cells from human pluripotent stem cells, the cells are differentiated in a medium as otherwise disclosed herein comprising effective amounts of a GSK3 inhibitor such as BIO, among others as described herein (about 0.1 to about 10 μM, about 1-5 μM, about 2 μM), a TGFβ/Activin A signaling inhibitor such as SB431542, (about 0.5 to about 100 μM, about 5.0 to about 50 μM, about 15-25 μM, about 20 μM) and a AMPK inhibitor such as Compound C, (about 0.05 to about 20 μM, about 0.1 to about 10 μM, about 0.5 to about 5 μM, about 1 μM), as otherwise disclosed herein.

As used herein, the term "differentiation agent" refers to any compound or molecule that induces a cell such as hPSCs (including hESC's) and BSCs to partially or terminally differentiate, wherein said differentiation is due at least in part to inhibition of GSK/GSK3, to the inhibition of TGFβ/Activan A signaling and inhibition of AMPK signaling to produce BSC cells, or the use of, for example, sonic hedgehog, TGFbeta3, ascorbic acid, cAMP and all-trans retinoic acid to produce neural cells, especially dopaminergic neurons and motor

neurons, among numerous others as described in greater detail herein. While the differentiation agent may be as described below, the term is not limited thereto. The term "differentiation agent" as used herein includes within its scope a natural or synthetic molecule or molecules which exhibit(s) similar biological activity.

The term "effective" is used to describe an amount of a component, compound or compositions which is used or is included in context in an amount and for a period sufficient to produce an intended effect, especially including the differentiation of a pre-rosette stage ectodermal progenitor cells (BSCs) to a mature cell. By way of example, an effective amount of a differentiation agent is that amount which, in combination with other components, in a differentiation medium will produce the differentiated cells desired.

The term "administration" or "administering" is used throughout the specification to describe the process by which a population of one or cells or cell-types according to the present invention are delivered to a patient for treatment purposes. Cells may be administered a number of ways including parenteral, intrathecal, intraventricular, intraparenchymal and intranigral which term allows the cells to migrate to the cite where needed.

The terms "grafting" and "transplanting" and "graft" and "transplantation" are used throughout the specification synonymously to describe the process by which cells according to the present invention are delivered to the site within the tissue or biological system (e.g. nervous system) where the cells are intended to exhibit a favorable effect, such as repairing damage to a patient's central nervous system, treating a neurodegenerative disease or treating the effects of nerve damage caused by stroke or other diseases and/or conditions for which the cells find use. Cells for use in the present invention may also be delivered in a remote area of the body, relying on cellular migration to the appropriate area in the patient's body, including the central nervous system, skin, bones, cartilage, etc. to effect transplanation.

The term "essentially" is used to describe a population of cells or a method which is at least 95+% effective, more preferably at least about 98% effective and even more preferably at least 99% effective. Thus, a method which "essentially" eliminates a given cell population, eliminates at least about 95+% of the targeted cell population, most preferably at least about 99% of the cell population.

The term "Non-tumorigenic" refers to the fact that the cells do not give rise to a neoplasm or tumor. BSC and related cells for use in the present invention are free from neoplasia and cancer.

The term "stable" when used to describe a cell population means that the population may be renewed over a number of generations (10 or more, 15 or more, 20 or more 25 or more). In addition, cells according to the present invention may be readily cryopreserved using methods which are standard in the art, thawed and renewed over a number of generations or differentiated as otherwise described herein.

The term "GSK inhibitor" is used to describe a compound which inhibits GSK (especially GSK3, including GSK3α or GSK3β). Examples of preferred GSK inhibitors for use in the present invention include one or more of the following, all available from Calbiochem:

BIO (2'*Z*,3'*E*)-6-Bromoindirubin-3'-oxime (GSK3 Inhibitor IX);

BIO-Acetoxime (2'Z,3'E)-6-Bromoindirubin-3'-acetoxime (GSK3 Inhibitor X);

(5-Methyl-1H-pyrazol-3-yl)-(2-phenylquinazolin-4-yl)amine (GSK3-Inhibitor XIII);

Pyridocarbazole-cyclopenadienylruthenium complex (GSK3 Inhibitor XV);

TDZD-8 4-Benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (GSK3β Inhibitor I);

2-Thio(3-iodobenzyl)-5-(1-pyridyl)-[1,3,4]-oxadiazole (GSK3β Inhibitor II);

OTDZT 2,4-Dibenzyl-5-oxothiadiazolidine-3-thione (GSK3β Inhibitor III);

α-4-Dibromoacetophenone (GSK3β Inhibitor VII);

AR-A014418 N-(4-Methoxybenzyl)-N'-(5-nitro-1,3-thiazol-2-yl)urea (GSK-3β Inhibitor VIII);

3-(1-(3-Hydroxypropyl)-1H-pyrrolo[2,3-b]pyridin-3-yl]-4-pyrazin-2-yl-pyrrole-2,5-dione (GSK-3β Inhibitor XI);

TWS119 pyrrolopyrimidine compound (GSK3β Inhibitor XII);

L803 H-KEAPPAPPQSpP-NH_2 or its Myristoylated form (GSK3β Inhibitor XIII); and

2-Chloro-1-(4,5-dibromo-thiophen-2-yl)-ethanone (GSK3β Inhibitor VI).

In addition, numerous wingless proteins or Wnt proteins function similar to GSK inhibitors and in particular, GSK inhibitors according to the present invention. They are therefore subsumed under the term GSK inhibitors. Exemplary Wnt proteins which may be used in the present invention include one or more of Wnt1, Wnt2, Wnt3, Wnt3a, Wnt4, Wnt10, Wnt 14, Wnt14b, Wnt15, and Wnt16, among other Wnt proteins. When a Wnt protein is used, the use of Wnt3a is preferred.

Preferred GSK inhibitors for use in the present invention include, BIO (GSK-3, Inhibitor IX).

GSK inhibitors are useful in all aspects of the invention which relate to the differentiation and formation of BSC cells. When used, they are used in effective amounts, in concentrations (depending upon the molecular weight of the inhibitors used) of about 0.001 to about 100μM or more, about 0.05 to about 75μM, about 0.1 to about 50 μM, about 0.25 to about 35 μM, about 0.5 to about 25 μM. In the case of the use of BIO, this GSK inhibitor is used in the differentiation medium in an amount ranging from about (about 0.05 to about 50 μM, about 0.1 to about 10 μM, about 1-5 μM, about 2 μM). When a Wnt protein is used, the amount of Wnt which is used ranges from about 1 to about 100 ng/ml, about 5 to about 50 ng/ml, about 10 to about 35 ng/ml, about 20 to about 30 ng/ml, about 25 ng/ml.

The term "Activin A inhibitor" or "TGFβ /ActivinA signaling inhibitor" is used to describe compounds or components which are added to a differentiation medium to inhibit the effects (singalling) of Activin A in the differentiation process and when used in combination with a GSK inhibitor and an inhibitor of AMPK signaling as otherwise described herein, produce BSCs from human pluripotent stem cells (hPSCs), including hESCs and hiPSCs. In order to produce BSCs from hPSCs, the differentiation agent comprises an effective amount of a GSK inhibitor (preferably, a GSK3 inhibitor, such as BIO or other GSK3 inhibitor), a AMPK signaling inhibitor such as Compound C and a TGFβ/ActivinA signaling inhibitor such as SB431542.

Exemplary Activin A inhibitors for use in the present invention include, for example, SB-431542 (Sigma), follistatin, follistatin gene related protein (FGRP, available from R and

D Systems), BMP and Activin Membrane Bound Inhibitor (BAMBI), anti-BAMBI (monoclonal antibody), Smad7 (Mothers Against Decapentaplegic Homolog 7) and TGF RI inhibitor (Calbiochem), among others. Activin A inhibitors are used in the present invention in effective amounts, generally within the range of about 0.001 to about 100μM or more, about 0.05 to about 75μM, about 0.1 to about 50 μM, about 1 to about 35 μM, about 5 to about 25 μM, about, about 20 μM. In preferred aspects, the TGFβ/ActivinA signaling inhibitor is SB431542.

The term "AMPK signaling inhibitor" is used to describe a differentiation agent which is combined with effective amounts of a GSK inhibitor as otherwise described herein in combination with a TGFβ/ActivinA signaling inhibitor in producing BSC cells from hPSCs according to the present invention. Exemplary AMPK signaling inhibitors include Compound C and STO-609 (available from Calbiochem). Preferably, Compound C is used to produce BSC cells according to the present invention.

The present invention relates to methods of producing an isolated population of pre-rosette stage ectodermal progenitor cells from human pluripotent stem cells (hPSCs) comprising exposing said pluripotent stem cells to a differentiation medium comprising effective amounts of a GSK inhibitor, a TGFβ/Activin A signaling pathway inhibitor and an AMPK signaling pathway inhibitor for a period of time sufficient to differentiate said stem cells to said pre-rosette stage ectodermal progenitor cells and isolating the progenitor cells. The isolated progenitor cells, which may be cryopreserved, are an additional aspect of the present invention.

Preferred Aspects and Examples of the Present Invention

Methods for generating a novel self-renewing cell type from hESCs

hESCs can be cultured as a stable self-renewing population as adherent monolayers in a defined media (DM) on Matrigel (1:10-1:1,000). Defined media is DMEM/F12, 2% Probumin (albumin; Millipore/Serologicals), 1x Pen/Strep, 1x NEAA, 1x Trace Elements A, B, C (Mediatech), 50ug/ml Ascorbic acid (Sigma), 10ug/ml Transferrin (Gibco), 0.1mM βMe (Gibco), 8ng/ml bFGF (Sigma), 200ng/ml LR-IGF (JRH Biosciences), 10ng/ml Activin A (R and D Systems) and 10ng/ml Heregulin (Peprotech).

Since the key factors required for hESC self-renewal in DM are Heregulin, Activin A, LR-IGF and Fgf2, this media is referred to as DM-HAIF. hESCs cells are Accutasepassaged as single cell suspensions at 1x106 cells/ 60mm dish (or equivalent). When hESCs are plated on Matrigel in DM lacking Activin A (DM-HIF) and in the presence of three small molecule compounds, they undergo a distinct morphological change (Figure 4). Furthermore, levels of stem cell marker transcripts and protein such as Oct4, Nanog significantly decrease, as judged by Q-RT PCR (Figure 5) and immunostaining (Figure 6,7), respectively. The morphology changes seen are maintained upon subsequent passaging and continuous culture (>30 passages; ~150 days). Typically, cells are passaged every five days in DM-HIF + inhibitors. The three small compounds referred to are GSK3 inhibitor IX, (also known as BIO [2μM]; Calbiochem, cat. #361550), SB431542 (20μM; an inhibitor of TGFβ/Activin A signaling; TOCRIS, cat. #1614) and Compound C (1μM; an inhibitor of AMPK signaling; Calbiochem, cat. # 171261). This inhibitor cocktail is referred to 'BSC' for BIO, SB431542 and Compound C. The new cell type arising from this culture is referred to as 'BSCs'. Typically, when BSCs were generated from hESCs, they were typically analyzed from P2 onwards to allow complete transition from the hESC to BSC state.

Characteristics of BSCs

To characterize BSCs in greater detail and to establish the lineage to which they were most closely associated with, we performed transcript microarray analysis, quantitative reverse transcriptase polymerase chain reaction (Q-PCR) assays (mRNA profiling), immunostaining and flow cytometry analysis to evaluate cell surface markers.

a. microarray analysis (summarized in Table 1, Figure 24)

b. Q-PCR analysis (Figures 5, 8A-C, 9A-C)

c. immunostaining (Figures 6,7,10)

d. flow cytometry (Figures 11-16, 23; summarized in Table 2, Figure 25)

2. BSCs Composition of matter

a. Global gene expression profiling (Table 1, Figure 24)

Microarray analysis was performed using GenChip® Human Genome U133 Plus 2.0 Arrays (Affymetrix). Two BSC-derived cell lines were analyzed- those derived from BG02 and WA09 hESCs. Each line was analyzed in biological triplicate (different passages of the same cell line). A list of transcripts which are significantly elevated in BSCs relative to hESCs genes is shown in Table 1. Several transcripts indicative of an ectoderm type cell were observed, based on transcript profiling. In particular, the expression profile indicated that BSCs are an early ectodermal cell type with characteristics consistent with them being a neural progenitor-like cell. To establish the identity of the aforementioned cell population (BSCs), microarray analysis was performed on treated cells generated from BG02 and WA09 hESC lines. Multiple passages of these cells were analyzed to obtain a comprehensive profile of this cell type. The analysis revealed that multiple genes associated with neural lineages were up-regulated in BSCs, relative to the parental hESC line (Table 1, Figure 24). Up-regulated genes include neuronal cell adhesion molecule (NRCAM), neurotrophic tyrosine kinase receptor 2 (NTRK2, Trk-B), synaptotagmin XI, N-Oct3 (Brain-2), hairy and enhancer of split (HES5), neurogenic differentiation 1 (NEUROD1) and PAX6.

b. Q-PCR (Figures 5, 8A-C, 9A-C)

BSCs do not express marker transcripts for hESCs (Nanog, Oct4; see Figure 5), mesoderm (FOXF1, MEOX1), definitive or extra-embryonic endoderm (SOX17, THBD, AFP, GSC, FOXA2). Sox1 mRNA was sometimes up-regulated in some later passage BSC cultures (WA09), but this was not consistently observed for all lines generated from different hESCs (ie BG02)- Figures 8,. Expression of Sox1 represented contamination with neural rosettes (Figure 10C) indicating that BSC can generate rosettes under some conditions. BSCs are therefore, pre-rosette stage ectoderm progenitors. Furthermore, Otx2 mRNA levels decrease significantly as hESCs transition to BSCs. The gene expression profile is therefore

distinctly different to any previously described neural progenitor, neural stem cell derived from pluripotent stem cells or neural stem/progenitors previously characterized in vivo. BSCs retained elevated Sox2 transcript levels- this marker is also expressed in pluripotent hESCs. Transcript levels for Zic1 are also elevated in BSCs.

c. Immunostaining (Figures 6,7,10,22)

Unlike hESCs, BG02 and WA09-derived BSCs do not express pluripotent markers such as Oct4 and Nanog (Figure 6,7). Immunostaining revealed that ~40-50% of cells in BSC cultures were Pax6+ (Figure 10) and ~20-30% β-tubulin positive (Figure 22).

d. Flow cytometry (Figures 11-16, 23).

Flow cytometry analysis was used to define a characteristic cell surface profile for BSCs generated form hESCs and human iPSCs. This analysis if summarized in Table 2, Figure 25.

In summary, the BSCs were generated from multiple hESC lines (WA01, 07, 09 and BG02) as well as iPSCs. Common to all BSC lines generated was the absence of the hESC markers SSEA3,4 (Figure 23, Table 2-Figure 25). They all expressed the early neural markers CD90 and CD56 (N-cam). In addition, greater than 65% of the cells expressed Trk-B and CXCR4 (Figures 11-16, and Table 2- Figure 25). The absence of CXCR4 on iPSCs derived BSCs is under investigation.

3. Utility of BSCs: *BSCs are neural progenitors that differentiate into cranial and spinal cord neurons*

To establish the ability of BSCs to generate cranial and spinal cord neurons we treated them with factors under conditions that have been reported to support differentiation of hESCs to these cell lineages.

1. dopaminergic neurons (modification of the method described by Cho et al., PNAS, 105, 3392-3397) -see Figure 17

Stage 1: BSCs were plated in neurobasal medium + N2 supplements, penicillin, streptomycin, L-glutamine, Fgf2 (20ng/ml), EGF (10ng/ml) for five days on poly-Lornithine

laminin coated plates.

Stage 2: Cells were then split onto poly-L-ornithine laminin coated plates in neurobasal media, N2 supplements, penicillin, streptomycin, L-glutamine, sonic hedgehog (SHH, 200ng/ml), TGFbeta3 (1ng/ml) for 4 days.

Stage 3: Media changed to neurobasal media, N2 supplements, penicillin, streptomycin, L-glutamine, SHH (200ng/ml), Fgf8 (100ng/ml), TGFbeta3 (1ng/ml), ascorbic acid (200microM) for 6 days.

Following this three stage differentiation regimen, BSCs were converted to a population of ~50% tyrosine hydroxylase positive (TH+) dopaminergic neurons (Figure 18-21). Cells differentiated along this pathway did not express motor neuron markers such as HB9 but ~30% of cells expressed β-tubulin (Figure 22).

2. motor neuron differentiation (modification of the method described by Li et al., 2005, Nat Biotech 23: 215-221)- see Figure 17

Stage 1: BSCs were plated in neurobasal medium + N2 supplements, penicillin, streptomycin, L-glutamine, Fgf2 (20ng/ml), EGF (20ng/ml) for five days on poly-Lornithine laminin coated plates.

Stage 2: Cells were then split onto poly-L-ornithine laminin coated plates in neurobasal media, N2 supplements, penicillin, streptomycin, L-glutamine, cAMP (1microM), alltrans retinoic acid (RA; 0.1microM) for 7 days.

Stage 3: media changed to in neurobasal media, N2 supplements, penicillin, streptomycin, L-glutamine, cAMP (1microM), RA (0.1microM), SHH (200ng/ml) for 14 days.

Stage 4: Media changed to neurobasal media, N2 supplements, penicillin, streptomycin, L-glutamine, cAMP (1microM), RA (0.1microM), SHH (50ng/ml), GDNF (10ng/ml), IGF (10ng/ml) for 7-10 days.

Following this four stage differentiation regimen ~40% of cells expressed the motor neuron marker HB9. The dopamineric neuron marker TH, was not detected by immunostaining in these cultures. ~30% of cells in these cultures expressed β-tubulin (Figure 22)

Claims:

1. A method of differentiating pre-rosette stage ectoderm progenitor cells (BSC cells) to mature cells selected from the group consisting of neural cells, glia cells, ectoderm progenitor cells, skin cells, neural crest cells, neural crest derivative cells, peripheral neurons, Schwann cells, smooth muscle cells, adiogenic (fat) cells, osteogenic (bone) cells, myogenic cells and chondrogenic (cartilage) cells comprising exposing said pre-rosette stage ectoderm progenitor cells to a differentiation medium comprising an effective amount of at least one differentiation agent effective to differentiate said ectoderm progenitor cells to said mature cells.

2. The method according to claim 1 wherein said ectoderm progenitor cells are isolated before differentiation.

3. The method according to claim 1 or 2 wherein said ectoderm progenitor cells are purified before differentiation.

4. The method according to claim 1 wherein said ectoderm progenitor cells are differentiated to neural cells.

5. The method according to claim 1-3 wherein said neural cells are dopaminergic neurons.

6. The method according to any of claims 1-3 wherein said pre-rosette stage ectoderm progenitor cells are differentiated to isolated dopaminergic neurons by exposing said progenitor cells to effective amounts of Fgf2 and EGF in a neurobasal medium in culture in a first stage, effective amounts of sonic hedgehog and TGFbeta in neurobasal medium in culture in a second stage and effective amounts of sonic hedgehog, Fgf8, TGFbeta and ascorbic acid in a neurobasal medium in a third stage to produce dopaminergic neurons followed by isolating said dopaminergic neurons from said culture.

7. The method according to any of claims 1-3 wherein said pre-rosette stage ectoderm progenitor cells are differentiated to isolated motor neurons.

8. The method according to any of claim 1-3 wherein said pre-rosette stage ectoderm progenitor cells are differentiated to isolated motor neurons by exposing said progenitor cells to effective amounts of Fgf2 and EGF in a neurobasal medium in culture in a first stage, effective amounts of cAMP and all-trans retinoic acid in a neurobasal medium in culture in a second stage, effective amounts of cAMP, all-trans-retinoic acid and sonic hedgehog in a neurobasal medium in culture in a third stage and effective amounts of cAMP, all-trans retinoic acid, and sonic hedgehog in a neurobasal medium in culture in a fourth stage to produce motor neurons, followed by isolating said motor neurons from said culture.

9. The method according to any of claims 1-3 wherein said pre-rosette stage ectoderm progenitor cells are differentiated to neural crest cells.

10. The method according to any of claims 1-3 wherein said pre-rosette stage ectoderm progenitor cells are differentiated to neural crest cells by exposing said progenitor cells to effective amounts of ascorbic acid, sonic hedgehog, Fgf8 and BCNF in a neurobasal medium in culture in a first stage and effective amounts of ascorbic acid, Fgf2, ascorbic acid, BDNF, and optionally, one or more of Wnt3a or Wnt1A, BMP4, FGF8, retinoic acid, sonic hedgehog, SU5402, Dkk1 and Noggin in a neurobasal medium in culture in a second stage to produce neural crest cells, followed by maintaining and/or isolated said neural crest cells.

11. The method according to claim 9 or 10 wherein said neural crest cells are further differentiatied into a cell selected from the group consisting of peripheral nerve cells, Schwann cells, adipogenic cells, chondrogenic cells, osteogenic cells and myogenic cells.

12. The method according to any of claims 1-3 wherein said pre-rosette ectoderm progenitor cells are differentiated to neural crest derivative cells.

13. The method according to claim 11 wherein said neural crest derivative cells is a peripheral nerve cell which is produced by differentiating neural crest cells in effective amounts of BDNF, GDNF, dbcAMP (in the absence of Fgf2 and EGF) in a neurobasal medium in culture to produce peripheral nerve cells which are optionally isolated from culture.

14. The method according to claim 11 wherein said neural crest derivative cells is a Schwann cell which is produced by differentiating neural crest cells in effective amounts of CNTF, neuregulin, Fgf2 and dbcAMP in a differentiation medium in culture to produce Schwann cells which are optionally isolated from culture.

15. The method according to claim 11 wherein said neural crest derivative cells is differentiated to mesenchymal lineage cells in αMEM in fetal calf serum.

16. The method according to claim 15 wherein said mesenchymal lineage cells are further differentiated to adipogenic cells by exposing said mesenchymal lineage cells in culture to effective amounts of dexamethasone, insulin and isobuytylxanthine in a differentiation medium to produce adipogenic cells which are optionally separated from said culture.

17. The method according to claim 15 wherein said mesenchymal lineage cells are further differentiated to adipogenic cells by exposing said mesenchymal lineage cells in culture to effective amounts of dexamethasone, insulin and isobuytylxanthine in a differentiation medium to produce adipogenic cells which are optionally separated from said culture.

18. The method according to claim 15 wherein said mesenchymal lineage cells are further differentiated to chondrogenic cells by exposing said mesenchymal lineage cells in culture to effective amounts of TGFbeta and ascorbic acid in in a differentiation medium to produce chondrogenic cells which are optionally separated from said culture.

19. The method according to claim 15 wherein said mesenchymal lineage cells are further differentiated to osteogenic cells by exposing said mesenchymal lineage cells in culture to effective amounts of beta-glycerophosphate, dexamethasone, and ascorbic acid in a differentiation medium to produce osteogenic cells which are optionally separated from said culture.

20. The method according to claim 15 wherein said mesenchymal lineage cells are further differentiated to myogenic cells (cardiac myocites) by exposing said mesenchymal lineage cells in culture to α-MEM for about 2-3 weeks followed by N2 medium to produce myogenic cells which are optionally separated from said culture.

21. The method according to claim 1 wherein said ectoderm progenitor cells are further differentiated into skin cells.

22. A method of treating a neurodegenerative disease in a patient comprising administering to said patient, optionally in the presence of a differentiation agent, an effective amount of pre-rosette stage ectoderm progenitor cells or neural cells, prepared from said pre-rosette stage ectoderm progenitor cells.

23. The method according to claim 22 wherein said neurodegenerative disease is Parkinson's disease, multiple sclerosis, Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis (ALS; Lou Gehrig's disease), primary lateral sclerosis (PLS) or progressive muscular atrophy (PMA).

24. The method according to claim 22 or 23 wherein neural cells are administered to said patient.

25. The method according to any of claims 22-24 wherein said administering occurs via transplantation.

26. The method according to any of claims 22-24 wherein said administering occurs by injection of neural cells into neural tissue.

27. A method of treating a patient for neurological damage which occurs secondary to stroke, head trauma or spinal cord injury, comprising administering to said patient, optionally in the presence of at least one differentiation agent, an effective amount of pre-rosette stage ectoderm progenitor cells or neural cells prepared from said pre-rosette stage ectoderm progenitor cells.

28. The method according to claim 27 wherein said injury occurs secondary to stroke.

29. The method according to claim 27 wherein said injury occurs secondary to head trauma.

30. The method according to claim 14 wherein said injury occurs secondary to spinal cord injury.

31. A method of treating an injury in a patient wherein said injury is a spinal cord injury comprising administering to said patient, optionally in the presence of at least one differentiation agent, an effective amount of pre-rosette stage ectoderm progenitor cells or neural cells prepared from said pre-rosette stage ectoderm progenitor cells.

32. A method of treating a patient suffering from an osteogenic or chondrogenic disease or injury comprising administering to said patient, in the presence of a differentiation agent, an effective amount of pre-rosette stage ectoderm progenitor cells or osteogenic or chondrogenic cells prepared from said pre-rosette stage ectoderm progenitor cells.

33. The method according to claim 32 wherein said patient has a bone disease and is administered osteogenic cells.

34. The method according to claim 32 wherein said patient has damaged bones and is administered osteogenic cells.

35. The method according to claim 32 wherein said patient has cartilage damage and is administered chondrogenic cells.

36. A method of treating a patient for damage to the patient's skin comprising administering to said patient, optionally in the presence of a differentiation agent, an effective amount of pre-rosette stage ectoderm progenitor cells or skin cells prepared from said pre-rosette stage ectoderm progenitor cells.

37. The method according to claim 36 which is a cosmetic procedure.

38. A method of treating a patient for motor neuron disease comprising administering to said patient, optionally in the presence of a differentiation agent, an effective amount of pre-rosette stage ectoderm progenitor cells or neural prepared from said pre-rosette stage ectoderm progenitor cells.

39. Use of an effective amount of pre-rosette stage ectoderm progenitor cells or neural cells prepared from said pre-rosette stage ectoderm progenitor cells, optionally in the presence of at least one differentiation agent, in the manufacture of a medicament for treating a neurodegenerative disease in a patient.

40. Use according to claim 39 wherein said neurodegenerative disease is Parkinson's disease, multiple sclerosis, Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis (ALS; Lou Gehrig's disease), primary lateral sclerosis (PLS) or progressive muscular atrophy (PMA).

41. Use according to claim 39 or 40 wherein said medicament comprises neural cells.

42. Use according to any of claims 39-41 wherein said medicament is adapted for administration via transplantation.

43. Use according to any of claims 39-41 wherein said medicament is adapted for administration by injection of neural cells into neural tissue.

44. Use of an effective amount of pre-rosette stage ectoderm progenitor cells or neural cells prepared from said pre-rosette stage ectoderm progenitor cells, optionally in the presence of at least one differentiation agent, in the manufacture

of a medicament for treating a patient for neurological damage which occurs secondary to stroke, head trauma or spinal cord injury.

45. Use according to claim 44 wherein said injury occurs secondary to stroke.

46. Use according to claim 44 wherein said injury occurs secondary to head trauma.

47. Use according to claim 46 wherein said injury occurs secondary to spinal cord injury.

48. Use according to claim 46 wherein said injury is a spinal cord injury.

49. Use of an effective amount of pre-rosette stage ectoderm progenitor cells or neural cells prepared from said pre-rosette stage ectoderm progenitor cells, optionally in the presence of at least one differentiation agent, in the manufacture of a medicament for treating a patient suffering from an osteogenic or chondrogenic disease or injury.

50. Use according to claim 49 wherein said patient has a bone disease and is administered osteogenic cells.

51. Use according to claim 49 wherein said patient has damaged bones and is administered osteogenic cells.

52. Use according to claim 49 wherein said patient has cartilage damage and is administered chondrogenic cells.

53. Use of an effective amount of pre-rosette stage ectoderm progenitor cells or neural cells prepared from said pre-rosette stage ectoderm progenitor cells, optionally in the presence of at least one differentiation agent, in the manufacture of a medicament for treating a patient for damage to the patient's skin.

54. Use according to claim 53 wherein said medicament is used in a cosmetic procedure.

55. Use of an effective amount of pre-rosette stage ectoderm progenitor cells or neural cells prepared from said pre-rosette stage ectoderm progenitor cells, optionally in the presence of at least one differentiation agent, in the manufacture of a medicament for treating a patient for motor neuron disease.

Figure 1

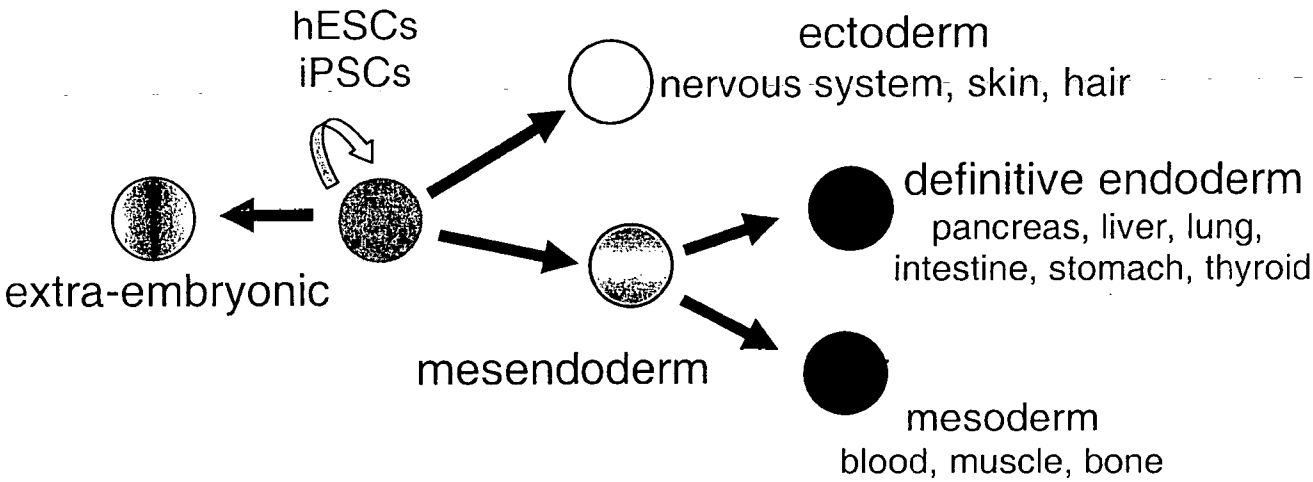

hESCs
iPSCs
ectoderm
nervous system, skin, hair
definitive endoderm
pancreas, liver, lung,
intestine, stomach, thyroid
extra-embryonic
mesendoderm
mesoderm
blood, muscle, bone

Figure 2

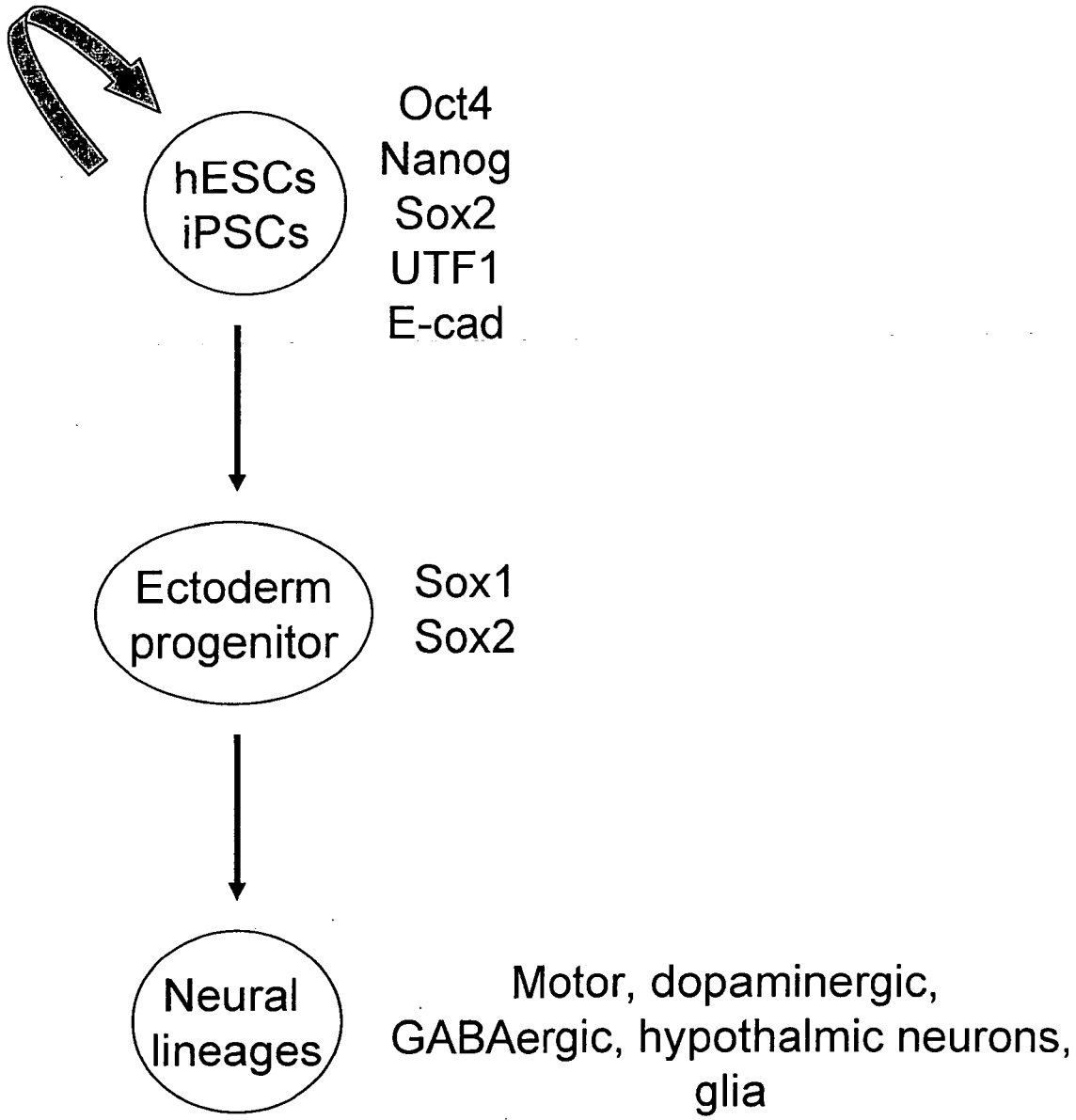

hESCs
iPSCs
Oct4
Nanog
Sox2
UTF1
E-cad
Ectoderm
progenitor
Sox1
Sox2
Neural
lineages
Motor, dopaminergic,
GABAergic, hypothalmic neurons,
glia

Figure 3

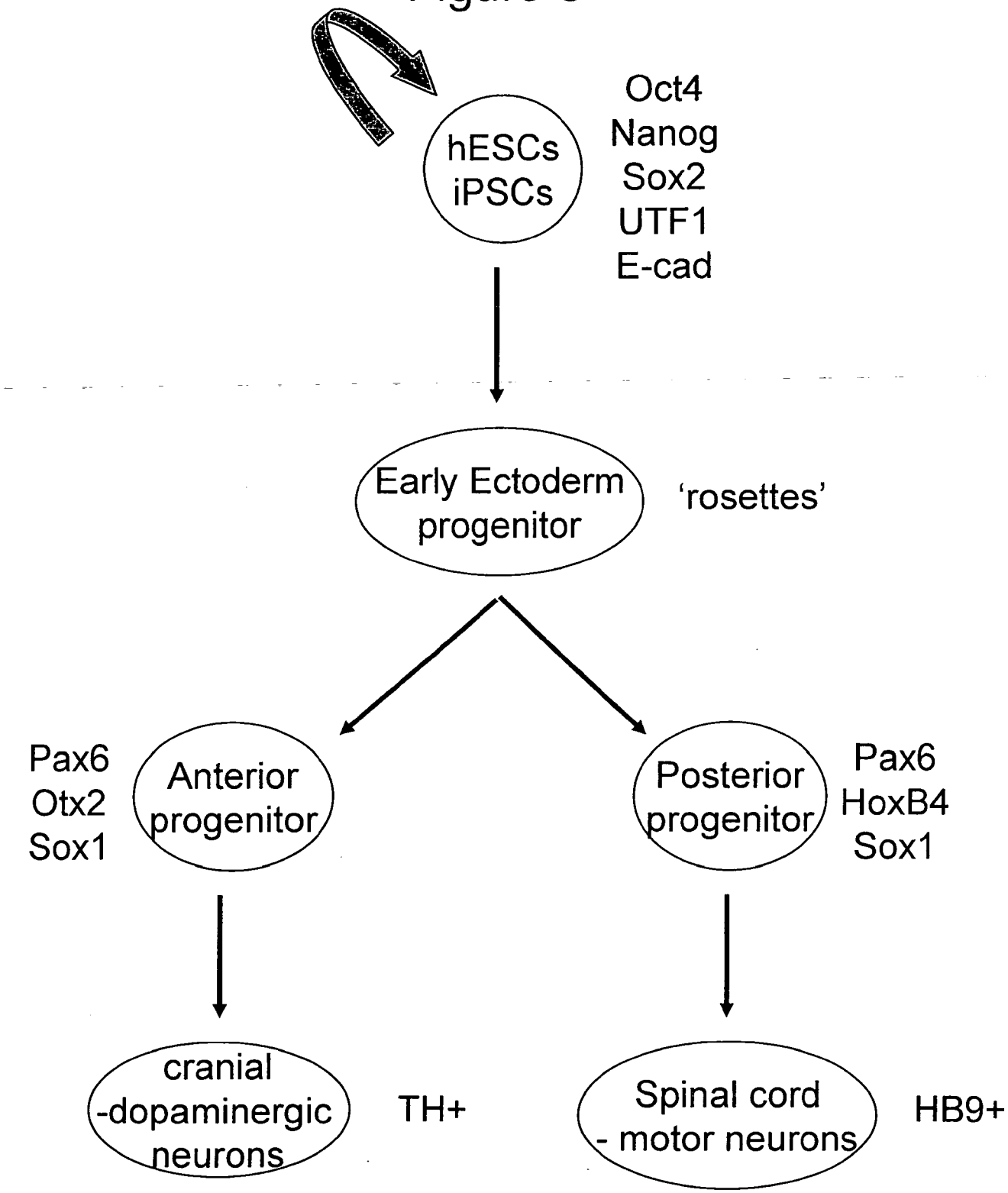

hESCs
iPSCs
Oct4
Nanog
Sox2
UTF1
E-cad
Early Ectoderm
progenitor
'rosettes'
Pax6
Otx2
Sox1
Anterior
progenitor
Posterior
progenitor
Pax6
HoxB4
Sox1
cranial
-dopaminergic
neurons
TH+
Spinal cord
- motor neurons
HB9+

Figure 4

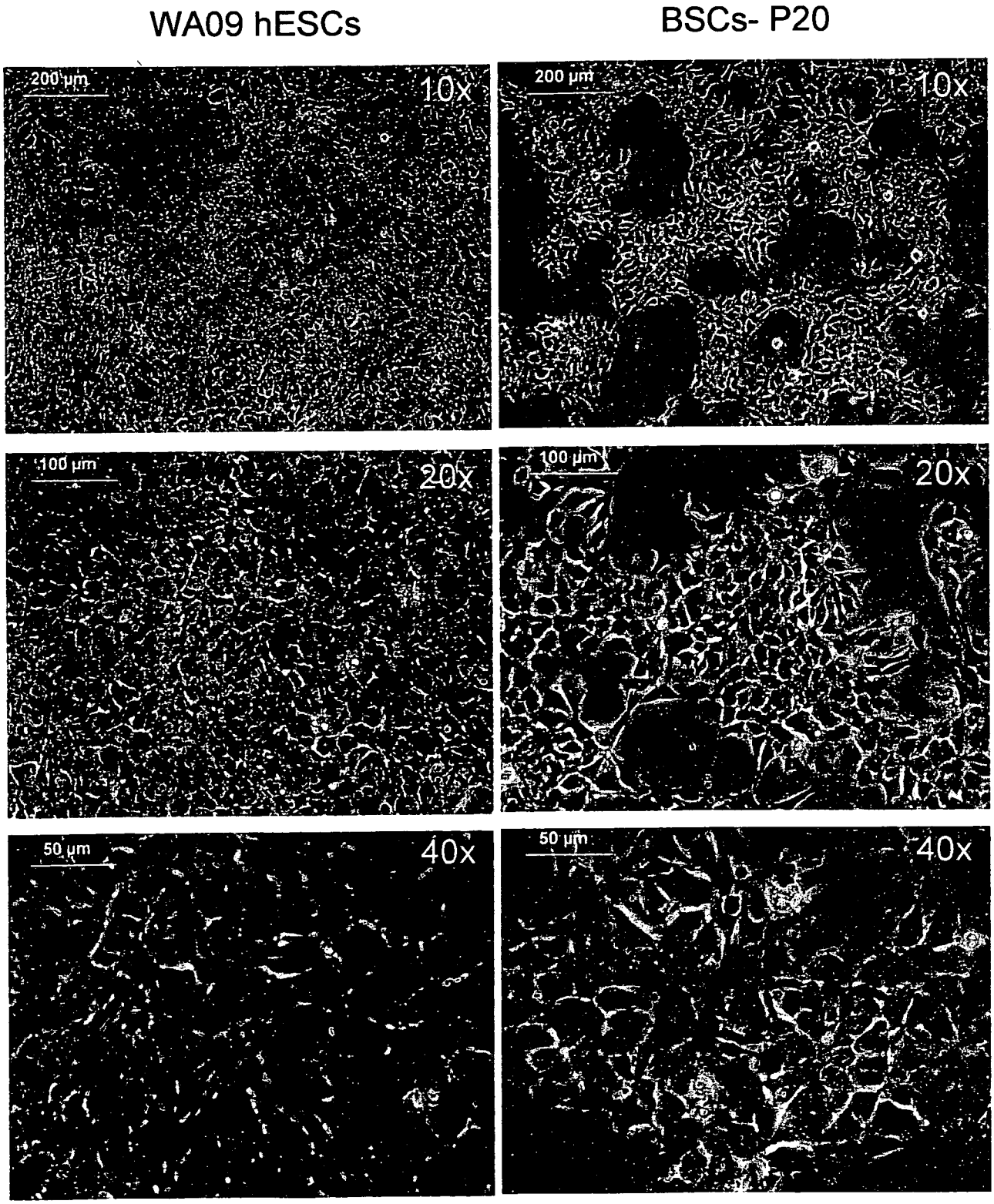
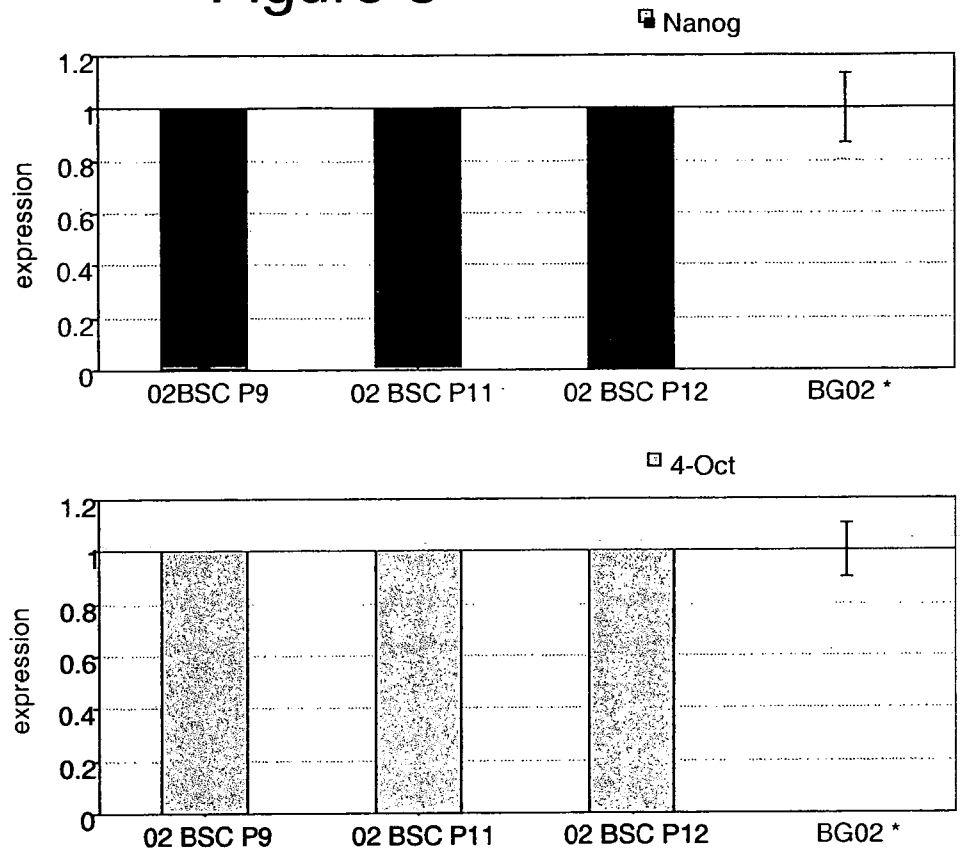

WA09 hESCs
BSCs- P20
200 µm
10x
200 µm
10x
100 µm
20x
100 µm
20x
50 µm
40x
50 µm
40x

Figure 5

BG02 hESCs

BSCs



WA09 hESCs

BSCs

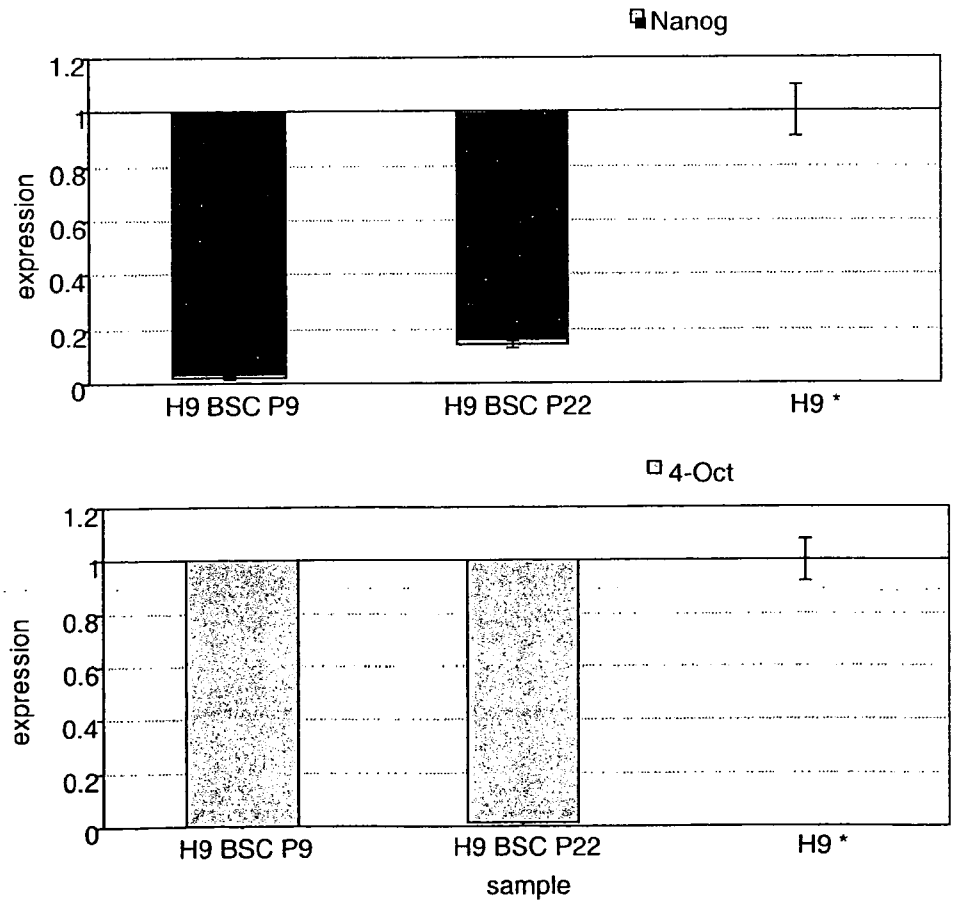


Figure 6

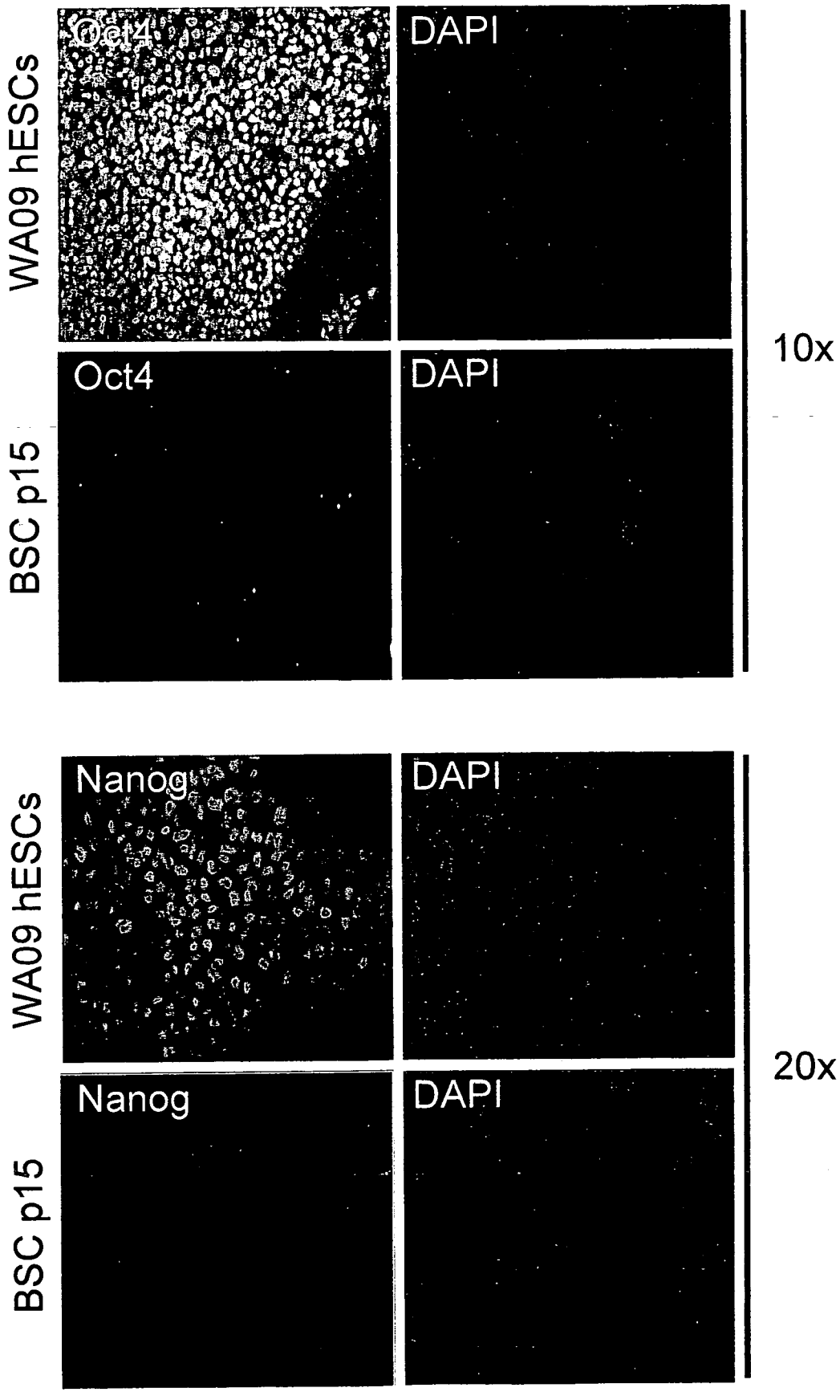

WA09 hESCs
Oct4
DAPI
BSC p15
Oct4
DAPI
10x
WA09 hESCs
Nanog
DAPI
BSC p15
Nanog
DAPI
20x

Figure 7

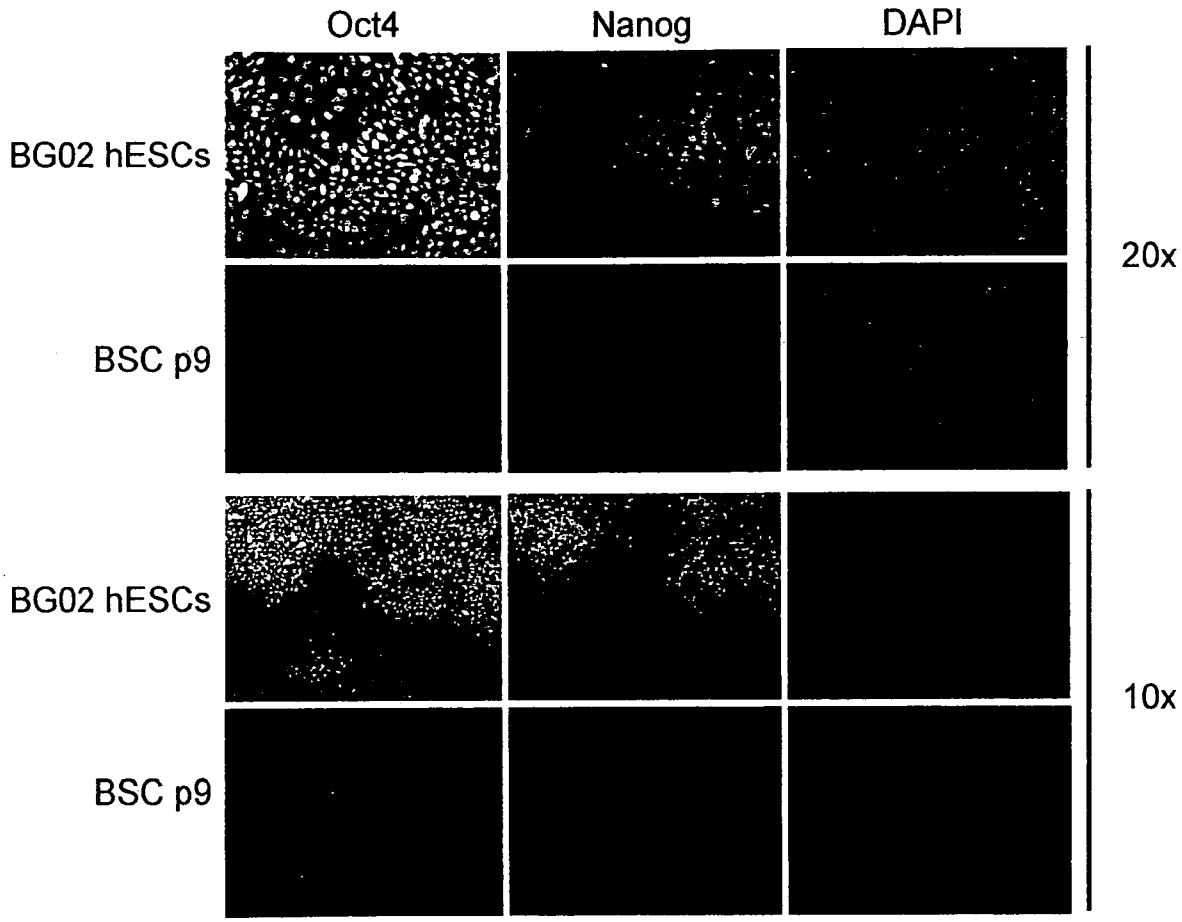


Figure 8A

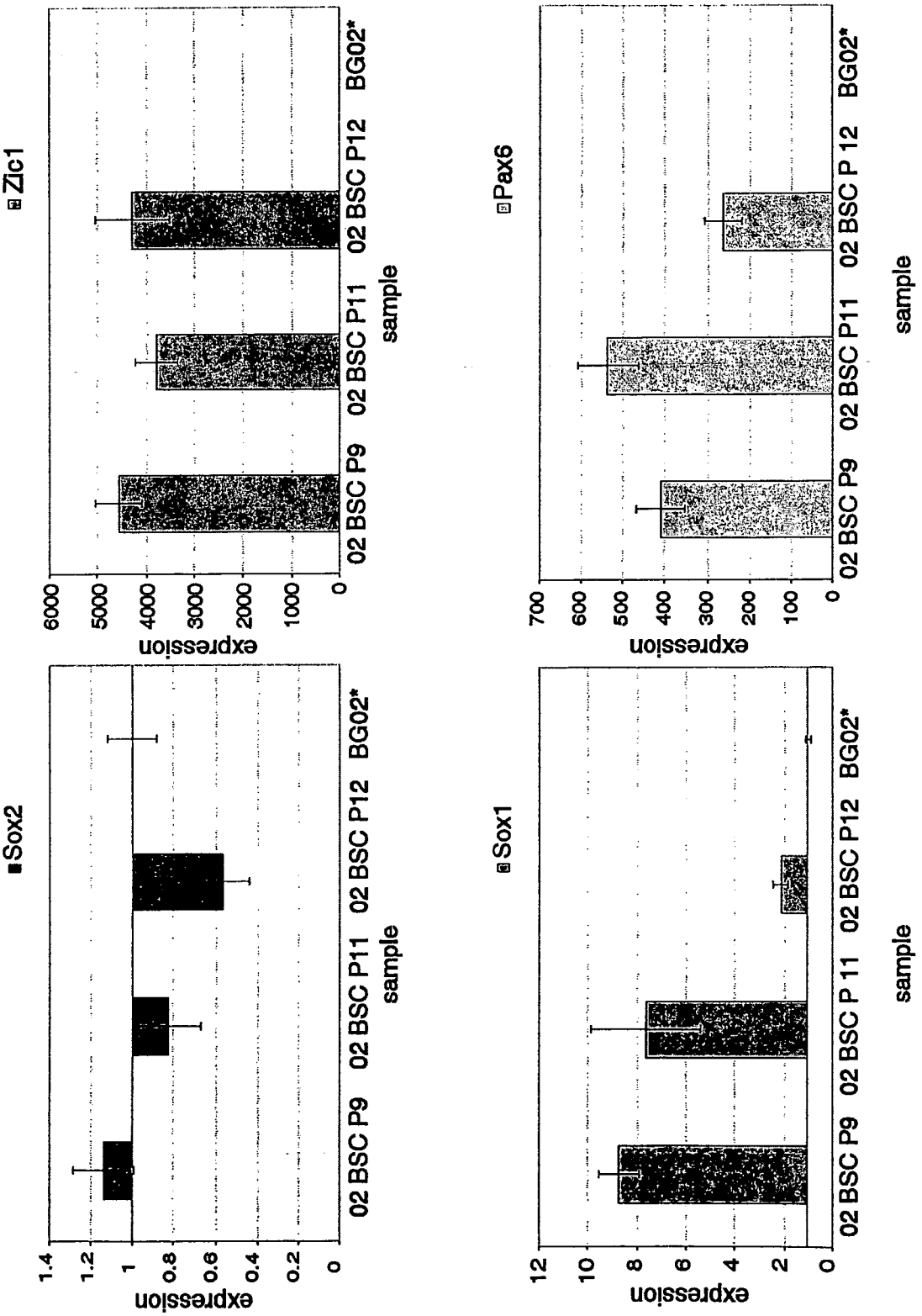
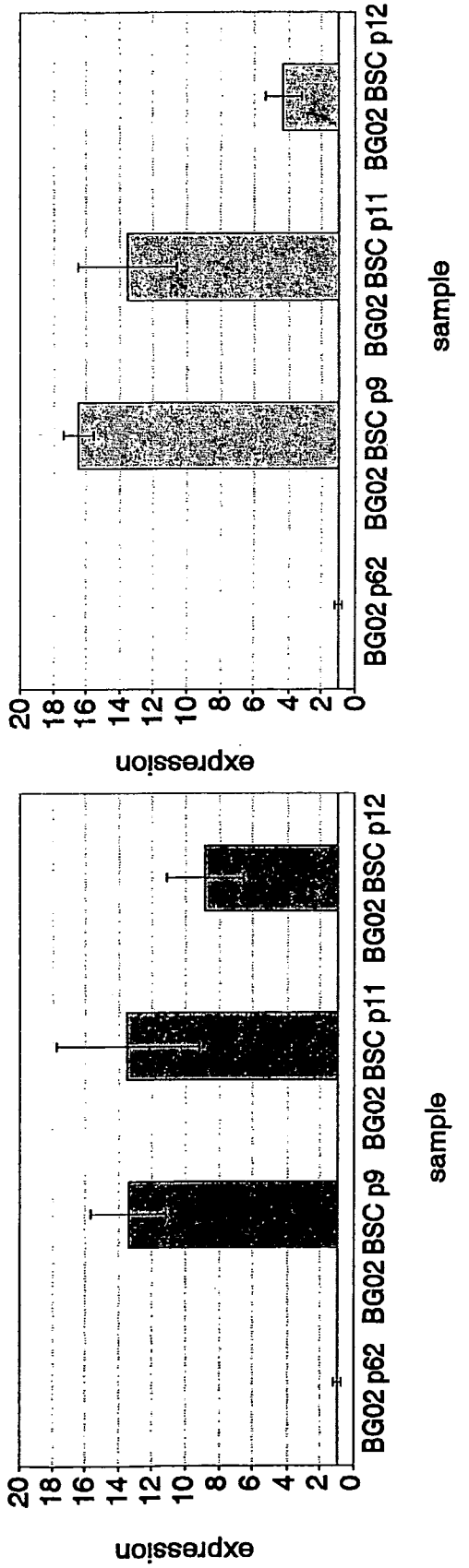

Zic1
Pax6
Sox2
Sox1
expression
sample
02 BSC P9
02 BSC P11
02 BSC P12
BG02*
02 BSC P 11
02 BSC P 12

Figure 8A (cont'd)



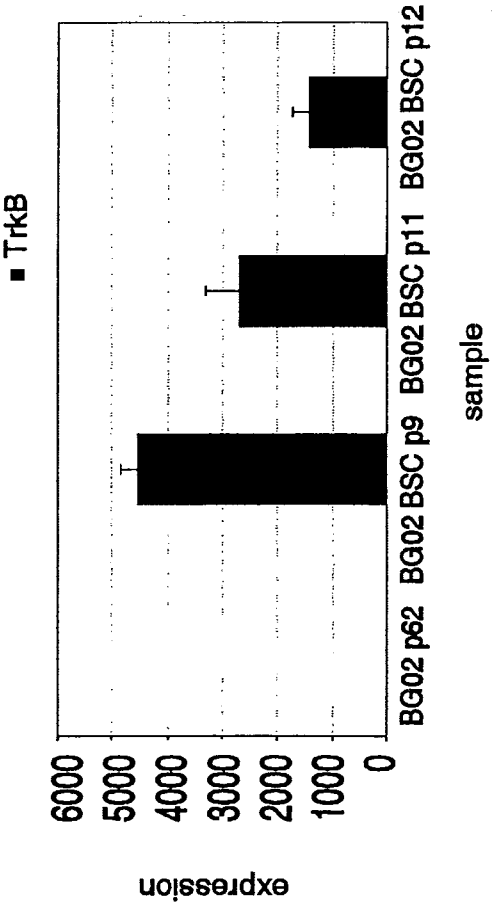


FIGURE 8B

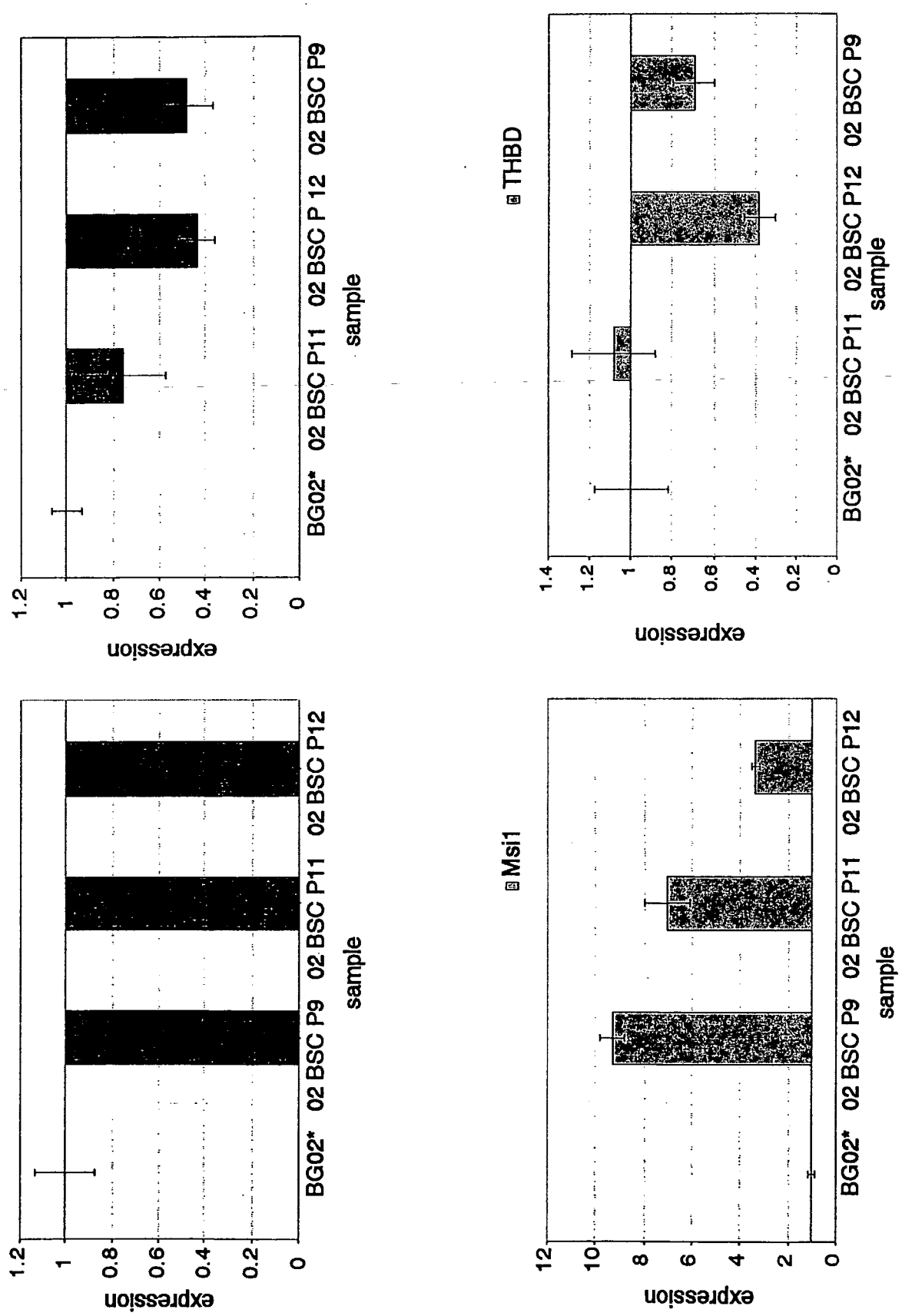

Sox17
expression
1.2
1
0.8
0.6
0.4
0.2
0
BG02*
02 BSC P9
02 BSC P11
02 BSC P 12
02 BSC P9
sample
THBD
expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
BG02*
02 BSC P9
02 BSC P11
02 BSC P12
02 BSC P9
sample
Otx2
expression
1.2
1
0.8
0.6
0.4
0.2
0
BG02*
02 BSC P9
02 BSC P11
02 BSC P12
sample
Msi1
expression
12
10
8
6
4
2
0
BG02*
02 BSC P9
02 BSC P11
02 BSC P12
sample

Figure 8B (Cont'd)

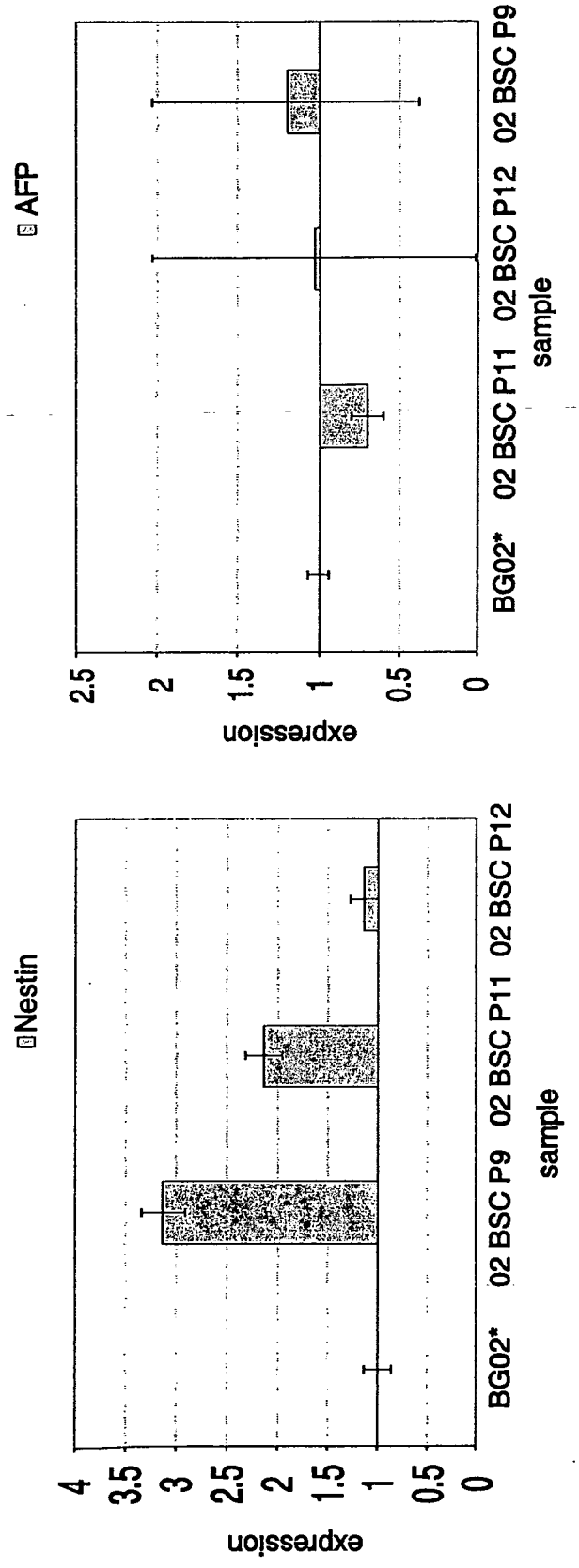


Figure 8C

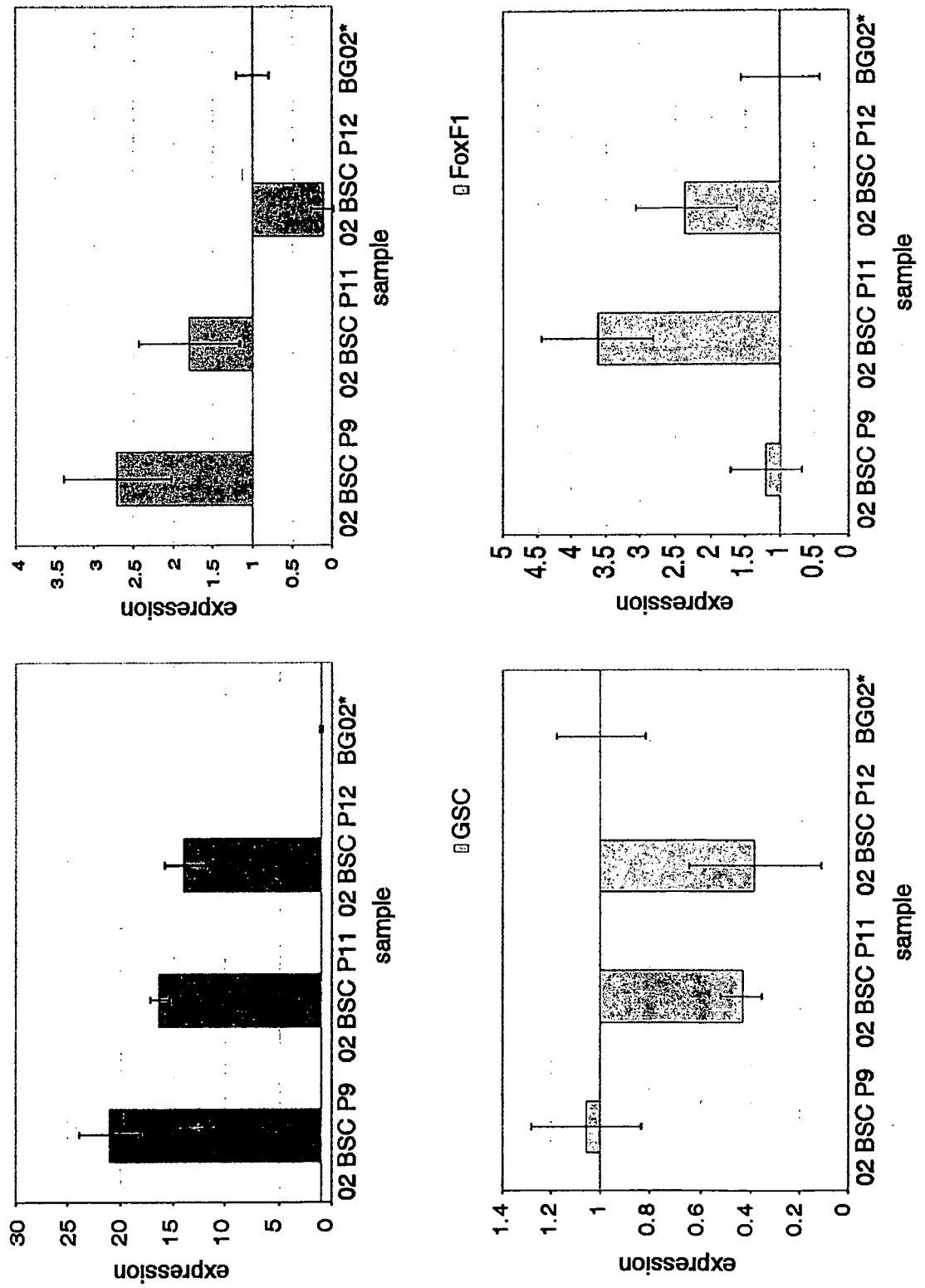

Meox1
expression
sample
02 BSC P9
02 BSC P11
02 BSC P12
BG02*
FoxF1
CXCR4
GSC

Figure 8C (Cont'd)

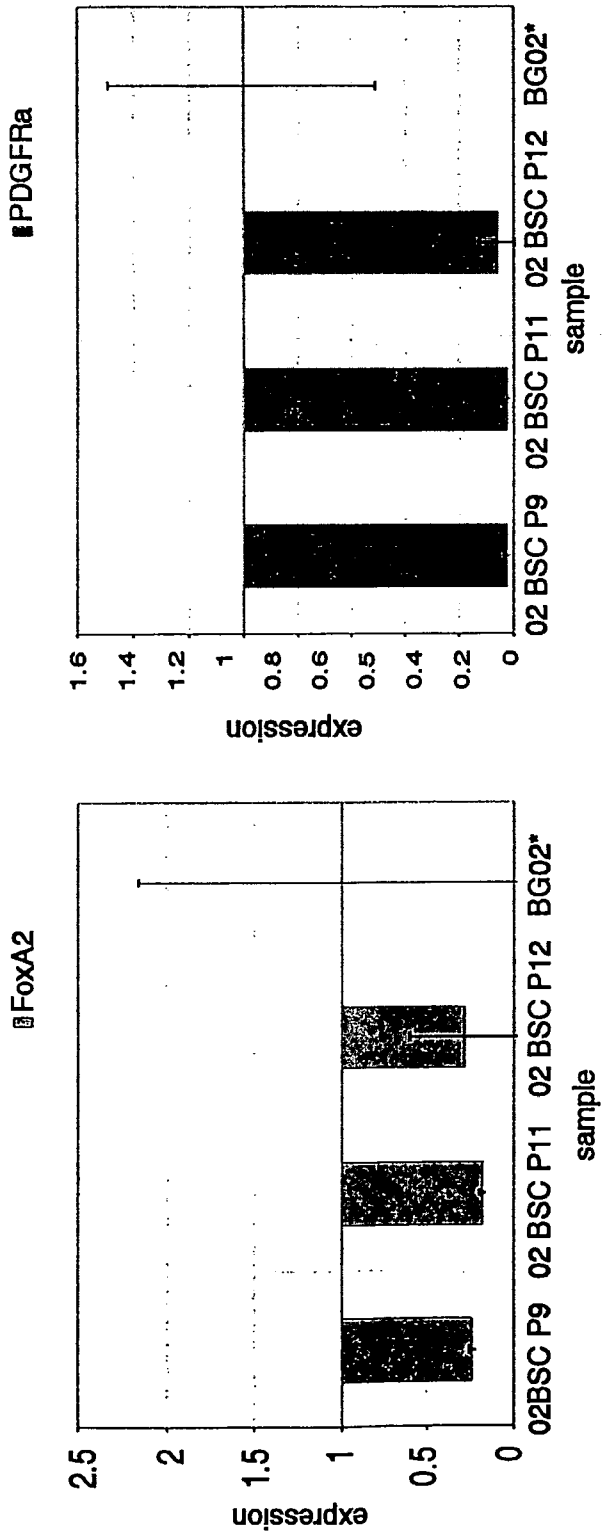


Figure 9A (Con't)

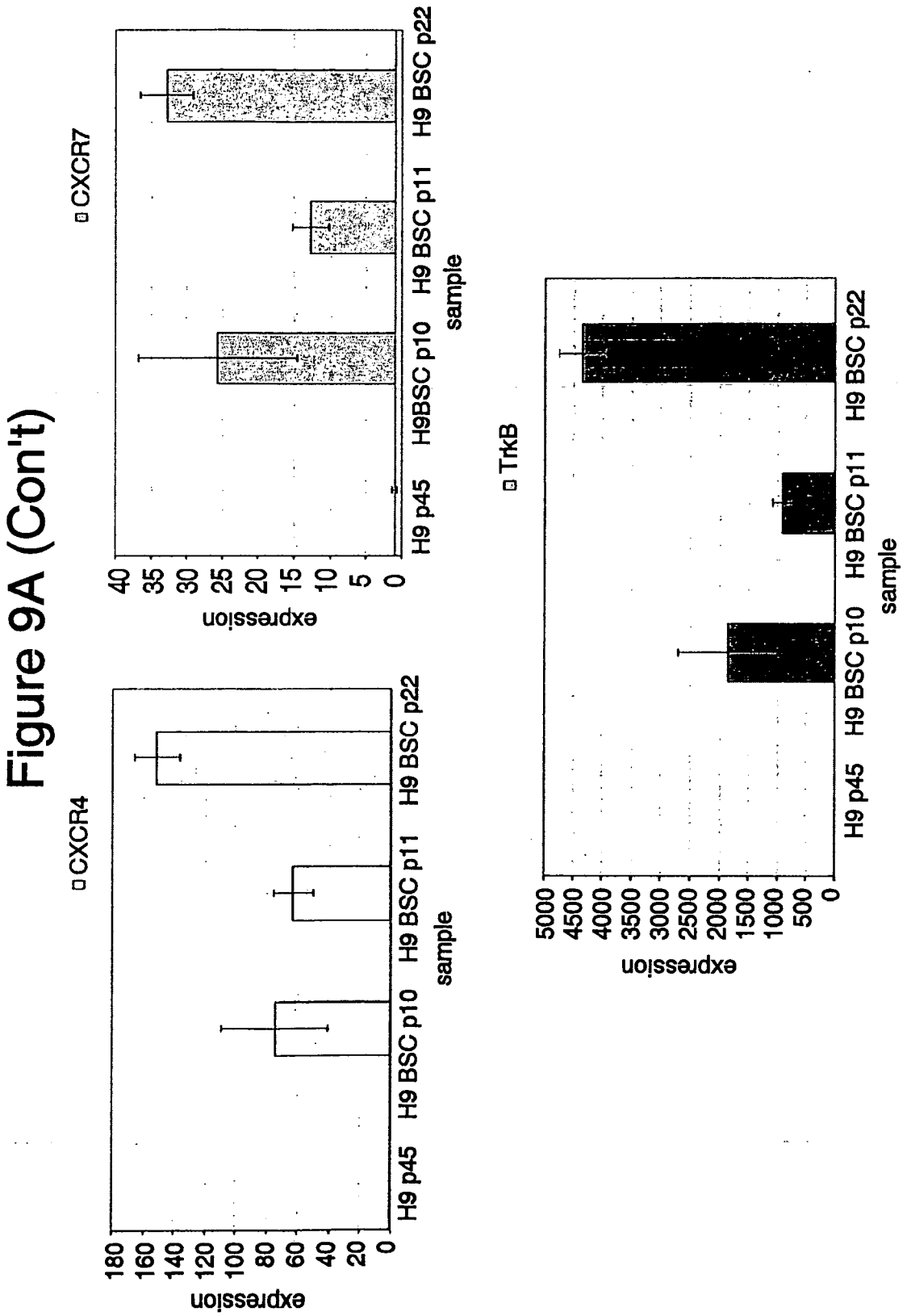


Figure 9B

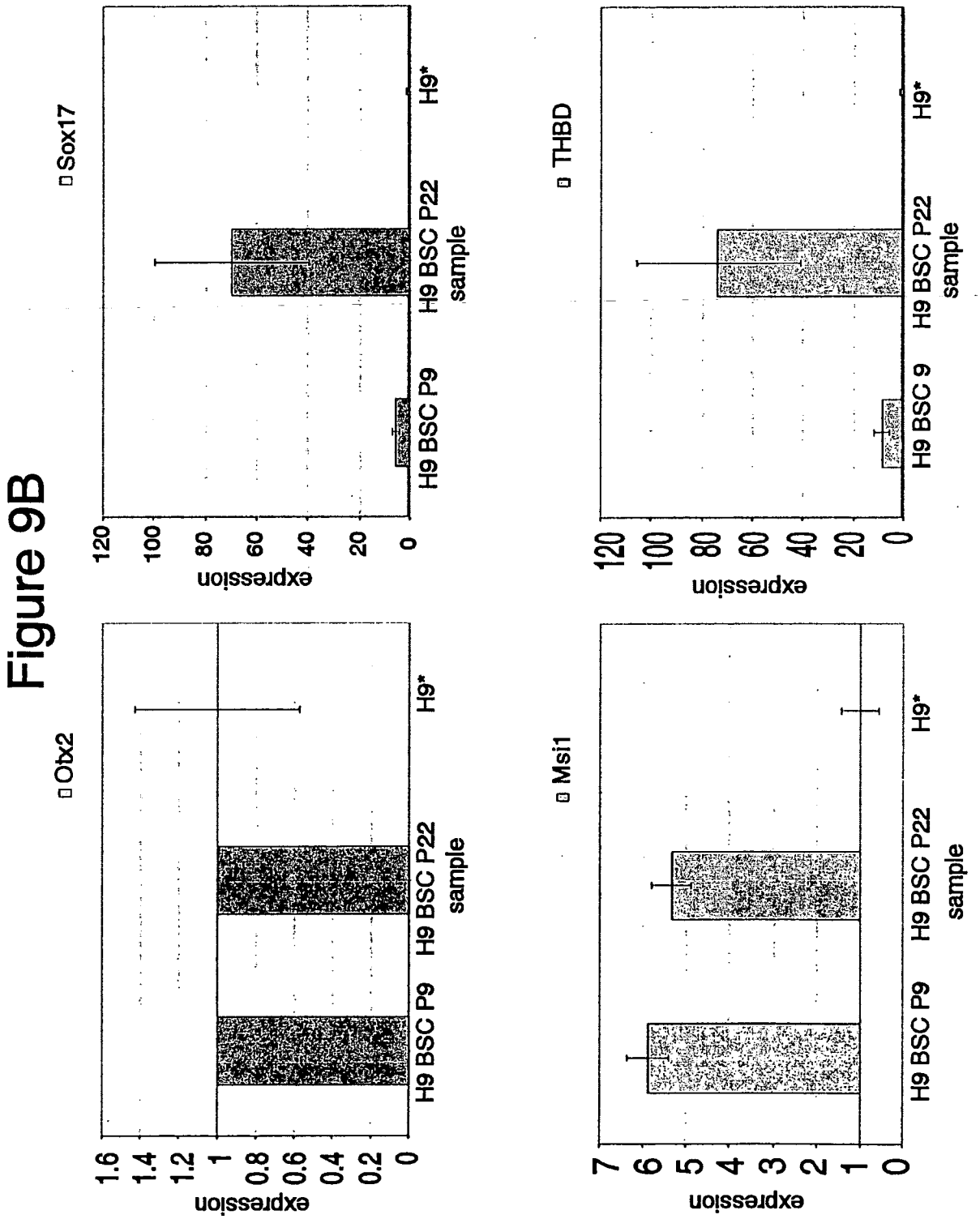

Sox17
expression
120
100
80
60
40
20
0
H9 BSC P9
H9 BSC P22
H9*
sample
THBD
expression
120
100
80
60
40
20
0
H9 BSC 9
H9 BSC P22
H9*
sample
Obx2
expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
H9 BSC P9
H9 BSC P22
H9*
sample
Msi1
expression
7
6
5
4
3
2
1
0
H9 BSC P9
H9 BSC P22
H9*
sample

Figure 9B (Con't)

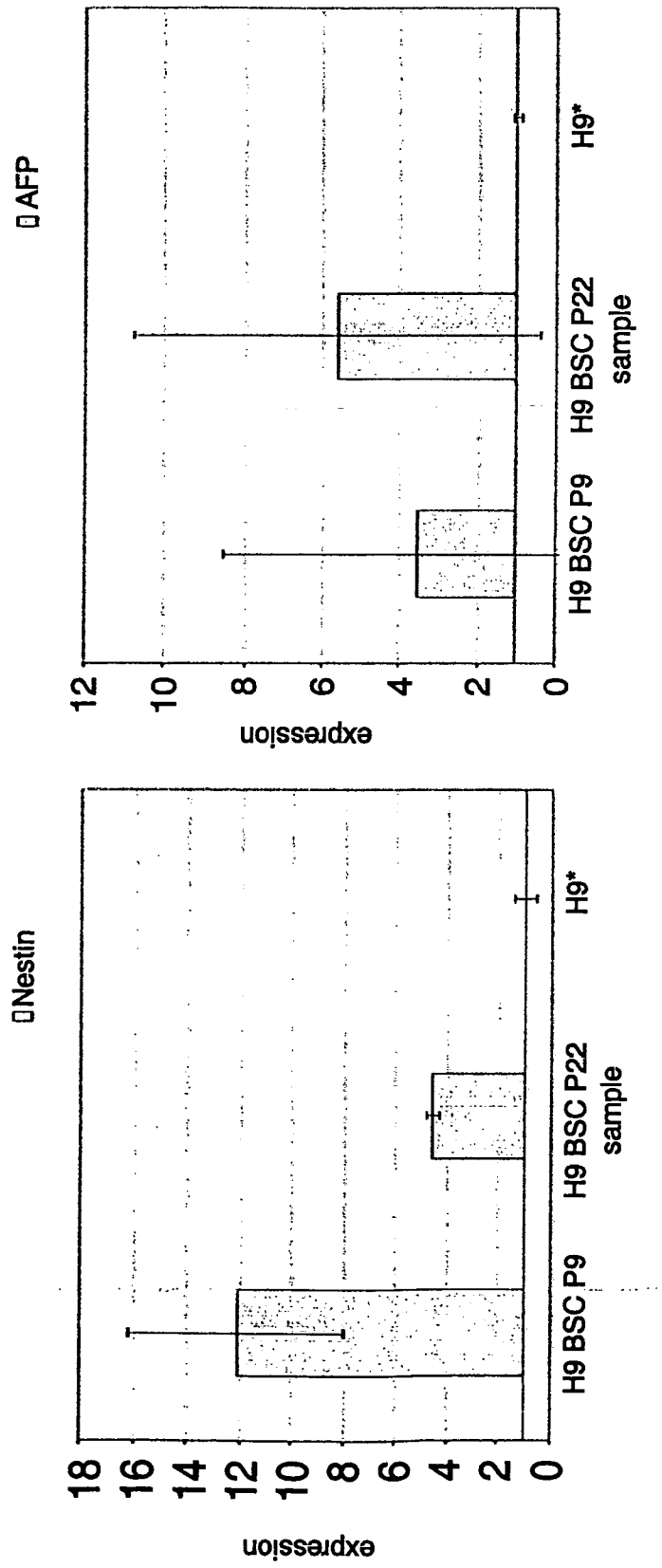


Figure 9C

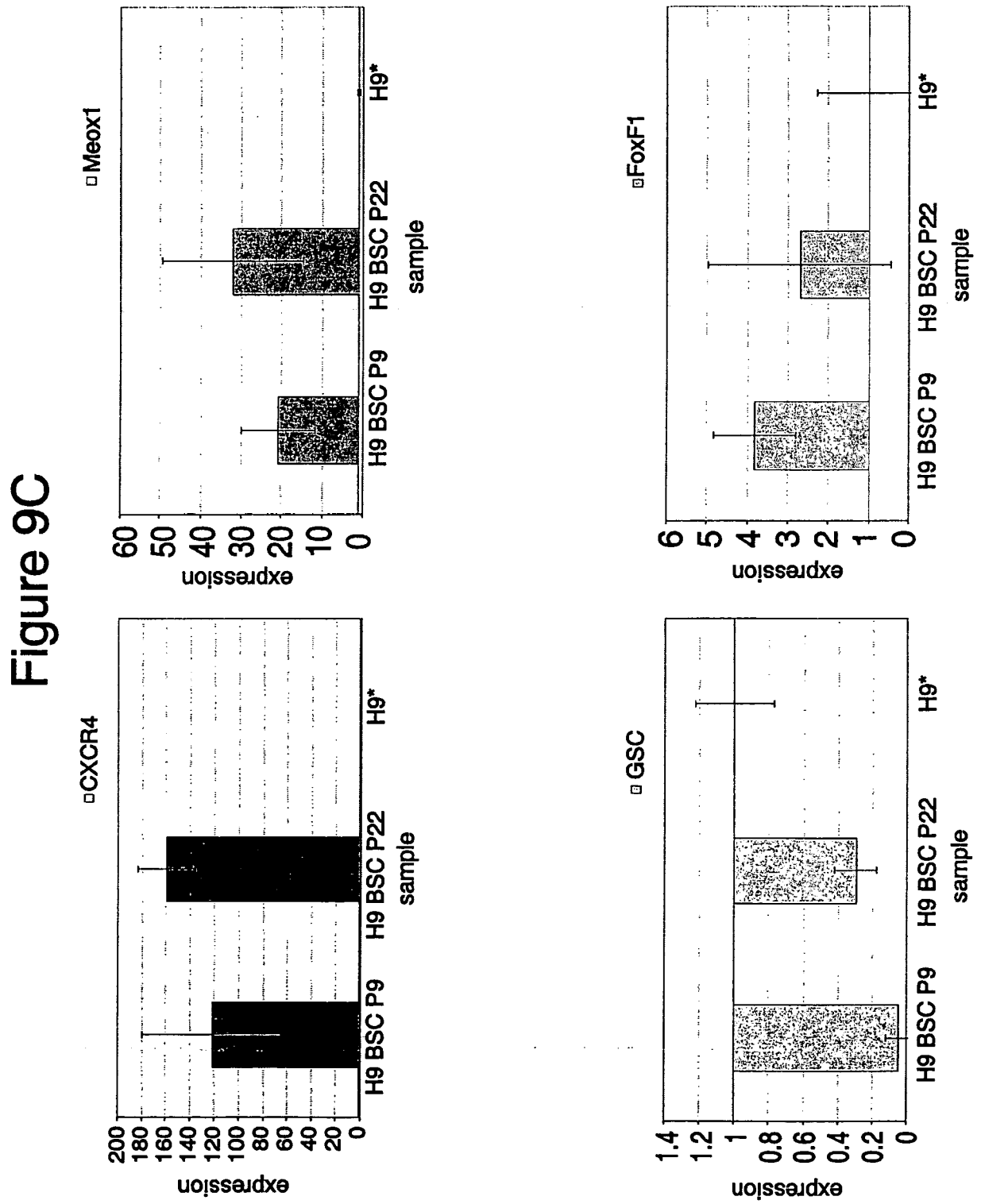

Meox1
expression
0
10
20
30
40
50
60
H9 BSC P9
H9 BSC P22
H9*
sample
FoxF1
expression
0
1
2
3
4
5
6
H9 BSC P9
H9 BSC P22
H9*
sample
CXCR4
expression
0
20
40
60
80
100
120
140
160
180
200
H9 BSC P9
H9 BSC P22
H9*
sample
GSC
expression
0
0.2
0.4
0.6
0.8
1
1.2
1.4
H9 BSC P9
H9 BSC P22
H9*
sample

Figure 9C (Cont'd)

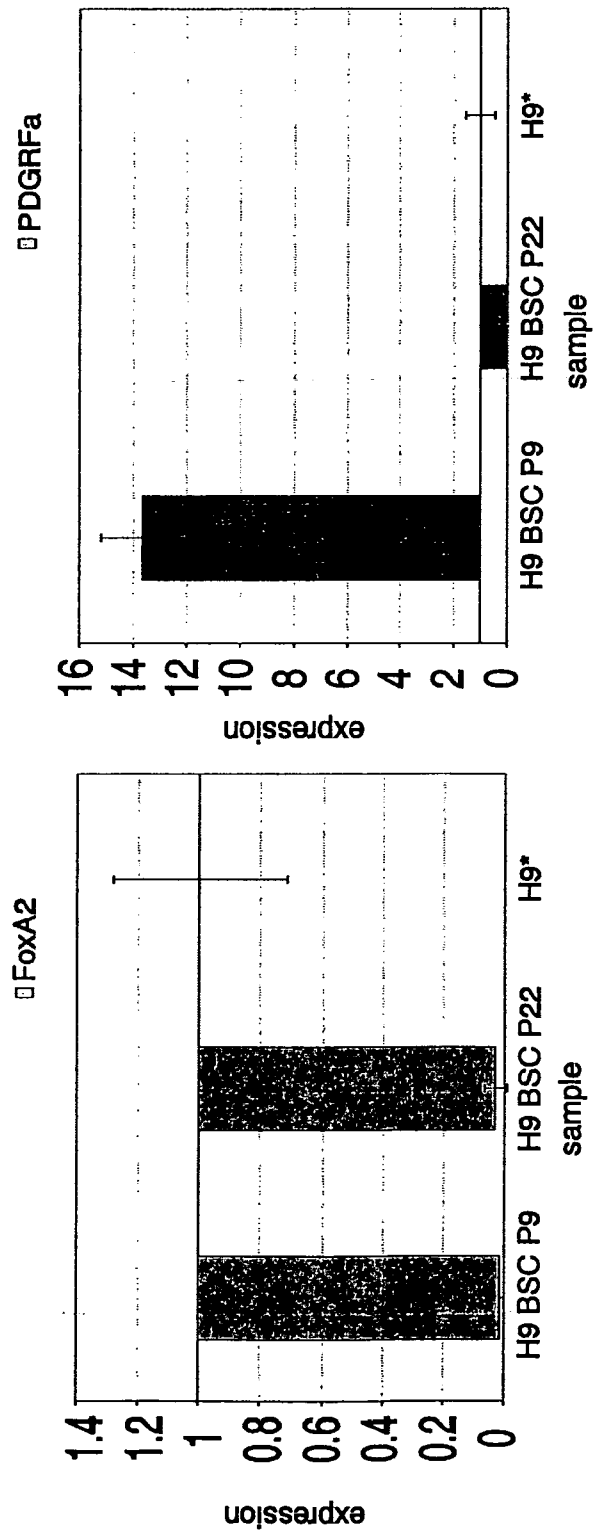


Figure 10A

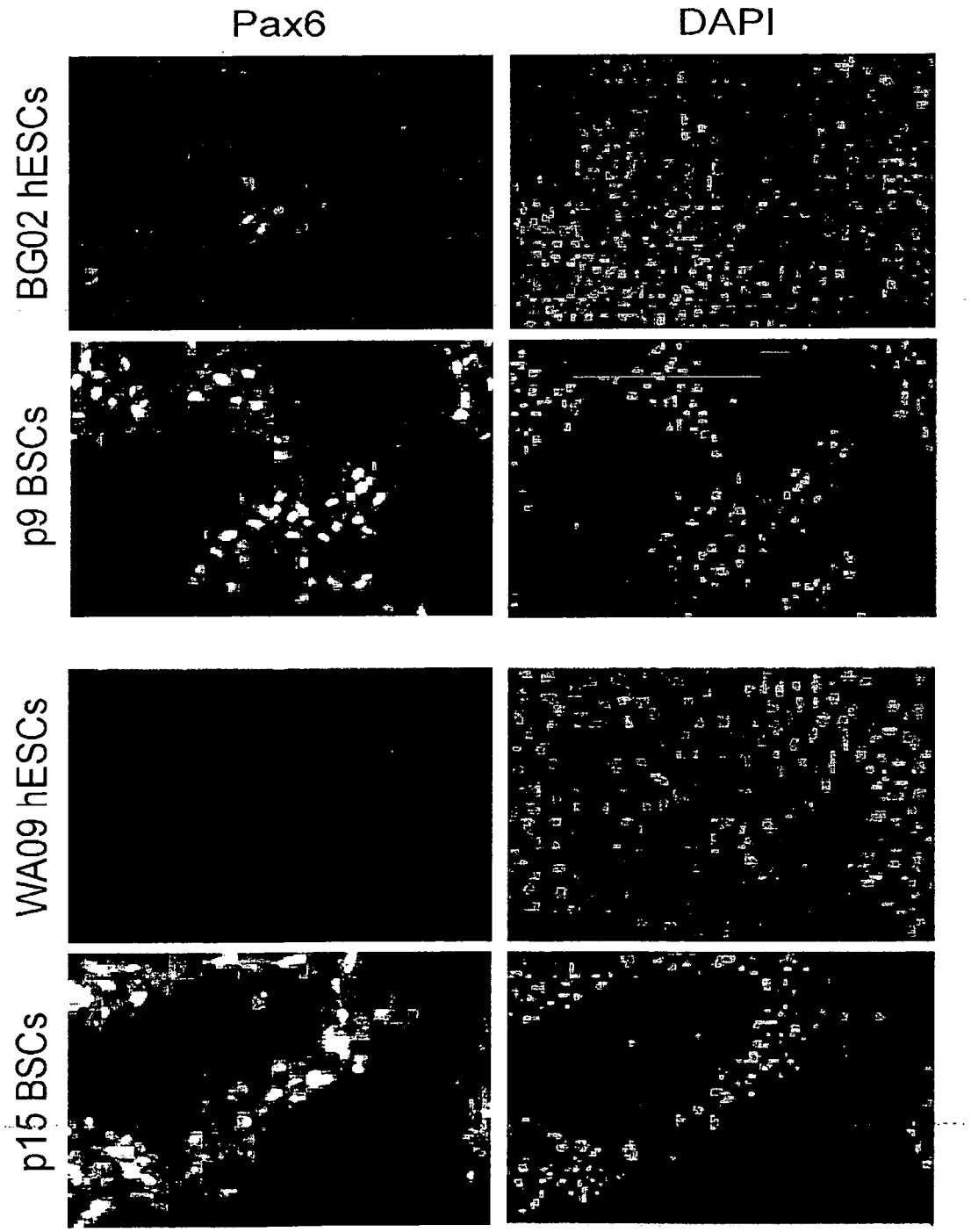


Figure 10B

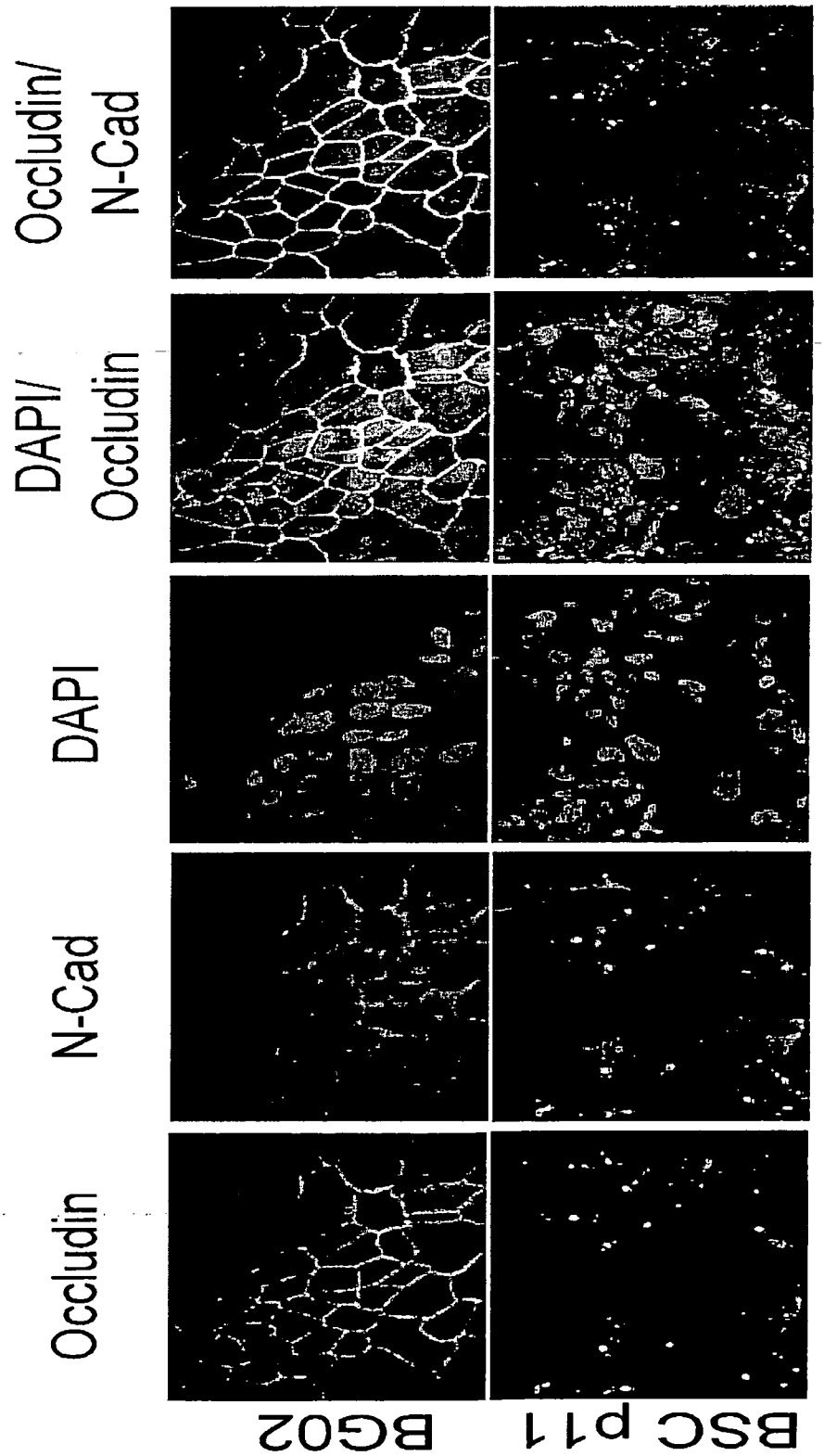

Occludin/ N-Cad
DAPI/ Occludin
DAPI
N-Cad
Occludin
BG02
BSC p11

Figure 10B (Con't)

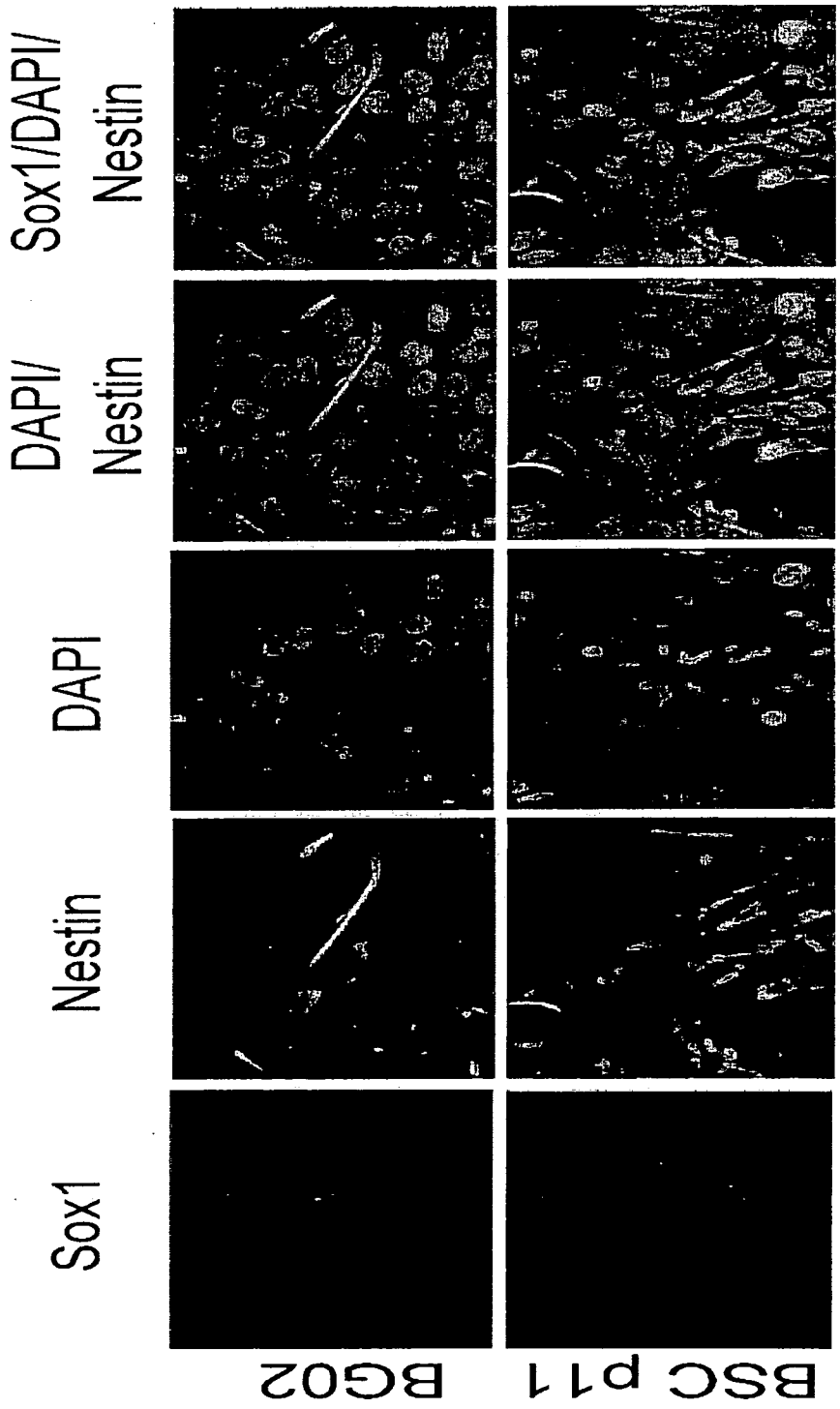


Figure 10B (Con't)

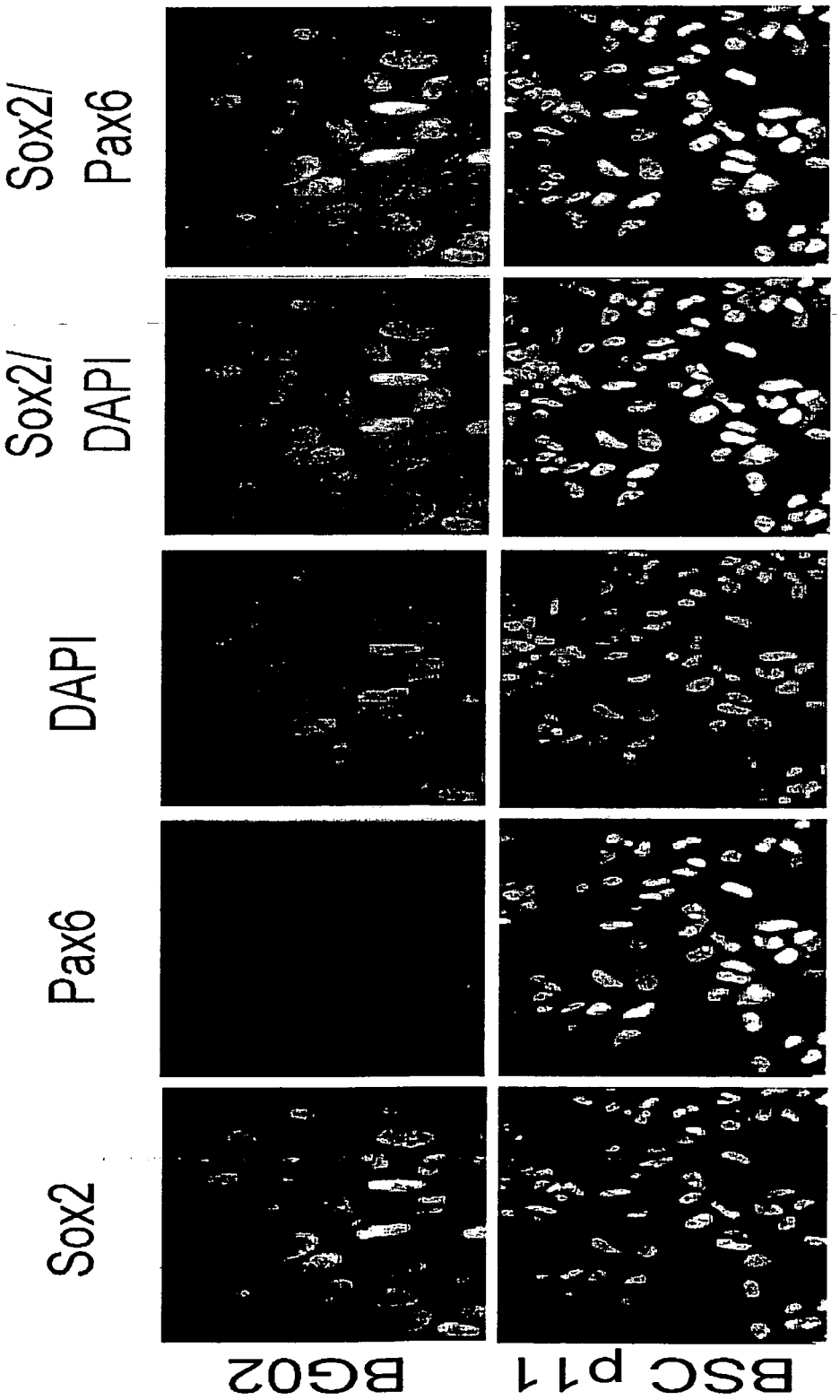


Figure 10C

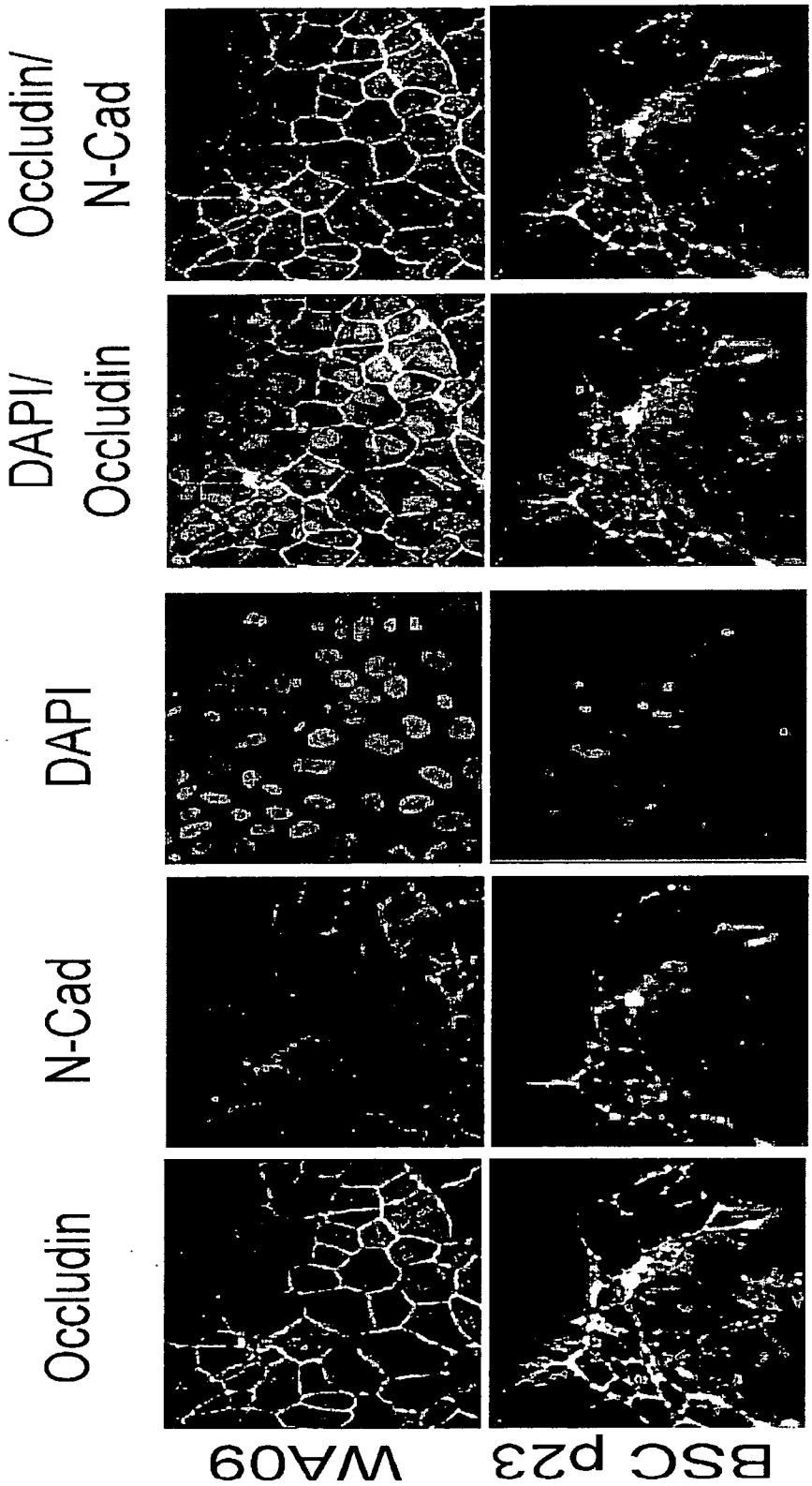

Occludin/ N-Cad
DAPI/ Occludin
DAPI
N-Cad
Occludin
WA09
BSC p23

Figure 10C (Con't)

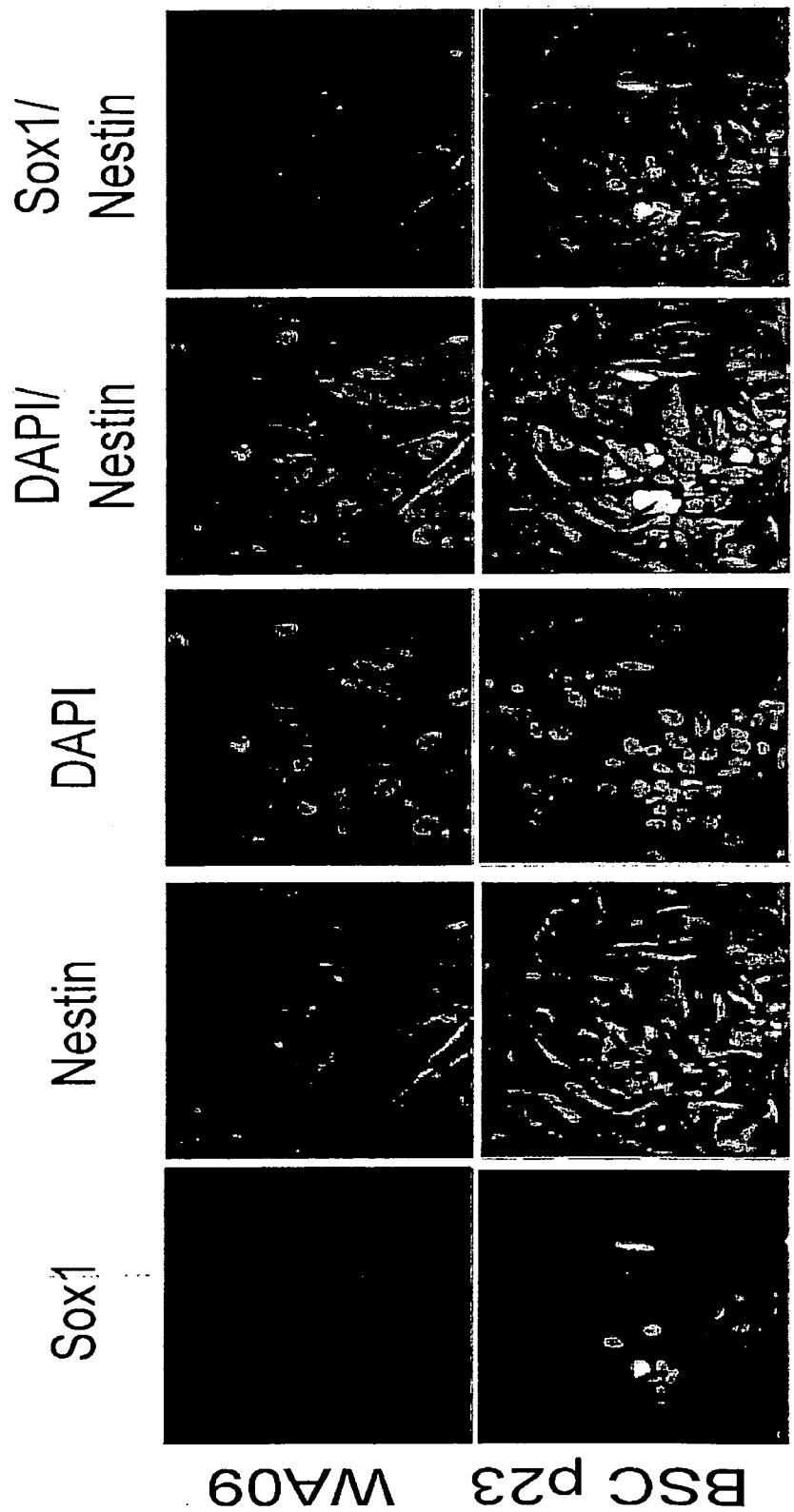


Figure 10C (Con't)

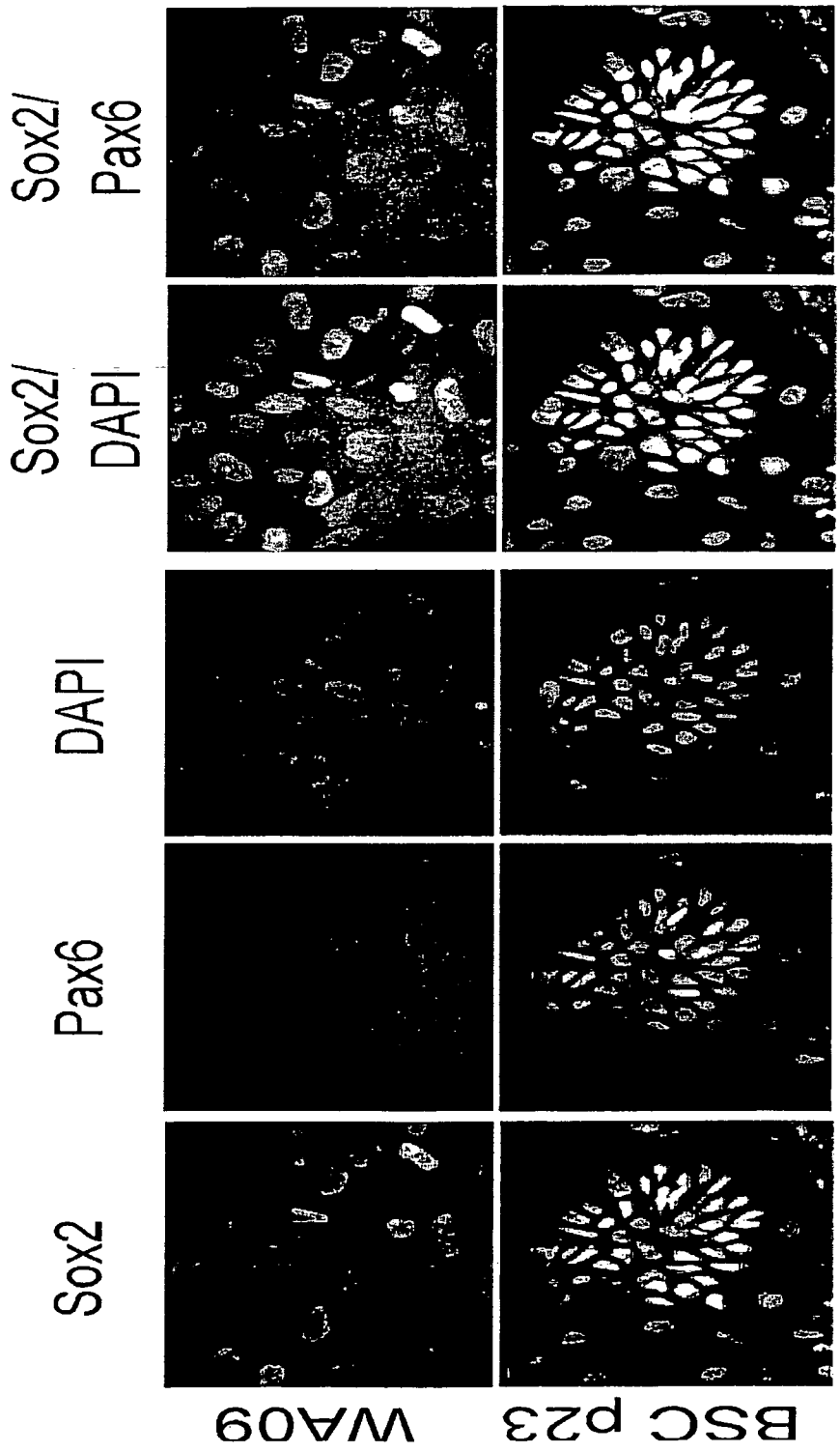


Figure 11

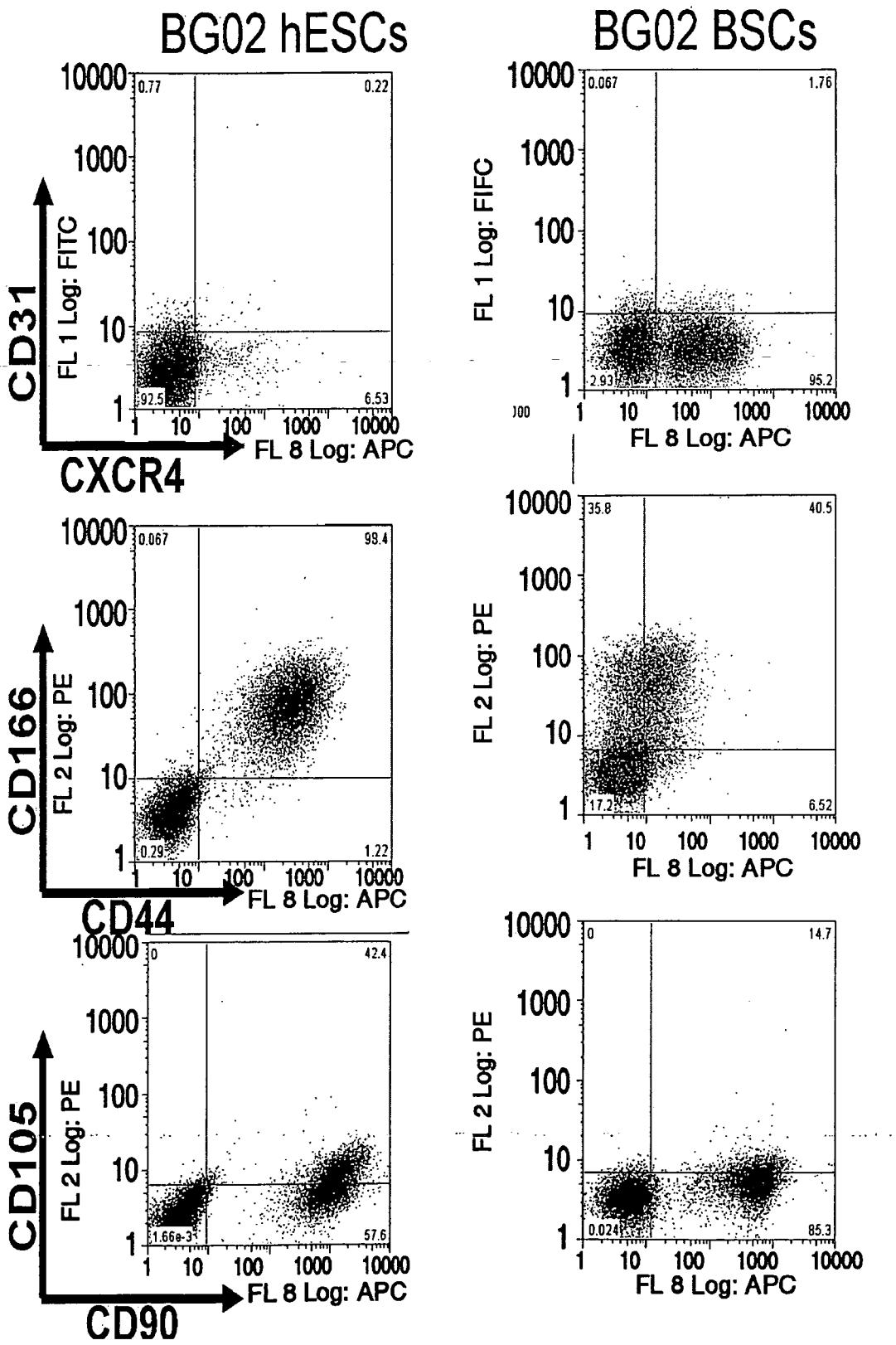

BG02 hESCs
BG02 BSCs
CD31
CXCR4
FL 1 Log: FITC
FL 8 Log: APC
0.77
0.22
92.5
6.53
FL 1 Log: FIFC
0.067
1.76
2.93
95.2
CD166
CD44
FL 2 Log: PE
0.067
99.4
0.29
1.22
35.8
40.5
17.2
6.52
CD105
CD90
0
42.4
1.66e-3
57.6
14.7
0.024
85.3
10000
1000
100
10
1
1
10
100
1000
10000

Figure 11 (Cont'd)

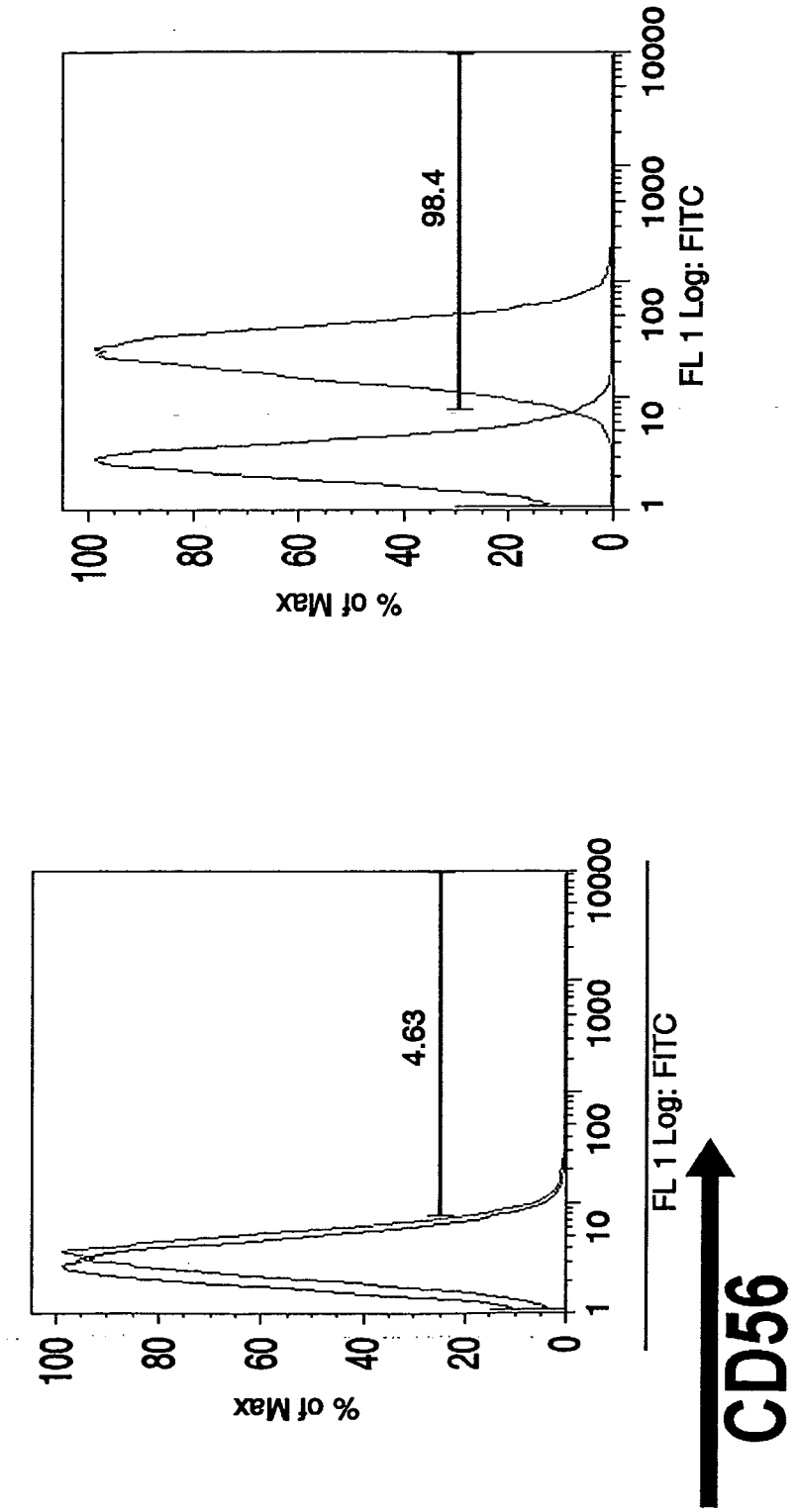


Figure 12

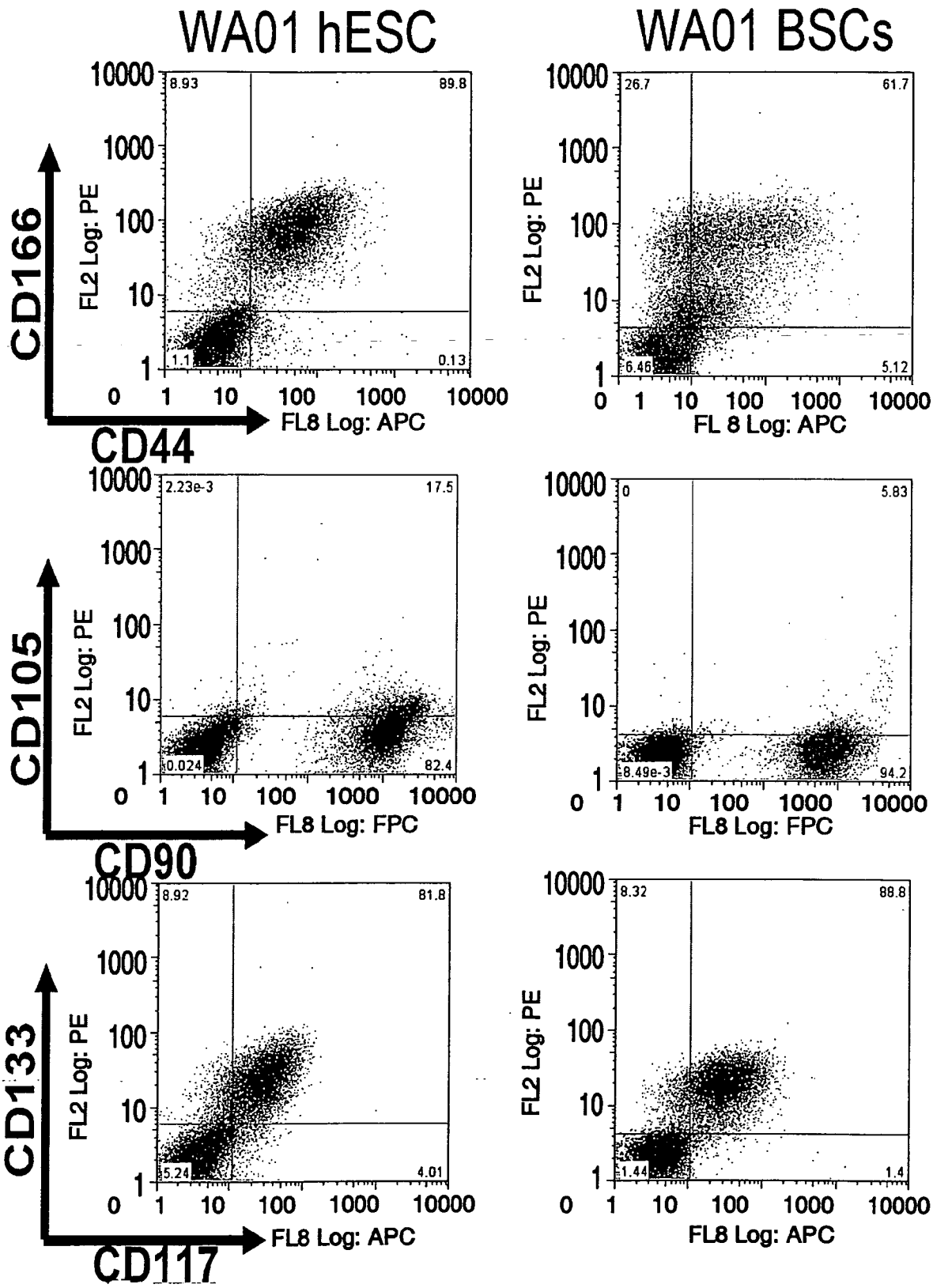

WA01 hESC
WA01 BSCs
CD166
CD44
CD105
CD90
CD133
CD117
FL2 Log: PE
FL8 Log: APC
FL 8 Log: APC
FL8 Log: FPC
8.93
89.8
1.1
0.13
26.7
61.7
6.46
5.12
2.23e-3
17.5
0.024
82.4
0
5.83
8.49e-3
94.2
8.92
81.8
5.24
4.01
8.32
88.8
1.44
1.4

Figure 12 (Cont'd)

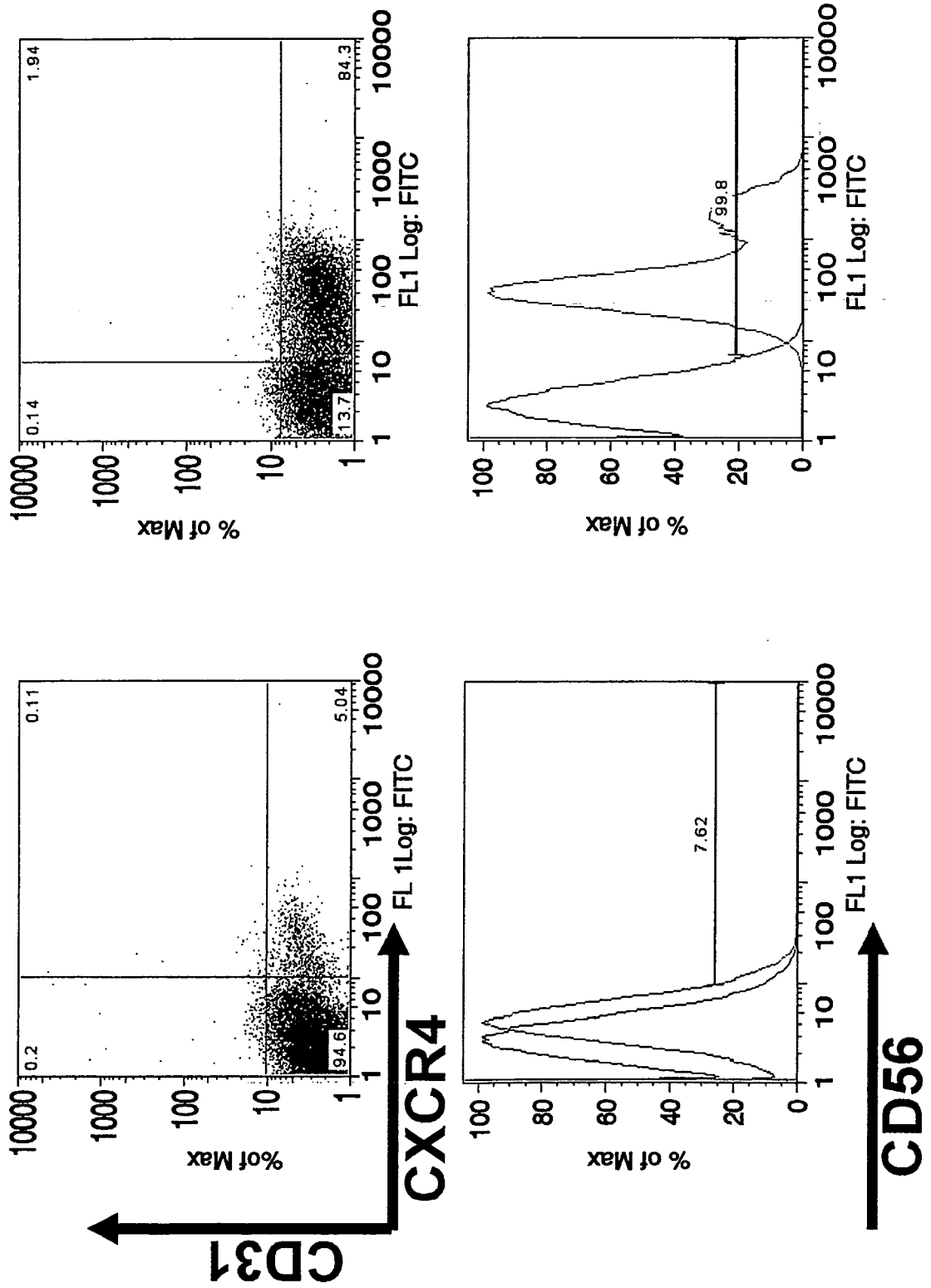


Figure 13

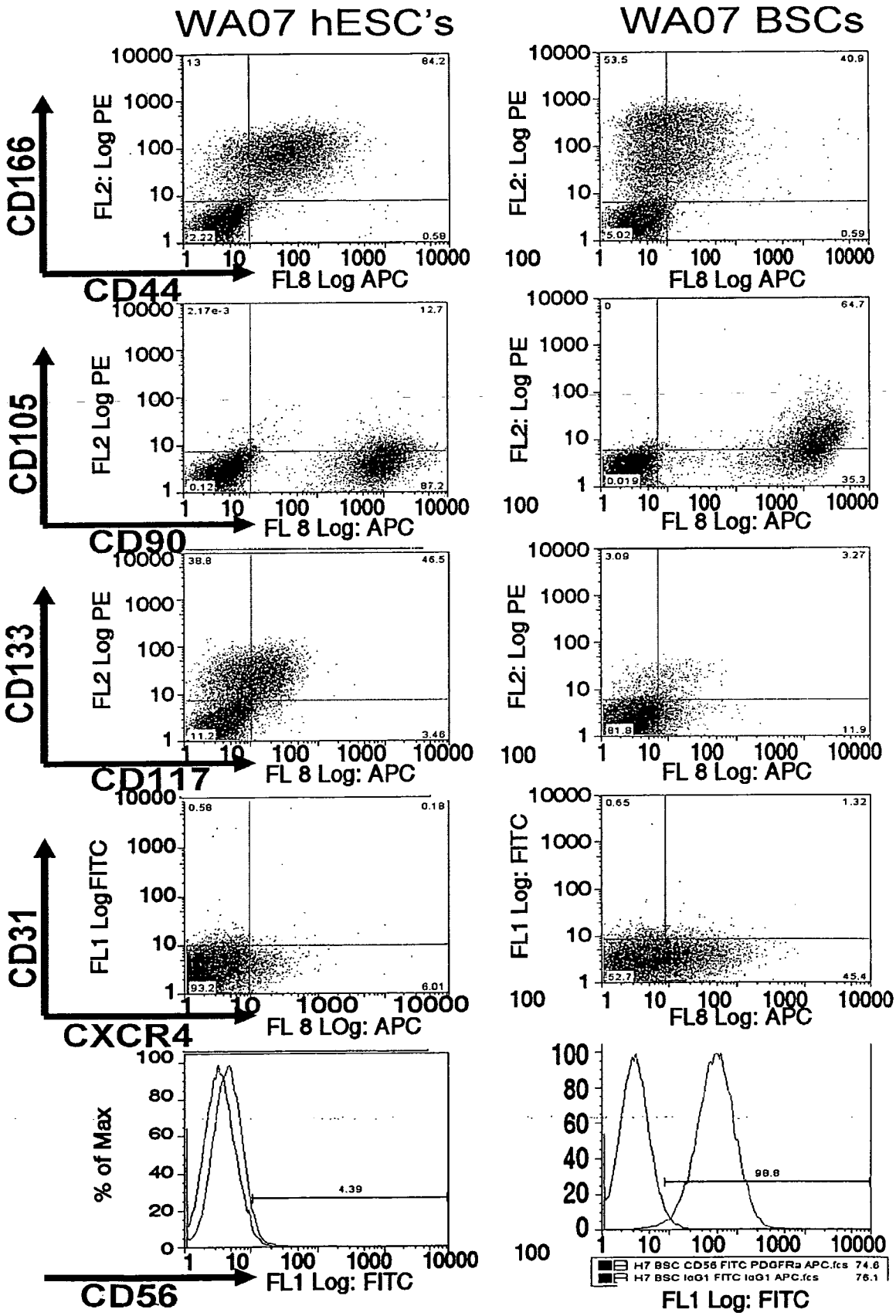

WA07 hESC's
WA07 BSCs
CD166
CD44
FL2: Log PE
FL8 Log APC
CD105
CD90
FL2 Log PE
FL 8 Log: APC
CD133
CD117
CD31
CXCR4
FL1 LogFITC
FL 8 LOg: APC
% of Max
CD56
FL1 Log: FITC
H7 BSC CD56 FITC PDGFRa APC.fcs 74.6
H7 BSC IgG1 FITC IgG1 APC.fcs 76.1

Figure 14

(hiPSCs) BSCs

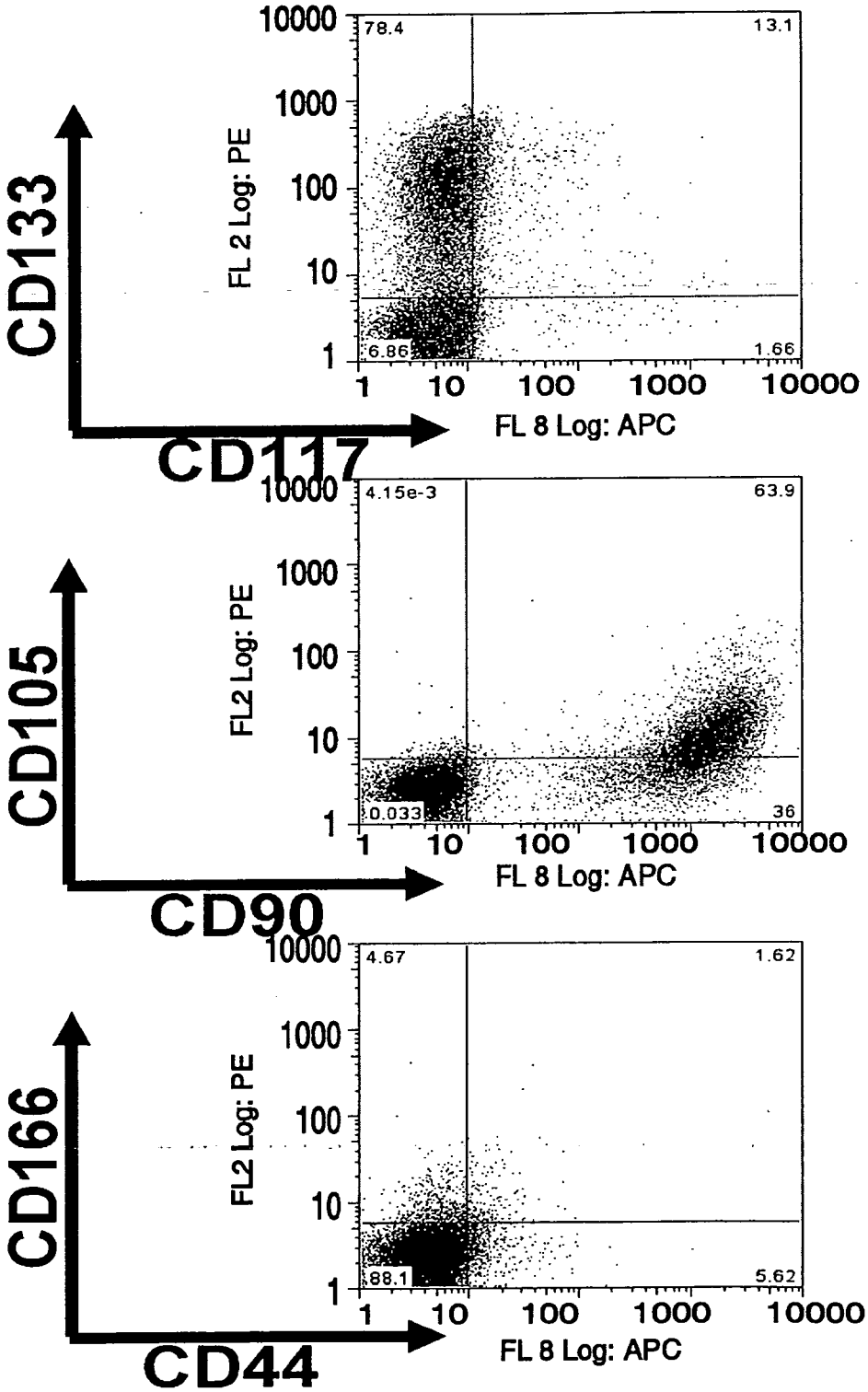


Figure 14 (cont'd)

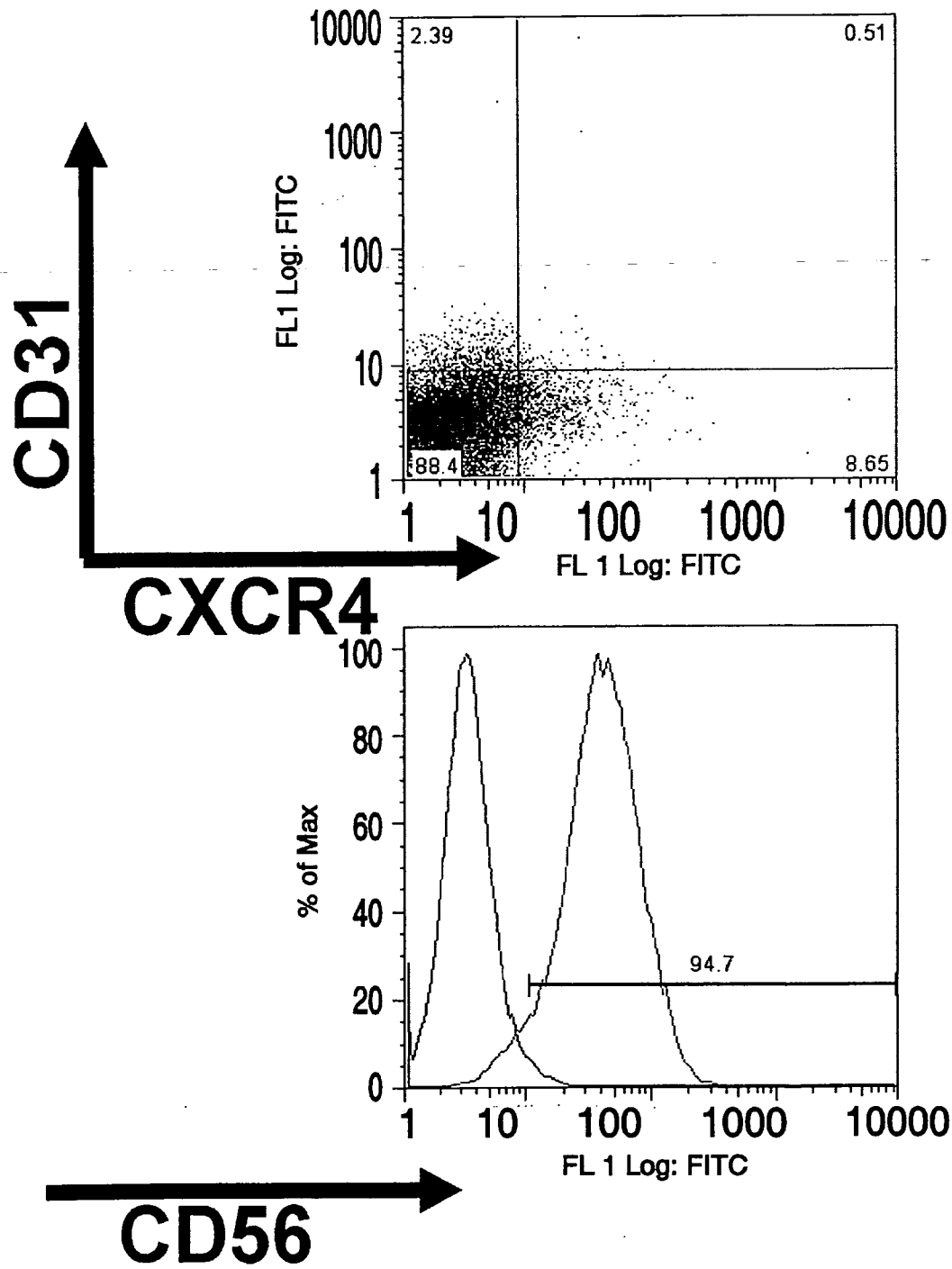


Figure 15

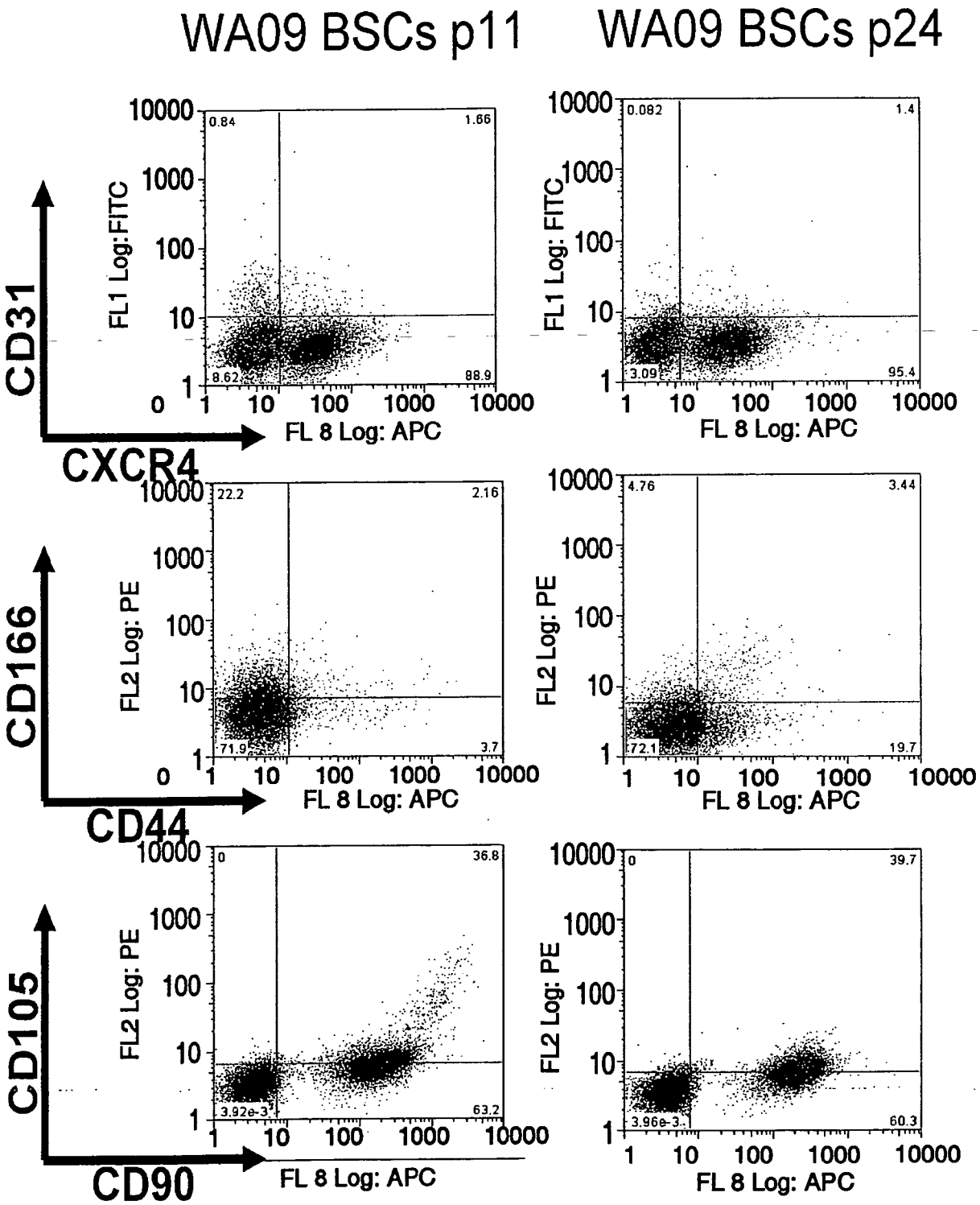

WA09 BSCs p11
WA09 BSCs p24
CD31
CXCR4
FL1 Log:FITC
FL 8 Log: APC
0.84
1.66
8.62
88.9
0.082
1.4
3.09
95.4
CD166
CD44
FL2 Log: PE
22.2
2.16
71.9
3.7
4.76
3.44
72.1
19.7
CD105
CD90
36.8
3.92e-3
63.2
39.7
3.96e-3
60.3

Figure 15 (con't)

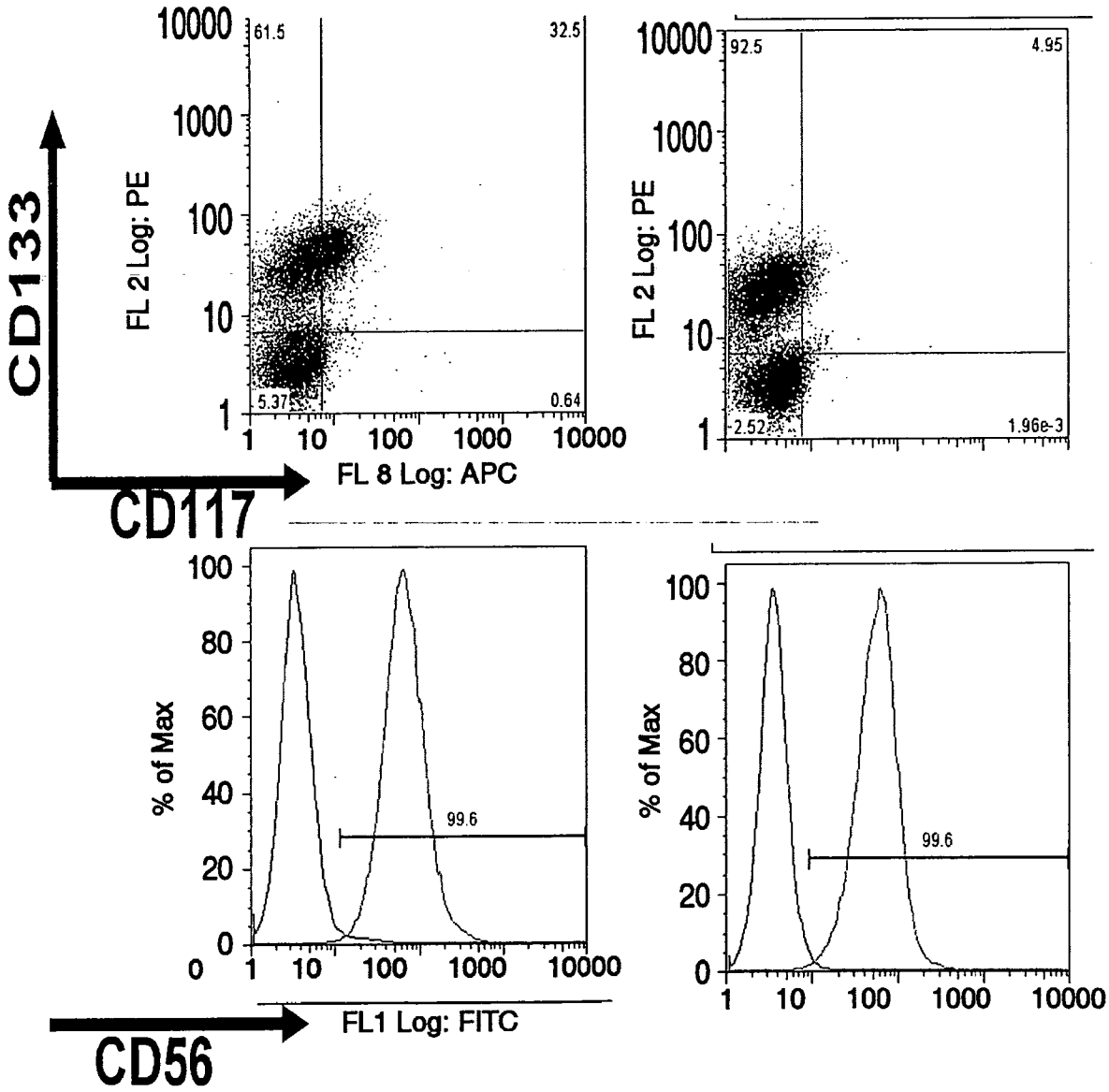


Figure 16

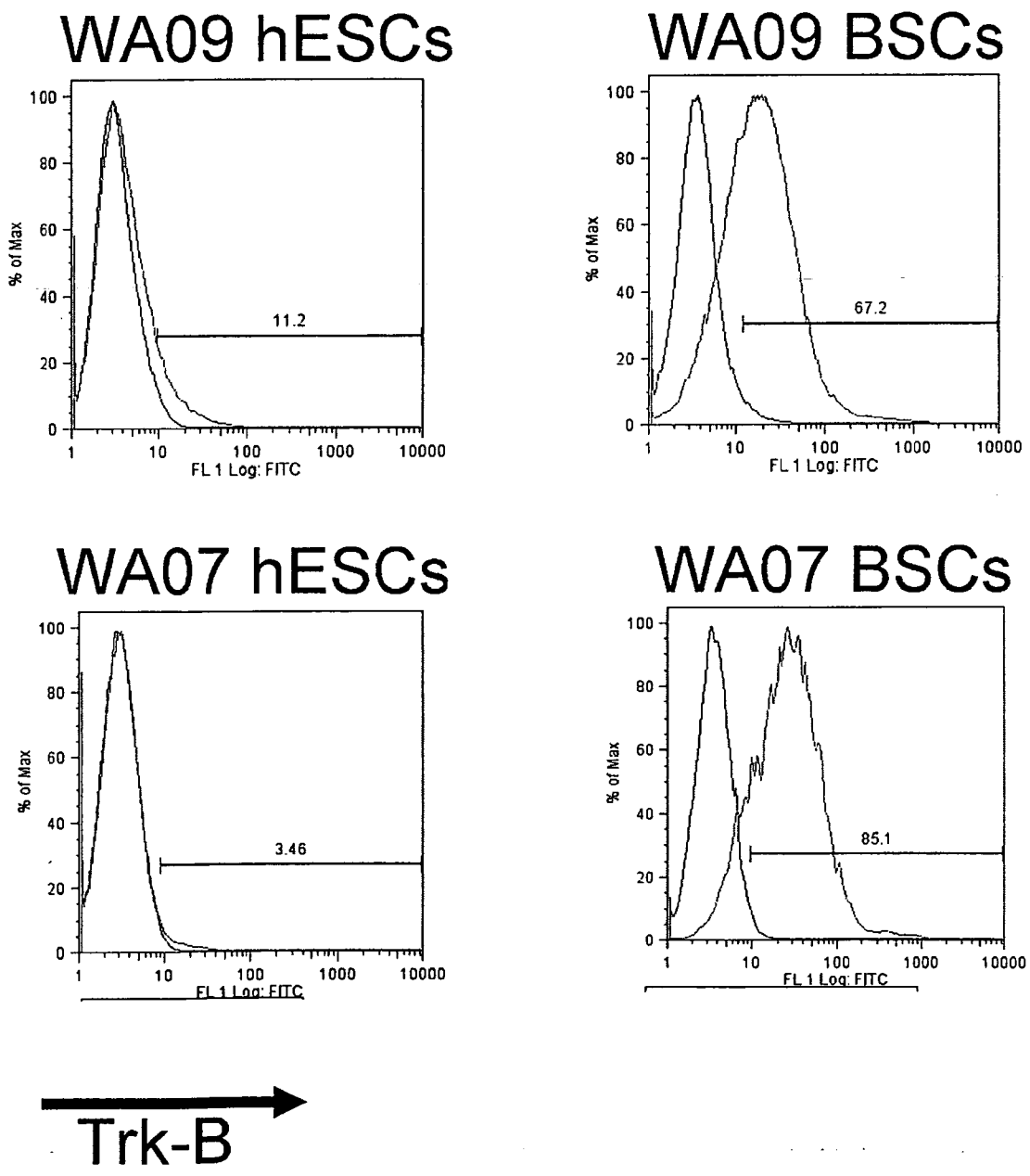

WA09 hESCs
WA09 BSCs
WA07 hESCs
WA07 BSCs
% of Max
FL 1 Log: FITC
11.2
67.2
3.46
85.1
Trk-B

Figure 17

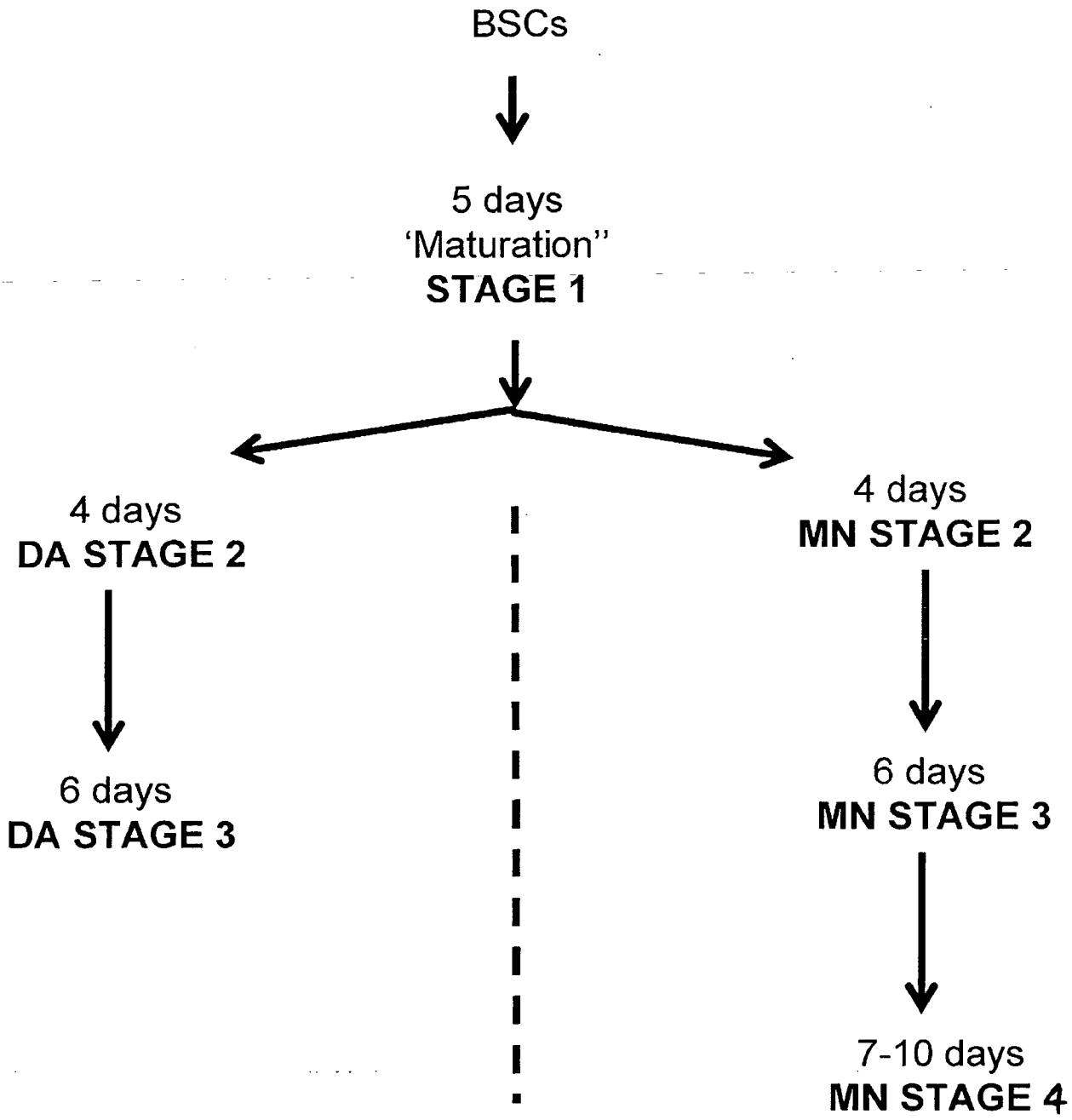

BSCs
5 days
'Maturation''
STAGE 1
4 days
DA STAGE 2
6 days
DA STAGE 3
4 days
MN STAGE 2
6 days
MN STAGE 3
7-10 days
MN STAGE 4

Figure 18

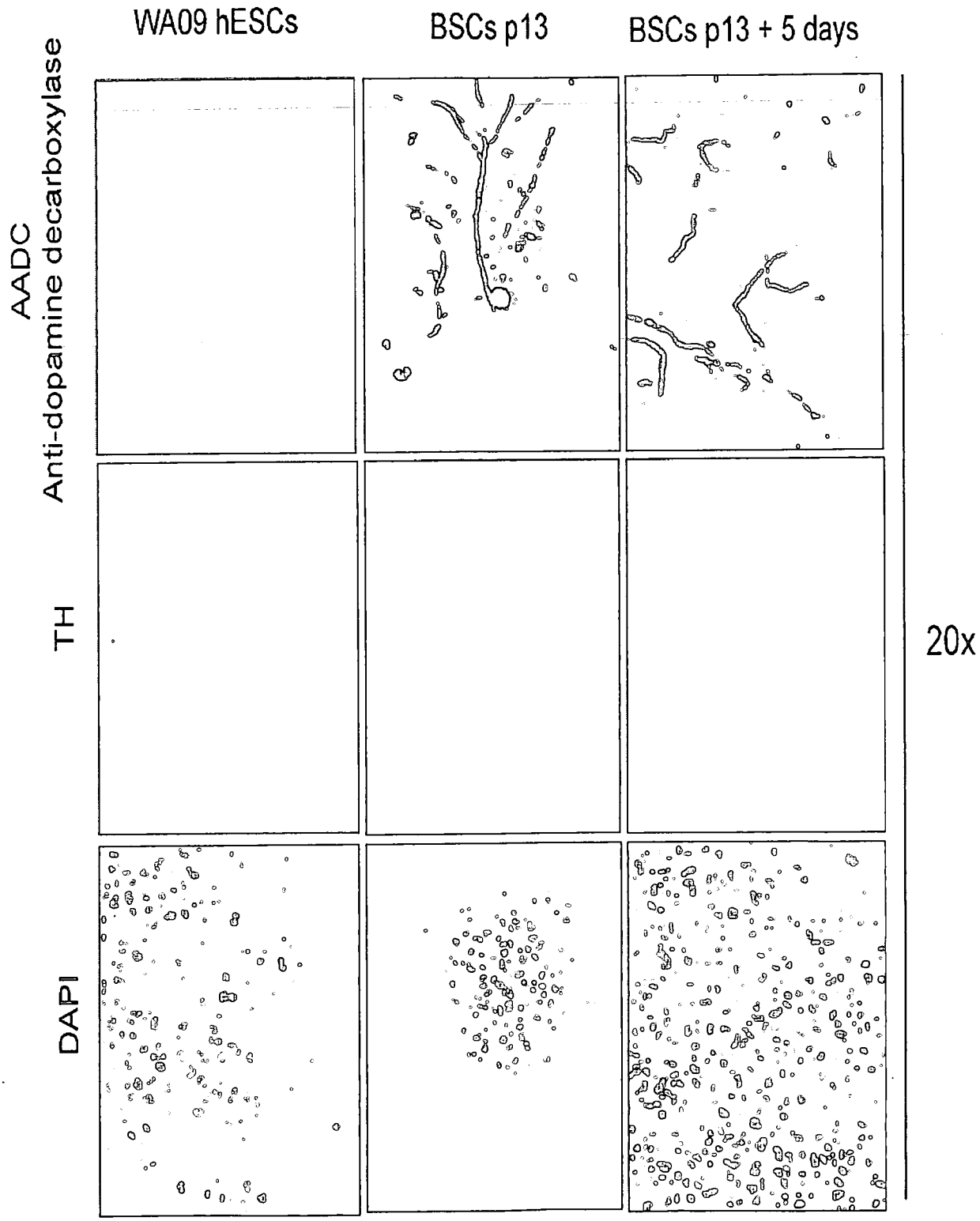

WA09 hESCs
BSCs p13
BSCs p13 + 5 days
AADC
Anti-dopamine decarboxylase
TH
DAPI
20x

Figure 19

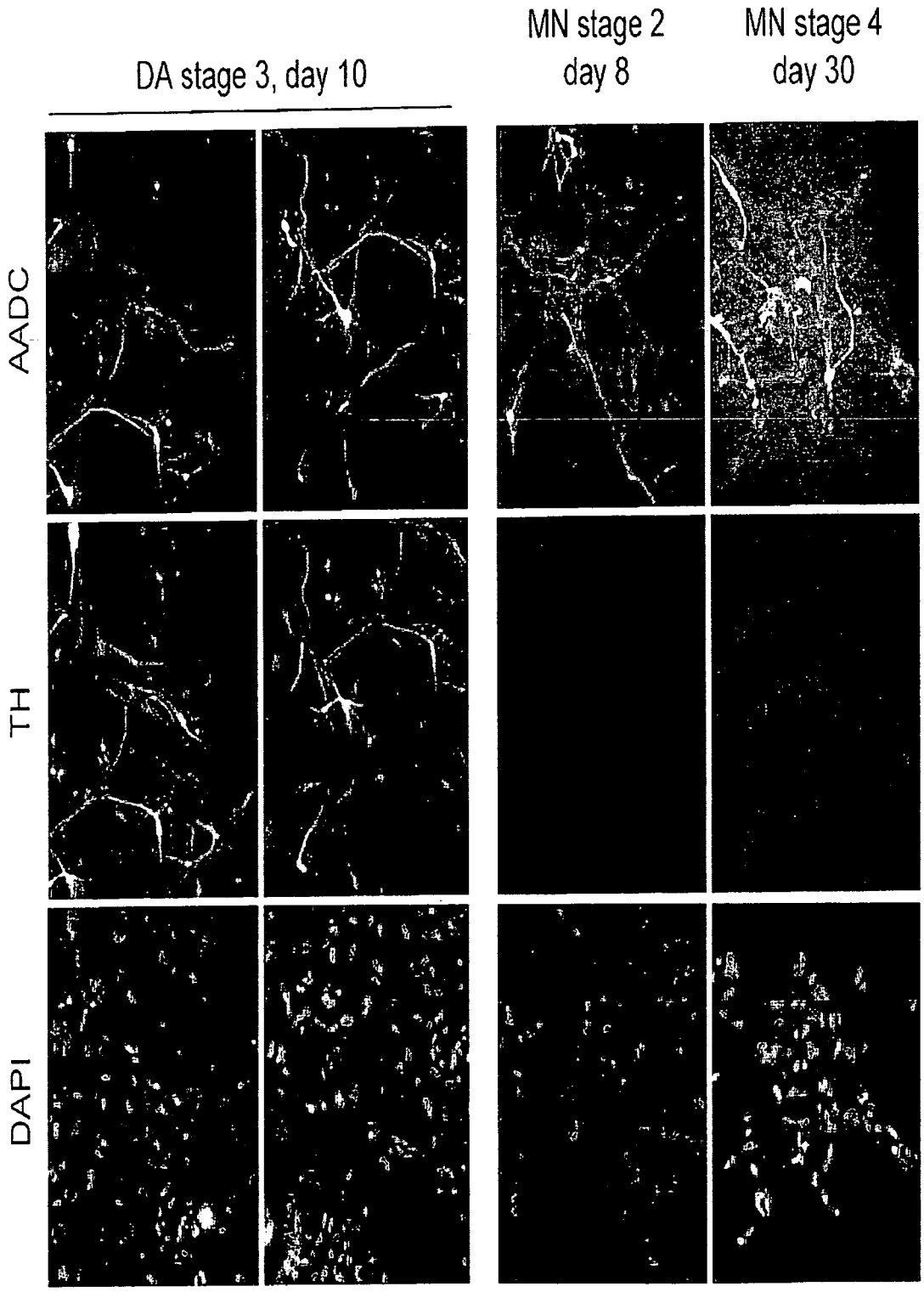


Figure 19 (con't)

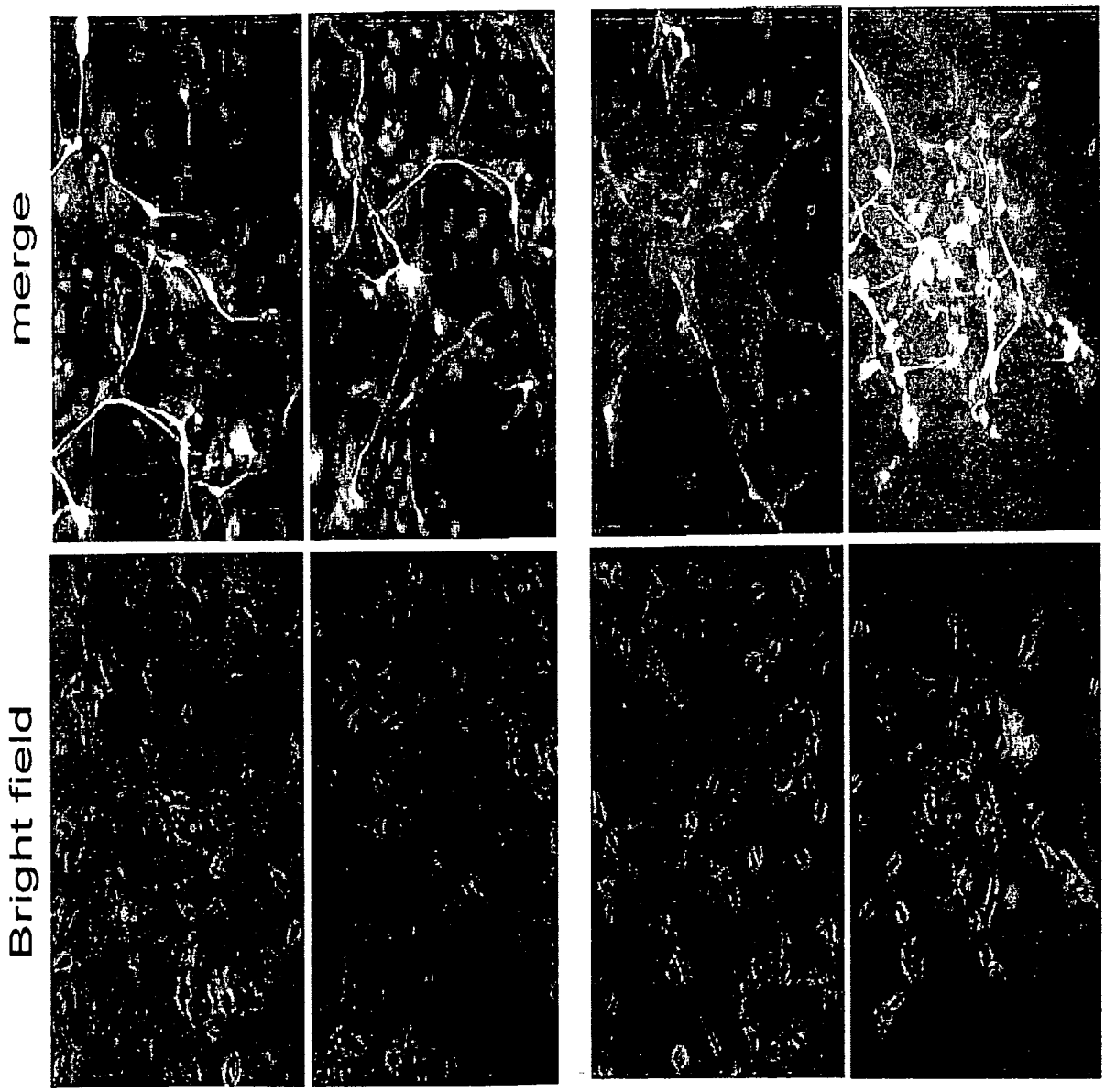


Figure 20

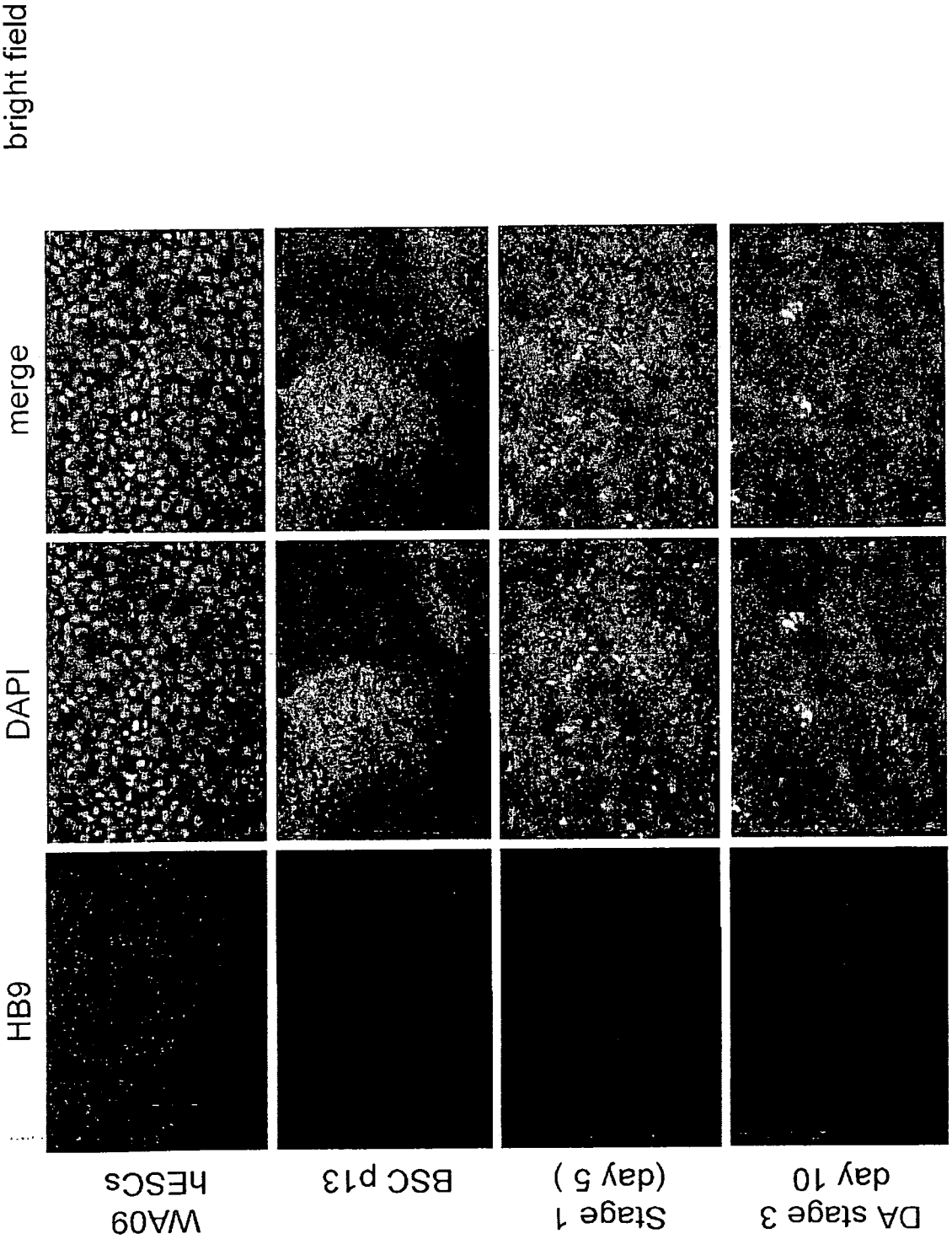

bright field
merge
DAPI
HB9
WA09 hESCs
BSC p13
Stage 1 (day 5)
DA stage 3 day 10

Figure 20 (cont'd)

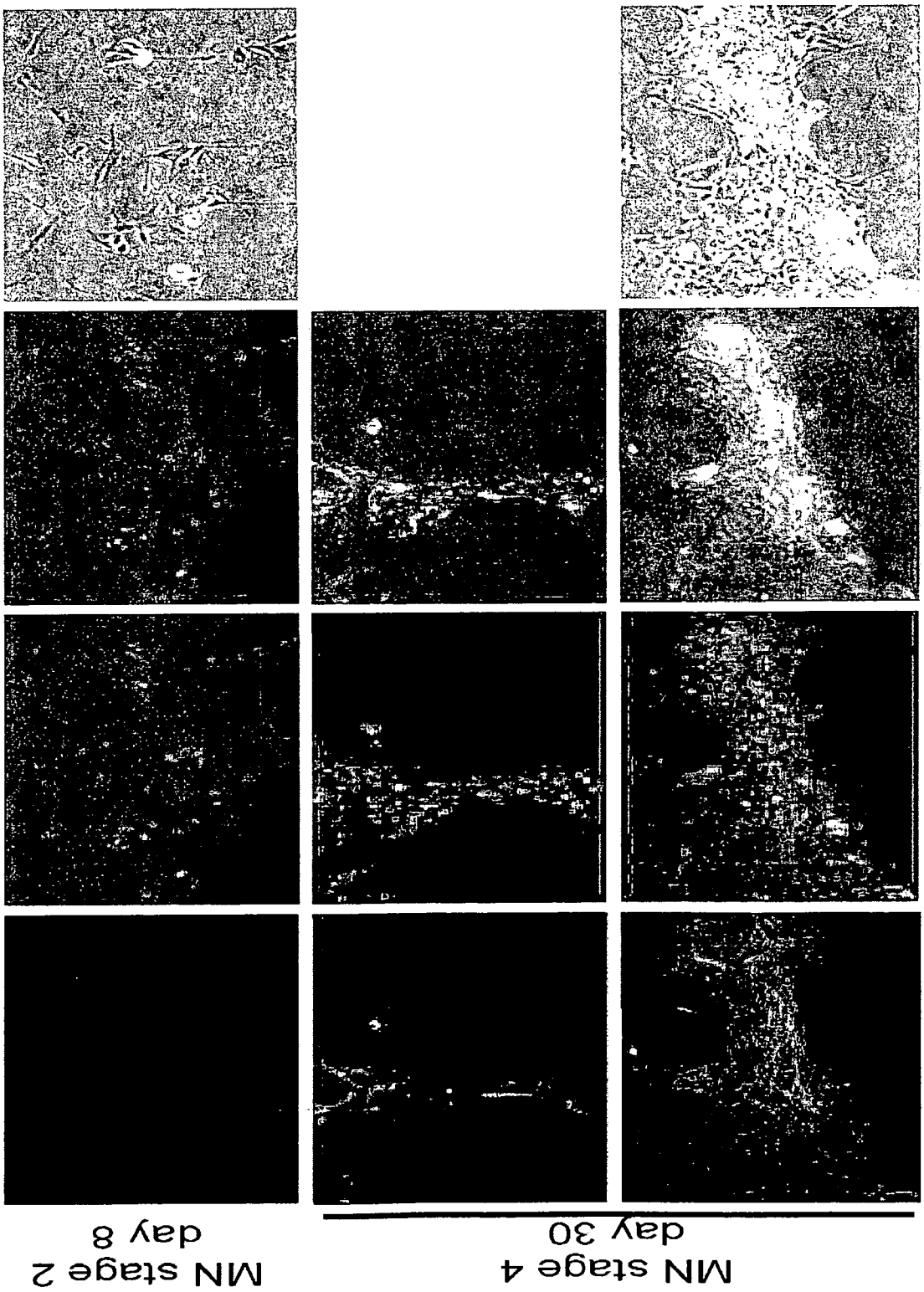


Figure 21

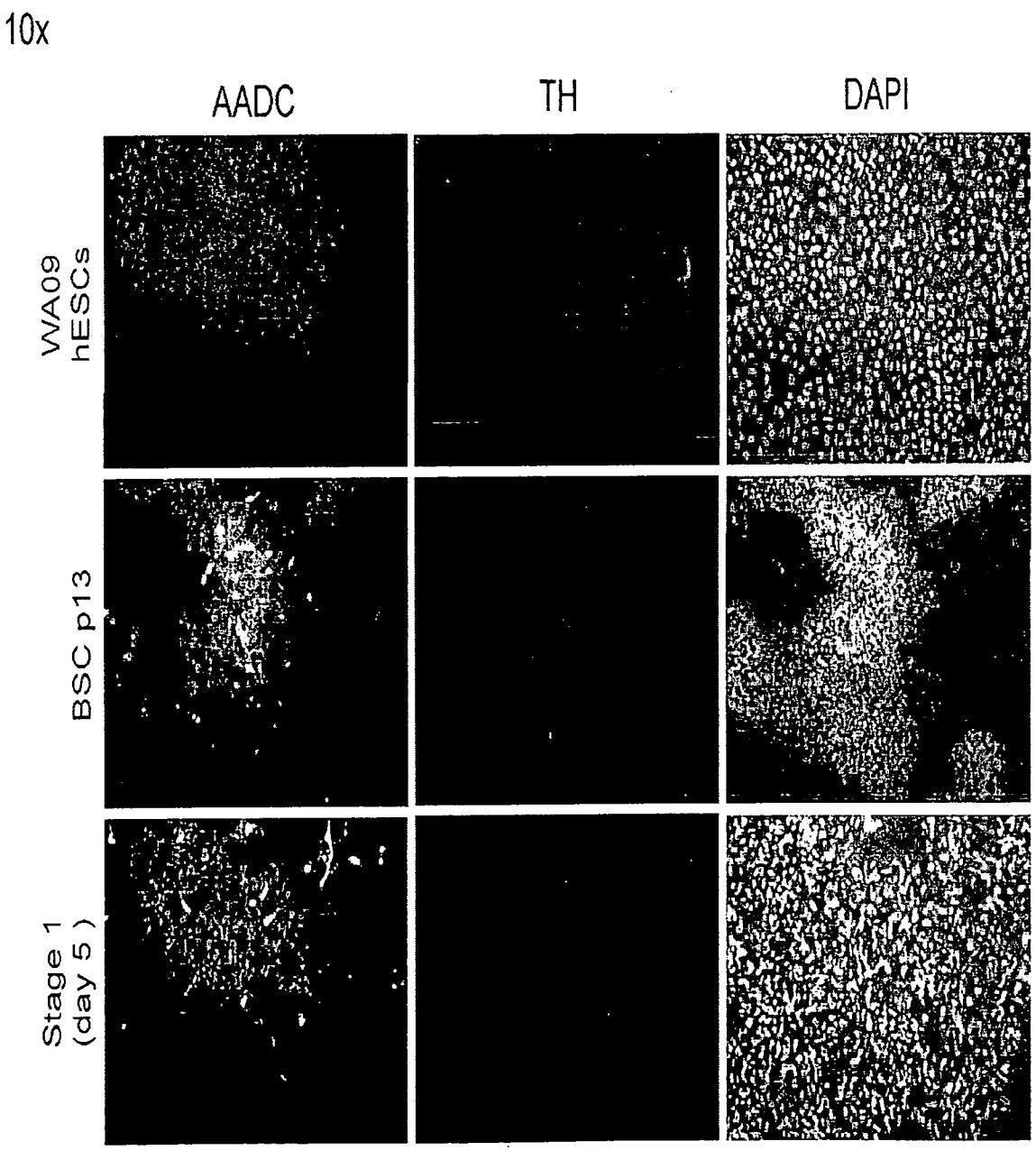


Figure 21 (con't)

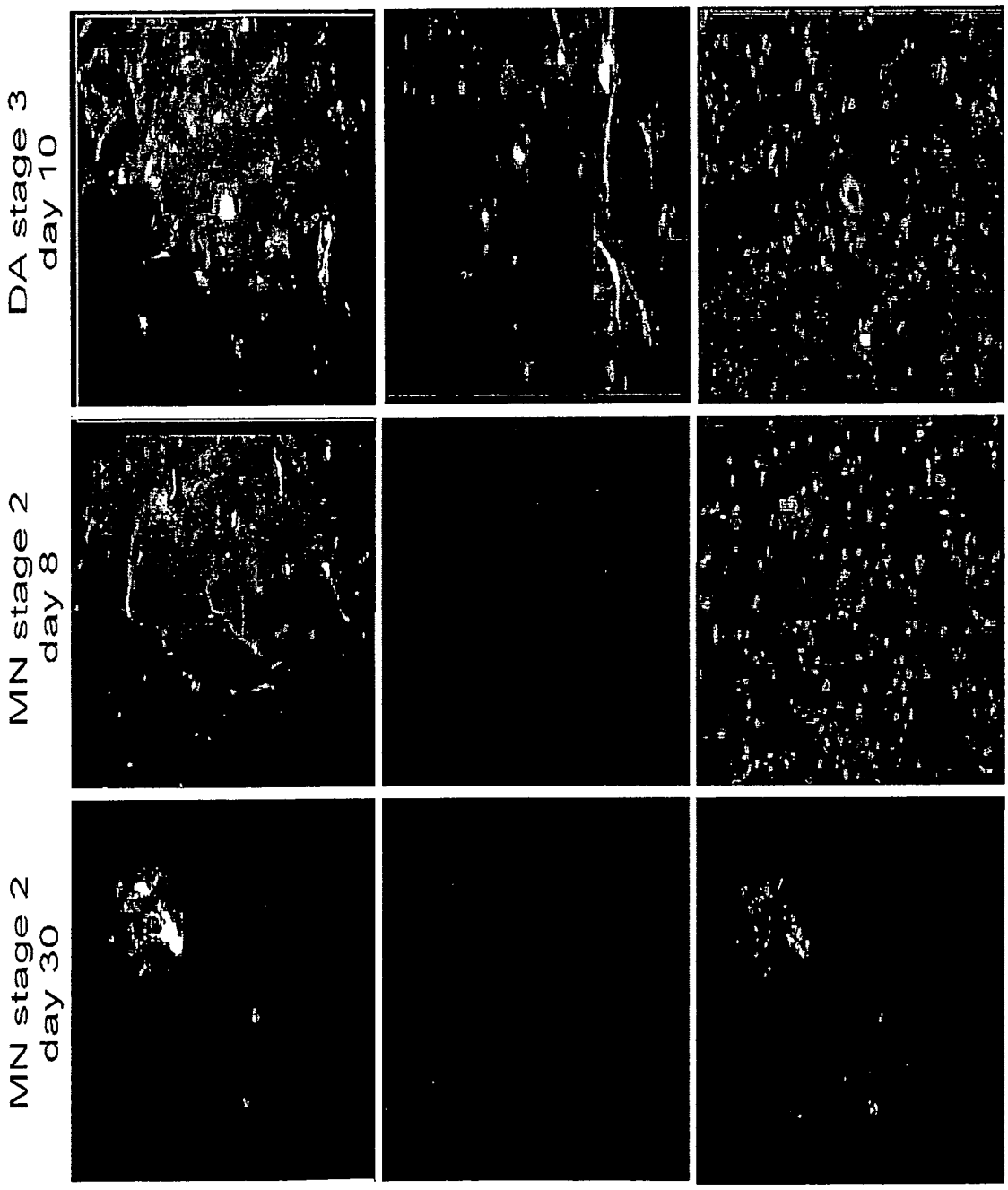


Figure 22

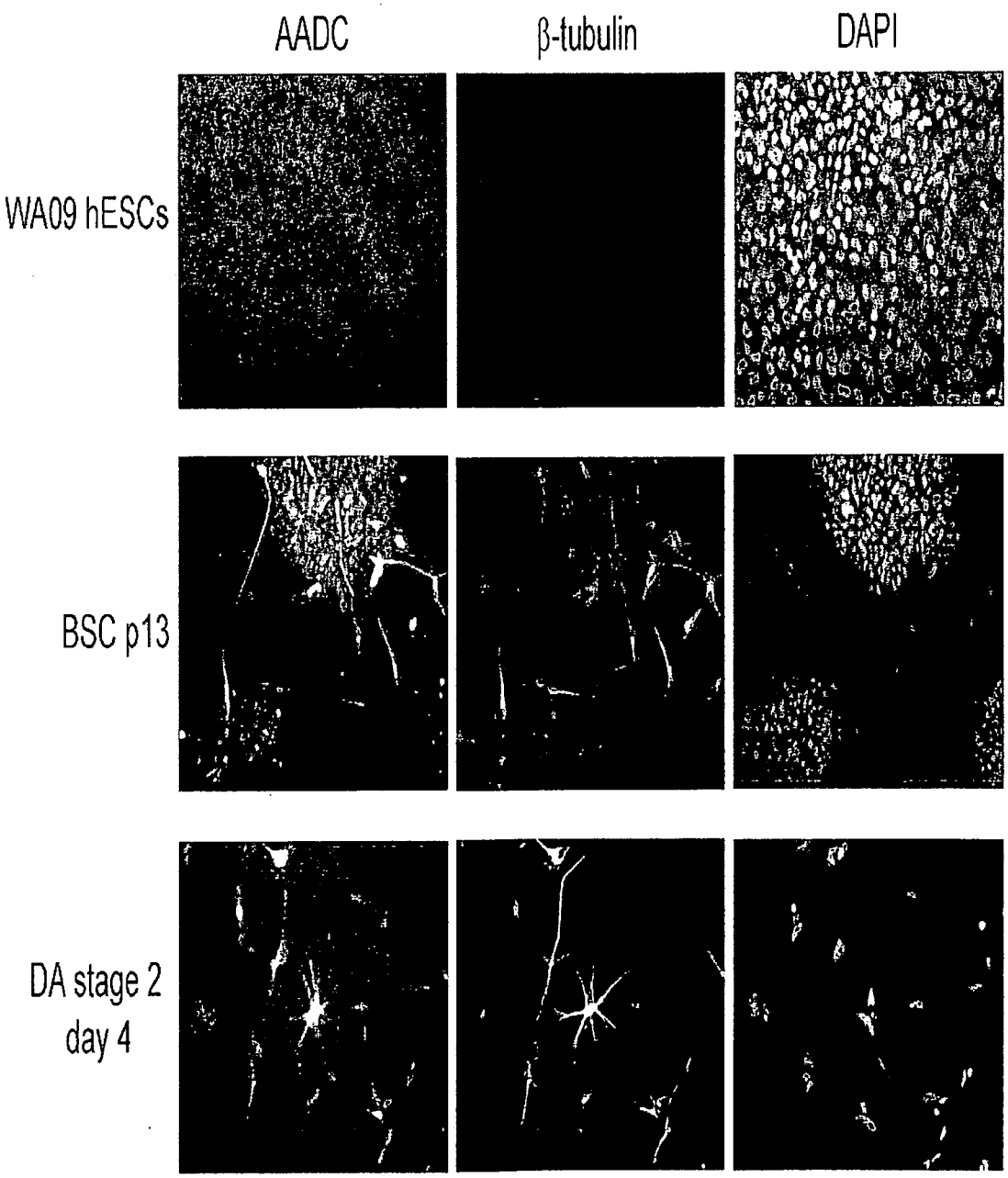


Figure 22 (con't)

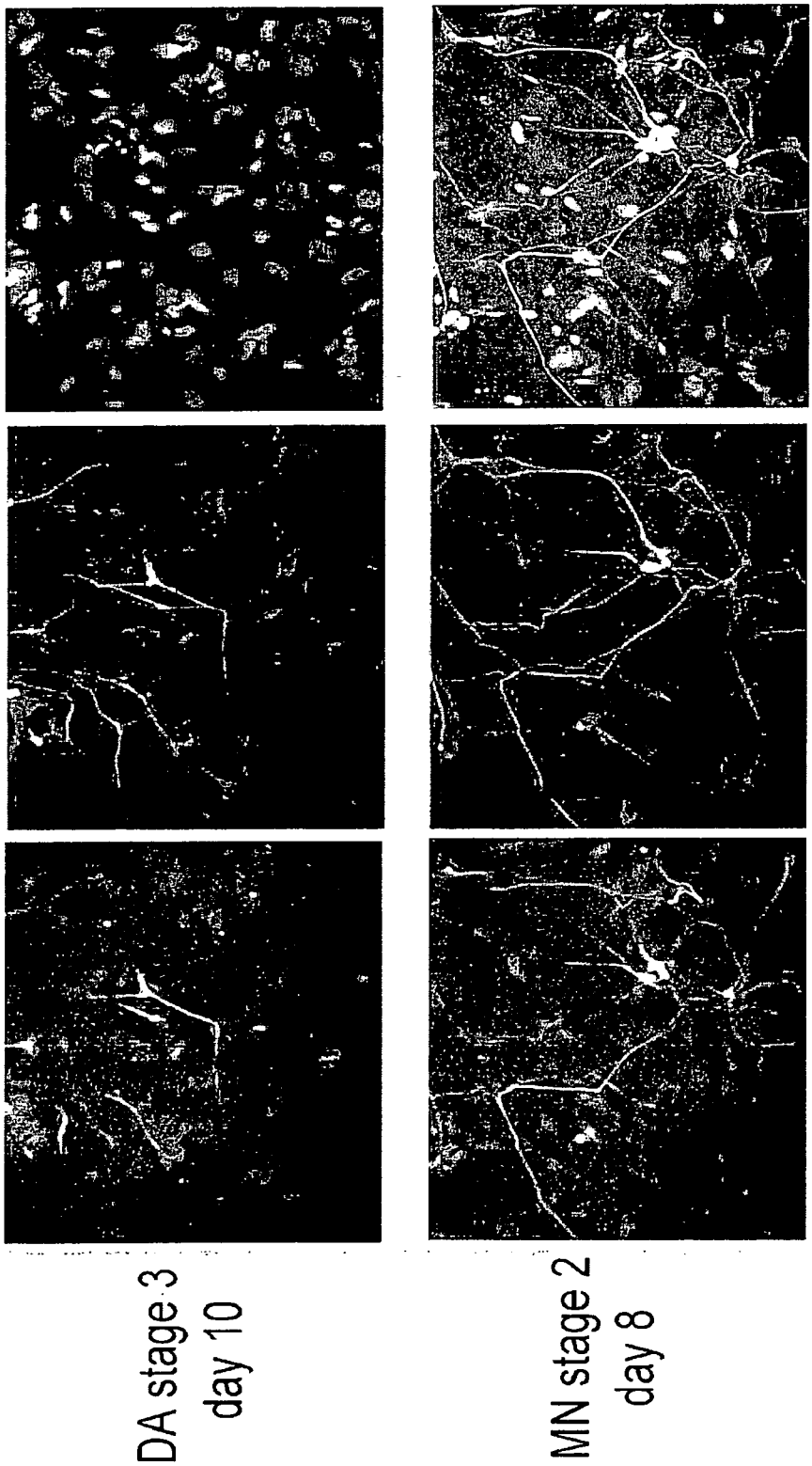


Figure 23

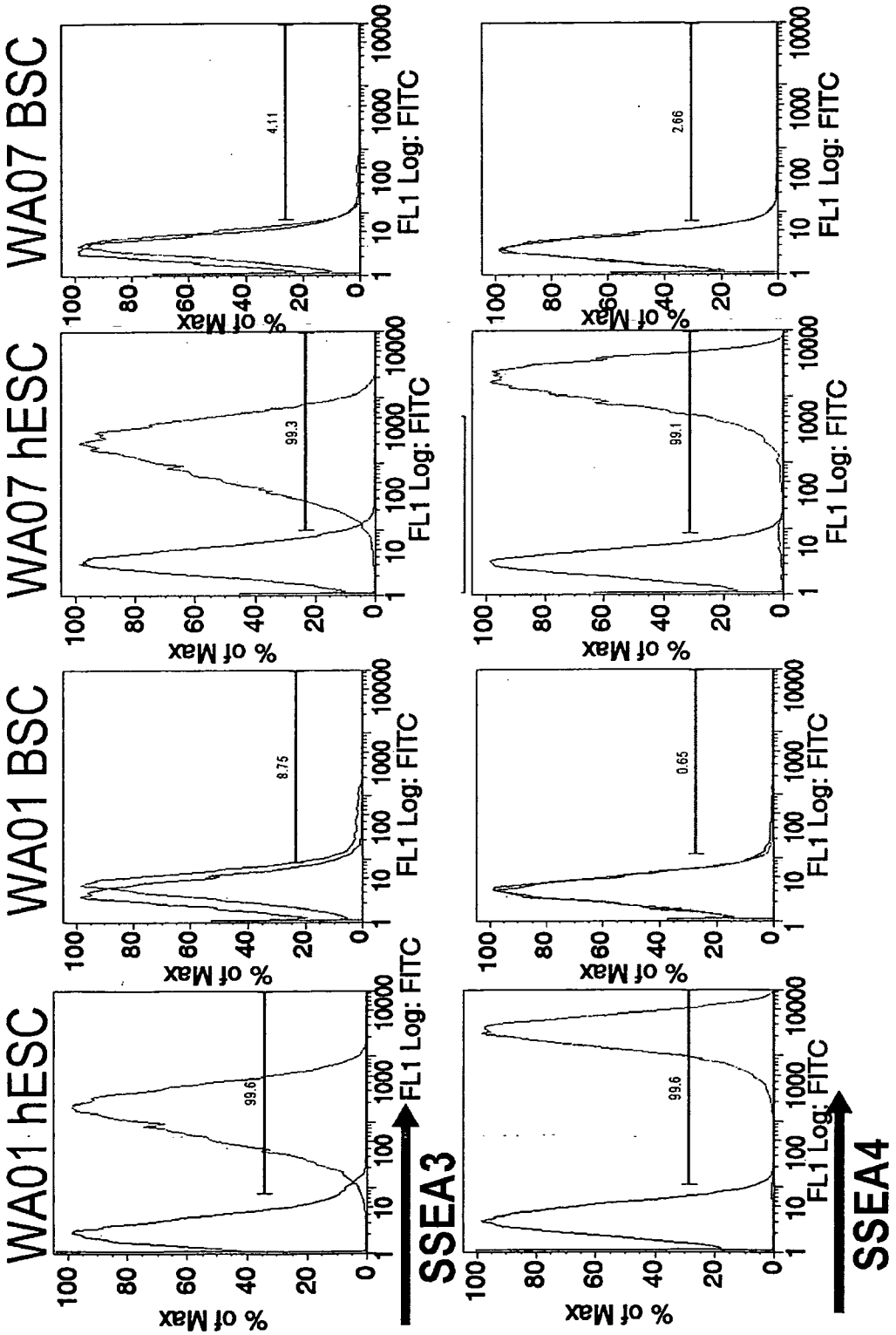

WA01 hESC
WA01 BSC
WA07 hESC
WA07 BSC
SSEA3
SSEA4
% of Max
FL1 Log: FITC
99.6
8.75
99.3
4.11
99.6
0.65
99.1
2.66

Figure 23 (con't)

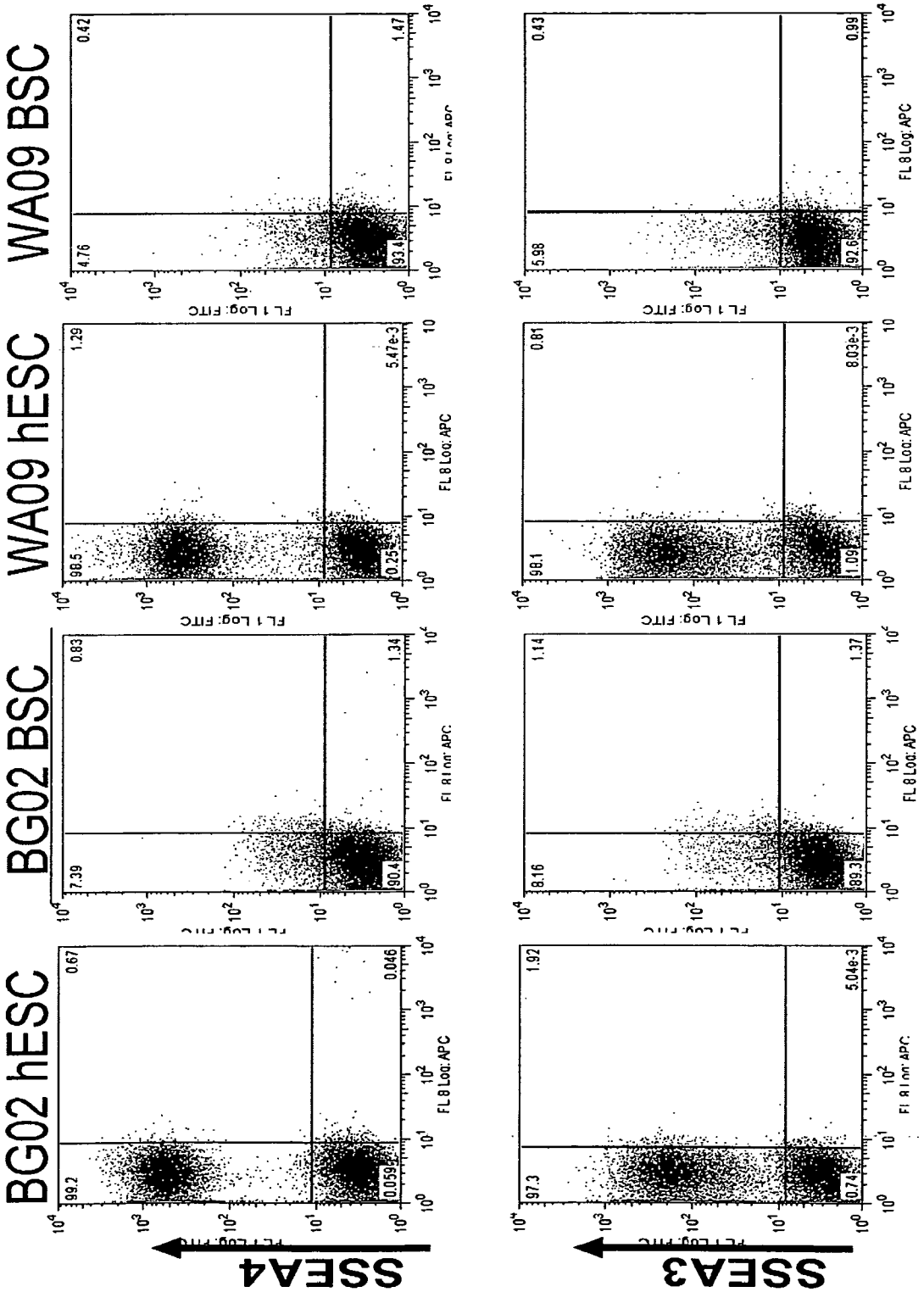


Table 1

Gene Symbol	Gene Name	Ave log fold H9/02	log replicates H9/H9/H9	02/02/02
NRCAM	neuronal cell adhesion molecule	3.7/2.4	3.8/3.2/4.0	2.8/2.5/1.9
PCDH17	protocadherin 17	4.8/4.6	5.4/3.8/4.5	4.4/5/4.4
PCDH8	protocadherin 8	8.5/4.9	8.7/7.8/9.1	6.0/5.7/3.1
NR2F2	nuclear receptor subfamily 2, group F, member 2., ARP1	12.8/9.2	13.1/12.5/12.9	9.5/9.2/9
CXCR4		4.9/3.7	4.3/4.8/5.5	3.5/3.3/4.2
NR2F1	nuclear receptor subfamily 2, group F, member 1, EAR-3	11.5/9.8	11.5/10.8/11.4	10.2/10.2/9
NCAM1	neural cell adhesion molecule 1, CD56	4.1/4.5	4.9/3.1/4.3	4.6/4.7/4.2
CXCR7		3.8/4.0	4/3.4/3.9	4.6/4.2/3..3
NTRK2	neurotrophic tyrosine kinase, receptor, type 2, Trk-B,	4.3/5.8	5.3/4.2/3.5	6.3/5.6/5.6
CDH7	cadherin 7, type 2	4.3/4.8	4.6/4.2/4	5.4/5.4/3.7
TNFRSF19	tumor necrosis factor receptor superfamily, member 19	5.7/6.6	5.1/6.4/5.6	6.6/5.9/7.3
RUFY3	RUN and FYVE domain containing 3, RIPX, Singar1	4.1/4.8	5.3/3.3/3.6	4.7/4.8/4.8
SCN3B	sodium channel, voltage-gated, type III, beta	5.3/6.6	6.1/5.2/4.7	7.1/7.0/5.7
HOXB2		7.6/6.5	8/6.9/8	6.8/6.8/6
DACH1	dachshund homolog 1 (Drosophila)	7.7/3.6	8.2/7.2/7.6	3.9/4.3/2.7
POU3F2	POU class 3 homeobox 2, Brain-2, N-Oct-3	7.1/7.6	7.5/6.5/7.4	7.7/7.8/7.3
SYT11	synaptotagmin XI	5.9/5.9	6.8/5.8/5.2	5.8/5.8/6.2
IRX5	iroquois homeobox 5	7.5/4.9	7.6/7/7.9	4.5/5/5.1
SCN3A	sodium channel, voltage-gated, type III, alpha subunit	6.2/4.3	7.4/5.2/6.1	4.9/4.7/3.4
KCNJ6	potassium inwardly-rectifying channel, subfamily J, member	3.4/4.2	2.9/3.4/3.9	4.2/3.7/4.7

Table 1 (cont'd)

TTYH1	tweety homolog 1 (Drosophila)	4.3/3.0	5.1/3.7/4.2	2.9/3.1/3.1
FZD10	frizzled homolog 10 (Drosophila), CD350	4.5/4.7	4.1/4.2/5.3	5.1/4.5/4.6
SMOC1	SPARC related modular calcium binding 1	5.2/5.6	5.6/5.1/5.1	5.8/5.8/5.3
TSHZ1	teashirt zinc finger homeobox 1	9.2/5.8	8.8/9.4/9.4	5.7/5.9/5.9
DLL1	delta-like 1 (Drosophila)	4.7/4.1	5.1/3.9/5.1	4.5/4.6/3.2
HES4	hairy and enhancer of split 4 (Drosophila)	6.4/3.3	6.2/6.5/6.6	3.4/3.4/3.2
HOXB3		9.4/6.4	9.4/8.6/10.1	6.7/6.6/5.8
IRX3	iroquois homeobox 3	5.2/4.2	5.4/4.7/5.5	4.2/4.5/4
IRX1	iroquois homeobox 1	3.6/3.2	3.3/3.8/3.7	3.3/3.3/3
PAX3		4.8/4.1	4.1/4.9/5.3	4.3/3.7/4.4
LIX1	Lix1 homolog (chicken)	5.1/4.9	4.8/6/4.4	5/4.1/5.5
HES5	hairy and enhancer of split 5 (Drosophila)	10.6/6.4	11.1/9.9/10.9	6.9/7.1/5.3
SDK2	sidekick homolog 2 (chicken)	5.8/5.2	6.8/5/5.5	5.4/5.8/4.3
CRB1	crumbs homolog 1 (Drosophila)	3.8/6.4	4.5/4.2/2.6	6.6/6.7/6
NEUROD1	neurogenic differentiation 1	4.9/6.4	5.3/4.6/4.7	7.1/6.8/5.3
PAX6		10.2/8	10.5/9.6/10.4	7.6/8/8.4
NRXN3	neurexin 3	3.8/4.6	3.3/4/4.2	4.1/4.6/5
NCALD	neurocalcin delta	5.1/5.7	5.6/4.8/5	5.8/5.8/5.4
CGI-38	TPPP3 , tubulin polymerization-promoting protein family me	6.1/6.7	6.8/6.3/5.1	6.8/7.1/6.3
BAALC	brain and acute leukemia, cytoplasmic	4.2/3	4.9/3.9/3.7	3.3/3.5/2.3
NPAS3	neuronal PAS domain protein 3	3.7/2.3	4.1/3.2/3.8	2.4/2.2/2.2

Table 2

	hESC				BSC				
	WA01	WA07	WA09	BG02	WA01	WA07	WA09	BG02	iPSC
SSEA3	++++	++++	++++	++++	-	-	-	-	NA
SSEA4	++++	++++	++++	++++	-	-	-	-	NA
CXCR4	-	-	-	-	++++	++	++++	++++	-
CD31	-	-	-	-	-	-	-	-	-
CD56	-	-	+	-	++++	++++	++++	++++	++++
CD44	+++	+++	++++	++++	++	++	-	++	-
CD166	++++	++++	++++	++++	++hi/ ++low	++hi/ ++low	+low	++hi/ +low	+++hi/ +low
CD133	+++	++++	++++	++++	++++	-	++++	++++	-
CD117	+++	++	++++	++++	++++	-	+	+	-
CD90	++++	++++	++++	++++	++++	++++	++++	++++	++++
CD105	+	+	+	++	-	+++	++	+	+++
Trk-B	-	-	-	NA	NA	++++	++++	NA	NA

WA01 had a high CD117 (c-kit) which may be due to a low passage number of BSC differentiation (P2).
IPS BSC did not express CXCR4.